Matrix comparison of isolation conditions for secondary metabolite producing marine sponge associated bacteria

By

Relebohile Matthew Matobole

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Department of Biotechnology

University of the Western Cape

Bellville

Supervisor: Prof. Marla Tuffin (Trindade)

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Declaration

I, Relebohile Matthew Matobole, declare that **"Matrix comparison of isolation conditions for secondary metabolite producing marine sponge associated bacteria"** is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Date: 01 June 2015

Signature:……..

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Dedication

This is for my family, more especially my mother for being the best mother in the world, Ntate, Rakhali Mary, Mpitsoana, Tlhang, Khothalang, Malintle, Mamosa, Karabo, Limpho Mpoi and ausi Tebello for the inspiration.

Abstract

The discovery of novel secondary metabolites has declined significantly in recent years whereas there is a rise in the number of multi-drug resistant pathogens and other types of diseases. The decline in natural product discovery was due to high rediscovery of already known compounds and the costs in developing natural products. As a result pharmaceutical companies lost interest in investing in natural product discovery. However, there is a renewed interest in marine sponge associated microorganisms as a rich and untapped source of secondary metabolites. The objective of this study was to design a matrix to investigate the extent to which the One Strain-Many Compounds (OSMAC) approach applies to a collection of marine sponge isolates harvested from two South African marine sponge samples. Terminal restriction fragment length polymorphisms (T-RFLP) analysis was used to investigate and ascertain the two marine sponges which hosted the highest microbial diversities to be used for further culture-dependent studies.

The culture-dependent studies, using 33 media which included liquid enrichment, heat treatments and antibiotic treatments, resulted in 400 sponge isolates from the two marine sponges *Isodictya compressa* and *Higginsia bidentifera*. Using antibacterial overlay assays, 31 dereplicated isolates showed antibacterial activity. Bioactivities were also exhibited against *E. coli* 1699 which is genetically engineered for resistance against 52 antibiotics which implies that some of the bioactive compounds could be novel. The 16S rRNA gene sequences revealed that the microbial phyla isolated from the marine sponges belonged to *Actinobacteria*, *Firmicutes* and *Proteobacteria* (*Alphaproteobacteria* and *Gammaproteobacteria*).

Thirty isolates were selected for an OSMAC-based matrix study, 17 of which showed no antibacterial activities in preliminary screening. The application of the OSMAC approach using

co-culture and 36 culture conditions resulted in 6 isolates showing antibacterial activities, three of which did not show activities in preliminary screening. One of these, a *Bacillus pumilus* isolated from *I. compressa* displayed antibacterial activity against 5 indicator strains whereas in preliminary screening it had not shown activity. The results show that marine sponges can host novel microbial species which may produce novel bioactive compounds. The results also confirm that traditional methods employing a single culture condition restricts the expression of some biosynthetic pathways of microorganisms and as a result many metabolites have yet to be identified.

Key words: Secondary metabolites, marine sponges, OSMAC, microorganisms, T-RFLP

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List of Abbreviations

×*g*	centrifugal force
µg/µL	microgram per microliter
µM	micromolar
BLAST	Basic local alignment sequencing tool
bp	base pairs
C.S	cold shock C.S
$CaCl_2$	calcium Chloride
CCR	catabolite repression
CTAB	cetyl-trimethyl-ammonium bromide
dH_2O	demineralised water
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylene diamine tetra acetic acid
et al.	*et alia* (and others)
FAM	fluorescein amidite dye
g	grams
g/L	grams per liter
gDNA	genomic DNA
H.S	heat shock
HMA	high microbial abundance
ie.	*id est* (That is)
kb	kilobase
KCl	potassium chloride
KH_2PO_4	potassium dihydrogen phosphate
LB	Luria-Bertani
LMA	low microbial abundance
mDNA	metagenomic DNA
mg	milligrams
mg/mL	milligrams per milliliter
$MgCl_2$	magnessium chloride
mL	milliliter
mM	millimolar
NA	not applicable
NaCl	sodium chloride
$NaNO_3$	sodium nitrate
ng	nanogram
ng/µL	nanogram per microliter
NH_4Cl	ammonium chloride

NH_4OAc	ammonium acetate
nMDS	non-metric multidimensional plot
^oC	degrees Celsius
OD	optical density
OSMAC	One Strain Many Compounds
OTU	operational taxonomic unit
PCR	polymerase chain reaction
R.T	Room temperature R.T
rpm	revolutions per minute
SDS	Sodium dodecyl sulphate
sp.	species .
TAE	tris-acetic acid
TE	tris-EDTA
T-RFLP	terminal restriction fragment length polymorphism
T-RFs	terminal restriction fragments
Tris	tris (hydroxymethyl)-aminomethane
UV	ultra violet
V	volts
v/v	volume per volume
w/w	weight per volume
µL	microliter

CHAPTER 1 INTRODUCTION

1.1 Overview: Lack of discovery of novel bioactive compounds

The discovery of penicillin as early as 1928 by Alexander Fleming, and its development in the 1940s, marked the beginning of the golden age of microbial natural compounds discovery (Demain & Vaishnav 2009; Demain 2014). Secondary metabolites or natural products are low molecular mass compounds from secondary metabolism which arise from intracellular intermediates which are condensed into complex structures through particular pathways (Ruiz *et al*. 2010; Nandhini & Selvam 2013). These natural products have many applications and include anti-infective drugs, pigments, anticancer, antiviral, anti-ageing agents and pesticides (Bhatnagar & Kim 2010; Fusetani 2010; Vaishnav & Demain 2010). Some of the compounds were discovered in small scale screenings in academic laboratories (Genilloud *et al*. 2011) and some through extensive screening processes at an industrial level (Yoon & Nodwell 2014). The discovery of natural products proceeded at an overwhelming rate until the 1970s. This was followed by a steady decline in the rate of discovery of novel compounds culminating in a significant decline in the 21st century (Demain & Vaishnav 2009; Demain 2014). The decline was mainly due to the loss of interest in bioactive compound discovery by major pharmaceutical companies.

The loss of interest in bioactive compound discovery by major pharmaceutical companies was fuelled by the poor profit margins on investment because of the high cost of drug development and the lengthy period it takes for new drugs to reach the market. Moreover preclinical tests and the clinical development of bioactive compounds takes years while patents granted by governments are of a short duration (Norrby *et al*. 2005; Li & Vederas 2009; Demain 2014). For

instance, the development of lovastatin was initiated in 1978 and it was only in 1987 that lovastatin was finally approved by the Federal Drug Administration after successful clinical trials on human beings (Demain 2014). Moreover, governments impose strict pricing controls on natural products which impact negatively on industrial investments (Norrby *et al*. 2005; Li & Vederas 2009). A further decline in research into natural product discovery occurred when pharmaceutical industries opted to invest in larger drug markets such as drugs which are taken on a daily basis by patients (Demain 2014). Furthermore, the rediscovery of already existing bioactive compounds diminished the interest in investing in natural product discovery programmes (Pettit 2011; Zotchev 2012).

Over the past decades there has been a worldwide increase in the number of antibiotic resistant strains of pathogens and the emergence of multi-drug resistant bacteria (Norrby *et al*. 2005; Bhatnagar & Kim 2010; Phelan *et al*. 2012). This is of great concern because new multi-resistant organisms (bacteria, parasites and fungi) are spreading globally. Infectious diseases which initially were easily treated become increasingly untreatable or even lethal (Norrby *et al*. 2005; Gomez-Escribano & Bibb 2011). Ultimately there are concerns that the world is facing an era where very few novel natural products are developed, resulting in challenges to develop novel therapeutic drugs (Bhatnagar & Kim 2010; Phelan *et al*. 2012).

It is therefore essential to search for novel natural compounds from underexplored environments (Bhatnagar & Kim 2010; Sun *et al*. 2010) and to develop novel strategies to investigate the bioactive potential of microbes (Gomez-Escribano & Bibb 2011; Ouyang *et al*. 2011). Microbiota from extreme environments, such as hyper-arid deserts, could be a rich potential source of secondary metabolites (Rateb *et al*. 2011). The marine environment hosts a rich and varied life (Krause & Tobin 2013). As a result, there is a renewed research interest in exploring

this environment as a source of novel bioactive compounds. Invertebrates such as tunicates and sponges harbour large microbial diversities with diverse chemical properties which can be exploited in different biotechnologies (Bhatnagar & Kim 2010).

1.2 Marine environment

Most of the earth's surface (approximately 70%) is covered by oceans. It is believed that life began in the ocean and as a result the ocean harbours a large diversity of organisms which serve as models for various studies (Bhatnagar & Kim 2010; Krause & Tobin 2013). Approximately 60% of marine diversity is constituted by invertebrates. The most dominant phyla in the marine environment are the Annelida, Arthropoda, Bryozoa, Cnidaria, Echinodermata, Mollusca and notably the Porifera (Leal *et al*. 2012). Most biodiversity is found in the deep ocean thermal vents as well as in the intermediate regions between the oceans and the land. Although the pelagic zone was presumed to be less habited than other marine zones (Simmons *et al*. 2005), whole-genome shotgun sequencing has revealed that substantial microbial communities live in these zones (Venter *et al*. 2004; Simmons *et al*. 2005).

Macroorganisms such as sponges, tunicates and macroalgae inhabiting the oceans are concentrated around the interface between the oceans and the land as well as in the deep ocean thermal vents where they experience intense competition for space and nutrients. Due to the sessile nature of these macroorganisms, they are prone to predation. As a result they have developed defense mechanisms which include, but are not limited to, chemical defense mechanisms which make sponges and other marine macroorganisms interesting for natural product research (Simmons *et al*. 2005; Bhatnagar & Kim 2010; Leal *et al*. 2012).

Marine organisms serve as rich arsenals of bioactive compounds (Zhang *et al*. 2009a; Sipkema *et al*. 2011; Bayer *et al*. 2013). Electron microscopy revealed that large bacterial communities live within the mesohyl of marine sponges (Hardoim *et al*. 2009; Hardoim *et al*. 2012). Furthermore these sponge symbionts are now known to produce the bioactive compounds which were initially thought to be of sponge origin (Sipkema *et al*. 2011; Hardoim *et al*. 2012; Ziemert *et al*. 2014). Thus there is a growing research interest in the marine-sponge associated microbiota due to the diversity of biologically active secondary metabolites that they produce (Hardoim *et al*. 2009; Zhang *et al*. 2009a; Taylor *et al*. 2011; Bayer *et al*. 2013).

1.2.1 Marine sponges

Sponges of the phylum *Porifera* are one of the oldest metazoans on earth, occur primarily in marine environments and are filter feeding sessile organisms (Hardoim *et al*. 2009; Bayer *et al*. 2013; Zeng *et al*. 2013). There are three classes of sponges, namely *Calcarea*, *Hexactinellida* and *Demospongiae* (Hentschel *et al*. 2006; Schmitt *et al*. 2007). In 2009, the number of recorded sponge species was 5500 with the most abundant species belonging to the *Demospongiae*, which constitutes around 95% of the recorded sponge species (Hardoim *et al*. 2009). Due to the growing interest in marine sponges and their potential applicability to pharmaceuticals, the number of recorded marine sponge species was 8553 in 2012. Of this number, the most abundant class was still the *Demospongiae* at 83%. A large number of sponges have survived in marine environments under immense competition and predation. This could be due to their adaptability and the microbial communities they harbour (van Soest *et al*. 2012). Figure 1.1 is an illustration of a sponge and its internal structure.

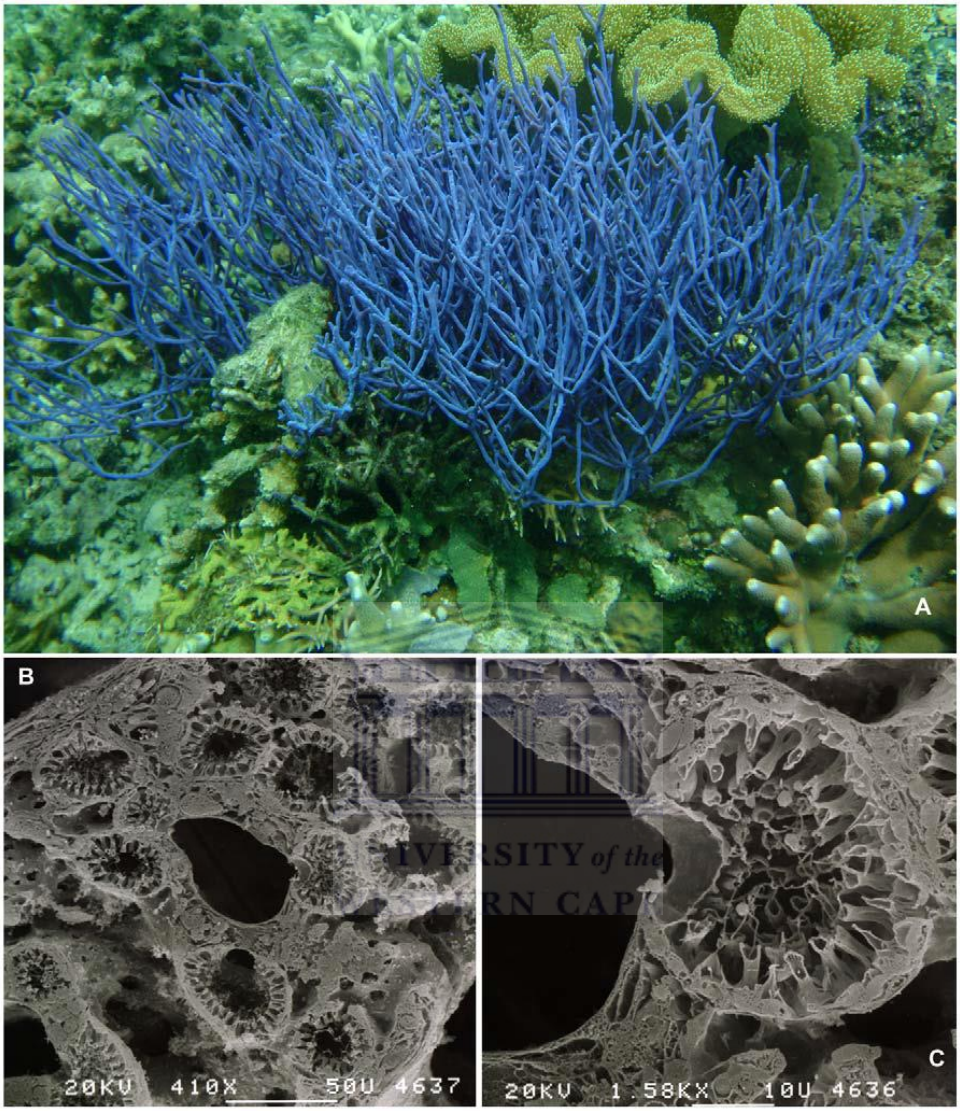


Figure 1.1 : Porifera morphology and internal structure. A. *Callyspongia (Callyspongia) samarensis* (Demospongiae: Haplosclerida); B. SEM image of cross section of mesohyl of the demosponge *Scopalina ruetzleri* obtained by freeze-fracturing technique; C. Detail of choanocyte chamber of *Scopalina ruetzleri* (Van Soest *et al*. 2012).

As filter feeders, marine sponges pump thousands of liters of water per day and use microorganisms as their main source of food (Fieseler *et al*. 2004; Hardoim *et al*. 2009). Sponges host a wide variety of microorganisms (Lee *et al*. 2011; Flemer *et al*. 2012; Walmsley *et al*. 2012; Zeng *et al*. 2013) or have mutualistic symbioses with them (Kennedy *et al*. 2009; Kamke

et al. 2010). Microorganisms can account for up to 40% of the sponge fresh weight (Fieseler *et al*. 2004; Hardoim *et al*. 2009; Webster & Taylor 2012; Zeng *et al*. 2013). Sponges with a thick mesohyl are classified as "high microbial abundance" (HMA) sponges and harbour dense and morphologically diverse microbial communities whereas "low microbial abundance" (LMA) sponges are those with well-developed aquiferous systems. Their low density mesohyl harbours few bacterial communities (Hardoim *et al*. 2009; Hardoim *et al*. 2012; Bayer *et al*. 2014). The vast microbial diversity they harbour is believed to serve as an evolutionary defense mechanism. Marine sponge associated microorganisms supply the sponge with nutrients, stabilize sponge skeletons and serve to protect sponges against predation (Zhang *et al*. 2009a; Zeng *et al*. 2013).

1.2.2 Marine sponge symbionts as a biotechnological resource

Sponges compete with other species by producing high levels of compounds which inhibit competitors from growing in close proximity to the sponges. Some of these compounds can result in clear zones around the sponge that produces these compounds. Potential drug leads from marine sponges include compounds that are likely to cure important diseases such as malaria, viral diseases and various malignant neoplasms (Perdicaris *et al*. 2013). Low concentrations of the compound Aeroplysinin-1 derived from the marine sponge *Verongia aerophoba* showed interesting activities including apoptosis of cancer cell lines (Haefner 2003). Some natural products exhibiting protease and antibiotic activities were derived from the marine sponge *Haliclona simulans* (Phelan *et al*. 2012). The marine sponge *Topsentia pachastrelloides* harvested from the South African coast, revealed some interesting natural product properties. The *in vitro* screening of extracts derived from *T. pachastrelloides* showed a potent inhibitory activity against methicillin-resistant *Staphylococcus aureus*. Some of the bioactive compounds

obtained from the extracts were identified as bis-indole alkaloids, topsentin (B, D) and hamacanthin C. The compounds showed very selective interesting activities with around 166-fold activities against methicillin-resistant *S. aureus* (Zoraghi *et al*. 2011). Tsitsikammamine B, isolated from a latrunculid sponge endemic to South Africa *Tsitsikamma favus* showed antifungal, cytotoxic and antimicrobial activities (Walmsley *et al*. 2012).

Although marine sponges also produce bioactive compounds (Perdicaris *et al*. 2013), there is growing evidence that a large proportion of these compounds are produced by the marine sponge associated microorganisms (Phelan *et al*. 2012; Walmsley *et al*. 2012). As a result the target of this study is on the marine sponge symbionts. Additional interest in marine sponge associated microorganisms is due to their novelty and complexity (Walmsley *et al*. 2012). Sponge associated bacteria are known to synthesize secondary metabolites with diverse and complex structures. These secondary metabolites therefore serve as potentially important pharmaceutical leads. The majority of the 18 000 recorded marine natural products are derived from marine sponge associated bacteria. Some of the compounds produced from the sponge *Theonella swinhoei* associated bacteria have antitumor, antifungal and antibacterial activities (Lavy *et al*. 2014). Microbial isolates from *H. simulans* were also reported to display potentially interesting biopharmaceutical, probiotic and biotechnological properties (Phelan *et al*. 2012).

A number of marine natural products are at pre-clinical and at clinical stages of investigation. Some of the natural products at pre-clinical stages include variolins from *Kirkpatrickia variolosa* and salicylihalimides A and B from *Haliclona* sp. Those that have entered phases of clinical tests include discodermolide from the sponge *Discodermia dissolute* (Simmons *et al*. 2005). Marizomib, which was obtained from the marine actinobacterium *Salinispora arenicola* reached phase 1 of clinical trials for its anticancer activity (Zhao 2011).

1.2.3 Marine bacteria

In the 19th century, studies on marine bacteria focused on obtaining pure cultures and characterization of the bacteria. Decades later microbial ecologists realized that only about 0.1% of the bacteria observed under the microscope could be brought into culture (Joint *et al*. 2010). This created the need for microbial ecologists to develop molecular techniques to characterize microorganisms. Molecular phylogeny for classification of microorganisms based on the evolutionary relatedness of ribosomal RNA was later developed (Woese 1987). Woese's discovery revolutionized microbial ecology since this method bypassed the need to culture microorganisms. As a result, microbes which had never been seen before could be classified based on their 16S rRNA gene sequences. The basis of this method is that the 16S rRNA gene sequences are mostly conserved in microbes. Molecular techniques revealed that microbial phyla and *Archaea* are more abundant than the animal phyla (Kirchman 2008).

Using molecular methods marine bacteria could be accurately classified. Some of the major clades in seawater are *Aphaproteobacteria*, *Gammaproteobacteria, Bacteriodetes*, *Cyanobacteria*, *Actinobacteria* and marine *Archaea* (Kirchman 2008). Some bacteria are called sponge specific clusters because they are only present in marine sponges whereas they are not found in other marine environments (Thiel *et al*. 2007; Simister *et al*. 2012b; Giles *et al*. 2013; Taylor *et al*. 2013). Sponge specific bacterial phyla are thought to be transmitted through vertical transmission (Lee *et al*. 2011; Taylor *et al*. 2013). One of the bacterial phyla believed to have been sponge specific is *Poribacteria* (Simister *et al*. 2012a), however, it has recently come to light that these phyla are not sponge specific but are found in other marine environments (Taylor *et al*. 2013). One of the largest sponge specific clusters is *Synechococcus spongiarum* which is a cyanobacterium. Marine sponges harbour large microbial diversities and to date 25 bacterial

phyla are known to have symbiotic relationships with sponges. Members of the three microbial domains *Eukarya*, *Bacteria* and *Archaea* all live symbiotically in marine sponges (Simister *et al*. 2012a).

Many studies have focused on marine *Actinobacteria* mainly due to their known and interesting bioactivities. Studies carried out in the mid-2000s indicated that of the 53 reported bacterial phyla from the marine environment, five of these produce anti-infective compounds. Of the five phyla reported, *Actinobacteria* of the order *Actinomycetales* contribute the highest number of natural products. *Streptomyces* account for around 80% of the natural products obtained from *Actinomycetes* (Jensen *et al*. 2005).

1.2.4 Methods used to culture marine bacteria

Microorganisms make up the highest proportion of the biodiversity of the planet. Despite this only a small fraction of samples obtained from different environments have been brought into culture using conventional laboratory methods. Around 1% of the microbes observed under the microscope form colonies on conventional cultivation media, a paradox coined "the great plate count anomaly". The general approach to cultivating microbes is to provide nutrients and conditions similar to their natural habitats, where different substrates and culture conditions may result in the isolation of different bacteria (Vartoukian *et al*. 2010).

Marine microbes are highly sought after for various reasons which include biodiversity research (Hardoim *et al*. 2009; Joint *et al*. 2010; Hardoim *et al*. 2013) and natural product discovery (Kennedy *et al*. 2008; Fusetani 2010; Phelan *et al*. 2012; Skariyachan *et al*. 2014). The latter is of significant interest due to the potential of discovering novel bioactive compounds from marine microbes, particularly sponge associated microbes (Bhatnagar & Kim 2010; Woodhouse *et al*.

2013; Skariyachan *et al*. 2014). As a result there have been different methods employed to bring a higher percentage of these microbes into culture so as to exploit their potential bioactivities.

Over the years, new methods were developed in the attempt to culture marine bacteria. The methods include the use of novel culture media such as very minimal nutrient content (Song *et al*. 2009) or application of diffusion chambers (Bollmann *et al*. 2007; Steinert *et al*. 2014) or microencapsulation (Joint *et al*. 2010). Genome sequencing gives an indication of the metabolic properties and capabilities of bacterial communities. This information may be used to develop conditions under which to grow individual bacterial species (Lavy *et al*. 2014). While most studies involving the cultivation of the sponge associated microbiota targeted *Actinomycetes* due to their proven capability to produce pharmaceutically applicable compounds (Kim *et al*. 2005; Xi *et al*. 2012), other studies focused on targeting all the representatives in a microbial community (Craney *et al*. 2013). Oligotrophic media, microencapsulation and *in vivo* cultivation of marine sponge symbionts represent some of the different methods used to target the total diversity (Lavy *et al*. 2014). Other unique methods such as floating disc filters have been employed resulting in higher microbial culture efficiencies (Sipkema *et al*. 2011).

Physiological and genomic information was used to enhance the growth of microbes associated with *Theonella swinhoei*. The study resulted in cultivation of previously cultured and uncultured bacteria, some of which represented new species and also probably new genera and families (Lavy *et al*. 2014). Another study resulted in the cultivation of rarely encountered bacteria such as *Verrucomicrobia*, *Deltaproteobacteria* and *Planctomycetes* from *Haliclona* (*gellius*) sp. using different culture methods which included floating filter cultures, liquid cultures and plate cultures. The media used to culture these *Haliclona* (*gellius*) sp. symbionts constituted

oligotrophic media including media with organic sponge extracts, bacterial signal molecules and siderophores (Sipkema *et al*. 2011).

1.2.5 South Africa's unique coastline

South Africa has a unique coastline (Figure 1.2) which is dominated by two major current systems: the cold Benguela current and the warm Agulhas current (Branch *et al*. 2010; Griffiths *et al*. 2010). As a result of the extreme physico-chemical differences introduced by the two currents the coastline has an exceptionally rich and varied marine life. The Agulhas current is one of the most powerful ocean currents, and it transfers warm nutrient-poor water from the equatorial Indian Ocean down to the east coast of South Africa (Neethling *et al*. 2008; Hutchings *et al*. 2009; Griffiths *et al*. 2010). Temperatures in this current range from 20^{o}C to 28^{o}C depending on the season (Griffiths *et al*. 2010) and temperatures are affected by the continental shelf that widens around East London where the water is forced offshore, resulting in the water cooling down.

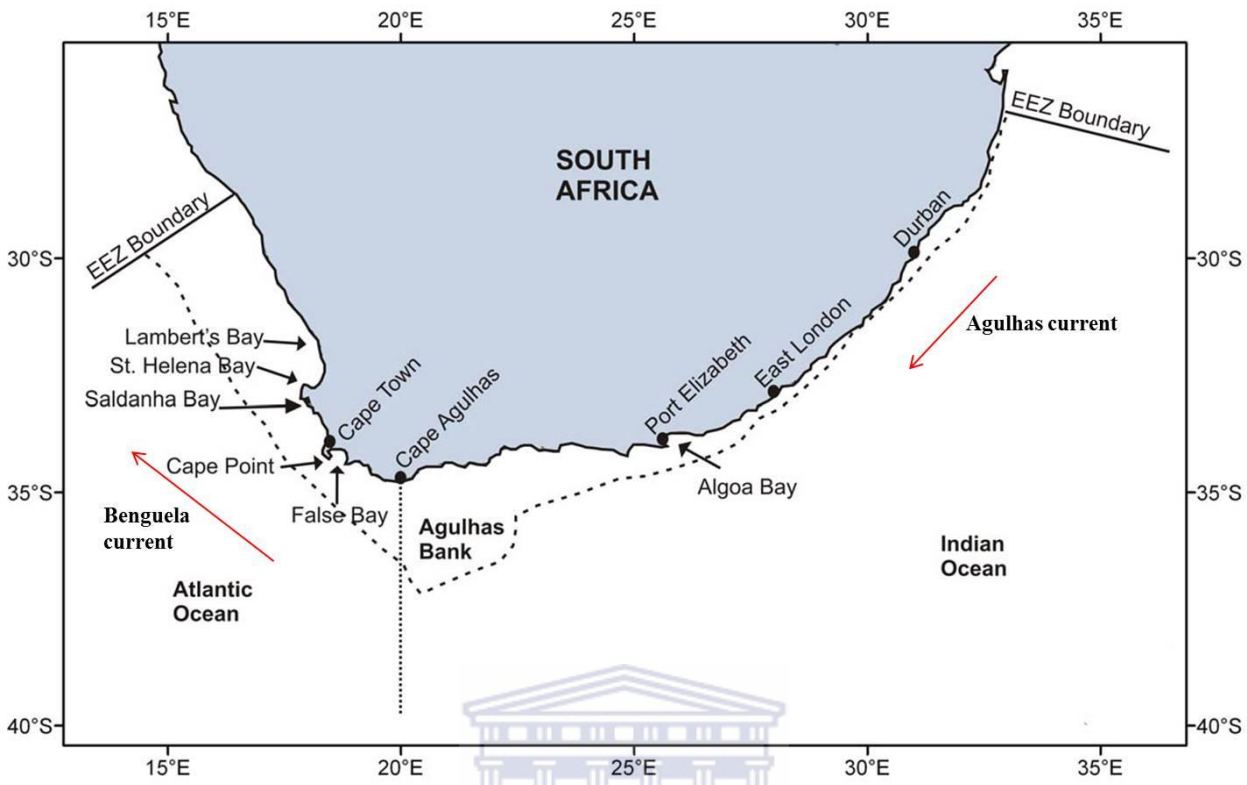


Figure 1.2: South Africa's unique coastline is dominated by two ocean currents: the Agulhas current in the Indian Ocean and Benguela current in the Atlantic Ocean. Adopted from (Griffiths *et al*. 2010).

The Benguela current comprises of two components (Griffiths *et al*. 2010). The first is the offshore oceanic flow that forms the eastern limb of the South Atlantic Subtropical Gyre. This has a broad and slow flow due to the northward drifting cold water from the South Atlantic Ocean. The second component is an inshore wind driven upwelling which is influenced by local weather systems and results in short-term upwelling cycles. The water from the deep South Atlantic is rich in nutrients and drives more productive biological systems which results in lucrative commercial fisheries (Branch *et al*. 2010; Griffiths *et al*. 2010; Garavelli *et al*. 2012). The west coast however harbours fewer species of macroorganisms than the east coast (Coetzee *et al*. 2008; Branch *et al*. 2010). This could be due to reduction of dissolved oxygen from the decaying organic matter from high productivity which sinks onto the wide continental shelf. The

low-oxygen conditions can reach the shoreline and cause mortalities to marine life (Griffiths *et al*. 2010).

The South African marine environment is rich in marine biota and remains largely unexplored. The South Africa's marine life consists of 12 914 recorded species, even though many taxa, especially those of small body size, are poorly documented and sampling of these areas was last conducted before the 1980s (Griffiths *et al*. 2010). These studies did not take the marine microorganisms into account. This makes it an ideal environment to explore for novel bioactive compounds of marine sponge associated microbial origin in this era of renewed interest in marine environments (Zhang *et al*. 2005). The potential of natural products obtained from the South African marine environment was partially revealed when compounds which showed potent bioactivities against methicillin-resistant *Staphylococcus aureus* (Zoraghi *et al*. 2011), cytotoxic, antimicrobial and antifungal activities (Antunes *et al*. 2004; Walmsley *et al*. 2012) were obtained from the South African marine sponges. Sampling for the discovery of chemical diversity of an environment can be done in three ways: firstly to explore geographical sources that remain largely untapped, secondly to explore the new classes of marine organisms and thirdly to combine the two sampling methods (Leal *et al*. 2012).

