THE INVESTIGATION OF NOVEL MARINE MICROORGANISMS FOR THE PRODUCTION OF BIOLOGICALLY ACTIVE METABOLITES

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Vanessa Ann Sunkel

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ABSTRACT

New drugs, particularly antibiotics, are urgently required to combat the increasing problem of antibiotic resistant human pathogens. Due to the scarcity of products available today, the pharmaceutical industry is now under pressure to reassess compounds derived from plants, soil and marine organisms. Pharmaceutical companies are showing renewed interest in marine biotechnology as the oceans represent a rich source of both biological and chemical diversity of novel molecular structures with anti-cancer, anti-inflammatory and antibiotic properties. Formerly unexplored locations, such as deep ocean sediments, show great potential as a source of genetically novel microorganisms producing structurally unique secondary metabolites.

In this research, a metabolite producing marine *Pseudoalteromonas* strain, known as AP5, was initially used to develop methods for the detection, optimisation of production and extraction of bioactive metabolites from other potentially novel marine isolates. Two hundred and seventy six (276) marine isolates from water and sediment samples from the Antarctic Ocean and Marion Island were isolated. Ten visually different isolates were screened for bioactivity against Gram-positive and -negative bacteria, fungi and yeast. Three out of the 10 isolates, WL61, WL114 and WL136, appeared to be novel *Streptomyces* spp. showing activity against different test organisms. Many of these marine microorganisms are difficult to culture in the laboratory, particularly when they are cultivated continuously in shake flasks as they can stop producing bioactive compounds.

The cultivation of marine isolates in bioreactors may be a more beneficial process for the optimisation of metabolite production compared to conventional liquid

fermentation techniques whereby the solid-liquid-air interface of membrane bioreactors can imitate the natural environment of microbes. The membrane bioreactor system is a stable growth environment with low shear that supports steady-state biofilm growth consisting of a high cell density due to a high mass transfer of nutrients and oxygen to the cells. This approach was employed and isolates WL61, WL114 and WL136 were immobilised onto ceramic membranes using Quorus single fibre bioreactors (SFR). The SFRs were used to establish the most suitable growth medium for continuous secondary metabolite production. The best growth conditions were applied to the Quorus multifibre bioreactor (MFR) for scale up of biologically active metabolites, highlighting the potential of bioreactor technology for use in bioprospecting for isolating and screening novel and known organisms for new and interesting natural products. Furthermore, the Quorus MFR was shown to be suitable for the production of high yields of antimicrobial metabolites and is an efficient new fermentation production system.

Purification by HPLC fractionation was used to characterise four major compounds from isolate WL114 extracts. NMR structure elucidation identified one of the two primary compounds as Bisphenol A. The complete chemical structure for the second potent bioactive compound could not be determined due to the low concentration and volume of material.

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LIST OF ABBREVIATIONS

%	percentage
δ	chemical shift
:	ratio (A is to B)
′	minutes
<	less than
>	greater than
±	plus minus (approximately)
°	degrees
°C	degrees celcius
$\mu g.l^{-1}$	microgram per litre
µl	microlitres
µm	micrometer
$\mu mol.kg^{-1}$	micromole per kilogram
μmol^{-1}	per micromole
^{13}C NMR	carbon 13 nuclear magnetic resonance
16S	16 subunit
^{1}H NMR	proton nuclear magnetic resonance
ACC	Antarctic Circumpolar Current
AHLs	acyl-homoserine lactones
AMP	adenosine mono-phosphate
APF	Antarctic Polar Front
AU	absorbance units
BLAST	basic local alignment search tool
C_{18}	carbon 18
cm	centimeters
COSY	two-dimensional correlation spectroscopy
CSTR	continuous stirred tank reactor
CTD	conductivity-temperature-depth
DCM	dichloromethane
DCW	dry cell weight
DNA	deoxyribonucleic acid
E	East

ECS	extracapillary space
EtOAc	ethyl acetate
Extra	extracellular
F	fraction
Fig.	figure
g	grams
G+ve	Gram-positive
GC	gas chromatography
GLS	Gas-liquid-solid
G-ve	Gram-negative
HCOOH	formic acid
HMBC	heteronuclear multiple bond correlation
HPLC	high performance liquid chromatography
hr	hour
hrs	hours
HSQC	heteronuclear single quantum coherence
HTC	high-throughput culture
HTS	high throughput screening
Hz	Hertz (measure of frequency)
Intra	intracellular
IR	infrared absorbance spectroscopy
kDa	kiloDalton (unit of mass)
KOH	potassium hydroxide
kPa	kilopascals (unit of pressure)
L	litre
LC	liquid chromatography
LiP	lignin peroxidase
m	meters
M1-M5	marine medium 1-5
MeOH	methanol
MFR	multifibre reactor
mg	milligrams
MGR	membrane gradostat reactor

min	minute
ml	millilitres
mm	millimetre
mm^2	millimetre squared
MnP	manganese peroxidase
MRSA	methicillin-resistant *Staphylococcus aureus*
MS	mass spectroscopy
NaOH	sodium hydroxide
NCBI	national centre for biotechnological information
nm	nanometer
OD_{600} nm	optical density at 600 nm
p	probability
Pa	Pascals (unit of pressure)
PCR	polymerase chain reaction
PDA	photodiodide array
PID	proportional integral differential
ppm	parts per million
PSu	polysulphone
QB	Quorus bioreactor
r	Pearson's correlation coefficient
R^2	R squared (goodness of fit)
rDNA	ribosomal deoxyribonucleic acid
rep-PCR	repetitive element PCR
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
s	seconds
S	South
SC	starch-casein
SD	standard deviation
SEM	scanning electron microscopy
SESS	marine sediment extract medium
SFR	single fibre reactor

spp. or sp.	species (plural or singular)
SSAF	Southern Sub-Antarctic Front
ST	station
T	time
TLC	thin layer chromatography
tRNA	transfer ribonucleic acid
UV-Vis	ultraviolet visible spectrum
v/v	volume per volume
vol/vol/hr	volume per volume per hour
w/v	weight per volume
X or x	times or multiply
XBT	expendable bathythermograph
α	alpha
γ	gamma
ΔP	delta P (change in pressure)
λ	wavelength

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CHAPTER 1

Literature Review

1.1. NATURAL PRODUCTS AND DRUG DISCOVERY

Natural products have played an important role in drug discovery and development throughout the 20th century (McGee, 2006). Between 1981 and 2002, 60% of new anticancer drugs and 75% of new anti-infectives, originated from natural sources. In addition, the period from 2001 to 2005 saw the introduction of 23 new drugs derived from natural products to treat a variety of disorders such as bacterial and fungal infections, cancer, diabetes, dyslipidemia, atopic dermatitis, Alzheimer's disease and genetic diseases such as tyrosinaemia and Gaucher disease. Furthermore, from 2003 and 2005, three additional drugs derived from natural products were approved, two as immunosuppressive agents and the third for pain management (Lam, 2007).

More than half the drugs on the market are natural products or derived from natural products (McGee, 2006). Despite this success, historically the drug discovery process was not well-suited to the screening of natural products because the high-throughput screening (HTS) or "blitz" screening approaches were unsuccessful in terms of the short timeline allowed - three months from start to finish (Lam, 2007). Due to the many diverse resources of natural products, pharmaceutical companies focused on too many areas simultaneously, leading to a perception within the industry that the discovery of a natural product for potential disease treatment was a costly and difficult challenge not worth the time and expense (Lam, 2007).

Competition between pharmaceutical companies in the mid 1980s to develop novel drugs prompted a renewed search to accelerate the drug discovery process where additional approaches to drug discovery, using technologies like synthetic chemistry libraries, combinatorial libraries, and high-throughput and ultra-high-throughput screening, were introduced (McGee, 2006; Lam, 2007). The screening of natural products has been revolutionized by such technological advances and the development of new methods, presenting a unique opportunity to re-establish natural products as a major source of new drug leads (Lam, 2007).

Today, the pharmaceutical industry is under more pressure than ever before, due to the urgent requirement of new drugs to combat the growing problem of antibiotic resistant human pathogens, and the scarcity of products currently available (Perić-Concha and Long, 2003; McGee, 2006). Many companies are therefore reassessing compounds that are derived from plants, soil and marine organisms as a source of new drugs (McGee, 2006).

Microbial natural products still emerge as the most promising source of future antibiotics. This may be attributed to the fact that natural antibiotics have been created by evolution, making them efficient at killing microorganisms. In terms of microbial diversity, the field of natural products research remains largely unexplored with between one and five percent of microorganisms having been studied. There is thus huge potential to exploit this untapped resource of natural products for antibiotic therapy, as well as the possibility of activating the expression of silent biosynthetic pathways through the manipulation of culture conditions (Peláez, 2006).

1.2. MARINE MICROBIAL NATURAL PRODUCTS AND BIOPROSPECTING

Bioprospecting is the search for valuable genetic and/or biologically active organic metabolites from nature which could be developed and used commercially (Davies-Coleman *et al.*, 2000). Although deep sea biodiversity research and marine natural product bioprospecting is still in its infancy, the marine environment has yielded a substantial number of potential drugs in the last three decades (Haefner, 2003). The total number of species and biochemical diversity in the oceans is higher than on land, increasing the probability of a larger number of marine natural products, or compounds derived from them, for the treatment of human illnesses. Many are predicted to be approved soon; however the majority of these molecules are still in preclinical development (Haefner, 2003). A few marine natural products are already on the market, for example, cytarabine (Ara-C), derived from a marine sponge, *Cryptotethya crypta*, and commonly used to treat acute myeloid leukaemia (Haefner, 2003). In addition, Prialt (ziconotide), a synthetic peptide from the marine snail, *Conus magus*, approved in 2004 and used for the treatment of pain (Vitale *et al.*, 2008); and Yondelis (Trabectedin, Ecteinascidin 743), derived from the marine tunicate, *Ecteinascidia turbinata*, used to treat soft tissue sarcoma and ovarian cancer (Allavena *et al.*, 2005), have both been successful marine natural products in

drug discovery. Some of the natural products isolated from marine invertebrates have been shown to be, or are suspected to be, of microbial origin, however only a small but growing fraction have been derived from marine microorganisms (Park *et al.*, 2002; Haefner, 2003).

Two novel anticancer agents, NPI-0052 (salinisporamide A) and NPI-2358, which entered phase I clinical studies in 2006, were developed as a result of investigating marine microorganisms for drug discovery. Due to the high frequency of novel bioactive metabolites detected in marine microbial fermentations, further studies on marine microorganisms would potentially deliver a steady supply of new drug leads (Lam, 2007). In particular, marine psychrophiles are proposed as a good quality source of polyunsaturated fatty acids for the pharmaceutical industry as well a good resource of novel therapeutic agents (Gomes and Steiner, 2004).

While the marine environment provides a rich source of novel and diverse chemistry, the opportunity for their use as drugs may be reduced as a result of their evolved potency. It is hypothesised that the reason marine natural products tend to be more bioactive and thus potentially more toxic than their terrestrial counterparts, is partly due to the fact that natural products are rapidly diluted by the surrounding seawater and therefore need to be highly potent as well as to maintain their potency to have any effect (Haefner, 2003; Bull and Stach, 2007). Toxic compounds are essential if organisms are to be successful in their micro-environment, for example, these compounds help them deter predators and defend themselves against their prey and competitors (Haefner, 2003). In this context, an approach of a low-dosage synergy programme, such as antifungal chemotherapy, could provide a potential means of making use of toxic natural products (Bull and Stach, 2007).

1.2.1. Marine biotechnology in the Pharmaceutical Industry

The Pharmaceutical and Fine Chemical industries have shown renewed interest in marine biotechnology, which uses marine organisms, including bacteria, fungi, algae and plants to discover new drugs and other useful products with anti-cancer, anti-viral, anti-inflammatory and antibiotic properties (Van der Wielen and Cabatingan, 1999; Adetunji, 2005). This interest was motivated by nature's diversity of novel molecular structures with unpredicted, but possibly advantageous functions and activities that were found in this environment (Van der Wielen and Cabatingan,

1999). This trend is highlighted in reports of novel natural products obtained over the past 20 years from terrestrial microorganisms which have remained unchanged, whilst those derived from marine microorganisms have increased linearly over the same period (Bull and Stach, 2007).

These bioactive compounds are representative of a wide range of polar (saponins, amino acids, alkaloid salts, polyhydroxy steroids), medium polar (peptides, peptide-derivatives, polyketides) and apolar (terpenes, terpenoids, cyclodepsipeptides, long chain fatty acids) molecules. Marine derived molecules have also found applications in the field of food additives as natural pigments and colourants, vitamins, antioxidants, stabilisers such as alginates and other biopolymers (Van der Wielen and Cabatingan, 1999).

1.3. MARINE MICROORGANISMS: A NEW RESOURCE FOR DRUG DISCOVERY

1.3.1. Marine microbes and their aquatic environment

The marine environment, made up of several habitats, starts from the sea surface microlayer of a few millimetres through the bulk water column to more than 10 000 m below the surface continuing further down to the habitats on and under the sea floor. Habitats on the sea floor include sediments of varying geology, mineral nodule fields, carbonate mounds (enormous deep water mounds of carbonate sediments associated with cold water corals, hydrocarbon seepage and water currents), cold seeps (a region on the surface of the ocean floor where hydrogen sulphide, methane and hydrocarbon-rich fluids leak), hydrocarbon seeps, saturated brines and hydrothermal vents (Ward and Bora, 2006). Marine snow and marine organisms, including actinobacteria, can be found in these various vertically established marine habitats. The marine environment is restricted by changing geophysical factors for example temperature, salinity, underlying geochemistry as well as ocean currents, thus sampling across this geographical distribution is still sparse (Ward and Bora, 2006).

It was previously thought that microbes were homogeneously distributed in seawater, but it was later found that microbes are dispersed heterogeneously (Long and Azam, 2001). Marine bacteria have been isolated from a range of nutrient rich and poor habitats, including the surface and interior spaces of marine invertebrates

and algae, deep and shallow oceanic sediments as well as in seawater (Davidson, 1995). Since nutrient concentrations in oligotrophic waters are extremely low, obtaining them is challenging for marine bacteria and for that reason any micro-source of nutrients is important to microbial survival. Evidence suggested that motility and chemotactic behaviour cause bacteria to exploit micro-environments such as marine snow particles and faecal pellets containing higher nutrient concentrations than that of the surrounding water (Goldman and Dennett, 2001).

1.3.1.1. Deep sea and cold water habitats

If the marine realm has been considered largely understudied as a source of chemical diversity, then the cold water habitats are truly an unexplored frontier. Traditionally it was difficult to comprehend extreme cold regions such as the Antarctic Ocean or deep sea habitats as suitable sources of biological or chemical diversity due to their harsh environments and remoteness (Lebar *et al.*, 2007). Interestingly, the bioactive compound producing organisms found in Antarctica, deep sea microbes, specifically invertebrate-associated microbe communities, were among the most diverse and robust (Lebar *et al.*, 2007). Even so, this diversity is greatly under-represented by the few species that have been isolated from these environments. It is thus necessary to use a wide range of culturing methods to generate culture collections from these diverse cold habitats (Cavicchioli *et al.*, 2002).

When drug discovery research with soil bacteria, particularly actinomycetes, was in progress, it was suggested that the most important groups of drug-relevant bacteria were not indigenous to the ocean. It is now well-known that the oceans are a highly complex microbiological environment with microbial abundances of 10^6 cells per ml in seawater and 10^9 cells per ml in ocean bottom sediments (Fenical and Jensen, 2006). In view of the fact that the area of the world's oceans are enormous (70% of the earth's surface area), it is astonishing that the extensive drug discovery efforts involving soil bacteria have not been duplicated in this ecosystem. However, sampling the ocean's diverse habitats requires specialised equipment, and in the case of deep sea environments, the development of new technologies (Fenical and Jensen, 2006).

1.3.1.2. Psychrophiles

A diverse range of microbes including representatives of the Bacteria, Eucarya and Archaea have been discovered in cold environments including the ocean, particularly deep sea. Most microorganisms isolated from cold environments are either psychrotolerant/psychrotrophic (cold-adapted), or psychrophilic (cold-loving). Psychrotolerant organisms grow well at temperatures close to the freezing point of water, but have fastest growth rates up to 20°C, whereas as psychrophilic organisms grow fastest at temperatures of 15°C or lower and are unable to grow above 20°C (Cavicchioli *et al.*, 2002).

Psychrophiles and their products were under-utilised in biotechnology and studies on cold adaptation lagged behind those of high-temperature adaptation. However, since then advances, especially with cold-active enzymes, indicated rapid expansion in this field. The Archaea, existing in cold aquatic environments, are diverse and abundant and in view of their unusual properties, represent an important source for biotechnological discovery and potential application, for example, the use of ether-linked lipids in the production of liposomes for vaccine and drug delivery (Cavicchioli *et al.*, 2002).

Despite the little explored and unusual nature of the deep sea environment, novel genera are being recovered from surface seawater and tidal mud flats (Bull and Stach, 2007). It is assumed that the shallow water can become dominated by microorganisms derived from terrestrial run-off. However, in response to convective mixing in the deep sea, nutrient availability is boosted and populations of planktonic actinobacteria are considerably increased, indicating the importance of temporal sampling. The enormity of the oceans has long been broadly appreciated by microbiologists, however serious exploration of their biology, specifically in the deep seas, has only been possible with recent innovations in marine engineering (Bull and Stach, 2007).

1.3.1.3. Marine bacterial diversity in seawater

Gram-negative heterotrophic bacteria are an essential part of marine microbial populations, who inhabit an array of diverse environments, namely coastal and open water areas, deep sea and hydrothermal basins, and marine sediments. These bacteria have attracted the attention of researchers in relation to the production of

various physiologically active compounds (Ivanova and Mikhailov, 2001). Gram-negative marine heterotrophic bacteria belong to the gamma (γ) subclass of the class *Proteobacteria*, for example, *Alteromonas, Pseudoalteromnonas, Idiomarina,* and *Colwellia*. Many of these bacteria have similar morphologies, physiologies, and biochemistries, making their identification difficult. Several novel species of marine *Proteobacteria* were described between the years 1996 and 2000, for example, *Alteromonas infernus* and *Alteromonas macleodii* subsp. *fijiensis*, isolated from deep sea hydrothermal basins, *Pseudoalteromonas antarctica* and *Pseudoalteromonas prydzensis*, isolated from Antarctic coastal waters, *Pseudoalteromonas bacteriolytica*, isolated from kelp, *Laminaria japonica* from the Sea of Japan, and *Pseudoalteromonas peptidolytica*, isolated from seawater (Ivanova and Mikhailov, 2001).

Ivanova *et al.* (2001) reported the isolation of a new genus of aerobic marine proteobacteria, *Idiomarina*, which incorporated two species namely, *I. abyssalis* and *I. zobellii*, from seawater samples at depths of 4000 and 5000 m, respectively. The species were phenotypically similar to bacteria of the genera *Alteromonas*, *Pseudoalteromonas*, and *Marinomonas*. Species of the genera *Alteromonas*, *Pseudoalteromonas*, and *Idiomarina* are heterotrophic, Gram-negative, rod-shaped, obligatory marine bacteria (Ivanova and Mikhailov, 2001).

Few bacilli of marine origin have previously been reported to produce biologically active compounds, thus the taxonomy of marine bacilli strains such as *B. badius*, *B. subtilis*, *B. cereus*, *B. lichenifirmis*, *B. firmus*, *B. pumilus*, *B. mycoides*, and *B. lentus*, from the Collection of Marine Microorganisms (KMM) of the Pacific Institute of Bioorganic Chemistry, were studied. These bacteria produced unusual biologically active metabolites that were different from those metabolites isolated from terrestrial bacteria. These metabolites included an antibiotic, 3-amino-3-deoxy-*D*-glucose, a new glucanase, as well as cyclic acylpeptides (Ivanova *et al.*, 1999).

Marine microbes play a significant role in marine biogeochemical cycling, however many of these organisms have not been cultivated as proven by molecular biological tools such as 16S rRNA gene cloning and sequencing. Pure cultures of marine microorganisms need to be obtained in order to learn about their physiology, ecology and taxonomy (Cho and Giovannoni, 2003). Furthermore, pure cultures can be used

to investigate the hypotheses rising from genome sequences. For example, oceanographers can gain knowledge of how nutrient limitation and other relevant variables in the ocean affect growth of individual species and possibly help in the identification of the dominant roles species play in geochemical cycles (Connon and Giovannoni, 2002).

The field of metagenomics studies the communities of microbial organisms in a specific environment by applying modern genomic procedures to 16S rRNA gene-based phylogenetic diversity. This field has revolutionized microbiology as it focuses on the estimated 99% of microbial species that cannot currently be cultivated, by diverting the attention away from clonal isolates (Chen and Pachter, 2005).

Craig Venter, president of the J. Craig Venter Institute, and his colleagues embarked on a pilot study in 2004 to shotgun sequence and clone microbial genomes from filtered seawater from the nutrient-poor waters of the Sargasso Sea (Venter *et al.,* 2004). Approximately 200 L of surface sea water, obtained from four sites in the Sargasso Sea was filtered, and matter above 3 µm and below 0.1 µm in diameter discarded, effectively leaving the remaining bacteria (Cherfas, 2006). This environmental investigation led to the discovery of 148 previously unknown bacterial phylotypes including 1800 new species of bacteria. More than 1.2 million new genes were identified revealing a level of microbial diversity previously only guessed at. This discovery included 782 new rhodopsin-like photoreceptors, which capture light energy from the sun and convert it to biological energy via biochemical pathways, proving that marine bacteria can capture sunlight independently of standard chlorophyll-based pathways (Venter *et al.,* 2004; Cherfas, 2006).

1.3.1.4. Marine bacterial diversity in sediment

It was found, with the aid of 16S phylogenetic diversity inventories as well as cultivation approaches, that deep sea sediments contained more than 1300 different actinobacterial taxonomic units. A large percentage of these are believed to represent novel species and genera (Fenical and Jensen, 2006). Intelligent approaches to sample handling and growth conditions were employed to complement this strategy and led to the recovery of many new taxa in laboratory cultures. Sampling for marine actinomycetes began in the late 1960s, but it was not until 2005 that the first seawater obligate marine actinomycete (*Salinispora* sp.) was

described and it was evident that these marine microbes were distinctly different from their terrestrial relatives (Fenical and Jensen, 2006). New species of known actinobacterial genera are being described on a regular basis; however this venture is hampered by the availability of qualified taxonomists. Other newly described marine genera besides *Salinispora* (Micromonosporineae) include *Demequina* (Micrococcinae), as well as others waiting formal taxonomic description, "*Marinispora*", "*Solwaraspora*" and "*Lamerjespora*" (Bull and Stach, 2007).

One of the many unique actinomycetes discovered from marine samples is undoubtedly *Salinispora* sp. The genus *Streptomyces*, which are readily cultured from marine samples, especially when collected near the shore, are also revealing new marine diversity (Fenical and Jenson, 2006). Most of these strains are identical to or closely related to strains isolated from land. However, in addition to strains that occur both on land and in the sea, distinct marine phylotypes are beginning to be documented. Chemical studies of these actinomycetes are particularly interesting and illustrate the correlation between phylogenetic diversity and secondary metabolite production (Fenical and Jenson, 2006). The MAR2 or "*Marinispora*" clade shows extensive phylogenetic diversity, indicating that it consists of multiple new species. Chemical studies on these strains have consistently yielded new polyketide-derived polyenes (Fenical and Jenson, 2006).

Actinobacteria are broadly distributed throughout the marine environment, although as a small but significant portion. The ubiquitous existence of actinobacteria is reflected by the presence of marine actinophages, as viruses reveal dominant organisms present in the sea. However, the degree of actinobacteria diversity, abundance and biogeography is still unclear as sampling remains low and inconsistent, techniques are often biased and actinobacterial identification is not clearly defined (Ward and Bora, 2006).

1.3.1.5. Attached and free-living microorganisms

Marine aggregates, consisting of detritus, inorganic particles, diatoms, and other microorganisms are referred to as marine snow and range in size from millimetres to centimetres. Bacteria colonize and solubilise sinking marine snow particles more rapidly than they can absorb the solutes. This results in a trail of dissolved organic matter seeping out behind the sinking aggregate promoting the growth of free-living

bacteria in the water column and plays an important role in the transfer of organic material to deep water and the ocean floor. Ocean carbon fluxes, atmospheric CO_2 and global climate are therefore impacted by the balance between the rate at which aggregates form and sink and the rate at which they are solubilised (Gram *et al.*, 2002). It is believed that bacterial abundance as well as bacterial species richness varies by millimetres in the seawater column, due to these nutrient-rich "hotspots". This variability increases with an increase in the concentration of particulate organic matter, for example, marine snow particles, in seawater (Long and Azam, 2001).

In previous studies, Nair and Simidu (1987) suggested that microbes isolated from marine particles were successful in inhibiting the growth of two human-pathogenic bacteria compared to free-living bacteria. Additionally, Long and Azam (2001) found that particle-attached bacteria were more likely to produce inhibitory compounds, and reported a 5- to 10-fold higher frequency of inhibition, in comparison to their free-living counterparts. This suggests that bacterium-bacterium antagonism in oligotrophic waters may be more common on particles where quorum sensing mechanisms have been discovered to occur (Gram *et al.*, 2002). This information may assist researchers to develop new strategies for searching for novel antibiotics of marine bacterial origin (Long and Azam, 2001).

The quorum sensing mechanism in Gram-negative bacteria allows the bacteria to communicate and sense their own density, triggering the expression of target genes at high cell densities, using chemical signals such as acylated homoserine lactones (AHLs). It has been shown that the association of AHLs allows marine snow bacteria to express antibiotic production only when reaching high bacterial cell densities on snow particles (Gram *et al.*, 2002). The oceans were originally thought to be predominately occupied by Gram-negative species however Gram-positive organisms have been equally acknowledged in natural products literature. This may be due to the fact that the early research in bacterial diversity focussed on nutrient poor seawater, as opposed to most chemical studies which focus on the isolation of bacteria from more nutrient rich sources which support Gram-positive organism growth. The published results only contain isolates that generate interesting metabolites in culture, thus may present a distorted distribution (Davidson, 1995).

1.4. MARINE ACTINOMYCETES

Terrestrial actinomycetes have played a major role in the pharmaceutical industry for the past 50 years due to their capacity to generate antibiotics such as actinomycin, streptomycin, and vancomycin. From the 100 strains of the marine version of these terrestrial bacteria that were isolated by William Fenical and colleagues from near-shore sediment samples, 80% were shown to produce molecules that inhibit cancer cell growth, and 35% had the capability to kill pathogenic fungi and bacteria (McGee, 2006). As the rate of research and development in marine microbiology increases, more novel actinobacterial metabolites are being described, several of which are currently in clinical trials (Bull and Stach, 2007). According to Fenical's group, the salinisporamide A produced from one of the marine actinomycetes they discovered, has been characterised as a "very potent" cancer inhibitor, including human colon carcinoma, non-small cell lung cancer, and breast cancer (McGee, 2006). This metabolite has entered phase I trials in under three years from the time of discovery (Bull and Stach, 2007).

1.4.1. Chemical ecology and biodiversity of actinobacteria

Even though the biology of marine bacteria is beginning to be understood, the biochemical activities of these organisms remain largely unexplored (Fenical and Jensen, 2006). Advances in marine microbial ecological analysis using molecular techniques and metagenomics, illustrate that actinobacteria are a significant, sometimes even dominant environmental clade. Cultivation methods and molecular techniques are both leading to new insights into marine actinobacterial biodiversity and biogeography, a hot topic for microbial ecologists. These approaches are essential to the development of natural product search and discovery tactics (Ward and Bora, 2006). Researchers are currently realising how complex, unique and highly adapted these organisms are, as DNA sequence-based methods are applied to the field of marine microbial ecology (Fenical and Jensen, 2006).

It has been acknowledged that actinomycetes can be isolated from the sea as well as the deepest known ocean trenches. Culturing spores derived from soil inhabiting strains may represent actinomycetes that have been washed into the marine environment (Fenical and Jensen, 2006). This issue has led to queries concerning the existence of marine actinomycetes and whether or not marine-derived strains are

metabolically active in the sea. However, it is now obvious that common soil-inhabiting actinomycetes can be isolated from marine samples (especially if water samples are collected in close proximity to the land), but it is clear that distinct marine populations do exist. This became evident in 1984 with the taxonomic description of the first marine actinomycete, *Rhodococcus marinonascens* (Fenical and Jensen, 2006).

1.4.2. Antibiotic-producing marine actinomycetes

The search for new antibiotics effective against multi-drug resistant pathogenic bacteria has been the focus of research (Sujatha *et al.*, 2004). Many marine free-living and sediment inhabiting bacteria produce secondary metabolites possessing antibacterial properties (Park *et al.*, 2002). The actinobacteria are especially distinguished for containing representatives producing approximately 10 000 diverse natural products (Bull and Stach, 2007). The genus *Streptomyces*, filamentous soil bacteria, is widely recognised as industrially important microorganisms for their ability to produce many kinds of novel secondary metabolites including antibiotics. Different *Streptomyces* species produce about 75% of commercially and medically useful antibiotics (Sujatha *et al.*, 2004).

Secondary metabolites, such as pigments, antibiotics and toxins are organic compounds produced in the idiophase of stationary growth of the organisms (Madigan *et al*, 1997). Secondary metabolites are not essential for growth, development or reproduction of organisms, but play an important role in organisms' defense mechanisms against predators, parasites, diseases, and for interspecies competition (Madigan *et al.*, 1997).

Although novel compounds have been isolated from marine microorganisms, there is still a necessity to develop and improve microbiological methods. Since less than 1% of seawater bacteria can be cultured in a laboratory, innovative microbiological techniques will permit the isolation and culture of unique marine microorganisms (Davidson, 1995; Park *et al.*, 2002). The culture of marine microorganisms in bioreactors can be used to optimise the production of bio-products which may be industrially viable (Yan *et al.*, 2003).

1.4.3. Production of secondary metabolites

Almost all marine environments contain low concentrations of substances that can be utilised for growth and metabolism. In contrast, natural surfaces have a tendency to accumulate and concentrate nutrients by charge-charge or hydrophobic interactions (Kwon *et al.*, 2002). Free-living microbes attach and eventually develop biofilms in the course of growth and division when they come into contact with suitable surfaces. Although the attachment is reversible to begin with, it becomes stronger and irreversible with time. During colonization, bacteria overproduce extracellular polymeric substances, which are the materials that create the biofilm matrix. This matrix has a multipurpose function for adhesion, immobilisation of cells on the colonized surface, protection, recognition and facilitating spatial arrangement of different species within the biofilm (Kwon *et al.*, 2002).

An example of a biofilm-forming marine bacterium is D2, isolated from the surface of an adult tunicate, *Ciona intestinalis* and shown to produce extracellular components able to kill the larvae of two marine invertebrates. Furthermore, D2 was found to produce a novel 190 kDa protein with antibacterial activity against a variety of marine and medically important bacterial species. In addition to this activity, the D2 bacterium is able to release products into its surrounding environment which are inhibitory against several major groups of biofouling organisms including algae and diatoms (James *et al.*, 1996).

The bacterial metabolism in flask cultures is very different to that in a biofilm. Marine bacterial isolates express various levels of activity, however when continuously cultivated in shake flasks in the laboratory, most stop producing bioactive compounds. This is due to the fact that suspension cultures in closed flasks represent artificial growth conditions that are very different from the natural environment (Yan *et al.*, 2003). The preferred growth of bacteria in their natural environment is as a biofilm attached to surfaces where growth conditions are usually heterogeneous. Further investigations have shown that biofilm formation and contact with air are required for eliciting production of bioactive compounds. Despite this, there have been few studies investigating the effect of growth as biofilms, particularly in the case of marine microorganisms, for the production of secondary metabolites (Yan *et al.*, 2003).

1.4.3.1. Bioreactors

Evaluation of the pharmaceutical, cosmetic, nutritional, and chemical potential of products derived from deep water organisms has been limited. Species from deeper water may have more critical growth requirements, such as high pressure and low temperature. Development of closed-system bioreactors for the culture of both shallow and deep water organisms has been a particularly challenging task for marine bioprocess engineers due to a lack of research into bioreactor engineering and fermentation procedure design in the field of marine bacterial antibiotic production (Pomponi, 1999; Marwick *et al.*, 1999). Most production approaches are carried out at the crude shake flask level, which cannot predict successful scale-up. The application of bioprocess intensification methods to the production of antibiotics, and other metabolites, from marine microbes will become an important technique for improving the supply of natural products for the pharmaceutical industry (Marwick *et al.*, 1999).

The aim of any bioreactor, producing secondary metabolites, is to maximise the amount of biomass produced in a short growth phase, while ensuring the best conditions for a high and sustained compound production phase. A number of different reactor configurations are used in the biotechnology industry, for example, the continuous stirred tank reactor (CSTR), the airlift reactor, three-phase fluidized beds, and membrane bioreactors. The reactor engineering determines the parameters essential to bioprocess intensification, namely oxygen and bulk media mass transfer, mixing patterns, shear stresses, and implementation of innovative fermentation strategies (Marwick *et al.*, 1999).

In marine biochemical systems, opportunities exist for the development of novel reactors or strategies, providing more efficient bioprocesses. One way to intensify marine bioprocess productivity is through induction strategies, for example, stress resulting in the expression of metabolites not otherwise possible (Wright *et al.*, 1999). Examples of stress applied to reactors are:

- Nutrient limitation

The formation of secondary metabolites is reliant on growth conditions for example culture medium composition, and is regulated by nutrients, growth rate, feedback control, enzyme inactivation, and enzyme induction (Madigan *et al*, 1997; Demain,

1998). Their formation is therefore linked to growth conditions or developmental stages. By way of illustration, most bacteria use basic metabolism to grow and reproduce when cultivated in nutrient rich media, but when nutrients are exhausted they start producing secondary metabolites. During secondary metabolism, nutrients, growth rates or inducer signals may activate a gene at the translation level by encoding a rare tRNA, or by encoding a positive transcription factor. Additionally, feedback regulation using low molecular weight compounds can control secondary metabolism (Demain, 1998).

- Temperature

Physical factors can also cause different effects on the growth and production phases of secondary metabolism (Marwick *et al.*, 1999). Microorganisms possess cellular mechanisms that react and adjust to high or low temperature shifts in their natural habitats (Phadtare and Inouye, 2007). A temperature change is a stress for all living organisms and can be used as a regulatory factor in secondary metabolism. For example, marine *Alteromonas* grows best at 28°C but yields more antiviral compound at 25°C. Similarly, *Lactobacillus* also increases the production of an exopolysaccharide when the temperature decreases from 37°C to 25°C at the beginning of the exponential phase (Marwick *et al.*, 1999; Phadtare and Inouye, 2007). Secondary metabolism can be induced by heat or cold shock. Heat shock can cause heat-shock proteins to induce the production of the polyketide, jadomycin B by *Streptomyces venezuelae* (Demain, 1998). The effect of cold-shock causes different parts of cells, for example, cytoplasmic membrane, ribosomes, nucleic acids, and proteins to respond at specific levels. Cold-shock proteins which play a significant role in the acclimation of cells to cold can also be induced by a temperature downshift (Phadtare and Inouye, 2007).

- pH

Maximum production of secondary metabolites depends on the optimisation of different variables. These include physicochemical conditions such as pH, temperature, osmolarity and the presence of activators/inhibitors (Bernal *et al.*, 2007). The pH level of the growth medium has a significant effect on secondary metabolite production, i.e. the production of compounds can decrease rapidly on either side of an optimum point. The marine bacterium *Alteromonas leuteoviolacea*, for example, shows a decrease in violacin production when the pH of the medium

increases from an optimum pH 7 to pH 9. A pH change can also be used as a technique to induce stress in *Streptomyces* sp. resulting in methylomycin synthesis (Marwick *et al.*, 1999).

- Salinity

Optimum salinity levels should be determined for both growth and production phases as some marine bacteria require salt for growth. The effect of salinity has not been comprehensively studied in secondary metabolism as high levels of salt may cause bioreactor corrosion and may also inhibit the dissolution of sparged oxygen into water-based growth media. Organism growth may be increased by reducing the salt level in seawater, possibly due to a decrease in the energy that is required to be channelled to cytoplasmic salt regulation (Marwick *et al.*, 1999).

- Mechanical stress

This includes agitation and shear stress. Yan *et al.* (2003) demonstrated that a modified roller bottle bioreactor, mimicking ocean intertidal wave action, induced the production of antimicrobial compounds of two marine isolates which, under normal planktonic growth conditions, had ceased producing secondary metabolites.

Thus, strategies that adapt a more heterogeneous environment, such as those simulating the natural environments of microorganisms using niche-mimicking bioreactors, may be used to elicit or enhance production of secondary metabolites by apparent non-producers (Yan *et al.*, 2003).

1.4.3.2. Membrane bioreactors

Membrane bioreactors are unique in that microbes may be cultured as biofilms in a controlled environment that simulates their natural growth conditions. They provide an inert membrane barrier or support on which immobilised biomass is retained or immobilised, respectively. These systems offer an environment where high cell density culture and thus improved productivity may be attained. Several membrane configurations have been tested with the hollow fibre/capillary membrane configuration emerging as the most appealing (Ntwampe *et al.*, 2007). The production of secondary metabolites from biofilms using membrane bioreactors has not been widely used for the production of low volume, high value bio-products such as pharmaceuticals, but more readily applied in waste water treatment (Ntwampe *et al.*, 2007).

One such example is the membrane gradostat bioreactor (MGR) used by Leukes *et al.* (1999) for the bioremediation of waste waters containing aromatic pollutants using the degradative ability of the filamentous fungus, *Phanerochaete chrysosporium.* This was achieved by nutrient limiting conditions to initiate secondary metabolism and the secretion of a group of hydrogen peroxide producing oxidases, for example lignin peroxidases (LiP), which are responsible for degrading a wide range of organic pollutants (Leukes *et al.*, 1999). The production of extracellular lignolytic enzymes by *P. chrysosporium* using the MGR has been evaluated by a number of researchers (Leukes *et al.*, 1999; Garcin, 2002; Fraser, 2005; Sheldon and Small, 2005; Ntwampe and Sheldon, 2006), as well as the non-specific oxidation of phenolic pollutants and industrial effluents by *Trametes versicolor* and *Neurospora crassa* (Ryan *et al.*, 1998; Luke and Burton, 2001).

Nevertheless, the use of membrane technology for biotechnological applications is under-utilised. Synexa Life Sciences (Pty) Ltd. has developed a MGR platform, the Quorus Bioreactor, and uses this technology in their Compound Production Unit to produce secondary metabolites for clients. The range of microbes cultured includes *Penicillium* spp., *Aspergillus* spp. and actinomycetes (Leukes and Fraser, 2007; Edwards *et al.*, 2007; Edwards and Leukes, 2007). Increasingly, with each new development and continued improvements, membrane technology is presenting itself as a cost-effective alternative approach to produce high value secondary metabolites (Ntwampe *et al.*, 2007).

Membrane bioreactors are very practical as they can operate continuously allowing culture conditions to change and/or be changed over time. They therefore represent a unique approach to screen for a diverse range of metabolites produced within the same experimental run by a stable biofilm (Fraser, 2006). The MGR system allows for the uninterrupted production of secondary metabolite(s) under aerobic conditions. The continuous removal of product from the biofilm avoids a negative product feedback mechanism. Furthermore, regular sampling of secreted product means a shorter exposure time to metabolically active cells and extracellular enzymes, thus limiting the decomposition of the product. It is therefore perceived to be a more beneficial process in comparison to conventional liquid fermentation techniques and

may be applied to bioprospecting for and screening of novel as well as known organisms for new and interesting natural products (Fraser, 2006).

1.5. OBJECTIVES OF THIS STUDY

In view of the remarkable potential of marine microorganisms to produce natural products with therapeutic potential and the challenges inherent in optimising the drug discovery process, the objectives for this study were:

- To develop efficient methods to isolate novel marine organism(s) from seawater and/or sediment samples from the Antarctic Ocean and Marion Island.
- To develop extraction methods for the isolation of new bioactive compound(s) from novel or known marine organisms.
- To utilise a membrane bioreactor, the Quorus bioreactor, for the screening and production of novel bioactive marine compound(s).
- To characterise and identify novel bioactive marine compound(s).

CHAPTER 2

Production of bioactive metabolites by the marine bacterium, *Pseudoalteromonas* strain AP5

2.1. INTRODUCTION

A marine strain of the genus *Pseudoalteromonas*, known as AP5, was investigated to develop methods for the detection, optimisation of production and extraction of bioactive metabolites from other marine microorganisms. *Pseudoalteromonas* spp., derived from the genus *Alteromonas*, can be isolated from marine animals, algae as well as seawater. Some strains are known to produce toxins, protease inhibitors or hydrolytic enzymes capable of degrading an array of polysaccharides, whereas others have been reported to synthesise various biologically active metabolites, targeting a range of organisms (Ivanova *et al.*, 1998; Holmström and Kjelleberg, 1999; Isnansetyo and Kamei, 2003; Kobayashi *et al.*, 2003; Kalinovskaya, 2004). Isnansetyo and Kamei (2003) discovered anti-methicillin-resistant *Staphylococcus aureus* (MRSA) activity from *Pseudoalteromonas phenolica*, while Sawabe and colleagues (1998) reported broad spectrum bacteriolytic activity against Gram-positive and –negative bacteria from *Pseudoalteromonas bacteriolytica*. While AP5 itself was an interesting candidate for drug discovery, the methods and experience derived from this work were to be used to establish a marine bioprospecting platform at Synexa Life Sciences (Pty) Ltd.

In a previous study, AP5 was isolated from the digestive tract of an adult common clownfish, *Amphiprion percula* and screened for potential probiotics that could be applied to eliminate fish pathogens during aquaculture (Vine, 2004). AP5 was shown to produce bioactive metabolite(s) against three aquatic pathogens, *Vibrio alginolyticus*, *Vibrio damsela* and *Carnobacterium piscicola*, however the chemistry of these bioactive compounds was not described. *V. alginolyticus* showed limited capability to attach to sites on the mucus of the gut wall of the clownfish when AP5 was added to intestinal mucus before the addition of the pathogens. Moreover, when AP5 was added to intestinal mucus after the addition of the pathogens, *V. alginolyticus* was out-competed for attachment sites to some extent (Vine, 2004).

In addition to the antibacterial activity described by Vine (2004), AP5 was also found to have antifungal activity at the onset of this project. The growth of AP5, stored on marine agar, was shown to have antagonistic activity towards a contaminating fungus, *Penicillium* sp. (Fig. 2.1). This fungus was then used as a test organism in further studies.

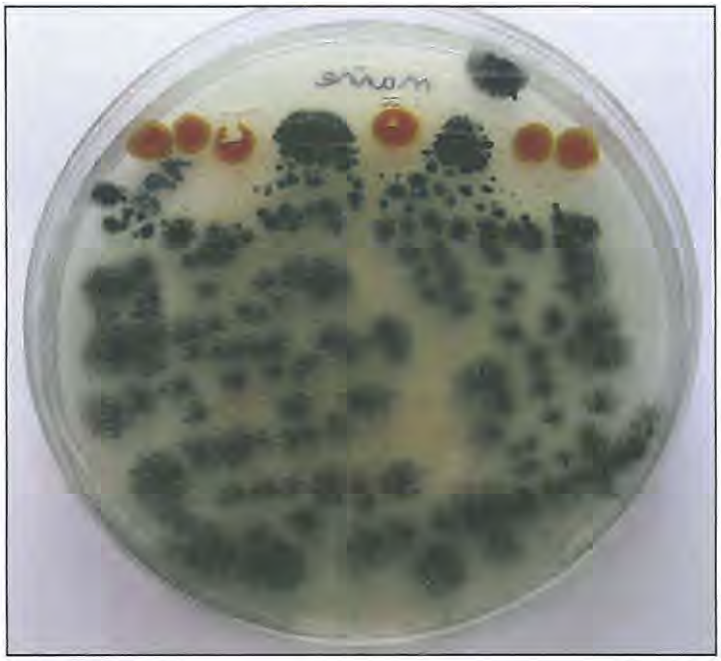


Fig. 2.1: Inhibition of *Penicillium* sp. by AP5 colonies

This chapter describes the optimisation of the production and isolation of the bioactive metabolites from AP5. Subsequently, the most appropriate methods resulting from these experiments will be applied to study the Marion Island and Antarctic Ocean isolates. The objectives were thus:

- To develop extraction methods for the isolation of the active compound(s).
- To characterise and possibly identify the active compound(s).

2.2. MATERIALS AND METHODS

2.2.1. Storage and maintenance of AP5 and test organisms

AP5 cells and the *Penicillium* sp. mycelia were stored in cryotubes (Greiner) in 50% glycerol (Kimix) in a -80°C freezer (Snijders). Additionally, the *Penicillium* sp. spores were aseptically harvested by scraping the spores from agar plates with a glass spreader and sterile water and stored in cryotubes at -80°C. Both organisms were

revived in marine broth (Difco) (see section 2.2.2) and maintained on marine agar (Difco).

2.2.2. Culture conditions

AP5 cells and the *Penicillium* sp. (from mycelia or spores) were grown in liquid culture using 40 ml marine broth in 100 ml Erlenmeyer flasks and incubated at 25°C with shaking at 200 rpm overnight in a shaking incubator (Labcon).

2.2.3. Elicitation responses of AP5 to the fungus, *Penicillium* sp.

2.2.3.1. Preparation of extracellular extracts

In order to assess whether the *Penicillium* sp. caused an elicitation response from the AP5 marine organism, 1 ml of a viable *Penicillium* sp. culture and 1 ml of a killed *Penicillium* sp. culture were each added to separate overnight AP5 liquid cultures (40 ml). These co-cultures (40 ml) and an overnight pure AP5 liquid culture (40 ml) were extracted with the cells (v/v) in 3 ml aliquots after three and 24 hr incubations using EtOAc (Kimix), Base EtOAc (0.5% NaOH) (Kimix) or DCM (Kimix). These extracts were concentrated ten-fold by evaporation under nitrogen in a turbovap (TurboVap LV). The concentrated samples were tested for bioactivity using the well diffusion bioassay method (Appendix A) with the *Penicillium* sp. as the test organism.

2.2.3.2. Preparation of intracellular extracts

The activity from an intracellular extraction of AP5 was investigated in a second experiment. A colony of AP5 cells was added to an overnight culture of the *Penicillium* sp. and incubated overnight with shaking at 200 rpm at 25°C. This co-culture and an AP5 pure culture (control) were harvested by centrifugation at 4000 rpm for 10 min (Hermle Z300K) and extracted on ice using MeOH (Romil) (w/v) and sonication (Branson Digital Sonifier) for 3 x 30 s bursts. The solvent extracts were concentrated ten-fold either by evaporation under nitrogen or air in a turbovap, or by rotary evaporation (drying under reduced pressure) (Laborota 4000 Heidolph Instruments). The concentrated samples were tested for bioactivity using the well diffusion bioassay method (Appendix A).

2.2.4. Optimisation of the production, extraction and concentration of the bioactive metabolites from AP5

The AP5 cells were cultured under different growth conditions for 24 hr to assess whether temperature (10 or 25°C), oxygen (stationary or shaking) and light (dark or light) affected the production of the bioactive metabolite(s). The whole broth cultures (40 ml) were extracted using EtOAc or MeOH solvents (v/v), respectively. MeOH and EtOAc extracted whole broth cultures were centrifuged at 4000 rpm for 10 min to remove biomass from the MeOH extracts and separate solvent layers from the EtOAc extracts. MeOH and EtOAc supernatant extracts were concentrated ten-fold in a turbovap using either air or nitrogen. The samples were screened for bioactivity against the *Penicillium* sp. using the well-diffusion bioassay (Appendix A).

2.2.5. Mechanism of action of the AP5 active metabolite(s) against the *Penicillium* sp.

Results from the extracellular extractions were not reproducible. It was thus decided to perform intracellular extractions on ice using sonication for 3 x 30 s bursts in the presence of MeOH to lyse AP5 cells in broth (v/v) or with AP5 cells concentrated by centrifugation (v/w). Similarly, intracellular extractions (sonication with MeOH) were also carried out on AP5 cells scraped off agar plates. The extracts (five-fold concentrated in a turbovap and unconcentrated) were screened using the well-diffusion bioassay method (Appendix A). The condition of the mycelia from fungal samples neighbouring the wells from the well-diffusion bioassay, were examined under 100X magnification using a light microscope in order to determine the action of the AP5 extracts against actively growing and established, stationary phase samples of the *Penicillium* sp.

2.2.6. Stability and extraction efficiency of the active compound(s)

The stability of the active metabolite(s) was assessed by exposing unconcentrated MeOH solvent extracts to different temperatures (-20°C, 10°C, 25°C and 40°C) as well as to diffused light and dark conditions. The extracts exposed to light were assayed every hour for 12 hrs. The extraction efficiency of the compound was assessed by adjusting the volume ratio of MeOH used to extract the cells (1:1, 1:2 and 1:3 wet weight cells:solvent).

2.2.7. Microtitre plate bioassay

The bioactivity of the compound(s) was assayed and quantified using a sensitive microtitre plate based bioassay where growth of the *Penicillium* sp. at 25°C was monitored at 600 nm for a period of 93 hrs (FLUOstar Optima BMG Labtech) (Appendix A). This was carried out at different dilutions (1, 2 and 5X) of 1:2 and 1:3 wet weight cells:MeOH solvent extracts (w/v). A series of higher MeOH dilutions of the extract (5, 10, 20, 50, 100, 500, 1000 and 2000X) was similarly tested to determine the percentage inhibition of the *Penicillium* sp. by the crude AP5 extracts.

2.3. RESULTS AND DISCUSSION

2.3.1. Elicitation responses

Elicitation experiments were carried out to assess whether competition or stress due to exposure of AP5 to the *Penicillium* sp. induced or enhanced bioactivity. No zones of inhibition in the well-diffusion bioassay were detected with samples from the co-cultures of AP5 and the *Penicillium* sp. Zones of inhibition were however, detected with samples from the control AP5 pure culture.

It was therefore concluded that the *Penicillium* sp. did not cause elicitation of AP5 and induce the production of the bioactive substance(s). Rather, co-cultures of AP5 and the *Penicillium* sp. appeared to inhibit the production of the bioactive compound(s). This was possibly attributed to the sharing of available nutrients resulting in a lower cell density of AP5 and/or a difference in the ratio of available nutrients that may have influenced the molecular regulation of secondary metabolite production.

2.3.2. Optimisation of the production, extraction and concentration of the bioactive metabolites from AP5

Concentrated AP5 cells extracted with MeOH (w/v) produced zones of inhibition, whereas no zones of inhibition were detected with EtOAc extracts. Thus it was concluded that the bioactive compound(s) were polar. The method of drying the extracted metabolite(s) (in a turbovap with air or nitrogen, or by rotary evaporation), did not affect the activity of the extracts. This suggests that the bioactive compound(s) are not easily oxidised.

Whole broth cultures extracted with MeOH (v/v) produced small or no zones of inhibition, thus all further extractions were carried out with concentrated cell samples. Extracts from concentrated AP5 cells harvested from agar plates produced larger zones of inhibition than the concentrated AP5 cells (centrifuged) from liquid cultures. The increased bioactivity from AP5 cells growing on agar plates suggests that either nutrient availability influences bioactive compound production or that the bioactive compound is induced by growth on solid surfaces.

In all further experiments the active metabolite(s) were extracted from cells scraped off agar and extracted by sonication using MeOH (w/v). Extracts were screened for activity against the *Penicillium* sp. using the well diffusion bioassay method (Appendix A).

2.3.3. Mechanism of action of the AP5 active metabolite(s) against the *Penicillium* sp.

The antifungal activity of AP5 extracts against the *Penicillium* sp. (cultivated from spores stored at -80°C) differed depending on the age of the fungus. The active compound(s) did not affect the appearance of the mycelia from the established *Penicillium* sp., but inhibited mycelial growth of the actively growing *Penicillium* sp. The fungal hyphae around the edge of the zones were sparse and showed delayed sporulation. Therefore only the actively growing *Penicillium* sp. was used for the bioassays.

2.3.4. Stability and extraction efficiency of the active compound(s)

The extracts exposed to diffused light over a 12 hr period changed colour from a dark yellow to a very pale/clear yellow colour and did not produce zones of inhibition. The control extracts kept in the dark over the same period and at the same temperature retained the dark yellow colour and produced zones of inhibition.

Fresh extracts, without the 12 hr holding period, were tested for bioactivity and showed zones of inhibition similar in size to those produced by the extracts stored in the dark for 12 hrs. Storage in the dark at 25°C for 12 hrs did not result in the inactivation of the active compound(s). It can therefore be concluded that the active compound(s) are inactivated by photolytic degradation. Furthermore, the rate of

degradation is rapid as exposure to light for 2 hrs resulted in total inactivation of the compound(s).

2.3.5. Microtitre plate bioassay

The well-diffusion bioassay is only qualitative and not as sensitive as the more recently developed high throughput screening (HTS) methods such as the microtitre plate based bioassay. A microtitre plate bioassay was developed for *Penicillium* sp. and used to assess the potential of these methods using filamentous organisms.

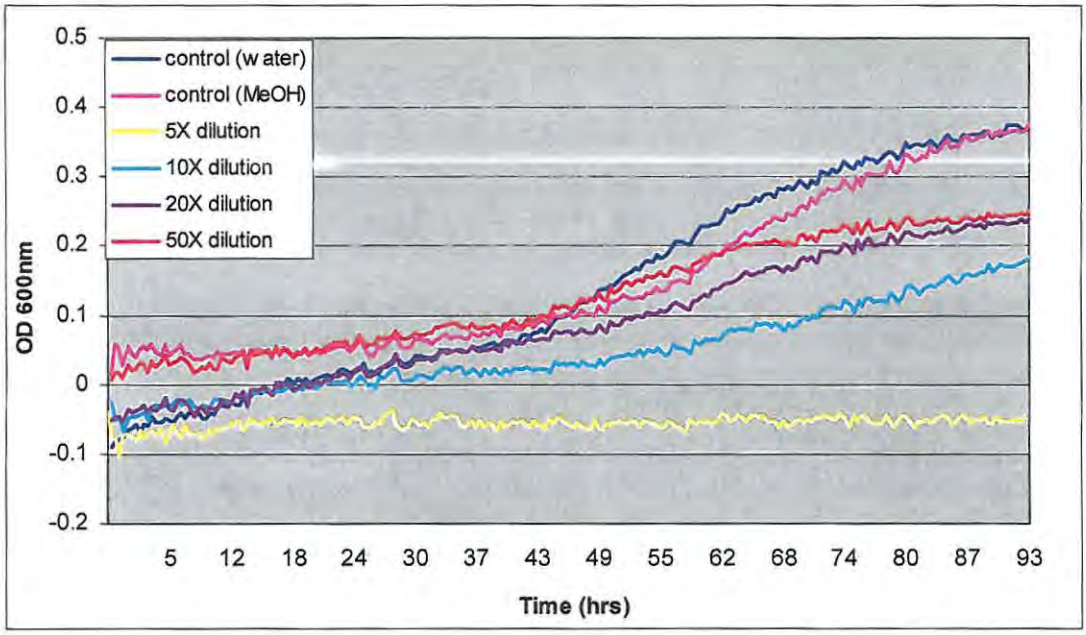


Fig. 2.2: Effect of diluted AP5 extracts on the growth of the *Penicillium* sp.

Fig. 2.2 shows that the *Penicillium* sp. grew in control 1 (water plus *Penicillium* sp. spores inoculated into the medium) and control 2 (MeOH plus *Penicillium* sp. spores inoculated into the medium), showing typical sigmoidal growth curves. Additionally, the results summarised in Table 2.1 below show that these controls did not inhibit the growth of the *Penicillium* sp. However, when the *Penicillium* sp. spores were added to the medium plus the 1:3 (w/v) extracted and diluted AP5 extracts (5, 10, 20 and 50X), the growth of the *Penicillium* sp. was inhibited to varying degrees.

In the 1:2 and 1:3 (w/v) extracted and diluted AP5 extracts (1, 2 and 5X), no growth of the *Penicillium* sp. was detected (data not shown), whereas when the dilutions of the active AP5 extracts increased, the percentage inhibition decreased (Fig. 2.2 and

Table 2.1). The 5X diluted extract resulted in 96% inhibition of growth of the *Penicillium* sp. The percentage decrease in the inhibition of growth by the 5 to 50X diluted extracts was linear with an R^2 value of 0.9959. Dilutions higher than 50X did not have an inhibitory effect against the *Penicillium* sp. (data not shown).

Table 2.1: Summary of microtitre plate and well-diffusion bioassays showing inhibition against *Penicillium* sp.

Dilutions	Slope of exponential growth phase (units/min)	% Inhibition (microtitre plate bioassay)	Zone of inhibition (well-diffusion bioassay)
Control 1 (water)	1.28×10^{-4}	0	0mm
Control 2 (MeOH)	1.33×10^{-4}	- 4	0mm
1x	0	100	4.5mm
2x	0	100	4mm
5x	4.71×10^{-6}	96	2.5mm
10x	5.51×10^{-5}	59	0mm
20x	8.63×10^{-5}	35	0mm
50x	9.60×10^{-5}	28	0mm

2.3.6. Loss of the production of bioactive compound(s) by AP5

Further attempts to produce and characterise the active metabolite(s) were unsuccessful as continuous sub-culturing of the AP5 cells resulted in the gradual loss of the ability of the AP5 cells to produce any bioactive compound(s). To overcome this problem, multiple cryotube stocks of the original isolated organism showing bioactivity should be prepared and cryopreserved for subsequent experiments.

2.4. CONCLUSION

AP5 did not require co-cultivation of the *Penicillium* sp. to induce an elicitation response for the production of active compound(s). Interestingly, co-cultures of AP5 and the *Penicillium* sp. appeared to inhibit the production of the bioactive compound(s). AP5 cells produced intracellular, polar active compound(s) that exhibited antifungal properties against the actively growing *Penicillium* sp. The active

compound(s) were photosensitive and more soluble in crude extracts when the AP5 cells were extracted with a larger volume of MeOH (1:3 ratio, w/v).

While the well-diffusion bioassay method is straightforward and rapid, the microtitre plate bioassay method proved to be more sensitive and quantitative. The latter method showed that the AP5 extract was still active and maintained some toxicity when diluted up to 50X (28% inhibition of growth) whereas no zones of inhibition were detected above 5X diluted extracts in the well-diffusion bioassay method.

It is well known that continuous sub-culturing of microorganisms, for example actinomycetes, which initially produce bioactive compound(s), can result in the loss of this ability (Taddei, 1998). AP5 was no exception and therefore further work on this initially promising source of bioactive compound(s) was discontinued. However, the knowledge and experience gained from these investigations was utilised in the study of the isolates from Marion Island and the Antarctic Ocean.

CHAPTER 3

Isolation of microorganisms from the Antarctic Ocean and Marion Island

3.1. INTRODUCTION

3.1.1. Sampling area for this study

Research in the Southern Ocean has focused on the changing climate at the Prince Edward Islands (Ansorge, 2006). It was found that the passing eddies have a marked effect on the climatic conditions which seem to be changing noticeably (Reason *et al.*, 2006). Large areas were previously permanently covered by snow or ice, but have melted since 1940 leaving the land free of such cover today (Ansorge, 2006; Reason *et al.*, 2006). Analysis of sea surface temperatures taken at Marion Island (one of the two islands forming the Prince Edward islands) has shown that temperatures have increased by 4.1°C over the past 50 years. A further analysis of wind and weather conditions has demonstrated that this climate change is most likely due to alterations in the semi-annual oscillation (Reason *et al.*, 2006).

The region where the Antarctic Circumpolar Current (ACC) crosses the south-west Indian ridge, upstream of the Prince Edward Islands, has been recognised as an important foraging ground for a number of predators inhabiting the islands. This region is thought to be an area of increased food accessibility as a result of meanders in fronts and counter-eddies which promote phytoplankton growth and thereby increase zooplankton biomass. Although this region plays a potentially important role in the island ecosystem, little research has been conducted in the vicinity of the south Indian ridge (Froneman *et al.*, 2002).

Several regions in the Southern Ocean have been acknowledged as "choke points" in the flow of the ACC, which results in wide ranging meso-scale activity and improved cross-zonal exchange of water masses. The hydrographic data collected during the survey suggest that the Andrew Bain Fracture Zone functions as a choke point to the flow of the ACC. The Antarctic Polar Front (APF) forms a wall between the Antarctic Zone to the south and the Polar Frontal Zone to the north. Surface temperatures across this front vary from 6°C to 2.8°C (Froneman *et al.*, 2002).

Froneman *et al.* (2002) reported concentrations of silicate ranging between 0.9 and 10.8 $\mu mol.kg^{-1}$, phosphate between 1.5 and 2.75 μmol^{-1} and total surface chlorophyll-*a* concentration between 0.09 and 0.38 $\mu g.l^{-1}$. The highest chlorophyll-*a* concentrations were recorded at stations positioned in the area of the APF and the SSAF (Southern Sub-Antarctic Front) probably due to elevated primary production in this region as a consequence of increased water column stability and nutrient availability, predominantly in the region of the APF. Total zooplankton abundance was also highest at stations in the immediate vicinity of these two zones. Enhanced biological activity is localised in the area of the convergence of these two frontal systems (Froneman *et al.*, 2002).

Sediment and seawater samples were collected from Marion Island and the Antarctic Ocean for this study. These sites have been described by climatologists (Ansorge, 2006; Reason *et al.*, 2006), but have not been intensively explored for the purpose of isolating and identifying novel marine psychrophilic microorganisms that may potentially produce new bioactive compounds such as antimicrobial agents, antiviral or anticancer drugs.

3.1.2. Laboratory cultivation difficulties

Psychrophilic marine microbes usually grow at temperatures of 15°C and below (Bowman, 1997) and are difficult to culture in the laboratory (Park *et al.*, 2002). Since the open ocean concentrations of organic substrates are often in the *pico* molar range, naturally occurring marine microbes are difficult to cultivate as most cannot adapt from the severe oligotrophy to the high nutrient concentrations of the growth media used in the laboratory (Bull, 2001). Furthermore, the risk of sample contamination by air is high due to the manner in which samples are collected and handled during processing.

Numerous bacteria survive in consortia where inter- and intra-population interactions are necessary. One organism may provide a crucial substrate or co-factor essential for growth of another member of the consortium, thus pure cultures will not provide the best approach to laboratory cultivation methods. Quorum sensing is also important because acyl-homoserine lactones (AHLs) play a key role in sensing, integrating and processing information from the inconsistent surroundings of aquatic

bacteria. Additionally, poor understanding of the basic physiology of aquatic microbes makes it difficult for scientists to design suitable culture media (Bull, 2001).

It is likely that most marine bacteria evolved in environments of inconsistent nutrient supply rates and low substrate concentrations, and thus, their uptake mechanisms can be expected to be extremely efficient (Bull, 2001). In nature, microbial species need diverse growth requirements; no single cultivation medium could be expected to culture a majority of these species. Seawater culture has yielded isolates of heterotrophic, oligotrophic bacteria that cannot be grown on organically rich marine media (Suzuki *et al.*, 1997). Furthermore, some bacteria have a short lag growth phase during laboratory cultivation after which they stop growing, preventing culture establishment. Strain selection processes can only result in improved growth and development of robust cultures after these initial difficulties have been overcome (Bull, 2001).

3.1.3. New cultivation strategies

Thus far, three cultivation approaches are worth particular attention: low nutrient media concentrations, dilution-to-extinction of environmental samples, and virus-depleted incubation conditions (Bull and Stach, 2007). A high diversity of isolates may well be obtained using minimal media with low nutrient concentrations, for example, media containing nutrients available in their natural environment, such as seawater which mimics the physical and chemical conditions found in the ocean. (Suzuki *et al.*, 1997; Connon and Giovannoni, 2002). Furthermore, the use of dilution-to-extinction cultures, whereby the dominant microbes are diluted out, is a method to isolate rare, more interesting strains. This method, often referred to as "extinction culturing", uses culture medium consisting of natural environmental seawater (Connon and Giovannoni, 2002).

Dilution-to-extinction in natural seawater media is a high-throughput culture (HTC) method that was developed for the isolation of a large number of microbes. The first cultured representative of the SAR11 clade and many novel strains of *Proteobacteria* were isolated using this approach. In addition, a number of novel bacteria associated with the α (alpha)- and γ-*Proteobacteria*, the families *Flavobacteriaceae* and *Nocardioidaceae* and the phylum *Cyanobacteria* were isolated using a similar

application of the HTC method, but in very low nutrient media (Cho and Giovannoni, 2003).

A high-throughput system involving micro-encapsulation of single cells also provides advantages for recovering slow-growing organisms. Media that can mimic the composition of the organisms' environment, for example, seawater, sediment or host organism extracts, can be added to the encapsulated cells to recover more isolates. This technique was used in a recent study and of the bacteria isolated, 42% were novel compared to the 7% isolated by the conventional method of spread plating samples directly on agar (Bull and Stach, 2007).

Methods have been established for the isolation of novel bacteria, including actinobacteria, using a variety of low nutrient media and different combinations of substrates added to diluted environmental samples. Such strategies have resulted in 23% cultivation efficiency from sub-surface coastal sediments. Furthermore, the effects of viral infection on bacterioplankton further enhance isolation, culturing rare planktonic group members, such as actinobacteria, as viral regulation may function at a broad taxonomic level rather than at individual strain levels. Additionally, if some or all of the characteristics of deep sea actinobacterial physiology can be defined, this could lead to a greater isolation efficacy (Bull and Stach, 2007).

The aim of the research reported in this chapter is to process and isolate microorganisms from deep and shallow seawater samples from the Antarctic Ocean and in the vicinity of Marion Island as well as sediment samples from Marion Island using various processing and culturing techniques in order to maximise the number and diversity of isolates obtained. This information would then be used to determine the most suitable microbial isolation techniques for the proposed marine bioprospecting platform.

3.2. MATERIALS AND METHODS

3.2.1. Sample collection procedures

A macro-scale hydrographic and biological survey *en route* and in the region of the south-west Indian ridge was carried out aboard the research and supply vessel MV

SA Agulhas by Fronemon and colleagues[1] during early autumn in April 2002. The survey consisted of a grid of seven north-south sections extending across the Andrew Bain Fracture Zone in the south-west Indian ridge between 49°00´- 51°45´S and 29°- 33°E (except the first two transects which only extended to 49°45´S). Water samples were collected at stations using a CTD (conductivity-temperature-depth) measure and an XBT (expendable bathythermograph) at intervals of 16 nautical miles. The CTD rosette contains a number of ten litre cylindrical containers and while lids remain open at both ends when lowered, they are closed and sealed after a particular depth is reached and raised to the surface. Salinity, temperature and density readings were attained, to a maximum depth of 1500 m, at each hydrographic station vertical profile. Water samples were collected by the researchers at ten standard depths, removed from each cylindrical container and analysed for dissolved oxygen, salinity, macronutrient (silicate, phosphate, nitrate and nitrite) concentrations, and microbial activity. A total of 135 hydrographic stations were studied during the survey (34 CTD/biological and 101 XB/surface chlorophyll-*a*) (Froneman *et al.*, 2002).

3.2.2. Processing of samples

Twenty six randomly chosen seawater samples, ranging between depths of ten to 5000 m, from the Antarctic Ocean and vicinity of Marion Island, and one randomly chosen surface dredge sediment sample from Marion Island, were processed and cultured under various conditions, after being stored at -20°C for four years. The combinations of all the variables (six variables at two levels (high and low)) namely, seawater sample depth (deep/shallow), defrosting technique (slow/quick), dilution factor (diluted/undiluted), culture matrix (agar/liquid broth), culture media (minimal/complex media), and replicate number (three/eight replicates), were depicted using a two-level factorial design technique, the Plackett-Burman experimental design tool (Design-Expert software version 6).

Following the Plackett-Burman experimental design, seawater samples were defrosted quickly in a waterbath (Shel Lab, Sheldon Manufacturing, Inc.) at 40°C for 10 min or slowly in a fridge (Bosch) at 7°C overnight. Sediment samples were

[1] Winston Leukes (WL) collected the samples during this voyage, but microorganism isolations for this research were done by Vanessa Sunkel.

defrosted slowly in a fridge at 7°C overnight. Seawater samples were used undiluted or diluted (10^{-1} to 10^{-3}) with filtered (0.45 µm MicronSep cellulosic membrane filters, Osmonics Inc.) and autoclaved seawater collected in Sea Point, Cape Town. Sediment samples were used undiluted or diluted (10^{-3} or 10^{-6}) with filtered and autoclaved seawater collected in Sea Point, Cape Town. The undiluted and diluted seawater and sediment samples were cultivated in three or eight replicates in liquid and agar media, reported to support the growth of marine microorganisms, described in Table 3.1.

Table 3.1: Complex and minimal growth media used for the isolation of marine microorganisms

Media for seawater samples		**Media for sediment samples**	
Marine agar/broth	complex	SC agar/broth	complex
$^1/_{10}$ marine agar	complex	$^1/_{10}$ R2A + seawater agar/broth	complex
$^1/_{10}$ R2A + seawater agar/broth	complex	SESS agar	minimal
R2A agar	complex	M3 agar/broth	minimal
Emerson's agar/broth	complex	M5 agar/broth	minimal
M1 agar/broth	complex		
M2 agar/broth	minimal		
M3 agar/broth	minimal		
M4 agar/broth	minimal		
M5 agar/broth	minimal		
Colloidal chitin agar/broth	minimal		

NOTE: Details of the media are given in Appendix B

3.2.3. Culture conditions for isolation

Aliquots of 100 µl of diluted and undiluted seawater samples, 100 µl of sediment samples (diluted) and dry sediment (undiluted), described in section 3.2.2, were spread onto the agar media as well as inoculated into 1 ml of the liquid media in 48-well microtitre plates (Greiner) in either three or eight replicates and incubated at 10°C (Thermal Control Incubator, Scientific Technology).

3.2.4. Sub-culturing and storage of isolates

All agar plates were checked twice weekly for one month during the incubation period and the colonies were counted. An estimate of the visual diversity of the

various colonies was also recorded. After one month incubation at 10°C, the agar plates were checked only once a week to detect the presence of slower growing organisms until the plates became overgrown. The microtitre plate liquid cultures were checked once a day for growth after which 100 µl from each well with visible growth was spread onto the same agar as the isolation broth and incubated at 10°C. These plates were then treated in the same manner as the agar isolation plates (described above).

Isolates were chosen according to their pigment, size, shape, texture and reappearance, and then sub-cultured onto the same agar as the isolation agar. These were re-incubated at 10°C until growth and then streaked to obtain pure cultures. Gram stains were performed on each of the pure colonies and examined microscopically to aid in the identification of the isolates. These were photographed using a microscope camera (Scopetek digital camera DCM 35 and MiniSee software) and stored in 50% (v/v) glycerol at -80°C. During this study, 276 isolates were isolated, purified and stored for further investigation.

3.3. RESULTS AND DISCUSSION

3.3.1. Processing and culturing of the Antarctic Ocean and Marion Island samples

The results for 14 deep water samples (350 – 5000 m), 12 shallow water samples (10 – 350 m) and one sediment sample (surface dredge) are summarised in Tables 3.2 and 3.3 below and discussed in greater detail in the text that follows.

Table 3.2: Processing techniques for water samples showing total colony count and visual diversity of colonies on complex agar media

Processing Technique			Replicate number	Total Colony Count on Complex Agar							
				1/10 R2A +seawater	R2A	1/10 Marine	Marine	Emerson's	M1	SC	Colony Diversity
Deep water	Slow defrost	Undiluted	3	503	1506	500	1011	1	507	/	**13**
	Quick defrost	Undiluted	8	3	509	2002	507	2511	5016	/	**20**
	Quick defrost	Diluted	3	/	/	/	/	/	/	/	/
Shallow water	Slow defrost	Undiluted	8	/	/	/	/	/	/	/	/
	Slow defrost	Diluted	3	0	1501	1000	504	1507	0	/	**5**
	Quick defrost	Diluted	8	/	/	/	/	/	/	/	/
Sediment	Slow defrost	1×10^{-3}	8	531	/	/	/	/	/	0	**4**
	Slow defrost	1×10^{-3}	3	500	/	/	/	/	/	500	**2**
	Slow defrost	1×10^{-6}	8	1019	/	/	/	/	/	0	**7**
	Slow defrost	1×10^{-6}	3	/	/	/	/	/	/	/	/
	Slow defrost	Undiluted	3	546	/	/	/	/	/	0	**4**

/ = Combination of processed samples not cultured on complex agar (Plackett-Burman)
0 = No growth

3.3.1.1. Deep seawater samples plated on complex agar media

The different combinations of processed deep water samples that were cultivated on complex agar (highlighted) all grew. The deep water, quick defrost, undiluted sample, plated on M1 agar with a replicate number of eight, resulted in the largest number of colonies (5016). Twenty visually different colony types were observed from this sample. The deep water, slow defrost, undiluted sample, plated on R2A agar with a replicate number of three, resulted in the largest number of colonies (1506). Thirteen visually different colony types were observed from this sample.

3.3.1.2. Shallow seawater samples plated on complex agar media

The different combinations of processed shallow water samples that were cultivated on complex agar (highlighted) all grew, with the exception of 1/10 R2A+seawater and M1 agar. The shallow water, slow defrost, diluted sample, plated on Emerson's agar

with a replicate number of three, resulted in the largest number of colonies (1507). Five visually different colony types were observed from this sample.

3.3.1.3. Sediment samples plated on complex agar media

The different combinations of processed sediment samples that were cultivated on complex agar (highlighted) all grew, with the exception of SC agar. The 10^{-3} diluted sediment samples, plated on 1/10 R2A+seawater with a replicate number of eight, resulted in the largest number of colonies (531). Four visually different colony types were observed from this sample. The same sample, but with a lower replicate number of three, plated on 1/10 R2A+seawater and SC agar, resulted in the same number of colonies (500). Two visually different colony types were observed from this sample. The 10^{-6} diluted sediment sample, plated on 1/10 R2A+seawater agar with a high replicate number of eight, resulted in the largest number of colonies (1019). Seven visually different colony types were observed from this sample. The undiluted sediment sample, plated on R2A+seawater agar with a replicate number of three, resulted in the largest number of colonies (546). Four visually different colony types were observed from this sample.

Table 3.3: Processing techniques for water samples showing total colony count and visual diversity of colonies on minimal agar media

Processing Technique			Replicate number	Total Colony Count on Minimal Agar						
				Colloidal chitin	M2	M3	M4	M5	SESS	Colony Diversity
Deep water	Slow defrost	Undiluted	3	/	/	/	/	/	/	/
	Quick defrost	Undiluted	8	/	/	/	/	/	/	/
	Quick defrost	Diluted	3	500	1000	7	24	4	/	4
Shallow water	Slow defrost	Undiluted	8	2506	11	0	1500	504	/	5
	Slow defrost	Diluted	3	/	/	/	/	/	/	/
	Quick defrost	Diluted	8	3000	1549	0	1003	0	/	12
Sediment	Slow defrost	1×10^{-3}	8	/	/	2508	/	16	1010	13
	Slow defrost	1×10^{-3}	3	/	/	/	/	/	/	/
	Slow defrost	1×10^{-6}	8	/	/	2003	/	2018	15	2
	Slow defrost	1×10^{-6}	3	/	/	2	/	1549	1	2
	Slow defrost	Undiluted	3	/	/	1001	/	516	5	7

/ = Combination of processed samples not cultured on minimal agar (Plackett-Burman)
0 = No growth

3.3.1.4. Deep seawater samples plated on minimal agar media

The different combinations of processed deep water samples that were cultivated on minimal agar (highlighted) all grew. The deep water, quick defrost, diluted sample, plated on M2 agar with a replicate number of three, resulted in the largest number of colonies (1000). Four visually different colony types were observed from this sample.

3.3.1.5. Shallow water samples plated on minimal agar media

The different combinations of processed shallow water samples that were cultivated on minimal agar (highlighted) all grew, with the exception of M3 and M5 agar. The shallow water, slow defrost, undiluted sample, plated on colloidal chitin agar with a replicate number of eight, resulted in the largest number of colonies (2506). Five visually different colony types were observed from this sample. The shallow water, quick defrost, diluted sample, plated on colloidal chitin agar with a replicate number of eight, resulted in the largest number of colonies (3000). Twelve visually different colony types were observed from this sample.

3.3.1.6. Sediment samples plated on minimal agar media

The different combinations of processed sediment samples that were cultivated on minimal agar (highlighted) all grew. The 10^{-3} diluted sediment sample, plated on M3 agar, resulted in the largest colony number (2508). Thirteen visually different colony types were observed from this sample. The 10^{-6} diluted sediment sample, plated on M5 agar with a replicate number of eight, resulted in the largest number of colonies (2018). Two visually different colony types were observed from this sample. The same sample plated on M5 agar, but with a lower replicate number of three, resulted in the largest number of colonies (1549). Two visually different colony types were observed from this sample. The undiluted sediment sample, plated on M3 agar with a replicate number of three, resulted in the largest number of colonies (1001). Seven visually different colony types were observed from this sample.

3.3.2. Organism characterisation

3.3.2.1. Water and sediment samples

A total of 276 marine microorganisms were isolated from shallow water, deep water and sediment samples. These isolates were characterised as Gram-negative, Gram-positive or fungal. Gram-negative bacteria occupy diverse habitats including coastal

and open water areas, deep sea and hydrothermal basins, and marine sediments (Ivanova and Mikhailov, 2001). Gram-negative bacteria were thought to dominate the oceans however Gram-positive organisms are now equally recognised in natural products literature (Davidson, 1995). This is illustrated in Fig. 3.1 which shows the distribution of organisms isolated and indicates that Gram-positive (G+ve) and Gram-negative (G-ve) bacteria were present in similar ratios in the shallow water samples at 47 and 49%, respectively; and deep water samples at 44 and 52%, respectively.

However, the sediment samples showed a higher percentage of Gram-positive organisms in comparison to the Gram-negative bacteria at 65 and 30%, respectively. A total of 23% of these were filamentous and thought to be actinobacteria, which according to literature, are broadly distributed throughout the marine environment, particularly in sediment/soil with a high level of diversity. A relatively small percentage (4 – 5%) of fungi was present in all samples.

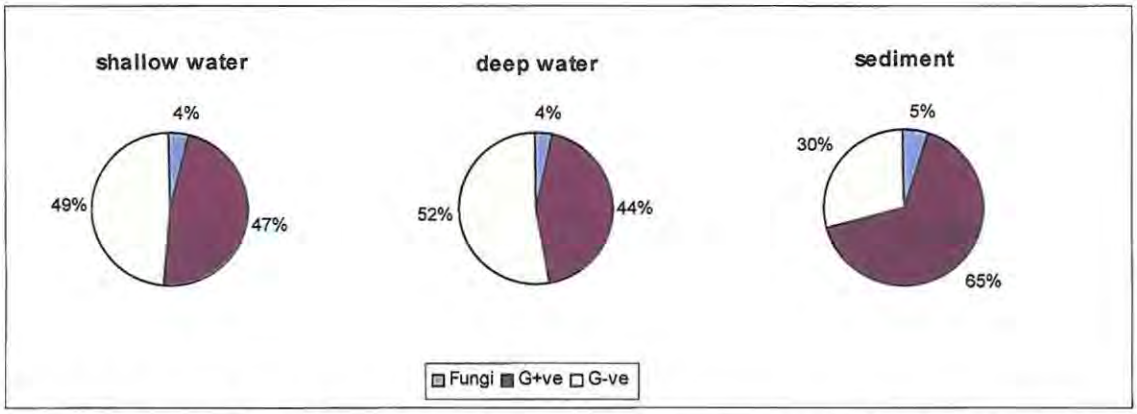


Fig. 3.1: Distribution of microorganisms isolated from seawater and sediment samples

3.3.3. Selection of the best methods for the isolation of marine microbes

Deep seawater samples yielded greater visual diversity and a higher total colony count, when processed using a quick defrosting processing technique at 40°C and cultured undiluted, on complex agar in replicates of eight (Table 3.2). Bowman and co-workers (1997) report that by defrosting the seawater samples quickly, hypotonic shock of halophilic organisms in the seawater samples may be avoided; results seem to indicate that this was indeed the case. In comparison, the thawing water during the slow defrost technique has lower salinity, since the salt remains in the ice fraction, thereby causing organisms to burst by osmosis. Additionally, the higher

replicate number increased the probability of isolating a greater number and diversity of organisms.

Similarly, the visual diversity of isolates from shallow seawater samples was greatest when using a quick defrosting processing technique at 40°C with replicates of eight. Conversely, these isolates were derived from diluted samples plated on minimal agar (Table 3.3). In comparison to the deep seawater samples, the shallow seawater samples yielded lower visual diversity for all combinations of processing conditions. This may be due to the higher microorganism abundance in shallow seawater samples as a result of higher concentrations of particulate organic matter (Long and Azam, 2001).

Lastly, the sediment samples yielded the lowest visual diversity of the three environments sampled. The highest isolate diversity was obtained from 10^{-3} diluted samples cultivated on minimal agar with a replicate number of eight, processed using a slow defrosting technique (Table 3.3). This may be due to the higher dilution (10^{-6}) being too dilute and diluting out the rare microorganisms. Furthermore, the minimal agar may also have selected more interesting slow growing organisms as a result of the limited nutrient concentration.

3.4. CONCLUSION

The best isolation methods for seawater and sediment samples differed. Greater visual diversity from deep and shallow seawater samples was obtained using a quick defrosting processing method, thus limiting hypotonic shock. Additionally, greater visual diversity was obtained from diluted shallow seawater samples and undiluted deep seawater samples. Possible explanations for these observations could be that the dominant microbes are diluted out from the shallow seawater, whereas the probability of attaining more viable organisms from undiluted deep seawater is increased. Furthermore, isolation on nutrient rich complex growth medium may have encouraged growth of isolates from deep seawater samples. Conversely, nutrient poor, minimal growth medium may have selected for more interesting and rare slower growing microorganisms from shallow seawater samples.

In contrast, the best isolation methods for sediment samples that showed increased diversity were obtained on minimal growth medium using a slow defrosting

processing method with a diluted sample (10^{-3}). A high replicate number also showed increased diversity from both seawater and sediment samples.

Cultivation independent procedures are also used to isolate novel marine microorganisms. Taxonomically similar isolates are assigned to groups using repetitive DNA fingerprinting (*rep*-PCR), a dereplication procedure useful for analyses of large numbers of strains, preventing excessive screening efforts. Further taxonomic studies on representative strains can then be carried out. Interestingly, extensive taxonomic phenotypic databases can assist in the formulation of selective media designed to isolate members of specific taxa (Pathom-aree *et al.*, 2006).

CHAPTER 4

Screening for biologically active metabolites and identification of selected isolates from Antarctic Ocean and Marion Island samples

4.1. INTRODUCTION

Many marine heterotrophic bacteria produce antibacterial secondary metabolites which may also be active against terrestrial pathogens (Nair and Simidu, 1987). Although a large number of biological assays are available, antibacterial screening of microbial secondary metabolites is an established method used to identify potential biologically active molecules. In order to prepare microbial fermentation extracts for bioactivity screening, growth conditions that support biosynthesis as well as appropriate extraction procedures of secondary metabolites produced, are necessary (Higgs *et al.*, 2001).

Several biological assay techniques have been developed to measure and monitor the effectiveness of bioactive compounds against microorganisms. These include well-diffusion, disk-diffusion and microtitre plate-based sensitivity tests. In antibiotic fermentations, the bioactivity of the broth is used as an indication of antibiotic production, where the size of the zone of inhibition against a test organism is qualitatively correlated to the amount of bioactive compound present in the fermentation broth (Casey *et al.*, 2004).

Historically, the identification of microorganisms from pure cultures, relied on phenotypic methods, however many bacterial and fungal isolates display unique or poorly distinctive phenotypic characteristics and thus cannot be identified by these conventional methods alone (Drancourt and Raoult, 2005; Petti *et al.*, 2007). Molecular tools such as PCR (Polymerase Chain Reaction) and the phylogenetic analysis of 16S rRNA gene (rDNA) sequences are thus becoming increasingly useful in characterising organisms that occur in microbial communities. The application of these molecular approaches to the investigation of microbial diversity in many different environments have identified new microorganisms that are abundant and/or physiologically significant (Lee *et al.*, 1999). Interestingly, culture dependent and

independent techniques have recovered actinomycetes from marine sediment samples at depths of 2000-6000 m (Pathom-aree *et al.*, 2006).

The aims of the research reported in this chapter were (i) to apply the same extraction methods optimised with marine bacterium, *Pseudoalteromonas* AP5 (Chapter 2), to ten marine microorganisms whereby intracellular and extracellular extracts were prepared using MeOH and EtOAc solvents. These organisms (isolates WL8, WL15, WL61, WL112, WL114, WL136, WL145, WL177, WL187, and WL195) were tested for antimicrobial activity against seven test organisms representing Gram-positive and -negative bacteria, yeast and fungi; and (ii) to identify those marine microorganisms that produced biologically active metabolites using 16S rRNA gene sequences.

4.2. MATERIALS AND METHODS

4.2.1. Selection of marine microorganisms for screening

Ten marine organisms from the 276 stored isolates obtained from the Antarctic Ocean and Marion Island were chosen based on interesting morphology, texture or colour.

4.2.2. Storage and maintenance of marine isolates and test organisms

Test organisms, Gram-positive bacteria (*Staphylococcus aureus* ATCC 6538P and *Bacillus subtilis*), Gram-negative bacteria (*Serratia marcescens* DSM 12481 and *Pseudomonas fluorescens*), yeast (*Schizosaccharomyces pombe* IFO 0347) and fungi (*Fusarium* sp. ABBAS and *Penicillium* sp.), were cultivated in 40 ml of broth (refer to Appendix B) in 100 ml Erlenmeyer flasks at 25°C with shaking at 200 rpm overnight. These test organisms were harvested by centrifugation at 4000 rpm for 10 min (Hermle Z300 K). All marine isolates and test organisms were stored in cryotubes in 50% glycerol at -80°C as well as maintained on agar (refer to Appendix B). Test organisms were used directly from cryotube stocks for well-diffusion bioassays. Marine isolates were revived as described in section 4.2.3.

4.2.3. Culture conditions

Marine isolates WL8, WL15, WL61, WL112, WL114, WL136, WL145, WL177, WL187 and WL195 were revived by inoculating 100 µl into 40 ml of its isolation broth, listed in Table 4.1 below, in 100 ml Erlenmeyer flasks. The cultures were

incubated at 10°C with shaking at 200 rpm until growth was observed. Revived isolates were sub-cultured and maintained on the respective agar at 10°C.

Table 4.1: Revival growth media for marine isolates

ISOLATE	MEDIA
WL8 and WL145	Emerson's broth
WL15, WL61, WL187 and WL195	Marine broth
WL112	M3 broth
WL136	SC broth
WL177	M1 broth
WL114	R2A + seawater broth

4.2.4. Preparation of extracellular extracts

Liquid broth cultures of each isolate were extracted with equal volumes (v/v) of MeOH or EtOAc solvent, vortexed for 10 s (Vortex mixer, VM-300) and centrifuged at 4000 rpm for 10 min to remove the biomass from MeOH extracts or to separate the EtOAc solvent layers.

4.2.5. Preparation of intracellular extracts

Cells of each isolate were harvested by centrifugation at 4000 rpm for 10 min. Harvested cells were extracted on ice using equal volumes (w/v) of MeOH or EtOAc and sonicated for 3 x 30 s bursts (Virsonic 60, Virtus), vortexed for 10 s, and centrifuged at 4000 rpm for 10 min to remove the biomass.

4.2.6. Bioassays and test organisms

Extracellular and intracellular MeOH and EtOAc extracts from each isolate were screened in duplicate for bioactivity against seven test organisms (described in section 4.2.2) using the simple and qualitative well-diffusion bioassay method (Appendix A). The bioassay plates were incubated at 25°C overnight. Zones of growth inhibition were measured from the edge of each well.

4.2.7. Identification of marine isolates

Identification of marine isolates by 16S ribosomal DNA sequencing (using ca 760 bp) was performed off site by a technician at Inqaba Biotechnical Industries (Pty) Ltd in Cape Town. Genomic DNA from biologically active isolates was extracted for

isolation using the ZR DNA II Kit and the ZR Fungal/Bacterial DNA kit. DNA was PCR amplified with Lucigen EconoTaq PLUS 2X Master Mix and Fermentas 2X Master Mix. This was followed by a purification step using the ZR Clean and Concentrator kit. The purified fragments underwent further PCR using two internal PCR primers. The forward primer used was PA, sometimes referred to as 27F and the reverse primer used was 1492R. These are "universal" bacterial PCR primers typically used for 16S rRNA sequence detection and analysis[II] (Larkin *et al.*, 2005).

The forward and reverse sequences were aligned using CLC Workbench 4 software and resulting consensus sequences compared to other nucleotide sequences using the National Centre for Biotechnological Information (NCBI) BLAST application (http://www.ncbi.nlm.nih.gov). Results obtained from the BLAST search were used for identification.

4.3. RESULTS AND DISCUSSION

4.3.1. Origin of ten marine isolates

The ten marine isolates selected for initial screening are described in Table 4.2 below.

[II] It was not possible to obtain any more details regarding the DNA sequencing procedure from Inqaba Biotec and thus considered as a preliminary identification.

Table 4.2: Marine origin, characterisation and processing of marine isolates

Isolate	Origin	Appearance	Isolation process	Image (Gram stain) 100X magnification
WL8	Deep water (3000 m) from Antarctica ST 8	White, irregular, undulate margin and crateriform, filamentous organism.	Quick defrost, undiluted and 8X replication on Emerson's agar.	
WL15	Deep water (3000 m) from Antarctica ST 8	Bright orange, rough texture, round, entire margin and umbonate, Gram-negative tiny bacilli.	Slow defrost, undiluted and 3X replication on Marine agar.	
WL61	Deep water (3000 m) from Antarctica ST 8	White, round, entire margin, flat, very hard on agar, filamentous organism.	Slow defrost, undiluted, and 3X replication on R2A agar.	
WL112	Dredge 12 sediment from the vicinity of Marion Island	White-yellow filamentous fungus, producing a red pigment on agar.	Slow defrost, 1000x diluted, and 8X replication on M3 agar.	
WL114	Dredge 12 sediment from the vicinity of Marion Island	Yellow, hard, round, entire margin, flat, small, filamentous organism.	Slow defrost, 1000x diluted, and 8X replication on 1/10 R2A + seawater agar.	
WL136	Dredge 12 sediment from the vicinity of Marion Island	Brown-red, hard, round with blue roots/hyphae, entire margin, raised, filamentous organism.	Slow defrost, 1000x diluted, and 3X replication on SC agar.	
WL145	Shallow water (200 m) from Antarctica ST 135 17	Yellow, round, entire margin, raised, bubbly appearance, Gram-negative bacilli.	Quick defrost, undiluted, and 8X replication on Emerson's agar.	
WL177	Deep water (1000 m) from Antarctica ST 125 12	Orange, tiny round, entire margin, flat, agar changes colour, Gram-negative small bacilli.	Quick defrost, undiluted, and 8X replication on M1 agar.	
WL187	Deep water (350 m) from Antarctica 126-01 17	Cream, round, undulate margin, hilly, Gram-negative diplococci/small bacilli.	Quick defrost, undiluted, and 8X replication on R2A agar.	
WL195	Shallow water (10m) from Antarctica ST 148 21	Yellow, round, entire margin, raised, producing red pigment on agar, Gram-negative small bacilli/diplococci.	Quick defrost, undiluted, and 8X replication on R2A agar.	

4.3.2. Screening for Bioactivity

A preliminary screen to assess the most promising isolates for further study was undertaken. In this work each microbe was cultured using the same medium with which it was isolated however, the growth rates and biomass obtained with these different media were variable.

Table 4.3 summarises the results obtained from the initial bioactivity screening against the seven test organisms. Numbers indicate the size of the zone of inhibition in mm and / indicates that no zone of inhibition was detected. Only isolates showing bioactivity are included as no bioactivity was detected for isolates WL112, WL145 and WL187 cultured in M3, Emerson's and Marine broth, respectively.

Isolates WL15 and WL195, cultured in Marine broth; isolate WL8 cultured using Emerson's broth, and isolate WL177 cultured in M1 broth showed limited bioactivity as small zones of inhibition against only one of the test strains were observed. Intracellular MeOH extracts from isolate WL15 were active against *S. aureus*, while intracellular EtOAc extracts from isolates WL195, WL8 and WL177 were active against *S. marcescens*, *P. fluorescens* and *Fusarium* sp., respectively.

Extracts from isolates WL61, WL114 and WL136 showed bioactivity against a number of test organisms. Specifically, the majority of extracts from isolate WL61 were active against three test organisms: *S. pombe*, *Fusarium* sp. and the *Penicillium* sp. Intra- and extracellular MeOH extracts produced larger zones of inhibition than the EtOAc extracts. This is possibly attributed to the fact that MeOH causes cells to lyse and release intracellular metabolites, consequently increasing the levels of extracellular metabolites. Intra- and extracellular extracts from isolate WL61 both show activity. Similarly, this may be due to the fact that whole broth cultures were extracted with MeOH or EtOAc solvents, extracting some intracellular compound(s) from the cells, thus increasing concentration levels of the extracellular active compound(s).

Table 4.3: Initial bioactivity screening

Isolate	Extracts	Test Organisms						
		S.marcescens	*S.aureus*	*B.subtilis*	*P.fluorescens*	*S.pombe*	*Fusarium* sp.	*Penicillium* sp.
WL15	MeOH intra	/	2 mm	/	/	/	/	/
	EtOAc intra	/	/	/	/	/	/	/
	MeOH extra	/	/	/	/	/	/	/
	EtOAc extra	/	/	/	/	/	/	/
WL195	MeOH intra	/	/	/	/	/	/	/
	EtOAc intra	4.5mm	/	/	/	/	/	/
	MeOH extra	/	/	/	/	/	/	/
	EtOAc extra	/	/	/	/	/	/	/
WL8	MeOH intra	/	/	/	/	/	/	/
	EtOAc intra	/	/	/	2 mm	/	/	/
	MeOH extra	/	/	/	/	/	/	/
	EtOAc extra	/	/	/	/	/	/	/
WL177	MeOH intra	/	/	/	/	/	/	/
	EtOAc intra	/	/	/	/	/	3.5 mm	/
	MeOH extra	/	/	/	/	/	/	/
	EtOAc extra	/	/	/	/	/	/	/
WL61	MeOH intra	/	/	/	/	10.5 mm	11.5 mm	6 mm
	EtOAc intra	/	/	/	/	5.5 mm	3.5 mm	2.5 mm
	MeOH extra	/	/	/	/	7.5 mm	7 mm	4.5 mm
	EtOAc extra	/	/	/	/	3 mm	1 mm	/
WL114	MeOH intra	/	/	1 mm	/	2 mm	3.5 mm	/
	EtOAc intra	/	/	/	/	/	/	/
	MeOH extra	/	1 mm	/	/	/	5 mm	/
	EtOAc extra	1 mm	/	/	/	/	/	/
WL136	MeOH intra	/	1.5 mm	1.5 mm	/	2 mm	/	/
	EtOAc intra	/	/	/	/	/	/	/
	MeOH extra	/	/	/	/	/	/	/
	EtOAc extra	1.5 mm	/	/	/	/	/	/

Extracts from isolate WL114 showed broad activity against five test organisms: *S. marcescens*, *S. aureus*, *B. subtilis*, *S. pombe* and *Fusarium* sp. While intracellular MeOH extracts showed more diverse activity against three test organisms, extracellular MeOH extracts were active against only two test organisms and extracellular EtOAc extracts active against only one test organism. Furthermore, intra- and extracellular MeOH extracts produced larger zones of inhibition than the extracellular EtOAc extracts. No activity was detected from the intracellular EtOAc extracts.

Similarly, intracellular MeOH and extracellular EtOAc extracts from isolate WL136 showed broad activity against four test organisms: *S. marcescens*, *S. aureus*, *B. subtilis* and *S. pombe*. Intracellular MeOH extracts showed more diverse activity against three test organisms in comparison to extracellular EtOAc extracts active against only one test organism. No activity was detected from intracellular EtOAc and extracellular MeOH extracts.

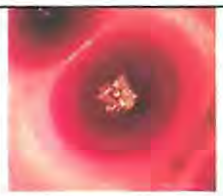
Isolates WL61, WL114, WL136 showed the highest and most diverse bioactivity, thus they were chosen for 16S rRNA sequencing and identification.

4.3.3. Identification of marine isolates WL61, WL114 and WL136

The 16S rRNA gene sequence of an organism indicates that it is either an unusual phenotype belonging to a known taxon or represents an unknown taxon. In addition to the fundamental role in the detection and initial identification of "as-yet-uncultured organisms", 16S rRNA gene sequences have successfully been used for the identification of slow-growing organisms and may also explain the inconsistency between phenotypic-based identification. Furthermore, sequencing of the 16S rRNA gene is useful for the identification of microorganisms that do not stain sufficiently or are poorly biochemically active (Drancourt and Raoult, 2005).

The highest percentage homologies of the three biologically active isolates, WL61, WL114 and WL136, were retrieved using the BLAST application (Table 4.4). The phylogenetic analysis of the 16S rRNA gene consensus sequences (Appendix C) showed that all three of the isolates belonged to the genus *Streptomyces* (order: Actinomycetales, class: Actinobacteria).

Table 4.4: Identification of marine isolates WL61, WL114 and WL136 using BLAST

Isolate		Identification	Query Coverage (%)	Maximum % Identification
WL61 (marine agar)		*Streptomyces* sp. A514Ydz-FQ	100	**100**
WL114 (marine agar)		*Streptomyces* sp. HBUM 74842	100	**96**
WL136 (SC agar)		*Streptomyces* sp. CNS-582_SD06	99	**99**

The 16S rRNA gene sequences identified by BLAST showed that isolate WL61 was phylogenetically close (100%) to *Streptomyces* sp. A514Ydz-FQ, isolated from the marine environment in China. Similarly, isolate WL114 was phylogenetically close (96%) to *Streptomyces* sp. HBUM 74842, isolated from soil in China, and isolate WL136 was phylogenetically close (99%) to *Streptomyces* sp. CNS-582_SD06, isolated from marine sediment off the coast of California, USA. This, together with the alignment results of the homologous regions for each isolate (Appendix C) and the phylogenetic trees, constructed using the neighbour-joining algorithm (Figs 4.1, 4.2 and 4.3), suggests that these isolates, particularly isolate WL114, may represent novel species.

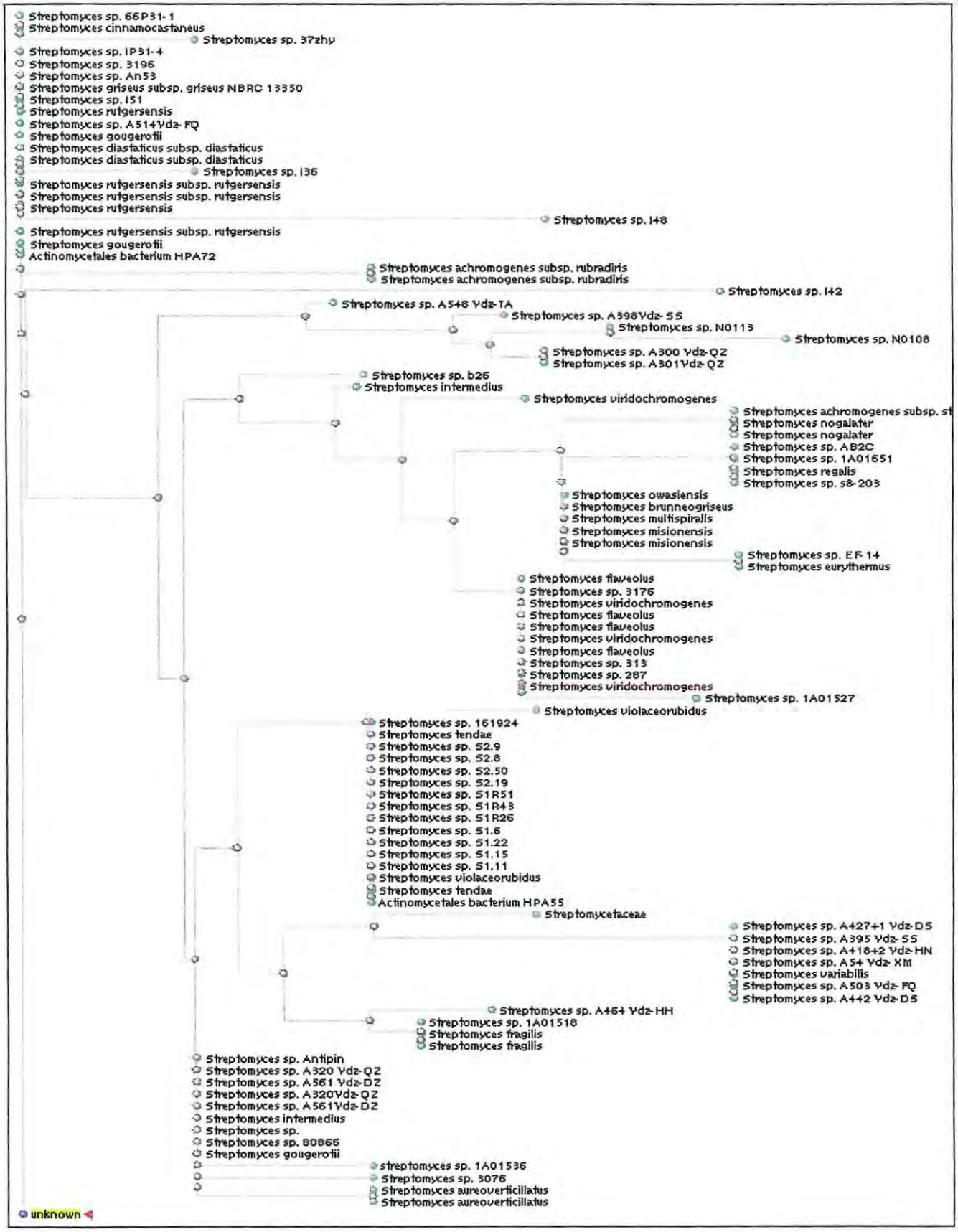


Fig. 4.1: Neighbour-joining phylogenetic tree for unknown isolate WL61

Fig. 4.2: Neighbour-joining phylogenetic tree for unknown isolate WL114

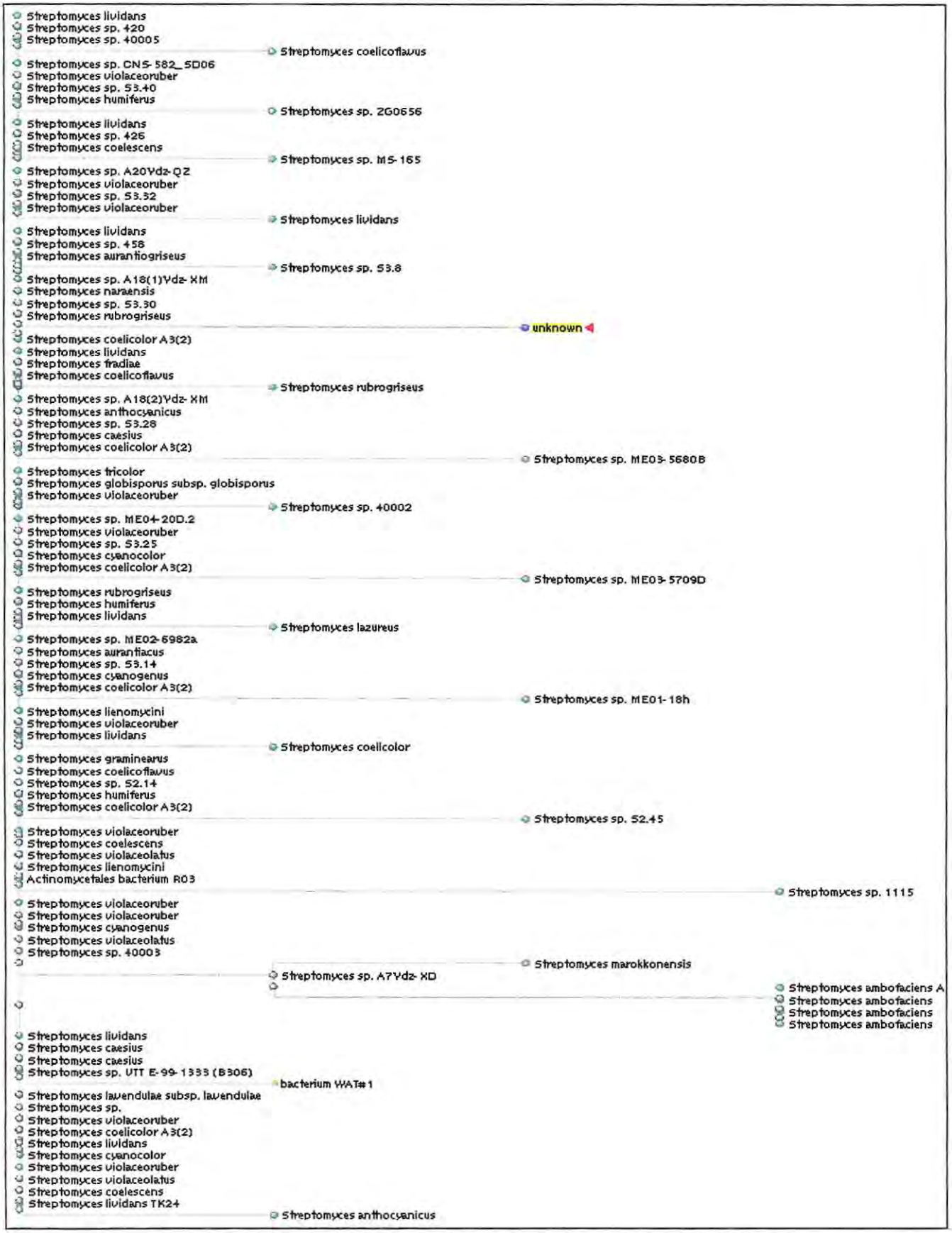


Fig. 4.3: Neighbour-joining phylogenetic tree for unknown isolate WL136

Phenotypic and genotypic characteristics of a newly described bacterial species should be compared to those of reference neighbouring species and genera to accurately identify phenotypic and genotypic characteristics that will distinguish the newly described bacterial species (Drancourt and Raoult; 2005). However, the 16S rRNA gene sequence may not be sufficient for the description of a new bacterial species. It has been suggested that two bacterial isolates would belong to different species if the similarity of their 16S rRNA gene sequences is less than 97%. Data suggests that an original 16S rRNA gene sequence exhibiting less than 97% similarity with its closest relative and containing less than 1% undetermined positions, is required to form the basis for the description of a new bacterial taxon (Drancourt and Raoult, 2005). Thus, a more conclusive description of the novelty of isolates WL61, WL114 and WL136 should be determined by sequencing longer fragments of the 16S rRNA genomic sequences using additional PCR primers.

4.4. CONCLUSION

Seven out of ten isolates examined in this preliminary screen showed antimicrobial activity. The low levels of activity detected in the intracellular extracts from isolates WL8, WL15, WL177 and WL195 may be improved by further optimising culture conditions and/or the extraction methods.

The active compound(s) produced by isolate WL61 had significant antifungal properties, whereas the active compound(s) produced by isolates WL114 and WL136 had antibacterial and antifungal activity. Additionally, MeOH extracts from isolates WL114 and WL136 were active against Gram-positive bacteria and fungi, whereas EtOAc extracts were active against Gram-negative bacteria, suggesting that these two isolates produced a polar and a non-polar active compound(s).

The differences in the range of antibacterial and antifungal properties from these three isolates against a selection of test organisms were noteworthy. Furthermore, the phylogenetic data indicated that they are *Streptomyces* spp., possibly novel strains, that are shown to be promising sources of bioactive compound(s) and were therefore selected for further study.