1.3 Bacteria as an alternative to overcome the natural product discovery bottlenecks

A challenge facing novel natural products discovery is the supply problem (Walmsley *et al*. 2012) and the high rediscovery rates of common natural products (Li & Vederas 2009; Craney *et al*. 2013). As a result, big biopharmaceutical companies have opted for different methods of drug

discovery such as combinatorial chemistry or high-throughput screening to avoid the pitfalls of natural product discovery. However synthetic chemistry has failed to produce the complexity and large diversity observed in natural products resulting in a significant decline in novel drug discoveries (Demain 2014).

The major bottleneck for novel drug discovery from natural environments is the limited biomass one is able to collect in order to generate sufficient amounts of the required compound, particularly from filter feeders such as marine sponges, bryozoans and tunicates (Piel 2006; Zhang *et al*. 2009b). For instance, 13 tons of *Bugula neritina* were required to get 18 g of bryostatin 1. Similarly 310 mg of halichondrin B were obtained from 1 ton of *Lissodendoryx* species (Piel 2006). The large quantities of marine invertebrates that were used would ultimately lead to their extinction. Aquaculture techniques have been developed to address this limited supply in a sustainable manner. However with marine sponges this has been particularly unsuccessful (Leal *et al*. 2013). With the recent confirmation that invertebrate associated microbes are the actual producers of many secondary metabolites previously identified from invertebrate samples (Walmsley *et al*. 2012), focus has shifted towards the microbes themselves (Zhang *et al*. 2009a). Alternative methods, which target invertebrate associated microbes, to harness natural products are being employed. There are several advantages to working with microbes which may alleviate the biomass bottleneck in drug discovery.

One of the major advantages of working with microbes is that they can be cultured in laboratories and at an industrial level and can hence be manipulated in various ways to benefit humankind (Diminic *et al*. 2014). One of the ways to manipulate microorganisms is through the One Strain Many Compounds approach (OSMAC) which exploits the microbial biosynthetic potential by activating their "cryptic" pathways (Bode *et al*. 2002). Additionally, microbial cells

can be modified by genetic engineering to overproduce some of the important bioactive compounds, through strain improvement, ribosome engineering (Ochi & Hosaka 2013) or by co-culturing microbes (Bertrand *et al*. 2013; Ochi & Hosaka 2013) to trigger expression of secondary metabolites. Other advantages include the ability for heterologous expression of biosynthetic gene clusters in microbial systems (Olano *et al*. 2008).

1.4 Microbial secondary metabolites

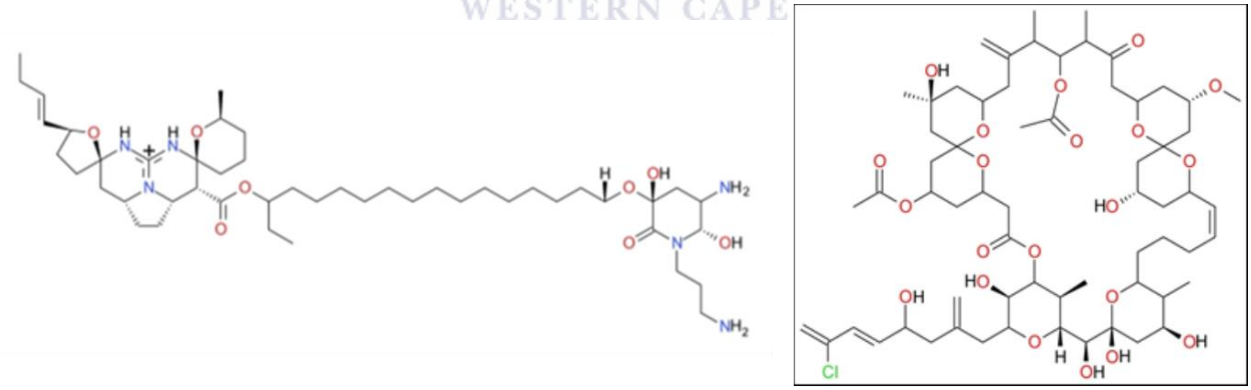
Secondary metabolites are low molecular mass organic compounds that are not directly involved in the normal growth (Ruiz *et al*. 2010; Sánchez *et al*. 2010), reproduction or development of an organism (Mi 2012; Craney *et al*. 2013). They arise from intracellular intermediates and are condensed into complex structures through particular pathways (Dunn *et al*. 2013; Fisch 2013). Until recently many of these bioactive compounds were largely isolated from organisms inhabiting terrestrial environments (Juntawong *et al*. 2013).

Secondary metabolites often have complex, diverse structures with potent bioactivities (Silakowski *et al*. 2001) and play an important role in the defense systems of different microbes against competing microorganisms (Bhatnagar & Kim 2010). Some of these compounds are able to inhibit the growth of other organisms or even to damage them. There is a particular interest in compounds which possess such activities since they are potential pharmaceutical leads (Bhatnagar & Kim 2010; Yoon & Nodwell 2014).

There are many classes of secondary metabolites which have been developed pharmaceutically such as polycyclic xanthones, terpenes, quinones, esters and alkaloids (Solanki *et al*. 2008). For instance, manzamines of the class alkaloids have antitumor, antimalaria and cytotoxic activities

(Yousaf *et al*. 2002). Much attention has been focused on those encoded by the polyketide synthase (PKS) and non-ribosomal peptide synthase (NRPS) pathways or hybrids of the two pathways. The pathways are organized into gene clusters and include resistance and regulatory components (Ziemert *et al*. 2014). As a result the two megaenzyme classes account for some of the most important diverse and complex structures of bioactive compounds (Fisch 2013).

Microorganisms have the capacity to produce a wide range of secondary metabolites although these compounds are not always expressed or are expressed at very minimal concentrations in laboratory cultures and they cannot be detected (Craney *et al*. 2013; Yoon & Nodwell 2014). The discovery that secondary metabolites remain unexpressed in microorganisms prompted studies such as the OSMAC approach as a way to unlock the expression of the biosynthetic pathways that remain unexpressed (Gross 2007; Demain 2014). Figure 1.3 shows the structures of two complex secondary metabolites produced by marine sponge associated microorganisms.



A **B**

Figure 1.3: Examples of secondary metabolites isolated from marine sponge isolated microorganisms. A: Monanchocidin, a polycyclic guanidine alkaloid that induces cell death in human monocytic leukemia and human cervical cancer cells. B: A macrocyclic lactone polyster which inhibits cytotoxic cell death in cancer cell lines (Perdicaris *et al*. 2013).

1.4.1 One Strain Many Compounds (OSMAC)

The school of thought in the 1990s was that secondary metabolite diversity and potential had been almost completely exploited and it was presumed that the discovery of new compounds would be difficult. Subsequently genome sequencing studies indicated that the biosynthetic potential of most microorganisms have been largely untapped (Scherlach & Hertweck 2009). As an example genome sequencing studies using *Streptomyces* species revealed that these organisms have the potential to produce around 20 to 40 different secondary metabolites (Yoon & Nodwell 2014). Through genome sequencing, *Streptomyces griseus* was shown to have 34 gene clusters for genes coding for various bioactive compounds. Most of these genes were inactive or poorly expressed in conventional culture conditions (Ohnishi *et al*. 2008; Scherlach & Hertweck 2009). *Streptomyces avermitilis* has approximately 25 gene clusters for the production of spore pigments, siderophores and secondary metabolites of polyketides or non-ribosomal peptide origin (Pettit 2011). A study of *Streptomyces coelicolor* A3 in 2002 indicated that this bacterium produces six secondary metabolites under different culture conditions. However, genome sequencing studies later revealed that the genome of this organism encodes for 31 secondary metabolites (Zhao 2011) further indicating that many microbes have a high potential for producing yet undiscovered secondary metabolites. Genes encoding biosynthetic enzymes in microorganisms outnumber those that are actively expressed using traditional cultivation methods. The unexpressed pathways are referred to as cryptic or "silent" pathways. There is therefore a need to design new strategies to unlock expression of these untapped bioactive compounds (Craney *et al*. 2013).

The findings that marine sponge associated microbes are the original producers of a large number of bioactive compounds (Phelan *et al*. 2012) make the OSMAC principle an ideal

approach to harness the observed chemical diversity from these organisms (Bode *et al*. 2002; Scherlach & Hertweck 2009). The OSMAC principle is ideal to use as an alternative strategy for discovering novel secondary metabolites because working with the microbes bypasses the biomass bottleneck. This approach exploits the fact that microorganisms produce secondary metabolites as a defense mechanism against other organisms in nature (Yoon & Nodwell 2014). Moreover, the concept exploits the fact that under stressful conditions, microbes tend to produce secondary metabolites either to adapt to the environment, for self-defense or for intercellular communication (Hutchinson 2003; Bhatnagar & Kim 2010). Thus the OSMAC principle has shown great success in enabling the expression of "silent/cryptic" biosynthetic pathways (Bode *et al*. 2002; Scherlach & Hertweck 2009).

1.4.1.1 Conditions employed in OSMAC

Different biotic and abiotic conditions have been used to test the efficacy of the OSMAC approach in eliciting the production of secondary metabolites by a wide range of microbes. Conditions which have been tested include carbon (Ruiz *et al*. 2010; Sánchez *et al*. 2010; Pettit 2011), phosphate and nitrogen sources, aeration levels (Bode *et al*. 2002), the effect of ethanol or organic compounds, heat shock (Doull *et al*. 1994; Nakata *et al*. 1999), the salinity (Nakata *et al*. 1999) the presence of precursors of secondary metabolites (Demain 1998) and the effects of co-culture (Bertrand *et al*. 2013).

The impact or success of the OSMAC approach was evident when more than 100 compounds were isolated from 6 different microorganisms. The compounds represented over 25 different structural classes of compounds. In the study the basic culture conditions (such as media

composition, pH, temperature, and oxygen supply) were altered to achieve this great diversity of secondary metabolites (Bode *et al*. 2002).

1.4.1.1.1 Carbon

Culture media composition and concentration can influence the production of secondary metabolites by microorganisms. The carbon source is one of the components of growth media which have been studied by different research groups and industry even though most of the media compositions involving alternate carbon sources have been carried out specifically on *Actinobacteria* (Gubbens *et al*. 2012). The impact that different carbon sources have on bioactive compound synthesis depends on how quickly the microbes utilize a preferred carbon source. Some studies have decreased carbon source repression or even totally prevented it through mutations (Ruiz *et al*. 2010; Gubbens *et al*. 2012).

Carbon sources frequently repress production of secondary metabolites in a process called carbon catabolite repression (CCR). CCR is a regulatory mechanism which ensures an organised and sequential use of carbon when two or more carbon sources are present in the growth medium (Sanchez & Demain 2002). During utilization of the most readily metabolizable carbon source there is a halt in the production of secondary metabolites whereas there is increased cell growth. After the preferred carbon has been exhausted the second best carbon source gets used and this often triggers the production of secondary metabolites (Demain 2014).

Different carbon sources have varied effects on secondary metabolite production in individual organisms (Sánchez *et al*. 2010). Glucose for some organisms is ideal for growth but interferes with the expression of bioactive compounds (Sánchez *et al*. 2010; Gubbens *et al*. 2012). Glycerol is known to suppress carbapenem production in *Erwinia carotovora* by repressing the

transcription gene responsible for synthesis of the quorum-sensing molecule which induces carbapenem synthesis. However, glycerol does not suppress carbapenem-like secondary metabolites in other organisms, even in close relatives such as *Erwinia herbicola* (Sánchez *et al*. 2010). Investigations on the production of prodigiosin by *Serratia marcesens* revealed that *S. marcesens* was able to produce prodigiosin using ethanol as the sole carbon source, while the addition of glucose (0.5% (v/v) or more) resulted in almost complete repression of prodigiosin production (Cang *et al*. 2000). The production of prodigiosin was also repressed by other carbon sources such as galactose, sucrose and fructose. One of the studies on *Serratia marcescens* UCP1459 revealed that production of prodigiosin was greatly enhanced when cassava liquid waste was supplemented with mannitol (De Araújo *et al*. 2010).

An investigation into the effect of carbon sources on the production of secondary metabolites by *Streptomycetes* stains showed that eliminating glucose from the culture media resulted in the production of secondary metabolites (Ruiz *et al*. 2010). In *Streptomyces*, the central protein that controls CCR is glucose kinase (Glk) and in these species the presence of glucose in the medium resulted in CCR. *Streptomyces coelicolor* and its mutant *S. coelicolor glk*A (Glk) were therefore examined for the production of secondary metabolites in the presence and absence of glucose using either mannitol or fructose as alternate carbon sources. The results indicated that glucose repression was a complex mechanism controlled by a Glk-dependent and Glk-independent manner (Gubbens *et al*. 2012) .

There was a poor production of jadomycin B by *Streptomyces venezuelae* ISP5230 when galactose was substituted with lactose or sucrose. However, substituting galactose with starch, maltose or glucose also resulted in a considerable difference in production of jadomycin B (Doull *et al*. 1994). A study conducted on *Streptomyces hygroscopicus* strain 111-81 showed that

when carbon is a limiting factor in the fermentation medium, microbial growth is limited whereas the carbon stress triggers secondary metabolism. Furthermore, when a mixture of carbon sources (fructose, glucose, sucrose, glycerol and/or lactose) were used in the fermentation medium, there was a minimal production of the antibiotic AK-111-81 but also to some extent favored the production of azalomycin B. The best carbon source for biosynthesis of AK-111-81 antibiotic was found to be lactose (Gesheva *et al*. 2005).

1.4.1.1.2 Nitrogen

Nitrogen regulation is of great importance since it is involved in the synthesis of the enzymes responsible for primary and secondary metabolism (Sanchez & Demain 2002). The nature of the compound (chemical structure) to be formed can be influenced by the nutrients used in the fermentation medium. Some nutrients such as nitrogen can be utilized as precursors for secondary metabolites and thus affect the secondary metabolite production. Nitrogen deficiency mostly halts microbial growth but triggers secondary metabolism (Gesheva *et al*. 2005). Most nitrogen sources which are favourable for growth, for instance, ammonium salts, tend to have a negative effect on most secondary metabolic pathways (Sanchez & Demain 2002; Gesheva *et al*. 2005). However ammonium succinate activated the production of a number of AK-111-81 antibiotics (Gesheva *et al*. 2005).

High concentrations of the nitrogen sources in the growth medium reportedly suppress secondary metabolite production. Thus different nitrogen sources, both from proteins and from slowly assimilated amino acids have been used in order to ensure antibiotic production (Sanchez & Demain 2002). This was shown in *Streptomyces griseus* which produces streptomycin when grown in the presence of soybean meal, proline and low concentration of ammonium salts (Zhu

et al. 2014). When *Streptomyces venezuelae* 1SP523 was cultured in increasing concentrations of the nitrogen source L-isoleucine (from 15 mM to 75 mM), there was an increased production of jadomycin B. Furthermore, the effect of using different nitrogen sources also resulted in modified jadomycin B products (Doull *et al*. 1994). On the other hand, the addition of nitrate and certain amino acids stimulated the production of aminoglycoside antibiotics whereas ammonium salts resulted in repression of the metabolites (Zhu *et al*. 2014).

1.4.1.1.3 Phosphate

Microbes are particularly interesting because they have adapted to harsh living conditions including low nutrient levels in the natural environment which impact on their proliferation. One of the major growth limiting factors for biological systems is the level of inorganic phosphorous available to the organism. As a result, microbes have evolved varied response mechanisms to modulate phosphorous utilization or phosphorous requirements for growth. Inorganic phosphates also affect the production of secondary metabolites and extracellular enzymes in microbial fermentations (Sanchez & Demain 2002). Easily metabolized phosphates tend to suppress secondary metabolite production and their depletion from culture medium triggers secondary metabolism (Martín 2004). Phosphate limiting concentrations of around 0.5 mM favour secondary metabolite production whereas higher concentrations suppress secondary metabolism but favour cell growth. In *Streptomyces lividans*, *S. coelicolor*, *S. griseus,* and *S. rumosus* antibiotic production is mediated by a two component system PhoR-PhoP (Zhu *et al*. 2014) which controls the gene *phoA*. Mutants lacking *phoP* overproduce actinorhodin and undecylprodigiosin whereas secondary metabolism was repressed in wild type *S. coelicolor* and *S. lividans*. Phosphate control thus appeared to repress all secondary metabolite gene clusters

(Sola-Landa *et al*. 2003; Zhu *et al*. 2014). Phosphate depletion in culture medium significantly limited *S. coelicolor* and *S. lividans* growth whereas secondary metabolite biosynthesis was triggered (Martín 2004).

When *B. subtilis* is cultured in phosphate limiting conditions, there was a 10-to-30 fold overproduction of extracellular enzymes (alkaline phosphodiesterase and acid phosphatases) (Sanchez & Demain 2002). The depletion of phosphate at a particular threshold increased secondary metabolite production. For example actinorhodin and undecylprodigiosin were overproduced by mutants with the *phoR-phoR* deletions and the production was almost insensitive to phosphate concentrations ranging from 1 mM to 10 mM (Martín 2004).

1.4.1.1.4 Heat Shock

The school of thought in the 1990s was that heat-shock-induced chaperones (*gro*EL-like genes found in *Streptomyces*) may play a role in multi-enzyme complexes for bioactive compound biosynthesis. Mesophilic cultures that were grown at 27 ºC produced virtually no jadomycin B whereas at elevated temperatures of 42 ºC there was a significant production of the compound. This therefore suggested that its production was a result of a heat-shock response. The stress response to heat shock reportedly results in the production of specific proteins (Doull *et al*. 1994). Investigations of the heat-shock effect in *Streptomyces venezuelae* revealed that the jadomycin B producing pathways were regulated by two pathway-specific transcription factors namely transcription activator gene *jadR1* and the repressor *jadR2* together with other regulatory genes. They also found that JadR2 negatively repressed *jadR1* and thus heat shock negated the repression to activate *jadR1* (Yang *et al*. 2001; Zhang *et al*. 2013; Yoon & Nodwell 2014). The effect of heat shock on *S. coelicolor* resulted in the production of the secondary metabolites 5-

hydroxyecotoine and ectoine confirming that heat shock induced the production of secondary metabolites (Yoon & Nodwell 2014).

1.4.1.1.5 Ethanol

The mechanism by which organic compounds such as ethanol and dimethylsulfoxide (DMSO) trigger secondary metabolism is not known. Nonetheless, they have been used in fermentation media to trigger secondary metabolite biosynthesis due to their inexpensive nature and ease of use. Some hypotheses presume that mistranslation resulting from organic compound stress triggers secondary metabolite production (Pettit 2011). Addition of 1% ethanol resulted in the production of the chlorinated antibiotic pestalone by a marine fungus *Pestalotia*. Pestalone has potential pharmaceutical properties as it demonstrates activity against methicillin resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium* (Cueto *et al*. 2001). A study carried out on *Pseudomonas fluorescens* S272 revealed that high ethanol concentrations (2%) result in the production of antibiotics with a concomitant inhibition of growth (Nakata *et al*. 1999). The production of jadomycin B was significantly induced in *Streptomyces venezuelae* ISP5230 when 6% ethanol was introduced in the culture medium (Doull *et al*. 1994; Pettit 2011; Yoon & Nodwell 2014).

1.4.1.1.6 Co-culture

Microbial co-culture aims to exploit quorum sensing (QS) used by microbial communities to communicate. Under QS, gene expression between members in a community can be simultaneously regulated through signaling molecules which diffuse between microbial cells

(Joint *et al*. 2010). QS may induce secondary metabolism in neighbouring microbes (Bertrand *et al*. 2013) or enhance proliferation of neighbouring microbes (Joint *et al*. 2010).

The co-culture study carried out on the fungi *Trichophyton rubrum* and *Bionectria ochroleuca* revealed that different compounds were produced in co-cultures which were not produced when the organisms were cultured independently (Bertrand *et al*. 2013). A new bioactive compound pastalone was produced by a marine fungus only when the fungus was co-cultured with marine bacterium strain CNJ-328 (Cueto *et al*. 2001). Similar results were also found when *S. coelicolor* was co-cultured with five other actinomycetes (Traxler *et al*. 2013).

1.5 Motivation for this study

The development of new drugs has declined significantly in recent years whereas there is a rise in the number of antimicrobial resistant pathogens and in other types and levels of human diseases. There is therefore a need to explore unexplored geographical environments and novel groups of microbes to provide novel bioactive compounds that would address these issues (Chairman *et al*. 2012; Leal *et al*. 2012; Subramani & Aalbersberg 2012). Marine natural products have unique and complex structures and often possess unusual chemistries and biological activities (Zhang *et al*. 2009b; Bhatnagar & Kim 2010; Leal *et al*. 2012). Marine sponges of the class Porifera are the leading species in novel bioactive compounds production (Leal *et al*. 2012). Since evidence shows that marine sponge associated bacteria are responsible for a range of chemical diversities (Zhang *et al*. 2009a; Craney *et al*. 2013), it is of great importance to be able to culture these microbes under laboratory conditions. Culturing these microbes would allow biopharmaceutical industries and researchers to investigate and exploit

microbial bioactivities and to develop and identify novel bioactive compounds (Sun *et al*. 2010; Craney *et al*. 2013).

1.5.1 Aims and objectives

The objective of this study was to design a matrix to investigate the extent to which one strain-many compounds (OSMAC) applies to a collection of marine isolates harvested from two South African marine sponge samples. The overall concept was to culture the organisms under a range of growth conditions and treatments in order to elicit secondary metabolite production. To achieve this objective, a number of preceding aims had to be achieved, and these were as follows:

(i) To determine which two sponge samples had the highest microbial diversity from five randomly picked sponge samples using Terminal Restriction Fragment Length Polymorphisms (T-RFLP).

(ii) To culture the marine sponge associated bacteria and screen for antimicrobial activity against five indicator organisms.

(iii) To identify the bacterial isolates that produce secondary metabolites through 16S rRNA gene sequence analysis

(iv) To investigate and compare whether the OSMAC approach (expanding growth conditions and treatments) affects production of secondary metabolites from a selection of the cultured isolates

CHAPTER 2 MATERIALS AND METHODS

2.1 General chemicals, enzymes and bacterial strains

Unless otherwise specified, chemicals and reagents used in this study were supplied by Sigma Aldrich Chemical Company (Deissenhofen, Germany), Merck Chemical and Laboratory Supplies (Darmstadt, Germany) and Kimix Chemical and Laboratory Supplies (South Africa). The enzymes, DNA size markers and polymerases were purchased from Fermentas Life Sciences Ltd (Vilnius, Lithuania). KAPA2G Fast, KAPA2G Robust polymerases were supplied by KAPA Biosystems (Cape Town, South Africa).

Buffers and stock solutions used in this study are listed in Appendix 1

General media and matrix media used in this study are listed in Appendix 2 and 3 respectively.

Primers and PCR cycling conditions are listed in Appendix 4

The bacterial strain for cloning purposes used in the study was *Escherichia coli* JM109. The genotype is shown in Table 2.1. The table also shows the *E. coli* 1699 strain engineered to show resistance against 52 antibiotics. The bacterium was used in antibacterial screening.

Table 2.1 *Escherichia coli* strains used in this study.

Bacterial strain	Genotype/description	Supplier
E. coli JM109	*end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 (rk–, mk+), *rel*A1, *sup*E44, Δ(*lac-pro*AB), [F´ *tra*D36, *pro*AB, *laq*IqZΔM15]	Promega
E. coli 1699	MG1655 *gyr*A *rps*L150 *rpoB*516 *met*E::*tet*A *latt*::*mini*Tn7*plus* D*csp*A::*aac*(3')-IV *cit*AB:(*neo ble*)	Cubist, USA

For antibacterial screening purposes five indicator strains were used. To screen for antibacterial activities for Gram positive strains *Bacillus cereus* ATCC10702 and *Staphylococcus epidermidis* ATCC14990 were used and for Gram negative activity the selected strain was *Pseudomonas putida* ATCC12633. *Mycobacterium smegmatis* LR222 was selected because it shares the same genus with the TB causing strain and *Escherichia coli* 1699 (Cubist, USA) was selected because it has been genetically engineered for resistance against 52 known antibiotics.

2.2 Sample collection and preparation

Marine sponges were collected by Dr Shirley Parker-Nance in January and April 2013 in Algoa Bay, Port Elizabeth, South Africa (longitude 34°00.366S and latitude 25°43.209E), and kept on ice in sterile sea water and shipped to Cape Town. On arrival the sponge material was stored at -20 ºC and -80 ºC in 20% glycerol until further processing.

2.2.1 Culturing to access maximum bacterial diversity

Thawed sponge material (1 g) was homogenised by grinding with a sterile pestle and mortar in 9 mL of sterile sea water. The sponge homogenates were serially diluted in sterile sea water to 10^{-6}, and from each dilution 100 µL aliquots were plated on 19 general media (Appendix 2). In addition, three aliquots of the 10^{-1} dilution underwent treatment processes (heat treatment, antibiotic treatment or liquid enrichment). The flow diagram in Figure 2.1 represents a summary of the culturing process inclusive of all treatment processes.

In addition, aliquots of the sponge homogenates underwent heat treatment to select for endospore forming microbes (Phelan *et al*. 2012) and to inhibit the growth of Gram negative fast growing

bacteria (Lavy *et al*. 2014). The heat treatment process was adapted from Phelan *et al*. (2012) with minor modifications. An aliquot of 100 µL of the 10^{-1} dilution of the sponge homogenate was incubated at 80 ºC for 10 minutes and serially diluted to 10^{-6}. Aliquots of 100 µL were aseptically plated on three media SNA, TSA and ZBA (Appendix 2).

Liquid enrichment homogenates were used to select for cyanobacteria (Atlas 2010) and nitrogen fixing microbes (Yang *et al*. 2013). For liquid enrichment cultures, 100 µL of the 10^{-1} dilutions of the sponge homogenate were added to 9 mL of ANFA, MMM and BG11 liquid media. Liquid enrichment cultures were incubated at 15 ºC for six weeks on a shaker (150 rpm). Subsequently, 100 µL of the liquid enrichment were plated on ZBA plates because ZBA is one of the generally rich media which can support proliferation of different microbes. In our laboratory, ZBA, TSA and GYM were selected as the standard media for isolation and characterization of microbial isolates.

For the antibiotic treated sponge homogenate, 20 µg/mL of streptomycin was added to the homogenate and mixed on a shaker (150 rpm) for 1 h at room temperature. The sponge homogenate was centrifuged at 11 000 ×*g* for 3 minutes and the supernatant discarded. Sterile sea water (1 mL) was used to re-suspend the pellet and the suspension was serially diluted to 10^{-6}. Aliquots of 100 µL of the 10^{-1} to 10^{-6} dilutions were plated on eight antibiotic treated media (OMA, OMA + salt, AIM, GYM, 172, MAA, YEME and SCN Appendix 2). Cyclohexamide (100 µg/mL) and 50 µg/mL nalidixic acid were added to each medium.

All plates were incubated at 15 ºC for four weeks. Colonies were examined and picked on a daily basis selecting for colonies with different morphologies (shape, colour, texture, and size).

Colonies were purified by re-streaking on 3 standard media (ZBA, GYM and TSA). Pure colonies from the standard media were characterised and Gram stained.

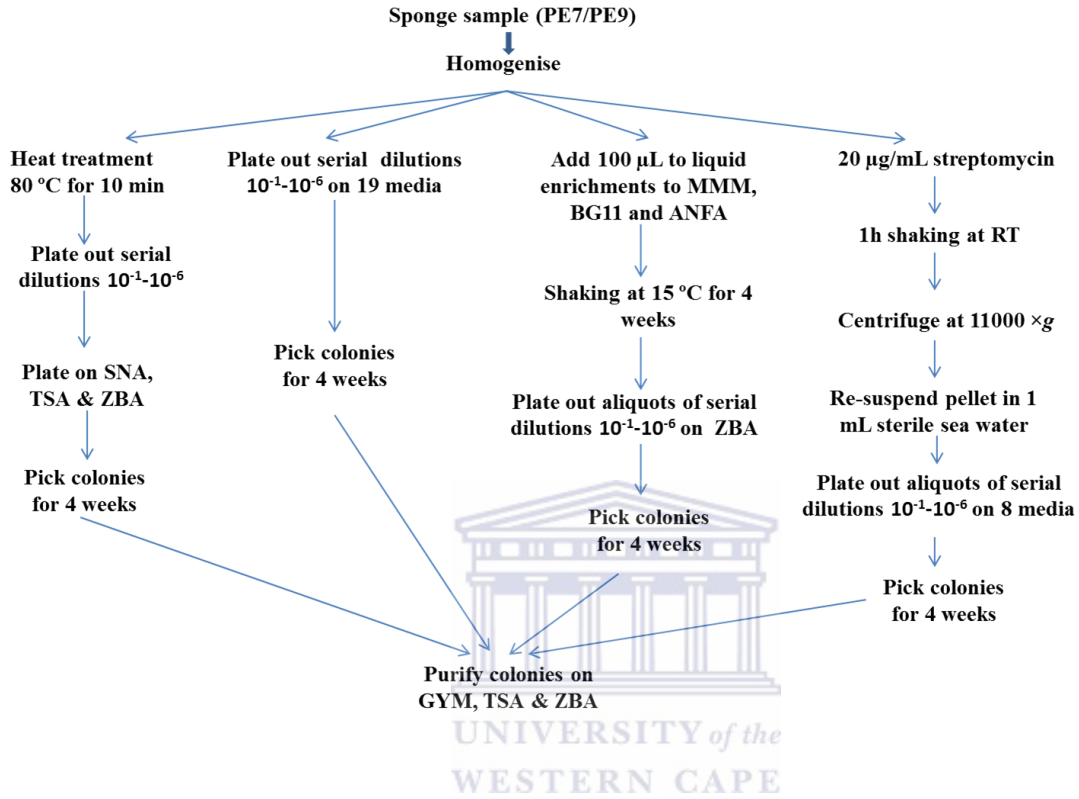


Figure 2.1 : Flow diagram for the culturing process using two marine sponge samples.

2.2.2 Glycerol stocks of bacterial isolates

Bacterial isolates were grown in 10 mL of liquid media (ZBA) and incubated at 15 ºC for a number of days until turbid. Liquid cultures which showed no growth after 3 days were incubated at room temperature on a shaker. Turbid liquid cultures were Gram stained before making freezer stocks. Freezer stocks were made in 200 µL 96 well plates by adding 50% glycerol to each well and a 150 µL aliquot of a bacterial culture. The plates were sealed and stored at -80 ºC.