CHAPTER 5

The effect of culture media and temperature on microbial growth and production of bioactive metabolites

5.1. INTRODUCTION

Deep sea marine heterotrophic bacteria are efficiently adapted to survive their unique and extreme environment which is characterised by high pressure, low temperature, absence of light as well as variable salinity and oxygen concentration. Sodium is essential for growth of most marine bacteria, maintaining the osmotic environment to protect cellular integrity. Additionally, adaptation to oligotrophy is essential as their activity is largely limited by substrate availability. Furthermore, bacterial distribution is dependent on temperature, salinity and other physicochemical parameter changes. For example, warm and slightly acidic soils are likely to contain a greater diversity of actinomycetes than water, thus there have been many studies on the isolation of actinomycetes from marine sediments (Das *et al.*, 2006). Temperatures below 4°C and pressures greater than 100 x 10^5 Pa, characterising nearly 75% of the marine environment, have resulted in the evolution of barophilic and psychrophilic bacterial species (Delong and Yayanos, 1987). Psychrophiles occupy environments that are constantly cold (15°C and lower) and rapid warming to ambient temperatures can kill them. Psychrotolerant microorganisms are much more widely distributed than psychrophiles as their optimum growth temperature is between 20°C and 40°C. However, they are also able to grow at 0°C, although not very well, as visual growth on culture media is only observed after several weeks of incubation (Madigan *et al.*, 1997).

Marine microbiology is developing strongly in many countries with particular attention directed at bioactive compounds (Bull and Stach, 2007). In India, most research on actinobacteria has focussed on the isolation, identification and maintenance of these organisms in different culture media. However, more recent studies are also focussing on their antagonistic properties against different pathogens (Sivakumar *et al.*, 2007). Marine isolates have been shown to produce antimicrobial compounds active against a wide variety of organisms as they can inhibit the growth of closely

related and taxonomically different microorganisms. This may be attributed to one strain producing more than one antibacterial compound, depending on the growth phase and growth medium (Nair and Simidu, 1987). Many natural products can only be produced under specific environmental conditions consequently several different growth media must be assessed when developing culture conditions (Larsen *et al.*, 2005).

Since psychrotrophic microorganisms have the ability to tolerate higher temperatures, they are isolated from permanently cold habitats like the deep-sea as well as from seasonally cold environments (Hamamoto *et al.*, 1994). The metabolic processes of psychrophiles and psychrotrophs are sensitive to changes in environmental temperatures, thus characterisation of bioactive metabolites produced by psychrophilic microbes requires fermentation facilities with low temperature control. In the absence of such facilities, it is necessary to evaluate cultures at ambient incubation temperatures. The ability of these microbes to grow efficiently at ambient temperatures would thus indicate that they are psychrotolerant (Hamamoto *et al.*, 1994; Madigan *et al.*, 1997).

This aims of the research presented in this chapter were to (i) evaluate the production of antimicrobial metabolites by isolates WL61, WL114 and WL136 using four different growth media only to complete a realistic number of experiments, and (ii) to evaluate the variation in growth of isolates WL61, WL114 and WL136 and secondary metabolite production of isolate WL61 with temperature. The specific objectives were thus:

- To cultivate the three marine isolates using two complex and two minimal media.
- To assess the effect on the growth rate of isolates WL61, WL114 and WL136 on agar plates incubated at 10 and 25°C.
- To monitor the production of bioactive metabolites from isolate WL61 incubated at 10 and 25°C using the well-diffusion bioassay method.

5.2. MATERIALS AND METHODS

5.2.1. Culture conditions

5.2.1.1. Effect of culture medium on growth and metabolite production of isolates WL61, WL114 and WL136

Isolates WL61, WL114 and WL136 were stored in 50% glycerol at -80°C and maintained on Marine agar, 1/10 R2A+seawater agar and SC agar, respectively. Aliquots of 200 µl of each isolate were inoculated into 40 ml of growth media, listed in Table 5.1, in 100 ml Erlenmeyer flasks. Cultures were incubated at 10°C with shaking at 200 rpm until growth was observed - varied from seven to 21 days. Test organisms were cultured as described in section 4.2.2.

Table 5.1: Growth media selected for production of metabolites

ISOLATE	COMPLEX MEDIA	MINIMAL MEDIA
WL61	Marine broth Emerson's broth	M2 broth M3 broth
WL114	1/10 R2A+seawater broth Emerson's broth	M2 broth M3 broth
WL136	Marine broth SC broth	M2 broth M3 broth

5.2.1.2. Effect of temperature on growth of isolates WL61, WL114 and WL136

Aliquots of 100 µl of glycerol stocks of isolates WL61, WL114 and WL136 were spread plated onto Marine, 1/10 R2A+seawater and 1/10 Marine, and SC agar, respectively and incubated at 10 and 25°C to simply and sufficiently indicate the differences in growth.

5.2.1.3. Effect of temperature on growth and bioactive metabolite production by isolate WL61

Due to time constraints, this study was carried out with isolate WL61 only. Pre-inoculum cultures of isolate WL61 were prepared by inoculating 400 µl of glycerol stock solution into 40 ml of Marine broth in 100 ml Erlenmeyer flasks. Cultures were incubated at 10 and 25°C with shaking at 200 rpm for four days. Aliquots of 200 µl of the 25°C pre-inoculum cultures and 400 µl of the 10°C pre-inoculum cultures were aseptically transferred into 40 ml of Marine broth in 100 ml Erlenmeyer flasks.

Cultures were incubated at 10 and 25°C with shaking at 200 rpm for 21 days. Test organisms were cultured and maintained as described in section 4.2.2.

5.2.2. Extraction processes and screening for bioactive compounds

5.2.2.1. Extractions after cultivation in different growth media

Extra- and intracellular extracts were prepared using EtOAc and MeOH solvents (described in sections 4.2.4 and 4.2.5, respectively) for each of the isolates in the different growth media. The qualitative well-diffusion bioassay was a simple and rapid method used to indicate the presence of bioactive compound(s) against four bacterial and three fungal test strains (described in section 4.2.6).

5.2.2.2. Extractions after cultivation at different temperatures

The flask cultures at both temperatures were extracted (in triplicate) every third day from the start of visual growth continuing for 21 days. Extra- and intracellular extracts were prepared using MeOH and EtOAc solvents as described in sections 4.2.4 and 4.2.5, respectively. The extracts were screened in duplicate using well-diffusion bioassays against seven test strains, as described in section 4.2.6, thereby producing six results/zones of inhibition for each sample at a particular time. No attempt was made to spend detailed time on growth curves. The controls used for the experiment involved adding the MeOH and EtOAc solvent to wells in plates seeded with the test organisms.

5.3. RESULTS AND DISCUSSION

5.3.1. Effect of culture medium on growth and metabolite production of isolates WL61, WL114 and WL136

The antimicrobial activity of isolates WL61, WL114 and WL136 under different fermentation conditions is highlighted in Table 5.2 below.

Table 5.2: Bioactivity after cultivation in different media

a) Isolate WL61

Growth media	Extract Type	*Penicillim* sp.	*Fusarium* sp.	*S. pombe*	*B. subtilis*	*P. fluorescens*
Marine broth	MeOH Intra	6.0 mm ± 0.7	5.7 mm ± 1.0	7.3 mm ± 2.0	No zones	No zones
	MeOH Extra	2.5 mm ± 0.0	1.5 mm ± 0.0	No zones	No zones	No zones
Emerson's broth	MeOH Intra	4.3 mm ± 0.5	2.7 mm ± 0.5	6.3 mm ± 0.2	1.5 mm ± 0.0	No zones
	EtOAc Intra	No zones	No zones	No zones	No zones	1.0 mm ± 0.0
	EtOAc Extra	No zones	No zones	No zones	No zones	2.0 mm ± 0.0
M3 broth	MeOH Intra	4.2 mm ± 1.7	6.5 mm ± 2.5	7.5 mm ± 0.5	No zones	No zones
	MeOH Extra	1.3 mm ± 0.5	No zones	3.5 mm ± 0.0	No zones	No zones
M2 broth	EtOAc Intra	No zones	No zones	No zones	No zones	2.0 mm ± 0.0
	EtOAc Extra	No zones	No zones	No zones	No zones	3.5 mm ± 0.5

b) Isolate WL114

Growth media	Extract Type	*S. pombe*	*B. subtilis*	*Fusarium* sp.	*S. aureus*	*P. fluorescens*
Emerson's broth	MeOH Intra	6.0 mm ± 0.0	2.5 mm ± 0.0	No zones	No zones	No zones
	MeOH Extra	7.5 mm ± 0.0	3.5 mm ± 0.0	No zones	No zones	No zones
	EtOAc Intra	5.5 mm ± 0.0	2.0 mm ± 0.0	No zones	No zones	No zones
	EtOAc Extra	7.0 mm ± 0.0	1.7 mm ± 0.3	No zones	No zones	No zones
1/10 R2A+ seawater broth	MeOH Intra	No zones	No zones	4.0 mm ± 0.7	3.0 mm ± 0.0	No zones
	MeOH Extra	No zones	No zones	2.8 mm ± 1.0	1.5 mm ± 0.0	No zones
M2 broth	MeOH Intra	No zones	No zones	3.0 mm ± 0.5	1.8 mm ± 0.6	No zones

c) Isolate WL136

Growth media	Extract Type	*Fusarium* sp.	*B. subtilis*	*S. aureus*	*P. fluorescens*
Marine broth	MeOH Intra	1.5 mm ± 0.0	No zones	No zones	No zones
	EtOAc Intra	No zones	No zones	No zones	1.5 mm ± 0.0
	EtOAc Extra	2.0 mm ± 0.0	No zones	No zones	1.8 mm ± 0.0
SC broth	MeOH Intra	No zones	1.8 mm ± 0.6	2.3 mm ± 0.5	No zones
	MeOH Extra	No zones	1.0 mm ± 0.0	1.0 mm ± 0.0	No zones
	EtOAc Intra	1.5 mm ± 0.0	2.5 mm ± 1.3	No zones	2.3 mm ± 0.6
	EtOAc Extra	1.0 mm ± 0.0	2.0 mm ± 0.0	No zones	2.5 mm ± 0.0
M2 broth	MeOH Intra	No zones	1.5 mm ± 0.0	No zones	No zones
	MeOH Extra	No zones	1.5 mm ± 0.7	No zones	No zones

It is interesting to note that isolate WL61 grew and produced antimicrobial compounds in both complex (particularly Marine broth) and minimal (particularly M3 broth) media. Furthermore, extracts produced by isolate WL61 were more active against the fungi, *Penicillium* sp., *Fusarium* sp., and *S. pombe* than the bacteria, *B. subtilis* and *P. fluorescens*. These antimicrobial compounds were detected primarily in the intracellular MeOH extracts as extracellular MeOH extracts only showed limited activity. No zones of inhibition were detected from the EtOAc extracts. This was consistent with previous observations (Chapter 4), however, activity against *P. fluorescens* was not observed in previous results (Chapter 4) indicating that different nutrient sources cause organisms to produce different and/or an increased number of secondary metabolites.

Similarly, isolate WL114 grew and produced antimicrobial compounds in both complex (particularly Emerson's broth) media, but only one of the minimal (M2 broth) media. Isolate WL114 cultivated in Emerson's broth produced extracts that were more active against both fungi and bacteria, *S. pombe* and *B. subtilis*, respectively when compared to those cultivated in 1/10 R2A+seawater and M2 broth which showed lower activity against *Fusarium* sp., (consistent with previous results - Chapter 4), as well as against *S. aureus*. These antimicrobial compounds were detected primarily in the intra- and extracellular MeOH extracts as EtOAc extracts showed lower activity.

Isolate WL136 grew and produced zones of inhibition in both complex (particularly SC broth) media, but only one of the minimal (M2 broth) media. These results differ from previous results (Chapter 4), most likely as a result of differences in growth phase - cultures in this experiment were allowed a longer growth period, possibly causing isolate WL136 to become slightly nutrient limited and therefore stressed. Extracts from isolate WL136 cultivated in Marine broth were active against fungi and bacteria, *Fusarium* sp. and *P. fluorescens*, respectively, while those from cultivation in SC broth, were active against *B. subtilis* and *S. aureus*, consistent with previous results (Chapter 4), as well as against *Fusarium* sp. and *P. fluorescens*. Those cultivated in M2 broth only showed activity against *B. subtilis*. These antimicrobial compounds were detected primarily in the intra- and extracellular EtOAc extracts as MeOH extracts showed lower activity.

5.3.2. Effect of temperature on growth of isolates WL61, WL114 and WL136

Growth of isolates WL61, WL114 and WL136 after one week of incubation at both 10 and 25°C is illustrated in Fig. 5.1.

(a) Isolate WL61 on Marine agar at 10 and 25°C

(b) Isolate WL114 **(i)** on 1/10 Marine agar at 10 and 25°C and **(ii)** on 1/10 R2A +seawater agar at 25°C

(c) Isolate WL136 on SC agar at 10 and 25°C

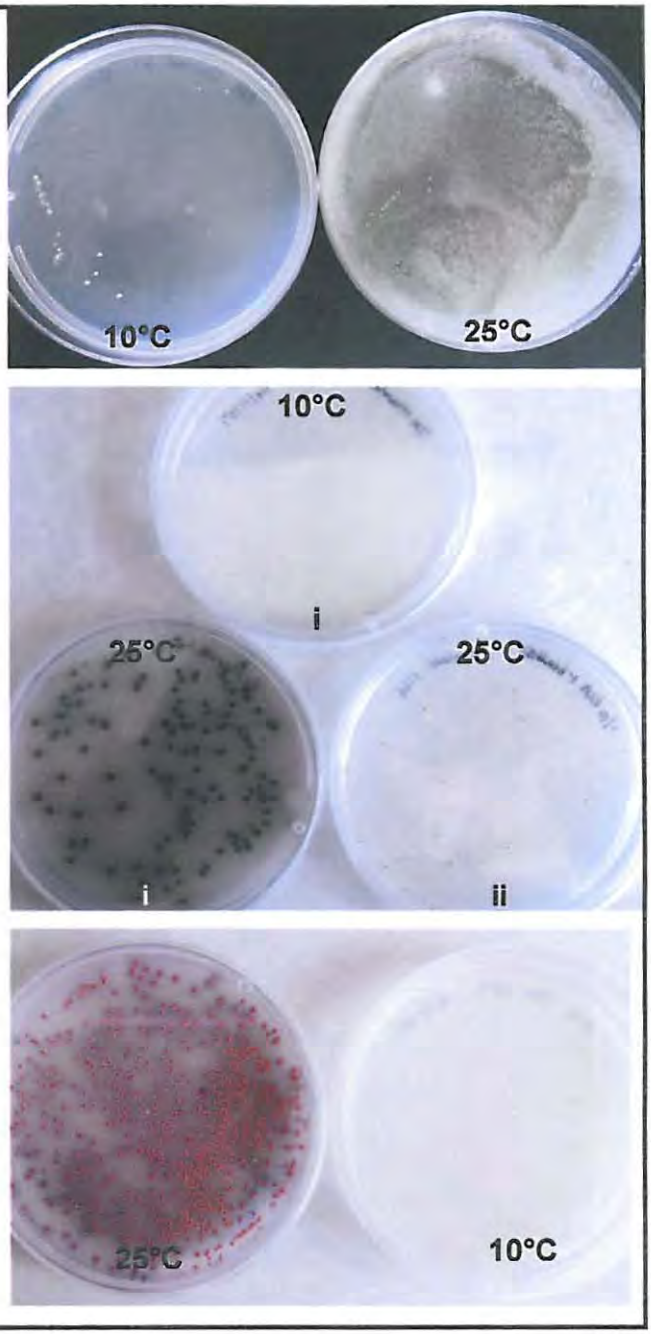


Fig. 5.1: Growth of the three marine isolates obtained after one week of incubation at each temperature

Growth of isolate WL61 sporulating on Marine agar was observed after one week of incubation at 25°C, while only limited growth was detected after incubation at 10°C for the same time period. No growth of isolate WL114 on 1/10 Marine agar was detected after incubation at 10°C, whereas good growth was observed on both 1/10 Marine and 1/10 R2A+seawater agar after incubation at 25°C. Similarly, no growth of isolate WL136 on SC agar was detected after incubation at 10°C, while a lawn of growth was observed after incubation at 25°C.

Isolate WL61 was isolated from a depth of 3000 m from the Antarctic Ocean, however it is highly possible that it may have originated from the terrestrial environment, since shallow water or water close to a land mass is likely to contain microorganisms derived from terrestrial run-off (Bull and Stach; 2007). Isolates WL114 and WL136 were isolated from sediment samples from Marion Island, where the constantly cool sub-Antarctic oceanic climate, high cloud cover and very high rainfall cause soils to experience low temperatures and extreme moisture conditions, limiting microbial activity. However, in a previous study, it was found that soil respiration rates in the cool, isothermal top soil layer did not differ with temperatures between 5°C and 20°C. This implies that the island's soil microbial populations are as sensitive to high temperatures as they are to the low temperatures to which they are normally exposed. This indicates a mixed community of soil microorganisms that have varying sensitivity to temperature (Smith, 2003). Isolates WL61, WL114 and WL136 were thus, not considered to be psychrophiles, but rather psychrotolerant microorganisms as all three isolates showed considerably greater growth at 25°C.

5.3.3. Bioactive metabolite production by isolate WL61 at 10 and 25°C

Activity from isolate WL61 incubated at 10 and 25°C is illustrated in Figs. 5.2 to 5.5 below. Only the test organisms sensitive to antimicrobial compound(s) were included. No zones of inhibition were detected from the solvent controls in all experiments.

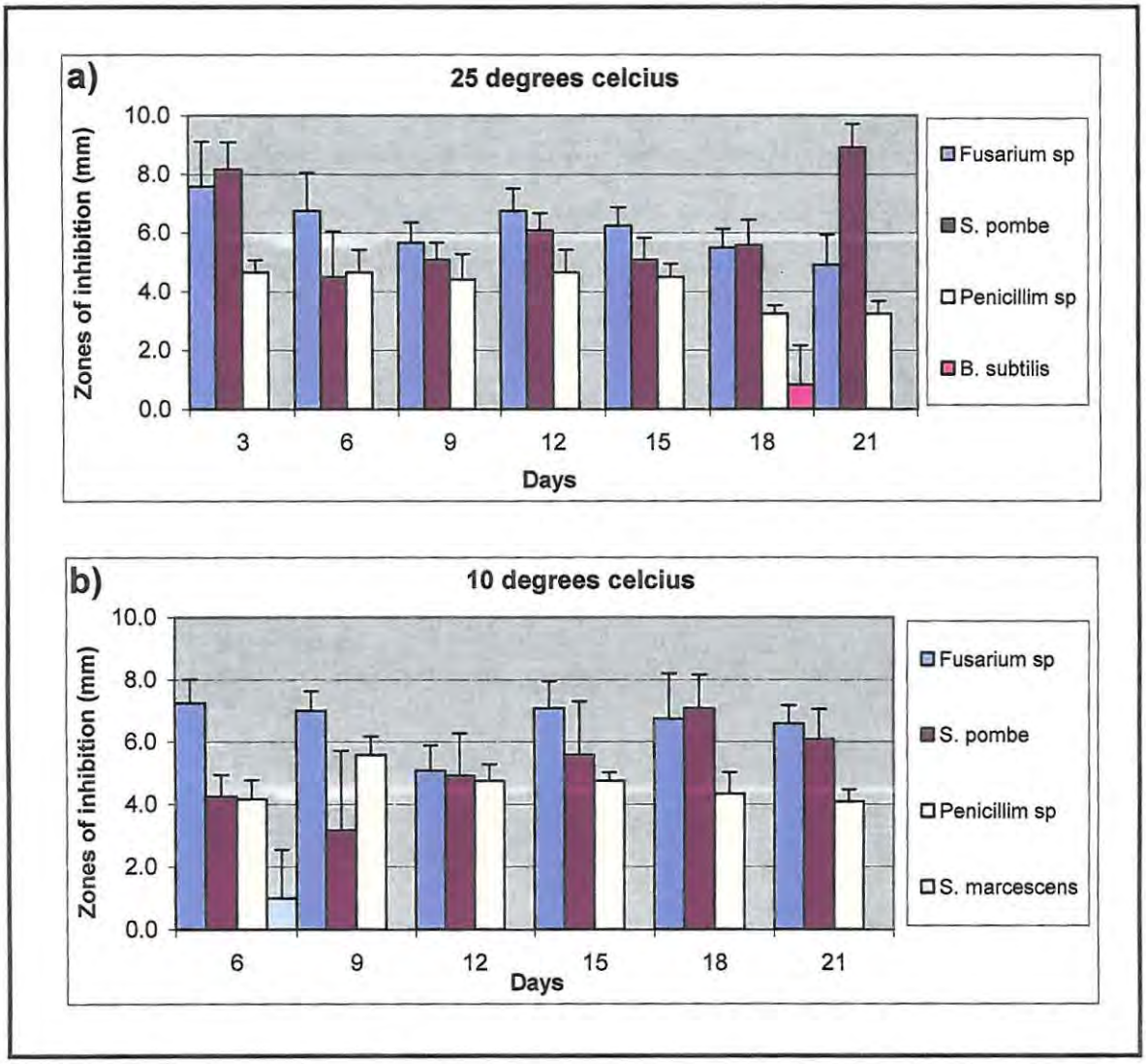


Fig. 5.2: Time-course study of bioactivity from intracellular MeOH extracts from isolate WL61 cultivated at different temperatures

Intracellular MeOH extracts showed the production of active compound(s) that were largely antifungal, consistent with previous results in Chapter 4, where *Fusarium* sp. and *S. pombe* were the most sensitive strains and the *Penicillium* sp. the least sensitive. Active compound(s) were detected from the intracellular MeOH extracts at both temperatures however, on average higher activities were detected in the cultures incubated at 25°C. Furthermore, extracts from the 10°C cultures showed slight activity on day 6 against *S. marcescens*, and extracts from the 25°C cultures showed slight activity on day 18 against *B. subtilis*.

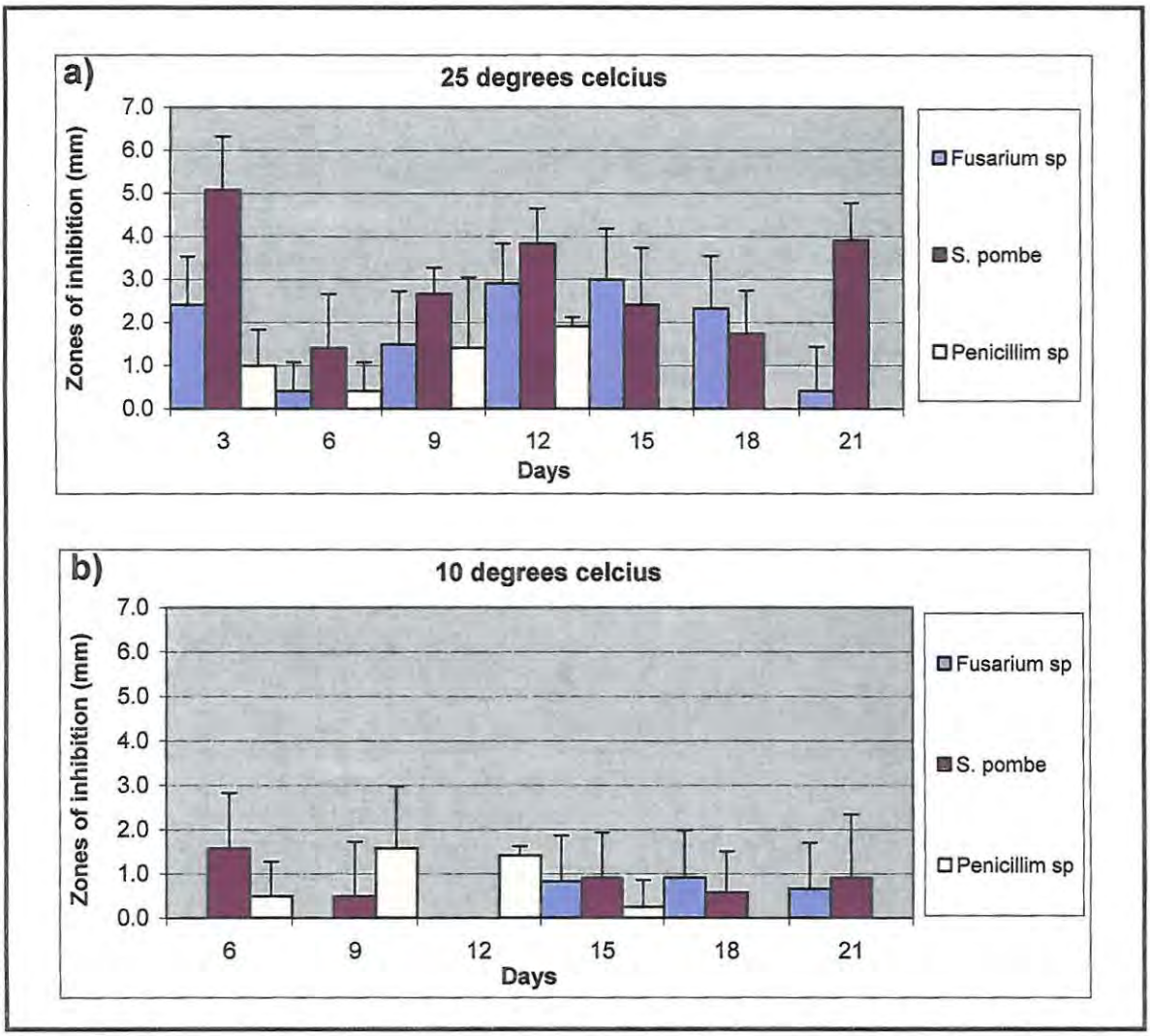


Fig. 5.3: Time-course study of bioactivity from extracellular MeOH extracts from isolate WL61 cultivated at different temperatures

Extracellular MeOH extracts also produced antifungal compound(s) against *Fusarium* sp., *S. pombe* and the *Penicillium* sp. at both incubation temperatures whereby *S. pombe* was the most sensitive strain and *Fusarium* sp. and *Penicillium* sp. were the least sensitive, consistent with results obtained in Chapter 4. However, the sizes of the zones of inhibition were much smaller and more variable when compared to zone sizes produced from intracellular MeOH extracts. Similarly, on average, higher activities were detected in the cultures incubated at 25°C.

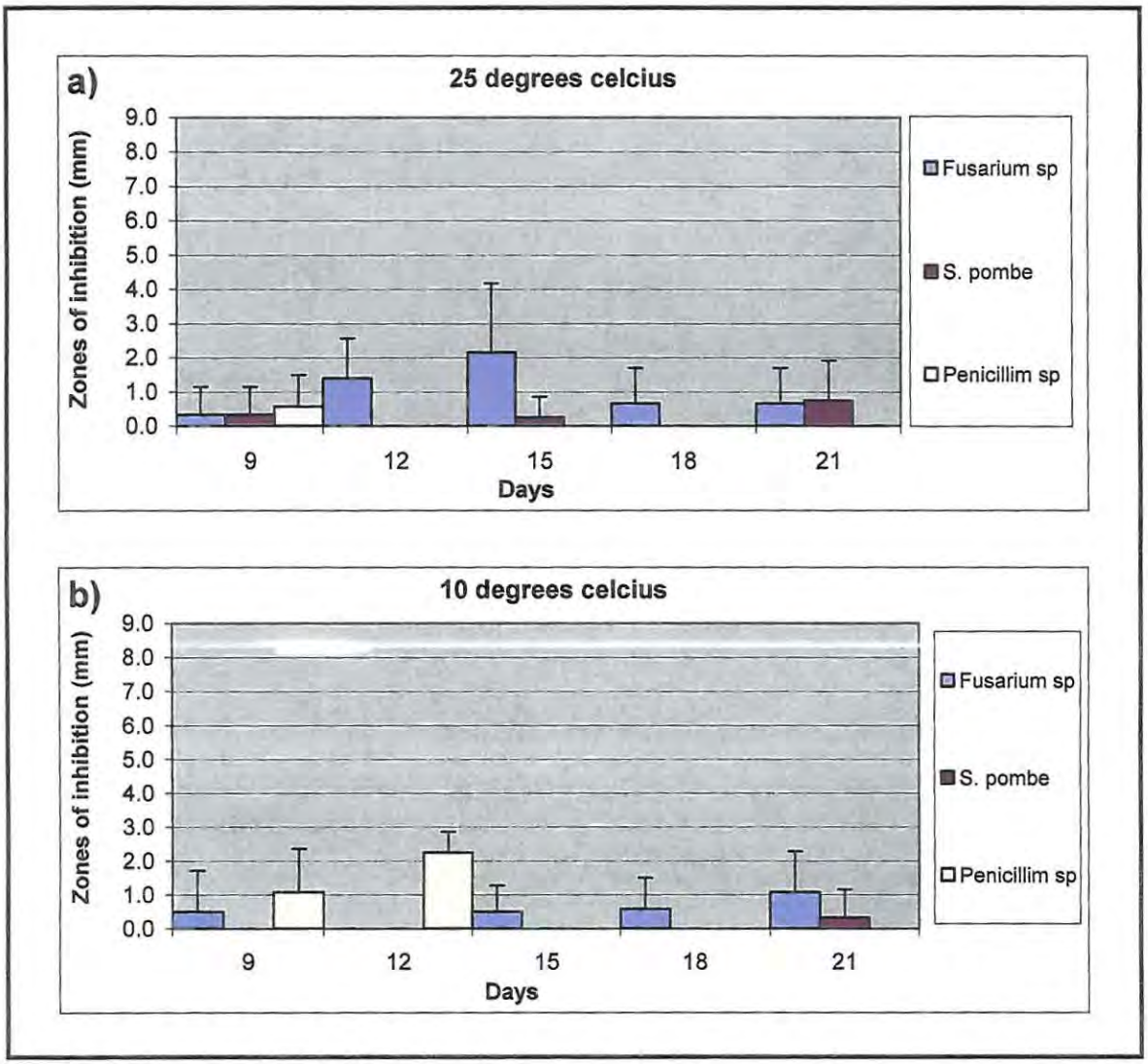


Fig. 5.4: Time-course study of bioactivity from intracellular EtOAc extracts from isolate WL61 cultivated at different temperatures

Similarly, antifungal compound(s) against *Fusarium* sp., *S. pombe* and *Penicillium* sp. were detected at both temperatures from intracellular EtOAc extracts, whereby the *Penicillium* sp. and *Fusarium* sp. were the most sensitive strains to extracts from the 10°C cultures and *Fusarium* sp. the most sensitive to extracts from the 25°C cultures. On average, higher activities were detected in cultures incubated at 25°C. However, the largest zone of inhibition was observed against *Penicillium* sp. in extracts from the 10°C cultures. The sizes, variability and frequency of the zones from these extracts were much smaller and fewer compared to those produced from intra- and extracellular MeOH extracts.

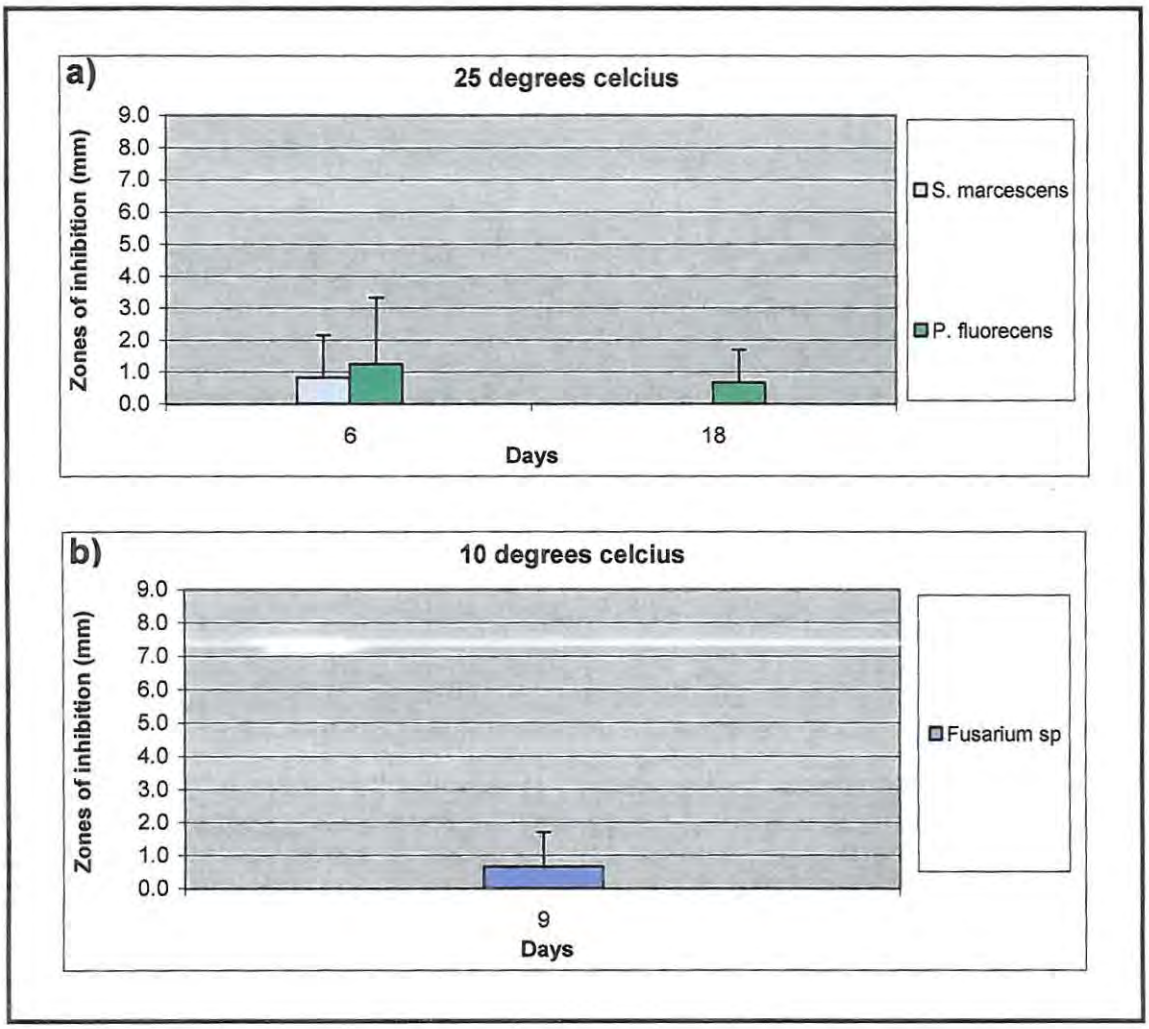


Fig. 5.5: Time course study of bioactivity from extracellular EtOAc extracts from isolate WL61 cultivated at different temperatures

Extracellular EtOAc extracts also produced antimicrobial compound(s) at both temperatures, however extracts from the 25°C cultures showed low activity detected against *S. marcescens* and *P. fluorescens*, with *P. fluorescens* being more sensitive than *S. marcescens*, whereas only the extracts from 10°C cultures were slightly active against *Fusarium* sp. *Fusarium* sp. was the only test organism sensitive to extracellular EtOAc extracts from the 10°C cultures. On average, higher activities were detected in the cultures incubated at 25°C.

5.4. CONCLUSION

In Marine broth, isolate WL61 extracts showed antifungal activity. Conversely, M2 broth extracts showed only antibacterial activity, while extracts from Emerson's broth showed both antifungal and antibacterial activity. The highest activity was detected from extracts produced by isolate WL61 cultivated in Marine broth. These results highlight the benefits of screening for bioactive metabolites using different nutrient media.

Antifungal and antibacterial activity was observed from extracts produced by isolate WL114 cultured in all three media however, extracts from isolate WL114 cultured in 1/10 R2A+seawater broth showed activity against a broader range of test organisms. This observation, once again, highlights the advantages of using both complex and simple growth media in drug discovery.

In Marine and SC broth, isolate WL136 extracts showed antifungal and antibacterial activity, whereas M2 broth extracts only showed antibacterial activity. There was no considerable difference in zone size between extracts from the different growth media used, however a broader range of activity was obtained with extracts from isolate WL136 cultivated in SC broth.

The differences in the production of bioactive compounds by the three isolates in the various media and the differences in the range of antibacterial and antifungal activity were considerable. These results demonstrate the importance of screening microorganisms for the production of bioactive compounds under a range of nutritional conditions. Furthermore, the broad spectrum of activity profiles against the test bacteria and fungi also suggest that the organisms produce more than one bioactive compound. It is proposed that at least two distinct antimicrobial compounds are produced by isolates WL61 and WL114, and three from isolate WL136.

In addition, isolates WL61, WL114 and WL136 were shown to be psychrotolerant microorganisms as growth occurred quickly at 25°C. Growth of these isolates may have been observed with a longer incubation period at 10°C, possibly between four and six weeks. Furthermore, Isolate WL61 grew and produced primarily intracellular polar (MeOH) biologically active compound(s) at both 10 and 25°C. However, it grew quicker and better at 25°C and maintained the production of antimicrobial

compound(s) consistently throughout the 21 day growth period, demonstrating its suitability for evaluation at ambient temperature in future experiments. Due to slower growth observed at 10°C, activities appeared to lag behind by approximately 3 days as zones of inhibition were only detected from day 6 for the 10°C cultures and from day 3 for the 25°C cultures. Although there was activity from extracellular extracts, this was minimal compared to that from the intracellular extracts, possibly due to the concentration of active metabolites in each of the solvent extracts. Intracellular metabolites are likely to be more concentrated in solvent extracts as metabolites are contained inside the cells, whereas extracellular metabolites are excreted into and further diluted by the growth medium, consequently lowering the metabolite concentration in solvent extracts. Additionally, due to flask culture variability, the growth/biomass between the triplicate flasks was different, thus the standard deviation between the six samples varied between the different extracts. This variability together with the fact that these isolates grow and produce biologically active compounds at 25°C, indicates that improved cultivation methods, such as bioreactor technology can be utilised for the production of marine bioactive compounds at ambient temperatures.

CHAPTER 6

Production of bioactive metabolites using the Quorus single fibre bioreactor (SFR)

6.1. INTRODUCTION

The single fibre membrane bioreactor (SFR) has been used as a tool to study biofilm growth and differentiation (Fraser, 2005) and to develop and optimise methods for the production of secondary metabolites (Leukes *et al.*, 1999; Garcin, 2002; Sheldon and Small, 2005; Ntwampe *et al.*, 2007). In its standard configuration, a single membrane is enclosed within a glass manifold consisting of inlet and outlet ports, illustrated in Fig. 6.1 below. Nutrients are supplied through the membrane lumen and humidified air is passed across the outer membrane surface. The permeable capillary membrane is used as a support for the biofilm growing at the air interface.

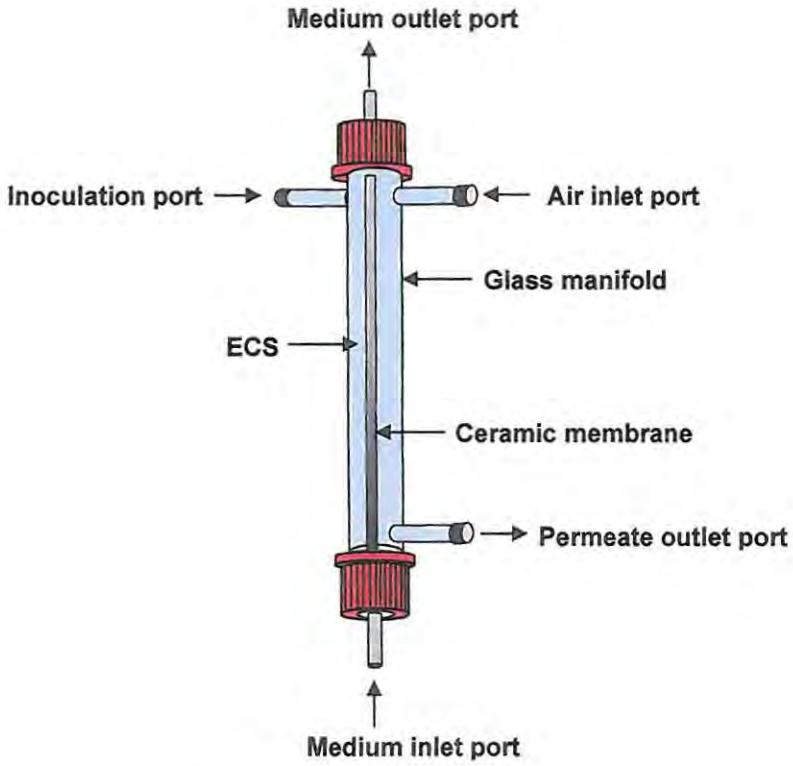


Fig. 6.1: The standard configuration of a single SFR

The humidified air allows good oxygen mass transfer into the wet biofilm, while nutrients, supplied to the biofilm from the lumen of the membrane, are metabolised by cells when flowing through the biofilm. This permits a constant oxygen and/or nutrient supply which facilitates steady-state, high cell density biofilm growth and differentiation in a continuous system. Opposing nutrient and oxygen gradients are established across the biofilm, resulting in the radial differentiation of growth and metabolism across the biofilm (Fraser, 2006 and 2007).

During operation, the growth medium is supplied at a rate which is high enough to sustain primary growth of microorganisms without dislodging the cells, but sufficiently low for a nutrient gradient to be established across the biofilm and to induce secondary metabolite production in response to nutrient limitation. While nutrients are readily available to the established biomass closest to the membrane (the nutrient rich zone), the biomass furthest away from the membrane surface (the nutrient poor zone) becomes nutrient starved and stressed, a requirement for secondary metabolite production (Fig. 6.2) (Ntwampe *et al.*, 2007). Morphological differentiation occurs radially due to the oxygen and/or nutrient concentration gradients and is associated with the production of biologically active secondary metabolites (Fraser, 2006 and 2007).

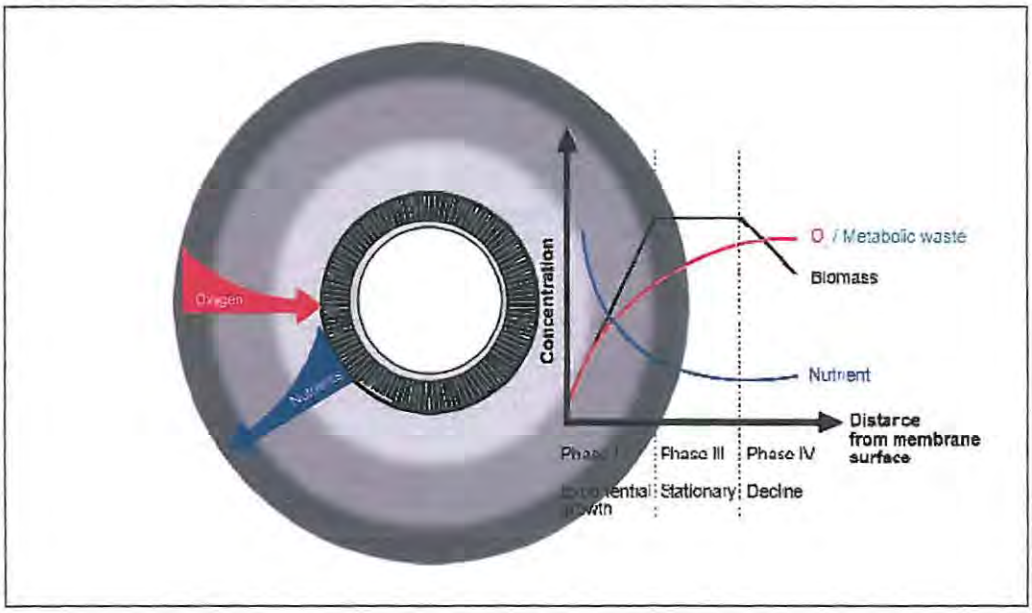


Fig. 6.2: Schematic representation of a ceramic membrane with nutrient and oxygen gradients (Fraser, 2006 and 2007)

Various support matrices (membrane types) have been used for biofilm growth, including polyurethane, silicone, polysulphone (PSu) and ceramic membranes (Sheldon and Small, 2005; Ntwampe and Sheldon, 2006). Membrane morphology is very significant for membrane bioreactor design and is the key for a successful process. The membrane should be developed to provide maximum surface area and a model environment for biofilm development. In 2005, Sheldon and Small tested different membranes including, an internally skinned capillary PSu membrane, two tubular aluminium oxide ceramic membranes (with pore sizes of 0.2 and 3 µm each) and one capillary titanium oxide ceramic membrane (with a pore size of 3 µm), for their suitability for fungal immobilisation, biofilm development and extracellular enzyme production by *Phanerochaete chrysosporium* (Sheldon and Small, 2005). These authors observed that the highest attachment and immobilisation of spores was obtained with capillary membranes, while the thickest biofilm growth was established on the 3 µm ceramic capillary membrane. A thicker biofilm enabled increased microbial stress due to enhanced nutrient gradients established across the biofilm (Sheldon and Small, 2005). Continuous peroxidase production was achieved with both the PSu and 3 µm ceramic capillary membrane reactors, however the PSu membrane lacked mechanical strength and sagged when operated in a horizontal position and could not be chemically or steam sterilised for re-use. The 3 µm ceramic capillary membrane was more rigid and had the advantage of chemical, thermal and mechanical stability (Sheldon and Small, 2005). Ntwampe *et al.*, (2007) also identified ceramic membranes as the most suitable for industrial production of secondary metabolites by *P. chrysosporium.*

Bioreactor orientation also plays an important role in biofilm growth. It was shown that *P. chrysosporium* biofilms cultured with the membrane in a horizontal orientation had uneven growth (Garcin, 2002; Fraser, 2005; Ntwampe *et al.*, 2007). This was attributed to droplet formation and biofilm detachment caused by gravitational forces, resulting in an uneven biofilm and mycelial growth in the recovered permeate that collects along the reactor length. Conversely, vertically orientated reactors showed more consistent biofilm development and thickness along the length of the membrane and could be operated for longer periods (Ntwampe *et al.*, 2007). In vertical orientation, the permeate does not collect as droplets, but rather flows down the length of the membrane uniformly making efficient use of essential nutrients

(Ntwampe *et al.*, 2007). Consequently, slight tapering of biofilm growth may be observed depending on the flow rate (Fraser, 2005).

Further improvements to bioreactor operation include the use of air pressure instead of mechanical pumps. Previously, accurated tubing and peristaltic pumps were used to deliver growth medium to SFRs. The walls of the tubing were prone to collapsing or tearing, limiting nutrient delivery or causing contamination of the SFRs, respectively. Pneumatically driven fluid delivery resolved problems experienced with changes in nutrient delivery over time as a result of the wear and tear of this tubing by peristaltic action (Garcin, 2002).

This part of the thesis aims to describe the use of an improved SFR Quorus optimisation rig, developed by Synexa Life Sciences (Pty) Ltd (Edwards *et al.*, 2007), to examine the growth and production of bioactive metabolites by marine streptomycetes, isolates WL61, WL114 and WL136. The objectives were thus:

- To select the most suitable growth medium for biofilm growth and scale-up of metabolite production for isolates WL61, WL114 and WL136.
- To determine the levels of bioactivity in different bioactive extracts in a time-course study for each isolate.

6.2. MATERIALS AND METHODS

6.2.1. Pre-inoculum culture conditions

Glycerol stocks of isolates WL61, WL114 and WL136 were inoculated in 200 µl aliquots into 20 ml of complex or minimal media in 100 ml Erlenmeyer flasks. Pre-inoculum flask cultures were cultivated in the same media that was used for growth on SFRs. Nutrient media, listed in Table 6.1 below, were chosen based on growth media used previously in shake flasks (Chapter 5) and the fact that particulate media are not suited to bioreactor operation as the lumen of the membrane becomes blocked, preventing flow of nutrients. Thus, M3 broth did not contain chitin. Flasks were incubated at 26°C with shaking at 220 rpm for seven days.

Table 6.1: Nutrient media supply for each isolate on SFR rigs

Bank	Marine isolate		
	WL61	**WL114**	**WL136**
1 (SFRs 1, 2, 3)	Marine broth	1/10 R2A+seawater broth	Marine broth
2 (SFRs 4, 5, 6)	R2A broth	SC broth	SC broth
3 (SFRs 7, 8, 9)	M2 broth	M2 broth	M2 broth
4 (SFRs 10, 11, 12)	M3 broth	M3 broth	M3 broth

* Refer to Appendix B for media composition

6.2.2. Reactor setup

SFR rigs were designed so that replicate bioreactors could be operated using similar operating conditions simultaneously, to optimise growth and process conditions at a 20 ml scale. Rigs were set up so that each SFR module was vertically orientated and manifolded together in series, resulting in a bank of SFRs. Each SFR rig included four separate banks of three SFR modules. Fig. 6.3 below shows a single bank of three SFR modules, where a compressor **(1)** was used to supply pressurised air, through regulator valves **(2)** and 0.22 µm filters **(3)**, to a sterile humidification vessel **(4)**. Humidified air was passed through the extracapillary space (ECS) (the space surrounding the membrane) of the SFR **(6)** at the air inlet port **(16)** and into the permeate collection vessel **(9)**, which was also used to collect the permeate/product. The permeate collection vessel, containing a vent filter **(10)**, allowed air to escape from the system through flow regulator nozzles **(11)**, which facilitated the control of the backpressure in the system.

A reticulation system of tubing connected by T-pieces **(12)** was used to distribute pressurised growth medium, from a medium supply vessel **(8)**, and pressurised air to each of the SFRs. The air and medium pressures were monitored using inline pressure gauges **(5)**. Each of the four banks was supplied with a different growth medium, listed in Table 6.1. A priming vessel **(7)** was used to prime medium through the lumen of the membrane, preventing air blocks. The prime line **(15)** was clamped closed during operation and the medium was forced from the membrane lumen, through the membrane wall and into the ECS. Permeate/sample was drained from the ECS into the collection vessel along with the air. The flow distribution of fluid between the three SFRs within a bank was evenly distributed by creating equal

backpressure, using flow regulator nozzles thereby providing resistance to the pressurised fluid flow and levelling out flow distribution patterns between SFRs. During various operating procedures, clamps were used to close tubing and prevent flow of air, medium or permeate.

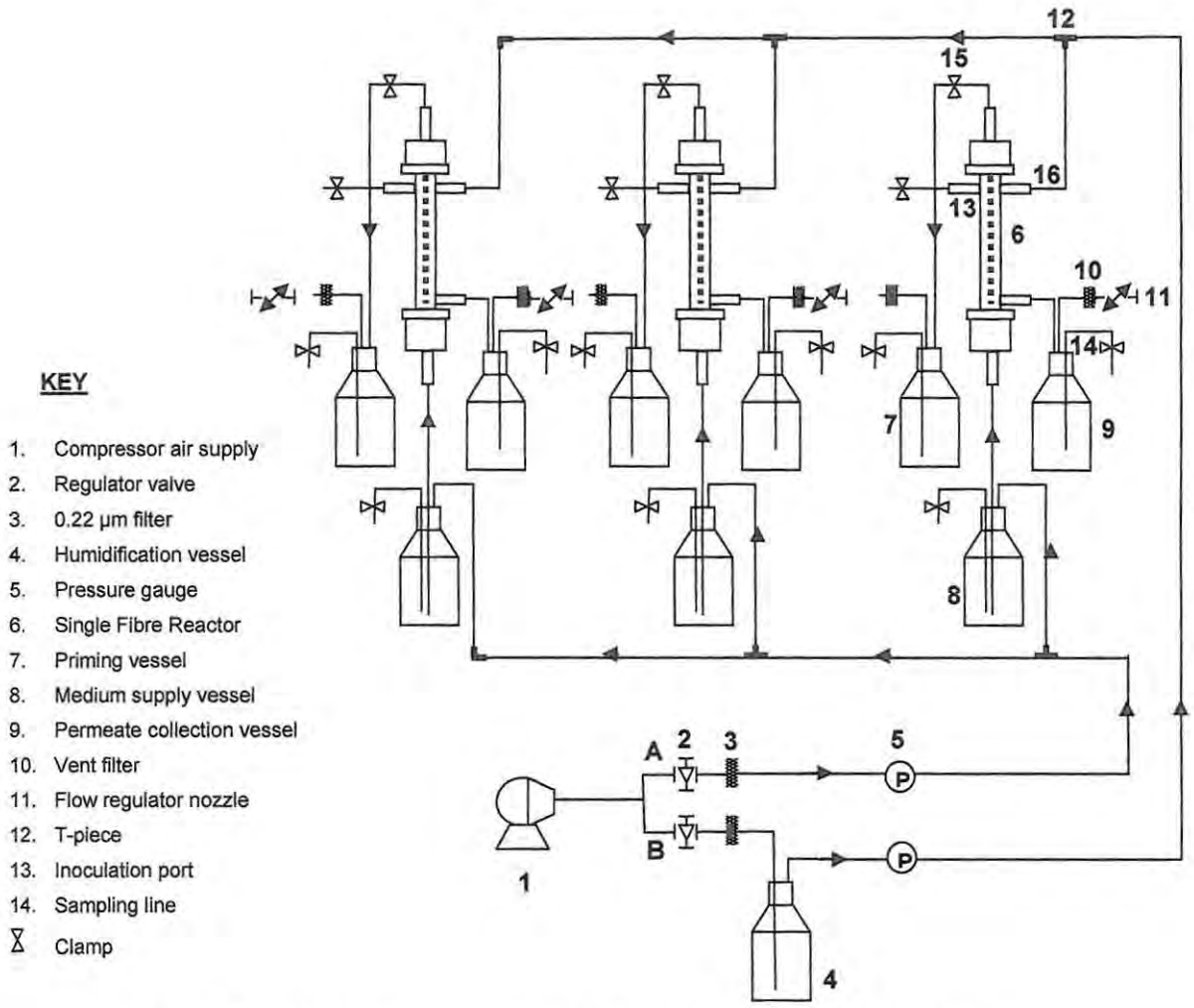


Fig. 6.3: Schematic representation of a bank of three SFR reactors

6.2.3. Sterilisation and inoculation of SFRs

The SFR rig was sterilised in an autoclave at 121°C for 20 min before inoculation. SFRs were inoculated by aseptically injecting 3 - 4 ml of pre-inoculum culture with a needle and syringe at the inoculation inlet port **(13)** (Fig. 6.3). Growth medium **(8)** was supplied from the lumen side of the membranes using dead-end flow. Dead-end flow was created by clamping the prime line (medium outlet line) **(15)**, forcing medium from the lumen through the pores of the membrane into the ECS allowing it

to fill with medium. The inoculum and nutrient medium was mixed by gently inverting each SFR a number of times. The medium inlet line was clamped to stop the flow of medium and the prime line was unclamped to allow immobilisation of microorganisms. Facilitated by the growth medium, microorganisms were immobilised onto the membrane surface, using air pressure **(16)** by reverse filtration, forcing cell free growth medium through into the membrane lumen and into the priming vessel **(7)**.

6.2.4. Aerobic operation of SFRs

The SFRs were operated aerobically using growth medium outlined in Table 6.1. The temperature was set at 26°C and maintained using a PID (proportional integral differential) control. The air pressure was set at 30 kPa and the air flow rate was set at 0.1 vol/vol/hr.

The flow rate (ml/hr) of growth medium determines the availability of nutrients to the biofilm, allowing the amount of productive biomass to develop or become nutrient limited consequently influencing metabolite production. This was regulated by the pressure differential (ΔP) between the medium supply in the lumen and the air supply at the biofilm surface within the ECS. The greater the ΔP, the higher the flow rate. The medium pressure was adjusted so that ΔP 1–2 kPa was obtained for the first three days following inoculation, allowing the biofilm to establish without washing the inoculum off of the membrane surface. As biofilm growth and resistance increased, the flow rate was adjusted by ± 2 kPa to obtain 5-20 ml of sample per day. If no growth occurred on the membrane surface after fourteen days, the medium for that particular bank was changed to an alternative medium promoting growth (this was only carried out with isolate WL61). Permeate samples were collected from the permeate collection vessels and flow rate was measured on a daily basis. Biological activity of extracts was screened every second day, described in section 6.2.5.

6.2.5. Extraction processes and bioassay screening

6.2.5.1. Preparation of extracellular extracts

Extracellular extracts were prepared from permeate samples from each reactor using an equal volume of EtOAc solvent (v/v). The samples were vortexed for 20 s and centrifuged for 5 min at 4000 rpm to separate the solvent layer and any particulate matter. The EtOAc solvent layer (non-polar constituent) and the water layer (polar constituent) were screened for bioactivity against four test organisms, *S. pombe*, *Fusarium* sp., *S. aureus* and *P. fluorescens*, using the well-diffusion bioassay method (Appendix A).

6.2.5.2. Preparation of intracellular extracts

One single fibre membrane from each bank of three SFRs was removed and sacrificed every seven days (on days 7, 14 and 21) and intracellular extracts were prepared. Previously, intracellular extracts were prepared by extracting the biomass from flask cultures using solvent according to weight (v/w). In this extraction process, biofilms on membranes were extracted on ice using 5 ml MeOH or 5 ml EtOAc solvents and sonicated for 3 x 30 s bursts to maximise and standardise the extraction method, using the immobilised biomass, as the weight of the immobilised cells on the membrane was difficult to quantify. Samples were centrifuged at 4000 rpm for 10 min and the supernatant was removed. Extracts were screened for bioactivity against four test organisms, *S. pombe*, *Fusarium* sp., *S. aureus* and *P. fluorescens*, using the well-diffusion bioassay method (Appendix A). Biomass was collected and dried in a Labcon oven at 70°C overnight after each extraction. Dry cell weight (DCW) was used to standardise and compare zone of inhibition results between SFRs according to mm zone per mg DCW.

6.3. RESULTS AND DISCUSSION

6.3.1. Growth and metabolite production of isolate WL61 on the Quorus single fibre bioreactor

6.3.1.1. Operating conditions

The average flow rate measured from days 8 to 21 for each bank using different growth media is listed in Table 6.2.

Table 6.2: Average flow rate of each bank for isolate WL61

Bank	Medium	Flow rate
1	Marine broth	0.22 ± 0.09 ml/hr
2	R2A broth	0.59 ± 0.13 ml/hr
3	M2 broth	0.41 ± 0.32 ml/hr
4	M3 broth	0.34 ± 0.16 ml/hr

On a given day, the differences in flow rates between SFRs within a given bank were relatively consistent, as made evident from the standard deviations (SDs). Within the time-course study, on average, the flow rate for each bank was only stabilised after eight days, illustrated in Fig. 6.4 below. The variability observed between banks operated at the same pressure, but using different media, further demonstrates the limitations of manual pressure control. The average flow rate for isolate WL61 cultivated on SFR in four growth media is illustrated in Fig. 6.4 and extracellular activity is discussed in more detail in section 6.3.1.3.

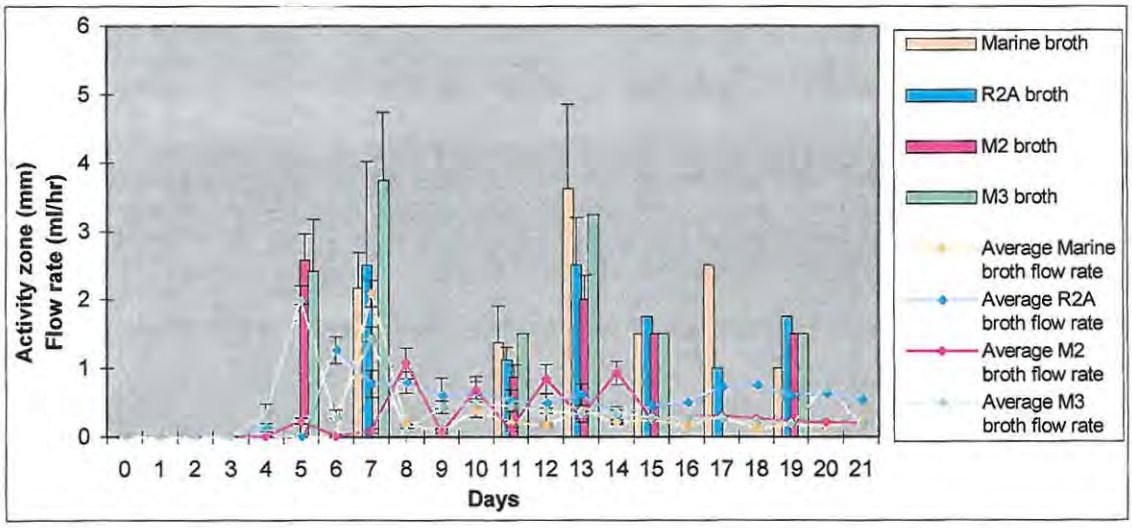


Fig. 6.4: The average flow rate and extracellular antibacterial activity of isolate WL61 against *P. fluorescens*

When using Marine broth, the flow rate for SFRs 1, 2 and 3 (bank 1) remained relatively constant, with the exception of day 7. With R2A broth, the flow rate for SFRs 4, 5 and 6 (bank 2) increased markedly on day 6, to a maximum of 1.26 ml/hr, decreasing again on day 7, but still remained high in comparison to the flow rate observed for the other banks. The flow rate for SFRs 7, 8 and 9 (bank 3), using M2

broth, increased slightly on day 5, decreasing on day 6, after which it fluctuated erratically until day 15. With M3 broth, the flow rate for SFRs 10, 11 and 12 (bank 4) increased markedly on day 5 to a maximum of 1.98 ml/hr and then fluctuated erratically until day 9, after which it remained relatively constant. The variations in flow rate corresponded to the manual adjustments of pressure while attempting to stabilise and standardise the flow rate between banks for the comparison of growth and activity using different media.

6.3.1.2. The effect of growth medium on biofilm growth

The biomass produced by isolate WL61 in complex and minimal growth media is illustrated in Fig. 6.5 and growth on SFRs in complex media is demonstrated in Fig. 6.6.

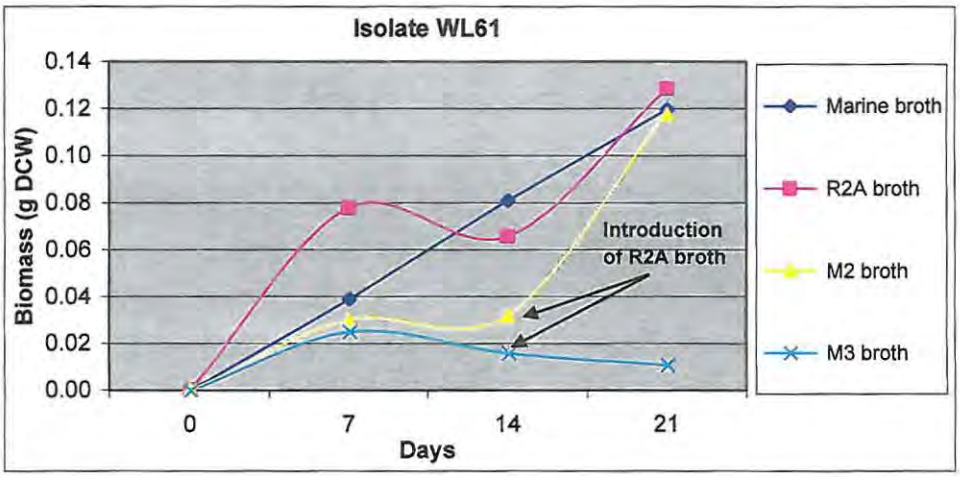


Fig. 6.5: Isolate WL61 cultivated in different growth media using SFRs

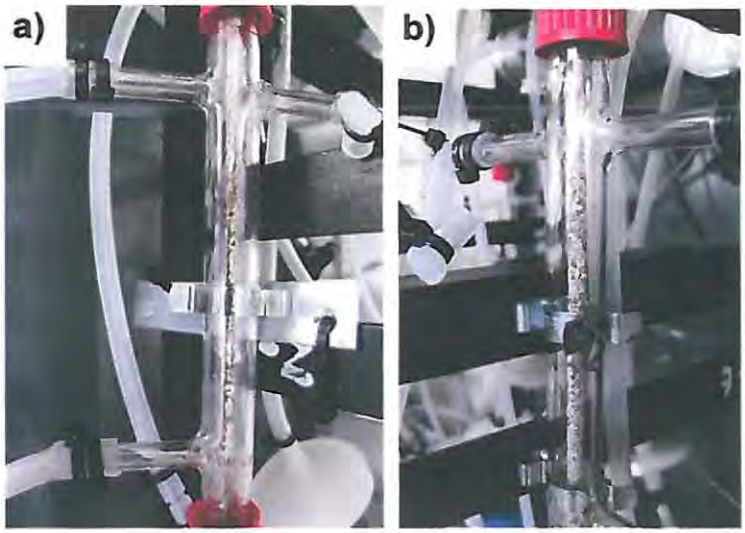


Fig. 6.6: Growth of isolate WL61 on SFR in **a)** Marine and **b)** R2A broth

Isolate WL61 grew better in complex growth media (Marine and R2A broth). The greatest biomass yield was obtained when Marine broth was used for growth (0.99 mg DCW/L medium) in comparison to R2A broth (0.44 mg DCW/L medium). Although the levels of biomass (DCW) were similar in Marine and R2A broth, the average flow rate for R2A broth was more than twice as high as that observed for SFRs using Marine broth (see section 6.3.1.1), allowing an increased nutrient supply.

This isolate showed poor biofilm growth in minimal growth media (M2 and M3 broth) and growth did not increase between days 7 and 14 in M2 broth, probably due to nutrient limitation. When R2A broth was introduced after 14 days to replace M2 broth and promote growth, the biomass of isolate WL61 increased markedly. Conversely, replacement of M3 broth with the complex medium, R2A broth, on day 14 did not revive biofilm growth.

6.3.1.3. Production of extracellular bioactive metabolites

The average flow rate (ml/hr) and activity from extracellular EtOAc extracts from isolate WL61 cultivated on SFRs in all the different growth media is illustrated in Fig 6.4 above. The permeate was sampled every second day from day 5 and bioactivity is expressed as the size of the zone of inhibition (mm).

Extracellular EtOAc extracts from isolate WL61 SFRs were active against *P. fluorescens* only. Antibacterial activity was observed when this isolate was cultured in all growth media throughout the 21 day growth period. These active extracts were detected against *P. fluorescens* on days 7, 11, 13, 15, 17 and 19 when using Marine and R2A broth. The largest zones of inhibition against *P. fluorescens* was observed on day 13 (3.63 ± 1.24 mm) with Marine broth and on days 7 (2.5 ± 1.52 mm) and 13 (2.5 ± 0.71 mm) with R2A broth. Slightly higher activity was observed when isolate WL61 was cultured in Marine broth.

Extracellular EtOAc active extracts were active against *P. fluorescens* on days 5, 11, 13, 15, and 19 with growth in M2 broth and on days 5, 7, 11, 13, 15, and 19, when M3 broth was used for growth. The largest zones of inhibition were observed on day 5 (2.58 ± 0.38 mm) and on day 7 (3.75 ± 1.0 mm) for M2 and M3 broth, respectively. Slightly lower activity was observed from growth in M2 broth than in Marine broth, but was similar to that in R2A broth. Conversely, higher activity was observed when isolate WL61 was cultured M3 broth. This may have been due to overfluxing (very high flow rate (ml/hr)) on days 5 (1.98 ml/hr) and 7 (1.43 ml/hr), causing:

1) All intracellular and biomass associated metabolites to be secreted by the cells as extracellular metabolites, increasing levels of extracellular active metabolites on days 5 and 7, resulting in no/insignificant levels of intracellular metabolites (section 6.3.1.4); or
2) Cells to be washed off of the membrane and into the permeate collection vessel, causing whole cells to be extracted, and resulting in cell-associated metabolites contributing towards extracellular activity on days 5 and 7. However, in this instance it is unlikely because the amount of biomass produced in M3 broth on day 7 (0.03 g), is not much lower than the amount of biomass produced with Marine broth (0.04 g), suggesting optimal biofilm growth in M3 broth at this time.

Isolate WL61 produced extracellular non-polar (EtOAc) active compounds in all growth media whereby the most consistently large zones of inhibition were observed with growth in Marine broth. No extracellular polar (MeOH) active compounds were detected throughout the 21 day growth period.

6.3.1.4. Production of intracellular bioactive metabolites

Bioactivity of intracellular MeOH extracts from isolate WL61 cultivated on SFRs in Marine and R2A broth is illustrated in Fig. 6.7 below. Biomass was collected every seven days and bioactivity is expressed as the size of the zone of inhibition (mm)/mg DCW.

Isolate WL61 produced intracellular polar (MeOH) antifungal compound(s) in all growth media however bioactivity (per mg biomass) was negligible in minimal media (data not shown). Furthermore, the extent of activity observed was dependent on the growth medium and flow rate conditions.

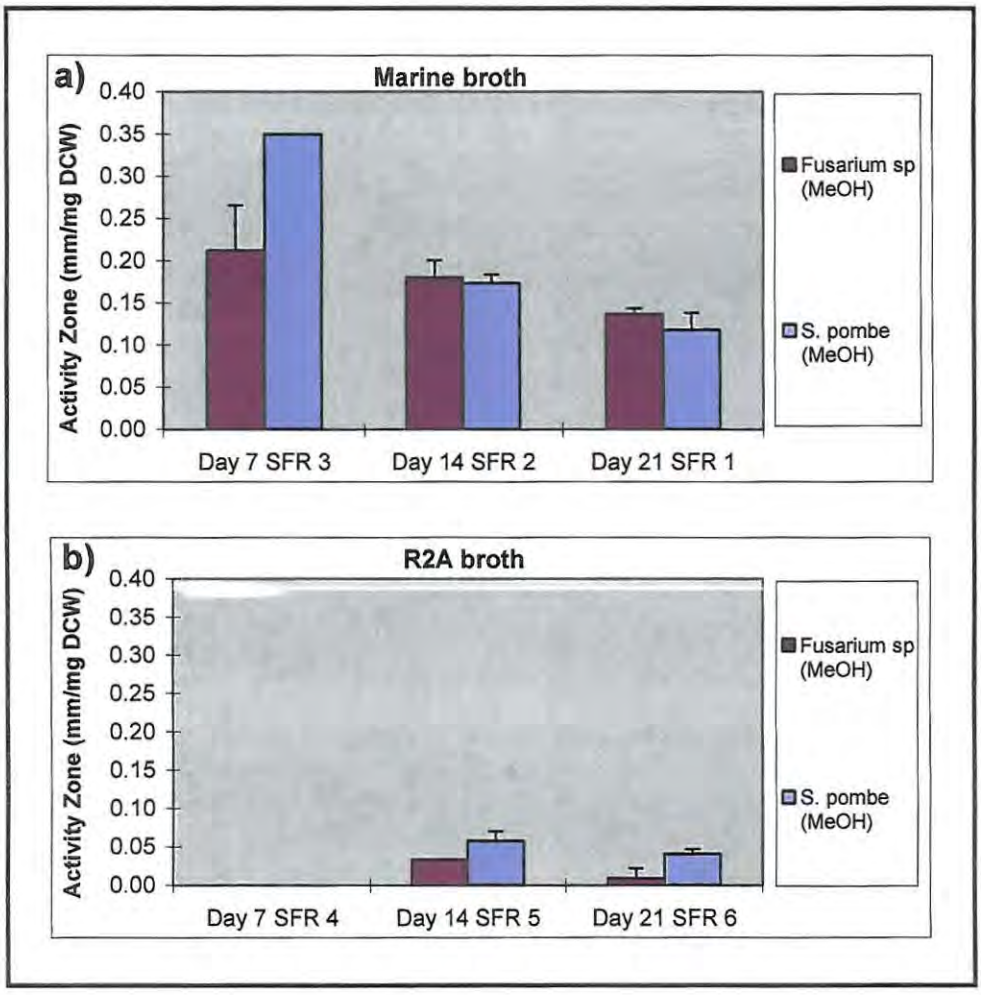


Fig. 6.7: Intracellular antifungal activity of isolate WL61 in complex media

MeOH extracts of isolate WL61 cultured in Marine broth produced higher activity against both *Fusarium* sp. and *S. pombe* than R2A broth. The level of antifungal compound(s), relative to the amount of biomass produced, decreased over time with

both growth media. While Marine broth showed activity from intracellular polar (MeOH) compound(s) detected against *Fusarium* sp. and *S. pombe* from day 7, activity was only detected from day 14 in R2A broth. This may be due to:

1) Insufficient nutrient limitation, due to the much higher average flow rate of R2A broth in comparison to Marine broth (section 6.3.1.1), thereby eliminating stress caused by nutrient limitation, and reducing the production of metabolites in response to this stress; or
2) Overfluxing may have caused water soluble (polar) cell-associated metabolites to be secreted by the cells within the biofilm as extracellular metabolites. However, in this instance this is unlikely as only antifungal activity was associated with isolate WL61 biofilms, and only non-polar (EtOAc) antibacterial activity was observed in extracellular samples (Fig. 6.4.)

This indicates that the late onset of production of bioactive metabolite(s) by isolate WL61 in R2A broth (bank 2) was most likely due to the different type of medium used and/or increased availability of nutrients as a result of a higher average flow rate, which was more than double the flow rate in comparison to the other banks (section 6.3.1.1). Different growth media have different nutrient content, concentrations and ratios of key nutrients that may be associated with the regulation of metabolite production at the level of gene expression.

6.3.1.5. Most suitable growth medium for MFR experiments

These experiments showed that the optimal nutrient requirement for the growth of isolate WL61 and the production of intracellular MeOH and extracellular EtOAc bioactive compound(s) was Marine broth. Marine broth will therefore be used on mutifibre bioreactors (MFR) to scale up the production of active compound(s) (Chapter 7).

6.3.2. Growth and metabolite production of isolate WL114 on the Quorus single fibre bioreactor

6.3.2.1. Operating conditions

The average flow rate measured from days 8 to 21 for each bank using different growth media is listed in Table 6.3.

Table 6.3: Average flow rate of each bank for isolate WL114

Bank	Medium	Flow rate
1	1/10 R2A+seawater broth	0.19 ± 0.11 ml/hr
2	SC broth	0.27 ± 0.07 ml/hr
3	M2 broth	0.33 ± 0.43 ml/hr
4	M3 broth	0.45 ± 0.65 ml/hr

On a given day, the differences in flow rates between SFRs within a given bank were highly variable, which is made evident from the large standard deviations (SDs) recorded. Within the time-course study, on average, the flow rate for each bank was only stabilised after eight days, as in section 6.3.1.1. The high SDs of flow rate recorded for banks 3 and 4 show that the flow rate for these two banks could not be sufficiently stabilised within the 21 day growth period, highlighting the limitations of manual pressure control. The average flow rate (ml/hr) for isolate WL114 cultivated in only three growth media on SFRs is illustrated in Fig. 6.8 below.

The flow rate for SFRs 1, 2 and 3 (bank 1), using 1/10 R2A+seawater broth, was at its highest on day 4 with a maximum of 0.70 ml/hr, decreasing irregularly until day 21, whilst the flow rate for SFRs 4, 5 and 6 (bank 2), using SC broth, had a relatively regular and constant flow rate throughout the 21 day growth period. With both M2 broth (SFRs 7, 8 and 9 (bank 3)) and M3 broth (SFRs 10, 11 and 12 (bank 4)) (data not shown), the flow rate was highly irregular throughout the 21 day growth period.

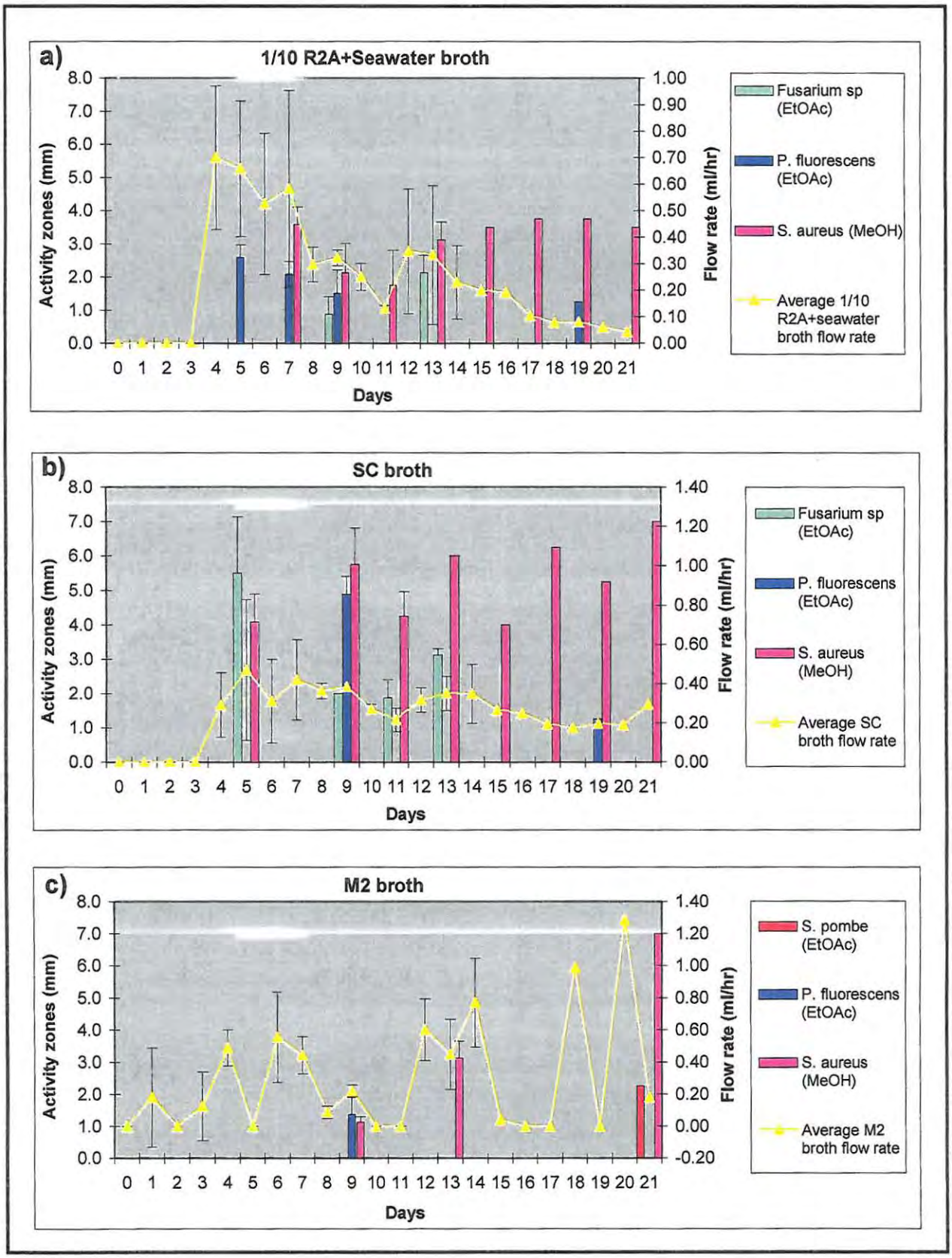


Fig. 6.8: The average flow rate and extracellular antifungal and antibacterial activity of isolate WL114

6.3.2.2. The effect of growth medium on biofilm growth

The biomass produced by isolate WL114 in complex and minimal growth media is illustrated in Fig. 6.9 and growth on SFR in complex and minimal media is demonstrated in Fig. 6.10.

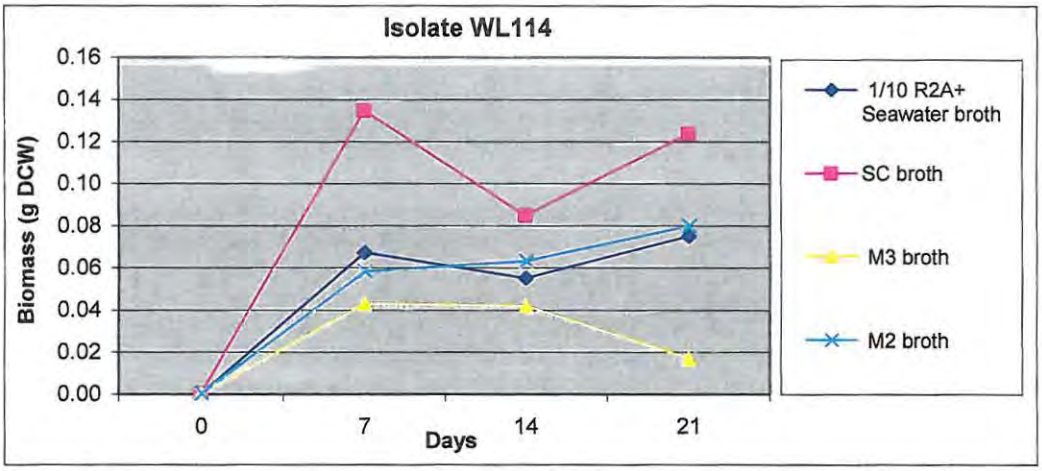


Fig. 6.9: Isolate WL114 cultivated in different growth media using SFRs

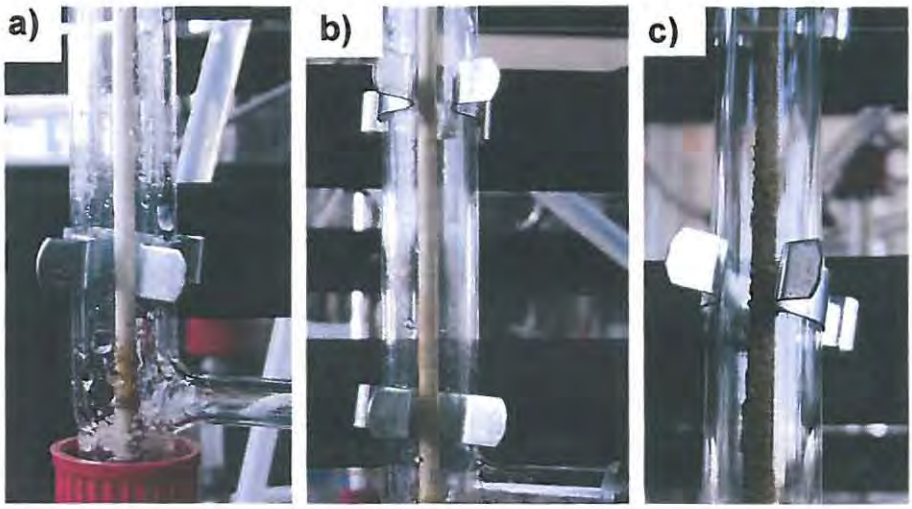


Fig. 6.10: Growth of isolate WL114 on SFR in **a)** 1/10 R2A+seawater **b)** SC and **c)** M2 broth

Isolate WL114 grew best in SC broth followed by M2 broth, but showed relatively poor biofilm growth in 1/10 R2A+seawater broth, while no visible growth was observed in M3 broth. Although the greatest biomass yield was obtained when SC broth was used for growth (0.71 mg DCW/L medium) in comparison to M2 broth (0.28 mg DCW/L medium), the average flow rate for M2 broth was slightly higher

than that observed for SFRs using SC broth (see section 6.3.2.1), allowing an increased nutrient supply.

Although the levels of biomass (DCW) were similar in 1/10 R2A+seawater and M2 broth, the average flow rate for 1/10 R2A+seawater broth was considerably lower, reducing nutrient supply. Levels of biomass (DCW) in 1/10 R2A+seawater broth were also considerably lower than in SC broth, most likely because the average flow rate for 1/10 R2A+seawater broth was two thirds that of SC broth (see section 6.3.2.1). This flow rate was reduced because the residual agar present in the 1/10 R2A+seawater broth (1.2 g) caused the membranes to foul as the agar solidified in the lumen of the membrane, blocking the flow of medium through the membrane. Attempts were made to unblock the SFR by dislodging and flushing out the solidified agar from the lumen twice a week by priming the medium through the membrane lumen. As a result of the solidified agar in the lumen of the membrane, the dry weight values representing the growth of isolate WL114 were largely increased. These values were corrected as follows:

> Assuming that all agar in the 1/10 R2A+seawater broth supplied to the reactor (measured as total permeate) precipitated on the lumen side of the membrane, DCW of the biomass was compensated for by subtracting the weight of agar present in the total permeate volume (ml) from the total dry weight. However, it should be noted that this may still not be a true reflection of DCW.

Maximum growth of isolate WL114 occurred in SC broth at day 7, after which biomass DCW appeared to stabilise. The same trend was observed with 1/10 R2A+seawater, M2 and M3 broth. In each medium, a stable biofilm was obtained after seven days growth, as the amount of biomass did not accumulate exponentially, but levelled off.

6.3.2.3. Production of extracellular bioactive metabolites

The average flow rate (ml/hr) and bioactivity from extracellular MeOH and EtOAc extracts from isolate WL114 cultivated on SFRs in three different media is illustrated in Fig. 6.8 above. The permeate was sampled every second day from day 5 and bioactivity is expressed as the size of the zone of inhibition (mm).

When 1/10 R2A+seawater broth was used for growth, extracellular EtOAc extracts were active against *P. fluorescens* on days 5, 7, 9, and 19, with the highest activity observed on day 5 (2.6 ± 0.4 mm); and against *Fusarium* sp. on days 9 and 13 with the highest activity observed on day 13 (2.1 ± 0.5 mm). Extracellular MeOH extracts were only active against *S. aureus* on days 7, 9, 11, 13, 15, 17, 19 and 21 with the highest activity observed on days 17 and 19 (3.8 ± 0.0 mm).
When SC broth was used for growth, extracellular EtOAc extracts were active against *P. fluorescens* on days 9 and 19 with the highest activity observed on day 9 (4.9 ± 0.5 mm); and against *Fusarium* sp. on days 5, 9, 11 and 13 with the highest activity observed on day 5 (5.5 ± 1.6 mm). Extracellular MeOH extracts were active against *S. aureus* on days 5, 9, 11, 13, 15, 17, 19 and 21 with the highest activity observed on day 21 (7.0 ± 0.0 mm). The production of extracellular compounds was considerably higher when isolate WL114 was cultivated using SC broth than 1/10 R2A+seawater broth. This was possibly due to the very low flow rate of SFRs using 1/10 R2A+seawater broth, due to fouling, preventing metabolites from being washed away from the biofilm.

When M2 broth was used for growth, extracellular EtOAc extracts were only active against *P. fluorescens* on day 9 (1.4 ± 0.5 mm); and against *S. pombe* on day 21 (2.3 ± 0.0 mm). Extracellular MeOH extracts were active against *S. aureus* on days 9, 13 and 21 with the highest activity observed on day 21 (7.0 ± 0.0 mm). The activity of these extracts was lower than that observed from SC broth and less consistent. This was probably due to the inconsistent nutrient supply and limitation, as a result of the very high and irregular flow rate of medium, eliminating stress caused by nutrient limitation and reducing the production of metabolites in response to this stress. The same was true for SFRs using M3 broth (data not shown).

Isolate WL114 produced extracellular polar (MeOH) and non-polar (EtOAc) active compounds in all growth media, whereby the amount of product produced was dependent on the type of growth medium and flow rate conditions. The most suitable nutrient requirements for biofilm growth and the production of extracellular active compound(s), with the exception of compounds active against *S. pombe*, are in SC broth. SC broth will therefore be used on MFR to scale up the production of extracellular active compound(s) (Chapter 7).

6.3.2.4. Production of intracellular bioactive metabolites

Bioactivity of intracellular MeOH and EtOAc extracts from isolate WL114 cultivated on SFRs in 1/10 R2A+seawater, SC, M2 and M3 broth is illustrated in Fig 6.11 below. Biomass was collected every seven days and bioactivity is expressed as the size of the zone of inhibition (mm)/mg DCW.

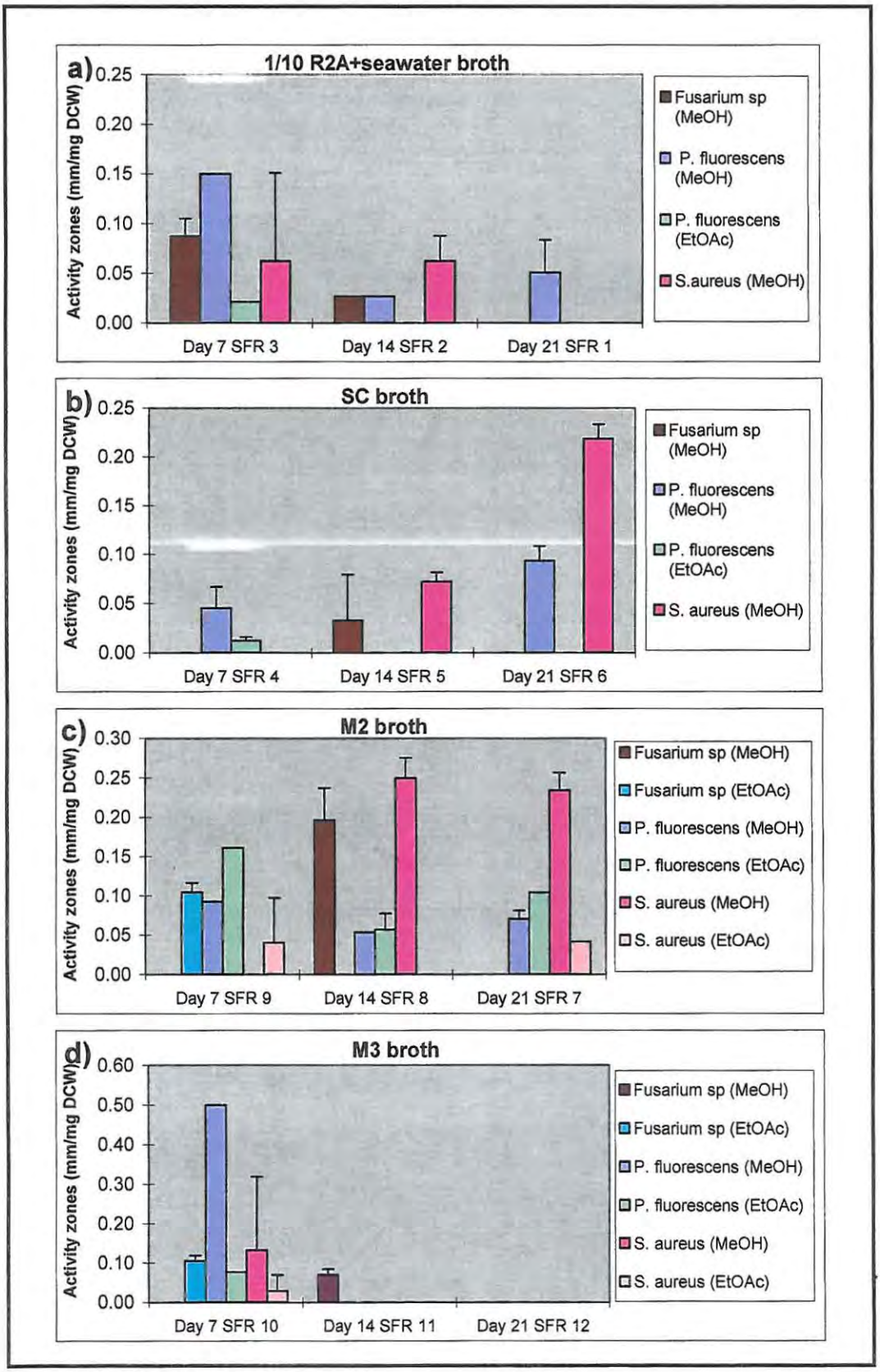


Fig. 6.11: Intracellular antibacterial and antifungal activity of isolate WL114

Isolate WL114 produced active compound(s) in all growth media. The extent of activity observed was dependent on the type of medium and flow rate conditions. Intracellular polar (MeOH) and non-polar (EtOAc) compound(s) active against *Fusarium* sp., *P. fluorescens* and *S. aureus* were detected on day 7 in 1/10 R2A+seawater broth. The level of activity was relatively low and the broad range of activity detected at day 7 was seen to decrease with time. This was probably due to fouling in the membrane lumen which resulted in a very low flow rate over time consistently changing nutrient gradients resulting in an unstable biofilm and limiting secondary metabolite production. The highest intracellular activity was observed from the MeOH extracts, against *P. fluorescens* (0.15 ± 0.0 mm/mg DCW).

When SC broth was used for growth, intracellular EtOAc extracts were shown to be active against *P. fluorescens* only, while MeOH extracts were active against *P. fluorescens, Fusarium* sp. and *S. aureus*. Contrary to 1/10 R2A+seawater broth, the range of activity detected within a single extract was lower, but the level of activity increased with time, with activity from polar (MeOH) compounds against *S. aureus* reaching a maximum of 0.22 mm/mg DCW on day 21. Overfluxing of SFRs on days 5 and 7 may have washed cell-associated metabolites away from the biofilm and into the permeate as extracellular metabolites, resulting in the detection of lower intracellular activity on these days. As the flow rate decreased and stabilised (section 6.3.2.1), a higher level of intracellular metabolites, produced in response to nutrient limitation, was able to accumulate within the biofilm over time.

Relatively high levels of intracellular polar (MeOH) and non-polar (EtOAc) compound(s) active against *P. fluorescens, Fusarium* sp. and *S. aureus* were detected from day 7 using M2 broth. The range and level of activity was greater than observed with more complex growth media. Intracellular EtOAc extracts were active against *Fusarium* sp. (0.1 ± 0.01 mm/mg DCW) and *S. aureus* (0.04 ± 0.06 mm/mg DCW), on day 7, for the first time. High levels of intracellular activity were detected from MeOH extracts against *Fusarium* sp. (0.20 ± 0.04 mm/mg DCW) and *S. aureus* (0.25 ± 0.03 mm/mg DCW) on day 14 as well as against *S. aureus* (0.23 ± 0.02 mm/mg DCW) on day 21. The production of a wide range of active compound(s) was most likely due to inconsistent nutrient supply and limitation caused by a very irregular flow rate of M2 broth in comparison to 1/10 R2A+seawater and SC broth

(section 6.3.2.1), thereby consistently changing nutrient conditions and the production of metabolites in response to these conditions.

When M3 broth was used for growth, the range of activity was fairly high, but the level of these activities was relatively low, with the exception of intracellular MeOH extracts active against *P. fluorescens* (0.50 ± 0.0 mm/mg DCW) on day 7. Intracellular EtOAc extracts were active against *Fusarium* sp. (0.11 ± 0.01 mm/mg DCW) and *S. aureus* (0.03 ± 0.04 mm/mg DCW) on day 7, as with M2 broth. While growth in M3 broth gave the widest range of intracellular activity within a single extract, the inconsistent nutrient supply and limitation, due to a very high and irregular flow rate, appears to have eliminated stress caused by nutrient limitation, reducing the production of metabolites from days 14 to 21 in response to this stress.

6.3.2.5. The most suitable growth medium for MFR experiments

The SFR experiments showed that the optimal nutrient requirement for the growth of isolate WL114 was in complex growth media however, growth in M2 broth produced a considerably wider range of intracellular activity against different test organisms as well as large zones of inhibition against *S. aureus*. Therefore, M2 broth will be used on MFR to scale up the production of intracellular active compound(s).

6.3.3. Growth and metabolite production of isolate WL136 on the Quorus single fibre bioreactor

6.3.3.1. Operating conditions

The average flow rate measured between days 8 to 21 for each bank using different growth media is listed in Table 6.4.

Table 6.4: Average flow rate of each bank for isolate WL136

Bank	Days	Medium	Flow rate
1	8 - 21	Marine broth	0.52 ± 0.26 ml/hr
2	8 - 21	SC broth	0.26 ± 0.17 ml/hr
3	10 - 21	M2 broth	0.45 ± 0.27 ml/hr
4	9 - 21	M3 broth	0.39 ± 0.25 ml/hr

In a time-course study, on a given day, the differences in flow rates between SFRs within a given bank were highly variable, which was made evident from the large standard deviations (SDs) recorded. On average, the flow rate for each bank was only stabilised after 8 to 10 days operation. The average flow rate (ml/hr) for isolate WL136 cultivated in different growth media on SFRs is illustrated in Fig. 6.12 below.

The flow rate for SFRs 1, 2 and 3 (bank 1) fluctuated irregularly until day 15 after which it remained constant until day 21 when using Marine broth. With SC broth, the flow rate for SFRs 4, 5 and 6 (bank 2) was relatively constant until day 13; it then decreased until day 15, remaining low until day 21. The flow rate for SFR 7, 8 and 9 (bank 3) was irregular during the entire 21 day period as it fluctuated erratically with M2 broth. The flow rate for SFRs 10, 11 and 12 (bank 4), using M3 broth, was relatively stable until day 13 after which it increased, but then decreased on day 15, remaining stable until day 21.

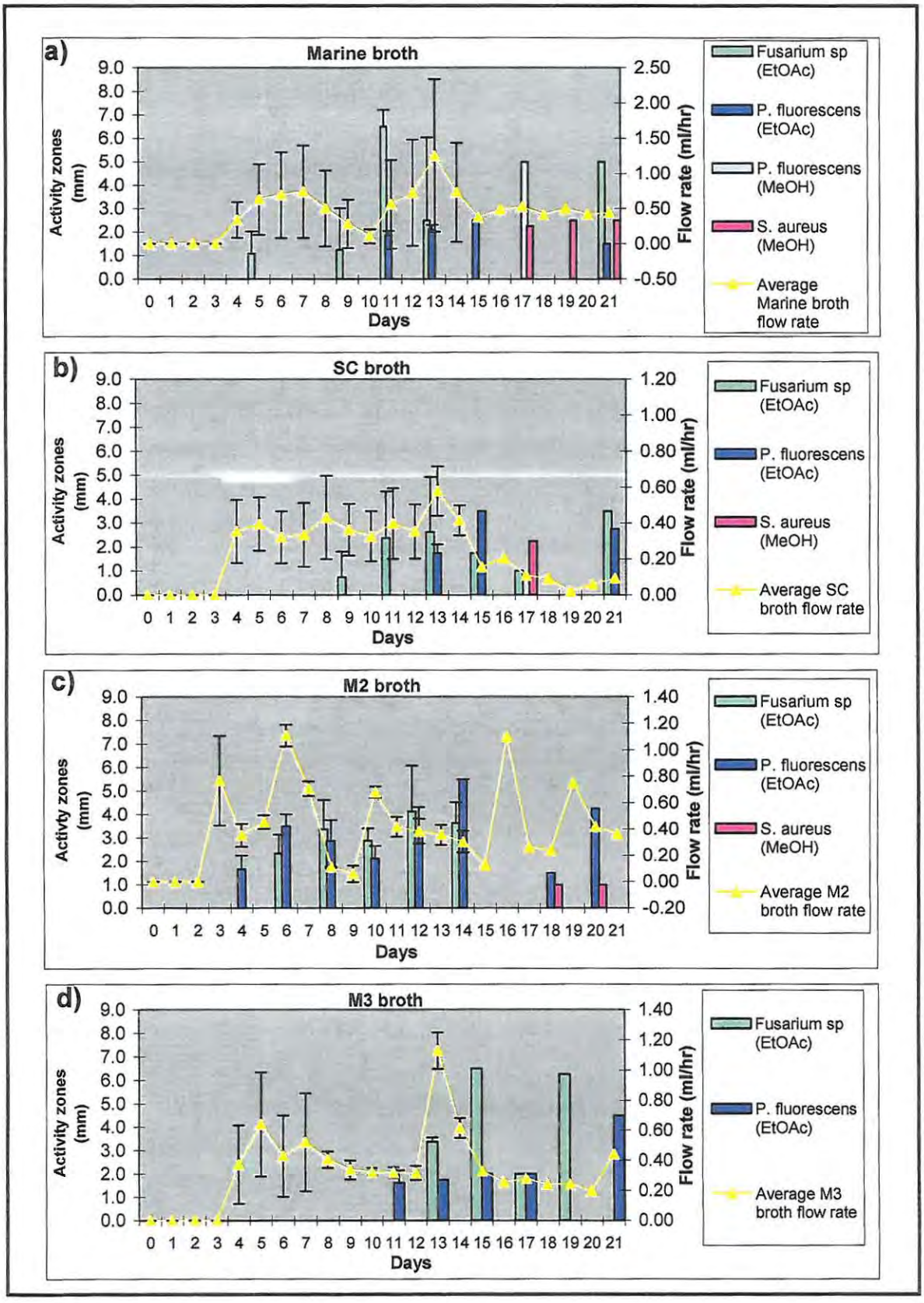


Fig. 6.12: Average flow rate and extracellular antifungal and antibacterial activity of isolate WL136

6.3.3.2. The effect of growth medium on biofilm growth

The biomass produced by isolate WL136 in complex and minimal growth media is illustrated in Fig. 6.13 and growth on SFR in complex and minimal media is demonstrated in Fig. 6.14.

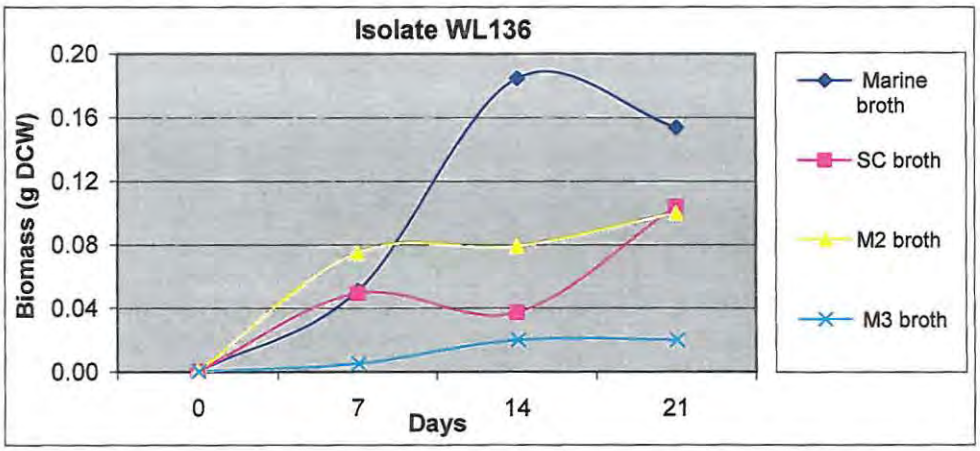


Fig. 6.13: Isolate WL136 cultivated in different growth media using SFRs

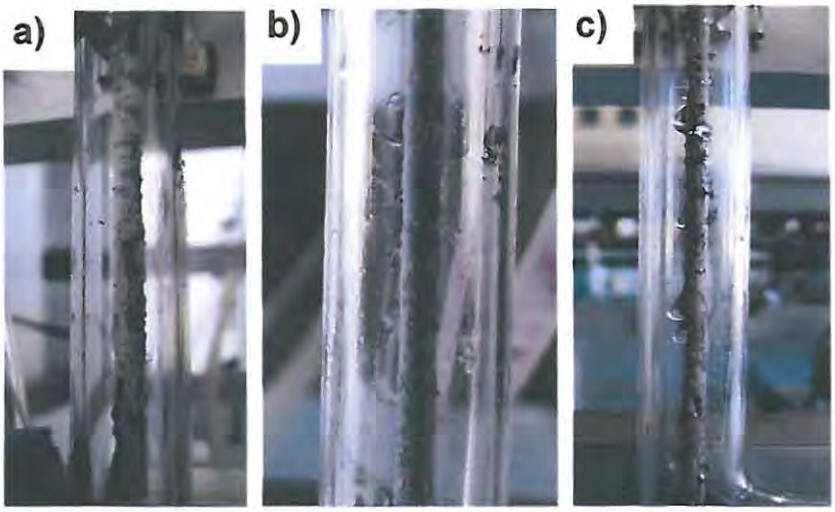


Fig 6.14: Growth of isolate WL136 on SFR in **a)** Marine **b)** SC and **c)** M2 broth

Isolate WL136 grew best in Marine broth (complex medium) followed by M2 broth (minimal medium) and SC broth (complex medium). Poor biofilm growth was obtained with the minimal medium, M3 broth, where no visible growth was observed.

The greatest biomass yields were obtained when SC and Marine broth were used for growth (1.12 mg DCW/L medium and 1.11 mg DCW/L medium, respectively)

followed by M2 broth (0.45 mg DCW/L medium). While the level of biomass (DCW) was higher with Marine broth than with SC and M2 broth, the average flow rate and nutrient supply for SFRs using Marine broth was twice as high as that observed for SC broth, and the flow rate for SFRs using M2 broth was also higher than that for SC broth, increasing nutrient supply (section 6.3.3.1).

Maximum growth of isolate WL136 occurred in Marine broth at day 14, after which biomass DCW decreased slightly on day 21. In SC broth, the growth increased until day 7 after which it decreased slightly between days 7 and 14, and then doubled between days 14 and 21. Growth in M2 broth increased until day 7 after which the growth levelled off between days 7 and 14, increasing only slightly between days 14 and 21. In M3 broth, the growth was poor and only increased slightly over the 21 day time period.

6.3.3.3. Production of extracellular bioactive metabolites

The average flow rate (ml/hr) and bioactivity from extracellular MeOH and EtOAc extracts from isolate WL136 cultivated on SFRs in four different media is illustrated in Fig. 6.12 above. The permeate was sampled every second day from day 5 (Marine, SC and M3 broth) and from day 4 (M2 broth) and bioactivity is expressed as the size of the zone of inhibition (mm). Both antibacterial and antifungal activity was observed with growth of isolate WL136 in complex and minimal growth media. Extracellular MeOH and EtOAc extracts were active against *P. fluorescens*, *Fusarium* sp. and *S. aureus*.

When Marine broth was used for growth, extracellular EtOAc extracts were active against *Fusarium* sp. on days 5, 9, 11, 13 and 21 with the highest activity observed on day 11 (6.5 ± 0.7 mm); and against *P. fluorescens* on days 11, 13, 15, 17 and 21 with the highest activity observed on day 15 (2.5 ± 0.0 mm). Extracellular MeOH extracts were active against *S. aureus* on days 17, 19 and 21 with the highest activity observed on days 19 and 21 (2.5 ± 0.0 mm); and against *P. fluorescens* on day 17 (5.0 ± 0.0 mm).

When SC broth was used for growth, extracellular EtOAc extracts were active against *Fusarium* sp. on days 9, 11, 13, 15, 17 and 21 with the highest activity observed on day 21 (3.5 ± 0.0 mm); against *P. fluorescens* on days 13, 15 and 21

with the highest activity observed on day 15 (3.5 ± 0.0 mm). Extracellular MeOH extracts were only active against *S. aureus* on day 17 (2.3 ± 0.0 mm). The activity of these extracts was lower than those from Marine broth, most likely due to the type of medium and/or reduced biomass levels as a result of the flow rates that were half of that observed for Marine broth (section 6.3.3.1).

When M2 broth was used for growth, extracellular EtOAc extracts were active against *Fusarium* sp. on days 6, 8, 10, 12 and 14 with the highest activity observed on day 12 (4.1 ± 1.9 mm); against *P. fluorescens* on days 4, 6, 8, 10, 12, 14, 18, and 20 with the highest activity observed on day 14 (5.5 ± 0.0 mm). Extracellular MeOH extracts were only active against *S. aureus* on days 18 and 20 (1.0 ± 0.0 mm). The level of activity was also slightly lower than in Marine broth. As with SC broth, this may be due to the type of medium and/or reduced biomass levels as a result of the flow rates that were lower than those observed for Marine broth (section 6.3.3.1).

When M3 broth was used for growth, only extracellular EtOAc extracts were active against *Fusarium* sp. on days 13, 15, 17 and 19 with the highest activity observed on day 15 (6.5 ± 0.0 mm); and against *P. fluorescens* on days 11, 13, 15, 17 and 21 with the highest activity observed on day 21 (4.5 ± 0.0 mm). The level of activity was similar to that observed when isolate WL136 was cultured in Marine broth. This was most likely due to overfluxing on day 13, causing the accumulated metabolites in the biofilm to be washed out into the permeate as extracellular metabolites.

Isolate WL136 produced extracellular active compounds in all growth media, whereby the amount of product produced was dependent on the type of growth medium and flow rate conditions.

6.3.3.4. Production of intracellular bioactive metabolites

Bioactivity of intracellular MeOH and EtOAc extracts from isolate WL136 cultivated on SFRs in Marine, SC, M2 and M3 broth is illustrated in Fig. 6.15 below. Biomass was collected every seven days and bioactivity is expressed as the size of the zone of inhibition (mm)/mg DCW.