2.2.3 Preliminary antibacterial screening of isolates (antibacterial overlay assay)

The marine sponge associated isolates were employed in an agar overlay assay to screen for antibacterial activity against the test strains *Escherichia coli* 1699 (Cubist, USA), *Mycobacterium smegmatis* LR222, *Pseudomonas putida* ATCC27853, *Bacillus cereus* ATCC10702, and *Staphylococcus epidermidis* ATCC14990. The four media (ZBA, GYM, TSA and ACM Appendix 2) were used for screening isolates as part of an undergoing collaboration on the PharmaSea project. Isolates were grown on the four media for two weeks at room temperature and on the day prior to carrying out overlays, the test strains were cultured overnight in 10 mL Luria Broth (LB) at 37 ºC with shaking at 250 rpm. To get the same concentration of cells (using OD_{600}) in sloppy agar, the following formulae were used per 6 mL sloppy agar overlay:

$OD_{600} \times X\ \mu L = 4$ (*E. coli*)

$OD_{600} \times X\ \mu L = 160$ (other test strains) (Mavengere, 2008)

The calculated volume of test strains was inoculated into sloppy agar and then overlaid on the isolates. These were incubated at 30 ºC and examined for zones of inhibition after an overnight incubation and after 2 days.

2.3 Matrix composition culturing

2.3.1 Selection of isolates for matrix

A total of 30 isolates were selected for the matrix based on bioactivities obtained in the antibiotic overlay assay (section 2.2.3). Thirteen isolates which gave positive hits against the panel of test

strains were chosen for the matrix. In addition 17 of the isolates which had not shown antibacterial activity were selected. These were either isolates which showed a resemblance to *Actinomycetes* or pigmented strains. Two strains of *Streptomyces coelicolor*, strain M145 and the mutant strain M1146, were used as controls for this work. The experiment was set up in a 96-well format on large petri dish plates (20 mm × 150 mm).

2.3.2 Matrix design and procedure

The matrix was designed to investigate the effects of carbon, nitrogen, phosphate sources and effect of heat and cold treatments as summarized in Table 2.2. The different conditions for this work were selected using the guidelines in literature pertaining to the OSMAC principle (Doull *et al*., 1994; Bode *et al*., 2002). To allow for the systematic alteration of different parameters, a minimal medium (Table 2.3) was used as the basic growth medium. Table 2.4 explains the abbreviations used for the media 36 media conditions used in the matrix. The matrix was designed using three carbon sources (mannitol, succinic acid and starch) used at a 5 mM concentration while the other factors/parameters were varied. The two nitrogen sources selected for the matrix were NH_4Cl (0.02%) and $NaNO_3$ (0.02%). For the phosphate source, two concentrations of KH_2PO_4 0.5 mM and 0.1 µM were used. Additional treatments that were investigated included heat and cold shock treatments. The matrix therefore represented 36 test conditions. Five plates were made for each of the 36 conditions for the five respective indicator/test strains. The overall work was repeated to verify the results for isolates which gave positive hits in the first round of screening.

Table 2.2: Parameters used in the design of the matrix.

MATRIX DESIGN			
	Mannitol	Succinic acid	Starch
NH_4Cl			
$NaNO_3$			
0.1µM KH_2PO_4			
0.5mM KH_2PO_4			
Heat shock			
Cold shock			

Table 2.3: Minimal medium composition.

Minimal media	
Component	g/L
NaCl	18
$MgCl_2$	2
KCl	0.525
$CaCl_2$	0.075
HEPES	2.38

Table 2.4: Media composition shorthand for the 36 conditions.

Abbreviation		Media constituents
Mannitol	A1M	Ammonium Chloride; 0.1 µM Phosphate; Mannitol
	A1HM	Ammonium Chloride; 0.1 µM Phosphate; Mannitol; Heat Shock
	A1CM	Ammonium Chloride; 0.1 µM Phosphate; Mannitol; Cold shock
	A5M	Ammonium Chloride; 0.5 mM Phosphate; Mannitol
	A5HM	Ammonium Chloride; 0.5 mM Phosphate; Mannitol; Heat Shock
	A5CM	Ammonium Chloride; 0.5 mM Phosphate; Mannitol; Cold shock
	Na1M	Sodium Nitrate; 0.1 µM Phosphate; Mannitol
	Na1HM	Sodium Nitrate; 0.1 µM Phosphate; Mannitol; Heat shock
	Na1CM	Sodium Nitrate; 0.1 µM Phosphate; Mannitol; Cold shock
	Na5M	Sodium Nitrate; 0.5 mM Phosphate; Mannitol
	Na5HM	Sodium Nitrate; 0.5 mM Phosphate; Mannitol; Heat shock
	Na5CM	Sodium Nitrate; 0.5 mM Phosphate; Mannitol; Cold shock
Succinic acid	A1Sa	Ammonium Chloride; 0.1 µM Phosphate; Succinic acid
	A1HSa	Ammonium Chloride; 0.1 µM Phosphate; Succinic acid; Heat shock
	A1CSa	Ammonium Chloride; 0.1 µM Phosphate; Succinic acid; Cold shock
	A5Sa	Ammonium Chloride; 0.5 mM Phosphate; Succinic acid
	A5HSa	Ammonium Chloride; 0.5 mM Phosphate; Succinic acid; Heat shock
	A5CSa	Ammonium Chloride; 0.5 mM Phosphate; Succinic acid; Cold shock
	Na1Sa	Sodium Nitrate; 0.1 µM Phosphate; Succinic acid
	Na1HSa	Sodium Nitrate; 0.1 µM Phosphate; Succinic acid; Heat shock
	Na1CSa	Sodium Nitrate; 0.1 µM Phosphate; Succinic acid; Cold shock
	Na5Sa	Sodium Nitrate; 0.5 mM Phosphate; Succinic acid
	Na5HSa	Sodium Nitrate; 0.5 mM Phosphate; Succinic acid; Heat shock
	Na5CSa	Sodium Nitrate; 0.5 mM Phosphate; Succinic acid; Cold hock
Starch	A1S	Ammonium Chloride; 0.1 µM Phosphate; Starch
	A1HS	Ammonium Chloride; 0.1 µM Phosphate; Starch; Heat shock
	A1CS	Ammonium Chloride; 0.1 µM Phosphate; Starch; Cold shock
	A5S	Ammonium Chloride; 0.5 mM Phosphate; Starch
	A5HS	Ammonium Chloride; 0.5 mM Phosphate; Starch; Heat shock
	A5CS	Ammonium Chloride; 0.5 mM Phosphate; Starch; Cold shock
	Na1S	Sodium Nitrate; 0.1 µM Phosphate; Starch
	Na1HS	Sodium Nitrate; 0.1 µM Phosphate; Starch; Heat shock
	Na1CS	Sodium Nitrate; 0.1 µM Phosphate; Starch; Cold shock
	Na5S	Sodium Nitrate; 0.5 mM Phosphate; Starch
	Na5HS	Sodium Nitrate; 0.5 mM Phosphate; Starch; Heat shock
	Na5CS	Sodium Nitrate; 0.5 mM Phosphate; Starch; Cold shock

2.3.3 Matrix set up in 96 well format

The method of plating in 96 well-formats was similar to that described by Bills *et al*. (2008) with some modifications. Deep 96 well plates containing 70 µL autoclaved ultra violet (UV) water were used in the study. Single colonies from the selected isolates and *S. coelicolor* controls were inoculated. The order of inoculation of isolates was interchanged between isolates that gave positive hits and those which did not. All isolates were inoculated in triplicate and therefore located at different positions resulting in different neighboring isolates which incorporated a co-culture setup. For uniform distribution, the 96 well plates were shaken at 100 rpm on a shaker for 10 minutes at room temperature. Cultures were later plated aseptically on the different matrix media (Appendix 3) using a 96 pin hedgehog and allowed to grow for 2 weeks at room temperature.

2.3.4 Matrix heat and cold shock treatments

On the day prior to performing overlays, cold and heat shock treatments were carried out. The heat shock method used was adapted from Nakata *et al*., (1999) with minor changes. Heat shock plates were incubated for 1 h at 42 ^{o}C whereas cold shock treatment was carried out at 4 ^{o}C for 1 h. The heat and cold shock treatments were repeated on the day of the antibiotic overlay assay.

2.3.5 Matrix media for antibacterial screening (antibacterial overlay assay)

The day prior to carrying out the overlay assay, single colonies for *E. coli* 1699 (Cubist, USA), *M. smegmatis* LR222, *P. putida* ATCC27853, *B. cereus* ATCC10702, and *S. epidermidis* ATCC14990 were inoculated into Luria Broth (10 mL) and the cultures were incubated at 37 ^{o}C

overnight while shaking at 250 rpm. The optical density (OD_{600} of the cultures were determined after which they were diluted to achieve the same overall concentration of cells (section 2.2.3). An overlay assay similar to that described in section 2.2.3 was carried out using 24 mL for the large format plates (20 mm x 150 mm).

2.4 DNA extraction

2.4.1 Metagenomic DNA (mDNA) extraction and purification from marine sponge associated bacteria

Several DNA extraction methods were employed for mDNA extraction of the marine sponge associated bacteria.

2.4.1.1 Modified Kennedy method (Kennedy et al. 2008)

Thawed sponge samples (1 g) were cut into small pieces with a sterile blade and ground to a fine powder under liquid nitrogen using a sterile pestle and mortar. The ground material was re-suspended in 8 mL of lysis buffer (15 mM Tris, 15 mM EDTA, 1.4 M NaCl, 1% CTAB, pH 8). A volume of 2 µL RNase A (10 µg/µL) and 100 µL lysozyme (25 mg/mL) were added to the samples and incubated for 2 h at 37 ºC in a water bath. A further 2 mL SDS (20% w/v) and 100 µL of proteinase K (20 mg/mL) were added and tubes were inverted once and incubated overnight at 65 ºC. The sample extracts were centrifuged at 10 000 × *g* for 15 minutes at 4 ºC. The supernatants were transferred to fresh tubes and equal volumes of phenol: chloroform: isoamyl alcohol (PCI) at a ratio of 25:24:1 were added and re-centrifuged. The process was repeated three times with the aqueous phase being extracted each time and transferred to a clean

tube. The DNA was precipitated by the addition of 0.7 × volume isopropanol and incubated at room temperature for 30 minutes. The DNA was collected by centrifugation at 10 000 × *g* for 30 minutes and the pellets were washed 3 times with 70% ethanol. The pellets were air dried and resuspended in 0.6 mL of TE (10 mM Tris, 1 mM EDTA, pH 8.0).

2.4.1.2 Extraction of mDNA using a commercial kit

Sponge samples were thawed on ice water and cut with a sterile blade into 1g aliquots for further processing. The UltraClean Soil DNA Isolation kit (MOBIO Laboratories) was used to isolate DNA according to the manufacture's protocol.

2.4.1.3 Agarose plug DNA purification

Molten agarose (2%) was cooled to about 45 ºC. Equal volumes of mDNA (500 µL) from (section 2.4.1.1) and the 2% agarose solution were mixed in 2 mL microcentrifuges tubes and cooled at 4 ºC for 1 h. A solution of 80% formamide/1.3 M NaCl (5 × volume) was added and the resultant solution was mixed by vortexing and incubated for 1 h at 15 ºC. The formamide/salt solution was decanted and the agarose plug was washed 3 times by adding 1× TAE (1 mL) followed by incubating for 30 minutes at 15 ºC. The NucleoSpin ® Gel and PCR Clean-up kit (Macherey-Nagel, Germany) was used as per the manufacturer's guidelines to extract the mDNA from the agarose plug. The resulting mDNA underwent an ethanol wash by adding 1 mL of 70% ethanol and centrifuging at 14 000 × *g* for 15 minutes. The ethanol wash was repeated three times and each time the supernatant discarded. The resulting pellet (mDNA) was allowed to air dry and was re-suspended in 200 µL TE.

2.4.2 Genomic DNA (gDNA) extraction from bacterial isolates

Several extraction methods were used for genomic DNA extraction.

2.4.2.1 Hard lysis gDNA extraction from Actinobacteria

Single colonies were picked and inoculated in liquid media (TSA) in 10 mL cultures and grown on a shaker (150 rpm) at room temperature until turbid. Cells were pelleted by centrifugation at 5000 × *g* for 10 minutes and the supernatant was discarded. The cell pellet was washed twice by adding 1 mL of TE (pH 8) buffer followed by centrifugation at 10 000 × *g* for 3 minutes at 4 ^{o}C and discarding the supernatant. The cell pellet was re-suspended in 200 µL breaking buffer and transferred to a 2 mL screw cap tube containing autoclaved acid-washed glass beads (212-300 µm, 50-70 U.S sieve). A volume of 200 µL phenol-chloroform-isoamyl alcohol (PCI) (25:24:1) was added to the tube under a fume hood. The solutions were vortexed for 2 minutes and 200 µL of ice cold TE buffer was added to the tube followed by centrifugation at 10 000 × *g* for 3 minutes. The supernatant was transferred to a pre-chilled 1.5 mL microfuge tube containing 1 mL of 96% ethanol and 10 µL of 3 M NH_4OAc solution and mixed by inverting several times. The solution was incubated at -20 ^{o}C for 20 minutes. The DNA was pelleted by centrifugation at 10 000 × *g* for 3 minutes at 4 ^{o}C and the supernatant was discarded using a pipette. The resultant pellet was dried overnight and re-suspended in 30 µL of TE (500 µL of TE mixed with 1 µL RNase A (10 mg/mL)).

2.4.2.2 gDNA extraction

Bacterial cells were grown in 10 mL of TSA or ZBA by incubation at room temperature on a shaker (150 rpm) until turbid. Cells were harvested by centrifugation at 10 000 × *g* for 5 minutes and the supernatant was discarded. Cells were re-suspended in 500 µL of lysozyme buffer (25 mM Tris-HCl pH8, 50 mM glucose, 10 mM EDTA and 25 mg/mL lysozyme) and 3 µL of RNase A (10 µg/mL) and incubated at 37 ^{o}C overnight. SDS 1% (v/v) was added to the tubes and incubated at 65 ^{o}C for 45 minutes. DNA liquid phase extraction was performed by adding an equal volume of PCI (25:24:1) and mixing thoroughly by vortexing briefly for 3 seconds. Centrifugation was carried out at 10 000 × *g* for 5 minutes and the aqueous phase transferred to a new microcentrifuge tube. An equal volume of chloroform/isoamyl alcohol (C:I) in the ratio of 1:1 was added to the aqueous phase and mixed thoroughly by briefly vortexing for 3 seconds followed by centrifuging at 10 000 × *g* for 5 minutes. The aqueous layer was transferred to a new microcentrifuge tube using cut tips. A 1/10 volume of 3 M NaOAc and a 2 × volume of 100% ethanol were added and the solution was mixed by inverting several times. The tube and contents were incubated for 1 h at -20 ^{o}C which was followed by centrifugation at 10 000 × *g* for 20 minutes at 4 ^{o}C. The supernatant was discarded and the pellet was washed once with 70% ethanol and dried overnight. The DNA was re-suspended in 30 µL TE.

2.4.2.3 gDNA extraction

A pure single colony was inoculated in 10 mL of TSA or ZBA broth and incubated at room temperature on a shaker (150 rpm) until turbid. The bacterial culture was harvested by centrifugation at 5000 × *g* for 10 minutes. The supernatant was discarded and the cell pellet was resuspended in 950 µL of TE buffer followed by addition of 50 µL of 10% SDS and 5 µL of 20

mg/mL proteinase K. These were mixed thoroughly and incubated for 1 h at 37 ^{o}C. A volume of 180 µL of 5 M NaCl was added and mixed thoroughly to which 150 µL of CTAB/NaCl solution was added. The solutions were incubated at 65 ^{o}C for 20 minutes. An equal volume of CI (24:1) was added and mixed vigorously. Centrifugation was carried out at 10 000 × *g* for 10 minutes and cut tips used to transfer the aqueous phase to fresh tubes. A second liquid extraction was carried out by adding an equal volume of PCI (25:24:1) and mixing thoroughly followed by centrifuging for 10 minutes at 10 000 × *g*. The aqueous layer was transferred to a fresh tube and 1/10 volume of 3 M NaOAc and a 2 × volume of 100% ethanol were added before incubation for 1 h at -20 ^{o}C. Centrifugation was carried out at 13 000 × *g* for 20 minutes at 4 ^{o}C and the supernatant was discarded. The DNA was dried overnight and resuspended in 30 µL of TE and incubated overnight at 60 ^{o}C.

2.4.2.4 gDNA extraction using the GeneJET Genomic DNA Purification Kit (Thermo Scientific)

A pure single colony was inoculated in 10 mL of tryticase soy broth (TSA) or ¼ strength Zobell broth (ZBA) and incubated on a shaker (150 rpm) at room temperature until turbid. DNA extraction was carried out according to the manufactures instructions for Gram negative bacteria. Minor modifications were carried out for gram positive bacteria where 200 µL of lysis buffer (25 mM Tris-HCl pH8, 50 mM glucose, 10 mM EDTA and 25 mg/mL lysozyme) was added to the pelleted bacterial cells and the solutions were incubated at 37 ^{o}C for 30 minutes. Thereafter the manufacturer's instructions were followed.

2.4.2.5 Plasmid extraction

Plasmid extraction was performed using the QIAGEN Plasmid Mini Kit (QIAGEN GmbH, Hilden, Germany) following the manufacturer's instructions.

2.5 Polymerase Chain Reaction (PCR)

Polymerase chain reactions (PCR) were performed using a Labnet MultiGene™ Gradient PCR Thermal Cycler (Labnet International, Inc.) or an automated thermal cycler (T100 Thermal cycler, BioRad). A volume of 50 µL reactions were used for PCR

2.5.1 PCR prior to T-RFLP PCR

The reaction mixture for Terminal Restriction Fragment Length Polymorphisms (T-RFLP) was carried out using Phusion polymerase (Fermentas, Lithuania). The reactions constituted 1 × Phusion Buffer, 0.2 mM dNTP mix, 0.5 µM of each forward and reverse primer E9F and U1510R (Appendix 4), 0.02 U/µL of Phusion DNA polymerase, varying concentrations of template mDNA and UV water. The following cycling conditions were used: initial denaturation of 98 ^{o}C for 2 minutes followed by 20 cycles of 98 ^{o}C for 20 sec, 54 ^{o}C for 30 sec, 72 ^{o}C for 1 minute and final elongation at 72 ^{o}C for 5 minutes.

2.5.2 Nested PCR for T-RFLP

The above amplicons were used to conduct a nested PCR using the FAM (fluorescent dye fluorescein amidite) labelled primers. These are the same sequence as the primers above, except that the 5' end of the E9F primer was labeled with FAM. The cycling conditions were increased

from 20 cycles to 35 cycles (Appendix 4). For samples which did not give problems with amplification using E9F_FAM, the template mDNA was used instead of PCR products and 35 cycling conditions were performed.

2.5.3 Amplification of 16*S* rRNA genes for isolate identification

PCR was carried out in 50 µL volumes which consisted of 1× Dream Taq buffer, 0.2 mM of dNTP mix, 1 µM of each of the reverse and forward primers, varying template DNA, 1.25 U DreamTaq DNA polymerase (Fermentas, Lithuania), and UV water. The primer set and cycling conditions are listed in Appendix 4. For samples which were difficult to amplify, different DNA polymerases were used, and included KAPA2G Fast PCR Kit (KAPA and KAPA2G Robust HotStart (KAPA Biosystems, Cape Town, South Africa). The reactions were conducted as per the manufacturer's instructions, further cycling condition details are summarized in Appendix 4.

2.5.4 PCR product purification

The NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Germany) was used to purify PCR products either directly or after PCR products were separated by electrophoresis on agarose gels. The manufacturer's instructions were followed.

2.6 Terminal restriction fragment length polymorphism analysis (T-RFLP)

The bacterial 16S rRNA genes were amplified from marine sponge mDNA as described in section 2.5.2 in duplicate, the reactions pooled and purified using a NucleoSpin® Gel and PCR

Clean-up kit (Macherey-Nagel, Germany) (section 2.5.4). The amplicon concentrations were normalized to 1 µg and restriction enzymes were used to double digest the amplicons (section 2.8.1) and the amplicons were re-purified. Capillary electrophoresis and analysis of the fluorescently labelled terminal restriction fragments (T-RFs) was carried out on an automated ABI3130XL genetic analyzer (Applied Biosystems, USA) as a service by the Central Analytical Facility at the University of Stellenbosch. The marker used for the analysis was GS500LIZ. T-RFLP profiles were analysed using freeware Peak Scanner™ Software Version 1.0 (Applied Biosystems). Peak height was used to characterize unique T-RFs. Peaks shorter than 25 bp and larger than 1200 pb were excluded from further analysis. Valid peaks were identified and aligned using the online T-REX software (http://trex.biohpc.org/ ; Culman *et al*. 2009) to create an operational taxonomic unit (OTU) data matrix. In theory, each OTU represents an individual T-RF where one T-RF represents one distinct ribotype (Blackwood *et al*., 2007). It can be noted however that, one peak may represent two different bacterial species or two peaks may be one bacterial species depending on the recognition site of restriction enzymes on the 16S rRNA gene sequence (Nocker *et al*. 2007; Schütte *et al*. 2008; Ramond *et al*. 2013). The OTU matrix was analysed using the Primer 6, Version 6.1.11 (Primer E, Plymouth, UK) software. Diverse function on Primer 6 software was used to calculate diversity indices for all the samples. Standardized T-RFLP profiles were used to calculate Bray-Curtis similarity coefficients (Bray & Curtis 1957) which were used to create similarity matrices of presence/absence transformed data. Similarity matrices were used to construct nonmetric multidimensional plots (nMDS). In nMDS plots, the distance between points reflects the degree of similarity between microbial community profiles in samples (Ramond *et al*. 2013).

2.7 Cloning of 16S rRNA genes

2.7.1 Preparation of electro-competent cells

A colony of *E. coli* JM109 was inoculated in 10 mL culture of Luria Broth (LB) and incubated overnight at 37 ^{o}C on a shaker at 150 rpm. Two conical flasks containing 500 mL of LB were inoculated with 5 mL of the overnight culture and incubated at 37 ^{o}C on a shaker. The OD_{600} was measured every hour until it reached between 0.6-0.7. The cells were immediately cooled on ice with occasional shaking for uniform cooling. Cells were harvested at 4000 × *g* for 20 minutes at 4 ^{o}C and the supernatant was discarded. Cells were re-suspended in 200 mL of ice cold 10% glycerol and harvested at 2500 × *g* for 10 minutes at 4 ^{o}C. The supernatant was decanted and the cells were re-suspended in 40 mL ice cold 10% glycerol. The re-suspended cells were transferred to two pre-chilled 50 mL tubes (20 mL in each). The cells were harvested by centrifugation at 2500 × *g* for 10 minutes at 4 ^{o}C. The supernatant was discarded and the pellet re-suspended in 1 mL ice cold 10% glycerol by gentle swirling. Aliquots of the cell suspension (70 µL) were dispensed into pre-chilled 1.5 mL microcentrifuge tubes and stored at -80 ^{o}C.

2.7.2 Ligation of PCR products

The CloneJET PCR cloning kit (Thermo Scientific) was used for the ligation of gel purified PCR products (section 2.5.4) according to the manufacturer's instructions with minor changes. Reagents were thawed on ice and 20 µL reaction volumes set up. The reactions consisted of 10 µL of 2 × T4 ligase buffer, 1 µL pJET1.2 blunt cloning vector (50 ng/µL), 1 µL T4 DNA ligase, 1 µL of purified PCR products (section 2.5.4) and UV water. The mixture was vortexed for 5 seconds and incubated for 20 minutes at room temperature.

2.7.3 Transformation of the ligation products

Electro-competent *E. coli* cells (section 2.7.1) were obtained from -80 ^{o}C and thawed on ice. Five microliters (5 µL) of the ligation mixture (section 2.7.2) was added to the thawed cells and mixed gently. The mixture was incubated for 30 minutes on ice and 50 µL was transferred to a pre-chilled 0.1 cm sterile electroporation cuvette (BioRad). Electroporation was performed using the following conditions: 1.8 kV, 25 µF and 200 Ω using a Biorad MicroPulser™ (USA). Immediately thereafter 950 µL LB broth was added to the cuvette and the mixture was transferred to a microcentrifuge tube and incubated at 37 ^{o}C for 45 minutes. A volume of 100 µL cells was plated on LB-agar plates supplemented with 100 µg/mL ampicillin and incubated overnight at 37 ^{o}C.

2.7.4 Sequencing

The sequencing reactions of purified 16S rRNA gene products were carried out as a service by the Central Analytical Laboratory at Stellenbosch University using an ABI PRISM 377 automated sequencer. The data from the sequencing unit was processed using ChromasPro software (version 1.5a) for alignment and manual editing of sequences. The contigs were determined by BLAST searches of the NCBI GenBank nucleotide database (http://www.ncbi.nlm.nih.gov/) using 16S ribosomal RNA sequences (Bacteria and Archaea).

2.8 Restriction enzyme digestion

2.8.1 Restriction enzyme digestion for T-RFLP

The PCR products from the nested T-RFLP analysis (section 2.5.2) were purified using a NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Germany) and quantified using a NanoDrop Spectrophotometer ND-1000 (section 2.9.1). The restriction enzyme digestions were carried out in 32 µL volumes containing 2 µL of 10 × Tango buffer, 2 µL of *Hha*I (10 U/µL), 4 µL *Hae*III (10 U/µL), 1 µg PCR products and UV water added to correct the volume. The reaction was carried out in a BioRad T100 Thermal Cycler overnight by incubation at 37 ºC which was followed by inactivation at 80 ºC for 20 minutes. The NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Germany) was used to clean up the double digestion products.

2.8.2 Restriction enzyme digestion analysis of transformants

Restriction enzyme digestion was carried out on plasmids isolated from transformants (section 2.7.3) in 20 µL reaction volumes. The reactions were set up on ice and comprised of 2 µL of 1 × buffer O, 5 µL transformed plasmid DNA, 0.5 µL *Swa*I (10 U/µL) and UV water. The reaction was incubated at 30 ^{o}C for 2 h or overnight in a Labnet MultiGene™ Gradient PCR Thermal Cycler (Labnet International, Inc.).

2.9 Analytical Techniques

2.9.1 Spectrophotometry

The DNA was quantified using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, USA) at 260 nm. The solvent used in DNA elution was used as a blank to determine the concentration of DNA present in the solvent.

2.9.2 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate DNA, mDNA, gDNA (section 2.4), PCR amplicons (section 2.5) and restriction digestion products (section 2.8.2) on 1% agarose gels prepared in 1× TAE buffer. Ethidium bromide (0.5 µg/mL), was added to the agarose gels during preparation to aid in visualization. Samples were prepared by mixing between 3-5 µL of DNA or PCR products with 3 µL of 6× loading dye (DNA tracking dye) and were loaded on agarose gels. Electrophoresis was performed in 1× TAE buffer at 85 V or 100 V. DNA sizes were determined by comparing band migration to that of the molecular weight marker bands (phage lambda DNA digested with *Hind*III or *Pst*I enzymes). Gels were visualised under ultraviolet illumination and photographed using a digital imaging system (AlphaImager 2000, Alpha Inotech, San Leandro, CA, USA).

CHAPTER 3 RESULTS AND DISCUSSION

3.1 INTRODUCTION

Marine sponges harbour a large diversity of microbes. Transmission electron microscopy (TEM) has been used to classify marine sponges based on their microbial abundances (Hentschel *et al*. 2006; Schmitt *et al*. 2007) into "low microbial abundance" or "high microbial abundance" sponges (Giles *et al*. 2013). Biodiversity studies on marine sponges carried out by several research groups (Hardoim *et al*. 2009; Hardoim *et al*. 2012; Lavy *et al*. 2014) concluded that culture-independent methods give access to wider microbial diversities than culture-dependent methods (Giles *et al*. 2013; Olson & Gao 2013; Lavy *et al*. 2014). However, previous studies have proposed that a combination of culture-independent methods and culture-dependent methods are useful for understanding the microbial diversities of marine sponges and provides access to higher microbial diversities (Giles *et al*. 2013; Lavy *et al*. 2014).

Culture-independent studies employ molecular fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) (Muyzer *et al*., 1993) or terminal restriction fragment length polymorphisms (T-RFLP) analysis (Liu *et al*. 1997) to investigate microbial diversity, and have been employed to determine microbial abundance and diversity in marine sponges (Lee *et al*. 2009; Hardoim *et al*. 2009; Olson & Gao 2013; Olson *et al*. 2014).

T-RFLP analysis is a high-throughput fingerprinting technique which was introduced by Liu *et al*. (1997). This technique is used to monitor and compare changes in the structure and composition of the dominant members in microbial communities (Schütte *et al*. 2008). Forward or reverse primers or both primers are fluorescently labelled and used to amplify a gene of interest. The 16S rRNA genes of microbial community DNA are commonly targeted. The

amplicons are digested with restriction enzymes, and the digestion products purified. The terminal restriction fragments (T-RFs) of different lengths result because of the sequence differences in the variable regions of the gene. The fragments are electrophoresed using capillary electrophoresis in a DNA sequencer and the sizes of the fluorescing fragments are determined. When the 16S rRNA gene is targeted, each T-RF peak on the resultant electropherogram is assumed to represent a phylogenetically different organism. The T-RFs are therefore referred to as operational taxonomic units (OTUs) (Ramond *et al*. 2013). T-RFLP is a rapid and relatively cheap method of analysis (Lueders & Friedrich 2003) which is not labor intensive (Schütte *et al*. 2008). For the purpose of this study T-RFLP analysis was used to determine the microbial diversities of five marine sponge samples in order to inform which contained the highest microbial diversity. The sponge associated microbes were used for culture-dependent analysis and for screening for antibacterial activities.