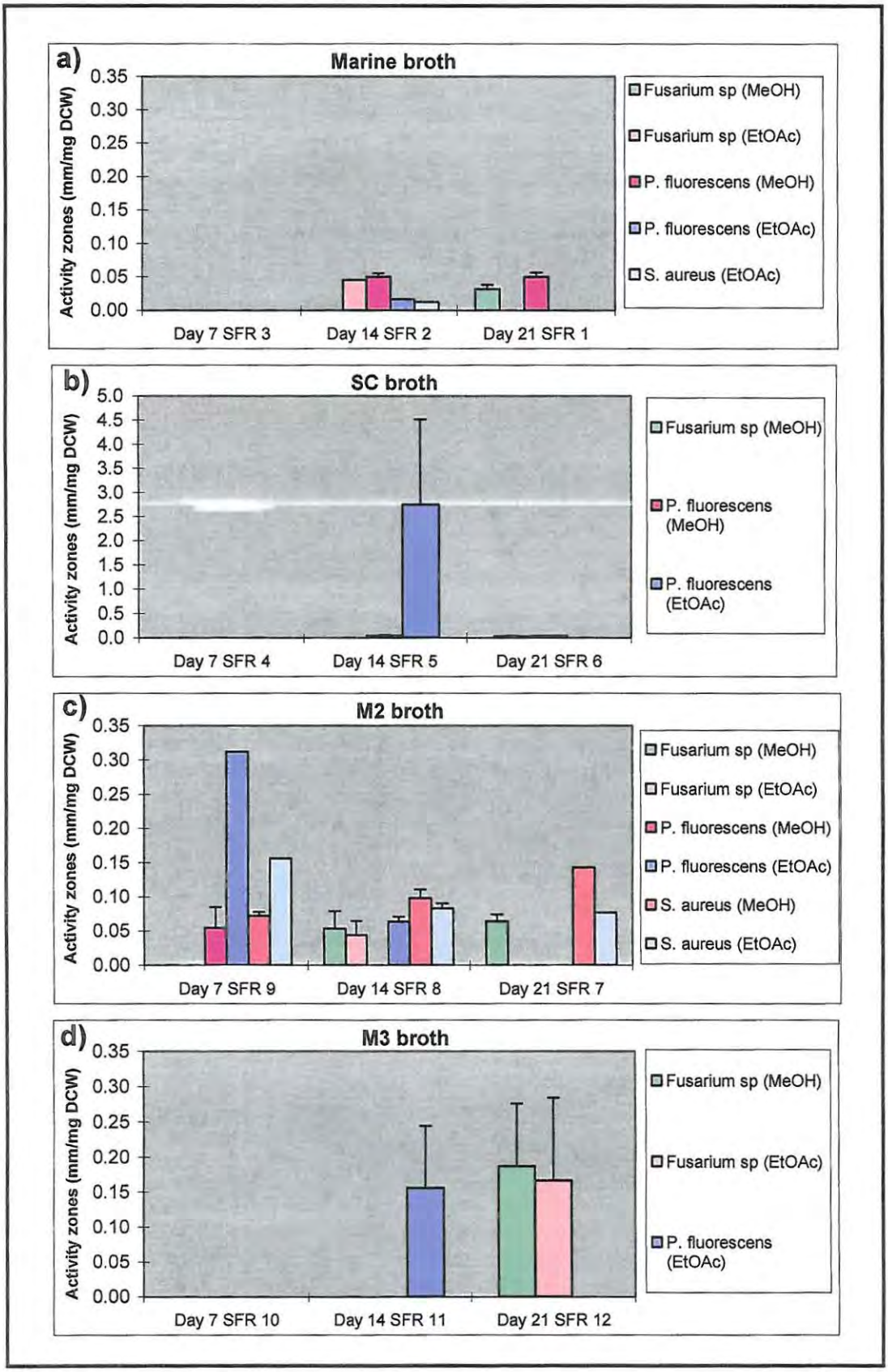


Fig. 6.15: Intracellular antifungal and antibacterial activity of isolate WL136

In Marine broth, intracellular activity was only detected from day 14. Intracellular MeOH and EtOAc extracts were active against *Fusarium* sp., *P. fluorescens* and *S. aureus*. Intracellular MeOH extracts showed the highest activity against *P. fluorescens* on days 14 and 21 (0.05 ± 0.01 mm/mg DCW). The level of activities was relatively low and the broad range of activity detected at day 14 was seen to decrease with time possibly due to:

1) Insufficient nutrient limitation due to the much higher average flow rate of Marine broth (section 6.3.3.1) thereby eliminating stress caused by nutrient limitation and reducing the production of metabolites in response to this stress; or
2) Overfluxing of SFRs using Marine broth between days 4 and 7, washing cell-associated metabolites away from the biofilm into the permeate as extracellular metabolites. But, because the average flow rate was considerably high (section 6.3.3.1), the polar (MeOH) and non-polar (EtOAc) metabolites may have been diluted further by the broth, resulting in low/insignificant levels of extracellular activity. However, in this case it is unlikely as it appears that within this period the biofilm had not accumulated sufficiently to become nutrient limited.

Similarly, intracellular activity was only detected from day 14 in SC broth. Intracellular MeOH extracts were active against *P. fluorescens* and *Fusarium* sp. and EtOAc extracts were only active against *P. fluorescens*. The level of activity on day 14 from MeOH extracts against *P. fluorescens* (0.05 ± 0.01 mm/mg DCW) was relatively lower than that observed from EtOAc extracts (2.75 ± 1.77 mm/mg DCW). The range of activity against the different test organisms was also very low as intracellular polar (MeOH) metabolites were only active against *Fusarium* sp. (0.03 ± 0.01 mm/mg DCW) and *P. fluorescens* (0.04 ± 0.0 mm/mg DCW), detected on day 21. This may possibly be due to the type of growth medium and the biphasic growth phase of isolate WL136 due to the depletion of nutrients after seven days and short stationary phase between days 7 and 14 causing an increase in metabolite production (bioactive metabolites and enzymes) as a result of gene expression. Enzymes may be responsible for the breakdown of different nutrients, increasing nutrient availability and allowing growth to continue.

In M2 broth, intracellular MeOH and EtOAc extracts were detected from day 7, active against *P. fluorescens* and *S. aureus*, and against *P. fluorescens*, *Fusarium* sp. and

S. aureus on day 14. The highest intracellular activity was observed from EtOAc extracts against *P. fluorescens* on day 7 (0.31 ± 0.0 mm/mg DCW). The level and range of activity was high, however the range of activity decreased as only activity against *Fusarium* sp. and *S. aureus* was detected on day 21. This was attributed to inconsistent nutrient supply and limitation due to a very irregular flow rate of M2 broth (section 6.3.3.1), thereby constantly changing the nutrient conditions and production of metabolites in response to these changing conditions.

When M3 broth was used for growth, only intracellular EtOAc extracts active against *P. fluorescens* was detected on day 14. The level of activity in this growth medium was high, but the range of activity was low as extracts were only active against *Fusarium* sp. on day 21. The highest intracellular activity was detected from MeOH extracts against *Fusarium* sp. on day 21 (0.19 ± 0.09 mm/mg DCW). This was most likely due to the very low growth of isolate WL136 in M3 broth resulting in no metabolite production from day 7. Flow rate decreased and stabilised between days 8 and 12 allowing metabolites to accumulate in the biofilm, which were probably washed out into the permeate as extracellular metabolites as a result of overfluxing on day 13. As flow rate decreased and stabilised between days 15 and 21, metabolite production increased from day 21 in response to nutrient limitation.

Isolate WL136 produced intracellular polar (MeOH) and non-polar (EtOAc) antibacterial and antifungal compound(s) in all growth media. The extent of activity observed was dependent on the type of medium and flow rate conditions.

6.3.3.5. The most suitable growth medium for MFR experiments

The SFR experiments showed that the most suitable nutrient requirement for biofilm growth and the production of a wider range of extracellular active compound(s) was in complex growth media, particularly in Marine broth. However, growth in M2 broth also showed relatively high and consistent production of extracellular active compound(s) and produced a considerably wider range of intracellular active compound(s) consistently against different test organisms. M2 broth was more cost effective than Marine broth, thus M2 broth was used on MFR to scale up the production of active compound(s) (Chapter 7).

6.4. CONCLUSION

In each case, SFR experiments demonstrated the limitation of using manually adjusted pressure control to regulate nutrient supply (flow rate). The variability in flow rate values was high between each bank operated at the same pressure, but using different growth media, complicating the analysis of bioreactor productivity under different conditions. These results emphasize the importance of maintaining a stable and constant flow rate for all banks supplied with different growth media, when interpreting and comparing the differences in activity or productivity.

Under the different growth conditions evaluated, isolate WL61 produced at least two distinct antimicrobial compounds. These include an extracellular non-polar (EtOAc) antibacterial compound against the Gram-negative bacterium, *P. fluorescens*, and an intracellular polar (MeOH) antifungal compound against *Fusarium* sp. and *S. pombe*. Marine broth was chosen for isolate WL61 as the preferred medium for bioactive compound production in MFR experiments (Chapter 7) as this produced a high yield of intracellular biologically active compound(s).

Similarly, isolate WL114 produced at least two antimicrobial compounds under the different growth conditions evaluated. These include a predominantly polar (MeOH) antibacterial compound against Gram-negative and Gram-positive bacteria, *P. fluorescens* and *S. aureus*, respectively, as well as an antifungal compound against *Fusarium* sp. and *S. pombe*. A similar range of activities was observed to a lesser extent from EtOAc extracts, suggesting that these are the same compound(s) which are more polar, but still show solubility in ethyl acetate. Both M2 and SC broth were chosen for isolate WL114 as the preferred media for bioactive compound production in MFR experiments. While M2 broth showed high yields as well as a broad range of intracellular activity against the test organisms, SC broth showed high yields of extracellular active compound(s).

Similarly, under the different growth conditions evaluated, isolate WL136 produced at least three broad spectrum antimicrobial compound(s). These include a predominantly extracellular non-polar (EtOAc) antibacterial and antifungal compound(s) against Gram-negative bacterium, *P. fluorescens* and *Fusarium* sp., respectively, also showing some solubility in methanol, and active against Gram-

positive bacterium, *S. aureus*; as well as a predominantly intracellular polar (MeOH) antifungal compound against *Fusarium* sp., with some solubility in ethyl acetate, and a non-polar (EtOAc) antibacterial compound(s) against Gram-positive and Gram negative bacteria, *S. aureus* and *P. fluorescens*, respectively, also showing some solubility in methanol. M2 broth was chosen for isolate WL136 as the preferred medium for bioactive compound production in MFR experiments as this produced a high yield of active compound(s) as well as a broad range of activity, and was more cost effective than Marine broth.

CHAPTER 7

Production of bioactive metabolites using the Quorus multifibre bioreactor (MFR).

7.1. INTRODUCTION

Membrane bioreactors can be increased in size from single fibre scale to multiple membrane bioreactors in order to produce increased levels of metabolites identified in initial SFR experiments. Once a set of operating conditions has been identified using SFR rigs, these may be directly applied to MFR operation.

In a previous scale-up experiment of the MFR for continuous enzyme production using *Phanerochaete chrysosporium* ME446, Govender *et al.* (2003) showed a volumetric scale-up from a 24 ml SFR to a 2.4 L MFR resulting in a 7-fold increase in manganese peroxidase (MnP) production. Furthermore, visual assessment and SEM (scanning electron microscopy) analysis of the biofilm by these authors, confirmed an increased and more uniform biofilm development using MFRs in comparison to the SFRs. No significant scale-up limitations were detected with the use of the MFR, which verified that the MFR, using *P. chrysosporium,* was a reproducible and scaleable process. It was concluded that this process, together with the necessary adjustments that suit the growth requirements, could also be applied to secondary metabolite production using other sporulating, filamentous fungi (Govender *et al.*, 2003). However, this bioreactor did not produce a linear scale-up of the secondary metabolites.

Synexa Life Sciences (Pty) Ltd has developed an improved scaleable process where the scale-up criteria from SFR to MFR are linear. The Quorus Bioreactor (QB) GLS (gas-liquid-solid) is a multi-membrane bioreactor for the culture of Synexa's microbial cells and is operated under optimal aerobic conditions. The new bioreactor shows uniform distribution of capillary membranes and aeration inlets which standardises oxygen delivery to the developing biofilm, irrespective of scale (Edwards and Leukes, 2007). The Quorus GLS module used in these experiments consists of 110 ceramic capillary membranes at a 2 L scale. A consistent supply of oxygen and nutrients to all membranes facilitates uniform and high cell density biofilm growth and

differentiation in a continuous bioprocess (Fraser, 2007). In this bioreactor, the scale-up criteria is defined by the number of membranes used and not volumetrically as described by Govender *et al.* (2003).

The MFR rig operation is fully automated, whereas the SFR rigs were operated manually. Therefore, the MFR rig permits constant operating conditions at set limits in a dynamic growth environment. Steady-state biofilm conditions are reached and sustained under optimised conditions. There are two separate fluid delivery chambers in the QB that allow controlled oxygen and nutrient supply to the organisms and for the collection of biomass-free secreted products from the organisms. The automated process design of the QB permits variation in pressure applied to the medium source in order to maintain a constant supply of nutrients to the biofilm, at a rate defined by the control flow rate set points, even as resistance to flow is increased by the growing biofilm. This allows for more reliable operating conditions than shown for SFRs. In the same way, variations in air flow velocity are regulated to offer a low-shear environment. The QB GLS is best suited to the growth of filamentous organisms and the production of toxic metabolites and/or recombinant products (Fraser, 2007).

The aim of the research presented in this chapter was to cultivate isolates WL61, WL114 and WL136 under controlled conditions on a MFR, the QB GLS, to produce large amounts of biomass (for intracellular product extraction) and permeate (for extracellular product extraction) for purification of larger levels of the active compounds observed in Chapter 6. Additionally, the relationship between the weights of biomass (g) extracted and the size of the zone of inhibition produced by the secondary metabolites from isolate WL61 in the well-diffusion bioassay was also evaluated. The objectives were thus:

- To produce large amounts of intracellular and extracellular active extracts for biological and subsequent chemical characterisation.
- To validate and standardise the well-diffusion bioassay method using isolate WL61.

7.2. MATERIALS AND METHODS

7.2.1. Pre-inoculum culture conditions

Pre-inoculum cultures of isolates WL61, WL114 and WL136 were prepared by inoculating 200 µl of glycerol stock solution into 3 x 40 ml of Marine broth (isolate WL61), M2 and SC broths (isolate WL114), and M2 broth (isolate WL136) in 100 ml Erlenmeyer flasks. Flasks were incubated at 26°C with shaking at 220 rpm for ten days. Test organisms were cultured and maintained as described in section 4.2.2.

7.2.2. Reactor setup

MFR rigs were set up for compound production at a 2 L scale. MFR modules are configured so that there are two separate fluid distribution systems. The first is defined as the extracapillary space (ECS), which is the space enclosed by a cylindrical glass manifold surrounding all the membranes. The ECS has two ports placed on the top and bottom head plates. A third air inlet is facilitated by air distribution lines, uniformly spread between the membranes improving oxygen delivery throughout the ECS. In its standard configuration, air enters through the air distribution lines and exits through the bottom ECS port.

A second fluid distribution system supplies the intracapillary space (lumen) of the membranes. In its standard configuration (Fig. 7.1), medium is supplied at the bottom medium inlet **(7)**, allowed to flow through the lumen of all membranes, and exit at the top medium outlet **(5)**. This top outlet is closed during operation (dead-end flow) **(mode 2 (5))**. The MFR module **(1)**, reticulation (tubing) and ancillaries is illustrated in Fig. 7.1. The ancillaries include the air humidification bottle **(A)** attached to the air distribution line, the inoculation bottle **(mode 1 (B))** and/or permeate collection vessel **(mode 2 (B))** attached to the bottom ECS outlet port **(2)**. The prime collection vessel **(mode 2 (C))**, attached to the medium outlet port **(5)**, the pressure gauges and the medium supply vessel **(mode 2 (D))** attached to the medium inlet port **(7)**, also form an essential part of the reticulation in the MFR rig.

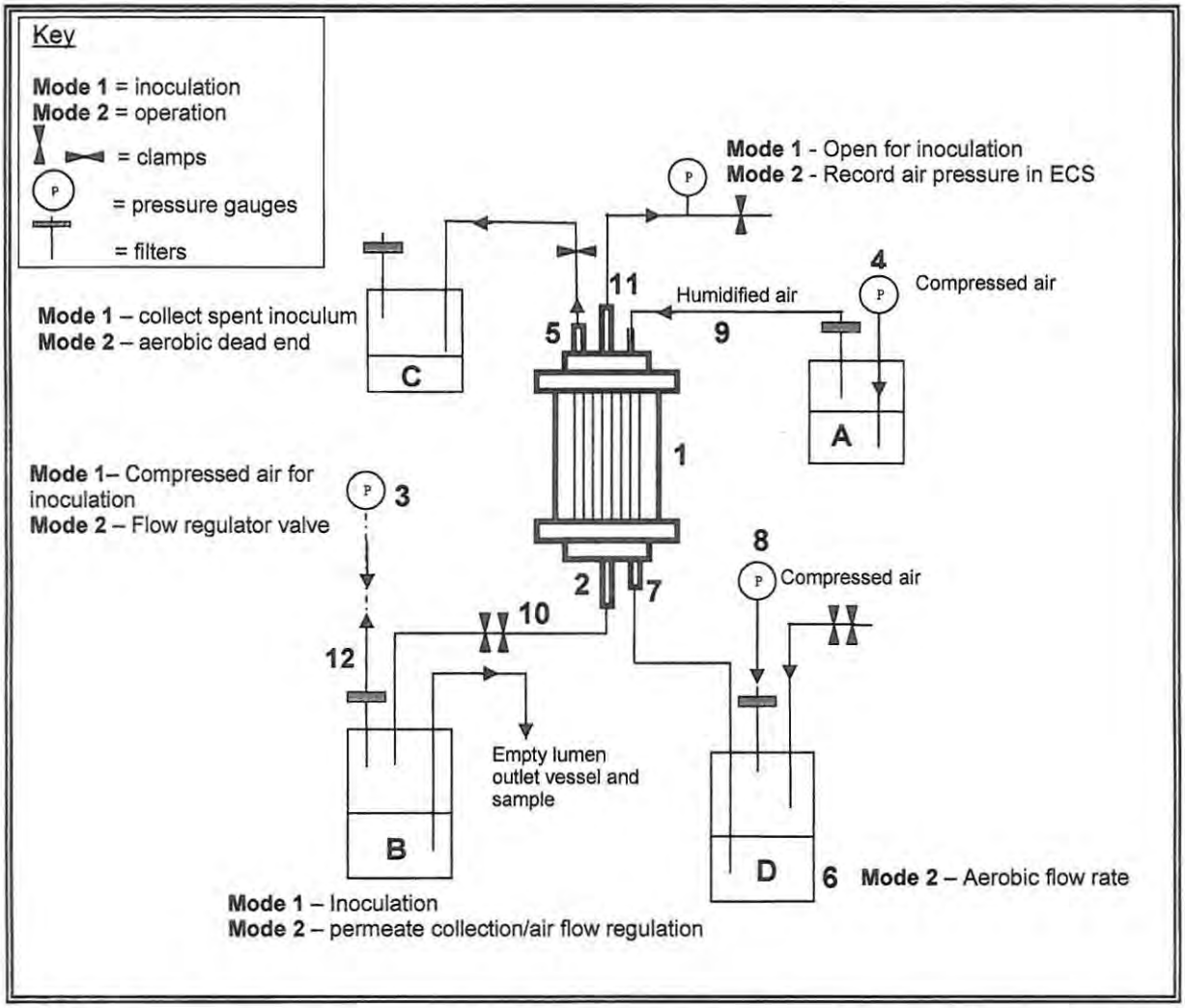


Fig. 7.1: Schematic representation of a MFR rig showing the inoculation **(mode 1)** and operation **(mode 2)** processes

7.2.3. Sterilisation and inoculation of MFR (mode 1)

The MFR, containing 110 membranes, was sterilised in an autoclave at 121°C for 20 min before inoculation. Fig. 7.1 shows the bioreactor setup for the inoculation process **(mode 1)** whereby the MFR **(1)** was inoculated by aseptically attaching a 2L inoculation bottle containing the relevant medium and 120 ml of pre-inoculum culture **(B)** to the inoculation line **(2)**. The inoculum culture was delivered into the reactor ECS using air pressure **(3)** until the ECS was filled. Air was allowed to exit via the ECS outlet port **(11)** after which this outlet was clamped shut. The inoculum culture was immobilised onto the membranes under pressure **(4)** by reverse filtration, whereby the inoculum suspension filling the ECS was displaced with air, forcing the cell free inoculum fluid through the membrane walls to be collected at the lumen side

of the membranes via the prime line **(5)** and into the prime collection vessel **(C)**. After inoculation, the inoculation bottle was removed and replaced with a permeate collection vessel for operation **(mode 2)**.

7.2.4. Aerobic operation of MFR (mode 2)

The MFRs were operated aerobically using the same medium as for the pre-inoculum cultures (section 7.2.1). Fig. 7.1 shows the operation process **(mode 2)** whereby the medium bottle **(D)** connected to the media inlet line, supplied medium to the biofilm from the lumen side of the membranes **(7)** using compressed air **(8)**. Fresh medium was primed through the lumen of the membranes into the prime collection vessel **(C)**, eliminating air blocks. The prime line **(5)** was then clamped closed during operation and the medium forced from the lumen side through the membrane walls and into the ECS. Compressed air **(4)**, supplying humidified air **(9)** from a sterile humidification vessel **(A)**, flowed through the ECS and expelled the permeate (medium filtrate) from the ECS **(10)** into the permeate collection vessel **(B)** along with the air **(12)**. During operation, the air flow rate was set to 2 ml/min using a flow regulator valve. The rate at which nutrients were supplied was set at 10 ml/hr until growth was established to prevent the biofilm from washing off of the membrane surface. The temperature was set at 26°C and the air pressure inside the ECS was set at 50 kPa to improve oxygen mass transfer and pressure control. As biofilm growth and resistance increased, the flow rate was monitored and adjusted accordingly to reflect SFR operating conditions. Daily samples of the permeate were collected from the permeate collection vessel **(B)** and pH and flow rates were recorded. Extracellular extracts were screened for antimicrobial activity as described in section 7.2.5.2. After 21 days the biomass was extracted and intracellular extracts were screened for antimicrobial activity as described in section 7.2.5.3.

7.2.5. Extraction processes and bioassay screening

7.2.5.1. Validation and standardisation of the well-diffusion bioassay using intracellular extracts from isolate WL61

The biomass obtained for isolate WL61 was divided into 0.1, 0.2, 0.5, 1.0 and 2.0 g fractions which were extracted on ice using 5 ml MeOH or 5 ml EtOAc solvent and sonication with a VirSonic 60 Virtis sonicator for 3 x 30 s bursts. The extracts were centrifuged at 4000 rpm for 10 min and supernatants screened for bioactivity against

Fusarium sp., *S. pombe* and *S. aureus* using the well-diffusion bioassay method (Appendix A). The sizes of the zones of inhibition for each of the fractionated weighed samples were compared to determine the quantitative status of the well-diffusion bioassay method.

Additionally, 2X, 5X, 10X and 50X dilutions of the 2.0 g biomass extract from isolate WL61 were analysed in order to validate these results. MeOH and EtOAc extracts were screened for bioactivity against *Fusarium* sp. and *S. pombe* using the well-diffusion bioassay method to investigate whether nutrients from the medium interfered with/absorbed to the active compounds, causing lower yields of the active compound, impacting the size of the zone of inhibition. All bioassays were carried out according to Appendix A.

7.2.5.2. Preparation of extracellular extracts

Permeate collected daily was extracted with equal volumes of EtOAc solvent (v/v). Extracts were combined at two or three day intervals and concentrated to 10 ml aliquots using a rotovap. Different test organisms, obtained from the well-diffusion bioassay results from SFR experiments (Chapter 6), were used to screen for bioactivity from each isolate. Concentrated extracts were screened for bioactivity against *P. fluorescens, Fusarium* sp. and *S. aureus* for isolate WL61; and *P. fluorescens*, *Fusarium* sp., *S. aureus, and S. pombe* for isolates WL114 and WL136, using the well-diffusion bioassay method (Appendix A).

7.2.5.3. Preparation of intracellular extracts

After 21 days growth, the membranes and biomass of isolates WL61, WL114 and WL136 were removed from the reactor and intracellular extracts were prepared. Isolate WL61 was extracted and screened for bioactivity as described in 7.2.5.1 above, while extracts of WL114 and WL136 were prepared from the biomass which was extracted using an Integral Systems Ultrasonicator bath for 20 min sessions in the presence of 300 ml MeOH or 300 ml EtOAc solvent. This was repeated three times, and for each isolate the combined volume of 900 ml solvent was concentrated 30-fold using a rotovap. Concentrated extracts were centrifuged at 4000 rpm for 10 min and the supernatant screened for bioactivity against *P. fluorescens*, *Fusarium* sp., *S. aureus, and S. pombe* using the well-diffusion bioassay method (Appendix A).

7.2.6. Substrate Analysis

Glucose substrate utilisation profiles were calculated from the level of residual sugars in the permeates obtained from isolates WL61, WL114 and WL136 (Appendix D) to monitor glucose utilisation during the time course study.

7.2.7. Statistical Analysis

Pearson's correlation analysis tool from the Analysis Toolpak provided by Microsoft Excel, to calculate the correlation between two measurement variables/parameters, was used to calculate the relationship between bioactivity from each isolate and flow rate, pH, or glucose concentration. Pearson's correlation coefficient values (r) were considered significant when the probability (p)> 0.95.

7.3. RESULTS AND DISCUSSION

7.3.1. Well-diffusion bioassay validation and standardisation

Theoretically, for a quantitative bioassay, the surface area (diffusion area) of the zone of inhibition should increase in a linear manner as the radius of the inhibition zone increases. The well-diffusion bioassay validation experiment was carried out using intracellular extracts from isolate WL61 to determine the quantitative status of the well-diffusion bioassay method. Theoretical zone values, listed in Table 7.1, were used to substitute real zone values so that the theoretical linear increase of the surface area could be determined, illustrated in Fig. 7.2 below.

Table 7.1: Theoretical zone size values

Well hole radius (mm)	Theoretical zone size (mm)	Total radius (mm)	Total area (mm^2)	Diffusion area (mm^2)
2.5			19.6	
	1	3.5	38.5	18.8
	2	4.5	63.6	44.0
	3	5.5	95.0	75.4
	4	6.5	132.7	113.0
	5	7.5	176.6	157.0
	6	8.5	226.9	207.2
	7	9.5	283.4	263.8
	8	10.5	346.2	326.6
	9	11.5	415.3	395.6
	10	12.5	490.6	471.0

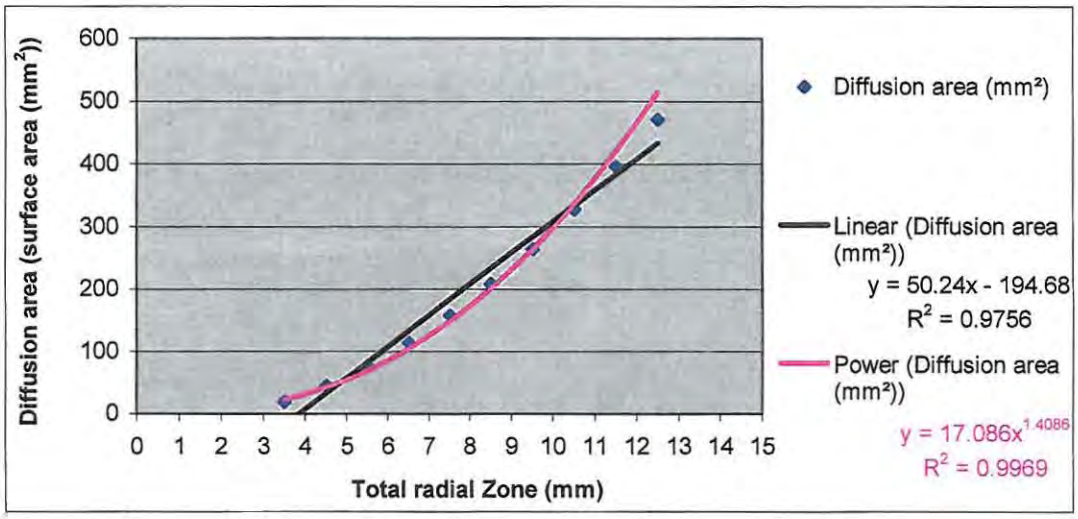


Fig. 7.2: Linearity of diffusion area of theoretical zones of inhibition

Zones of inhibition plotted against surface area show that the theoretical linear graph ($y = mx + c$) had an R^2 value of 0.975, whereas the theoretical power graph showed an R^2 value of 0.996. The R^2 value for the linear graph is very close to 1, thus it can be classified as a semi-quantitative bioassay as the surface area increased when the radius of the zone of inhibition increased. Since this increase was linear when using the equation: $y = mx + c$, it was expected that the same would be true when the real zone values were used. Real zone of inhibition values against *Fusarium* sp. and *S. pombe* are illustrated in Figs 7.3 and 7.4, respectively, below.

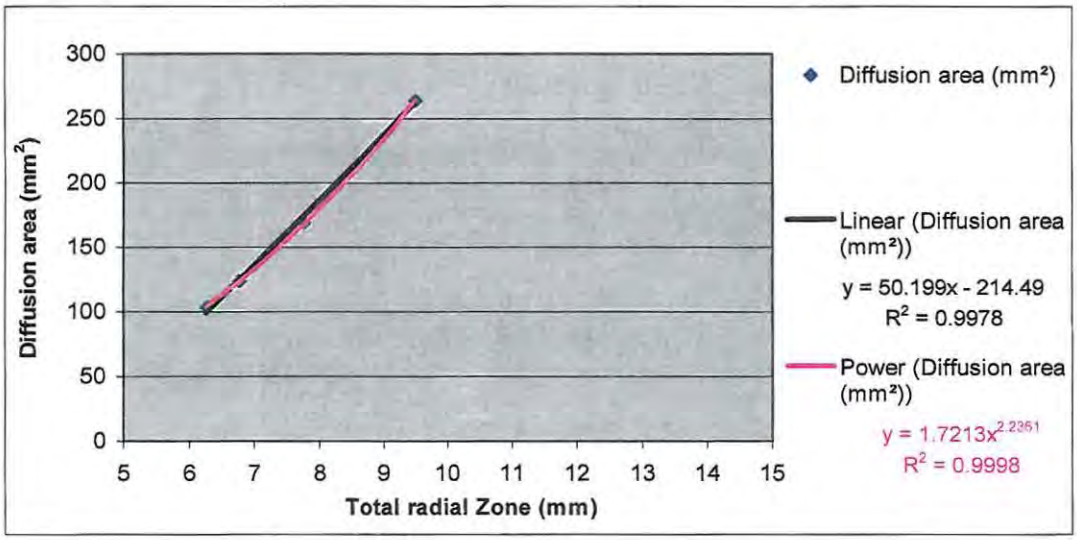


Fig. 7.3: Linearity of diffusion area of zones of inhibition against *Fusarium* sp.

Intracellular MeOH extracts active against *Fusarium* sp. showed an R^2 value of 0.997 for the linear graph and an R^2 value of 0.999 for the power graph. The radius of the zones increased as diffusion area increased and 0.997 is very close to 1, thus the increase was linear.

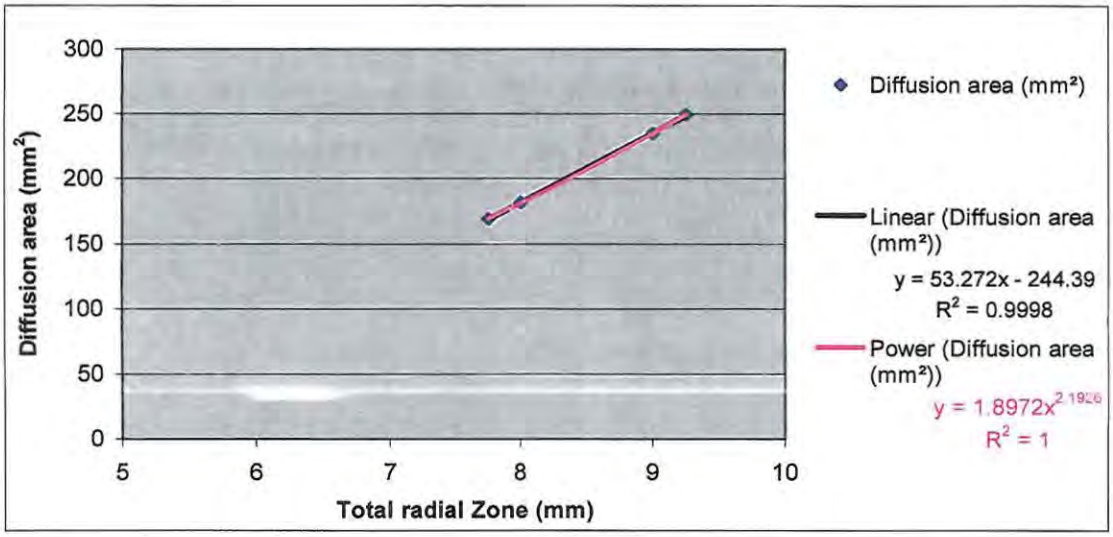


Fig. 7.4: Linearity of diffusion area of zones of inhibition against *S. pombe*

Intracellular MeOH extracts active against *S. pombe* showed an R^2 value of 0.999 for the linear graph and an R^2 value of 1 for the power graph. Similarly, the diffusion area increased as the radius of the zone increased and 0.999 is very close to 1, thus the increase was also linear.

Additionally, the relationship between biomass (DCW) of isolate WL61 and size of the zone of inhibition was investigated and summarised in Table 7.2 and illustrated in Figs. 7.5 and 7.6 below.

Table 7.2: Activity zones from intracellular extracts from isolate WL61 biomass on MFR

DAY	WET WEIGHT (g)	TEST ORGANISM	EXTRACT	DRY WEIGHT (g)	ZONE (mm)	SD
21	0.1	*Fusarium* sp.	Intracellular EtOAc	0.029	0.75	0.53
	0.2			0.051	0.75	0.53
	0.5			0.094	0.75	0.53
	1.0			0.164	0.50	0.35
	2.0			0.417	0.50	0.35
	0.1	*Fusarium* sp.	Intracellular MeOH	0.016	3.75	0.18
	0.2			0.023	4.25	0.18
	0.5			0.056	5.25	0.18
	1.0			0.134	7.00	0.35
	2.0			0.268	7.00	0.35
	0.1	*S. pombe*	Intracellular MeOH	0.016	5.25	0.18
	0.2			0.023	5.50	0.35
	0.5			0.056	6.50	0.00
	1.0			0.134	6.75	0.18
	2.0			0.268	6.50	0.35

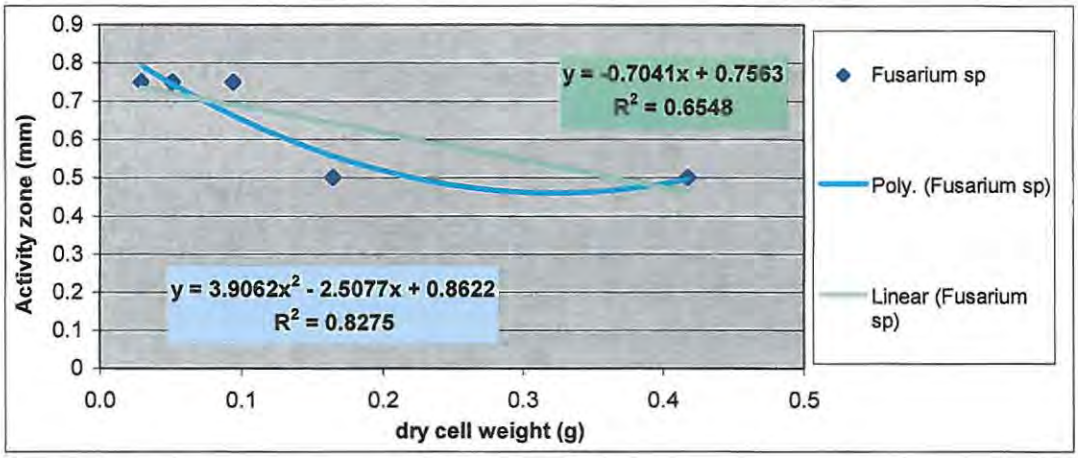


Fig. 7.5: Activity zones from isolate WL61 biomass (DCW) against *Fusarium* sp.

Zones of inhibition observed from intracellular EtOAc extracts against *Fusarium* sp. decreased as the DCW (g) increased. The R^2 value obtained from the polynomial graph (poly.) was 0.827 and the linear graph showed an even lower R^2 value of 0.655. The relationship between biomass (DCW) and size of the zone of inhibition was thus, not linear. This could be due to the fact that the single 5 ml volume of solvent used to extract the biomass (particularly 1.0 g and 2.0 g fractions) became saturated, causing a significantly lower yield of active compound. However, this could also be due to nutrient interference from the growth medium, still present in the

biomass, absorbing the active compound during the extraction process. In further experiments, all intracellular extracts were prepared by extracting the biomass three times with fresh solvent.

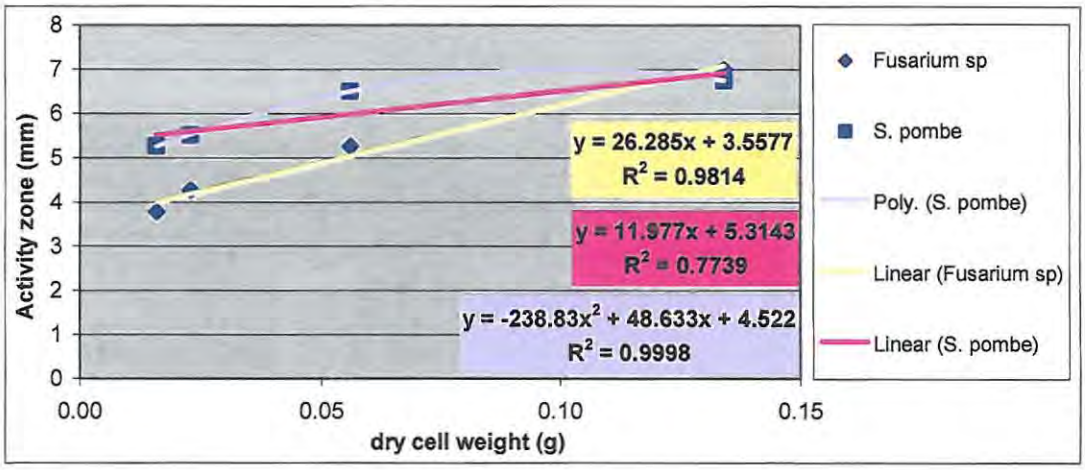


Fig. 7.6: Activity zones from isolate WL61 biomass (DCW) against *Fusarium* sp. and *S. pombe*

Zones of inhibition observed from intracellular MeOH extracts active against *Fusarium* sp. and *S. pombe* both increased as DCW (g) increased. The R^2 values obtained from the linear graph for *Fusarium* sp. and *S. pombe* was 0.981 and only 0.774, respectively. The R^2 value obtained from the polynomial graph for *S. pombe* was 0.999. This indicates that the relationship between the biomass (DCW) and zone sizes against *Fusarium* sp. was linear as 0.981 is very close to 1. Conversely, this relationship was not linear for *S. pombe* as the polynomial graph illustrated the best fit (seen by the R^2 value of 0.999).

Nutrient interference and solvent saturation limits may impact the yield of biologically active compound(s) during the extraction process resulting in smaller zones of inhibition. This was investigated by diluting the active extracts, thus further diluting nutrients from the growth medium that may possibly absorb active compound(s), illustrated in Fig. 7.7 below.

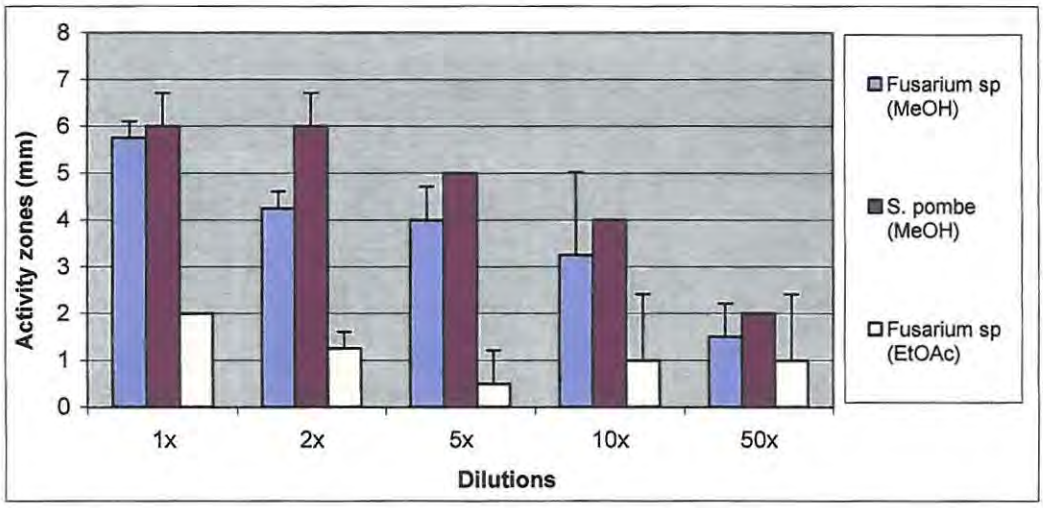


Fig. 7.7: Activity zones from diluted MeOH and EtOAc extracts from 2.0 g isolate WL61 biomass

Zones of inhibition, produced from 2.0 g fraction intracellular MeOH extracts active against *Fusarium* sp. and *S. pombe*, decreased steadily until 50X diluted. Activity was still detected in the 50X diluted extract, indicating that the active compound(s) is either a potent antimicrobial metabolite and/or the metabolite may have been masked by other compounds present in the extract, before being diluted 50X.

Zones of inhibition observed from intracellular EtOAc extracts active against *Fusarium* sp. decreased steadily until 5X diluted. The slight increase in zone size from 10 and 50X diluted extracts was negligible. The zones of inhibition decreased as the dilutions of the extracts increased, thus the nutrients from the growth medium did not interfere with/absorb the intracellular active compound(s). The low yield of intracellular active compound(s) was therefore attributed to saturation of the extraction solvents which could not extract additional active compound(s).

7.3.2. Cultivation of isolates WL61, WL114 and WL136 on MFR

Four MFRs were used to cultivate isolates WL61 using Marine broth; WL114 using M2 and SC broths; and WL36 using M2 broth. Growth of these isolates on MFRs is demonstrated in Figs. 7.8 - 7.10.

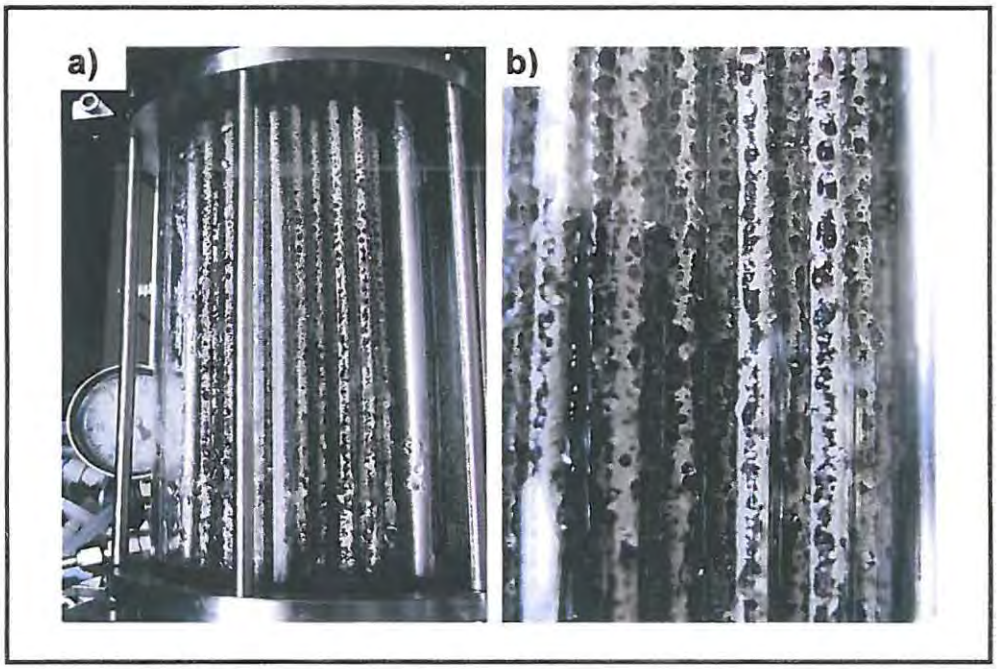


Fig. 7.8: Growth of isolate WL61 on MFR in Marine broth showing sporulating cells

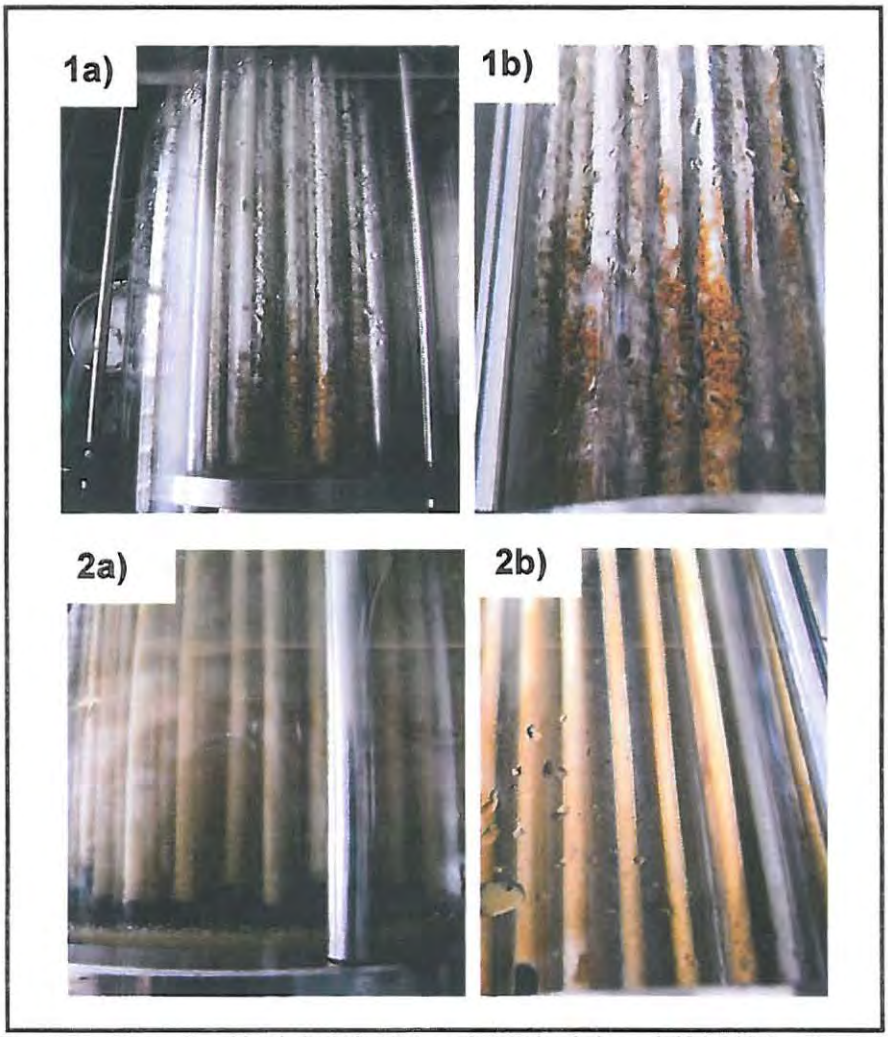


Fig. 7.9: Growth of isolate WL114 on MFR in **1a)** and **1b)** M2 broth and in **2a)** and **2b)** SC broth

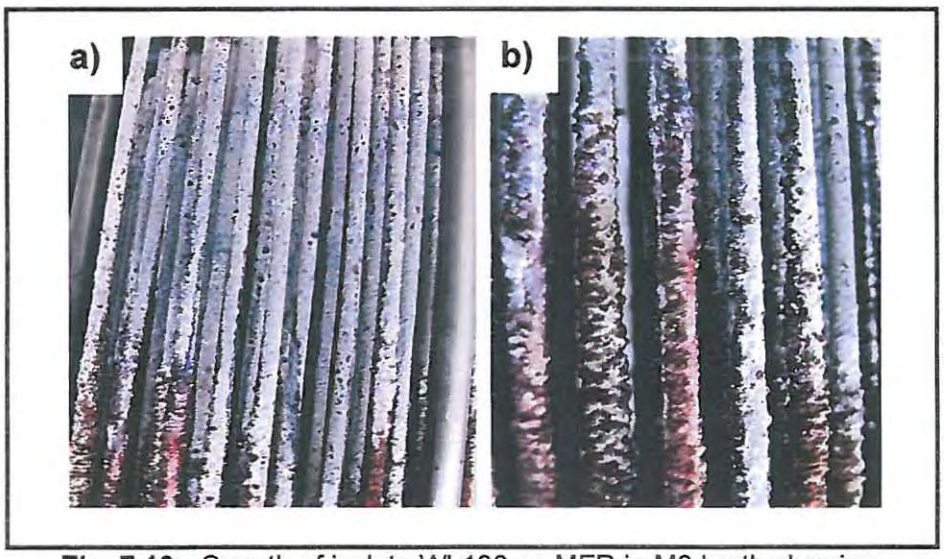


Fig. 7.10: Growth of isolate WL136 on MFR in M2 broth showing sporulating cells

7.3.2.1. Extracellular activity of isolate WL61

Bioactivity of extracts from each isolate is described below. The flow rate for isolate WL61 cultured on MFR using Marine broth and the pH of the permeate were monitored daily. Extracts were combined as outlined in Table 7.3 and bioactivity is illustrated in Fig. 7.11.

Table 7.3: Combined extracts from MFRs

DAYS POST INOCULATION	SAMPLE
1 - 3	1
4 - 6	2
7 - 8	3
9 – 10	4
11 - 13	5
14 - 15	6
16 - 17	7
18 - 19	8
20 - 21	9

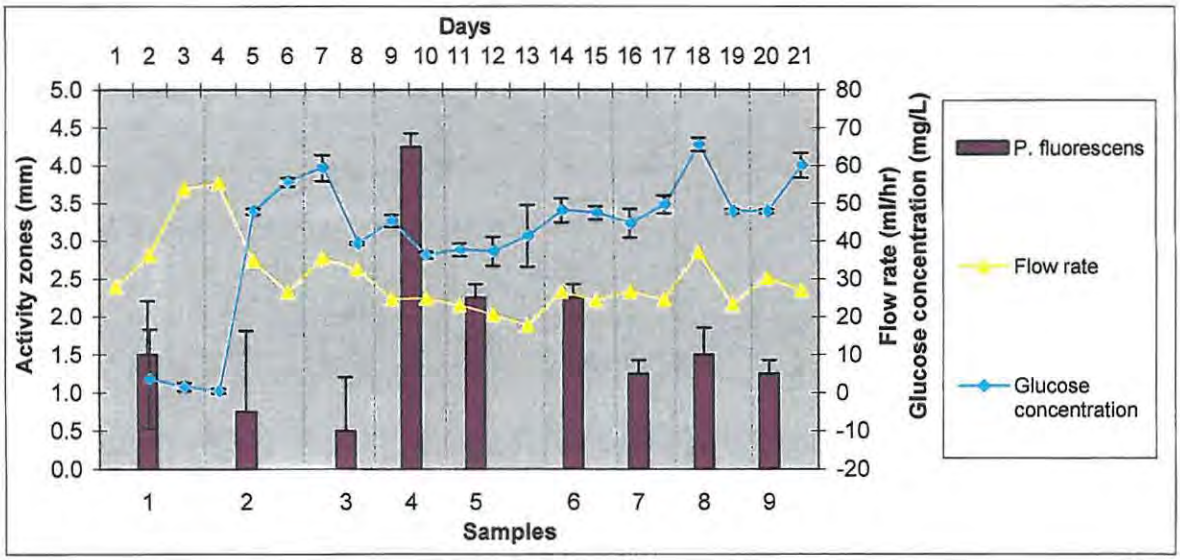


Fig. 7.11: Extracellular bioactivity from isolate WL61 cultivated on MFR in Marine broth

While no activity was detected from MeOH extracts over the 21 day time-course study, extracellular EtOAc extracts (samples 1 – 9) were active against *P.*

fluorescens only. Bioactivity against *P. fluorescens* is expressed as the size of the zone of inhibition (mm).

Smaller zones of inhibition were detected against *P. fluorescens* between days 1 and 8 (samples 1 - 3) where sample 3 showed the lowest activity (0.50 ± 0.71 mm). This was attributed to the high flow rate between days 3 (53.88 ml/hr) and 4 (55.32 ml/hr), resulting in insufficient nutrient limitation, eliminating stress caused by nutrient limitation and reducing the production of metabolites. While the flow rate was high between days 2 and 4, the level of glucose was low as isolate WL61 exhausted residual carbohydrates in the medium. Isolate WL61 most likely produced enzymes, degrading complex carbohydrates in the medium into reducing sugars for metabolism, thereby increasing the glucose concentration level from day 5. The level of activity from extracellular EtOAc extracts was greatest between days 9 and 15 (samples 4 - 6) as the flow rate stabilised (23.1 ml/hr). The flow rate then increased slightly and fluctuated, resulting in lower levels of active compound(s) from day 16 to 21 (samples 7 – 9), due to insufficient nutrient limitation.

Table 7.4 shows the correlation between bioactivity from isolate WL61 and flow rate, pH or glucose concentration. Although none of these were considered significant ($p < 0.95$), the parameter most closely linked to bioactivity was flow rate ($r = -0.57$). This relationship shows a trend towards increased bioactivity with a decrease in flow rate. The pH of the permeate was relatively constant, increasing gradually from pH 7.84 on day 1 to pH 8.30 on day 21 with the highest value of 8.37 on day 13 (data not shown).