3.2 RESULTS

3.2.1 Metagenomic DNA (mDNA) extraction

Sponge samples were collected from Algoa Bay, Port Elizabeth, and were labeled "PE" followed by an identity number. Metagenomic DNA (mDNA) of the marine sponge associated bacteria was extracted using two methods: the modified Kennedy method (section 2.4.1.1) and the Mobio Ultraclean Soil DNA kit (section 2.4.1.2). mDNA from PE05 was generously provided by Dr. Lucas Black. The modified Kennedy method initially yielded higher concentrations of mDNA than commercial DNA extraction kit (data not shown) but further purification using the agarose plug method (section 2.4.1.3) was necessary to remove impurities which inhibited downstream

PCR amplifications. The mDNA final concentrations obtained using the two extraction methods are shown in Table 3.1. Tissue from sponge PE07 was not able to undergo the Mobio Ultraclean Soil DNA extraction kit process as glutinous agglomerate was formed during the first stage of the protocol which clogged the purification columns.

Table 3.1: Concentration of mDNA from sponge samples using the two extraction methods

Sample		Mobio Ultraclean Soil DNA kit	Modified Kennedy method
		Conc. ng/µL	Conc. ng/µL
PE05	*Waltherarndtia caliculatum*	15	-
PE07	*Higginsia bidentifera*	-	49
PE08	*Spongia* sp. 001RSASPN	43.1	21.4
PE09	*Isodictya compressa*	106.1	42.9
PE11	*Axiinella* sp. 007RSASPN	14.2	153.4

The mDNA extracted from the marine sponge sample was electrophoresed on a 1% agarose gel. High molecular weight mDNA with an approximate size of about 23 kb was extracted (Figure 3.1).

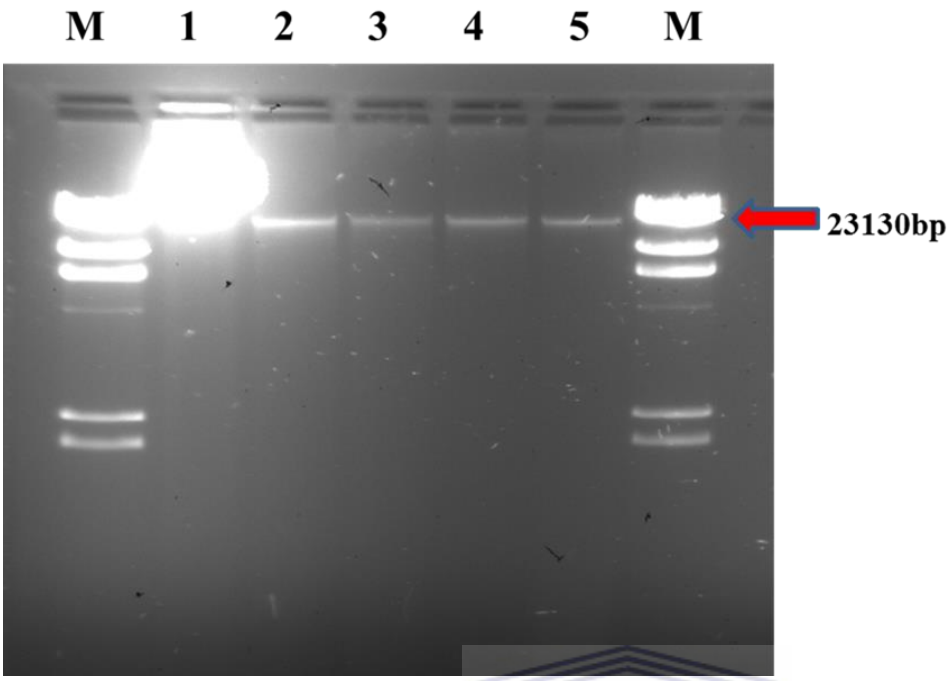


Figure 3.1: mDNA extracted from marine sponge associated bacteria, electrophoresed on a 1% agarose gel. Lane M is phage λ DNA digested with *Hind*III, Lane1 PE05, Lane 2 PE07, Lane 3 PE08, Lane 4 PE09 and Lane 5 PE11.The mDNA extracted using commercial kit are in lanes 3 and 4 and that from the modified Kennedy method are in lanes 2 and 5. Lane 1 is the mDNA supplied by Dr Lucas Black.

3.2.2 Terminal restriction fragment length polymorphisms (T-RFLP) analysis

3.2.2.1 16S rRNA gene amplification

With the exception of those extracted from sponge sample PE11, the 16S rRNA genes of the microbial communities present in the sponge samples could not be amplified directly using the fluorescently labelled E9F_FAM primer. A nested PCR approach was used on the mDNA of the four marine sponge samples by first amplifying with the E9F and U1510R primers, followed by a second amplification using the E9F-FAM and U1510R primers. PCR products of approximately 1.5 kb were obtained (Figure 3.2). The PCR products were digested with

restriction enzymes *Hha*I and *Hae*III to release the T-RFs and subjected to capillary electrophoresis. Peaks representing the predominant ribotypes were sequenced.

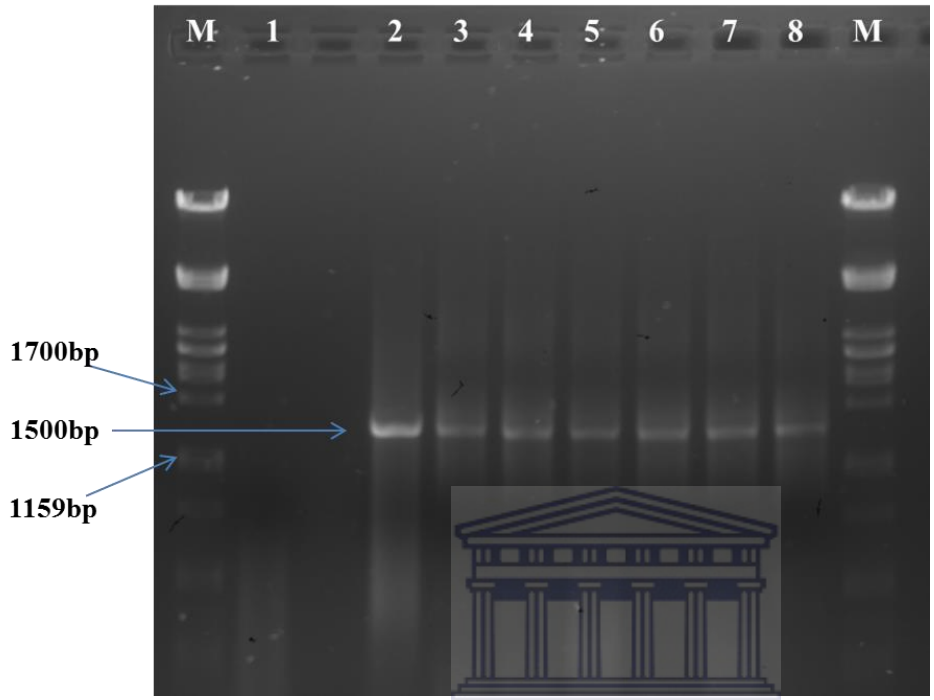


Figure 3.2: Agarose gel (1%) showing the 16S rRNA gene PCR amplification products (direct and nested PCR) of mDNA from five marine sponge samples using E9F_FAM and U1510R primers. Lane M is phage λ DNA digested with *Pst*I; Lane 1 negative control; Lane 2, positive control (*E. coli* gDNA generously provided by Dr William Mavengere); lane 3, PE05; lane 4, PE07; lane 5, PE08; lane 6, PE09; lane 7, PE11 and lane 8, PE11 (direct).

3.2.2.2 Analysis of T-RFLP profiles

T-RFLP analysis was used to determine the diversity of marine sponge associated bacteria. The diversity indices used in this study were the species richness (S) and the Simpson index (1 – λ) (Clarke & Warwick, 2001). Species richness theoretically counts the total number of observed OTUs present in a sample. The Simpson index on the other hand is a measure of the evenness or dominance of different OTUs. Simpson index can be represented in a number of forms:

$$\lambda = \sum p_i^2$$

or:

$1 - \lambda = 1 - (\sum p_i^2)$,

where $\{p_i\}$ comes from proportions for biomass or standardized abundance (Clarke & Warwick, 2001).

A uniform starting sponge sample (1g) used for mDNA extraction yielded different number of OTUs for each of the five samples. The T-RFLP analyses resulted in a combined diversity of 179 OTUs from the five marine sponges (Table 3.2). Some of the OTUs were shared across the five sponges resulting in a total of 90 unique OTUs (Figure 3.3). The sponge sample containing the highest number of microbial OTUs was PE09 (44) and the lowest bacterial OTUs were recorded from sample PE08 (24). The second highest number of bacterial OTUs originated from PE07 (43). This sample also showed the highest level of evenness (1-λ) of 0.9308. The Simpson index (1 – λ) of the bacterial species from PE05 (0.6923) was the least even, indicating the presence of more dominating OTUs in this sample.

Table 3.2: Diversity indices of the sponge associated microbial populations.

Sample ID	Marine sponge ID	S	1-λ
PE05	*W. caliculatum*	36	0.6923
PE07	*H. bidentifera*	43	0.9388
PE08	*Spongia (spongia)* sp. 001RSASPN	24	0.8514
PE09	*I. compressa*	44	0.9306
PE11	*Axiinella* sp. 007RSASPN	32	0.7706

Index: species richness (S) = OTUs and Simpson index (1-λ) = evenness

Figure 3.3 is a Venn diagram illustrating the distribution of the sponge associated microbial OTUs present in all the 5 sponges which also highlight their overlapping OTUs. The T-RFLP profiles revealed that there were a total of 90 unique OTUs from the 5 sponges with the highest number of OTUs (29 OTUs) shared between samples PE07 and PE09. The majority of the OTUs were shared between two or more sponges. Studies have classified microbial OTUs as species specific OTUs (defined as OTUs unique to a certain sponge species) and sponge specific OTUs (defined as OTUs found in sponges but not found in other environments) (Schmitt *et al*. 2012a; Taylor *et al*. 2013). Notably, 5 of the OTUs were shared among all the five sponges indicating that the representative OTUs could be sponge specific (Schmitt *et al*. 2012a; Simister *et al*. 2012a; Simister *et al*. 2012b). Sponge sample PE09 had the highest number of unique OTUs (14) with the second highest being PE05 with 11 OTUs. Although PE07 had the second highest number of OTUs only 3 OTUs were specific to this sample which indicated that fewer species specific OTUs were isolated from this sample.

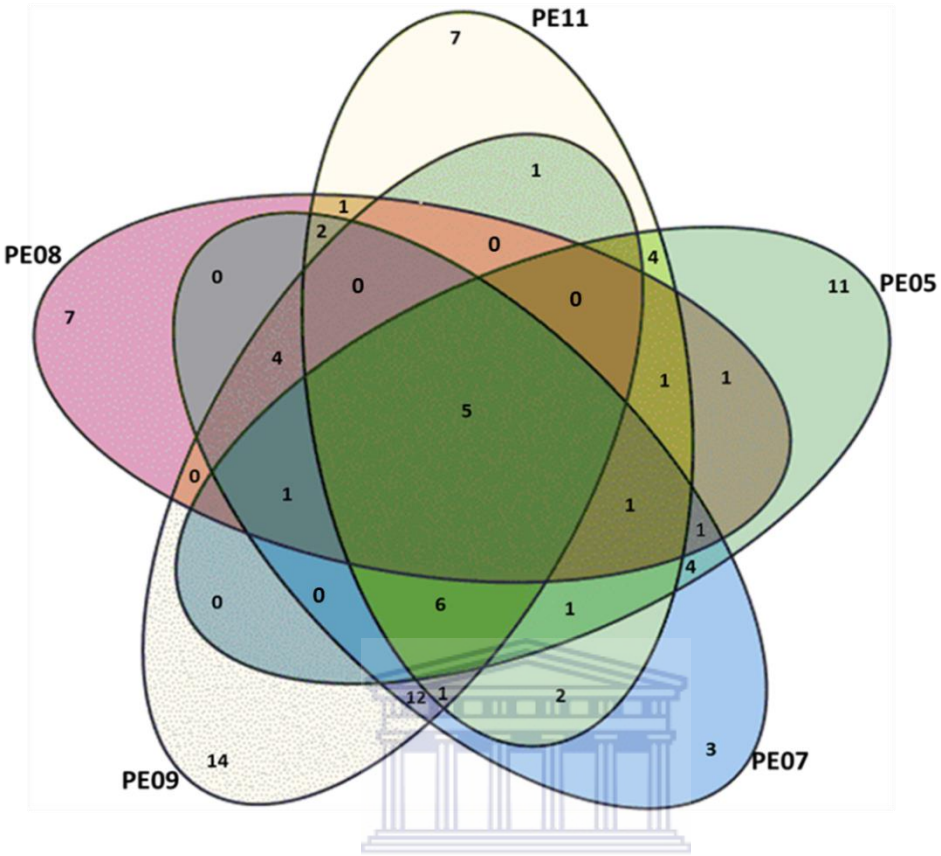


Figure 3.3: Venn diagrams illustrating the distribution of marine sponge associated bacterial OTUs present in 5 marine sponges.

The nMDS plot in Figure 3.4 shows the presence-absence of marine sponge associated bacterial OTUs in the 5 sponge samples. The plot indicates that marine sponge symbionts from PE05 and PE11 were similar given their closeness on the plot. In a similar way, the plot also revealed that OTUs from PE07 and PE09 were also similar. The sample whose symbionts were dissimilar to all four sponges was from sponge PE08. The stress value for this analysis was at 0.01 indicating that the results from the plot were reliable since they were closer to zero on the non-parametric regression line from which sample dissimilarities are calculated (Clarke & Warwick, 2001).

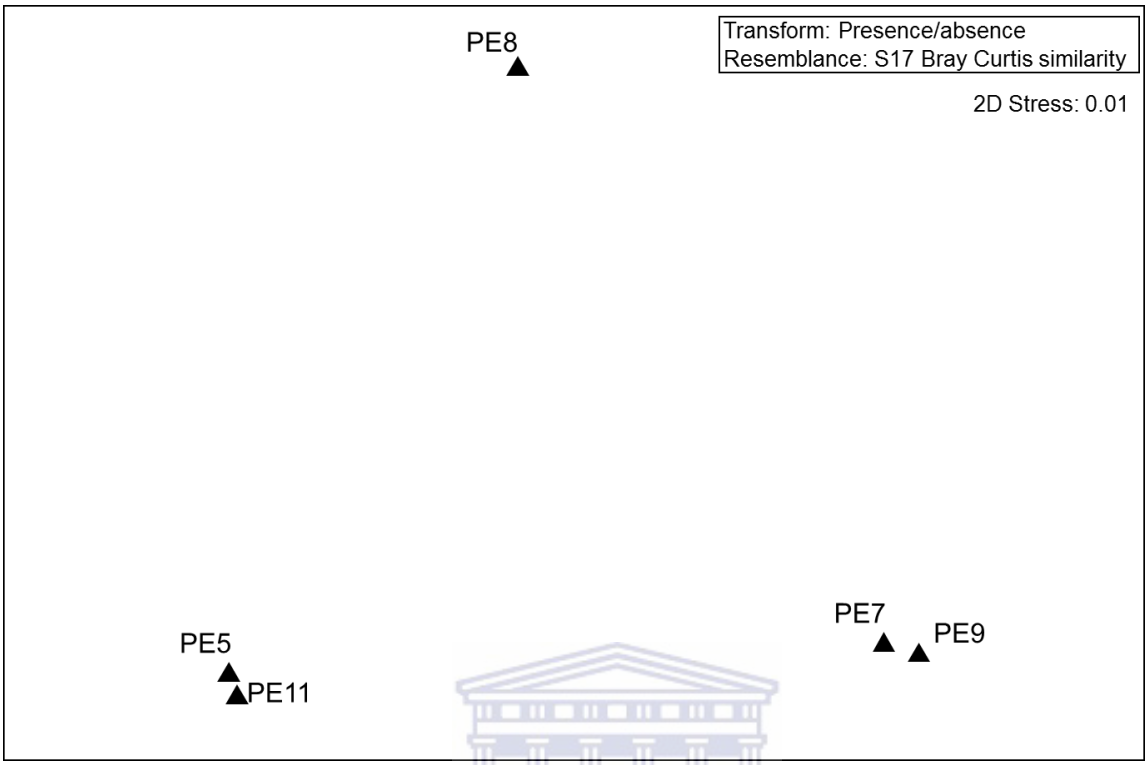


Figure 3.4: 2D-Monmetric multi-dimensional scaling (nMDS) plot of the Bray Curtis similarity (presence/absence transformation) of bacterial community structures determined by T-RFLP analysis of 16S rRNA genes associated with 5 marine sponges.

The two marine sponges which harboured the highest microbial diversities were PE07 and PE09 with 43 and 44 OTUs respectively. The OTUs from the present study were similar to T-RFLP OTUs from other studies where 45 and 32 OTUs were identified in *H. heliophila* and 36 and 22 OTUs were identified in *H. tubifera* depending on the restriction enzymes used (Erwin *et al.* 2011). The two marine sponges PE07 and PE09 in the present study are of the phylum Porifera and class Demospongia. Images of the sponges are shown in Figure 3.5.

PE07: *Higginsia bidentifera*

PE09: *Isodictya compressa*

Figure 3.5: Marine sponges PE07 and PE09 obtained from Algoa Bay, Port Elizabeth, South Africa.

3.2.3 Preliminary culturing

3.2.3.1 Culturing of marine sponge associated bacteria

Microorganisms are the most abundant organisms on the planet but only around 1% can be cultured using conventional laboratory conditions. Different culture methods were developed in an attempt to access the biodiversity that is observed in nature (Kim & Fuerst 2006; Joint *et al*. 2010). Diffusion growth chambers were used to successfully cultivate 15 previously uncultured bacteria associated with marine sponges (Steinert *et al*. 2014). Another cultivation study used 42 different media treatments where 22 novel species of bacteria were successfully cultivated some of which may represent new genera or families (Lavy *et al*. 2014). The current study also employed a wide variation of media conditions and treatments (antibiotic treatments, heat treatments, liquid enrichment) resulting in a total of 33 different conditions (Appendix 2). This

study therefore, aimed to bring into culture a high diversity of marine sponge symbionts by employing a wide variety of cultivation conditions.

The fewest number of isolates were obtained from sample growing in liquid enriched media and from the heat treatment media, which yielded 5 and 12 isolates respectively. This could be due to the selectivity in the liquid enrichment medium which selected for nitrogen fixing bacteria. In the case of heat treatment the low microbial culture indicated that the number of spore-forming bacteria is low in marine sponges (Phelan *et al*. 2012). The antibiotic treated media yielded 37 isolates while the non-treated media resulted in 346 bacterial isolates. These 346 isolates were cultivated on different media which contained varied carbon and nitrogen sources together with other nutrients which could support the proliferation of the different bacterial colonies (Sipkema *et al*. 2011; Lavy *et al*. 2014). A total of 400 bacteria were isolated from the two sponge samples with PE09 yielding 273 isolates and 127 bacterial isolates from PE07.

The pie chart in Figure 3.6 summarises the overall number of bacterial isolates cultured per medium. GYM was the medium which resulted in the highest number of bacterial isolates (40) which accounted for 10% of the total isolates. AIM and ZBA resulted in 39 (10%) and 37 (9%) isolates respectively. GYM, AIM and ZBA (Appendix 2) are nutrient rich media in which could be the reason for high isolate yields. The media which resulted in the fewest bacterial isolates were MMM and SWA since each contributed only 1 bacterial isolate. Both media discourage growth of fast growing bacteria thus resulting in fewer numbers of microbial isolates.

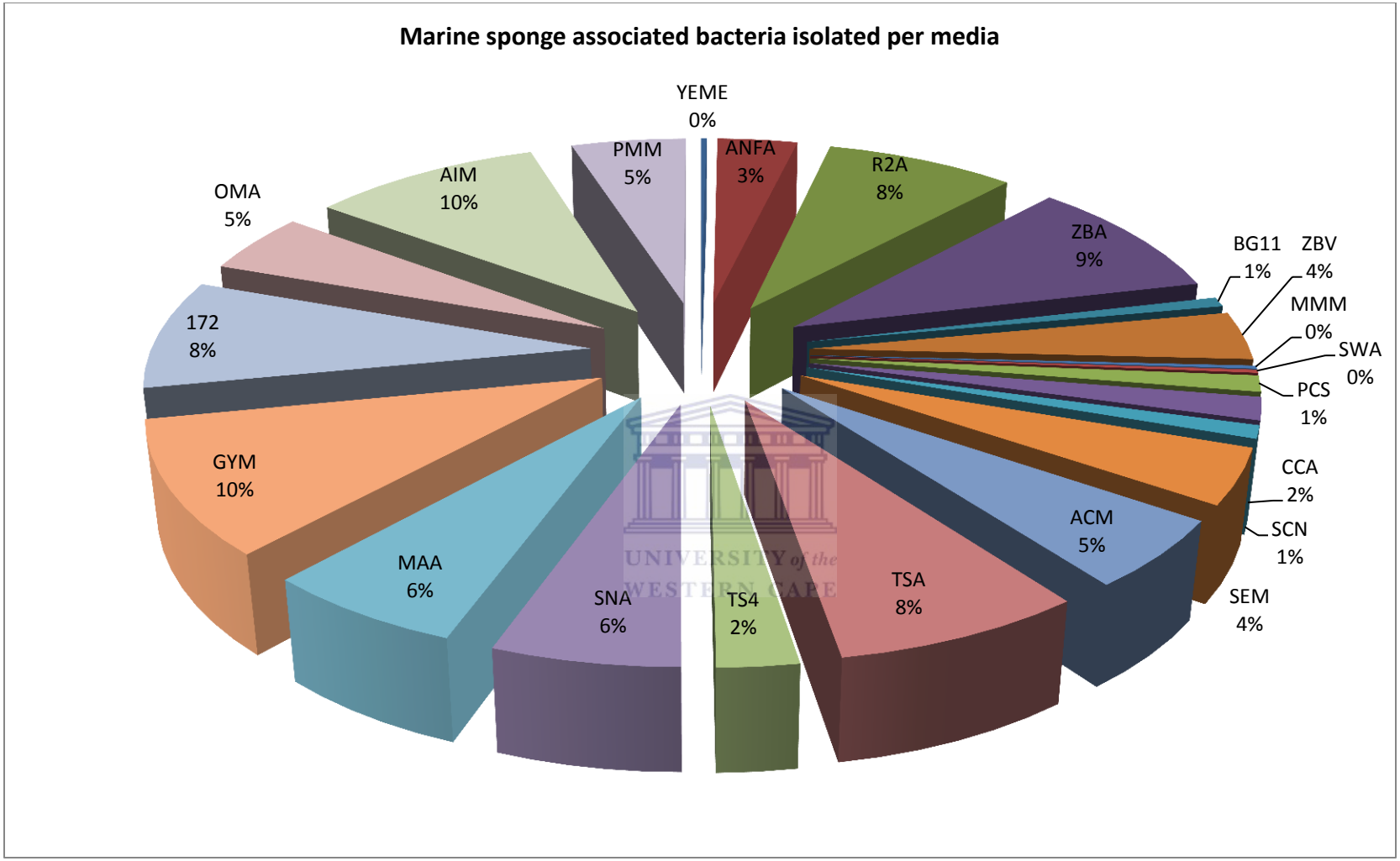


Figure 3.6: Pie chart showing the overall bacterial isolates obtained per medium. Each medium contribution is represented in percentage. All the bacterial isolates originated from the two sponge samples PE09 (*Isodictya compressa*) and PE07 (*Higginsia bidentifera*).

3.2.3.2 Isolation and characterization of bacteria

After the initial isolations, three media GYM, TSA and ZBA were selected as the "standard media" for routine culturing and characterization of the bacterial isolates. Isolates from TS4, TSA and SNA inclusive of those which underwent different treatments were cultured on TSA. Isolates from MAA, GYM, 172, OMA, AIM, and PMM along with their different treatments were cultured on GYM. Isolates from the other 13 media were also cultured on ZBA.

3.2.4 Preliminary antimicrobial overlay assay

Marine sponges harbor large diversities of microorganisms which serve as a chemical defense for these sessile invertebrates. As a result, marine sponge associated microorganisms have attracted attention to screen for and harness their bioactive compounds (Zhang *et al*. 2009a; Skariyachan *et al*. 2014). The antibacterial properties of microbial isolates from the marine sponges PE09 (*I. compressa*) and PE07 (*H. bidentifera*) were investigated in an agar overlay assay against five indicator strains: *Escherichia coli* 1699, *Mycobacterium smegmatis* LR222, *Pseudomonas putida* ATCC12633, *Bacillus cereus* ATCC10702 and *Staphylococcus epidermidis* ATCC14990. The antibacterial activities were indicated by clear zones of inhibition around the bacterial isolate as depicted in Figure 3.7. The clearing around the bacterial isolate indicates that the organism was producing compounds that inhibit proliferation of the indicator strains.

Four standard media (GYM, TSA, ACM and ZBA see Appendix 2) were used in the agar overlay assay. The results indicated that not all bacterial isolates portray identical properties on all four standard media, and that differential antimicrobial activities were observed in relation to the media the isolates were cultured on (Table 3.3). For instance, the isolate PE07_5, showed antibacterial activities against *E. coli* and *M. smegmatis* in GYM medium and only against *B.*

cereus on ZBA media. This is a well-known phenomenon regarding microbial secondary metabolite production (Muscholl-Silberhorn *et al*. 2008; Rateb *et al*. 2011).

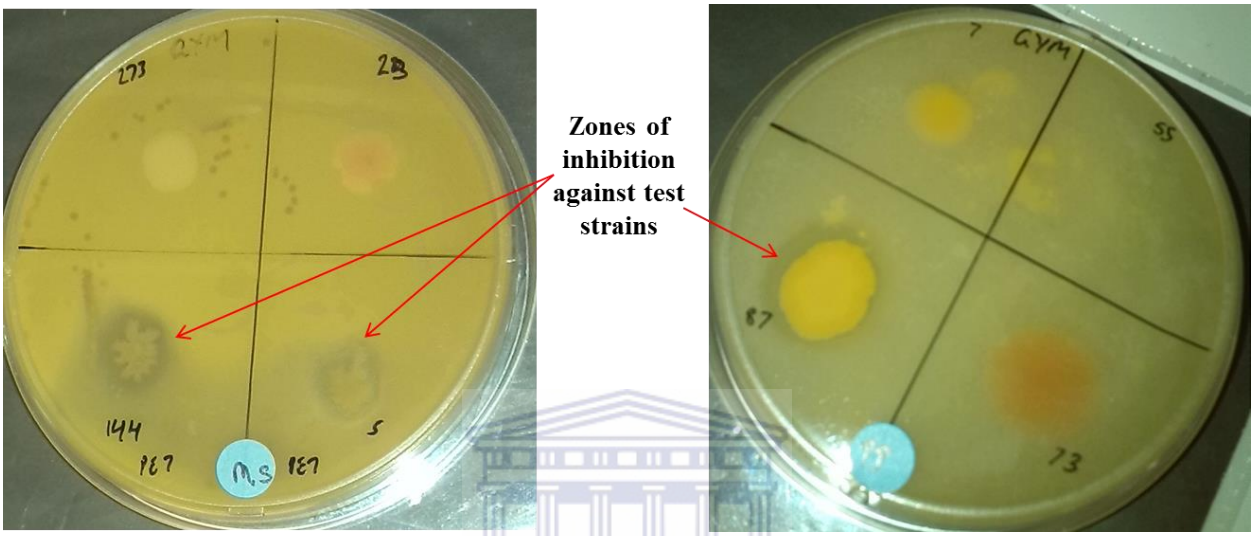


Figure 3.7: Preliminary screening of the antibacterial activities of marine sponge associated bacterial isolates against indicator strains in agar overlay assays. Isolates with antibacterial activities have zones of clearing around them and the arrows in the picture points out to the isolates which showed antibacterial activities.

Table 3.3: Screening of bacterial isolates showing antibacterial activities against indicator strains. The table indicates the media on which the antimicrobial properties were exhibited

Isolate	*E. coli*	*M. smegmatis*	*P. putida*	*B. cereus*	*S. epidermidis*
PE09_7					TSA
PE09_73			ACM		
PE09_87		GYM	GYM		GYM
PE09_100				ZBA	
PE09_105	GYM TSA	TSA GYM			TSA
PE09_108				GYM	
PE09_110	ZBA				
PE09_119		ZBA GYM		TSA ZBA	ACM TSA
PE09_124		ACM			
PE09_140		ACM			
PE09_142				ACM	
PE09_168				ACM	
PE09_197				ACM	
PE09_210				ACM	
PE09_213				ACM	
PE09_221				ZBA ACM	
PE09_228				ACM	
PE09_229		ACM		ACM	
PE09_235				ACM	
PE09_266				ZBA	
PE07_5	GYM	GYM		ZBA	
PE07_7				TSA ZBA	TSA ZBA
PE07_86				TSA	
PE07_106	GYM				
PE07_133	GYM				
PE07_143				TSA ZBA	
PE07_144	GYM	GYM			
PE07_172					TSA
PE07_200			GYM		
PE07_201			GYM		
PE07_204			GYM		
PE07_207	GYM			GYM	

Key	
media	
ACM	
TSA	
GYM	
ZBA	

Table 3.4 summarises all the antibacterial activities for all isolates per sponge material and indicates the number of microbes which exhibited multiple activities. Bacterial isolates from PE07 showed a slightly higher percentage (8.7%) of antibacterial properties whereas PE09 had 7.7% of its bacterial isolates showing antibacterial properties. Three bacterial isolates from each of the two sponges PE09 and PE07 showed multiple antibacterial activities which suggested broad-spectrum antibacterial activity. This could mean that more than one bioactive compound with different modes of action are produced by these isolates. It was interesting to notice that isolates showed antibacterial activities against the multidrug-resistant *E. coli* 1699 strain. This could indicate that the bioactive compounds produced from these isolates could be novel.

Table 3.4: Summary of the results of the screening of marine sponge associated microbial isolates using an antibiotic overlay assay against a panel of five indicator strains.

Sample	Isolates	Total number of activities	Indicator strains					
			E. coli	*M. smegmatis*	*P. putida*	*B. cereus*	*S. epidermidis*	Multiple activity
PE9	273	21	2	7	2	12	4	3
PE7	127	11	4	1	3	5	2	3

3.2.5 Identification of bacterial isolates using 16S rRNA genes

3.2.5.1 16S rRNA genes PCR analysis

Several genomic DNA (gDNA) extraction methods (section 2.4.2) were employed for different range of bacteria that gave positive hits in the antibiotic overlay assay and those that were selected for the matrix study (chapter 4). The gDNA was amplified using E9F and U1510R primers resulting in approximately 1500 bp amplicons (Figure 3.8). Some of the extracted gDNA

such as for isolate PE9_151 (Figure 3.8) could not amplify and different polymerases were used, different DNA extraction as well as colony PCR but amplification failed. Spiking the positive control gDNA with gDNA from other isolates resulted in failed amplification indicating that some of the gDNA had impurities which inhibited amplification. Some isolates which would not amplify could have been Archaea (Hentschel *et al*. 2006; Klindworth *et al*. 2013). The amplified PCR products were gel purified and sequenced to identify the bacterial isolates.

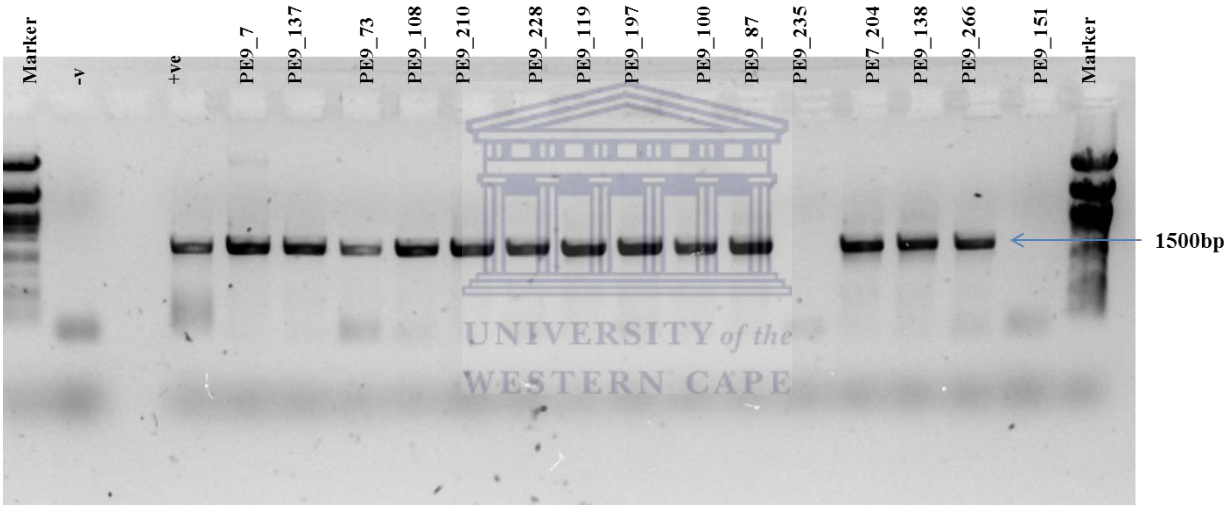


Figure 3.8: A representative agarose gel (1%) showing the 16S rRNA gene PCR amplification product (approximately 1.5 kb) from gDNA from marine sponge associated bacteria using E9F and U1510R primers. The lane labelled marker is phage λ DNA digested with *Pst*I; Lane –ve is the negative control; Lane +ve is the positive control, and the other labelled lanes indicate different bacterial isolates from which 1.5 kb 16S rRNA gene products resulted.

3.2.5.2 Cloning in pJET 1.2/blunt vector

The 16S rRNA genes PCR products were cloned into the pJET 1.2/blunt vector. The gel image in Figure 3.9 illustrates the transformed plasmid and insert after digestion with *Swa*I restriction enzyme (section 2.8.2) resulting in bands of approximately 2.4 kb and 2.1 kb. The plasmid DNA

was extracted and sequenced directly. For plasmid DNA with low concentrations, PCR amplification was carried out using pJET 1.2 Forw and pJET 1.2 Rev vector specific primers and the amplicons were sequenced directly.

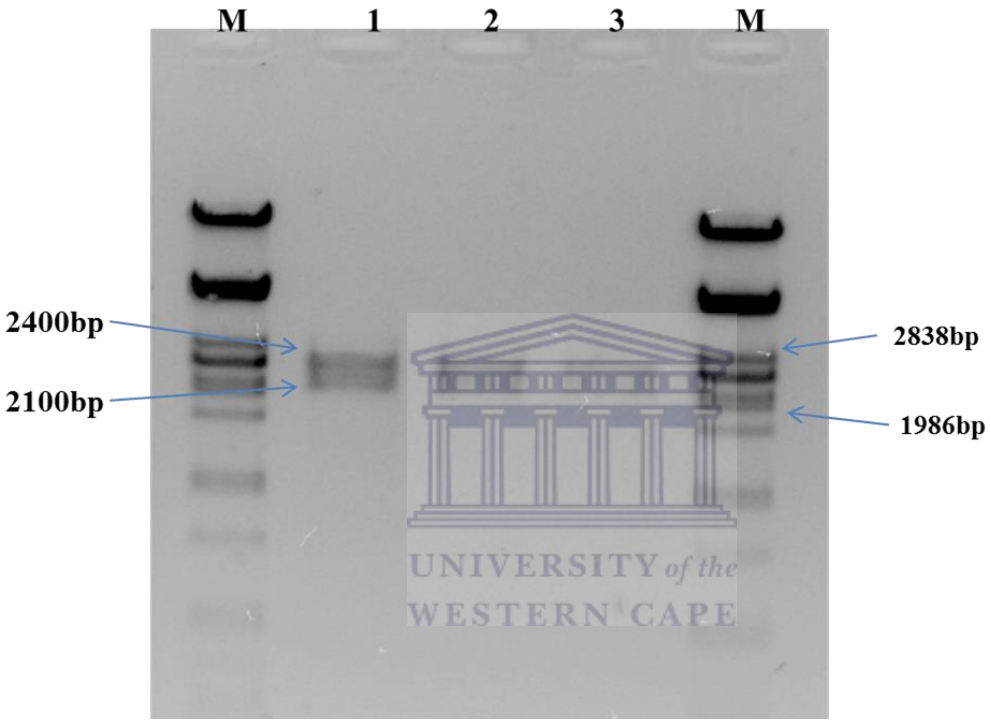


Figure 3.9: Agarose gel (1%) showing the transformed pJET 1.2/blunt and 1.5 kb insert digested with *Swa*I restriction enzyme which resulted in DNA fragments of about 2.4 kb and 2.1 kb. Lane M is phage λ DNA marker digested *Pst*I; Lanes 1-3 show digestion fragments from the recombinant plasmids.

3.2.5.3 Phylogenetic analysis

The 16S rRNA gene sequences were aligned using ChromasPro software and blasted against the NCBI database (BLASTn) to obtain the phylogenetic similarities to the sequences on the GenBank database. Table 3.5 shows the identified closest relatives (type strains). The identities

ranged from 97% to 100% similarity. An interesting isolate was PE09_221 which had a 97% identity to the *Actinobacterium Aeromicrobium erythreum* which implies that the isolate could be a new species (Lavy *et al*. 2014). Some 16S rRNA gene sequences showed 99% similarities and were de-replicated to become one OTU. In some instances, isolates shared 99% 16S rRNA gene sequence similarities whereas their bioactivity profiles differed. As a result these were considered to be different OTUs. An example of this was observed with isolates PE09_100 and PE09_110 which were both identified as *Citricoccus nitrophenolicus* strain PNP1 (Table 3.5) whereas their bioactivities (Table 3.3) differed. Similar de-replication procedures have been carried out in other studies (Vynne *et al*. 2012; Duncan *et al*. 2015). The sequenced bacteria belonged to the following phyla: *Actinobacteria*, *Firmicutes* and *Proteobacteia* (*Alphaproteobacteria* and *Gammaproteobacteria*).

Table 3.5: Identification of de-replicated marine sponge associated bacteria with antibacterial activity by 16S rRNA gene sequencing analysis in a BLAST search against NCBI database and the closest relatives (type strains).

Isolate	Identity	Closest hit	Phylum
PE09_110	99%	*Citricoccus nitrophenolicus* strain PNP1	*Actinobacteria*
PE09_124	99%	*Kushneria indalinina* strain CG2.1	*Gammaproteobacteria*
PE09_140	100%	*Pseudomonas cremoricolorata* strain NBRC 16634	*Gammaproteobacteria*
PE09_221	97%	*Aeromicrobium erythreum* strain NRRL B-3381	*Actinobacteria*
PE09_229	99%	*Halomonas titanicae* BH1	*Gammaproteobacteria*
PE07_5	99%	*Bacillus aquimaris* strain TF-12	*Firmicutes*
PE07_133	99%	*Micrococcus yunnanensis* strain YIM 65004	*Actinobacteria*
PE07_172	99%	*Sporosarcina aquimarina* strain SW28	*Firmicutes*
PE07_200	99%	*Staphylococcus warneri* SG1 strain SG1	*Firmicutes*
PE07_7	99%	*Bacillus marisflavi* strain TF-11	*Firmicutes*
PE07_103	99%	*Bacillus kochii* strain WCC 4582	*Firmicutes*
PE07_106	99%	*Oceanobacillus picturae* strain R-5321	*Firmicutes*
PE07_201	99%	*Staphylococcus epidermidis* strain Fussel	*Firmicutes*
PE07_204	99%	*Staphylococcus cohnii* strain GH 137	*Firmicutes*
PE09_7	99%	*Curtobacterium oceanosedimentum* strain ATCC 31317	*Actinobacteria*
PE09_73	99%	*Kushneria indalinina* strain CG2.1	*Gammaproteobacteria*
PE09_87	99%	*Psychrobacter alimentarius* strain N-154	*Gammaproteobacteria*
PE09_100	99%	*Citricoccus nitrophenolicus* strain PNP1	*Actinobacteria*
PE09_119	99%	*Arthrobacter citreus strain* DSM 20133	*Actinobacteria*
PE09_168	99%	*Brevibacterium luteolum* strain CF87	*Actinobacteria*
PE09_197	100%	*Pseudomonas cremoricolorata* strain NBRC 16634	*Gammaproteobacteria*
PE09_210	99%	*Halomonas profundus* strain AT1214	*Gammaproteobacteria*
PE09_228	99%	*Halomonas titanicae* BH1	*Gammaproteobacteria*
PE09_266	99%	*Kushneria indalinina* strain CG2.1	*Gammaproteobacteria*

Table 3.5 Continued: Identification of selected marine sponge associated bacteria by 16S rRNA gene sequencing analysis in a BLAST search against NCBI database and the closest relative (type strains). These isolates showed no activity in antibacterial screening but were selected for Matrix work (chapter 4).

Isolate	Identity	Closest hit	Phylum
PE09_55	99%	*Pseudovibrio ascidiaceicola* strain NBRC 100514	*Alphaproteobacteria*
PE09_72	99%	*Bacillus pumilus* SAFR-032 strain SAFR-032	*Firmicutes*
PE09_163	99%	*Staphylococcus saprophyticus* strain ATCC 15305	*Firmicutes*
PE09_180	99%	*Marinilactibacillus psychrotolerans* strain NBRC 100002	*Firmicutes*
PE09_222	99%	*Pseudovibrio ascidiaceicola* strain NBRC 100514	*Alphaproteobacteria*
PE09_250	99%	*Paenibacillus glucanolyticus* strain NBRC 15330	*Firmicutes*
PE07_37	99%	*Bacillus hwajinpoensis* strain SW-72	*Firmicutes*
PE07_72	99%	*Brevibacterium frigoritolerans* strain DSM 8801	*Firmicutes*
PE07_103	99%	*Bacillus kochii* strain WCC 4582	*Firmicutes*
PE07_124	99%	*Brevibacterium frigoritolerans* strain DSM 8801	*Firmicutes*
PE07_136	99%	*Lysinibacillus fusiformis* strain NBRC15717	*Firmicutes*

The media on which the identified members of the different bacterial phyla were isolated is summarized in Figure 3.10. Around 78% of all *Actinobacteria* were isolated on ZBA. ZBA is generally a rich medium on which a large diversity of microbial phyla was expected to grow. *Firmicutes* were the phyla which were cultured almost evenly on GYM and ZBA with minimal isolates obtained from TSA. None of the *Alphaproteobacteria* were obtained from ZBA whereas *Gammaproteobacteria* were obtained from TSA.

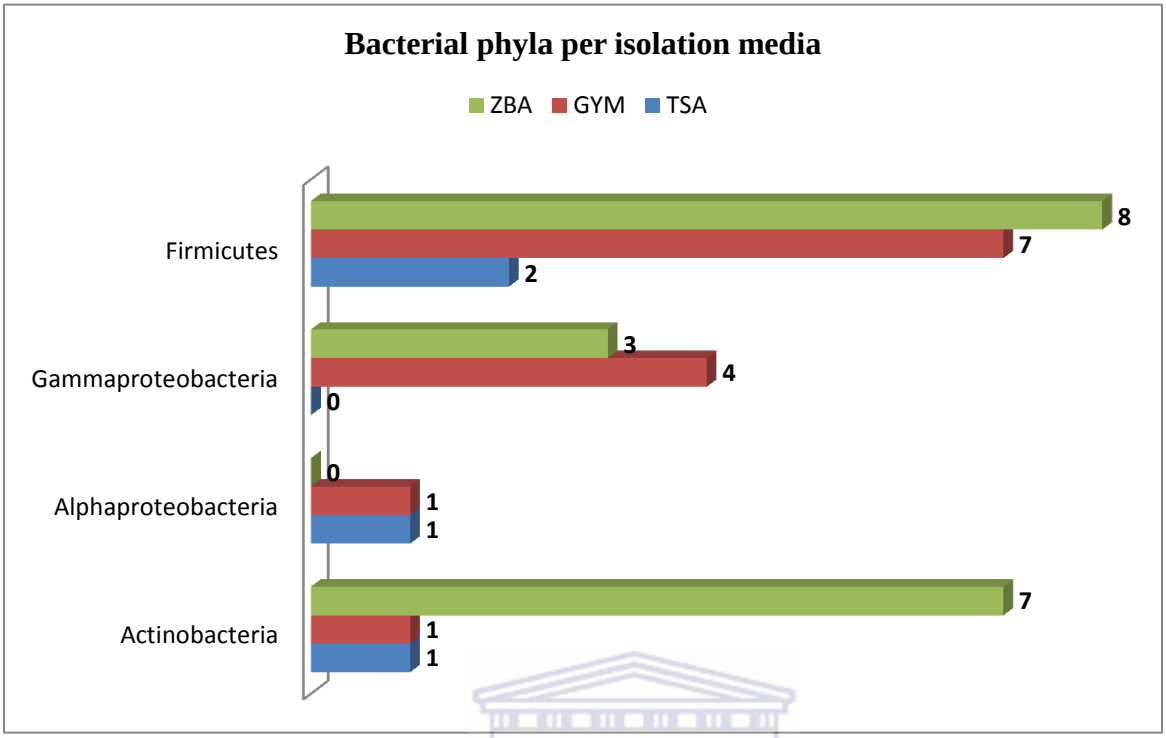


Figure 3.10: Bacterial phyla associated with marine sponges based on 16S rRNA gene sequences. The media on which the members of each phylum were isolated are shown in the key.

3.3 DISCUSSION

3.3.1 DNA extraction and T-RFLP analysis

DNA extraction: Good quality and quantity DNA and its amplification success can be influenced by several factors which span from storage of samples, length of storage, to extraction methods (Salgado *et al*. 2007). Two different extraction methods (commercial kit and the modified Kennedy method) were used in this study which resulted in different DNA yields. Although 1g was used as the uniform starting material per sponge sample, the DNA yields varied per sponge material. As Salgado *et al*. (2007) indicated, the different extraction methods may have influenced the quantity of DNA. Furthermore, under the same DNA extraction methods, the DNA yields varied between sponges, which could be a reflection of the associated microbial biomass. It is well known that marine sponges have been termed low microbial abundance (LMA) or high microbial abundance (HMA) sponges depending on the microbial biomass and diversity they host in their mesohyl matrix (Hentschel *et al*. 2006; Kennedy *et al*. 2014). The sponge samples were collected at different times in the year as a result, their preservation times differed. This could have also contributed to the different DNA yields. A study carried out by Salgado *et al*. (2007) revealed that preservation methods have an effect on the DNA yields. Salgado's group indicated that after one week of preservation the sponge DNA yields decrease.

T-RFLP: Understanding microbial abundances and communities is important for microbial ecologists (Prabhakar & Bishop 2013) and T-RFLP serves as a tool that monitors the structural composition of these communities (Schütte *et al*. 2008). T-RFLP analysis is used in many studies to monitor community changes (Dunbar *et al*. 2001; Stres 2006; Prabhakar & Bishop 2013; Ramond *et al*. 2013). T-RFLP was used to compare microbial diversity in the Caribbean sponge *Aplysina cauliformis* over a period of 2 years. However, the resolution of T-RFLP is superficial,

and typically is only successful in comparing the most dominant organisms in highly diverse samples (Olson *et al*. 2014). In the present study T-RFLP was used as a method to evaluate major differences in the dominant microbes associated with 5 taxonomically different sponges and to ascertain which harboured the most diverse communities.

PCR tends to be biased towards dominant groups in a community which could be due to the difference in DNA purities (Stres 2006) or DNA yields and amplification efficiencies which may affect the downstream statistical analysis of relative abundance (Lavy *et al*. 2014). High DNA yields however are not necessarily a good indicator of the total diversity accessed (Maropola 2014). T-RFLP analysis can detect 0.1-1% of the bacterial group in a community which results in apparent loss of fragments or appearance in T-RFLP finger prints (Dunbar *et al*. 2001). Furthermore, different bacterial populations may share the same recognition site resulting in underestimation of microbial diversity in samples (Schütte *et al*. 2008). Therefore, the observed bacterial diversities could be affected by PCR and T-RFLP biases thus affecting the observed bacterial diversities (OTUs). For this study however, the aim was to get a basic understanding of the bacterial diversities to enable the selection of sponge samples which harboured greater diversities, it was not intended to explain ecological context. T-RFLP analyses are most useful for examining changes in community composition thus this technique is appropriate for the purpose of this study (Olson *et al*. 2014). T-RFLP also provides large amount of easily analysed data on microbial community structure (Stres 2006).

Some studies have compared the effect of extraction protocols on the resulting microbial diversities (Stres 2006; Maropola *et al*. 2015). Studies indicated that fidelity in sample preparation and DNA extraction are crucial in minimizing possible misinterpretation of T-RFLP results (Stres 2006). Contrarily in this study, DNA proved to be difficult to amplify, as a result

different extraction methods were used for DNA extraction. In a similar study, it was reported that DNA from marine sponges is rarely suitable for downstream enzymatic reactions such as PCR amplification (Salgado *et al*. 2007). In the current study the DNA extracted from the different sponges was therefore, purified using agarose plugs. Similar PCR amplification problems from marine sponge DNA were encountered from DNA extracted from *Haliclona simulans* which was purified using commercial kits (Kennedy *et al*. 2008). Other studies used polyvinylpyrrolidone purification process for DNA that could not amplify (Maropola 2014). Inhibition of PCR amplification could be due to the presence of acidic polysaccharides in marine sponges (Salgado *et al*. 2007; Demeke & Jenkins 2010). Moreover, the potent secondary metabolites resulting from marine sponge associated bacteria may inhibit PCR amplification (Vargas *et al*. 2012).

There have been suggestions to carry out numerous replicate samples or replicated DNA extractions for T-RFLP analysis (Stres 2006). However, this suggestion has not been adhered to in different studies. Studies have either used classical methods (Liu *et al*. 1997) or commercial kits (Stomeo *et al*. 2012) or both methods (Ramond *et al*. 2013; Maropola *et al*. 2015) for DNA extractions. In this study, both commercial kits as well as classical methods were used for DNA extractions. Duplicate PCR products of the samples were pooled together in order to compensate for the potential misinterpretation of the resulting T-RFLP downstream analysis. The PCR products were normalized for all marine sponge samples before digestion with restriction enzymes. A similar procedure was reported where duplicate PCR products were pooled together before a restriction digest (Verbruggen *et al*. 2012).

In the context of this study, the sponge samples with the highest number of OTUs (PE09 and PE07 with 44 and 43 OTUs respectively) were selected for further culturing studies. The range

of OTUs from the present study (24 to 44 OTUs) was consistent with the OTUs found in other studies. The T-RFLP analyses on 3 marine sponges *Plakortis angulospiculatus, Agelas conifer,* and *Xestospongia muta* revealed that the average microbial OTUs in these sponges ranged from 19 to 108 depending on the restriction enzyme used. These marine sponges harboured the "core" microbial communities regardless of time, space, biotic and abiotic factors (Olson & Gao 2013). Moreover, T-RFLP analyses in this study were in a similar range to those found for the marine sponges *H. heliophila* and *H. tubifera* which reportedly resulted in 45 and 36 OTUs respectively (Erwin *et al*. 2011). The OTUs obtained from the marine sponge *Aplysina cauliformis* were in the range of 14 to 111 depending on time, space and disease status on the sponges. *Aplysina cauliformis* maintained the same "core" bacterial communities over time, space and health status (Olson *et al*. 2014). As a result, the T-RFLP results obtained in this study were regarded as sufficient to ascertain and select the two sponges which showed higher microbial diversities based on the observed diversity (OTUs). Other studies have reported higher OTUs from *Ircinia* spp. A study carried out on *I. fasciculata,* resulted in 139 OTUs, 108 in *I. oros,* 140 in I. *variabilis* (Pita *et al*. 2013).

The difference in restriction enzymes result in different OTUs for the same sponge sample and some restriction enzymes yield higher OTUs (Erwin *et al*. 2011; Erwin *et al*. 2012). In the present study, a double digest of enzymes was used which resulted in a similar range of OTUs as those reported in different studies where single restriction enzymes were used (Erwin *et al*. 2011; Erwin *et al*. 2012). There was however, a significant difference in OTUs on T-RFLP analyses from *Ircinia* spp. which yielded slightly higher OTUs (Pita *et al*. 2013).

The sponge sample with the lowest diversity (24 OTUs) was PE08. It was also interesting that the microbial symbionts in this sample were relatively distinct from other samples as illustrated

by nMDS analysis (Figure 3.4). This could be an indication that this sample could be regarded as a low microbial abundance (LMA) sponge. A preliminary trend is that LMA sponges tend to host specialist microbial communities (Erwin *et al*. 2011) and host little microbial diversity (Hardoim *et al*. 2009; Pita *et al*. 2013). High microbial abundance (HMA) sponges are reportedly generalist sponges and host bacterial communities that are not necessarily host specific (Erwin *et al*. 2011; Pita *et al*. 2013). Under the same notion, the marine sponge PE07 shared most of its OTUs (93%) with other sponges hence it could be speculated that this sample is a HMA sponge.

Five OTUs were observed in all of the five sponges (Figure 3.3) and these OTUs can be regarded as sponge specific clusters since they appear in one or more host species (Erwin *et al*. 2011; Schmitt *et al*. 2012a; Schmitt *et al*. 2012b; Olson *et al*. 2014). Similar studies using five Mediterranean sponges revealed that some bacterial OTUs were specific to marine sponges and they are not found in surrounding water or other marine habitats (Schmitt *et al*. 2012b). Over 12 million 16S rRNA gene sequences from marine environments were analysed and of those, 96 of the 173 which were reportedly sponge specific clusters were not found in other environments but specifically in marine sponges (Taylor *et al*. 2013). Analysis of over 7500 publicly available 16S rRNA gene sequences obtained from clone libraries, microbial isolates and DGGE data further supported the notion that some OTUs are sponge specific (Schmitt *et al*. 2012b). The OTUs identified in this study however were from T-RFLP analysis and the taxonomic information is limited. Therefore, it cannot be conclusively stated that the 5 OTUs are sponge specific. Contradicting conclusions were reached when T-RFLP analysis was employed on 3 *Ircinia* spp. to investigate species specificity of microbial OTUs analysed over time and distance in the marine environment. Certain OTUs were stable and specific to *Ircinia* spp. whereas these OTUs were absent in surrounding water (Pita *et al*. 2013).

3.3.2 Culturing marine sponge associated bacteria

A number of studies have used varying culture methods as a means of increasing access to some of the previously uncultured microbes (Muscholl-Silberhorn *et al*. 2008; Steinert *et al*. 2014). In this study, culture-dependent methods were employed to culture bacterial isolates from two marine sponges (*I. compressa* and *H. bidentifera*) in order to screen for antimicrobial and other secondary metabolite activities. The two marine sponges belong to the class *Demospongia* and they are indigenous to South Africa (taxonomically classified by Dr Shirley Parker-Nance; WoRMS 2015). Other studies conducted on the South African marine sponges mention the genus *Higginsia* and *Isodictya* but not particularly the sponge species in the present study (Branch *et al*. 2010; Samaai *et al*. 2010). Both studies were not based on sponge microbial diversities and composition but rather on the marine sponge richness of the South African marine environment. It is important to understand the diversity, abundance and specificity of marine sponge associated bacteria in order to exploit them for applications which benefit humankind. Studies of the South African marine sponges and their associated microbial diversities and their bioactivities have mainly focused on latrunculid sponges *Tsitsikamma favus* (Walmsley *et al*. 2012), *Tsitsikamma scurra* and *Latrunculia sp*. (Walmsley 2013).

The use of the same procedural methods for culturing marine bacteria routinely results in the isolation of the same bacterial species. Therefore, a variety of culture media ought to be employed in order to access higher bacterial diversities (Lavy *et al*., 2014). Following the same hypothesis, media in this study comprised of liquid enrichments, antibiotics as well as heat treatments. The media which was treated with antibiotics resulted in few numbers of bacterial isolates. A similar study where media supplemented with antibiotics was used also resulted in the

growth of a low microbial diversity (Sipkema *et al*. 2011). Another study conducted on marine sponge *Haliclona simulans* was aimed at culturing and assessing the biodiversity of endospore-forming bacteria associated with this sponge. To select for endospore forming bacteria, heat treatment and ethanol treatments were employed. A relatively high number of endospore forming bacteria were isolated from *H. simulans*, and represented 1% of the total culturable bacteria (Phelan *et al*. 2012). In the present study although fewer bacteria were isolated following heat treatment, they also represented around 1% of the total culturable bacteria.

Studies have indicated that rich media does not necessarily yield higher microbial diversities. Some of the media used is novel and may constitute extremely low-nutrient content and different growth conditions such as atmospheric pressure or oxygen levels (Lavy *et al*., 2014). Moreover, a high number of marine bacterioplankton were cultured from marine environment using very low nutrient media. The very low nutrient media successfully cultured previously uncultured bacterial lineages as well as four undescribed/novel cell lineages belonging to marine *Proteobacteria* clades (Connon & Giovannoni 2002). The application of different culturing techniques has an effect on the cultivation of marine sponge associated bacteria. A range of diverse media and conditions that included aerobic and microaerophilic culture conditions and the addition of antibiotics to the media were designed to culture bacteria from Rea Sea sponge *Thionella swinhoei* (Lavy *et al*., 2014). Lavy's group was able to culture higher bacterial diversities and isolated novel marine sponge symbionts as well as sponge specific bacteria. Culturing studies on the marine sponge *Haliclona* (*gellius*) sp. using floating filters, liquid cultures and agar plates constituting of a wide range of media including oligotrophic media resulted in large bacterial diversities of about 3900 isolates (Sipkema *et al*. 2011). In a similar manner in this study diverse culture media (heat treatment, antibiotic treated, liquid enrichment,

rich and low nutrient content media) were used resulting in 400 bacterial isolates based on morphological characterization.

3.3.3 Phylogenetic analysis of marine sponge associated bacteria

A small fraction of the total bacterial isolates which showed antibacterial activities were identified using 16S rRNA gene sequence analysis. The phylogeny of the bacterial isolates were consistent with those phyla identified from previous studies where *Proteobacteria* (*Gamma* and *Alpha*) *Firmicutes*, and *Actinobacteria* were isolated from sponges (section 3.2.5.3) (Taylor *et al*. 2004; Taylor *et al*. 2007; Muscholl-Silberhorn *et al*. 2008; Jackson *et al*. 2012; Steinert *et al*. 2014). Studies carried out on marine sponges from different parts of the world tend to harbour the same bacterial phyla thus indicating that certain bacterial phyla could be sponge specific. Bacterial isolates from the sponge *Aplysina fulva* obtained from Brazil hosted similar bacterial phyla (Hardoim *et al*. 2009), similar bacterial phyla were isolated from marine sponges in China (Li 2009), India (Chairman *et al*. 2012) and the Mediterranean Sea (Muscholl-Silberhorn *et al*. 2008). Studies also show that there are bacterial phyla that are specific to sponges or marine invertebrates only. *Poribacteria* is one bacterial phylum which is unique to marine sponges (Fieseler *et al*. 2004). Other research groups have been able to culture more bacterial phyla such as *Bacteroidetes* (Bergman *et al*. 2011; Jackson *et al*. 2012; Steinert *et al*. 2014). Some studies successfully cultured the rarely cultured *Planctomycetes*, *Verrucomicrobia*, and *Deltaproteobacteria* bacterial phyla using a variety of media (Sipkema *et al*. 2011).

Culture-independent methods revealed more diversities amongst marine sponge associated bacteria than the diversities revealed using culture dependent methods. Pyrosequencing (Jackson

et al. 2012), DGGE (Walmsley *et al*. 2012) and clone libraries (Kennedy *et al*. 2008; Giles *et al*. 2013) have been used in studies to determine the phylogeny of marine sponge associated bacteria. Some of the bacterial phyla determined using pyrosequencing included *Acidobacteria*, *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, *Cyanobacteria*, *Nitrospira* and *Proteobacteria* from the marine sponges *Raspailia ramosa* and *Stelligera stuposa* (Jackson *et al*. 2012). DGGE analyses of microbial diversities from the Indian Ocean sponge *Tsitsikamma favus* included *Proteobacteria, Acidobacteria*, *Chlamydiae*, *Planctomycetes, Verrucomicrobia* and *Spirochaetes* (Walmsley *et al*. 2012). Analyses of the microbial diversity associated with *Haliclona simulans* using clone libraries identified the phyla *Planctomycete, Verrucomicrobia*/*Lentisphaerae, Actinobacteria, Bacteroidetes, Cyanobacteria, Proteobacteria and Spirochaetes* (Kennedy *et al*. 2008). Similar clone library analyses resulted with *Cyanobacteria*, *Chloroflexi*, *Gamma*- and *Alphaproteobacteria*, *Actinobacteria*, *Bacteroidetes,* and *Acidobacteria* among the microbial diversity associated with the marine sponge *Aplysina fulva* (Hardoim *et al*. 2009).