Table 7.4: Pearson's Correlation coefficients (r) for isolate WL61

Parameters	Bioactivity against *P. fluorescens*	Flow rate	pH	Glucose concentration
Flow rate	-0.57	1.00		
pH	0.48	-0.94	1.00	
Glucose concentration	0.10	-0.58	0.62	1.00

7.3.2.2. Bioactivity of isolate WL114

The flow rate and bioactivity of extra- and intracellular extracts from isolate WL114 cultured on MFR using M2 and SC broth is illustrated in Fig. 7.12 below. The flow rate (ml/hr) and the pH of the permeates were monitored daily. Extracts were

combined every third day from day 1 to 21 (samples 1 – 7) and bioactivity is expressed as the size of the zone of inhibition (mm).

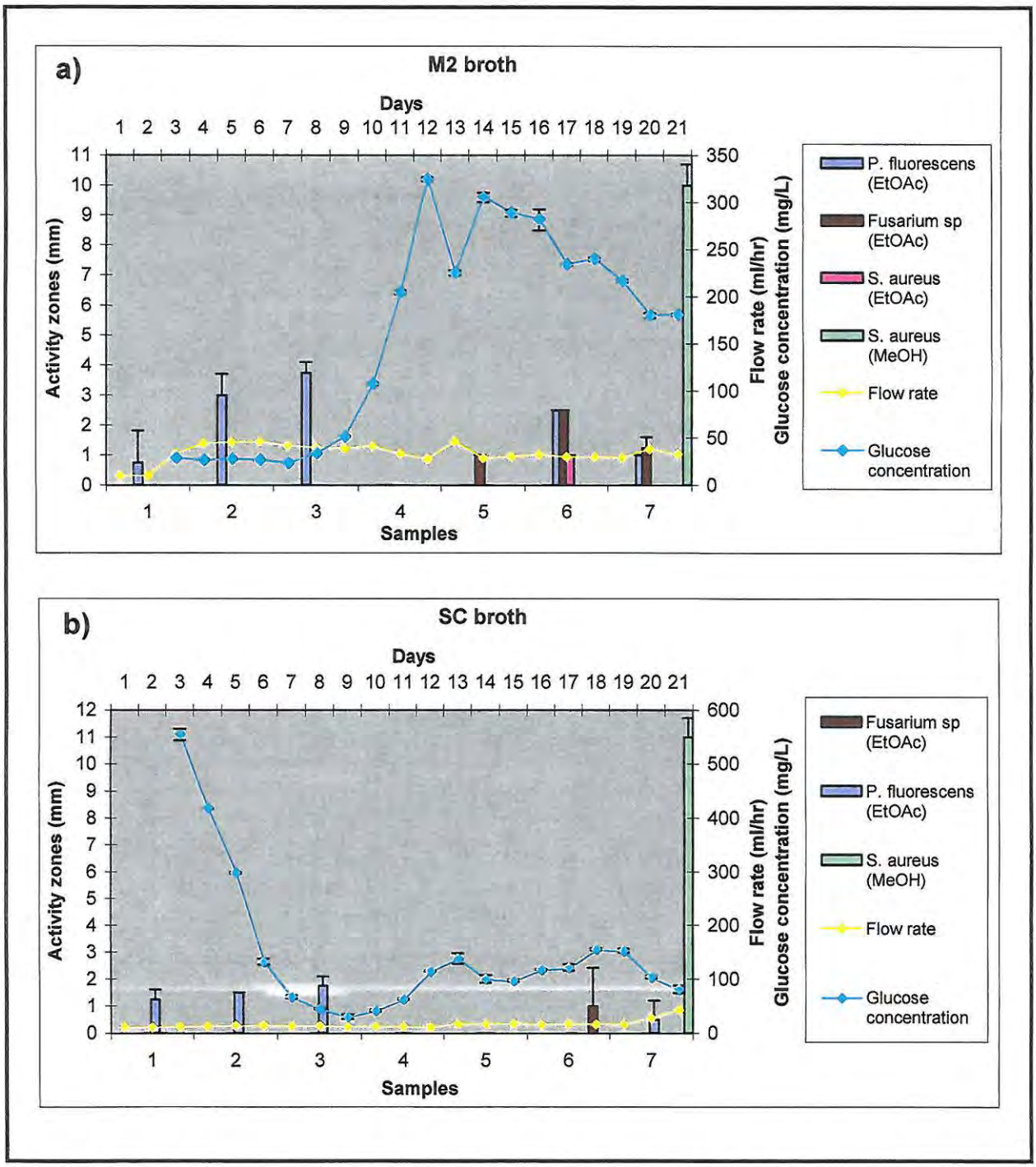


Fig. 7.12: Extracellular (EtOAc) and intracellular (MeOH) bioactivity from isolate WL114 cultivated on MFR in M2 and SC broth

Extracellular EtOAc extracts from isolate WL114 cultured in M2 broth were active against *P. fluorescens*, *Fusarium* sp. and *S. aureus,* whereas activity from growth in SC broth was only detected against *P. fluorescens* and *Fusarium* sp. Intracellular MeOH extracts, from growth in M2 and SC broths (samples 7 on day 21), were both active against *S. aureus* only.

When M2 broth was used for growth, activity from extracellular EtOAc extracts was detected against *P. fluorescens* between days 1 and 9 (samples 1 - 3) with the highest activity from sample 3 (3.0 ± 0.71 mm). This was most likely due to nutrient limitation between days 3 and 8, resulting in stress and the production of metabolites in response to this stress. This was supported by the lower levels of glucose present in the permeate during this period. The glucose concentration increased considerably after day 9, possibly due to enzyme degradation of glycerol, present in M2 broth, into glyceraldehyde-3-phosphate which enters the gluconeogenesis pathway, and converted into glucose. A lower level of activity against *P. fluorescens* was observed again after the flow rate decreased between day 16 and 19 (samples 6 and 7). As the biofilm continued to grow, the glucose levels decreased. Table 7.5 supports the observed correlation between bioactivity from isolate WL114 in M2 broth and glucose concentration ($r = -0.62$). This correlation is however not significant ($p<0.95$), but does show a trend towards an increase in bioactivity against *P. fluorescens* with a decrease in glucose concentration.

EtOAc extracts active against *Fusarium* sp. were detected from days 13 to 21 (samples 5, 6 and 7). Activity against *Fusarium* sp. was highest from sample 6 (2.5 ± 0.0 mm), possibly due to nutrient limitation and an increase in stress after the flow rate decreased, and/or growth of the biofilm with M2 broth had reached steady state. Although the relationship between bioactivity against *Fusarium* sp. and flow rate was not significant, it shows a tendency towards an increase in bioactivity with a decrease in flow rate ($r = -0.50$) and elevated glucose levels ($r = 0.63$) (Table 7.5). The production of compound(s) active against *Fusarium* sp. may have been due to nutrient limitation by a nutrient source other than glucose, such as phosphate, sulphate or nitrogen. Additionally, the relationship between bioactivity and pH showed a trend towards an increase in bioactivity with a decrease in pH ($r = -0.59$), probably due to the production of organic acids by isolate WL114 as it metabolised

glucose and complex carbohydrates in the medium. The pH of the permeate decreased gradually from pH 8.00 on day 1 to pH 7.29 on day 21 with the lowest value of 6.57 on day 14 (data not shown).

Table 7.5: Pearson's Correlation coefficients (r) for isolate WL114 cultured in M2 broth

Parameters	Bioactivity against *P. fluorescens*	Bioactivity against *Fusarium* sp.	Bioactivity against *S. aureus*	Flow rate	pH	Glucose concentration
Flow rate	0.42	-0.50	-0.28	1.00		
pH	0.59	-0.59	-0.16	0.68	1.00	
Glucose concentration	-0.63	0.63	0.22	-0.74	-0.95	1.00

Similarly, when SC broth was used for growth, extracellular EtOAc activity was detected against *P. fluorescens* between day 1 and 9 (samples 1 - 3) with the highest activity from sample 3 (1.75 ± 0.35 mm). The flow rate for this MFR was very low throughout the 21 day growth period in comparison to the flow rate observed for the MFR using M2 broth. While the lower flow rate should have resulted in more concentrated extracellular extracts, this was not the case. Lower flow rates may have resulted in washing less metabolite(s) away from the biofilm into the permeate as extracellular compound(s), resulting in lower levels of activity against *P. fluorescens* and *Fusarium* sp. Alternatively, the nutrient content of SC broth may not have been optimal. In contrast to M2 broth, activity against *P. fluorescens* corresponded with higher levels of glucose remaining in the growth medium, therefore the production of extracellular compound(s) active against *P. fluorescens* was not induced by glucose limitation. Table 7.6 shows that none of the parameters measured corresponded with bioactivity, although pH and bioactivity showed a trend towards increased bioactivity when pH was higher (r = 0.65). Low levels of activity were observed against *P. fluorescens* again between days 19 and 21 (sample 7).

Low levels of activity from EtOAc extracts were observed against *Fusarium* sp. between day 16 and 18 (sample 6) only. Table 7.6 confirmed that the relationship between bioactivity against *Fusarium* sp. and the parameters measured were not significant, although there was a trend towards increased bioactivity with a decrease in pH (r = -0.73) and increased flow rate (r = 0.63). The pH of the permeate decreased gradually from pH 6.80 on day 1 to pH 6.02 on day 21 with the lowest value of 5.91 on day 19 (data not shown). Therefore, the production of metabolites

active against *Fusarium* sp. may be linked to the production of organic acids by isolate WL114 and/or limitation of nutrients other than glucose.

Table 7.6: Pearson's Correlation coefficients (r) for isolate WL114 cultured in SC broth

Parameters	Bioactivity against *P. fluorescens*	Bioactivity against *Fusarium* sp.	Flow rate	pH	Glucose concentration
Flow rate	-0.22	0.63	1.00		
pH	0.65	-0.73	-0.59	1.00	
Glucose concentration	0.29	-0.15	-0.21	0.36	1.00

Activity from intracellular MeOH extracts was detected against *S. aureus* only. Large zones of inhibition were observed from biofilms cultured using both M2 broth (10 mm) and SC broth (11 mm) on day 21 (samples 7). This suggests that the active compound(s) detected in extracellular extracts were actively secreted by isolate WL114 biofilms.

Extracts of the highly active intracellular compound(s), as well as extracts from the less active extracellular compound(s) produced, were kept for chemical analysis as described in Chapters 8 and 9.

7.3.2.3. Bioactivity of isolate WL136

The flow rate and bioactivity of extra- and intracellular extracts from isolate WL136 cultured on MFR using M2 broth is illustrated in Fig. 7.13 below. The flow rate (ml/hr) and the pH of the permeates were monitored daily. Extracts were combined every third day from day 1 to 21 (samples 1 – 7) and bioactivity is expressed as the size of the zone of inhibition (mm).

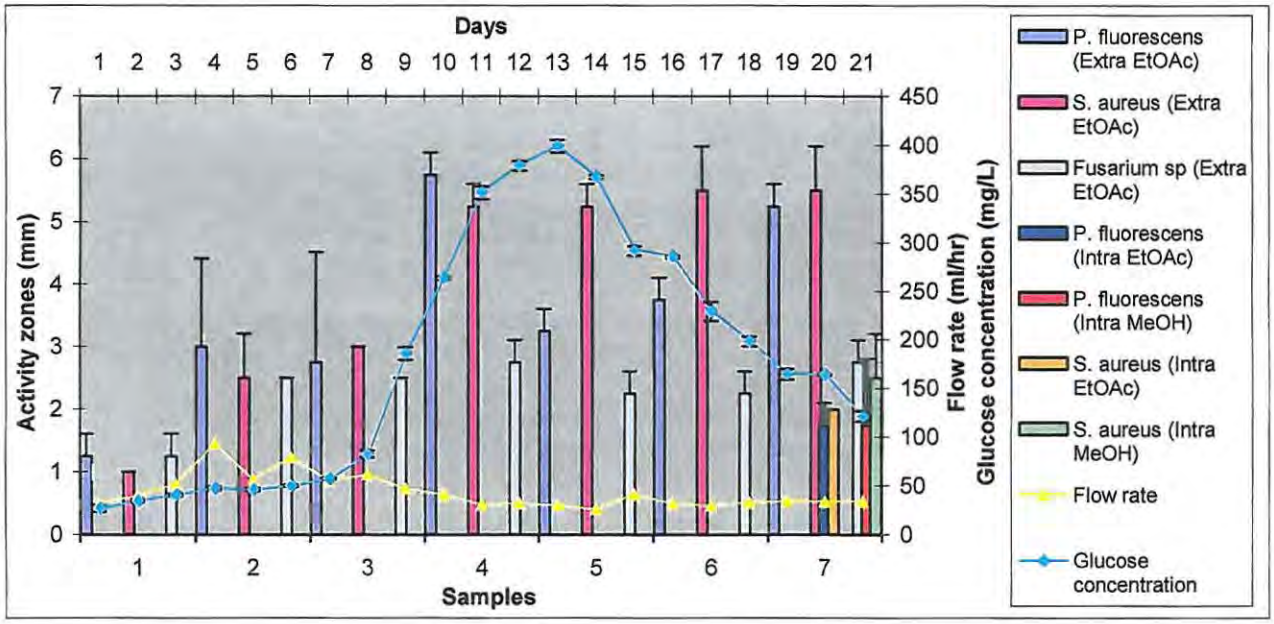


Fig. 7.13: Extracellular EtOAc and intracellular MeOH and EtOAc bioactivity from isolate WL136 cultivated on MFR in M2 broth

Extracellular EtOAc extracts were active against *P. fluorescens*, *S. aureus* and *Fusarium* sp. where higher activity was observed against *P. fluorescens* and *S. aureus* than against *Fusarium* sp. Intracellular MeOH and EtOAc extracts were both active against *P. fluorescens* and *S. aureus* only on day 21 (samples 7).

Activity against *P. fluorescens* were detected throughout the 21 day period (samples 1 - 7) whereby zones of inhibition increased in size between day 1 and 12, decreasing considerably between day 13 and 15 then increased slightly until day 21. The highest level of activity against *P. fluorescens* was detected between day 10 and 12 (sample 4) (5.75 ± 0.35 mm). Lower levels of activity from extracellular EtOAc extracts were detected between day 1 and 9 (samples 1 – 3) due to the early growth phase of isolate WL136 and very high flow rate on days 4 (93.3 ml/hr), 5 (56.1 ml/hr), 6 (79.1 ml/hr), 7 (54.8 ml/hr) and 8 (61.7 ml/hr), eliminating stress thereby limiting metabolite production. However, higher flow rate and greater nutrient delivery has been shown to increase growth rate and biomass formation, which in turn impacts on the amount of product produced. The increased bioactivity against *P. fluorescens* was possibly due to nutrient limitation as the flow rate decreased and stabilised from day 9, resulting in steady-state growth, nutrient gradients and stress, increasing the production of metabolites in response to this stress. The glucose levels increased

from day 7, probably due to the production of other secondary metabolites such as enzymes, which degrade the glycerol present in M2 broth, into glyceraldehyde-3-phosphate, which enters the gluconeogenesis pathway and is converted into glucose. The relationship between glucose concentration and bioactivity against *P. fluorescens* in Table 7.7 was not significant, but shows a trend towards increased bioactivity with an increase in glucose concentration (r = 0.57). Increased bioactivity after day 7 was possibly due to nutrient limitation of another nutrient source other than glucose, such as phosphate, sulphate or nitrogen. The relationship between pH and bioactivity against *P. fluorescens* shows a trend towards increased bioactivity with a decrease in pH (r = -0.65), probably due to the production of organic acids by isolate WL136 as it metabolised glucose in the medium. The pH of the permeate decreased gradually from pH 7.94 on day 1 to pH 4.54 on day 21 (data not shown).

Activity against *Fusarium* sp. was detected throughout the 21 day period (samples 1 - 7) whereby zones of inhibition increased in size between day 1 and 12, decreasing slightly between day 13 and 18, then increased slightly until day 21. The highest level of activity against *Fusarium* sp. was detected between day 10 and 12 (sample 4) and day 19 and 21 (sample 7) (2.75 ± 0.35 mm). Lower levels of activity from extracellular EtOAc extracts were detected between day 1 and 9 (samples 1 – 3) due to the early growth phase of isolate WL136 and very high flow rate on days 4 (93.3 ml/hr), 5 (56.1 ml/hr), 6 (79.1 ml/hr), 7 (54.8 ml/hr) and 8 (61.7 ml/hr), eliminating stress thereby limiting metabolite production, as described with *P. fluorescens*. Similarly, the increased bioactivity against *Fusarium* sp. was possibly due to nutrient limitation as the flow rate decreased and stabilised from day 9, resulting in steady-state growth, nutrient gradients and stress, increasing the production of metabolites in response to this stress. Table 7.7 shows that none of the parameters measured corresponded to bioactivity against *Fusarium* sp.

Activity against *S. aureus* was detected throughout the 21 day period (samples 1 - 7) whereby zones of inhibition increased in size between day 1 and 21. The highest level of activity against *S. aureus* was detected between day 16 and 21 (samples 6 and 7) (5.50 ± 0.71 mm). Lower levels of activity from extracellular EtOAc extracts were detected between day 1 and 9 (samples 1 – 3) due to the early growth phase of isolate WL136 and very high flow rate on days 4 (93.3 ml/hr), 5 (56.1 ml/hr), 6 (79.1

ml/hr), 7 (54.8 ml/hr) and 8 (61.7 ml/hr), eliminating stress thereby limiting metabolite production, as described with *P. fluorescens* and *Fusarium* sp. Similarly, increased bioactivity against *S. aureus* was possibly due to nutrient limitation of another nutrient source other than glucose, such as phosphate, sulphate or nitrogen as the flow rate decreased and stabilised from day 9, resulting in steady-state growth, nutrient gradients and stress, increasing the production of metabolites in response to this stress. While the relationships between the parameters measured were not significant, it does show a trend towards increased bioactivity against *S. aureus* with an increase in glucose concentration (r = 0.78) and decrease in flow rate (r = -0.55) and pH (r = -0.90) (Table 7.7). The glucose levels increased from day 7, due to enzyme degradation of glycerol present in M2 broth, into glyceraldehyde-3-phosphate, which enters the gluconeogenesis pathway and converted into glucose, as described previously. The production of organic acids by isolate WL136 was probably due to the metabolism of glucose in the medium. The pH of the permeate decreased gradually from pH 7.94 on day 1 to pH 4.54 on day 21 (data not shown).

Table 7.7: Pearson's Correlation coefficients (r) for isolate WL136

Parameters	Bioactivity against *P. fluorescens*	Bioactivity against *Fusarium* sp.	Bioactivity against *S. aureus*	Flow rate	pH	Glucose concentration
Flow rate	-0.33	0.07	-0.55	1.00		
pH	-0.65	-0.43	-0.90	0.66	1.00	
Glucose concentration	0.57	0.37	0.78	-0.61	-0.60	1.00

Activity from MeOH and EtOAc intracellular extracts was detected against *P. fluorescens* and *S. aureus* on day 21 (sample 7). These zones of inhibition were considerably smaller in comparison to extracellular EtOAc extracts. Lower or more consistent flow rates and nutrient limitation allow metabolites to accumulate intracellularly, washing them away from the biofilm at the same rate. This indicates that isolate WL136 produced a lower level of intracellular metabolites or that the activity of extracellular non-polar (EtOAc) compound(s) was more potent.

Extracts of active extra- and intracellular compound(s) produced by isolate WL136 were kept for chemical analysis as described in Chapters 8 and 9.

7.4. CONCLUSION

The well-diffusion bioassay validation experiment using isolate WL61 showed that an increase in DCW did increase the size of the zone of inhibition in a linear manner for intracellular MeOH extracts against *Fusarium* sp. only. Furthermore, as the radius of the zone of inhibition increased, the surface area (diffusion area) of the zone also increased in a linear manner. The results obtained from the intracellular EtOAc active extracts were characterised by large SDs and were non-linear, possibly due to the fact that EtOAc is not miscible with water and thus does not penetrate wet biomass sufficiently to break open cells and extract metabolites. Consequently no further experiments were carried out using intracellular EtOAc active extracts from isolate WL61. Thus, measuring zones of inhibition from well-diffusion bioassays can be utilised as a semi-quantitative method to determine optimum conditions for the production of biologically active compound(s).

In comparison to previous shake flask and SFR experiments (Chapters 5 and 6), the production of extracellular active compound(s) by isolate WL61 was more consistent on MFRs. Extracellular active compound(s) produced from SFRs and MFRs were both active against the same test organism, *P. fluorescens* and the sizes of zones of inhibition were similar in Marine broth.

Similarly, the production of active compound(s) from isolate WL114 on MFR using M2 broth was more consistent. The average flow rate of SFRs using M2 broth was 0.33 ml/hr (Chapter 6, Table 6.3), thus the average flow rate of MFR should have been 36.3 ml/hr for linearity of scale-up from one membrane to 110 membranes. However, the average flow rate of this MFR was 33.7 ml/hr, relatively close to the flow rate for SFRs. Conversely, the average flow rate of MFR with isolate WL114 using SC broth should have been 29.7 ml/hr for linearity of scale-up from one membrane to 110 membranes, instead it was considerably lower (15.3 ml/hr) than for SFRs (0.27 ml/hr) (Chapter 6, Table 6.3), resulting in less consistent production of active compound(s) from on MFR over 21 days. This lower flow rate was caused by fouling of the membranes due to starch present in the SC growth medium, thus the same growth conditions for metabolite production could not be obtained on the MFR using SC broth.

The production of active compound(s) from isolate WL136 on MFR using M2 broth was more consistent than SFR experiments since the higher flow rate produced a thicker biofilm, which in turn provided more consistent gradients. The average flow rate of SFRs using M2 broth was 0.45 ml/hr (Chapter 6, Table 6.4), thus the average flow rate of MFR should have been 49.5 ml/hr for linearity of scale-up from one membrane to 110 membranes. However, the average flow rate of this MFR was 43.4 ml/hr, relatively close to the flow rate for SFRs. Additionally, the sizes of the zones of inhibition were similar from MFR experiments in comparison to SFR experiments.

The MFR was successful in generating large amounts of intracellular and extracellular active product from biomass and permeate extracts produced from all three isolates, under similar preliminary SFR conditions, allowing further analysis and metabolite profiling of active compound(s). These bioactive extracts will be examined in more detail in Chapters 8 and 9.

CHAPTER 8

Stability of active compound(s) from extracts from isolates WL61, WL114 and WL136 and their mode of action (biocidal or biostatic)

8.1. INTRODUCTION

Isolates WL61, WL114 and WL136 were shown to produce bioactive compound(s) with antifungal and antibacterial properties against different test organisms in well-diffusion bioassays. Many bioactive compounds are known to lose activity if exposed to elements such as heat or light. Typically, ambient temperatures and storage conditions can result in loss of activity of active compounds over time (Catron and Mann, 2000). By way of illustration, the active compound(s) produced by the marine bacterium, *Pseudoalteromonas* AP5 was shown to be highly photosensitive as there was rapid loss of activity after only two hours exposure to light (described in Chapter 2). It was therefore necessary to assess the stability and degradation rate of the active compound(s) from extracts produced by marine isolates WL61, WL114 and WL136 over 24 hrs.

Microorganisms are capable of producing substances which are used in human and animal medicine, however most microbial products are inclined to be toxic, or have side effects, and for that reason cannot be used in clinical practice (Běhal, 2006). To reduce the side effects of antibiotics and other bioactive metabolites, their mode of action should also be studied. A biostatic compound/metabolite is known to inhibit further growth of microorganisms without killing them. These compounds act by, for example, binding weakly to ribosomes and inhibiting protein synthesis. However, when the concentration of the biostatic compound is lowered, the compound is freed from the ribosome and growth of the microorganism continues. A biocidal compound or metabolite is known to kill cells as they bind strongly to their cellular targets preventing the growth of the microorganism (Madigan *et al.*, 1997).

The knowledge of the mode of action of bioactive substances provides the potential mode of damage to the macroorganism as well as assisting in the identification of enzyme reaction sequences in metabolic pathways. Similarly, molecular biology

studies can assess the mode of action of bioactive substances at a molecular level (Běhal, 2006).

The research presented in this chapter aims to assess the stability of the active compound(s) from isolates WL61, WL114 and WL136 by exposing the extracts to different temperatures and light and dark conditions over 24 hrs. Additionally, the biocidal or biostatic mode of action of the active compound(s) for each extract was established.

8.2. MATERIALS AND METHODS

8.2.1. Temperature stability

Intracellular extracts from isolates WL61, WL114 and WL136 obtained from MFR experiments on day 21, as well as extracellular extracts from isolate WL136 from MFR experiments obtained from days 10-12 (sample 4), were aliquoted into eppendorf tubes and covered in foil to protect them from the light. These extracts were placed at various temperatures (-20°C, +10°C, +25°C and +50°C) for 24 hrs. Controls of each were stored at -9°C (standard storage temperature of the extracts). Extracts from isolates WL61, WL114 and WL136 were screened against *S. pombe* and *Fusarium* sp.; *S. aureus*; and *S. aureus*, *Fusarium* sp. and *P. fluorescens*, respectively using the well-diffusion bioassay method (Appendix A).

8.2.2. Light stability

Similarly, each of the extracts (as above) were aliquoted into eppendorf tubes and exposed to lamp light at 25°C. A 200 µl sample was removed from each tube at time zero (T=0) and screened for activity. Thereafter, 200 µl samples were removed from each tube exposed to light every two hours for eight hours (T=2, T=4, T=6, T=8) and screened for activity. Similarly, extracts were screened for activity after 24 hrs exposure to light. Controls of each extract were kept in the dark for 8 and 24 hrs at 25°C. Extracts were screened for bioactivity against the same test organisms as described in section 8.2.1.

8.2.3. Biocidal or biostatic mode of action

Mode of action was assessed with the rapid and commonly used well-diffusion bioassay method (Appendix A). Well-diffusion bioassay plates were prepared with test organisms (described in section 8.2.1) and allowed to grow overnight at 25°C to

obtain established cultures (stationary phase cultures). Extracts from each of the isolates were aliquoted into the wells and the bioassay plate was re-incubated at 25°C overnight. A control bioassay plate was prepared using actively growing test organisms (Appendix A). The nature of the cells of the established test organisms surrounding the wells was examined at 100X magnification using a light microscope to determine whether or not the cells had been damaged.

8.3. RESULTS AND DISCUSSION

8.3.1. Temperature stability

The activity of each extract from isolates WL61, WL114 and WL136 after storage at different temperatures is summarised in Table 8.1.

Table 8.1: Summary of activity from extracts after storage at different temperatures

Isolate	Extract	Test organisms	Zones of inhibition (mm)				
			Control (-9°C)	-20°C	10°C	25°C	50°C
WL61	MeOH Intra	*Fusarium* sp.	9.00	9.00	9.00	8.75	7.25
	MeOH Intra	*S. pombe*	11.00	10.25	10.75	8.00	7.75
WL114	MeOH Intra (M2 broth)	*S. aureus*	6.50	6.25	6.50	6.00	6.00
	MeOH Intra (SC broth)	*S. aureus*	6.50	6.50	6.50	6.50	6.50
WL136	MeOH Intra	*S. aureus*	2.50	1.50	1.50	1.75	1.50
	EtOAc Intra	*S. aureus*	2.00	2.00	1.75	1.50	1.50
	EtOAc Extra	*S. aureus*	5.25	5.50	5.50	5.75	5.00
	MeOH Intra	*P. fluorescens*	1.75	0.00	0.00	0.00	0.00
	EtOAc Intra	*P. fluorescens*	6.00	6.00	6.00	5.00	5.00
	EtOAc Extra	*P. fluorescens*	5.00	4.25	4.00	2.75	3.00
	EtOAc Extra	*Fusarium* sp.	3.50	3.75	3.75	2.75	3.00

8.3.1.1. Isolate WL61

Activity was observed from isolate WL61 extracts, after storage at all temperatures, against *Fusarium* sp. and *S. pombe*. While the zones of inhibition against *Fusarium* sp. from extracts stored at higher temperatures (25 and 50°C) decreased slightly from 9.00 mm (control) to 8.75 mm and 7.27 mm, respectively, activity from the control extracts and those stored at the lower temperatures (-20 and 10°C) did not decrease.

Similarly, zones of inhibition against *S. pombe* decreased with an increase in temperature (25 and 50°C) from 11.00 mm (control) to 8.00 mm and 7.75 mm,

respectively. This suggests that the active compound(s) produced by isolate WL61 was relatively stable at extreme temperatures as there was little inactivation at 50°C.

8.3.1.2. Isolate WL114

The activity against *S. aureus* observed from isolate WL114 extracts, stored at all temperatures, was similar to that observed from the controls. While the extracts from cultivation in SC broth showed consistent activity, extracts from cultivation in M2 broth, stored at 25 and 50°C, showed that the zones of inhibition against *S. aureus* decreased slightly from 6.50 mm (control) to 6.00 mm. This suggests that the active compound(s) produced by isolate WL114 was stable as there was relatively little inactivation at 50°C.

8.3.1.3. Isolate WL136

Activity was observed against *S. aureus*, *P. fluorescens* and *Fusarium* sp. from isolate WL136 extracts after storage at all temperatures, except against *P. fluorescens* from intracellular MeOH extracts. Intracellular MeOH extracts stored at all the temperatures resulted in a decrease in the size of the zones of inhibition against *S. aureus* from 2.50 mm (control) to 1.50 or 1.75 mm. No activity, besides the control, was detected against *P. fluorescens*. This indicates that the active compound(s) produced from the intracellular MeOH extracts from isolate WL136 was unstable at higher temperatures.

Zones of inhibition detected against *S. aureus* from isolate WL136 intracellular EtOAc extracts decreased slightly from 2.00 mm (control) to 1.50 mm as temperature increased to 25 and 50°C. Similarly, zones of inhibition decreased slightly against *P. fluorescens* from 6.00 mm (control) to 5.00 mm. This also indicates that the active compound(s) from the intracellular EtOAc extracts was slightly inactivated at higher temperatures.

Activity against *S. aureus* from extracellular EtOAc extracts, stored at all temperatures, remained relatively constant in comparison to the control and only decreased slightly from 5.25 mm (control) to 5.00 mm at 50°C. Activity detected against *P. fluorescens* decreased with an increase in temperature. Zones of inhibition from extracts stored at 25°C decreased from 5.00 mm (control) to 2.75 mm and those stored at 50°C decreased to 3.00 mm.

Similarly, activity detected against *Fusarium* sp. from extracellular EtOAc extracts decreased slightly with an increase in temperature. Zones of inhibition from extracts stored at 25°C decreased from 3.50 mm (control) to 2.75 mm and those stored at 50°C decreased slightly to 3.00 mm. This suggests that the active compound(s) in the extracellular EtOAc extracts was slightly inactivated against *S. aureus* and *Fusarium* sp. at higher temperatures, but was more unstable against *P. fluorescens*.

8.3.2. Light stability

The activity of each extract from isolates WL61, WL114 and WL136 after exposure to lamp light for various lengths of time is summarised in Table 8.2.

Table 8.2: Summary of activity from extracts after light exposure at two hour intervals

Isolate	Extract	Test organisms	Zones of inhibition (mm)						
			Control (dark)	T=0	T=2	T=4	T=6	T=8	T=24
WL61	MeOH Intra	*Fusarium* sp.	9.50	9.50	9.00	7.50	7.50	6.75	8.00
	MeOH Intra	*S. pombe*	11.25	11.50	10.25	9.50	9.25	8.25	8.00
WL114	MeOH Intra (M2 broth)	*S. aureus*	9.00	9.00	9.00	9.00	9.00	9.00	9.50
	MeOH Intra (SC broth)	*S. aureus*	10.00	10.00	10.00	10.00	10.00	10.00	9.00
WL136	MeOH Intra	*S. aureus*	2.50	2.25	2.25	1.75	2.00	1.50	1.50
	EtOAc Intra	*S. aureus*	2.00	2.00	1.75	1.75	2.00	2.00	2.25
	EtOAc Extra	*S. aureus*	5.25	5.00	5.00	4.75	5.00	5.00	4.50
	MeOH Intra	*P. fluorescens*	1.75	0.00	0.00	0.00	0.00	0.00	0.00
	EtOAc Intra	*P. fluorescens*	5.00	5.00	5.25	5.75	5.50	5.50	5.75
	EtOAc Extra	*P. fluorescens*	5.00	5.00	5.00	4.75	4.75	4.75	4.50
	EtOAc Extra	*Fusarium* sp.	3.50	3.75	3.75	3.75	3.50	3.50	3.50

8.3.2.1. Isolate WL61

Activity against *Fusarium* sp. from isolate WL61 extracts decreased with every 2 hr interval of light exposure. The zones of inhibition decreased from 9.50 mm (control and T=0) to 6.75 mm (T=8). However, after 24 hours of light exposure, the zones of inhibition only decreased to 8.00 mm (T=24). Similarly, the zones of inhibition against *S. pombe* decreased from 11.50 mm (T=0) and 11.25 mm (control) to 8.25 mm (T=8) and 8.00 mm (T=24). This indicates that the active compound(s) produced from isolate WL61 was sensitive to light as there was a decrease in activity.

8.3.2.2. Isolate WL114

Activity against *S. aureus* was constant and zones of inhibition were similar in size to the controls. However, the extract from cultivation in SC broth showed that the zones of inhibition against *S. aureus* decreased slightly from 10.00 mm (control) to 9.00 mm (T=24). This suggests that the active compound(s) produced by isolate WL114 was relatively stable after storage in the light.

8.3.2.3. Isolate WL136

Activity was observed against *S. aureus*, *P. fluorescens* and *Fusarium* sp. from isolate WL136 extracts after long periods of exposure to light, except against *P. fluorescens* for intracellular MeOH extracts. Zones of inhibition detected against *S. aureus* from intracellular MeOH extracts after exposure to light decreased slightly from 2.50 mm (control) to 1.50 mm (T=8 and T=24), whereas no activity was detected against *P. fluorescens* after exposure to light. This indicates that intracellular MeOH extracts produced by isolate WL136 are sensitive to storage in light.

Activity against *S. aureus* from intracellular EtOAc extracts remained relatively constant after exposure to light and zones of inhibition were similar in size to the controls (2.00 mm) and 2.25 mm (T=24). Similarly, activity against *P. fluorescens* was relatively constant and zones of inhibition were similar in size to the controls (5.00 mm) and 5.75 mm (T=24). This indicates that intracellular EtOAc extracts produced by isolate WL136 are stable with storage in light.

Activity detected against *S. aureus*, *P. fluorescens* and *Fusarium* sp. from extracellular EtOAc extracts remained relatively constant after exposure to light and zones of inhibition were similar in size to the controls. While zones of inhibition only decreased slightly from 5.25 mm (control) to 4.50 mm (T=24) and against *P. fluorescens* from 5.00 mm (control) to 4.50 mm (T=24), zones of inhibition against *Fusarium* sp. did not decrease after exposure to light. This suggests that intracellular EtOAc extracts produced by isolate WL136 are stable with storage in the light.

8.3.3. Biocidal or biostatic mode of action[III]

The activity of each extract from isolates WL61, WL114 and WL136 against established and actively growing test organisms is summarised in Table 8.3 below.

Table 8.3: Summary of activity against actively growing or established cultures

Isolate	Extract	Test organisms	Zones of inhibition (mm)	
			Control *	Established growth
WL61	MeOH Intra	*Fusarium* sp.	9.00	0.00
	MeOH Intra	*S. pombe*	11.00	6.00
WL114	MeOH Intra (M2 broth)	*S. aureus*	9.00	4.50
	MeOH Intra (SC broth)	*S. aureus*	10.00	5.00
WL136	MeOH Intra	*S. aureus*	2.50	0.00
	EtOAc Intra	*S. aureus*	2.00	0.00
	EtOAc Extra	*S. aureus*	5.25	2.50
	MeOH Intra	*P. fluorescens*	1.75	0.00
	EtOAc Intra	*P. fluorescens*	4.25	0.00
	EtOAc Extra	*P. fluorescens*	5.00	1.25
	EtOAc Extra	*Fusarium* sp.	3.00	2.75

* Bioassay with actively growing cells

8.3.3.1. Isolate WL61

No visible zones of inhibition against established (stationary phase) *Fusarium* sp. were observed from isolate WL61 extracts as the mycelia close to the wells of the bioassay plate appeared healthy and undamaged. However, these extracts were active against actively growing cells of *Fusarium* sp. as short and broken hyphae were observed when the cells within the zone of inhibition were examined microscopically. Conversely, these extracts produced zones of inhibition against both actively growing and established *S. pombe* cells. Microscopic observation of the *S. pombe* cells within the zone of inhibition of the established cells indicated that the cells had been damaged as they appeared smaller and wrinkled.

It was concluded that the bioactive compound(s) produced from isolate WL61 is biocidal against *S. pombe* and biostatic against *Fusarium* sp. The mode of action against *Fusarium* sp. and *S. pombe* is different, suggesting that there are at least two different relatively stable active metabolites in the extracts from isolate WL61 or that a single compound is acting differently in the two organisms.

[III] The designation of biostatic or biocidal mode of action is considered to be preliminary data and would need to be confirmed by additional experiments.

8.3.3.2. Isolate WL114

Active extracts from isolate WL114 produced zones of inhibition against both actively growing and established *S. aureus* cells. Microscopic observation of the *S. aureus* cells within the zone of inhibition of the established cells indicated that the cells had been damaged and they appeared swollen. It was concluded that the bioactive compound(s) from isolate WL114 cultivated in both M2 and SC broths is a relatively stable active metabolite(s) biocidal against *S. aureus*.

8.3.3.3. Isolate WL136

No visible zones of inhibition against *S. aureus* and *P. fluorescens* were observed from intracellular MeOH and EtOAc extracts from isolate WL136 as the cells close to the wells of the bioassay plate appeared healthy and undamaged. These extracts were only active against actively growing cells of *S. aureus* and *P. fluorescens* and was therefore concluded that the bioactive compound(s) from intracellular MeOH and EtOAc extracts from isolate WL136 is biostatic. Conversely, the active extracellular EtOAc extracts produced zones of inhibition against both actively growing and established *S. aureus, P. fluorescens* and *Fusarium* sp. strains. Microscopic observation of these test organisms within the zone of inhibition of the established cells indicated that the cells had been damaged. It was concluded that only the extracellular non-polar (EtOAc) active compound(s) from isolate WL136 is biocidal against *S. aureus, P. fluorescens* and *Fusarium* sp.

8.4. CONCLUSION

The active compound(s) from isolate WL61 was relatively stable after storage in the light for more than 24 hrs as well as with storage in the dark at different temperatures, as there was only slight inactivation at higher temperatures. The mode of action of the active compound(s) was biocidal and biostatic as the healthy cells of *S. pombe* were damaged after 48 hrs growth and further growth of *Fusarium* sp. and *S. pombe* was inhibited after 24 hrs.

The active compound(s) from isolate WL114 cultivated in M2 and SC broths was relatively stable with storage in the dark at different temperatures and was not inactivated after storage in light over an eight hour period, but was slightly sensitive after storage in light over a 24 hr period (isolate WL114 cultivated in SC broth). The

mode of action of the active compound(s) was biocidal and biostatic as the healthy cells of *S. aureus* were damaged after 48 hrs growth and further growth of *S. aureus* was inhibited after 24 hrs.

The active compound(s) from intracellular MeOH extracts from isolate WL136 was slightly sensitive with storage in the dark at different temperatures, but was unstable after storage in the light for 8 and 24 hr periods. The active compound(s) from intracellular EtOAc extracts from isolate WL136 was relatively stable with storage in the dark at different temperatures as well as after storage in the light over a 24 hr period. The mode of action of the active compound(s) from these two extracts was biostatic as only the healthy and actively growing cells of *S. aureus* and *P. fluorescens* were damaged after 24 hrs.

The active compound(s) from extracellular EtOAc extracts from isolate WL136 was relatively stable against *S. aureus* and *Fusarium* sp. with storage at different temperatures in the dark, but was inactivated slightly against *P. fluorescens.* However, the active compound(s) remained stable after storage in the light over a 24 hr period. The mode of action of the active compound(s) was biocidal and biostatic as the healthy cells of *S. aureus, P. fluorescens* and *Fusarium* sp. were damaged after 48 hrs growth and further growth of these strains was inhibited after 24 hrs.

CHAPTER 9

Metabolite Profiling, purification and structure of active metabolite(s) from *Streptomyces* sp., isolate WL114

9.1. INTRODUCTION

Marine microorganisms, such as marine actinomycetes, produce natural products that have unique structural features in comparison to those derived from terrestrial environments. However, many promising bioactive marine compounds can only be isolated in very low yields due to the difficulties associated with culturing these organisms by standard fermentation techniques (Larsen *et al.*, 2005).

Separation and detection procedures, allowing rapid characterisation of the biologically active components, are challenging due to the chemical diversity of natural product extracts (Strege, 1999). Traditionally, indirect methods of identifying secondary metabolites involved, for example, the colour of diffusible pigments, odour of cultures, the KOH test and filter paper methods. The introduction of separation methods such as thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), gas chromatography (GC), and the use of a wide selection of detectors, has since made it possible to isolate and identify individual natural products (Larsen *et al.*, 2005).

TLC is a rapid screening method used to detect and separate small amounts of polar or non-polar compounds in an extract as a result of the type of stationary phase used (Chong, 2006). In addition, the separation of components and assessment of their biological activity can be combined in a TLC bioassay method called bioautography. Bioautography is a bioactivity-related detection method for TLC allowing direct tracking of bioactive compounds in complex mixtures using small amounts of sample (Wennberg, 2006). The method involves overlaying the TLC plate with media seeded with a test organism and observing zones of inhibition of growth as test organisms do not grow where bioactive compound(s) are present. Although bioautography only provides qualitative results, this method is useful in verifying the presence of bioactive compound(s) in an extract (Soylu *et al.*, 2002; Horváth, 2002; Wennberg, 2006).

TLC is considered a pilot procedure prior to HPLC as it is more flexible, rapid and less expensive than column techniques. Additionally, several mobile phase systems can be studied simultaneously using inexpensive equipment. However, the comparability of these two methods is often case-specific depending on the nature of the mobile phase or the properties of the solute (Wennberg, 2006).

HPLC in conjunction with photodiode-array UV-Vis absorbance detection has been very useful and is described as an essential analytical and preparative chromatographic tool used in the pharmaceutical industry for the characterisation of natural product extracts (Strege, 1999; Guttman, 2004). GC is another separation technique that has been used to analyse volatile natural products, but approximately 80% of all known natural compounds are non-volatile or thermally unstable and consequently incompatible with GC methods (Strege, 1999). Novel compounds can be isolated after HPLC and milligram quantities used for both secondary biological activity evaluation and structure elucidation by infrared absorbance (IR) spectroscopy and nuclear magnetic resonance (NMR), a technique providing information about the carbon-hydrogen framework of a molecule (McMurry, 1996; Strege, 1999).

NMR is the most commonly used spectroscopic technique for the structure determination of complex natural products. Two-dimensional homonuclear correlation spectroscopy (COSY), allows for the quick determination of the connectivity of a molecule by determining the correlation between two coupled protons. Two-dimensional heteronuclear NMR experiments, such as heteronuclear single quantum coherence (HSQC), detects the correlation between primarily attached pairs of protons and carbons, while heteronuclear multiple bond correlation (HMBC) is useful for long range correlations of protons and carbons two or three bonds away, confirming structural moieties (Holtman, 2003).

The development of a range of simple analytical methods based on the use of mass spectrometry (MS), UV and NMR data, in combination with modern informatics tools, is important for rapid bioassay screening and for characterising the nature of mixtures of compounds in crude extracts. Similarly, the use of hyphenated analytical techniques, for example GC-MS(-MS), LC-UV, LC-MS(-MS) and LC-NMR are

relevant approaches for compound dereplication and to simplify natural product discovery (Larsen *et al.*, 2005).

In this chapter, chemical analysis was carried out on extracts from *Streptomyces* sp., isolate WL114, as this isolate was shown to be the most novel according to the BLAST analysis (Chapter 3). Bioautography, HPLC and NMR methods were used to characterise the active compound. Thus, the objectives were:

- To separate the active compound(s) from the extract from *Streptomyces* sp., isolate WL114, using TLC and confirming the presence of the bioactive compound(s) by observing zones of inhibition against *S. aureus* in well-diffusion bioassays.
- To determine the purity and metabolite profile of the active compound(s) using HPLC on a C_{18} column.
- To determine the structure of the active compound(s) using NMR.

9.2. MATERIALS AND METHODS

9.2.1. Development of an HPLC method for the profiling of extracts from *Streptomyces* spp., isolates WL61, WL114 and WL136

The metabolite profiles of the extracts from isolates WL61, WL114 and WL136 and the spots from the TLC plate from isolate WL114 where determined using HPLC (2695 Separations Module, Waters) and a PDA detector (2996 PDA, Waters) at wavelengths 190-800 nm with an Xterra C_{18} HPLC column using Empower software (1154, 2002 Waters) to verify the best separation method as follows:

- An isocratic method using 20, 40, 60, 80 or 100% MeOH at a flow rate of 1.0 ml/min for 30 min each. Depending on the results from these isocratic chromatograms:
- A set of different gradient methods was carried out by adjusting/changing the time, flow rates and slopes of the gradients according to the results obtained. The metabolite profiles of the extracts from isolates WL61, WL114 and WL136 were determined using the gradient method.

9.2.2. TLC analysis of intracellular MeOH extracts from *Streptomyces* sp., isolate WL114

Aliquots of 20 µl of extract from isolate WL114 cultivated in M2 broth and SC broth were loaded onto pre-coated silica gel TLC plates ($60F_{254}$, Merck) (3 x 6 cm). The most suitable mobile phase was determined by developing the plates in the following solvents:

a) Chloroform : MeOH (90:20, v/v)
b) Chloroform : Hexane (90:10, v/v)
c) MeOH (0.1% HCOOH)
d) MeOH (0.1% NaOH)
e) 50% MeOH (0.1% HCOOH)
f) 50% MeOH (0.1% NaOH)
g) EtOAc

The solvents were allowed to run to 0.5 cm from the top of the plate after which the plates were removed and left to air dry. The plates were examined under short (254 nm) and long (365 nm) wavelengths using a UV light box (Spectroline Fluorescence Analysis cabinet, Spectronics Corporation) to detect visible spots. Duplicate plates were developed using the most suitable solvent (mobile phase). One plate was used for the TLC bioassay and the other plate for HPLC analysis of the visible spots.

9.2.3. Bioautographic analysis of intracellular MeOH extracts from *Streptomyces* sp., isolate WL114

Aliquots of 200 µl of overnight stock cultures of *S. aureus* were suspended in 40 ml sloppy 117 *Micrococcus* agar and a thin layer (± 2 ml) was overlayed on the TLC plate which was then incubated for 48 hrs at 25°C. If any zones of inhibition were detected, the equivalent spots from the duplicate TLC plate were removed and extracted with 100 µl 100% MeOH for HPLC analysis (see section 9.2.1). The metabolite profiles of isolate WL114 extracts and TLC plate spots were compared and common or new peaks were identified by their UV spectrum.

9.2.4. Isolation of active compound(s) from intracellular MeOH extracts from *Streptomyces* sp., isolate WL114

Column chromatography was used to fractionate the major peaks detected in the intracellular MeOH extract from *Streptomyces* sp., isolate WL114 cultivated in SC

broth. A 1 g column (Oasis HLB LP extraction cartridge, Waters) was conditioned with 5 ml MeOH followed by 5 ml distilled water under a vacuum in a manifold (Visiprep, Supelco). A 2 ml aliquot of isolate WL114 extract was loaded into the matrix of the column with 20 ml 20% MeOH (0.1% HCOOH). The column was eluted with 40 ml mobile phase consisting of 80% MeOH (0.1% HCOOH) followed by 20 ml mobile phase consisting of 100% MeOH and collected in approximately 2 ml fractions. The eluent from each 2 ml fraction was assayed for bioactivity using the well-diffusion bioassay method (Appendix A) with *S. aureus* as the test organism and analysed on an Xterra C_{18} HPLC column using the optimised gradient method to obtain the best peak separation (see section 9.2.1).

Semi-pure fractions from the peaks of interest were collected by combining the fractions where peak concentrations were high and did not overlap with each other. These combined fractions were dried using a turbovap, reconstituted with 1 ml 100% MeOH and dried in a desiccator overnight. The purity of the four peaks of interest was analysed using HPLC.

9.2.5. Purification and structure elucidation of active compound(s) from intracellular MeOH extracts from *Streptomyces* sp., isolate WL114

9.2.5.1. Purification by HPLC fractionation

Crude intracellular MeOH extracts from isolate WL114 were analysed using HPLC (1525 binary HPLC pump, Waters) and Dual λ absorbance detector (2487, Waters) at wavelengths of 278 and 323 nm with an Xterra C_{18} HPLC column using Breeze software (version 3.30 SPA, 2000 Waters). Injection volumes (20, 30, 50, 60, 80 and 100 µl) were used after the HPLC gradient method was adjusted by only changing the time of the gradient from 20% MeOH (0.1% HCOOH) to 70% MeOH (0.1% HCOOH) from 0 - 31 min; 100% MeOH for 10 min before the column was reconditioned with 20% MeOH (0.1% HCOOH) for 10 min before the next run. The four peaks of interest were collected from the HPLC in different fractions and the purity of each fraction was determined.

9.2.5.2. Structure elucidation using NMR spectroscopy

Purified peak fractions were concentrated by rotary evaporation (Buchi Rotavapor R-215) and prepared for NMR analysis by reconstituting the dried samples in

deuterated chloroform. Samples were transferred into NMR tubes for ^{1}H NMR and ^{13}C NMR analysis (Bruker NMR) to provide structural information on the active compound(s). ^{1}H NMR was also carried out on the crude intracellular MeOH extract from isolate WL114 and compared with the NMR spectra of the purified fractions. The well-diffusion bioassay method (Appendix A) was used to screen the purified peaks against *S. aureus* to confirm which peak contained the active compound.

9.3. RESULTS AND DISCUSSION

9.3.1. HPLC analysis of extracts from *Streptomyces* spp., isolates WL61, WL114 and WL136

The chromatograms from the different isocratic methods resulted in poor separations (data not shown). Gradient HPLC methods gave better elution profiles of these crude extracts (data not shown) with the optimal method consisting of a linear slope from 20% MeOH (0.1% HCOOH) to 100% MeOH over 50 min followed by 100% MeOH for 5 min before the column was reconditioned with 20% MeOH (0.1% HCOOH). The gradient was operated at 25°C at a flow rate of 0.8 ml/min.

9.3.2. TLC analysis and bioautography of intracellular MeOH extracts from *Streptomyces* sp., isolate WL114

The best TLC separation was obtained with 50% MeOH (0.1% HCOOH) solvent. Three distinct spots were visualised when the plates were examined under short (260 nm) and long (340 nm) wavelengths. Fig. 9.1 illustrates the three spots (spot 1, 2 and 3 (solid circles)) that were identified before the TLC plate was overlayed with *S. aureus* in sloppy agar.

Large zones of inhibition (dashed circles) from intracellular MeOH extracts from isolate WL114 grown in either M2 or SC broth were detected as a clearing of growth against *S. aureus* after 48 hrs incubation compared to the visible orange colour of *S. aureus*. The zone of inhibition on the TLC plate was larger from the SC extract, consistent with previous well-diffusion bioassays using these extracts (Chapter 7). The zone of inhibition overlapped spots 1 and 3. Since spot 1 was at the centre of the zone, it is suggested that it contained the bioactive compound.

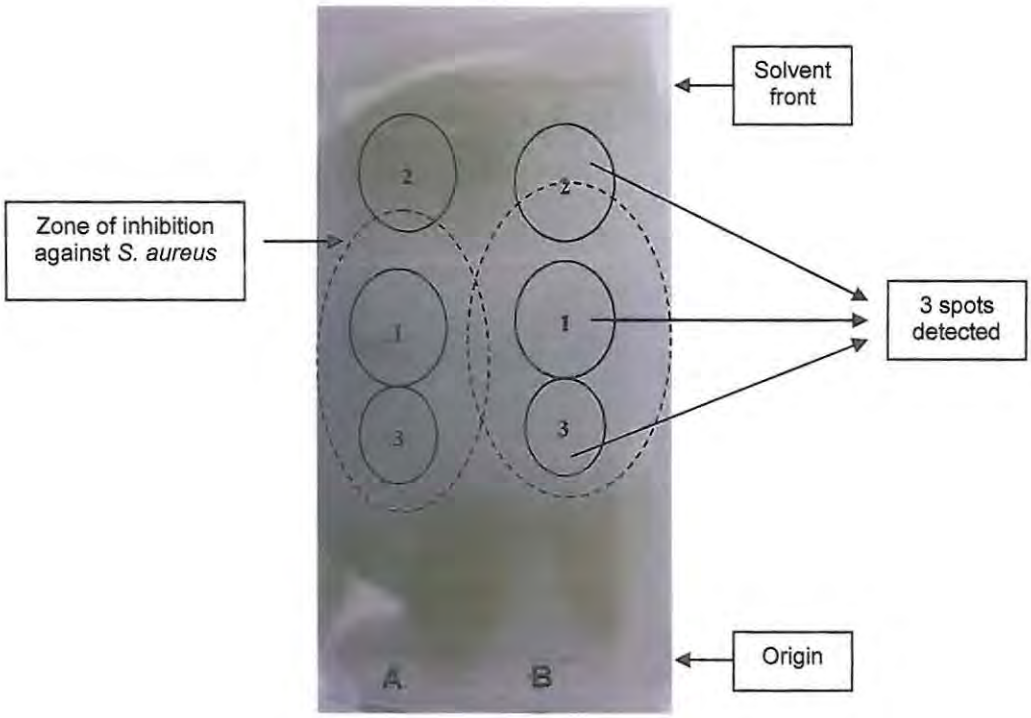


Fig. 9.1: Bioautographic analysis of intracellular MeOH extracts from isolate WL114 grown in M2 broth (A) and SC broth (B)

9.3.2.1. Analysis of EtOAc extracts

The maximum absorbance profile (Maxplot) for the EtOAc blank from wavelengths 215-600 nm was determined (Fig. 9.2). The peaks observed in this chromatogram were excluded when analysing the different HPLC metabolite profiles obtained from the intracellular and extracellular EtOAc extracts from isolates WL61, WL114 and WL136 (Figs. 9.3 to 9.6).