An interesting result was found for one of the bacterial isolates PE09_221 which had a 97% identity to the closest relative on the NCBI database. This result could indicate a novel bacterial species. Studies indicate that bacterial species with identities of less than 97% are indicative of undescribed or novel bacteria (Kennedy *et al*. 2008; Lavy *et al*. 2014). About 9% of the microbial isolates in the current study were identified using 16S rRNA gene sequences of which one bacterium showed some novelty. This could indicate that if more isolates were identified there is a chance that more isolates would show some novelty.

Microorganisms inhabit every possible niche and they are the most diverse and abundant life forms on earth (Rinke *et al*. 2013). This microbial diversity is better observed in culture-

independent studies because culture-dependent methods are known to access only a small fraction of the diversity (Connon & Giovannoni 2002; Jackson *et al*. 2012; Rinke *et al*. 2013). Four bacterial phyla (*Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes*) dominate the cultured microbes by representing more than 88% of all cultured bacterial isolates (Rinke *et al*. 2013). For the purpose of this study, the focus was to identify bacterial isolates which showed antibacterial activities as well as those selected for the matrix study. In a similar manner, in our study, three of dominant bacterial phyla (*Proteobacteria*, *Firmicutes*, and *Actinobacteria*) were cultured. This could be an indication that the three bacterial phyla dominate the cultured bacterial isolates in marine sponges as well. The results were also consistent with studies carried out on *Haliclona simulans* where the four dominant bacterial phyla (*Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes*) were cultured (Kennedy *et al*. 2009). The present study was contradictory to the findings on other marine sponges (Sipkema *et al*. 2011; Lavy *et al*. 2014) that using a wide variety of media access a wider microbial diversity.

In the present study some of the bacterial 16S rRNA gene sequences which showed 99% similarity were considered to represent different OTUs based on their bioactivity profiles (section 3.2.5.3). Similar analyses of five strains of *Pseudoalteromonas luteoviolacea* revealed that the strains were closely related based on 16S rRNA gene sequences; however, they produced different bioactivity profiles (Vynne *et al*. 2012). Similarly analysis of *Actinomycetes* from marine sediments revealed that 16S rRNA gene sequences are limited in resolving phylogenetic variation within subclades (Duncan *et al*. 2015). Similar reports in *Actinomycetes* studies revealed that 16S rRNA gene sequencing is inefficient in resolving intraspecies heterogeneity. This required the use of alternative de-replication tools to assess the uniqueness of the strains (Genilloud *et al*. 2011). Under the same notion, in the present study, bioactivity profiles were

used to resolve OTUs which resulted in different bioactivities whereas their 16S rRNA gene sequences were similar. Phylogenetic clustering based on bioactivities of species was also carried out on *Pseudoalteromonas luteoviolacea* where 16S rRNA gene sequence was inefficient in resolving species phylogenies (Vynne *et al*. 2012). This therefore indicates that alternative de-replication methods are necessary where 16S rRNA gene sequence analysis prove insufficient in resolving phylogenies.

3.3.4 Antibacterial activities

The marine environment has been identified as a rich source of bioactive compounds with interesting chemical diversities (Piel 2006). The diverse marine sponge symbionts are the source of many unique secondary metabolites. Some of these metabolites have the potential to be pharmaceutical compounds because of their pronounced activities (Skariyachan *et al*. 2014). A lot of studies have thus focused on the marine environment with the aim to harness novel bioactive compounds (Muscholl-Silberhorn *et al*. 2008; Chairman *et al*. 2012; Skariyachan *et al*. 2014). There is evidence that the secondary metabolites are of microbial origin (Muscholl-Silberhorn *et al*. 2008; Walmsley *et al*. 2012). Most of the studies on marine sponge associated bacteria focused on *Actinobacteria*. This was due to the fact that *Actinobacteria* have the ability to produce a wide range of bioactive compounds (Fiedler *et al*. 2005; Lam 2006; Sun *et al*. 2010; Ouyang *et al*. 2011; Xi *et al*. 2012).

While secondary metabolites from marine sponge associated bacteria are sought after, their production is largely hampered by the lack of these bacteria in culture collections (Bergman *et al*. 2011). It is therefore important to develop cultivation techniques for these marine sponge symbionts in order to access and exploit their secondary metabolite capabilities (Steinert *et al*.

2014). Different methods for screening for antibiotic activities used in different studies include antibiotic overlay assay (Skariyachan *et al*. 2014) or disc diffusion method (Chairman *et al*. 2012).

The present study used antibiotic overlay assay to screen for production of bioactive compounds from marine sponge associated bacteria. Zones of clearing around bacterial isolates were indicative of bioactive compound production. This method of screening is dependent on the secondary metabolites being extracellular or the lysis of the producing organism. Certainly it is known that some bioactive compounds are extracellular (Skariyachan *et al*. 2014).

Recent studies revealed that other marine sponge associated bacteria apart from *Actinobacteria* also produce a wide varied of important bioactive compounds (Muscholl-Silberhorn *et al*. 2008; Lavy *et al*. 2014). Studies carried out on the sponge *Erylus discophorus* showed that *Alphaproteobacteria, Gammaproteobacteria, Actinobacteria* and *Firmicutes* exhibit bioactivities against indicator strains (Graça *et al*. 2013). The results from the present study concurred with the study that the bacterial phyla which showed bioactivities (*Alphaproteobacteria, Gammaproteobacteria, Actinobacteria* and *Firmicutes*) fell in the same phyla as those reported from a study carried out on the marine sponge *Erylus discophorus* (Graça *et al*. 2013). In the present study, *Firmicutes* were the bacterial phyla which resulted in most bioactivities. Similarly, *Firmicutes* obtained from other marine sponges showed antimicrobial activities (Muscholl-Silberhorn *et al*. 2008; Phelan *et al*. 2012). Six bacterial isolates from the two marine sponges (PE07 and PE09) showed activity against more than one type of indictor strain. Similar results were seen from the bacteria associated with the marine sponge *Erylus discophors* (Graça *et al*. 2013).

CHAPTER 4 OSMAC APPROACH

4.1 INTRODUCTION

The One Strain Many Compounds (OSMAC) approach has been successfully applied in microbial studies which assess the effect of systematically altering culture conditions on the production of secondary metabolites (Bode *et al*. 2002; Gesheva *et al*. 2005; Rateb *et al*. 2011; Zhang *et al*. 2013). The culture conditions that trigger secondary metabolism for each organism are unpredictable and there are no common rules that can be applied for all microbes. The hypothesis that with regard to secondary metabolism triggers, "anything goes" as long as the culture conditions ultimately trigger secondary metabolism are accepted (Bode *et al*. 2002) arose after the induction of secondary metabolism in *Streptomyces* strains by using low concentration of dimethyl sulfoxide (DMSO) (Xue & Sherman 2000). Some of the conditions which have been successful include altering the culture media composition (Rateb *et al*. 2011) altering easily accessible parameters such as pH and aeration (Bode *et al*. 2002) and co-culturing microorganisms (Scherlach & Hertweck 2009; Bertrand *et al*. 2013; Ola *et al*. 2013; Dashti *et al*. 2014).

Secondary metabolism in bacterial cells is triggered by stress when the carbon (Gubbens *et al*. 2012), phosphate (Lounès *et al*. 1996; Sola-Landa *et al*. 2003) or nitrogen (Gesheva *et al*. 2005; Martín *et al*. 2011) are limited in the culture media. The aim of this chapter therefore was to establish whether a similar approach could be employed to induce a larger antibacterial capacity in the organisms isolated and screened in section 3.2.4. A detailed account on how the matrix study was set up and undertaken is given in Chapter 2 (section 2.3). In brief, selection of the culture conditions was based on the conditions used in previous studies to induce the

biosynthesis of secondary metabolites. The choice in the carbon source concentration of 5 mM was adapted from production of antimicrobial activities from marine bacteria where starch was used as the carbon source (Heindl *et al*. 2012). Other studies have used different carbon sources such as mannitol (Ruiz *et al*. 2010; Zhu *et al*. 2014) and succinic acid in the form of ammonium succinate (Gesheva *et al*. 2005). Nitrogen sources which have been employed in other studies which were used in the present study were NH_4Cl and $NaNO_3$ (Wang *et al*. 2013). Studies have also indicated that phosphate limiting conditions trigger secondary metabolism (Lounès *et al*. 1996; Sola-Landa *et al*. 2003; Martín 2004) as a result two different concentrations of phosphate (0.1 µM KH_2PO_4 and 0.5 mM KH_2PO_4) were selected for this study. The effect of temperature, in the form of a cold or heat shock treatment, has also yielded production of secondary metabolites. Heat shock treatments at 42 ºC successfully induced secondary metabolism in other mesophiles with optimal growth conditions at 28 ºC (Doull *et al*. 1994; Liao *et al*. 2009). The marine sponge isolates for the present study were obtained from Algoa Bay which is in the path of the warm Agulhas current where seasonal temperatures vary from 20 ºC to 28 ºC. It is known that warmer surface water generally overlies cooler water (Griffiths *et al*. 2010). As such, 42 ºC was selected as the heat shock temperature and cold shock treatments were conducted at 4 ºC.

Furthermore the matrix setup in 96-well plate format adapted from Bills *et al*. (2008) for the present study incorporated the co-culture effect (Figure 4.1). Each sponge isolate in this study had a minimum of 3 isolates with which it could interact and hence co-culture induction contributed to the overall setup. Co-culturing organisms is known to trigger the expression of biosynthetic pathways resulting in novel compounds which are not produced in pure culture (Oh *et al*. 2007; Bertrand *et al*. 2013; Traxler *et al*. 2013).

4.2 RESULTS

A panel of 30 isolates selected for the matrix work (Table 4.1) included 13 isolates with prior antibacterial activities in the preliminary screening (chapter 3, section 3.2.4) and 17 isolates which did not exhibit any antibacterial activities. The phylogenetic analysis (section 3.2.5.3) revealed that the marine sponge associated microbes selected for this chapter were members of the phyla *Actinobacteria, Firmicutes, Alpha and Gammaproteobacteria* (Table 4.1).

Table 4.1: A panel of 30 isolates selected for matrix study which showed antibacterial activities at preliminary screening and those which did not show any bioactivities.

	Isolate	Identity	Closest hit	Phylum
Activities in previous screening	PE09_64	NA	NA	
	PE09_110	99%	*Citricoccus nitrophenolicus* strain PNP1	*Actinobacteria*
	PE09_124	99%	*Kushneria indalinina* strain CG2.1	*Gammaproteobacteria*
	PE09_140	100%	*Pseudomonas cremoricolorata* strain NBRC 16634	*Gammaproteobacteria*
	PE09_142	NA	NA	
	PE09_221	97%	*Aeromicrobium erythreum* strain NRRL B-3381	*Actinobacteria*
	PE09_229	99%	*Halomonas titanicae* BH1	*Gammaproteobacteria*
	PE07_5	99%	*Bacillus aquimaris* strain TF-12	*Firmicutes*
	PE07_105	NA	NA	
	PE07_133	99%	*Micrococcus yunnanensis* strain YIM 65004	*Actinobacteria*
	PE07_144	99%	*Bacillus aquimaris* strain TF-12	*Firmicutes*
	PE07_172	99%	*Sporosarcina aquimarina* strain SW28	*Firmicutes*
	PE07_200	99%	*Staphylococcus warneri* SG1 strain SG1	*Firmicutes*
No prior activities	PE09_4	99%	*Staphylococcus saprophyticus* strain ATCC 15305	*Firmicutes*
	PE09_55	99%	*Pseudovibrio ascidiaceicola* strain NBRC 100514	*Alphaproteobacteria*
	PE09_72	99%	*Bacillus pumilus* SAFR-032 strain SAFR-032	*Firmicutes*
	PE09_77	NA	NA	
	PE09_103	99%	*Pseudovibrio ascidiaceicola* strain NBRC 100514	*Alphaproteobacteria*
	PE09_116	NA	NA	
	PE09_163	99%	*Staphylococcus saprophyticus* strain ATCC 15305	*Firmicutes*
	PE09_180	99%	*Marinilactibacillus psychrotolerans* strain NBRC 100002	*Firmicutes*
	PE09_222	99%	*Pseudovibrio ascidiaceicola* strain NBRC 100514	*Alphaproteobacteria*
	PE09_250	99%	*Paenibacillus glucanolyticus* strain NBRC 15330	*Firmicutes*
	PE09_267	NA	NA	
	PE09_274	99%	*Staphylococcus saprophyticus* strain ATCC 15305	*Firmicutes*
	PE07_37	99%	*Bacillus hwajinpoensis* strain SW-72	*Firmicutes*
	PE07_72	99%	*Brevibacterium frigoritolerans* strain DSM 8801	*Firmicutes*
	PE07_103	99%	*Bacillus kochii* strain WCC 4582	*Firmicutes*
	PE07_124	99%	*Brevibacterium frigoritolerans* strain DSM 8801	*Firmicutes*
	PE07_136	99%	*Lysinibacillus fusiformis* strain NBRC15717	*Firmicutes*

Index: NA = isolates whose gDNA could not be amplified and thus could not be sequenced

4.2.1 Agar overlay assay using the OSMAC approach

For ease to reference, the matrix media which made up 36 culture conditions are listed in Table 4.2. As it was already indicated in section 2.3.2 matrix media were designed based on minimal media to allow alterations of the carbon sources, nitrogen and phosphate concentrations. The "standard" media (media selected in our laboratory to be the general isolation media) (Appendix 2, ZBA, TSA, GYM, ACM) were also used as controls to verify viability of isolates with the concurrent matrix media screening. It was observed that on "standard" media (Figure 4.1 B), the isolates grew faster and generated more biomass as opposed to the growth observed in matrix media (Figure 4.1 A). Moreover some isolates produced very little biomass on the matrix media even after the 2 weeks prior to carrying out the agar overlay assays.

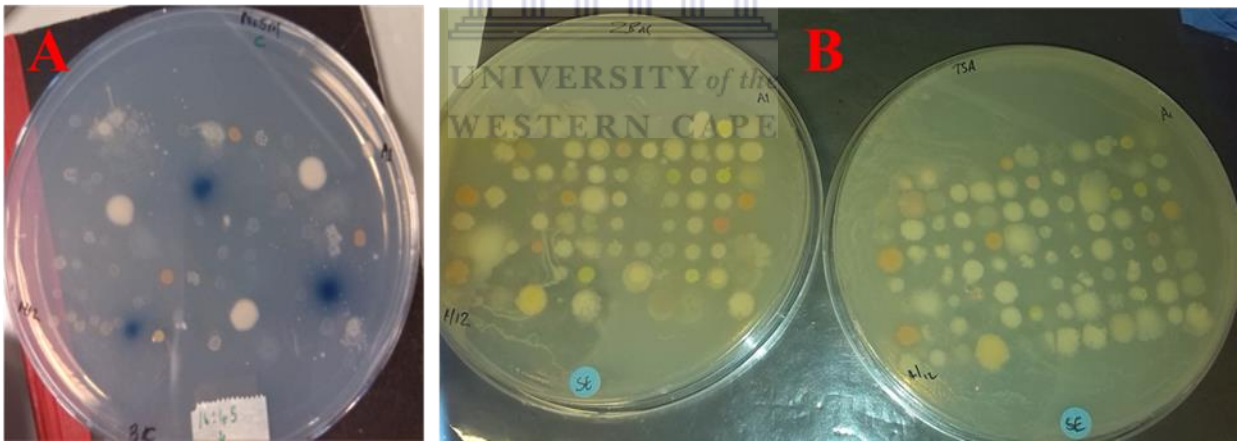


Figure 4.1: Bacterial isolates showing differential growth on matrix media (A) and standard media (B). The images also demonstrate the experimental setup allowing for co-culturing effects to impact on the secondary metabolite induction.

Table 4.2: Matrix media composition and shorthand for the 36 conditions investigated in this study.

Abbreviation		Media constituents
Mannitol meida	A1M	Ammonium Chloride; 0.1 µM Phosphate; Mannitol
	A1HM	Ammonium Chloride; 0.1 µM Phosphate; Mannitol; Heat Shock
	A1CM	Ammonium Chloride; 0.1 µM Phosphate; Mannitol; Cold shock
	A5M	Ammonium Chloride; 0.5 mM Phosphate; Mannitol
	A5HM	Ammonium Chloride; 0.5 mM Phosphate; Mannitol; Heat Shock
	A5CM	Ammonium Chloride; 0.5 mM Phosphate; Mannitol; Cold shock
	Na1M	Sodium Nitrate; 0.1 µM Phosphate; Mannitol
	Na1HM	Sodium Nitrate; 0.1 µM Phosphate; Mannitol; Heat shock
	Na1CM	Sodium Nitrate; 0.1 µM Phosphate; Mannitol; Cold shock
	Na5M	Sodium Nitrate; 0.5 mM Phosphate; Mannitol
	Na5HM	Sodium Nitrate; 0.5 mM Phosphate; Mannitol; Heat shock
	Na5CM	Sodium Nitrate; 0.5 mM Phosphate; Mannitol; Cold shock
Succinic acid media	A1Sa	Ammonium Chloride; 0.1 µM Phosphate; Succinic acid
	A1HSa	Ammonium Chloride; 0.1 µM Phosphate; Succinic acid; Heat shock
	A1CSa	Ammonium Chloride; 0.1 µM Phosphate; Succinic acid; Cold shock
	A5Sa	Ammonium Chloride; 0.5 mM Phosphate; Succinic acid
	A5HSa	Ammonium Chloride; 0.5 mM Phosphate; Succinic acid; Heat shock
	A5CSa	Ammonium Chloride; 0.5 mM Phosphate; Succinic acid; Cold shock
	Na1Sa	Sodium Nitrate; 0.1 µM Phosphate; Succinic acid
	Na1HSa	Sodium Nitrate; 0.1 µM Phosphate; Succinic acid; Heat shock
	Na1CSa	Sodium Nitrate; 0.1 µM Phosphate; Succinic acid; Cold shock
	Na5Sa	Sodium Nitrate; 0.5 mM Phosphate; Succinic acid
	Na5HSa	Sodium Nitrate; 0.5 mM Phosphate; Succinic acid; Heat shock
	Na5CSa	Sodium Nitrate; 0.5 mM Phosphate; Succinic acid; Cold hock
Starch media	A1S	Ammonium Chloride; 0.1 µM Phosphate; Starch
	A1HS	Ammonium Chloride; 0.1 µM Phosphate; Starch; Heat shock
	A1CS	Ammonium Chloride; 0.1 µM Phosphate; Starch; Cold shock
	A5S	Ammonium Chloride; 0.5 mM Phosphate; Starch
	A5HS	Ammonium Chloride; 0.5 mM Phosphate; Starch; Heat shock
	A5CS	Ammonium Chloride; 0.5 mM Phosphate; Starch; Cold shock
	Na1S	Sodium Nitrate; 0.1 µM Phosphate; Starch
	Na1HS	Sodium Nitrate; 0.1 µM Phosphate; Starch; Heat shock
	Na1CS	Sodium Nitrate; 0.1 µM Phosphate; Starch; Cold shock
	Na5S	Sodium Nitrate; 0.5 mM Phosphate; Starch
	Na5HS	Sodium Nitrate; 0.5 mM Phosphate; Starch; Heat shock
	Na5CS	Sodium Nitrate; 0.5 mM Phosphate; Starch; Cold shock

The plating system in 96 well-format was similar to that described by Bills *et al*. (2008). The 30 isolates were plated out in triplicate along with two strains of *Streptomyces coelicolor* (M145 and the mutant strain M1146). The gene clusters which encode the four main biosynthetic pathways are deleted in strain M1146 (Coze *et al*. 2013). It is noteworthy that although triplicates are reported for each isolate on a single plate, they are not true replicates because they were ordered in such a manner that they were co-cultured with different isolates. However, duplicate plates were also setup to evaluate reproducibility. The antibacterial activity was adjudged based on the presence or absence of bioactivities (indicated by zones of inhibition) surrounding the isolates. In some cases, some of the triplicate isolates did not grow or they produced very little biomass (Figure 4.1 A). This may result in misrepresentation of the observed overall bioactivities of isolates. The isolates that were judged as "positive hits" were those that showed antibacterial activity in at least two or three instances depending on the frequency at which each isolate grew. Interestingly under the matrix study certain bacterial isolates which did not show activity in preliminary screening (section 3.2.4) started showing antibacterial activities. Figure 4.2 shows some of the antibacterial activities from certain isolates resulting from the matrix media A5CM and Na1HM (Table 4.2). The most noteworthy isolate was PE09_72 which showed multiple activities on different matrix media whereas in preliminary screening (section 3.2.4) it did not show any antibacterial activities. In a similar manner, the *S. coelicolor* control strains also showed different activities under the different media compositions. For example, on A5CM *S. coelicolor* did not show any activities whereas it showed activity on Na1HM. This was an indication that the media composition and co-culture influences the metabolic profiles when employing the OSMAC approach in a matrix of culture conditions, as anticipated.

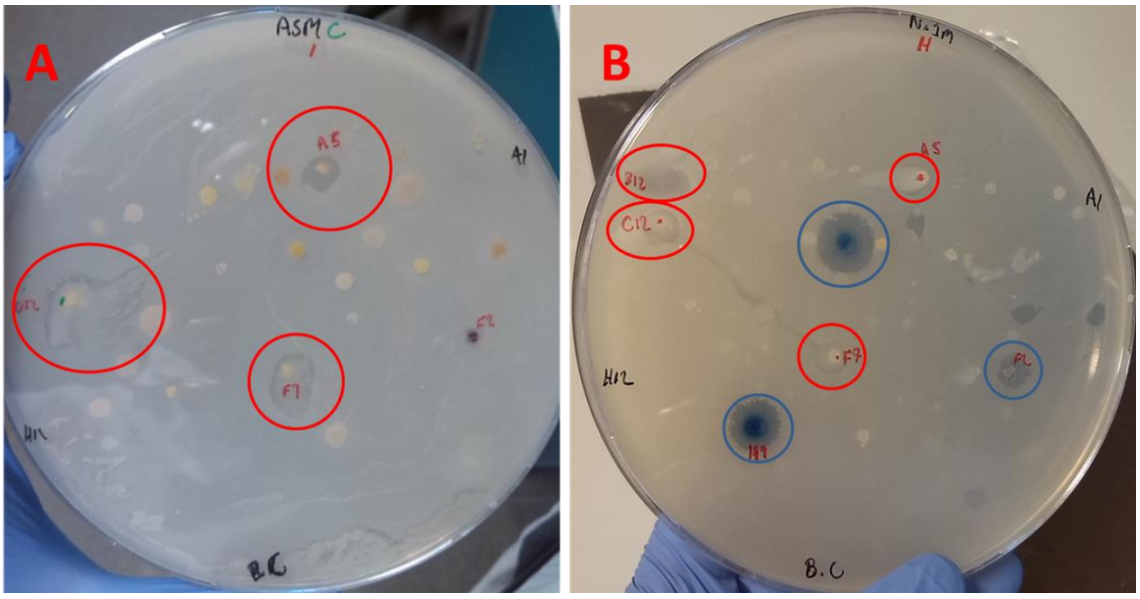
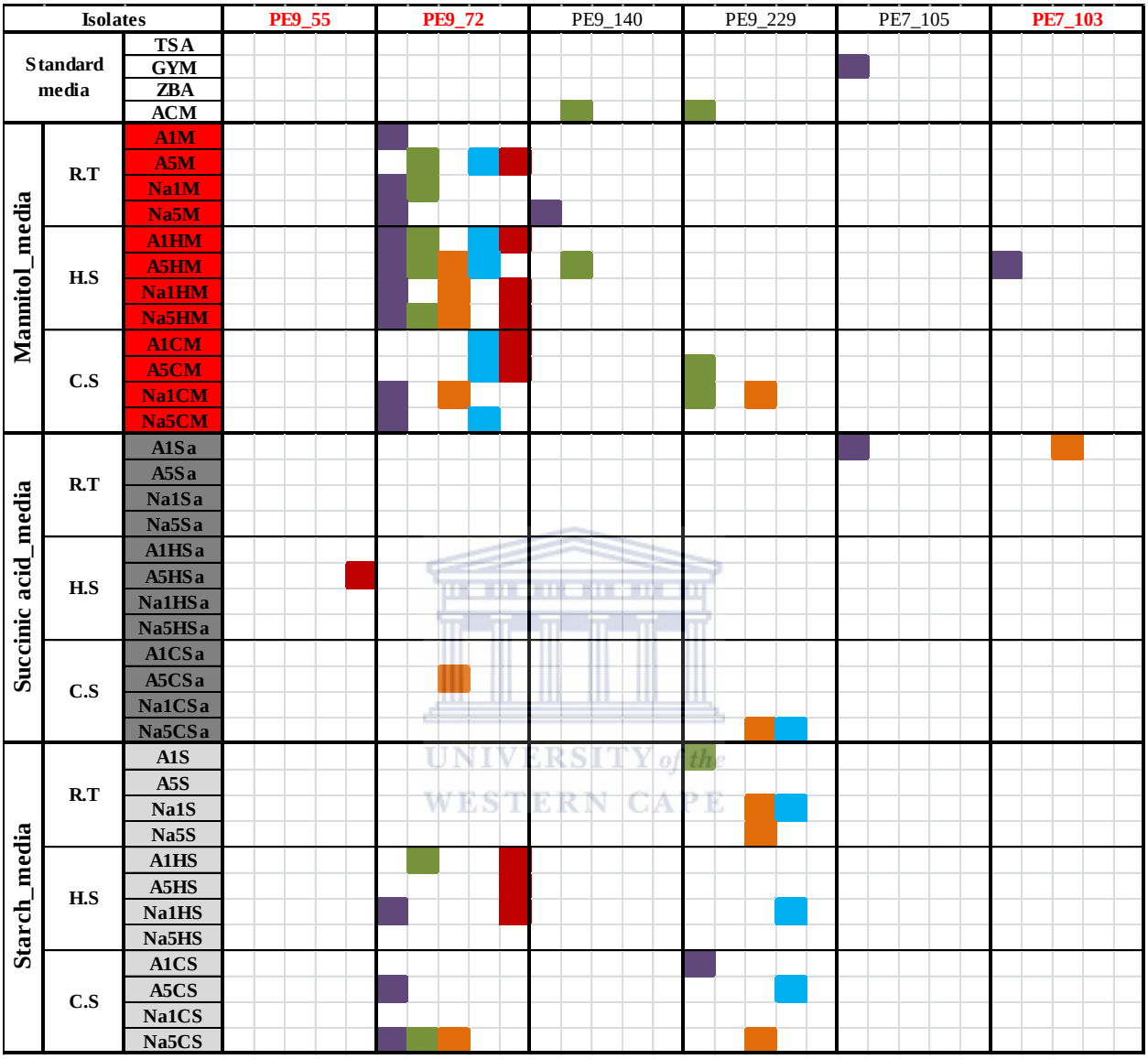


Figure 4.2: OSMAC based screening for antibacterial activity for 30 bacterial isolates. The red circles indicate zones of inhibition shown by isolates which did not show antibacterial activity in preliminary screening whereas the blue circles indicate zones of inhibition from the *S. coelicolor* strains. A: isolates growing on A5CM. B: isolates growing on Na1HM.

Out of the 30 microbial isolates screened in a matrix of 36 culture/treatment conditions and co-culture, a total of 6 isolates showed antibacterial activities (Table 4.3). One of the 30 isolates, PE9_64 with prior activity (section 3.2.4), stopped growing on matrix media and on standard media. As a result it was disregarded in further analyses. Although only 6 isolates showed activity in the matrix, very little to no growth was observed for some isolates under some of the conditions. This may have resulted in some of the antibacterial activities being missed.

Table 4.3: Bioactivities of 6 marine sponge associated isolates which showed activities against 5 indicator strains under the matrix of 36 conditions. Details of the 36 matrix media are provided in table 4.2.



Pre-treatment	Key
R.T	~27℃
H.S	Heat shock
C.S	Cold shock

Test Strain	Key
E. coli 1699	
M. smeg	
S. epi	
P. putida	
B. cereus	

Key	Bacterial isolates
Red	No prior bioactivities
Black	Prior bioactivities

TSA	Trypticase Soya Agar
GYM	Glucose Yeast Medium
ZBA	Zobell Agar ¼ strength
ACM	Activated Charcoal Medium

Out of the 12 sponge isolates which showed activity in previous screening (with PE9_64 excluded), 3 isolates (PE9_140, PE9_229 and PE7_105) continued to show activities in the matrix media. This could be an indication that the biosynthetic pathways from some of these isolates were likely constitutively expressed. It is notheworthy that 3 isolates (PE9_55, PE9_72 and PE7_103) started showing antibacterial activities under the matrix conditions whereas in previous screening they did not exhibit any activities. These isolates are indicated by bold red text in Table 4.3. This may indicate that certain biosynthetic pathways were induced under some of the matrix conditions and not in standard media. Moreover, the isolates which had shown antibacterial activities in preliminary screening also began to exhibit differential bioactivities as shown in Table 4.3. The exception was PE7_105 which showed the same bioactivity against *E. coli* 1699. The differential expression of bioactivities is illustrated using PE9_140. PE9_140 had shown antibacterial activity against *M. smegmatis* in the previous chapter whereas under the matrix conditions this isolate showed activity against both *M. smegmatis* and *E. coli* 1699.

The two isolates which are worth specific mention are PE9_72 and PE9_229 which showed multiple activities against most of the indicator strains. PE9_72 showed activities against all the five indicator strains (*E. coli* 1699, *M. smegmatis*, *S. epidermidis*, *P. putida*, and *B. cereus*) whereas PE09_229 showed activity against four (Table 4.3). It was also interesting that 5 isolates (PE9_72, PE9_140, PE9_229, PE7_105 and PE7_103) showed activity against the genetically engineered indicator strain *E. coli* 1699 (Cubist, USA) which is multi-resistant to 52 known antibiotics. This could be an indication that some of the bioactive compounds produced by these microbial isolates are novel. The differential bioactivities from microbes which showed activities initially as well as those isolates which started showing activities under the matrix indicated that some of the stress conditions were able to trigger secondary metabolism from certain pathways.

It was also clear from Table 4.3 that the different media compositions were organism and likely pathway specific as opposed to having a global effect.

Further analysis will be centered on Table 4.3 in later discussions. For all analysis to be discussed from this chapter, there are considerations that co-culture, carbon sources, temperature treatments, different nitrates, and phosphate concentrations had an influence on the overall bioactivities. Although the analysis examines each parameter individually, the other factors are not overlooked and are acknowledged to have influenced the activities observed.

4.2.1.1 Co-culture in 96 well format

The experimental setup in the 96 well-plate-format is shown in Figure 4.3, where every isolate had a minimum of 3 "immediate" neighbouring isolates that could likely interact with it. By way of illustration PE9_72 will be presented. PE9_72 is recorded as presenting activity against 4 indicator strains on Na5HM media (Table 4.3). However, when analysing how this organism was arranged in the 96 well-plate-format, it became apparent that the antibacterial activities were not consistent. Figure 4.3 gives an illustration of the positioning of PE9_72 in relation to the neighboring isolates, and the 3 different positions are referred to as group 1, 2 and 3 respectively. In group 3 PE9_72 had 4 "immediate" neighbouring isolates (PE7_5, PE7_72, PE9_110 and PE9_124) whereas in group 1 and 2 it had 3 "immediate" neighbours. PE9_110 and PE7_5 were common "immediate" neighbours whilst PE9_124 was the third neighbour of PE9_72 in group 1 and PE7_72 in group 2.

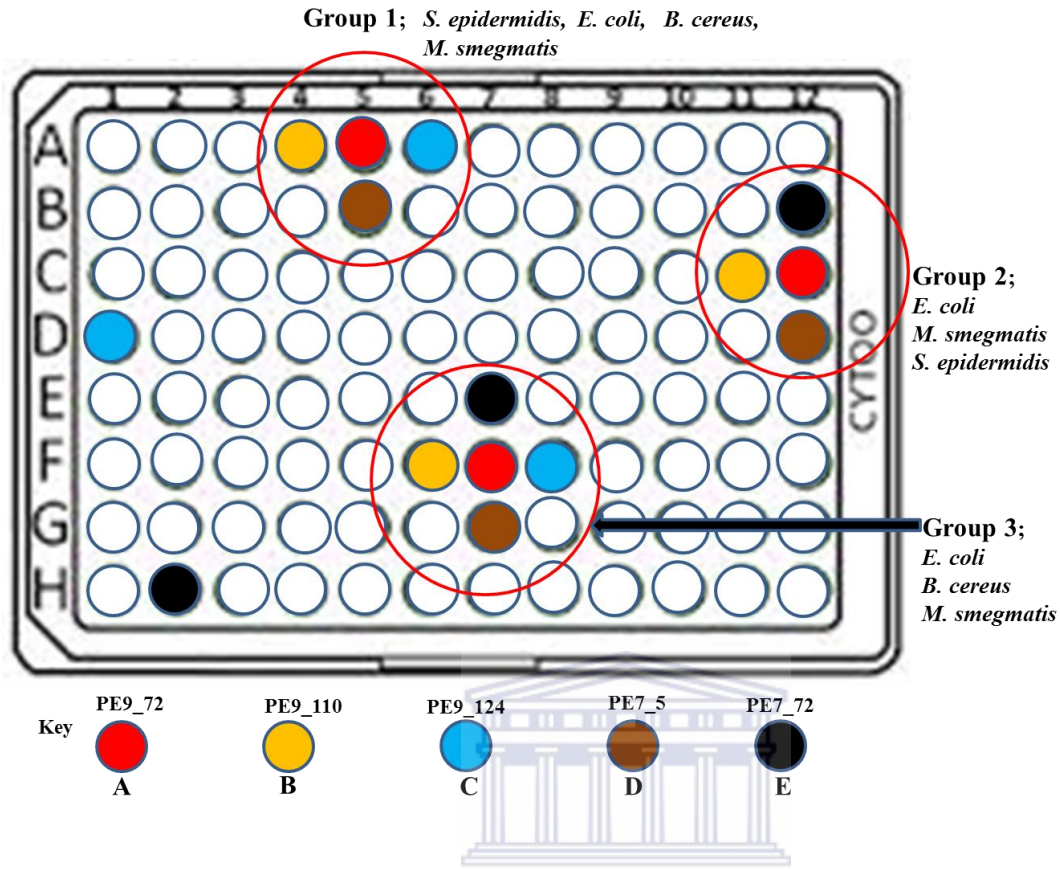


Figure 4.3: Bioactivities of the isolate PE9_72 (A) when cultured on Na5HM under co-culture conditions with 3 and 4 "immediate" neighbouring isolates in 96 well-plate setup. The red circles indicate the co-culture groups to aid to identify action of a pattern of co-culture induction. The isolates were renamed A-E as shown in the key. The activities which PE9_72 showed against indicator strains are shown next to each group.

For simplicity, the isolates have been renamed A-E, as shown in the key in Figure 4.3. Table 4.4 summarises the antibacterial activities for PE9_72 in relation to its positioning (ie. group 1, 2 and 3) in the matrix co-culture setup. In addition the table summarises all the antibacterial activities of the isolates 4 isolates in closest proximity to PE9_72 (isolate A) in the matrix. Section 1 in the table represents bioactivities recorded during the preliminary screening (section 3.2.4), section 2 represents bioactivities in the matrix setup per isolate and section 3 represents the bioactivities for isolate A in relation to the neighbouring isolates (group 1, 2 and 3, Figure 4.3). Isolate A and

E showed no activity in preliminary screening. However, in the matrix study only isolate A showed activities against 4 indicator strains on Na5HM medium. Isolates B, C and D showed activities in preliminary screening but not in the matrix screening.

In all the three group scenarios, isolate A showed activity against *E. coli* 1699 and *M. smegmatis*. However in group 1, which included isolate C as an additional "immediate" neighbour, isolate A demonstrated activity against *S. epidermidis* and *B. cereus* as well. In group 2, where the effect of isolate C was absent from the co-culture but isolate E was present, isolate A did not show activity against *B. cereus* while the activity against *S. epidermidis* was still exhibited. This could imply that the interaction of isolate C with B and D appeared to induce activity of isolate A against *B. cereus*. An alternative argument is that the compound/s which confers activity against *B. cereus* were not expressed in isolate A when grown in close proximity to isolate E with the exclusion of isolate C from the co-culture. In group 3, the activity of isolate A against *B. cereus* reappeared whereas it did not show activity against *S. epidermidis*. This could be an indication that the activity against *B. cereus* was induced by the presence of isolate C. The introduction of both isolates C with E to the co-culture resulted in isolate A not showing activity against *S. epidermidis*. The examples explained in Figure 4.3 and Table 4.4 illustrates the complexity of the mechanisms required to trigger expression of biosynthetic pathways in co-culture scenarios for any microorganism.

Table 4.4: The bioactivities of all the 5 isolates during preliminary screening (section 1), during matrix screening in co-culture (section 2) and in group scenarios (section 3). Effects of co-culture in group 1, 2 and 3 scenarios show different bioactivity patterns on medium Na5HM where PE9_72 showed activities against 4 indicator strains.

Shorthand for the 5 isolates			Original identities	Indicator strains				
				E. coli 1699	*B. cereus*	*P. putida*	*S. epidermidis*	*M. smegmatis*
Previous screening	Section 1	A	PE9_72	-	-	-	-	-
		B	PE9_110	+	-	-	-	-
		C	PE9_124	-	-	-	-	+
		D	PE7_5	+	-	-	-	+
		E	PE7_72	-	-	-	-	-
Matrix screening	Section 2	A	PE9_72	+	+	-	+	+
		B	PE9_110	-	-	-	-	-
		C	PE9_124	-	-	-	-	-
		D	PE7_5	-	-	-	-	-
		E	PE7_72	-	-	-	-	-
	Section 3	A,B,C,D	Group 1	+	+	-	+	+
		A,B,D,E	Group 2	+	-	-	+	+
		A,B,C,D,E	Group 3	+	+	-	-	+

Index: section 1 = preliminary screening activities per isolate (chapter 3), section 2 = matrix screening activities per isolate, section 3 = same as section 2 however PE9_72 (A) as the point of reference , (+) activity, (-) no activity

4.2.1.2 Carbon sources

The effect of the three carbon sources in the growth medium on secondary metabolism of the marine sponge associated isolates was investigated. It should however be noted that although the effect of carbon sources are compared, the inherent nitrates, phosphates and temperature treatments, as well as co-culturing effects play a role in the reported outcome/s and or observed bioactivities. Figure 4.4 summarises the effects of the three carbon sources on the bioactivity of 6 isolates. The activities are reported as a fraction based on the number of bioactivities per culture condition with a specific carbon source. For example in Table 4.3 when looking at PE9_55 this would be reported as a 1/12 of activities observed under the succinic acid as the sole carbon

source. Figure 4.3 therefore indicates that for PE9_72, mannitol was the best carbon source for inducing bioactivities (12/12 = 1) whereas for PE9_229, starch induced more activities (7/12). This indicates that certain biosynthetic pathways were triggered by different carbon sources per organism. Furthermore, PE9_72 also showed only 1 activity against *S. epidermidis* when using succinic acid as the carbon source; see Table 4.3, indicating that succinic acid was not as favourable for inducing secondary metabolism in this organism, at least with respect to antibacterial metabolites against the indicator strains being tested for in this study .

Although there appears to be carbon source related influences, for some isolates it was observed that it was not a prevailing inducer. This indicates that the activities shown under the matrix growth conditions were influenced by the combination of the other factors such as nitrates, phosphates, co-culture or temperature treatments which resulted in the activation of different pathways. For example, for PE9_140, mannitol induced two bioactivities, against *E. coli* 1699 and *M. smegmatis* (Table 4.3). However the activity against *E. coli* 1699 occurred in the presence of sodium nitrate and high phosphate conditions (Na5M) only. None of the other Na5-carbon conditions induced this activity. Furthermore, the activity against *M. smegmatis* was specific to the combination of ammonium nitrate, high phosphate and a heat treatment and likely co-culture (Table 4.3). These results not only demonstrate the combinatorial requirement, but they also imply that one or more biosynthetic pathways are induced in PE9_140 resulting in 2 different bioactivities.

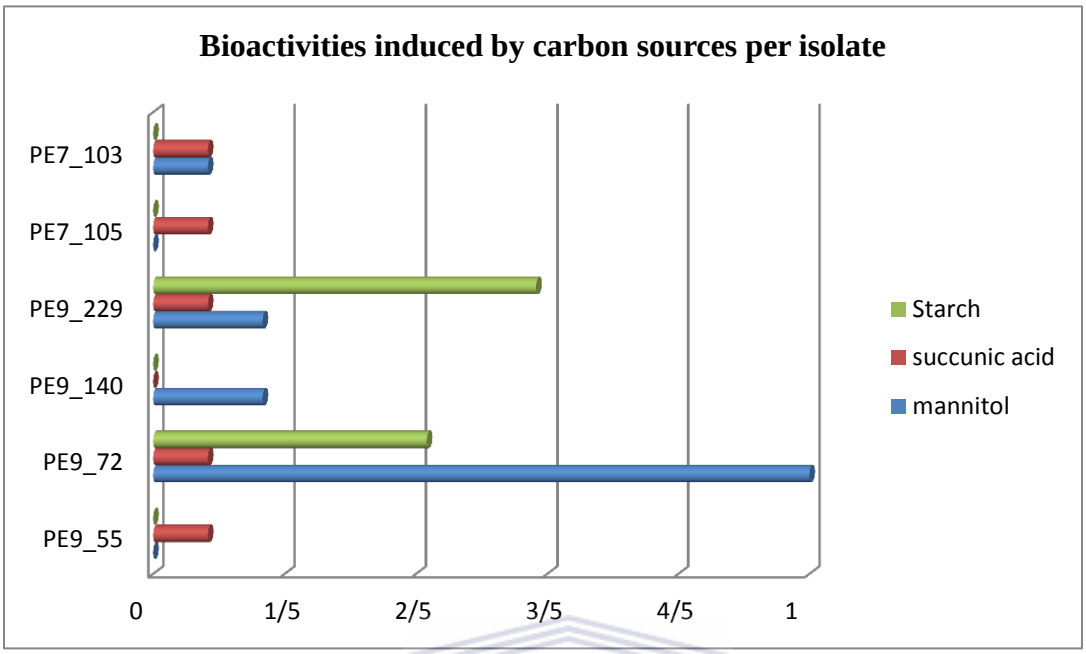


Figure 4.4: The effects of the carbon source in the growth medium on the bioactivities of 6 marine sponge associated bacteria. The activity is reported as a fraction based on the number of bioactivities observed when growing on a carbon source.

4.2.1.3 Temperature as a trigger for secondary metabolism

The temperature effect on inducing production of secondary metabolites from individual microbial isolates was investigated by incorporating three different treatments: room temperature (R.T, essentially representing no treatment), heat shock (H.T) and cold shock (C.S). Based on the results presented in Figure 4.5, it is not possible to identify whether temperature treatments have a global effect in inducing biosynthetic pathway expression. For example, if one looks at isolate PE7_103, it showed antibacterial activities with the combination of "mannitol + heat shock" and "succinic acid + room temperature". However the heat shock activity was against *E. coli* 1699 whereas under room temperature, the activity was against *S. epidermidis* (Table 4.3). This was an indication that the different culture conditions were responsible for activating possibly different

biosynthetic pathways in this organism. Similar observations were made for temperature treatments for PE9_72, PE9_140 and PE9_229 where different heat treatments induced different bioactivities. To further illustrate temperature effects, PE9_72 is discussed. Table 4.3 shows that under "mannitol + room temperature" this organism showed antibacterial activities against 4 indicator strains (*E. coli* 1699, *M. smegmatis*, *P. putida* and *B. cereus*) whereas under "mannitol + heat shock", this organism showed activities against all 5 indicator strains. Different bioactivities were also observed for this organism under "mannitol + cold shock" where activities were exhibited against 4 indicator strains with the exception of *M. smegmatis*. This was an indication that although PE9_72 showed activities under mannitol (room temperature, heat shock and cold shock) (Figure 4.5) the bioactivities were not identical (Table 4.3). This further indicated that different biosynthetic pathways were activated by the different temperature treatments hence the differential activities. However, it is apparent that cold shock inhibits the expression or the activity of the metabolite responsible for activity against *M. smegmatis*. Moreover, activities were observed only against *S. epidermidis* under cold shock treatment when succinic acid was the carbon source (Table 4.3). Heat shock and cold shock were also observed to induce some bioactivities in PE9_72 when cultured using starch whereas there was no induced activity at room temperature (Table 4.3 and Figure 4.5).

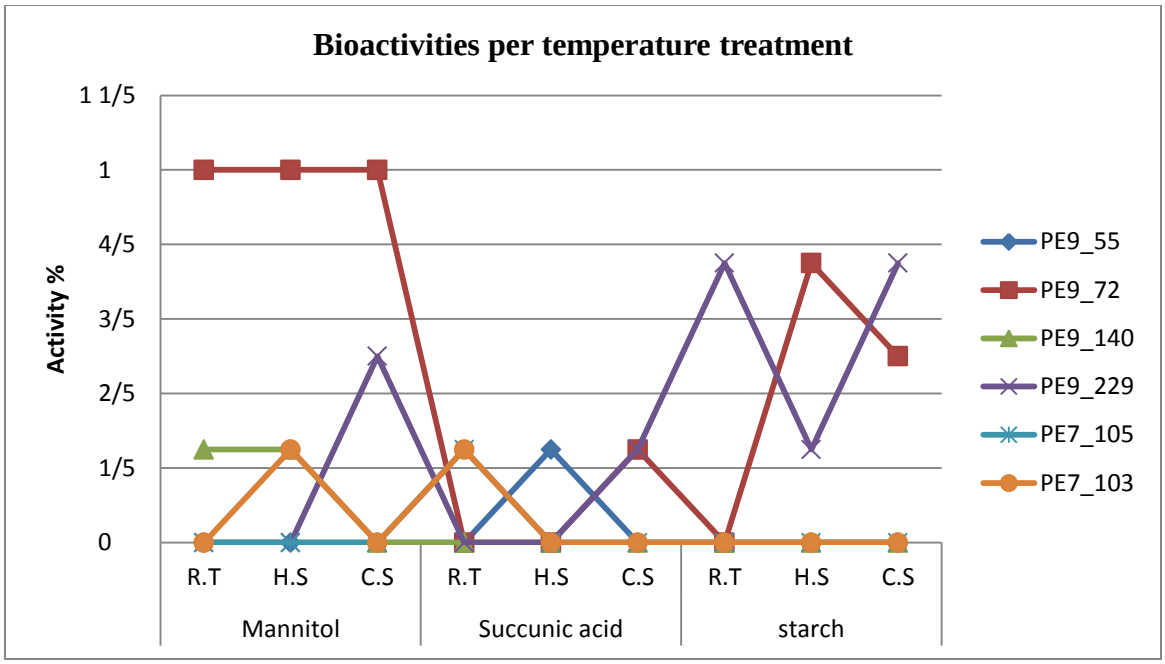


Figure 4.5: The effects of heat shock (H.S), cold shock (C.S) and room temperature (R.T) treatments on production of secondary metabolites by marine sponge associated bacteria. Activities are recorded as fractions in percentages out of 4 media compositions per temperature treatment. The carbon sources used in the media are shown.

4.2.1.4 Phosphate and Nitrogen as triggers for secondary metabolism

Looking at Table 4.3 it was difficult to determine a pattern for nitrogen source or phosphate concentrations as inducing factors of secondary metabolism in the 6 marine sponge isolates. This indicates the complexity of the induction mechanisms which are involved in the activation of pathways in individual microbes. However, some interesting bioactivities were displayed on 3 different media conditions (A1HM, A5HM and Na5HM, see Table 4.3) where PE9_72 showed 4 different bioactivities. Under all conditions variable activities against the indicator strains were noted. For example, using A1HM medium, PE9_72 did not show activity against *S. epidermidis*, whereas on A5HM medium the only activity that was not expressed was against *B. cereus*. Furthermore, the activity against *P. putida* was not expressed on Na5HM medium. For these results it is not possible to determine if the phosphate concentrations or different nitrate sources

were responsible for the activities against the 4 indicator strains. Moreover, since the bioactivities are also a function of co-culture, carbon sources and temperature treatment, it is not possible to pinpoint the factor/s which induced the different bioactivities.

Furthermore, the activity against *E. coli* 1699 was induced under all the mannitol+HS scenarios (Table 4.3), and appears to be independent of the nitrogen source and phosphate concentration. However, heat shock did not induce this activity when cultured in succinic acid as the carbon source, demonstrating carbon source specificity in addition to heat treatment. However, induction of this activity using cold shock treatment (+ mannitol) was dependent on the nitrogen source, occurring only in the presence of sodium nitrate (Na5CM and Na1CM). Whether the same anti-*E.coli* 1699 agent is induced in the heat shock versus cold shock scenarios is impossible to determine from these results, and it could be entirely possible that these activities are actually a result of different compounds. This analysis extends to the other isolates as well (although a case by case analysis is not presented), and draws attention to the intricacies associated with OSMAC-based studies and the difficulty in interpreting the effects. The results in this study indicate that the OSMAC approach is organism and pathway specific hence there cannot be any standard media or stress conditions which can be incorporated as a general/global inducer for all microbes.

4.2.1.5 Antibacterial capacities

Figure 4.6 presents an overall picture of individual media compositions which induced bioactivities in the individual isolates. The capacities to produce bioactivities were indicated by a percentage out of all the 5 indicator strains. For example, PE9_55 and PE7_105 showed one

activity each (Figure 4.6) which is shown as 20% capacity. Both organisms showed the least number of activities in the matrix study. PE9_72 showed the highest antibacterial capacities when cultured on media compositions A1HM, A5HM and Na5HM where bioactivities were against 4 variable indicator strains (80% activities). Although bioactivities against all 5 indicator strains were shown in this study (Table 4.3) not a single medium composition induced activities against all five indicator strains. The antibacterial capacities were also interpreted by an organism which had its pathways induced by most media conditions. In this case the isolate that had most of its bioactivities induced under the 36 conditions was PE9_72, where 18 of the 36 conditions were able to induce bioactivities in this isolate. PE9_72 (*B. pumilus*) was therefore the most "talented" isolate with regard to antibacterial activities against all indicator strains used and across all media compositions. PE9_229 showed the second highest antibacterial activities across a range of matrix culture conditions from which 10 activities were induced.

Alternatively, *E. coli* 1699 (Cubist, USA) which is resistant to 52 antibiotics can serve as a priority antibacterial strain with regard to potentially novel bioactive compounds. In this regard, PE7_105 could be regarded as talented although it only showed one activity. In a similar way, PE9_229 would have the same capacities as with PE7_105 and PE7_103 since they all showed one activity against the multi-resistant *E. coli* 1699 strain.

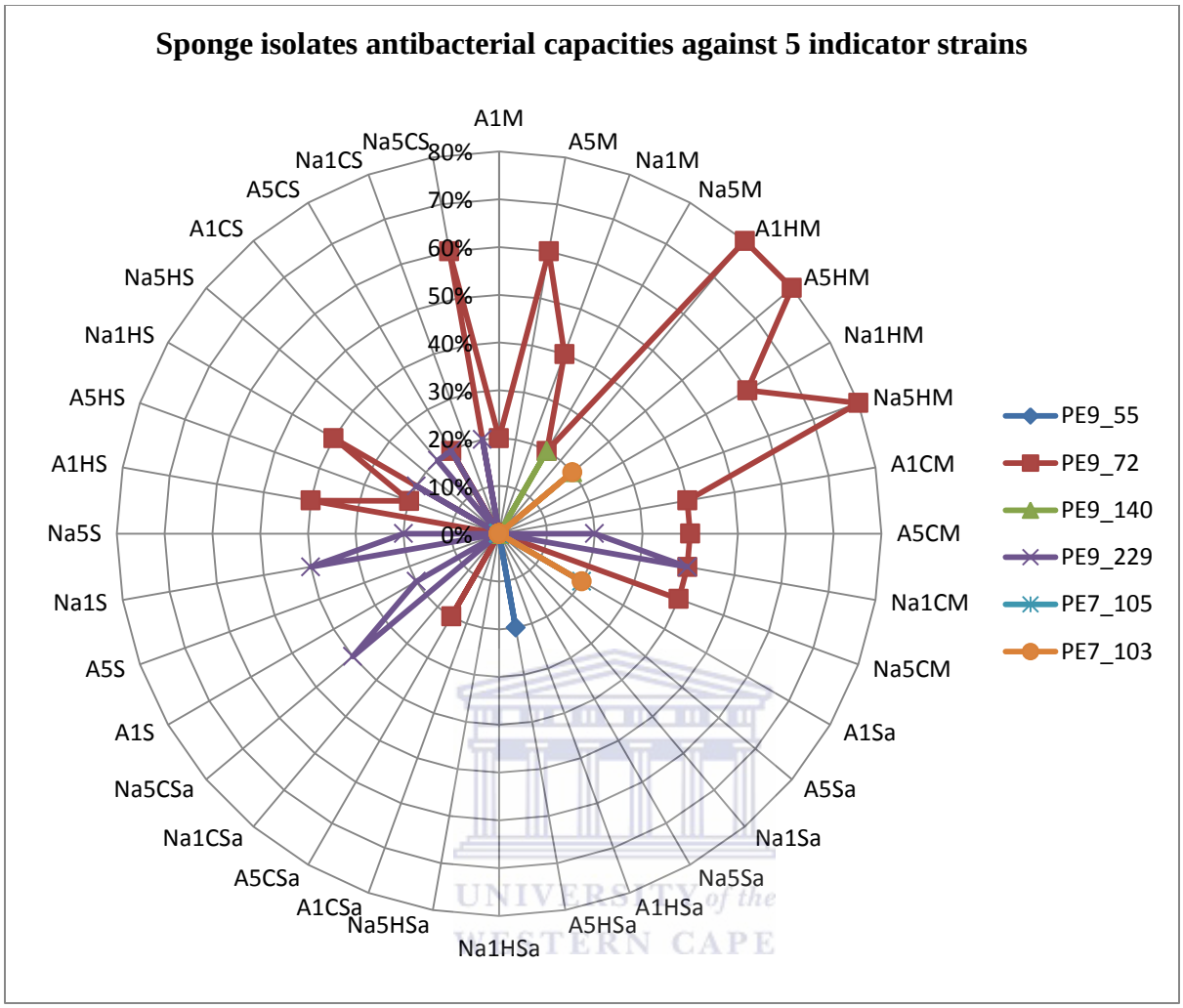


Figure 4.6: Radial representation of the bioactivities exhibited by the microbial isolates against 5 indicator strains. The capacities to produce bioactivities are indicated by a percentage out of all the 5 indicator strains.

4.3 DISCUSSION

An improvement in the ability to culture previously uncultured microorganisms could yield a much wider and diverse range of novel bioactive secondary metabolites of pharmaceutical interest (Lavy *et al*. 2014). OSMAC is a random approach and affects different microbes differently; as a result, no common rules can be followed for this approach for all microbes. Alterations of every parameter are allowed as long as the chosen conditions lead to discovery of a new secondary metabolite (Bode *et al*. 2002). The application of the OSMAC approach in this study has thus resulted in production of antibacterial compounds. Microbial isolates which initially did not show bioactivities could be induced to express certain activities as a result of changed growth parameters. Previous studies have shown that growth conditions which altered carbon sources (Ruiz *et al.* 2010; Sánchez *et al*. 2010; Gubbens *et al*. 2012), phosphates (Sola-Landa *et al*. 2003; van Wezel & McDowall 2011), nitrogen (Bibb 2005; Reddy *et al*. 2011), heat, phage infection (Doull *et al*. 1994) as well as aeration levels (Bode *et al.* 2002) elicit such responses.

A study of the conditions leading to the production of secondary metabolites by *Streptomyces* sp. strain C34 showed that the overall media composition results in the production of secondary metabolites (Rateb *et al*. 2011). In such cases, it is difficult to identify which components in the media were responsible for triggering expression of biosynthetic pathways. When *Streptomyces* sp. strain C34 was cultured on a range of 8 media (simple, complex, enriched and defined media), the organism produced novel natural compounds. The different media not only induced secondary metabolism but also the quantity of the secondary metabolites was increased. This was an indication that employing a wider range of culturing media activated additional biosynthetic pathways and may have overexpressed some previously unknown pathways (Rateb *et al*. 2011).

In a similar manner in the present study, some of the activities that will be discussed may have been a result of the incorporation of alternative media compositions.

The presence of carbon sources in culture media plays a major role in secondary metabolite induction. Over 30 examples of secondary metabolism are repressed by carbon with particular mention of glycerol, xylose, mannose, maltose and glucose being amongst those known to impair synthesis of secondary metabolites (Ruiz *et al*. 2010). The choice of carbon source in a growth medium is thus very important when attempting to induce of secondary metabolite production. Reports also indicate that suboptimal microbial growth is usually associated with production of secondary metabolites (Doull *et al*. 1994; Bibb 2005). Moreover carbon sources which are not easily metabolized tend to trigger production of secondary metabolites (Ruiz *et al*. 2010; Demain 2014). Under this notion it could be that the low microbial biomass on most of the matrix media in this study could have triggered biosynthesis of secondary metabolites. The media composition which resulted in most bioactivities per isolate (PE9_72, PE9_140, PE9_229 and PE7_103) was media with mannitol as the sole carbon source. It cannot be overlooked however that other components in the media such as phosphates, temperature treatments, nitrates and co-culture effects contributed to triggering production of secondary metabolism in these isolates. The antibacterial activity from the aforementioned marine sponge associated microbes was exhibited against a panel of 5 indicator strains (Table 4.3). This could suggest that for each microbe expression of one or more biosynthetic pathways could have resulted in the observed bioactivities. Mannitol reportedly is a non-repressing (catabolite) carbon source (van Wezel & McDowall 2011). Mannitol was reported to trigger secondary metabolite related pathways in *S. coelicolor* (Zhu *et al*. 2014). The results in the present study also support this notion since most

of the bioactivities were exhibited when some of the marine sponge microbes were cultured on mannitol media.

The frequency of bioactivities induced using succinic acid was few compared to the bioactivities observed under mannitol and starch. PE9_55, PE9_72, PE9_229, PE7_105 and PE7_105 were the isolates which exhibited bioactivities under succinic acid as the sole carbon source. Moreover, although the bioactivity frequencies were few, succinic acid secondary metabolite induction as the sole carbon source was spread out amongst several individual microbes (Table 4.3). Similar studies where the carbon source used was ammonium succinate resulted in significant production of secondary metabolites in *Streptomyces hygroscopicus* 111-81 whereas growth rate of *S. hygroscopicus* was low. The bioactivity induction however was a combination of a carbon source (succinate) and nitrogen source (ammonium) (Gesheva *et al*. 2005). This was consistent with the present study since the bioactivities were a result of a number of factors (nitrates, phosphates, temperature treatments, and co-culture) at a time.

Starch is one of the polysaccharides that are often preferred by microbial systems in production of secondary metabolites. As a result, starch is used in many fermentation media in industry for production of secondary metabolites (Sanchez & Demain 2002; Zhu *et al*. 2014). In the present study, the effect of starch on secondary metabolism was notable on the two isolates PE9_72 and PE9_229. Most bioactivities were observed for PE9_229 indicating that starch was ideal for triggering antibacterial activity in this isolate (Table 4.3) compared to just one bioactivity on standard media (ACM Appendix 2). The bioactivities were also affected by other media compositions and experimental setup as previously explained. The frequency of bioactivities on PE9_72 when starch was the sole carbon source was fewer than those observed under mannitol. This was an indication that starch did not induce as many biosynthetic pathways as in PE9_72

with mannitol. It could however be noted that when starch was the sole carbon source, more activities were observed over succinic acid. This could imply that starch was more preferable for secondary metabolite production as opposed to succinic acid in this oganism. Similar studies have revealed that starch was a more preferable secondary metabolite inducer compared to maltose in production of jadomycin B from *Streptomyces venezuelae* ISP5230 (Doull *et al.* 1994). Furthermore, unusual steroids dankasterone A and B were produced from *Gymnascella dankaliensis* when soluble starch was used in modified malt extract media (Amagata *et al*. 2007). Other studies have used different carbon sources such as ethanol to elicit secondary metabolite production which resulted in significant production of antibiotics (Nakata *et al.* 1999; Pettit 2011). Some studies have used a mixture of carbon sources to induce secondary metabolism as in the case of *Pseudomonas aeruginosa* which produced antimicrobial compounds when a mixture of oils (50% olive oil and 50% corn oil) were used as the sole carbon source (Wang *et al*. 2013). The use of one-factor-at-a-time approach carried out on *Xenorhabdus bovienii* YL002 revealed that glycerol was the best carbon source for antibiotic production in the presence of soytone as the nitrogen source (Wang *et al*. 2011). This further indicated that not one factor is responsible for the bioactivities exhibited per organism.