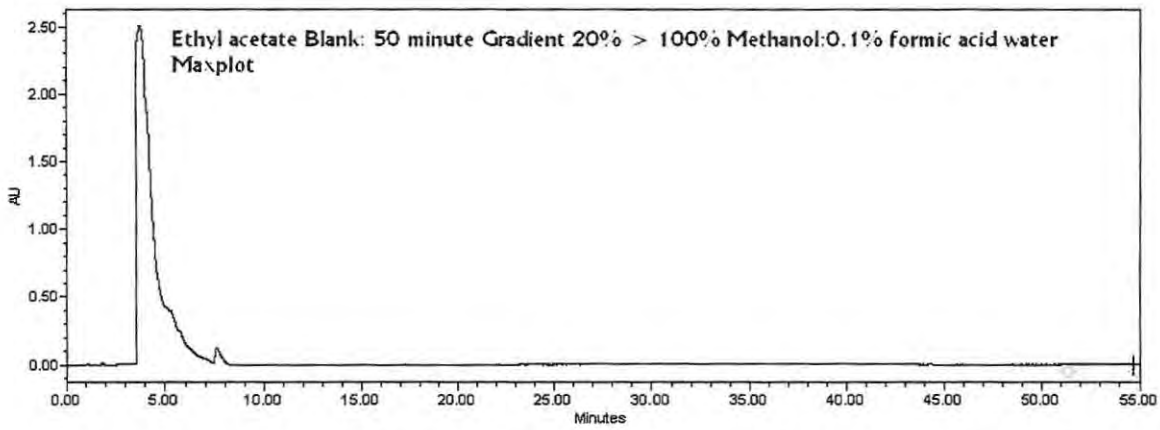


Fig. 9.2: HPLC Maxplot profile of EtOAc blank

The HPLC Maxplot of the intra- and extracellular EtOAc extracts from isolate WL61 are shown in Fig. 9.3.

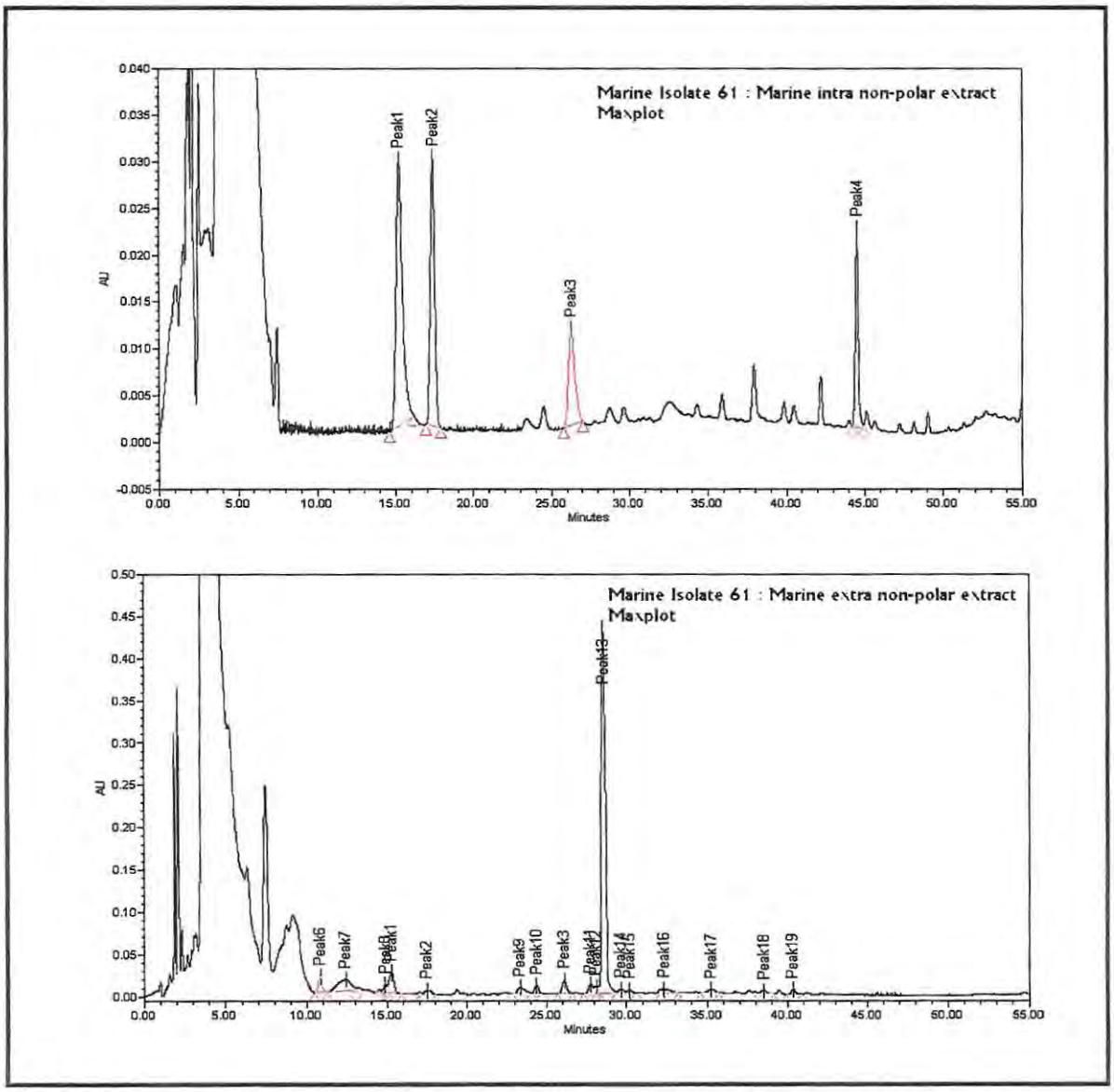


Fig. 9.3: HPLC Maxplot profiles of intracellular and extracellular EtOAc extracts from isolate WL61 grown in Marine broth

Peaks 1, 2, 3, 4 and 13 were the most significant peaks in these extracts. Peaks 1, 2 and 3 were present in both intra- and extracellular extracts, although in different relative proportions. Peak 4 was present in the intracellular extracts, but not detected in the extracellular extracts, and peak 13 was present in large concentrations in the extracellular extracts, but was not detected in the intracellular extracts. The UV spectra for these 5 peaks ranged between 240-265 nm (data not shown).

Extracellular EtOAc extracts from isolate WL61 were consistently active against *P. fluorescens* (see Chapters 6 and 7) whereas intracellular EtOAc extracts from isolate WL61 were slightly active against *Fusarium* sp. (see Chapter 6). This suggests that the active compound in the extracellular EtOAc extracts from isolate WL61 may possibly be represented by peak 13. The different peaks in these extracts would need to be purified further to confirm that peak 13 is the active compound.

Figs. 9.4 and 9.5 below show many significant peaks present in both the intra- and extracellular EtOAc extracts from isolate WL136 however more peaks were detected from extracellular EtOAc extracts. Peaks 1, 2, 4, 9 and 11 were the most significant and largest peaks in intracellular extracts. In extracellular extracts, peaks 29, 30 and 36 were the most significant and largest peaks. Peaks from the extracellular extracts were much greater than those observed in the intracellular extracts.

In addition to the maxplots, the UV spectra for all these peaks present in the intra- and extracellular EtOAc extracts are shown in Figs. 9.4 and 9.5 in the PDA contour plots. These plots highlight the many different UV spectra between 215 and 600 nm. The different peaks could be further analysed based on the UV spectra.

The EtOAc extracts from isolate WL136 grown in M2 broth were active against the same test organisms, *P. fluorescens, S. aureus* and *Fusarium* sp., however zones of inhibition were consistently larger with the extracellular EtOAc extracts (see Chapters 6 and 7). This suggests that the active compound from intracellular EtOAc extracts from isolate WL136 may possibly be represented by either of peaks 1, 2, 4, 9 or 11 and extracellular EtOAc extracts may possibly be represented by either of peaks 29, 30 or 36.

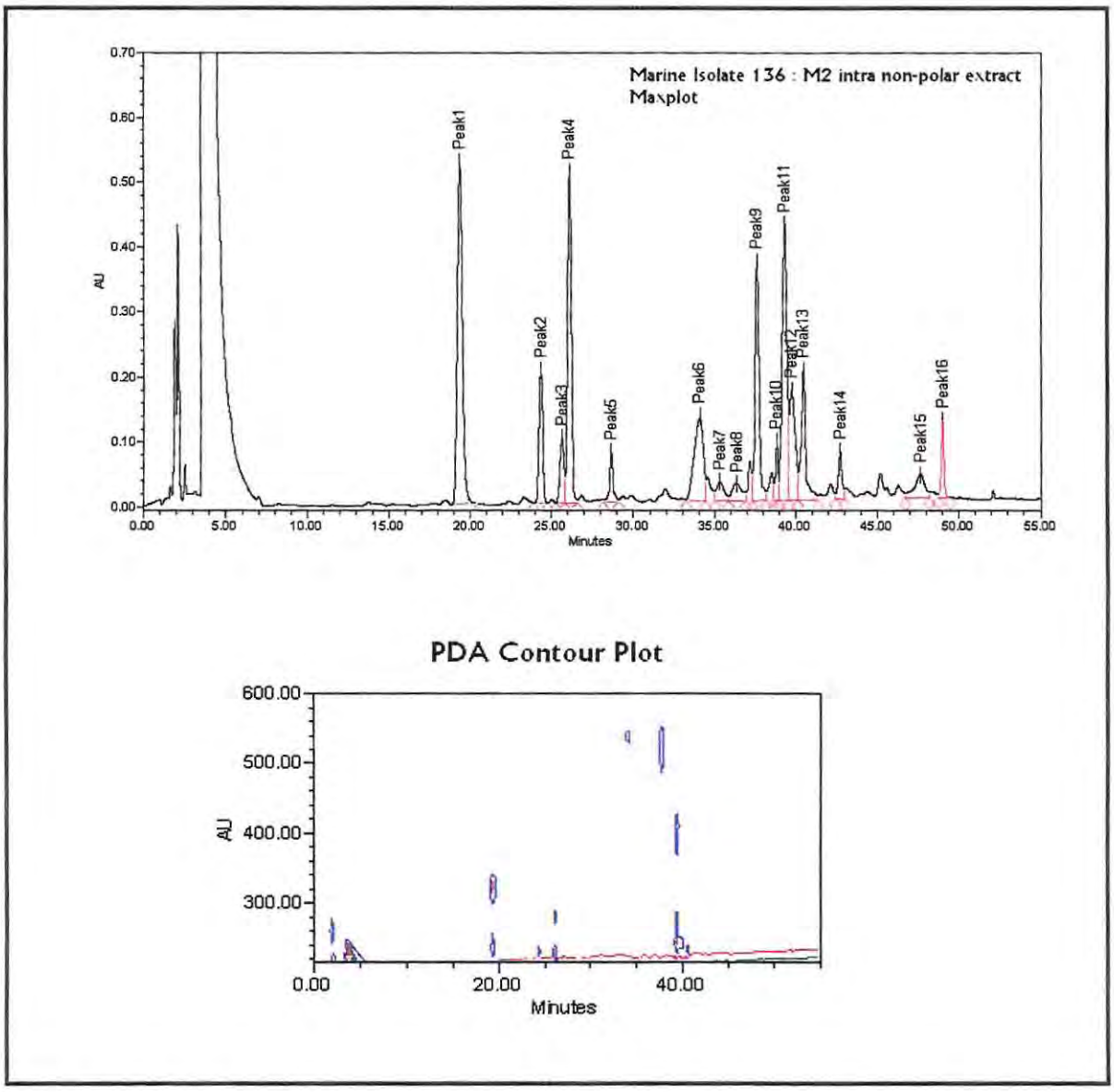


Fig. 9.4: HPLC Maxplot profile and contour plot of intracellular EtOAc extracts from isolate WL136 grown in M2 broth

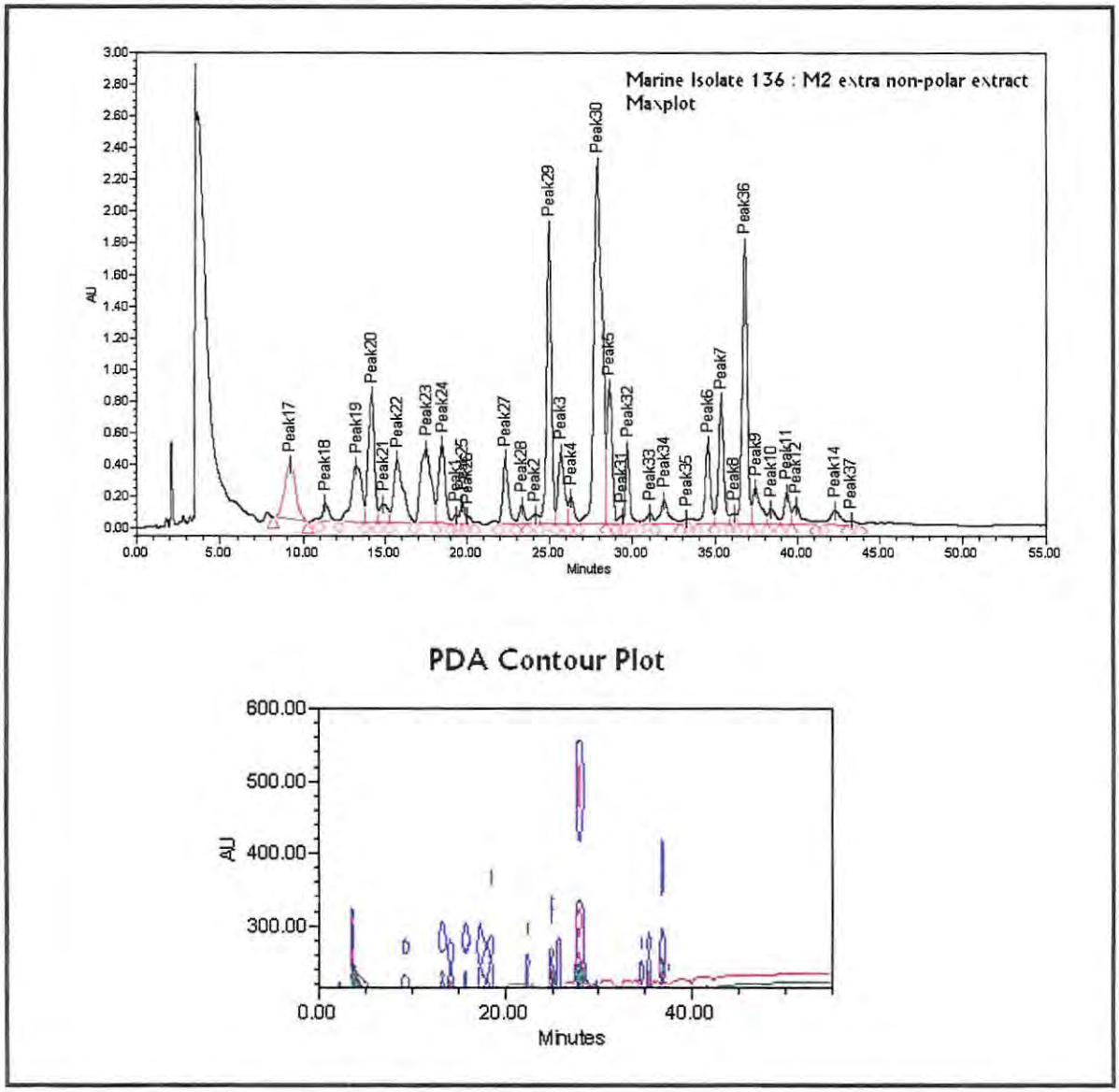


Fig. 9.5: HPLC Maxplot profile and contour plot of extracellular EtOAc extracts from isolate WL136 grown in M2 broth

The different peaks in these extracts would need to be better separated, fractionated and purified further to confirm the active compound(s) peak against all three test organisms.

The metabolite profile of the extracellular EtOAc extract from isolate WL114 is highlighted in Fig. 9.6. These extracts were analysed after the HPLC gradient method was adjusted to 70% MeOH (0.1% HCOOH) over 31 min followed by 100% MeOH for 10 min before the column was reconditioned with 20% MeOH (0.1%

HCOOH) for 10 min using HPLC (1525 binary HPLC pump, Waters) and Dual λ absorbance detector (2487, Waters) at 278 and 323 nm wavelengths with an Xterra C_{18} HPLC column using Breeze software (version 3.30 SPA, 2000 Waters).

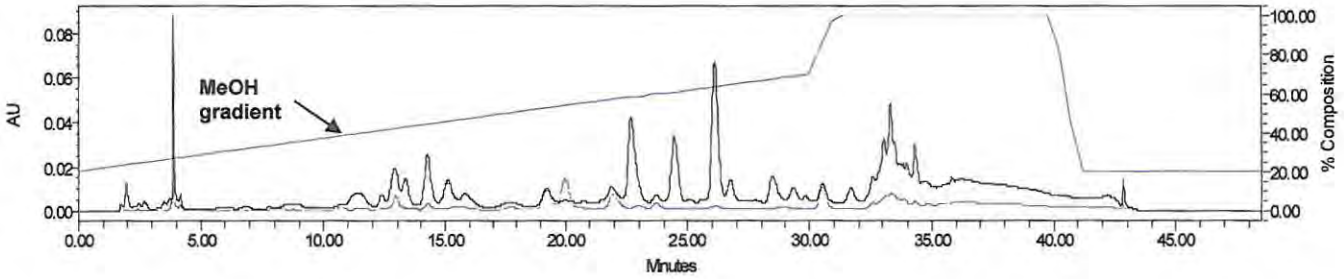


Fig. 9.6: HPLC profile of extracellular EtOAc extracts (20 µl) from isolate WL114 grown in SC broth

The gradient method used also showed fairly good separation of the different peaks, but no peaks were fractionated and collected from this sample as no activity was detected from well-diffusion bioassays.

9.3.2.2. Analysis of MeOH extracts

The maximum absorbance profile (Maxplot) for the MeOH blank from wavelengths 215-600 nm is illustrated in Fig. 9.7.

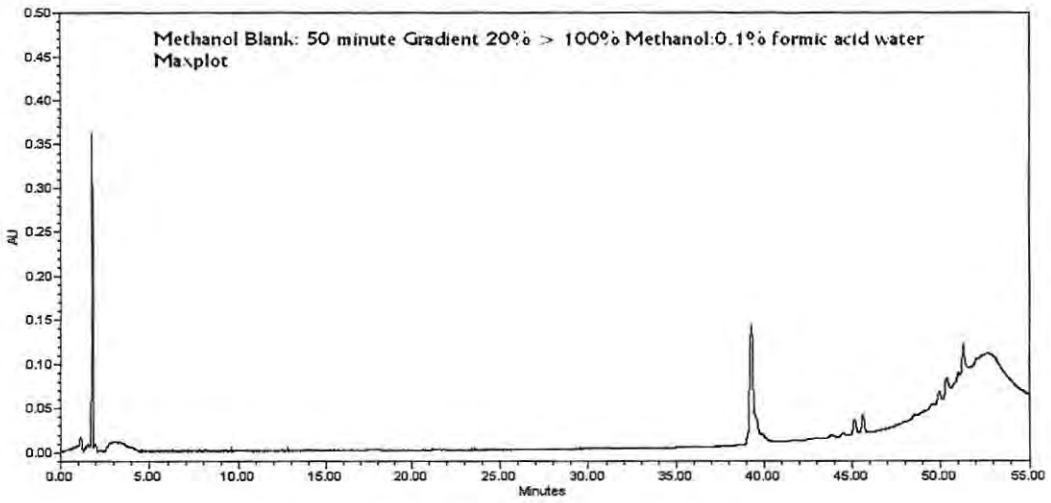


Fig. 9.7: HPLC Maxplot profile of MeOH blank

The peaks observed in this chromatogram were excluded when analysing the different HPLC metabolite profiles obtained from the intracellular MeOH extracts from isolates WL61, WL114 and WL36 (Figs. 9.8 to 9.10).

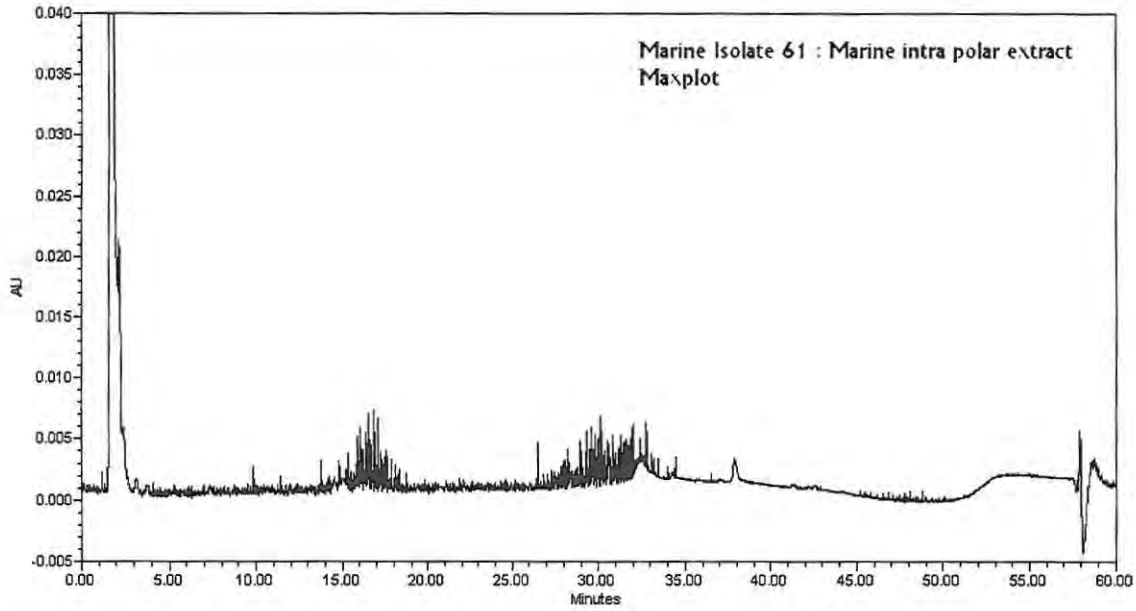


Fig. 9.8: HPLC Maxplot profile of intracellular MeOH extracts from isolate WL61 grown in Marine broth

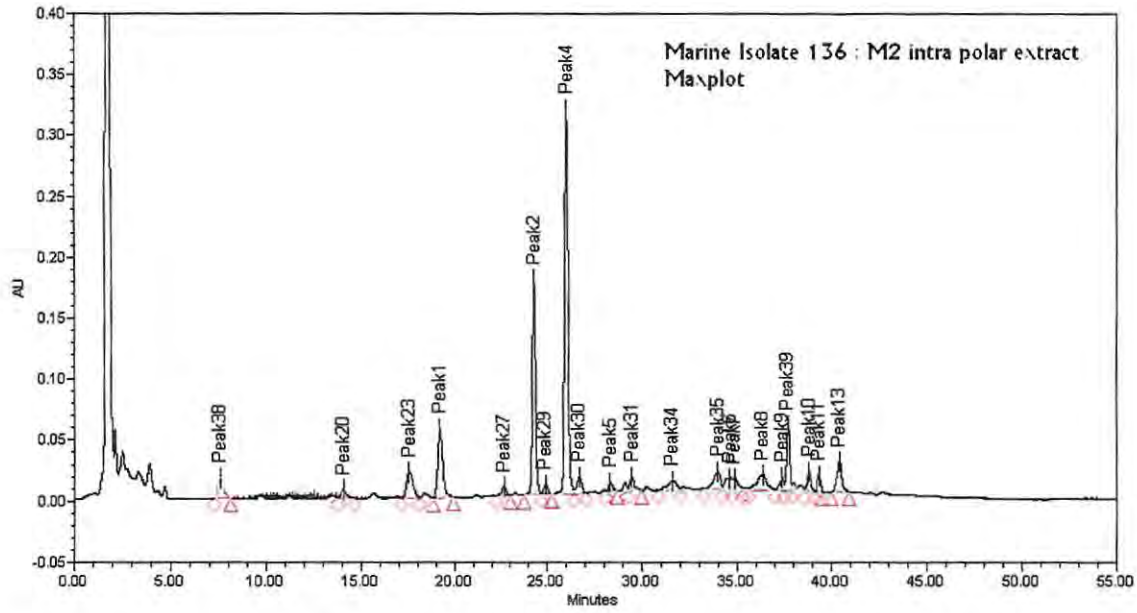


Fig. 9.9: HPLC Maxplot profile of intracellular MeOH extracts from isolate WL136 grown in M2 broth

Fig. 9.10 shows that most of the same peaks were present in both the extracts. Peaks 29, 17, 30, and 35 were the most significant peaks in both extracts. In the SC broth extract, peaks 29, 17 and 35 were greater than those observed in the M2 broth extract, while peak 30 was much lower.

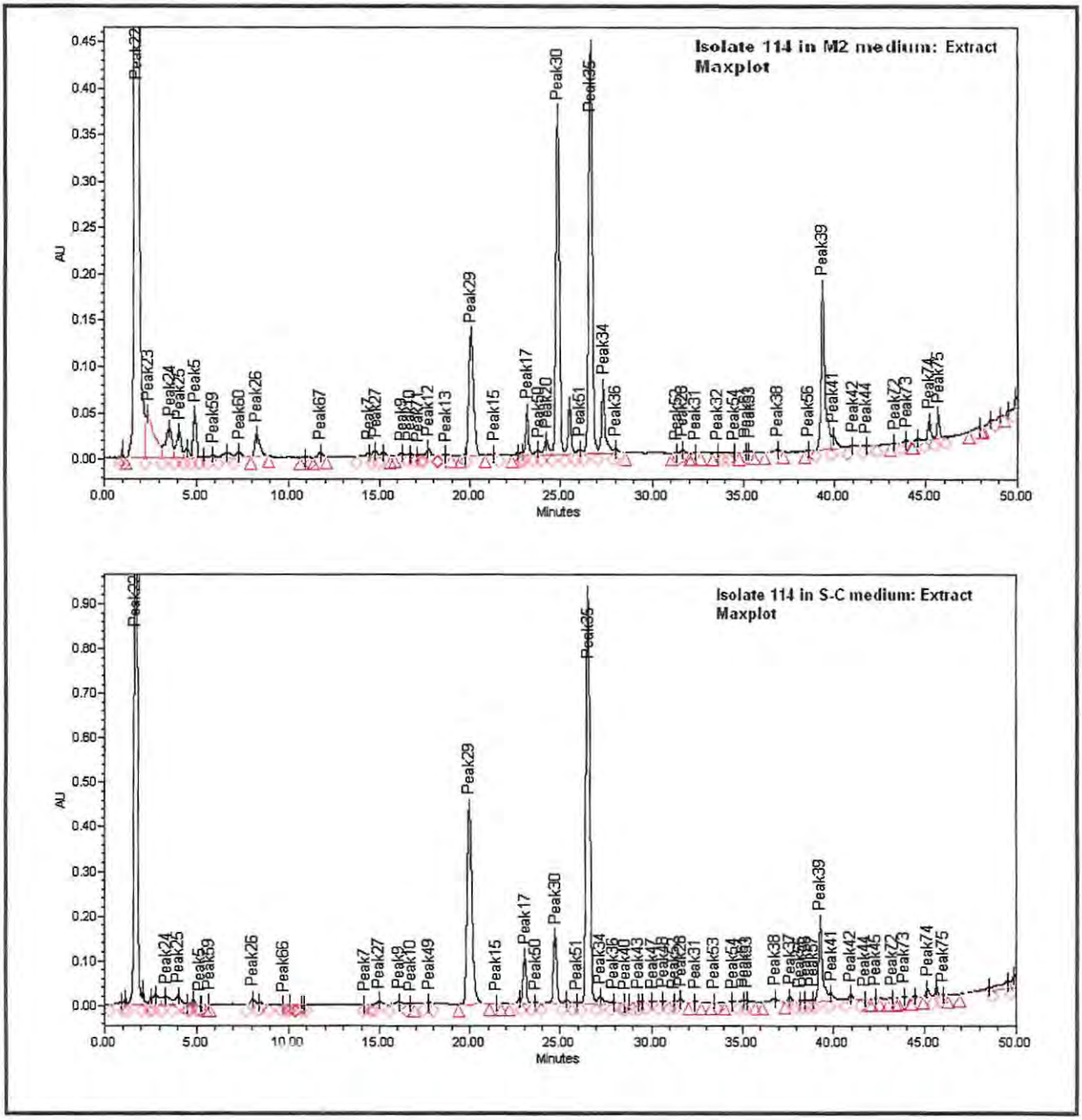


Fig. 9.10: HPLC Maxplot profiles of intracellular MeOH extracts from isolate WL114 grown in M2 and SC broths

The UV spectra for these peaks are illustrated in Fig. 9.11 below. Based on the UV spectrum of these major peaks, further HPLC chromatograms were analysed at 278 and 323 nm in addition to the Maxplot.

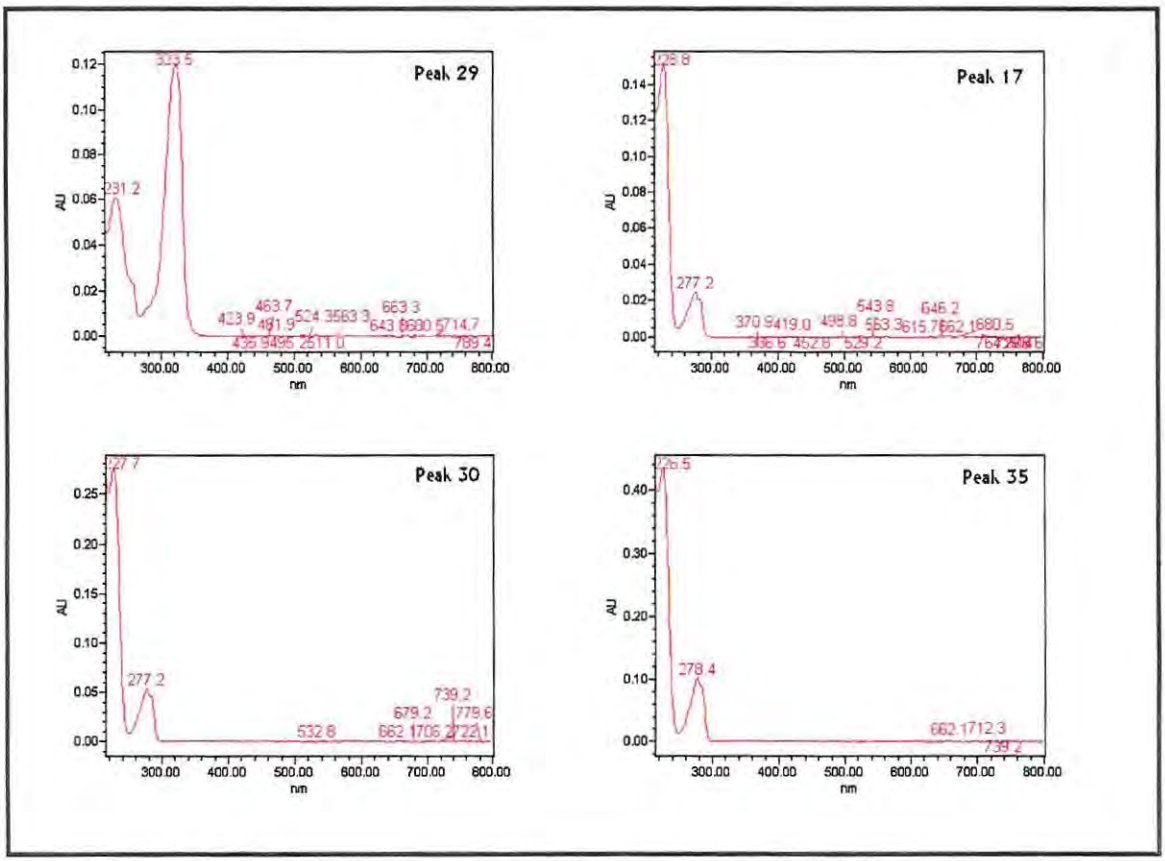


Fig. 9.11: UV spectra of each peak from the intracellular MeOH extract from isolate WL114 grown in SC broth

Both M2 and SC broth intracellular extracts where active against the same test organisms, however well-diffusion bioassays showed that isolate WL114 cultivated in SC broth consistently produced much larger zones of inhibition in comparison to those detected in M2 broth (also observed in Chapter 7 section 7.3.2.2). This suggests that the concentration of active compound(s) in SC broth intracellular extracts was higher than in M2 broth extracts. Peak height and area is an indication of concentration of a metabolite within each extract. Peak 29 and peak 35 were both observed to be significantly higher in the SC broth extracts which suggests that either of these peaks were responsible for the differences in the level of activity found between these two extracts.

9.3.2.3. Analysis of TLC spots

The HPLC chromatograms of the three TLC spots identified (Fig. 9.1) are shown in Figs. 9.12 to 9.14.

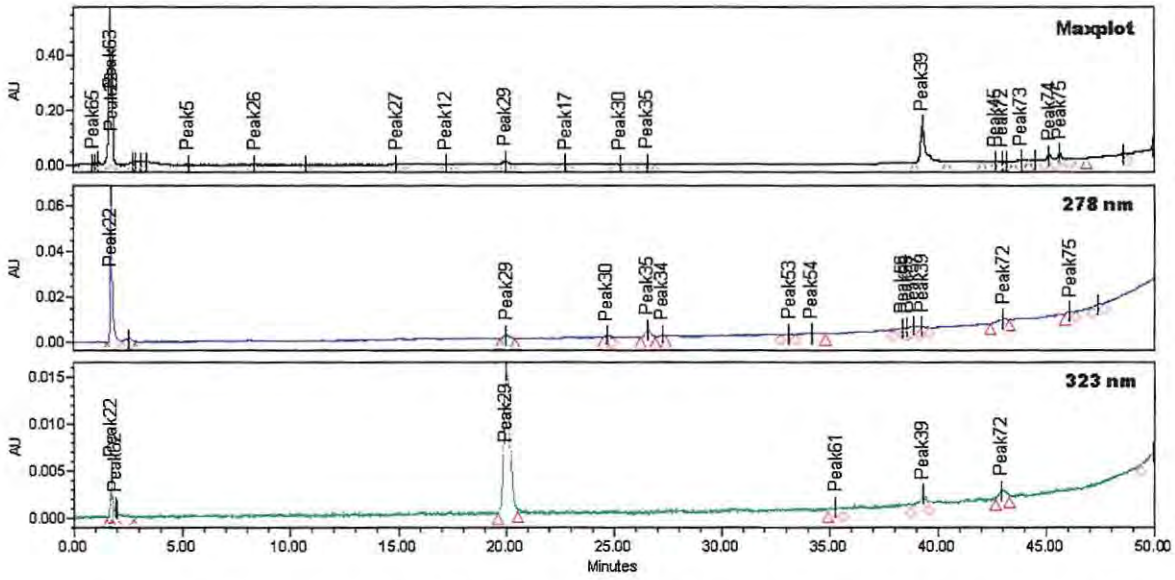


Fig. 9.12: Intracellular MeOH extract from TLC plate spot 1 from isolate WL114 grown in SC broth

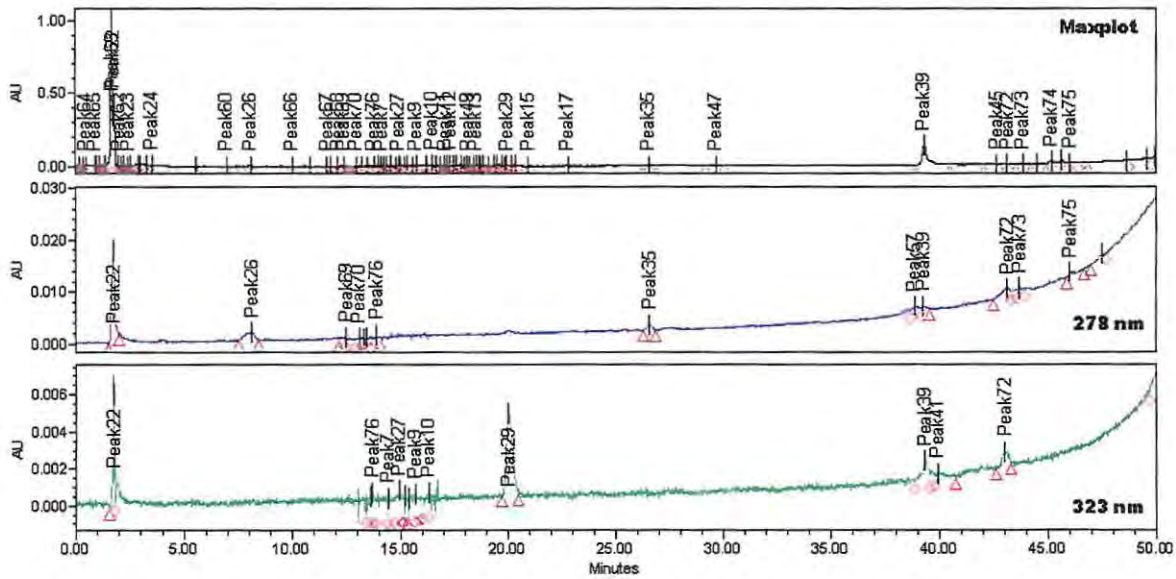


Fig. 9.13: Intracellular MeOH extract from TLC plate spot 2 from isolate WL114 grown in SC broth

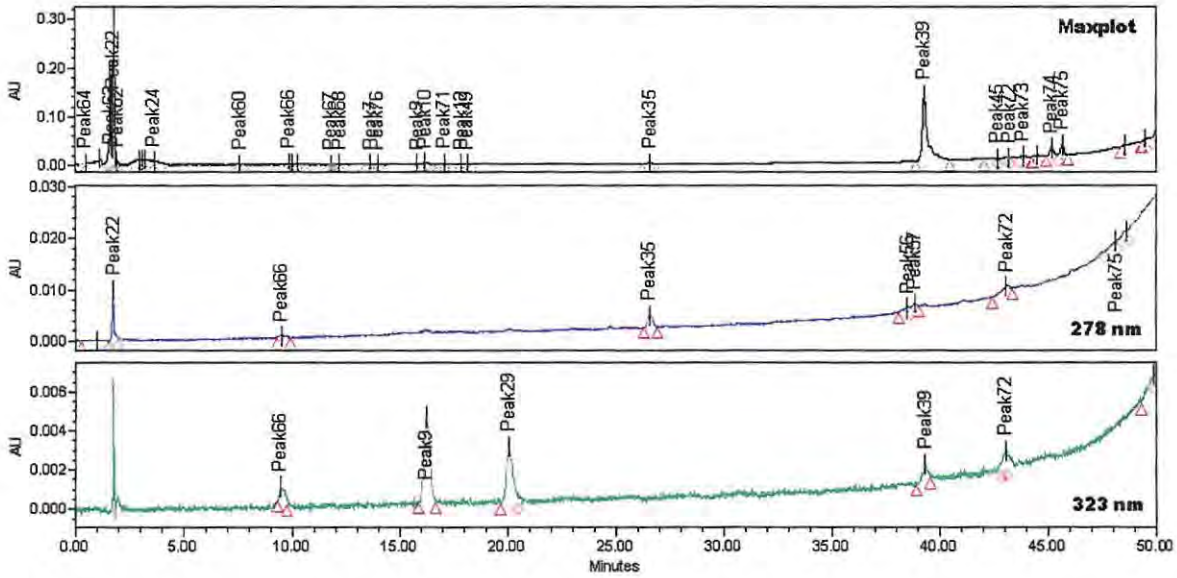


Fig. 9.14: Intracellular MeOH extract from TLC plate spot 3 from isolate WL114 grown in SC broth

Fig. 9.12 shows that peak 29 was the dominant peak in spot 1, although it was also present in spots 2 and 3, but at much lower concentrations (Figs. 9.13 and 9.14). Similarly, peak 35 was detected in spots 1, 2 and 3, but at very low concentrations (Figs. 9.12 to 9.14); while very low levels of peak 30 were only observed in spot 1 (Fig. 9.12), peak 17 was not detected in any of the spots.

9.3.3. Isolation of active compound(s) from intracellular MeOH extracts from *Streptomyces* sp., isolate WL114

The fractions collected from the silica column chromatography of isolate WL114 (see section 9.2.4) were analysed by HPLC and for activity against *S. aureus*. Zones of inhibition against *S. aureus* (blue curve) and metabolite profiles of the different compounds in each HPLC fraction of the extract from isolate WL114 are illustrated in Fig. 9.15 below. These peaks were correlated to the size of the zones of inhibition against *S. aureus* in well-diffusion bioassays (Fig. 9.16).

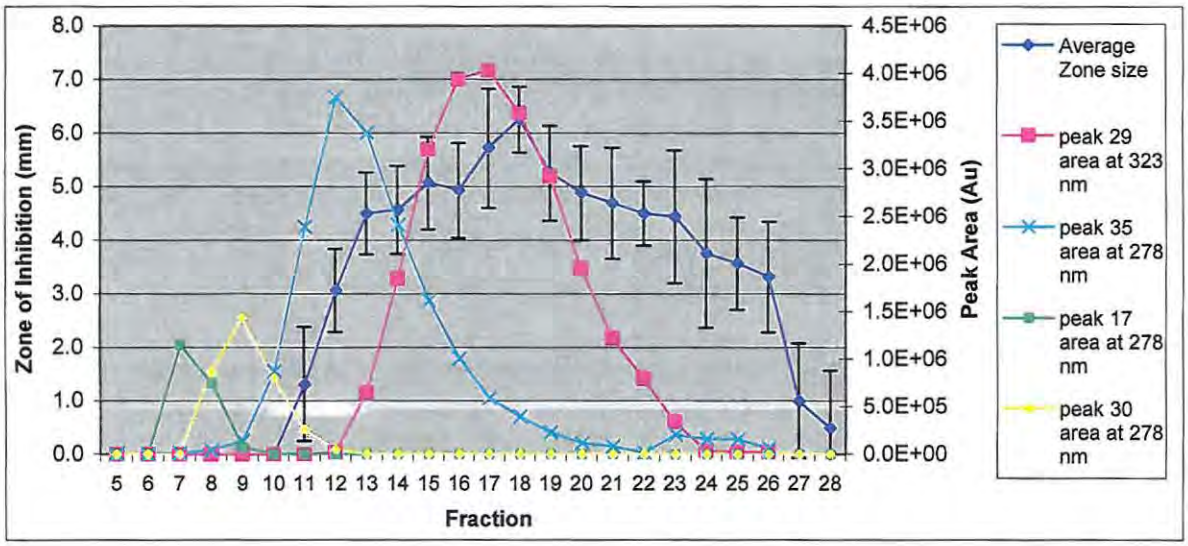


Fig. 9.15: Biological activity of the major peaks detected in each HPLC fraction from isolate WL114 extracts

Two compounds (peaks 17 and 30) were eluted first with 80% MeOH (0.1% HCOOH) in fractions 5 to 12. Maximum levels of peaks 17 and 30 were detected in fractions 7 and 9, respectively, but did not produce zones of inhibition. A third compound, peak 35, was detected in fractions 7 to 28. Maximum levels of peak 35 were detected in fractions 11 to 12 after which the concentration decreased gradually towards fraction 22 and nominal levels were detected up until fraction 28.

The lack of correlation between peak 35 and the size of the zones of inhibition indicated that peak 35 was not associated with antimicrobial activity. The fourth compound, peak 29, was detected in fraction 12 and increased in concentration until fraction 17 after which it decreased until fraction 26. The curve showing the concentration level of peak 29 in each fraction, and the size of the zones of inhibition for the corresponding fractions, showed the more obvious correlation to antimicrobial activity.

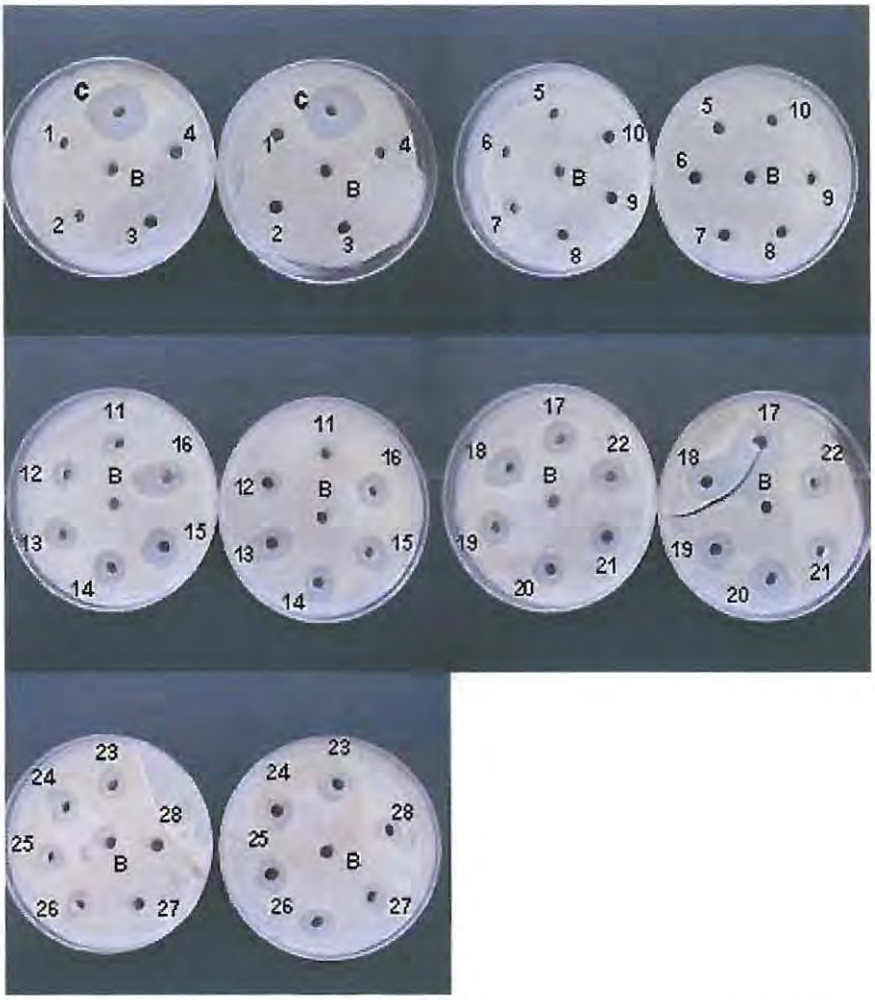


Fig. 9.16: Zones of inhibition against *S. aureus* from corresponding HPLC fractions of isolate WL114 extract. C=crude extract control, B= MeOH solvent blank, fractions 1-4 = 20% MeOH, fractions 5-20 = 80% MeOH, fractions 21-28 = 100% MeOH.

The separation of the four major compounds detected needs to be improved as the peaks for each compound overlapped, however it was clear that the active compound was represented by peak 29. This peak was shown to have a unique spectrum that was different from the spectra of the other three peaks (Fig. 9.11).

9.3.4. Purification and structure elucidation of active compound(s) from intracellular MeOH extracts from *Streptomyces* sp., isolate WL114

9.3.4.1. Purification by HPLC fractionation

The two peaks of interest, peaks 29 and 35 were separated by HPLC fractionation and collected from the crude intracellular MeOH extract from isolate WL114 in fractions 2 and 4 (Fig. 9.17a). A higher injection volume of 60 µl was used to collect

as much of the pure fractions as possible in the shortest time for NMR analysis. However, the shape and retention time of peaks 29 and 35 shifted slightly as a function of concentration due to overloading the column (Fig. 9.17b).

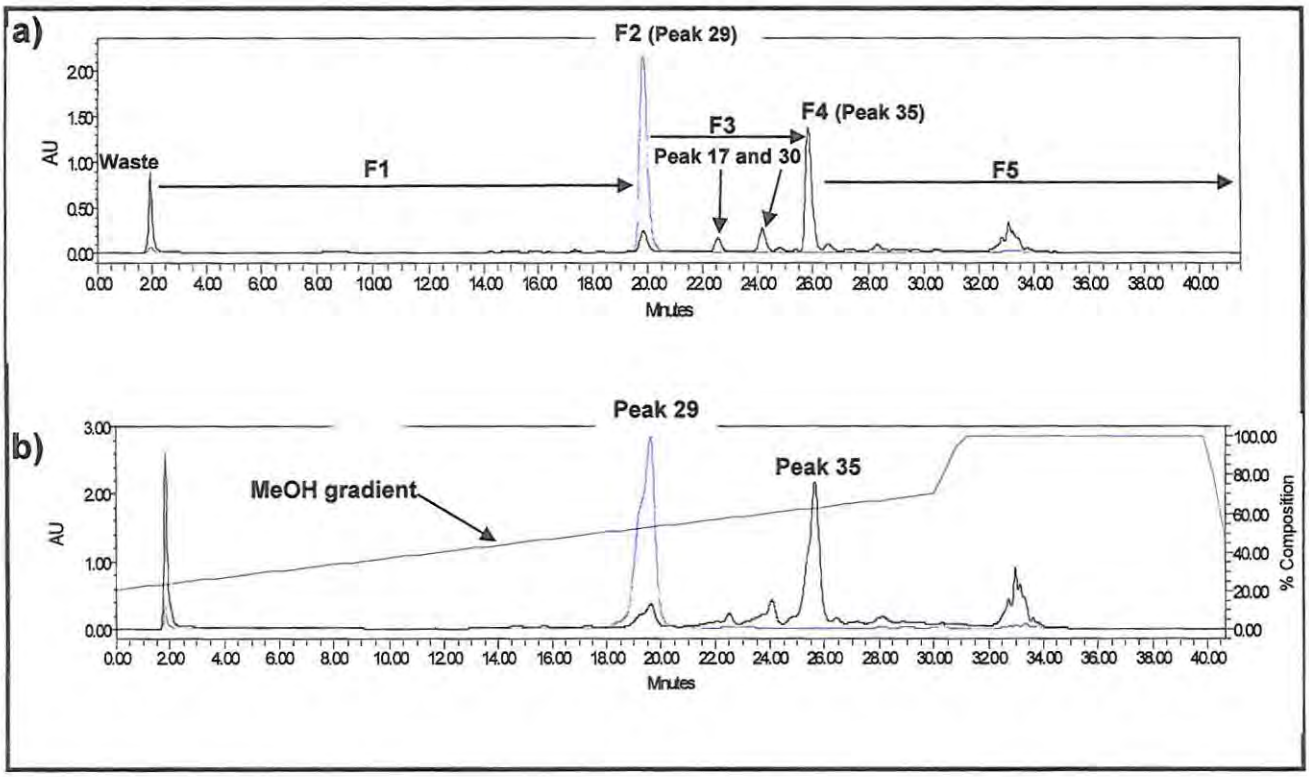


Fig. 9.17: HPLC profiles of **a)** 20 µl and **b)** 60 µl injection volumes of intracellular MeOH extracts from isolate WL114 grown in SC broth

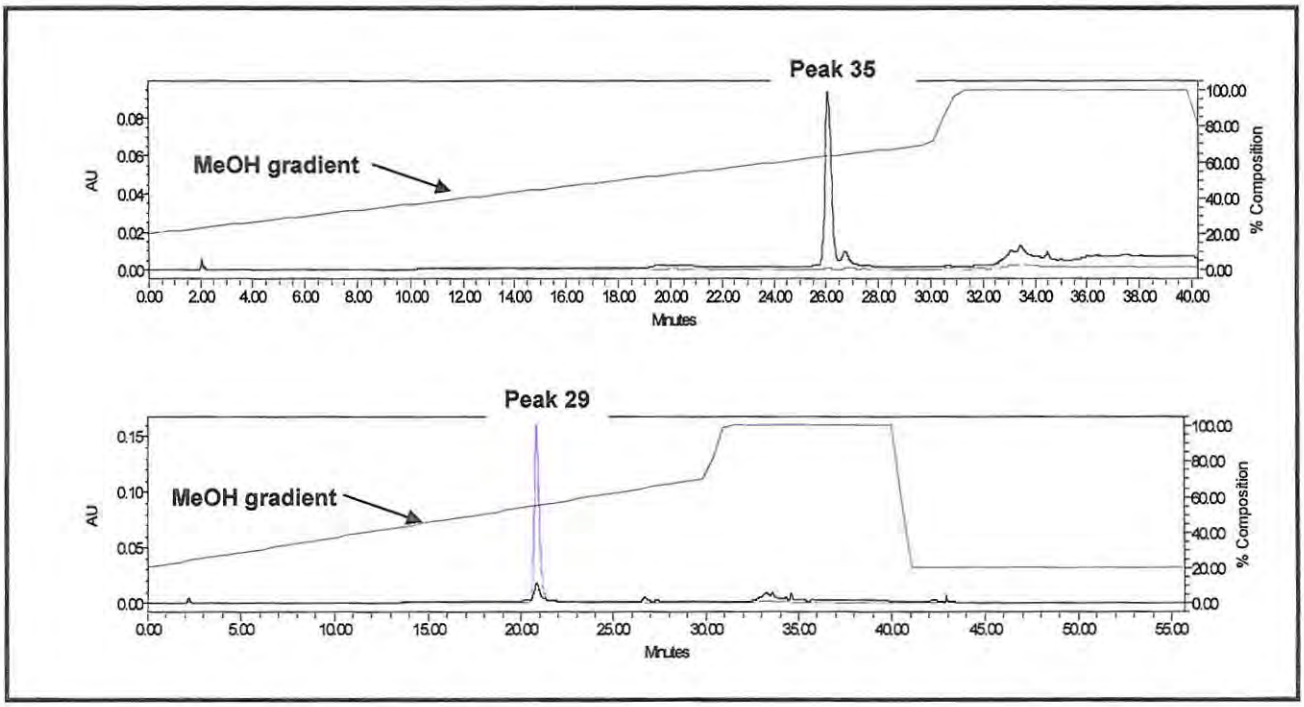


Fig. 9.18: Purity of peaks 35 and 29 after HPLC fractionation (50 µl)

The purity of fractions 2 (peak 29) and 4 (peak 35) were confirmed by HPLC (Fig. 9.18). Fraction 3 was not a pure fraction as both peaks 17 and 30 were collected together (Fig. 9.17a).

9.3.4.2. NMR structure elucidation of the compound in peak 35

Peak 35 gave the simplest ^{1}H NMR spectrum and was analysed first. The structure of this compound was determined from one- (^{1}H and ^{13}C) and two-dimensional (^{1}H-^{1}H COSY, HSQC and HMBC) NMR data (Figs. 9.19 – 9.22).

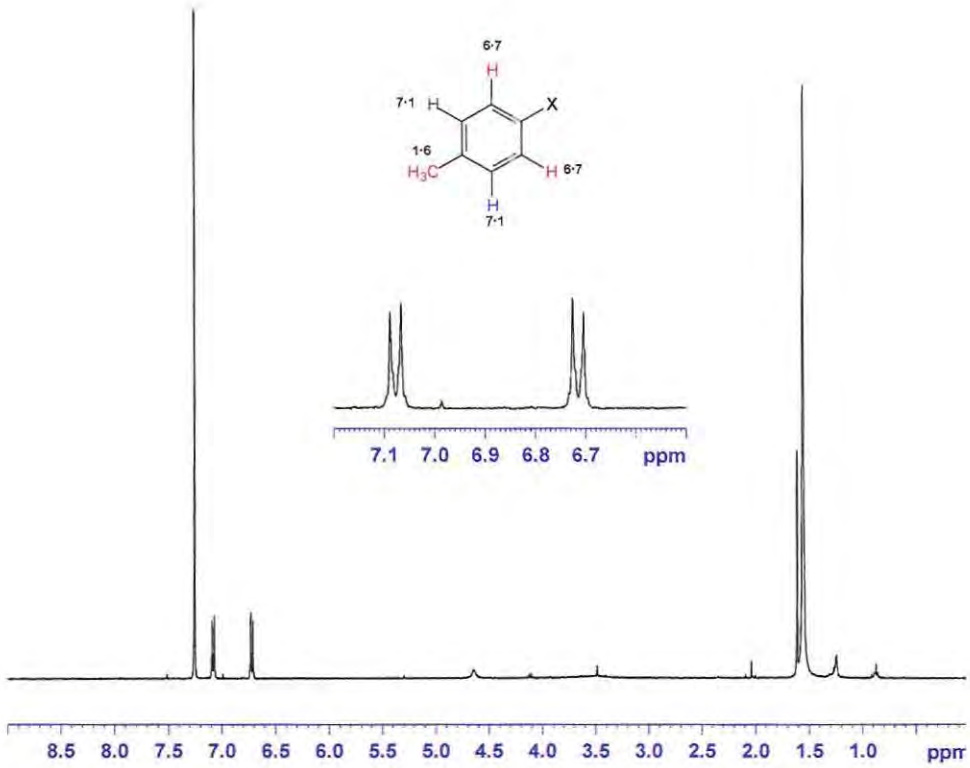


Fig. 9.19: ^{1}H NMR spectrum of the compound in peak 35

The ^{1}H NMR spectrum appeared to be deceptively simple with a *para*-substituted benzene moiety at chemical shift δ 6.7 (*d*, *J* = 8 Hz) and 7.1 (*d*, *J* = 8 Hz) and a methyl group at δ 1.6 (Fig. 9.19).

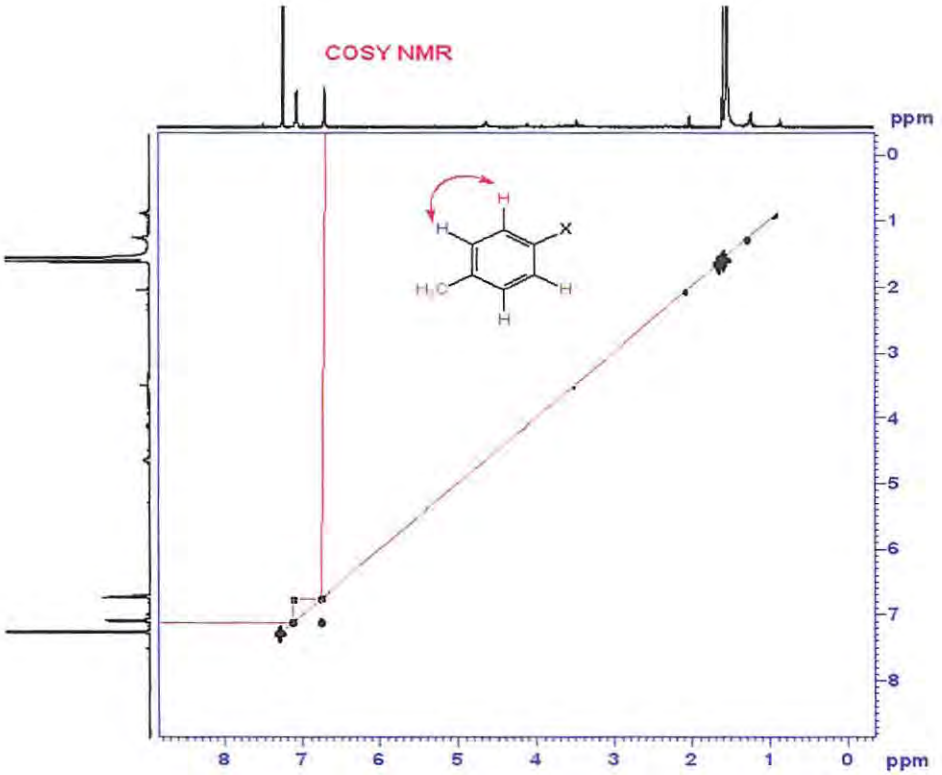


Fig. 9.20: ^{1}H-^{1}H COSY NMR spectrum of the compound in peak 35

The ^{1}H-^{1}H COSY NMR spectrum showed that the hydrogen atoms at positions 6.7 and 7.1 ppm were on neighbouring carbon atoms as they were spin-spin coupled to each other (Fig. 9.20). The symmetrical cross-peaks that appeared next to the diagonal line indicated which hydrogen atoms were spin-spin coupled to each other by matching the center of a cross-peak to the center of each of two corresponding diagonal peaks. The peaks on the diagonal line, when matched with the cross-peaks, were therefore coupled to each other.

The HSQC spectrum (Fig. 9.21) indicated which protons and carbon atoms were connected while the HMBC spectrum (Fig. 9.22) indicated which protons were connected to carbon atoms two or three bonds away.

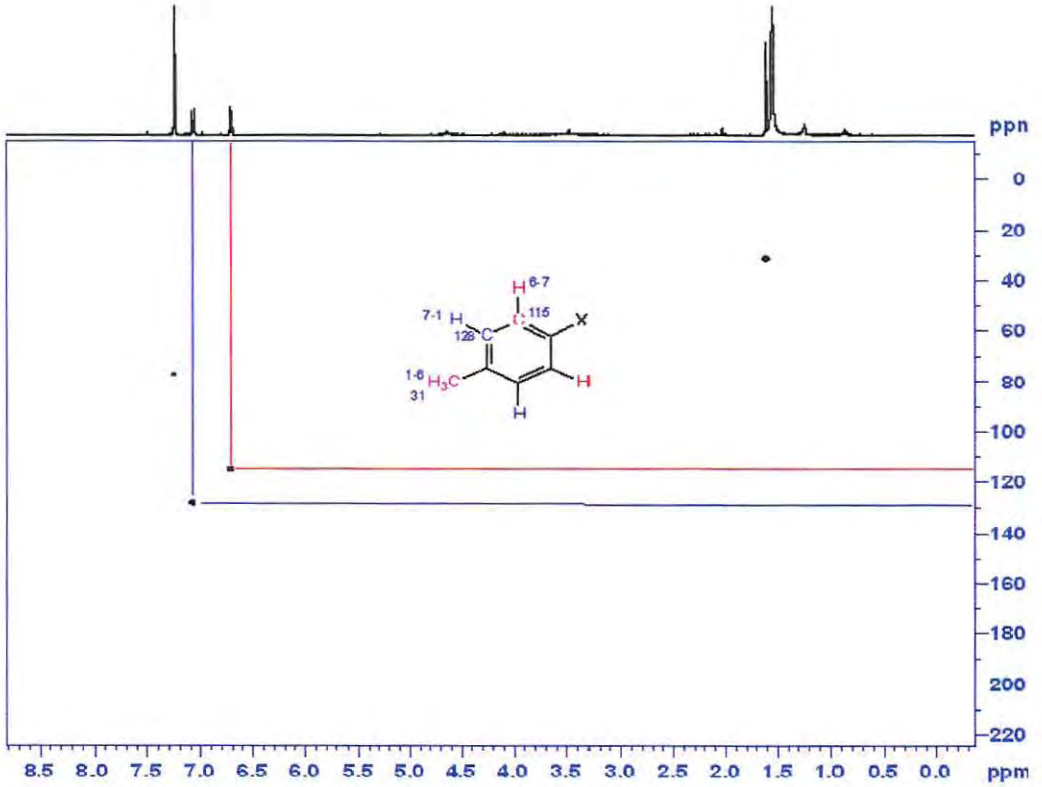


Fig. 9.21: HSQC NMR spectrum of the compound in peak 35

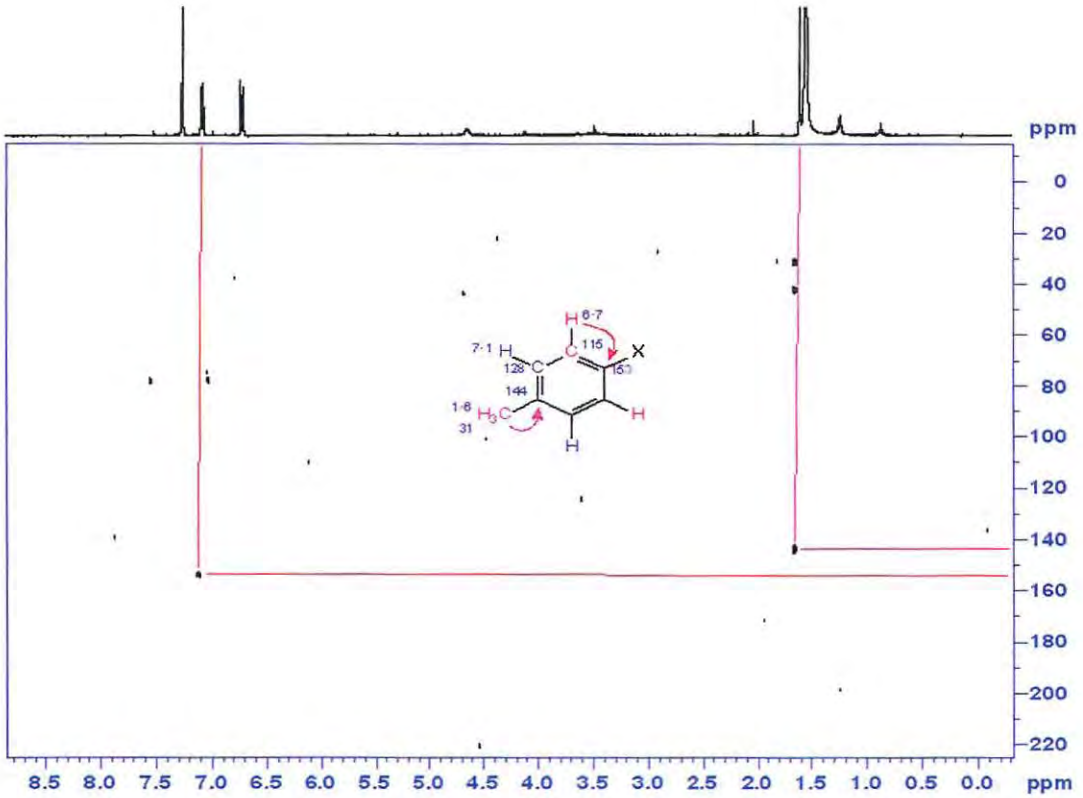


Fig. 9.22: HMBC spectrum of the compound in peak 35

Further analysis of the one and two dimensional NMR data, and comparison with literature values, suggested that peak 35 (found in fraction 4) is an industrial chemical called Bisphenol A. Bisphenol A ($C_{15}H_{16}O_2$) is a precursor used for the synthesis of polycarbonate and epoxy resins (Fig. 9.23) (Lobos *et al.*, 1992).

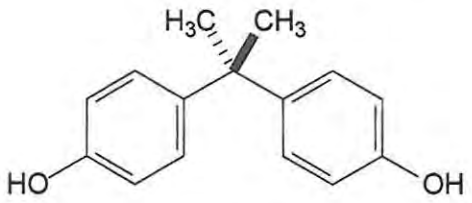


Fig. 9.23: Chemical structure of Bisphenol A

The use of these resins in many consumer products, including food-contact plastics, has resulted in extensive scientific debates about the significance of this compound to humans (Haighton *et al.*, 2002). Bisphenol A is known to be an endocrine-disrupting chemical, imitating the action of oestrogen, thus interfering with mammalian development (Sakai *et al.*, 2007). Chemical manufacturing plants generate considerable quantities of waste containing Bisphenol A, of which trace levels are discarded into terrestrial, aquatic, and marine environments, and leach out from resins used in plastics (Lobos *et al.*, 1992; Sakai *et al.*, 2007). Bisphenol A in surface water and sediments from diverse locations degrade rapidly, indicating that microorganisms capable of degrading this compound are ubiquitous in the environment, however the biodegradation of Bisphenol A in seawater is not well understood (Sakai *et al.*, 2007). The biological activity of Bisphenol A (peak 35) and purified peak 29 against *S. aureus* is demonstrated in Fig. 9.24.

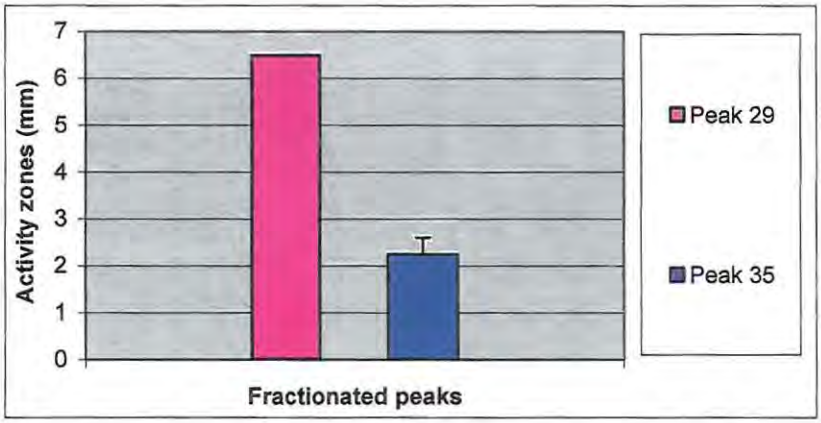


Fig. 9.24: Biological activity of peaks 29 and 35 against *S. aureus*

The largest zone of inhibition corresponded to peak 29 (6.50 mm), confirming that the active compound is present in peak 29, while the zone of inhibition for Bisphenol A was only 2.25 mm. This may be attributed to the fact that Bisphenol A together with other environmental pollutants, wash into the sea from soils and river water as contaminants (Sakai *et al.*, 2007), and was consequently detected in extracts from *Streptomyces* sp., isolate WL114, after cultivation in seawater media containing this pollutant.[IV]

9.3.4.3. NMR structure elucidation of the compound in peak 29

The increased complexity and low concentration of peak 29 necessitated the use of a higher resolution NMR spectrometer. Thus all spectra for peak 29 were obtained on a 600 MHz Bruker NMR spectrometer.

Electron clouds surrounding nuclei have a magnetic moment causing the magnetic field created by these electrons to impact the chemical environment of individual nuclei, called shielding. Electron-withdrawing groups decrease shielding, increasing resonance frequency, resulting in a higher chemical shift (Holtman, 2003). The ^{1}H NMR spectrum of peak 29 shows the presence of several aromatic moieties in the region δ 6.5 - 8.0 (J values ~ 8 Hz) (Fig. 9.25). The deshielded nature of some resonances (δ 7.8 - 7.3) are suggestive of electron withdrawing substituents on the aromatic nucleus. The integration values for individual peaks indicate significant overlap of signals or the presence of impurities.

[IV] Storage tubes (Greiner) did not contain Bisphenol A as based on information supplied by the manufacturer. Thus, Bisphenol A may be present as an unexplained artifact.

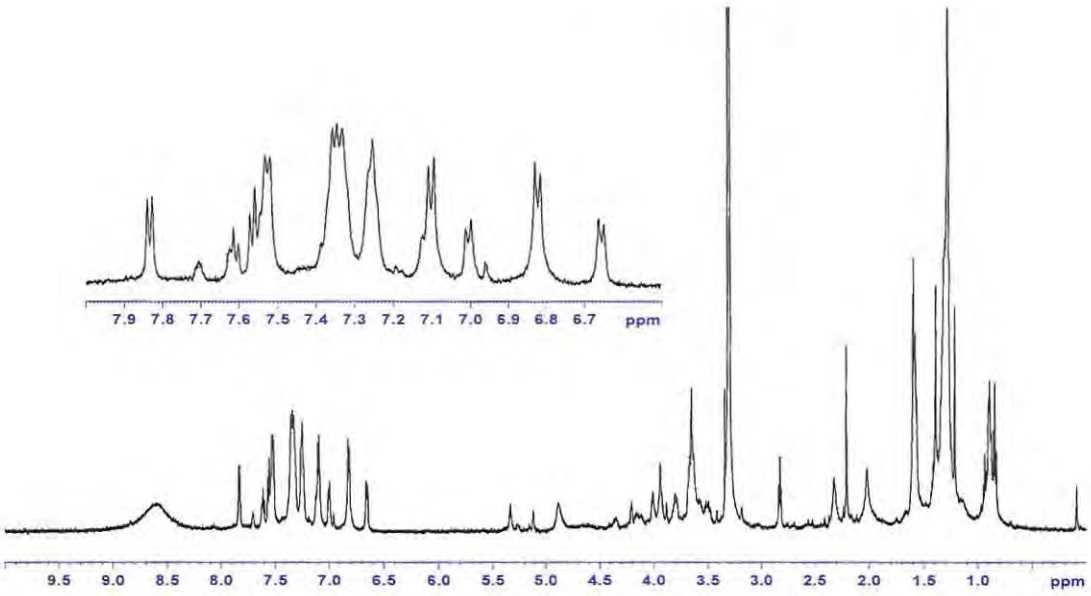


Fig. 9.25: ^{1}H NMR spectrum of the compound in peak 29 with presaturation (ns 80)

^{13}C NMR spectra could not be obtained due to the low concentration of the compound in peak 29 consequently carbons present in the compound were determined from the HSQC and HMBC spectra (Fig. 9.26).

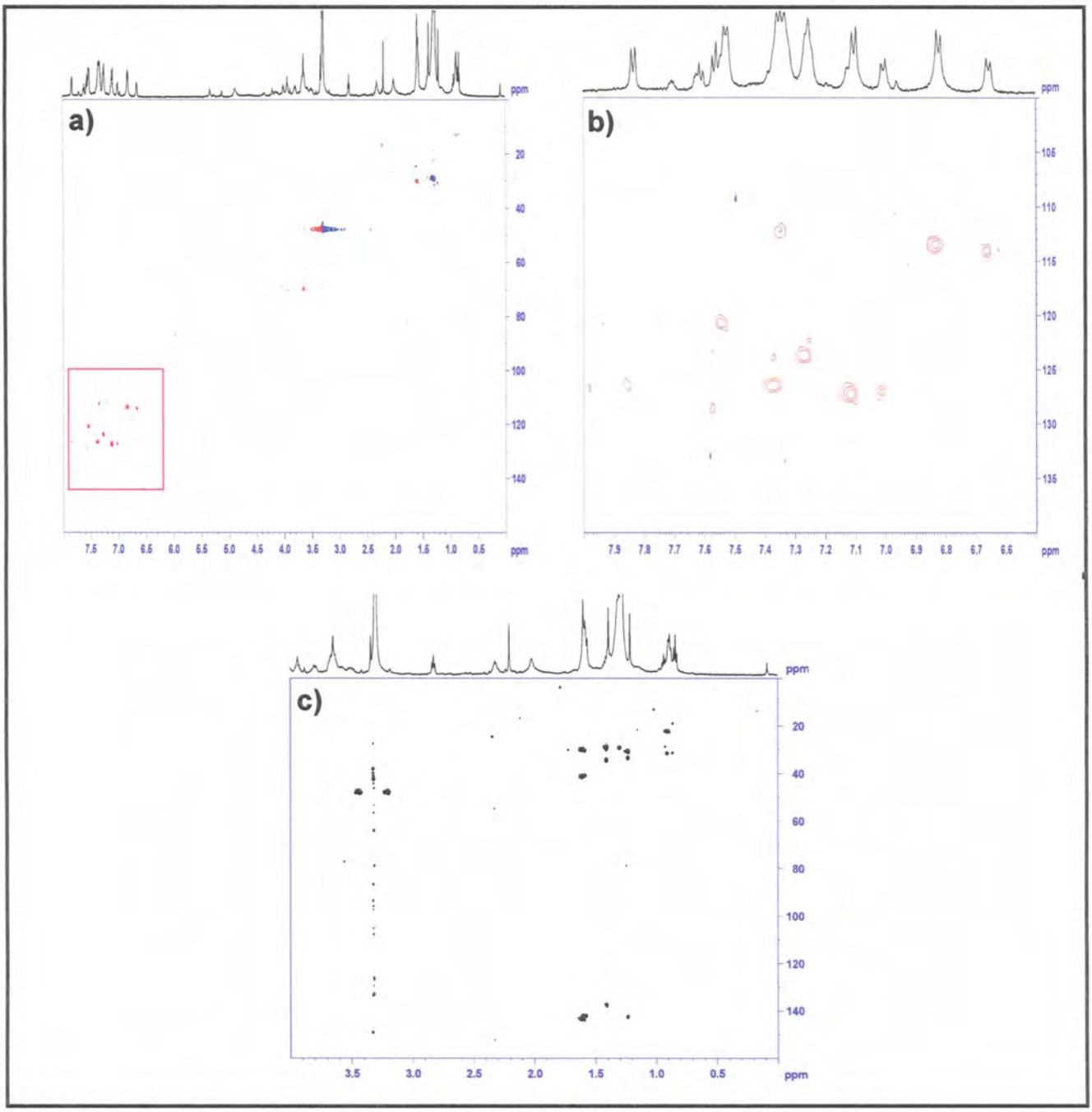


Fig. 9.26: a) HSQC spectrum of the compound in peak 29 and **b)** HSQC spectrum expanded in the aromatic region and **c)** HMBC spectrum expanded

The most valuable structural information was obtained from the COSY spectrum (Fig. 9.27). Additionally, a composite all NMR information for the compound in peak 29 is represented in Fig. 9.28. However, it is clear that in order to confidently assign the full structure of the compound in peak 29, more of the compound in peak 29 will have to be isolated.

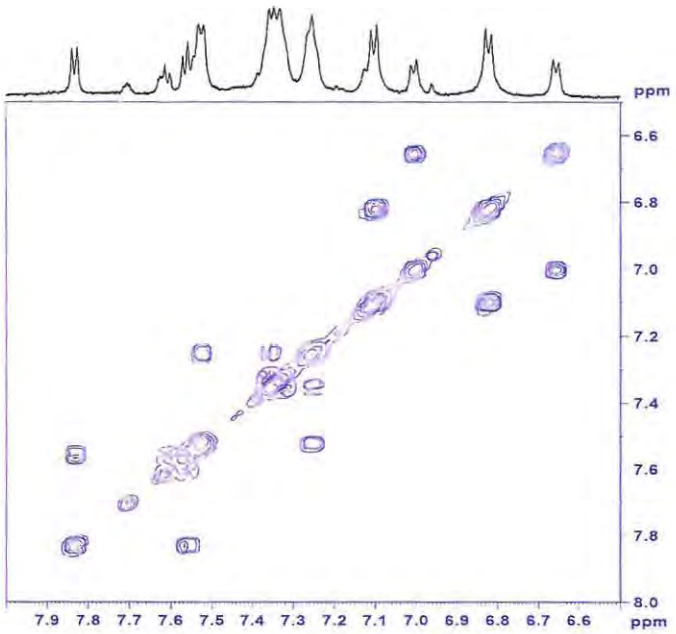


Fig. 9.27: ^{1}H -^{1}H COSY spectrum of the compound in peak 29

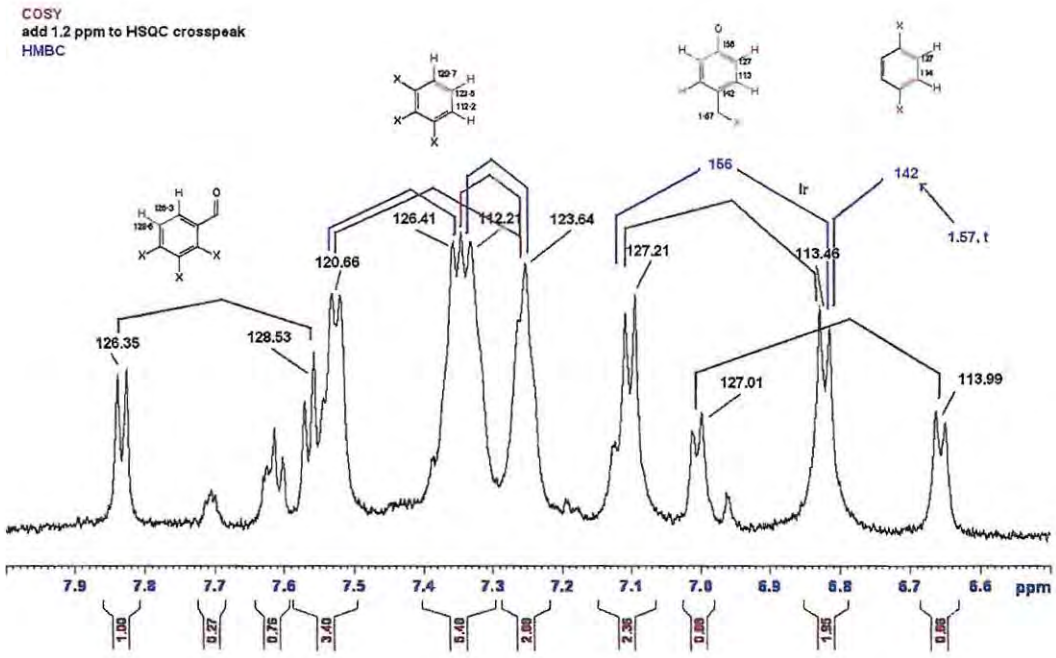


Fig. 9.28: A composite of all NMR data for the compound in peak 29

9.4. CONCLUSION

It is evident that the metabolite responsible for the bioactivity of *Streptomyces* sp., isolate WL114, was found in peak 29. This compound showed higher levels in the more active of the two extracts examined (SC broth extract), and was also extracted from TLC spot 1 which corresponded to the zones of inhibition observed with the TLC bioassay (bioautographic analysis). Additionally, HPLC analysis of the metabolite profiles of significant peaks in these intracellular MeOH extracts and well-diffusion bioassays showed a distinct correlation between peak 29 and antimicrobial activity against *S. aureus*.

Furthermore, NMR structure elucidation established that HPLC fractionated peak 35 was Bisphenol A, a possible artifact, while biological activity against *S. aureus* confirmed that the active compound was represented by HPLC fractionated peak 29. However, due to the low concentration and volume of this potent active compound, the chemical structure could not be fully characterised.

CHAPTER 10

Conclusions

10.1. Overview and conclusions

The aim of this study was to investigate novel marine microorganisms for the production of biologically active metabolites. It involved the development of efficient methods for the isolation of marine microorganisms from seawater and/or sediment from the Antarctic Ocean and Marion Island, and the extraction methods for the isolation of novel bioactive compound(s) from these marine microorganisms. The methods used to investigate the marine microorganisms were developed from the experience gained from a previously isolated marine microorganism, *Pseudoalteromonas* AP5, with known antibacterial activity. The photosensitive metabolite from AP5 exhibited antifungal activity, but the active metabolite(s) could not be characterised as AP5 gradually lost the ability to produce metabolite(s) due to continuous cultivation in shake flasks.

The Plackett-Burman experimental design tool, which depicted the combination of different processing techniques together with a range of complex and simple growth media, assisted in the successful isolation of 276 marine microorganisms from deep and shallow seawater and sediment samples. These isolates included a small percentage of fungi, similar percentage ratios of Gram-negative and -positive bacteria from water samples, and an increased percentage of Gram-positive bacteria from sediment samples of which 23% where filamentous bacteria, possibly actinomycetes. Actinomycetes are widely distributed in the world's oceans and many microorganisms, such as actinomycetes and fungi, are washed or blown into the sea from the shore, however true indigenous marine actinomycetes, such as *Salinispora*, have recently been reported (Larsen *et al.*, 2005; Pathom-aree *et al.*, 2006).

Initial bioactivity screening of ten marine microbes showed that three isolates, WL61, WL114 and WL136, exhibited bioactivity against different test organisms. Isolate WL61 was isolated from the Antarctic Ocean at a depth of 3000 m, isolates WL114 and WL136 were both isolated from sediment samples from Marion Island. 16S ribosomal DNA sequencing and phylogenetic relationships confirmed that these

isolates were *Streptomyces* spp. However, a more conclusive description of the novelty of these isolates must be carried out, particularly for isolates WL61 and WL136, as percentage homologies were high (100%). It is beneficial to know the species of the microorganism under investigation since the optimal growth medium used for maximum metabolite production differs between species (Larsen *et al.*, 2005).

A novel technique, using Quorus membrane bioreactors, was utilised for nutrient medium selection, screening and production of marine bioactive compound(s). It is well-known that the medium composition and culture conditions affect the growth and production of secondary metabolites, thus several media containing different nutrients were investigated. In addition to general factors such as carbon, nitrogen, trace elements, temperature, aeration and time of cultivation, some microorganisms may need stimulation by the addition of factors such as pyruvate, cyclic AMP and AHLs to detect their full metabolic potential (Larsen *et al.*, 2005).

Results obtained from SFR experiments demonstrated the value of screening microorganisms under a range of nutritional conditions, for the production of bioactive compounds. All three of the *Streptomyces* spp. produced one or more biologically active metabolite(s) active against a small selection of Gram-positive and -negative bacteria, yeast and fungi. MFR experiments, using optimal media determined from the SFR experiments, were successful in generating large amounts of intra- and extracellular bioactive extracts which were relatively stable when stored in the light and at varying temperatures. Novel Quorus bioreactor technology can thus be used successfully in future research and as a production facility for novel or known marine metabolites. Furthermore, the active compounds produced by each isolate had either a biostatic or a biocidal effect on the cells of the test strains. Further research, using biological assays to determine the affects of these active compounds on immune function, anti-inflammatory responses, anti-cancer and antiviral activities, will be carried out in the laboratories of Synexa Life Sciences BioAnalytical Unit.

Bioautographic analysis of intracellular MeOH extracts from isolate WL114 showed very large zones of inhibition against *S. aureus*. The HPLC metabolite profile of this extract resulted in four interesting peaks (peaks 17, 29 30 and 35). Larger peaks, peaks 29 and 35, overlapped and all peaks were purified by HPLC fractionation. Only

peak 29 resulted in a zone of inhibition in well-diffusion bioassays. However, due to the complexity and low concentration and volume of the bioactive compound in peak 29, a full chemical structure could not be confidently assigned to this potent metabolite until more material is isolated. Structure elucidation confirmed that peak 35 was Bisphenol A. Interestingly, Bisphenol A present in the extract was not a contaminant from leaching of the plastic materials during the extraction procedures, but rather an unexplained artifact. While bioactive compound(s) from only one of the *Streptomyces* spp., isolate WL114, was chemically characterised and identified, active compounds from isolates WL61 and WL136 will also be analysed in the laboratories of Synexa life Sciences Analytical Unit together with the pharmaceutical chemistry laboratories of the Faculty of Pharmacy at Rhodes University.

Advances in and the application of new methods in separation science and analytical chemistry techniques, together with targeted mechanism-based screens will improve the rate at which active compounds from natural product extracts are isolated and identified, making natural products valuable components of modern drug discovery (Harvey, 2000; Fenical and Jensen, 2006). While the ecology, biogeographic distribution and evolutionary history of actinobacteria in the marine environment are still significant areas of research, it is becoming progressively more apparent that marine actinomycetes specifically, represent a major resource for biotechnological search and discovery.

10.2. Advances and new interest in natural product research

Mention has been made about the shortage of new antibiotics, the reason for their necessity reaching disturbing proportions as well as the rationale for the withdrawal of many big pharma companies from this field. However, it is apparent that there is renewed interest in natural product-based drug discovery and there have been encouraging signs of the newer biotechnology companies developing an interest in marine organisms.

Attention has moved to smaller high-quality libraries making natural product leads a justifiable starting template for combinatorial chemistry allowing novel biologically active analogs with improved properties or new activities to be discovered (Lam, 2007). Furthermore, high-speed approaches to dereplication could eliminate technical barriers while making use of libraries of natural products in high-throughput

screening processes. Cloning and genetic engineering offer alternative methods for the production of diverse natural compounds. This can be accomplished by cloning the relevant metabolic pathways from unculturable organisms into a suitable host species, significantly expanding the range of available compounds. Similar techniques can be used to "mix-and-match" enzymes in artificial arrangements whereby combinational chemistry is used to modify a natural structure, resulting in even more novel structures (Harvey, 2000; McGee, 2006).

Genome mining and the existence of gene sequences involved in secondary metabolite production, have confirmed that soil actinomycetes will continue to be a source of new secondary metabolites. This is largely due to the diversity of actinobacteria, particularly from unexplored sites or extreme habitats, such as deep ocean sediments. Structurally unique secondary metabolites produced by these microorganisms, further support the fact that understudied habitats are a rich source of novel actinomycetes for bioprospecting (Pathom-aree *et al.*, 2006; Fenical and Jensen, 2006). It is evident, from the analysis of one low-density marine actinomycete taxon (the genus *Salinispora*), that the pattern of secondary metabolite production can be highly complex and that the drug discovery process can be supported by phylogenetic studies (Fenical and Jensen, 2006).

The process of antibiotic discovery from natural products has made considerable progress over time. It is this progress that has assisted researchers to understand the main features required for success, therefore any new work in this field will also gain from this understanding, as well as from the new technologies that contribute to the development of the efficiency of the process. The resources needed for this may appear to be major, but the reward for the companies that are prepared to take the risk is worthwhile as it may be the solution to one of the most severe health threats that we may face in the future (Peláez, 2006). Medical necessity and business opportunity could be the driving force for investment in natural product drugs (Fenical and Jensen, 2006; Bull and Stach, 2007). The question that should be asked is not whether natural products should be screened for drug discovery, but whether we can afford not to screen natural products (Lam, 2007).

APPENDIX A

A. Well-diffusion Bioassay Method

1. Spread 100 µl of each test organism onto the appropriate agar plate in duplicate.
2. Aseptically punch wells into the agar using the back of a glass Pasteur pipette and remove the agar plug.
3. Add 100 µl of extract or MeOH or EtOAc solvent (control) to the relevant wells.
4. Incubate plates at 25°C overnight or until growth or inhibition thereof.
5. Measure zones of inhibition of growth from the edge of each well (mm).

B. Microtitre plate Bioassay Method

1. Add 260 µl of Marine broth to all the wells in a sterile 96-well microtitre plate (Greiner) plus 30 µl of 1 X 10^6 *Penicillium* sp. spores (OD_{650}) to all the wells containing Marine broth, excluding the wells for the blanks.
2. Add 10 µl of the different dilutions of AP5 extract to six replicate wells designated for samples.
3. Add 10 µl of sterile distilled water (control 1) or 10 µl MeOH (control 2) to six replicate wells designated for the two controls.
4. Add 40 µl sterile distilled water (blank 1) or 30 µl sterile distilled water plus 10 µl MeOH (blank 2) to six replicate wells designated for the two blanks.
5. Monitor the growth curve of *Penicillium* sp. over 48-96 hours at 28°C using a plate reader (FLUOstar Optima, BMB Labotech).

APPENDIX B

A. Growth media for marine microorganisms:

1. **Marine Agar/Broth** (complex media) (Yoshikawa *et al.*, 2000)

37.4g	Marine broth powder (Difco)
18g	Agar (Biolab)
1L	Distilled water

2. **1/10 Marine Agar/Broth** (complex media).

3. **R2A Agar** (complex media) (Zengler *et al.*, 2002).

15.2g	R2A powder (Merck)
6g	Agar (Biolab)
1L	Distilled water

4. **1/10 R2A Agar/Broth + Seawater** (complex media).

5. **M1 Agar/Broth** (complex media) (Mincer *et al.*, 2002).

10g	Starch (Kimix)
4g	Yeast (Biolab)
2g	Peptone (Biolab)
18g	Agar (Biolab)
1L	Filtered Seawater

6. **M2 Agar/Broth** (minimal media) (Mincer *et al.*, 2002).

6mL	Glycerol (100%) (Saarchem)
1g	Arginine (Aldrich)
1g	K_2HPO_4 (Saarchem)
0.5g	$MgSO_4.5H_20$ (Saarchem)
18g	Agar (Biolab)
1L	Filtered Seawater

7. **M3 Agar/Broth** (minimal media) (Mincer *et al.*, 2002).

6g	Glucose (Kimix)
2g	Chitin (Sigma)
18g	Agar (Biolab)
1L	Filtered Seawater

8. **M4 Agar/Broth** (minimal media) (Mincer *et al.*, 2002).

2g	Chitin (Sigma)
18g	Agar (Biolab)
1L	Filtered Seawater

9. **M5 Agar/Broth** (minimal media) (Mincer *et al.*, 2002).

18g	Agar (Biolab)
1L	Filtered Seawater

10. **Colloidal Chitin Agar/Broth** (minimal media) (Hsu and Lockwood, 1975).

0.7g	K_2HPO_4 (Saarchem)
0.3g	KH_2PO_4 (Saarchem)
0.5g	$MgSO_4.5H_20$ (Saarchem)
0.01g	$FeSO_4.7H_20$ (Saarchem)
0.001g	$ZnSO_4$ (Saarchem)
0.001g	$MnCl_2$ (Saarchem)
0.4g	Chitin (Sigma)
1L	Distilled water
pH	7.0 with 1M NaOH

11. **Emerson's medium Agar/Broth** (complex media) (Del Frate and Caretta, 1990).

4g	Yeast extract (Biolab)
15g	Soluble starch (Kimix)
1g	K_2HPO_4 (Saarchem)
0.5g	$MgSO_4.5H_20$ (Saarchem)
16g	Agar (Biolab)
1L	Distilled water

12. **Starch-Casein Agar/Broth** (complex media) (Kokare *et al.*, 2004).

10g	Soluble Starch (Kimix)
1g	Casein (Sigma)
0.5g	KH_2PO_4 (Saarchem)
0.5g	$MgSO_4.5H_20$ (Saarchem)
30g	NaCl (Kimix)
18g	Agar (Biolab)
500ml	Filtered Seawater
500ml	Distilled water
pH	7.6 with 1M NaOH

13. **SESS Agar/Broth** (minimal media) (Sobecky *et al.*, 1997).

200g	Marine sediment
1L	Filtered Seawater
	Allow to settle and use supernatant
18g	Agar (Biolab)

B. Growth media for test organisms:

1. **117 *Micrococcus* medium (Agar/broth) for *S. aureus*** (ATTC medium)

5g	Peptone (Biolab)
3g	Yeast extract (Biolab)
1.5g	Beef extract (Biolab)
1g	Glucose (Kimix)
1L	Distilled water
18g	Agar (Biolab)
pH	pH 7.4 with 1M NaOH

2. **Nutrient Agar/broth for *P. Fluorescens, S. marcescens* and *B. subtilis***

16g	Nutrient broth powder (Biolab)
18g	Agar (Biolab)
1L	Distilled water

3. **Potato Dextrose Agar/Malt extract Broth for *Fusarium* sp.**

39g	Potato Dextrose Agar (Biolab)
3g	Agar (Biolab)
1L	Distilled water

4. **MYSA Agar/broth for *S. pombe***

10g	Malt extract (Biolab)
1g	Yeast extract (Biolab)
3g	Sucrose (Kimix)
18g	Agar (Biolab)
1L	Distilled water

5. **Marine Agar/broth for *Penicillium* spp.**

   As above

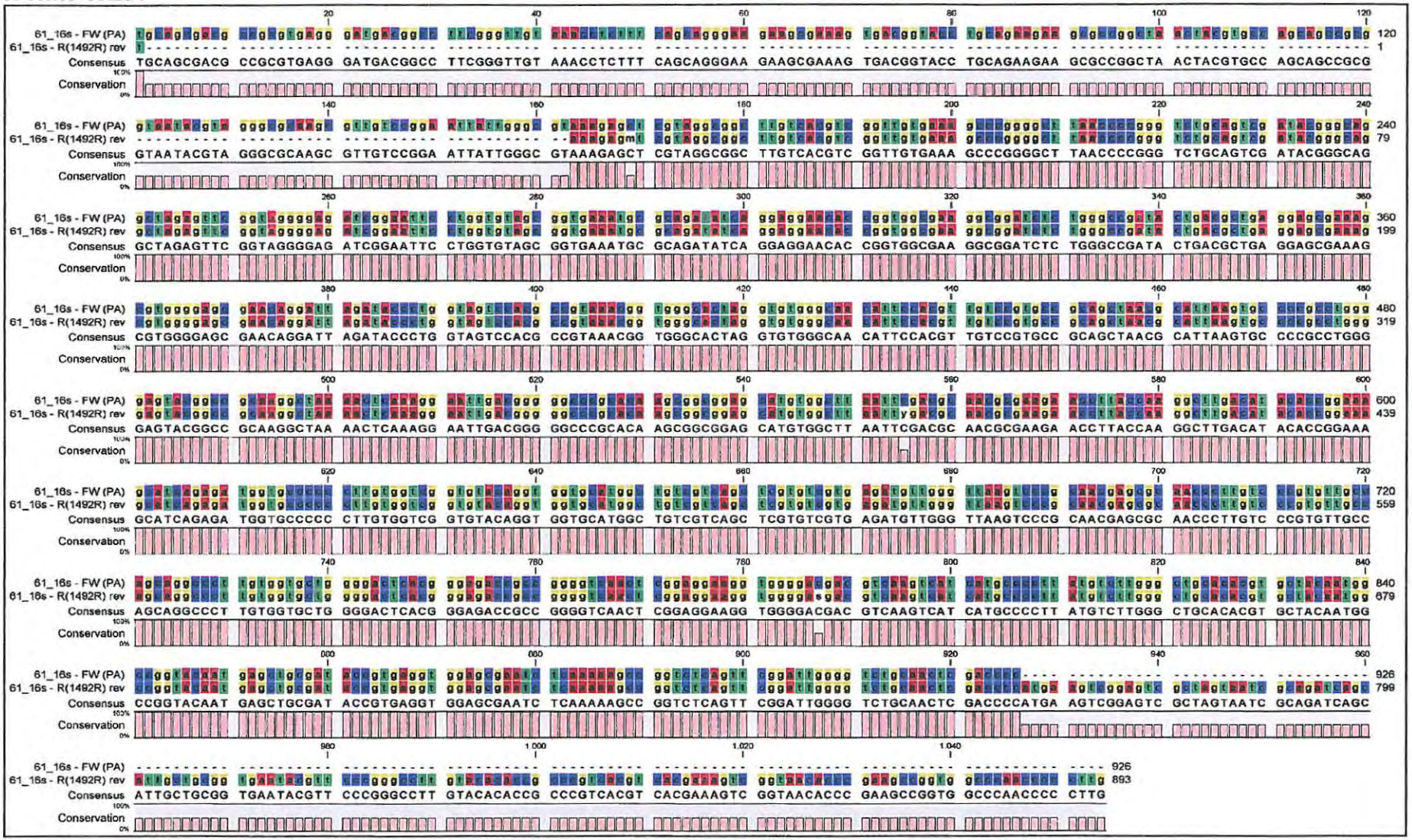
All media was autoclaved for 20 min at 121°C.

NOTE:

- Difco Marine broth (MB 2216) was obtained from Becton Dickinson, The Scientific Group, South Africa.
- All Biolab and Saarchem products were obtained from Merck, South Africa.
- R2A powder was obtained from Merck, Germany.
- All Kimix products were obtained from South Africa.
- Arginine was obtained from Sigma-Aldrich, Germany.
- Chitin was obtained from Sigma-Aldrich, USA.
- Casein was obtained from Sigma-Aldrich, New Zealand.

APPENDIX C (Sequence alignments of isolates WL61, WL114 and WL136 to obtain the consensus sequences for BLAST)

Isolate WL61



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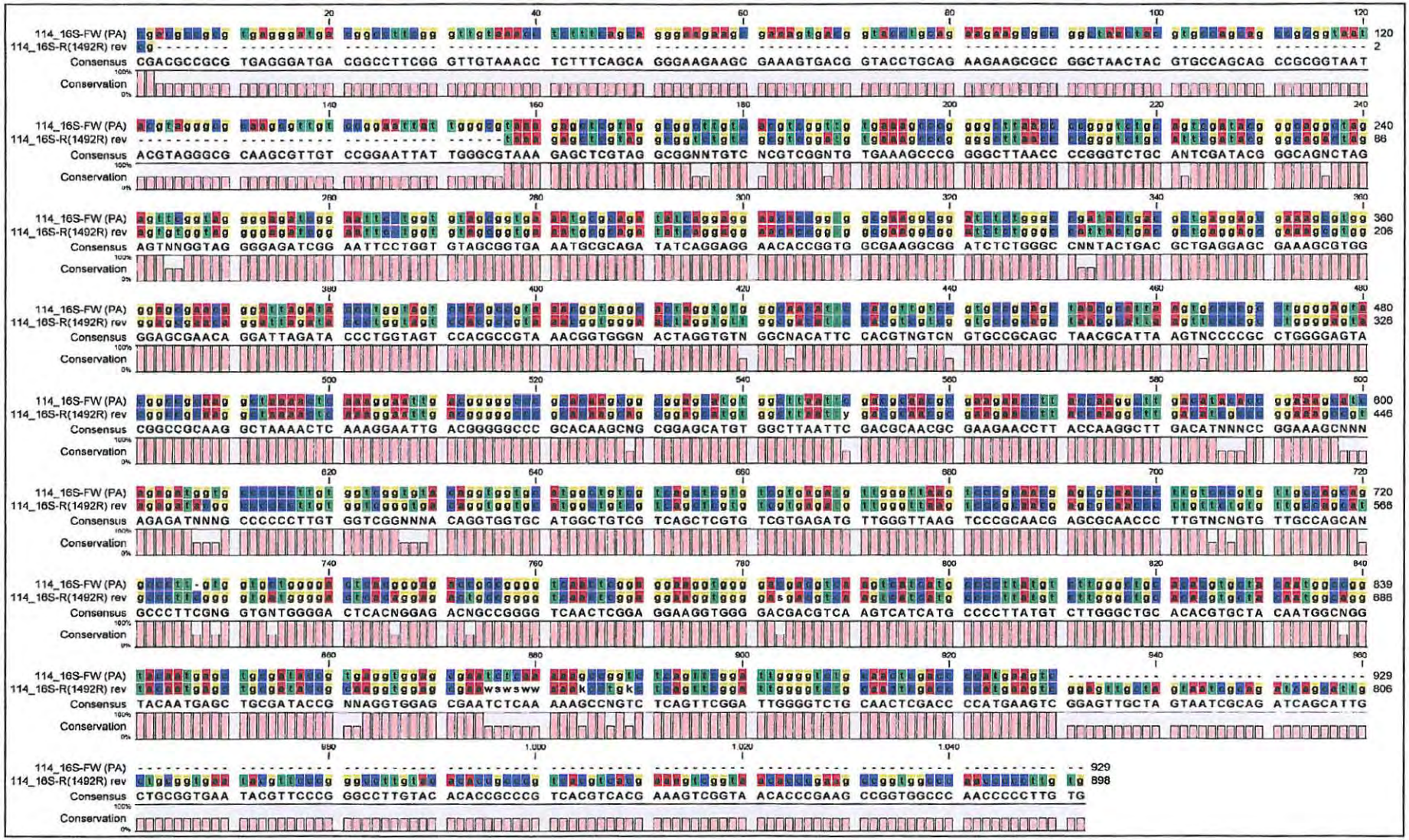
Sequence alignment of isolate WL61 to Streptomyces sp. A514Ydz-FQ

Isolate WL114



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Query 841    TACAATGAGCTGCGATACCGNNAGGTGGAGCGAATCTCAAAAAGCCNGTCTCAGTTCGGA  900
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Query 1021      ACACCCGAAGCCGGTGGCCCAACCCCCTTGTG                                1052
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Sbjct 1375      ACACCCGAAGCCGGTGGCCCAACCCCCTTGTG                                1406
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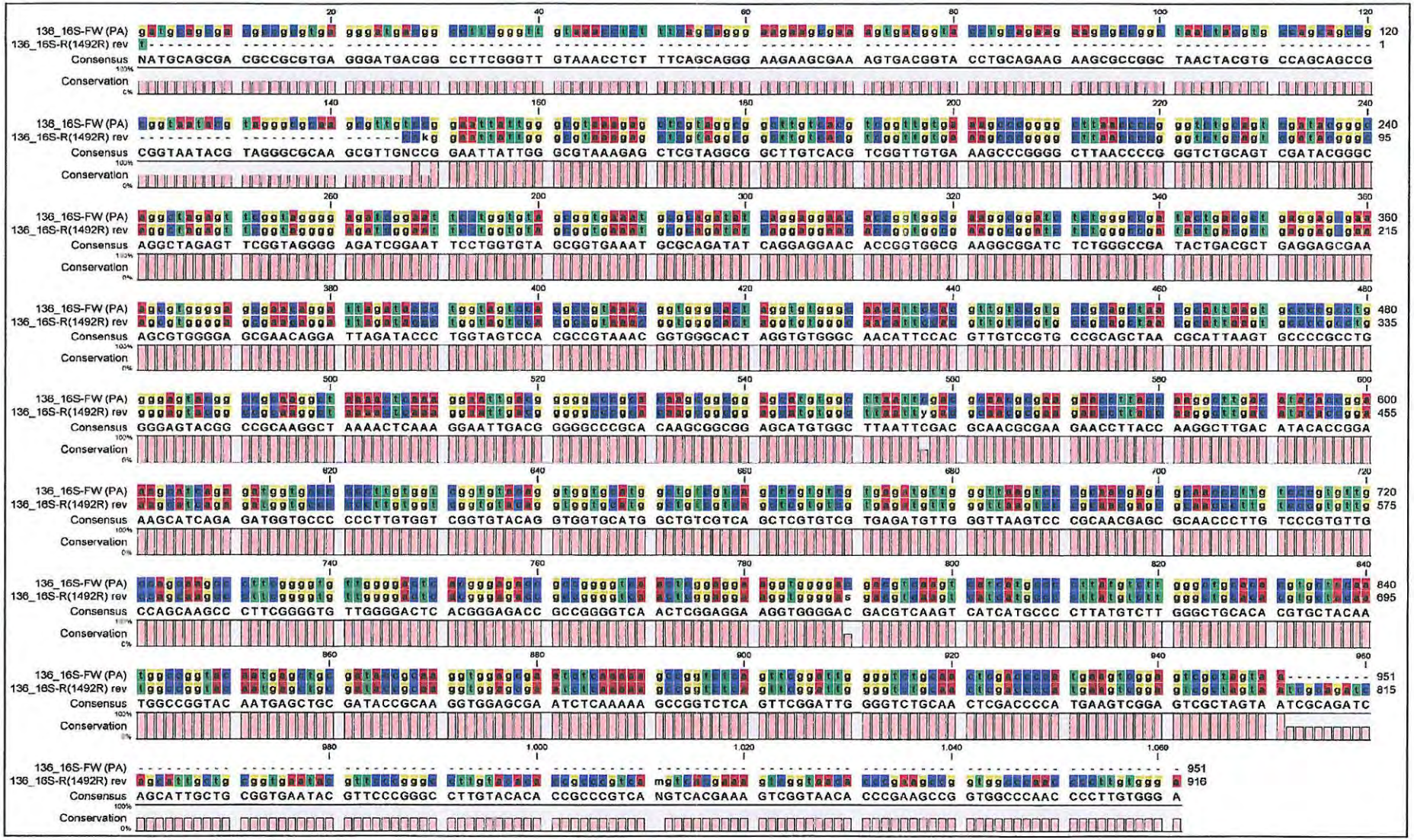
Sequence alignment of isolate WL114 to Streptomyces sp. HBUM 74842

Isolate WL136



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Query  182   CTTGTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCA  241
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Query  662   CTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGC  721
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Query  722   CAGCAAGCCCTTCGGGGTGTTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAA  781
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Sbjct  1200  GGCCGGTACAATGAGCTGCGATACCGCAAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAG  1259

Query  902   TTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCA  961
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Query  962      GCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCANGTCACGAAAG     1021
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Sbjct 1320      GCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAG     1379

Query 1022      TCGGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGGGA                         1061
                |||||||||||||||||||||||||||||||||||||||
Sbjct 1380      TCGGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGGGA                         1419
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Sequence alignment of isolate WL136 to Streptomyces sp. CNS-582_SD06

APPENDIX D

Glucose Assay method (Miller, 1959)

1. **DNS (Dinitrosalicylicacid) Reagent:**

5.0 g	DNS
1.0 g	Phenol
0.25 g	Sodium sulphite
5.0 g	NaOH
500 ml	Distilled water

2. **Glucose standard curve concentrations using a 5 g/L glucose standard solution:**

Glucose standard solution (µl)	Distilled water (µl)	Concentration (mg/L)
1000	0	1.0
900	100	0.9
800	200	0.8
600	400	0.6
400	600	0.4
200	800	0.2
100	900	0.1

3. Add 500 µl of permeate (sample) or water (blank) or glucose standards (standard curve) to 500 µl of DNS reagent in eppendorf tubes and vortex.

4. Heat the sample and standard mixtures at 90°C in a waterbath for 10 min (turns a brown-red colour).

5. Place on ice to stop the reaction and add 167 µl of a 40% potassium sodium tartrate solution and vortex.

6. Add 300 µl of sample, blank and standards to duplicate wells in a 96-well microtitre plate and measure absorbance at 590nm using a microtitre plate reader.

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