Temperature treatment studies have paid more attention on heat shock induction with high induction successes reported by many groups (Doull *et al*. 1994; Nakata *et al*. 1999; Liao *et al.* 2009; Zhou *et al*. 2012). Heat tends to result in cell envelope stress and as a result induces secondary metabolism (Doull *et al*. 1994; Zhou *et al*. 2012). In the present study, heat induction on secondary metabolism was notable for isolate PE9_72 (Table 4.3 and Figure 4.5) although it could not be conclusively pinpointed that heat was the sole inducer of expression of bioactivities. Other microbial bioactivities with regard to temperature treatments seemed random whereas for

PE9_229 the bioactivities seemed to be evenly distributed when starch was the carbon source. For PE9_72 with mannitol and starch as carbon sources, there were higher frequencies of bioactivities coupled with heat shock. This was an indication that heat shock induced more activities in PE9_72 by triggering expression of some biosynthetic pathways which subsequently resulted in antibacterial activities. The results from this study were similar to those reported for *S. venezuelae* ISP5230 under heat shock. *S. venezuelae* ISP5230 was initially known to produce chloramphenicol, but when exposed to elevated temperatures (42 ^{o}C) for 1 h and shifted back to 27 ^{o}C, it produced high levels of novel bioactive compounds. This indicated that the bioactive compounds produced were linked to heat shock response (Doull *et al.* 1994; Yoon & Nodwell 2014). Additionally heat shock treatments on *Streptomyces hygroscopicus* 5008 resulted in enhanced productivity of validamycin A (Liao *et al.* 2009). Other studies were able to quantify the antibiotics produced from heat shock. *Pseudomonas fluorescens* S272 was able to produce 2 to 3 fold more of the same bioactive compounds under heat shock at 50^{o}C (Nakata *et al.* 1999). A study carried out using *Streptomyces coelicolor* A3(2) also revealed that heat stress (39^{o}C) on this bacterium resulted in expression of higher quantities of secondary metabolites (Bursy *et al.* 2008). Additionally the temperature treatments in the present study did not function independently of the carbon, nitrogen and phosphates. All the activities could have been a combinatorial effect from co-culture, carbon sources, nitrates, phosphates and/or temperature treatments.

Under cold shock, it was notable that two isolates (PE9_72 and PE9_229) showed antibacterial activities. The former previously did not show any antibacterial activities in preliminary screening whereas the latter did show initial activities. PE9_229 showed different activities under cold shock in addition to activities shown in preliminary screening. The results from this

study therefore, indicate that under cold shock, differential expressions of biosynthetic pathways were triggered resulting in new activities in some of the bacterial isolates. This concurred with the notion that "anything goes" as indicated in other studies (Chen *et al.* 2000). Bioactive compound production was also reported in *Bacillus circulans* and *Streptomyces* spp. using dimethylsuloxide (DMSO) in culture media (Chen *et al*. 2000; Pettit 2011). Six novel secondary metabolites were produced from *Paraphaeosphaeria quadriseptata* in response to changing the water used in culture media from tap water to distilled water (Scherlach & Hertweck 2009; Pettit 2011). Moreover, heavy metals ions such as Cu^{2+} were reported to increase production of secondary metabolites in *Paraphaeosphaeria quadriseptata* (Pettit 2011) which further supports the "anything goes" hypothesis.

Nitrogen sources are known to trigger secondary metabolite production. Growth suppression due to nitrogen depletion in the media allows for expression of microbial biosynthesis of secondary metabolites (Doull *et al*. 1994; Reddy *et al.* 2011). When the culture media was manipulated to comprise about 1% of peptone, there was a high production of bioactive compounds from *Streptomyces rochei* MTCC 10109 (Reddy *et al.* 2011). *Streptomyces coelicolor* A3(2) was also found to produce secondary metabolites under nitrogen limiting conditions (Bibb 2005). The best production of antibacterial compounds in *Pseudomonas aeruginosa* was when peptone was used as a nitrogen source together with carbon source as an oil mixture (Wang *et al*. 2013). Furthermore, ammonium salts reportedly do not favor production of secondary metabolites such as novobiocin, actinomycin, neomycin, kanamycin from *Streptomyces hygroscopicus* (Lee *et al*. 1997). Contrastingly, ammonium salts such as ammonium chloride or acetate were found to stimulate the formation of some components of polyether IM-111-81 and azalomycin B antibiotics from *Streptomyces hygroscopicus* (Gesheva *et al*. 2005). This serves as an indication

as to how unpredictable changes in culture media can influence different bioactive compound production. In a similar manner, in the present study no pattern could be drawn as to whether ammonium nitrate or sodium nitrate was a more favourable secondary metabolite inducer in most of the microbes under study. However, PE9_72 seemed to show activity under sodium nitrate as the nitrogen source coupled with co-culture, cold shock and mannitol in bioactivities against *E. coli* 1699. Interestingly, microbes which did not show prior activities in preliminary antibacterial screening started showing activities under the matrix. Furthermore, isolates that had previously shown antibacterial activities in previous screening showed different activity profiles under the matrix. This was an indication of expression of different biosynthetic pathways under the matrix culture conditions and co-culture effect. This further supported the notion by (Bode *et al*. 2002) that no common rules can be formulated for all microbes. Therefore, "anything goes" as explained by (Bode *et al*. 2002) is permitted as long as the alterations in culture conditions unlock expression of biosynthetic pathways.

In a similar manner, although phosphate limiting concentrations (0.1µM or 0.5 mM) were used (Lounès *et al*. 1996; Sola-Landa *et al*. 2003), no observable influences could be assigned to any of the two concentrations. The bioactivities for each microbe appeared to be random. For instance, PE9_55 only showed activity on media composition A5HSa (Table 4.3), hence the activity cannot be conclusively ruled to be a result of higher phosphate concentration (0.5 mM). Phosphates are known to be responsible for induction of selected biosynthesis using high or low-phosphate media (Bode *et al*. 2002; Martín 2004). There is more evidence however that under phosphate limiting conditions, secondary metabolites are produced (Sola-Landa *et al*. 2003; Martín 2004; van Wezel & McDowall 2011). It could be speculated that the phosphate limiting conditions (together with co-culture effects, carbon sources, nitrates and temperature treatments)

in the matrix study could have induced some of the bioactivities although no specific concentration could be pin pointed per microbe.

Most studies have screened microbes for bioactivities in pure cultures (Doull *et al*. 1994; Bode *et al*. 2002; Rateb *et al.* 2011; Zhou *et al*. 2012) whereas in nature, bioactive compounds are produced after specific signals such as stress or the presence of community members (Bertrand *et al*. 2013; Bertrand *et al*. 2014; Zhu *et al*. 2014). One of the methods reported to successfully elicit biosynthetic pathways to produce secondary metabolites is microbial co-culture (Cueto *et al*. 2001; Oh *et al*. 2007; Bertrand *et al*. 2013; Traxler *et al*. 2013).

When *Streptomyces coelicolor* was co-cultivated with 5 other *Actinomyces* spp., the compounds produced by *S. coelicolor* were unique (Traxler *et al*. 2013). When *Streptomyces endus* S-522 was co-cultured with *Tsukamurella pulmonis* TP-B0596 a novel compound alchivemycin A was produced (Onaka *et al*. 2011). Moreover, the marine fungus from the genus *Pestalotia* produced a novel compound (pestalone) in co-culture with the marine bacterium strain CNJ-328 (Cueto *et al*. 2001). Other studies were conducted on fungal co-cultures where *Trichophyton rubrum* and *Bionectria ochroleuca* resulted in different bioactive compounds (Bertrand *et al.* 2013). In a similar manner, the co-culture results in the present study concurred with reported studies where the co-culture interaction between one or more microorganisms resulted in bioactivities. For example, in the previous chapter when PE9_72 was screened in pure culture, it did not show any bioactivities whereas under the matrix in co-culture it started to show multiple activities. This was an indication that the certain biosynthetic pathways were expressed in the matrix co-culture.

Marine sponge-associated microbes produce a wide range of secondary metabolites which are structurally and chemically diverse and have interesting pharmacological and biotechnological

properties. Secondary metabolites with interesting properties include peptides, (Macintyre *et al*. 2014) bacteriocins, (Desriac *et al*. 2010), terpenes (Pandey *et al*. 2014), alkaloids (Antunes *et al*. 2004) nonribosomal peptides and polyketides (Woodhouse *et al*. 2013). Some of these compounds have been isolated from *Actinobacteria*, *Firmicutes*, *Alpha* and *Gammaproteobacteria* which is consistent with the microbial phyla obtained in the present study. The isolate which showed the most bioactivities/"talented" under the matrix was PE9_72 (*Bacillus pumilus*) which is consistent with reports that the genus *Bacillus* are known for production of diverse bioactive compounds with relevant pharmaceutical and biotechnological properties (Bhosale *et al*. 2002; Stein 2005; Phelan *et al*. 2013). *Bacillus pumilus* isolated from the sponge *Haliclona simulans* is also renowned for a wide range of bioactivities (Phelan *et al*. 2012). Another isolate with interesting bioactivities in the present study was PE9_229 which is *Halomonas titanicae.* A study carried out on *Haliclona simulans* (marine sponge) recovered some *Halomonas* sp. which had some PKS genes and displayed antimicrobial activities (Thomas *et al*. 2010). *Halomonas titanicae* isolated from a bryozoan *Membranipora membranacea* also exhibited some antibacterial activities (Heindl *et al*. 2012). Whether the same activities and pathways responsible for the bioactivities in other *H. titanicae* are the same as those detected in the present study has yet to be determined. The 6 isolates which exhibited antibacterial activities support the notion that marine sponge associated microbes are capable of producing a wide range of secondary metabolites (Muscholl-Silberhorn *et al*. 2008; Desriac *et al*. 2010).

Triggers of expression of biosynthetic pathways are complex and organism specific as well as pathway specific. The same gene has been reported to be induced in two alternative ways by changing from producing a 12 membered macrolactone methymycin to a 14 membered

narbomycin as a result of culturing *Streptomyces venezuelae* on two different media (Xue & Sherman 2000). It has been reported that nitrogen regulators are controlled by phosphate regulators in *Streptomyces coelicolor*. On the other hand the interaction between carbon and phosphate show that carbon influences expression of some phosphate genes such as *glpQ1* and *glpQ2* genes of *S. coelicolor*. Similar carbon and phosphate interactions also occur in *S. lividans*. The same interaction between carbon sources influencing expression of phosphate genes was also reported in *Vibrio vulnificus* and *Bacillus subtilis* (Martín *et al*. 2011). This indicates the complexity of regulation mechanisms which are triggered by culture conditions in expression of biosynthetic pathways. The matrix study was able to unlock expression of some pathways in some of the sponge isolates under study although in most cases no factor would be singled out to be responsible for the observed bioactivities. This could have been due to the usage of a lot of factors at a time on a number of isolates which could have resulted in masking some overall influences of each parameter under study.

CHAPTER 5 GENERAL DISCUSSION AND CONCLUSION

Chapter 1 gave an overview on marine natural products with particular focus on the renewed interest in marine sponge associated microorganisms as a rich and untapped source of secondary metabolites (Piel 2006; Zotchev 2012). For this study, emphasis was on the marine sponges based in the South African coastline which is dominated by two contrasting ocean currents resulting in high marine biodiversities (Griffiths *et al*. 2010). Marine sponges from this environment present an interesting area of study because it is largely unexplored and could likely result in novel secondary metabolites which can be developed to benefit mankind (Skariyachan *et al*. 2014; Steinert *et al*. 2014).

In order to investigate and potentially exploit the biosynthetic profiles of marine sponge associated microbes, one of the first steps is to bring these microbes into culture (Skariyachan *et al*. 2014). It is well known that microbes are difficult to culture with over 99% of total microbial population from any environmental sample remaining uncultured using conventional culturing techniques (Duncan *et al*. 2015). Some studies based on culturing marine sponge associated bacteria have used rich culture media or aerobic media (Flemer *et al*. 2012; Lavy *et al*. 2014; Steinert *et al*. 2014) with particular targets being *Actinobacteria* (Genilloud *et al*. 2011; Zotchev 2012). In chapter 3, a culture-independent technique (T-RFLP), was employed to investigate, and ascertain marine sponges which hosted the highest microbial diversities to be used in further culturing studies. While *Actinomycetes* are known to be a prolific source of bioactive compounds (Kim *et al*. 2005; Genilloud *et al*. 2011; Subramani & Aalbersberg 2012), other bacterial phyla also need to be targeted because they produce essential bioactive compounds (Gross 2007; Craney *et al*. 2013). A lot of effort is required to culture a larger diversity of other phyla to screen for secondary metabolite production (Lavy *et al*. 2014). The phylogenetic analyses in

section 3.2.5.3 also revealed that the cultured microorganisms were of the phyla *Actinobacteria*, *Firmicutes*, *Alpha* and *Gammaproteobacteria* and the three phyla exhibited antibacterial activities. One of the isolates PE9_221 showed 97% identity to the closest relative in the NCBI database which could mean that it represents a new species (Lavy *et al*. 2014; Ramasamy *et al*. 2014).

The OSMAC principle indicates that shifting minor culture conditions can result in substantial changes in metabolic profiles of microbes (Bode *et al*. 2002; Genilloud *et al*. 2011). Under stressful conditions, microbes tend to produce compounds which are thought to be a defense mechanism or a way of adapting to the environment (Hutchinson 2003; Graça *et al*. 2013). Thus culture conditions which are easily manipulated are systematically altered to identify conditions that elicit expression of "silent"/unexpressed biosynthetic pathways (Bode *et al*. 2002; Pettit 2011). In chapter 4, the OSMAC approach was employed using a matrix of 36 different culture conditions (mannitol, succinic acid, starch, $NaNO_3$, NH_4Cl, KH_2PO_4, room temperature, heat shock and cold shock) with the experimental setup incorporating co-culturing. The application of the OSMAC principle was able to unlock expression of biosynthetic pathways from microbes which did not show antibacterial activities in section 3.2.4 as well as those which showed activities.

MARINE ENVIRONMENT

The ocean covers around 70% of the earth's surface (Bhatnagar & Kim 2010; Krause & Tobin 2013) and therefore serves as a niche for large biodiversities (Krause & Tobin 2013). The growing need for pharmaceutically relevant compounds to address the increase in multi-resistant pathogens and other illnesses makes the marine environment an ideal area to search for novel bioactive compounds (Phelan *et al*. 2012; Monciardini *et al*. 2014). Marine sponges in particular

are known to host large microbial diversities which are responsible for the high chemical diversities of natural products (Krause & Tobin 2013; Skariyachan *et al*. 2014). As a result marine sponge symbionts need to be explored as a source of new pharmaceutical leads. However, other studies indicated that marine sponge symbionts are generally sponge specific (Thiel *et al*. 2007; Lee *et al*. 2011; Erwin *et al*. 2012; Simister *et al*. 2012a; Pita *et al*. 2013; Taylor *et al*. 2013) (microbial communities repeatedly found in marine sponges around the world but not detected in other marine environments) (Schmitt *et al*. 2012a; Olson & Gao 2013; Taylor *et al*. 2013). Moreover, sponges obtained from different parts of the world, Brazil (Hardoim *et al*. 2009), China (Xi *et al*. 2012), India (Chairman *et al*. 2012) and Portugal (Graça *et al*. 2013) tend to harbour similar bacterial phyla. This goes against the renewed interest of exploring underexplored marine environments such as the South African marine environment and other marine environments which are known to host high macroscopic and microscopic biodiversities in the quest for novel bioactive compounds. In the present study the microbial phyla obtained from the two marine sponges were similar to those found in aforementioned parts of the world.

Extracts from the latrunculid sponges endemic to the South African coast have shown hope in discovering novel bioactive compounds (Antunes *et al*. 2004; Davies-Coleman & Beukes 2004). The pyrroloiminoquinones alkaloid Tsitsikammamine B has interesting bioactivities and it has only been found in *Tsitsikamma favus* sponges. Additionally the *Proteobacteria* phyla derived from *T. favus* showed some novelty when compared to other sequences from other sponge symbionts. This is the only sponge that hosts *Betaproteobacteria* as the dominant microbial taxon (Walmsley *et al*. 2012). Such studies indicate that unexplored marine environments focusing on marine sponges can yield some novel bioactive compounds and potentially novel microbial communities.

Over the past decades screening programs at an industrial level using millions of strains failed to deliver novel compounds from the predominant microbial species. As a result the probability of discovering novel compounds from common species is not worth the effort (Genilloud *et al*. 2011). This extends to the present study where the three common microbial phyla (*Actinobacteria*, *Firmicutes*, *Alpha* and *Gammaproteobacteria*) were discovered. Moreover, *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes* account for over 88% of cultured microbial phyla. This is indicative of the limitations of exploiting the potential of the rest of the uncultured microbes (Rinke *et al*. 2013) to produce compounds that would serve as new pharmaceutical leads. This calls for development of new methods in improving culture techniques such as using genomic information to have an insight into uncultured microbial species growth requirements in order to culture novel species (Lavy *et al*. 2014). Alternatively, different avenues needed to exploit the potential of the microbes that are already in culture stocks. One of the methods that resulted in high success rates in production of novel bioactive compounds is the OSMAC principle (Bode *et al*. 2002; Demain 2014; Hewage *et al*. 2014; Marmann *et al*. 2014).

OSMAC

The search for secondary metabolites has been through screening of a huge number of microorganisms for natural products discovery (Bérdy 2005; Baltz 2008; Genilloud *et al*. 2011). This approach was expensive and led to high rediscovery rates of bioactive compounds already in use which resulted in big pharmaceutical industries losing interest in investing in natural product discovery (Li & Vederas 2009; Demain 2014). One of the avenues aimed at improving natural product discovery came to light around the mid-1990s and gained momentum in the early 2000s is the OSMAC principle (Bode *et al*. 2002). The OSMAC approach has been successful in

inducing the production of novel compounds from one or few microbes using systematic alteration to culture conditions (Rateb *et al*. 2011; Zhang *et al*. 2013; Hewage *et al*. 2014). In the present study, the effect of the OSMAC principle was studied for 30 marine sponge isolates using co-culture effect and 36 culture conditions. Each culture condition showed different induction efficiencies per isolate with the most bioactivities induced in the isolate PE9_72 identified as a *Bacillus pumilus*. The metabolic profiles of *B. pumilus* changed greatly under the matrix conditions (chapter 4) whereas in the preliminary screening process, this isolate did not exhibit any antibacterial activities (chapter 3). Although this study was not able to conclusively pinpoint the precise conditions which triggered the sudden bioactivities, every media composition while using mannitol as the sole carbon source (together with co-culturing effects, temperature treatments, phosphates, and nitrates) induced at least one antibacterial activity in *B. pumilus*. This was an indication that mannitol (together with already mentioned conditions) was the best carbon source for inducing activities in *B. pumilus*. Notably, under the mannitol media compositions, *B. pumilus* exhibited bioactivities against the 5 indicator strains indicating that the matrix conditions probably activated expression of several biosynthetic pathways in this bacterium. This was an indication that culture conditions are organism and pathway specific (Xue & Sherman 2000). The interactions between nitrogen and carbon sources along with phosphates are reportedly poorly understood since each component has been studied individually. However, in *S. lividans* and *S. coelicolor*, carbon sources affect expression of some phosphate genes. Additionally, phosphates affect expression of genes involved in nitrogen assimilation and transport but also directly oversee nitrogen regulators in *S. coelicolor*. Transcriptomic data indicated that under phosphate limiting conditions, there was an increase in expression of nitrogen metabolism genes (Martín *et al*., 2011). Similar arguments about the

carbon, nitrogen and phosphate interaction could have also influenced the bioactivities in *B. pumilus* since each media composition constituted carbon, nitrogen and phosphate sources.

The present study could not go as far as identifying the antibacterial compounds which were produced. This translates into how the OSMAC approach is laborious and time consuming. Since pharmaceutical industries are driven by desire for high profit margins, chances are they would not find it worthwhile to engage in this kind of drug discovery (Harvey 2008; Li & Vederas 2009; Demain 2014). Additionally, pharmaceutical industries stopped investing in natural products discovery because it takes years of preclinical and clinical tests to develop natural compounds whereas the patenting of a novel compound is a relatively shorter process (Li & Vederas 2009; Demain 2014). The OSMAC approach therefore entails the same problems that drove major pharmaceutical industries to no longer invest in natural product discovery. Traditional drug discovery methods involve many steps such as making crude extracts which are prescreened for bioactivities before fractionation, isolation and identification. Furthermore despite the time spent on resources there are no guarantees that the compounds would indeed be novel or useful drugs (Krause & Tobin 2013). There is however recent advancements in screening processes as explained in the next paragraph to persuade major pharmaceutical industries to reinvest in natural product discovery.

DEREPLICATION

Natural product screening programs have resulted in high rediscovery of identical microbes being rescreened which is a waste of time and resources (Bérdy 2005). In order to reduce screening costs and to save time, it is important to discriminate between previously encountered and screened microorganisms as early as possible (Brandão *et al*. 2002). For example analysis of microorganisms from marine environments could be analysed for early stage dereplication on the

basis of their 16S rRNA gene sequence identities. However, 16S rRNA gene sequence dereplication is limited in resolving phylogenetic variation within subclades of microbes (Duncan *et al*. 2015). Moreover 16S rRNA gene dereplication of *Puedoalteromonas luteoviolacea* strains proved to be inefficient, as a result metabolic profiles of *P. luteoviolacea* strains were used in dereplication methods (Vynne *et al*. 2012). In the present study, similar 16S rRNA gene dereplication limitations were encountered and bioactivities of some isolates were used to dereplicate marine sponge derived OTUs. It can be concluded that metabolic dereplication is better than 16S rRNA gene dereplication (Vynne *et al*. 2012; Duncan *et al*. 2015). Metabolic profile dereplication is important in natural products discovery in order to identify microbes which produce novel secondary metabolites. Alternatively, pyrolysis mass spectrometry is a whole cell fingerprinting technique which showed some success in microbial dereplication and it has the advantage that it is fully automated. It has also shown high discriminatory capacity at intraspecies level (Brandão *et al*. 2002).

Moreover, it is essential to identify and dereplicate secondary metabolites as early as possible to avoid wasting time and resources only to realize later that developments are being done on already known compounds (Genilloud *et al*. 2011). However compound dereplication has been largely unsuccessful (Bérdy 2005). Another problem of secondary metabolite dereplication is mainly due to published literature giving numbers to novel bioactive compounds (Bode *et al*. 2002), thus making the communication about novel compounds difficult. Additionally, there is uncertainty about antibiotic nomenclature which also adds to dereplication problems. For example, identical actinomycin C complexes have about 15 different names (Bérdy 2005). One of the dereplication methods that has been used but still had high rediscovery limitations is the empirical screening method. Other analytical advancements to overcome dereplication problems

include an Liquid Chromatography-Mass Spectometry (Genilloud *et al*. 2011). One of the advancements in analytical and spectroscopic methods involves identification and structural elucidation of secondary metabolites which are in crude extracts. Additionally, this method simultaneously profiles bioactivities of these compounds in bioassays which is a great advantage in natural products discovery (Krause & Tobin 2013).

LIMITATIONS TO THE STUDY

One of the limitations to this study was failed PCR from mDNA extracted from marine sponges (section 3.2.2.1). This is a frequently encountered problem when mDNA from marine sponges is as the template for PCR (Kennedy *et al*. 2008; Vargas *et al*. 2012) with the likely causes being the age of sponge material (Salgado *et al*. 2007) or the secondary metabolites found in marine sponges (Vargas *et al*. 2012). Further purification methods were employed as a result of PCR failures which let to successful PCR amplifications. T-RFLP analysis is known to achieve superficial results and mainly detects dominant taxa in diverse samples which oversimplify diversities in samples. As a result, duplicate PCRs were carried out for all samples and the amplicons were pooled together in efforts to reduce biases introduced by PCR (Stres 2006) and T-RFLP analyses (Schütte *et al*. 2008). However, these techniques remain useful and continue to be used in different diversity studies (Erwin *et al*. 2012; Pita *et al*. 2013; Olson *et al*. 2014; Maropola *et al*. 2015).

The name "One Strain Many Compounds" is self-explanatory and indicates that this principle is best applied to the analysis of a single microbe or a minimal number of microbes. The principle aims to culture microbes under different conditions to ultimately trigger expression of cryptic biosynthetic pathways (Bode *et al*. 2002). While many studies have focused on *Actinobacteria* as a major potential source of novel compounds (Xi *et al*. 2012; Zotchev 2012; Diminic *et al*. 2014;

Duncan *et al*. 2015), other studies have focused on other microbes (Phelan *et al*. 2012; Lavy *et al*. 2014). Genome sequencing has shown that "silent" biosynthetic pathways in other microbes such as cyanobacteria, fungi and myxobacteria are in a similar range or even more than those of *Actinobacteria* (Gross 2007). While the OSMAC approach aims to focus on a single or very few microbes and to exploit all the conditions that would eventually trigger expression of some of the biosynthetic pathways. The present study was ambitious and used 30 sponge isolates. Moreover, co-culture along with 36 culture conditions were used in this study whereas 3 to 4 conditions are regarded as sufficient for application of the OSMAC approach (Genilloud *et al*. 2011).

Furthermore, had the matrix study being performed using a one-factor-at-a-time approach (Wang *et al*. 2011), the total number of matrix conditions would have entailed the use of 180 different media conditions (mannitol permutations for NH_4Cl: 6^2-6 = 30; mannitol permutations for $NaNO_3$: 6^2-6 = 30; overall mannitol permutations; 30 + 30 = 60; therefore, permutation for 3 carbon sources (mannitol, succinic acid and starch); would be 60 × 3 = 180). This translates into 1800 screening processes for all indicator strains (180 conditions × 5 indicator strains × 2 biological repeats = 1800). This would be extremely laborious and time consuming. However, an alternative method to overcome this bottleneck would be to employ automation using 96-deep well plates. This high throughput method has been reported in some studies and used in industry (Sánchez-Hidalgo *et al*. 2012: Graça *et al*. 2013). The present study however aimed to use a selected number of conditions (36 conditions and co-culture) to get an idea of the conditions that would likely induce bioactivities for individual sponge isolates. To ascertain if the bioactivities of PE9_72 resulted from the co-culture effects with isolates (PE9_110, PE9_124, PE7_5 and PE7_72) the five isolates can be screened on the same media Na5HM individually in pure

culture, in the same three group scenarios (Figure 4.3) and in co-cultures of PE9_72 with each of the isolates in a two organism co-culture.

The molecular triggers responsible for alternative expression of one gene which was able to change from producing a 12 membered macro-lactone methymycin to a 14 membered narbomycin by simply switching cultivation of *S. venezuelae* between two media remains unknown (Xue & Sherman 2000). This indicates that the conditions that trigger expression of secondary metabolism are very organism and pathway specific. Genome sequencing analyses revealed that some microorganisms can have over 20 uninduced biosynthetic pathways (Gross 2007; Ohnishi *et al*. 2008; Yoon & Nodwell 2014). This indicates that no matrix would be big enough to incorporate all the signals to induce activities in a huge range of microbes because the signals to induce secondary metabolism are organism and pathway specific. This also translates that even for one microbe it would be difficult to devise a matrix which would induce all the activities present in an organism that has the genetic capacity to produce 20 to 40 natural compounds (Yoon & Nodwell 2014).

One of the limitations to the present study was that all the 30 microorganisms under study had different growth rates such that during the screening process, some of their biomasses were very low. Moreover, in some cases not all isolates grew on the matrix media which could have led to a misrepresentation of the overall activities observed under the matrix conditions.

It requires a lot of effort to set up the present study which ultimately could not identify the compounds produced or if there were any novel bioactive compounds being produced. Therefore, this type of study (using a huge number of conditions in the form of a matrix on a huge number of microorganisms) is unnecessary and alternative and more informed methods are required for discovery of novel compounds. One such method would be the use of genome

sequencing to identify then select a novel pathway and subsequently induce the particular pathway under different stress conditions. Real time-PCR (RT-PCR) would be used to ascertain if the pathway is being expressed. A similar genome sequencing study was used to express genes in *S. griseus* whereupon total RNA was extracted and RT-PCR used to quantitate expression levels or selected regulator genes (Ohnishi *et al*. 2008). Another informed approach would be to select an interesting microbial isolate and select a model indicator strain to which one needs to express activity against. Thereafter apply the OSMAC approach using a different culture conditions that trigger expression of bioactivities against the model indicator strain.

CONCLUSION AND PERSPECTIVES

In the present study, culture-independent and culture-dependent studies were used to select and culture marine sponge associated bacteria. The culture-independent technique T-RFLP was successfully employed to ascertain and select two marine sponges which hosted the highest microbial diversities. Subsequently the two marine sponges were used in microbial culture studies to obtain the highest cultural diversity using a range of media treatments. The resulting microbial isolates were screened in an antibiotic overlay assay against a panel of 5 indicator strains. Some of these isolates exhibited some antibacterial activities and were selected for the second component of this study along with other microbes which did not show any activities but had interesting morphological characteristics (pigmented or looked like *actinomycetes*).

The second component of this study was to employ the OSMAC approach using a matrix of culturing conditions to determine conditions which would induce secondary metabolite production from individual isolates from previous screening. Some of the culture conditions under the matrix, especially mannitol (along with co-culture, phosphates, nitrates and heat shock)

induced most of the bioactivities in one of the isolates, PE9_72 which was identified at a *B. pumilus* in 16S rRNA gene sequencing. The matrix approach for the present study resulted in a limited number of antibacterial activities in the isolates under study, thus indicating that this approach using a lot of parameters and large number of microbes is not worthwhile to undertake.

Subsequent studies would be to use a more informed approach such as selecting a single isolate and searching for conditions that induce activity against a model indicator strain instead of a panel of indicator strains. Another option would be to use genome sequencing to identify a novel pathway and induce the pathway under different stress conditions and use RT-PCR to determine if the pathway is being induced.

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Appendices: Buffers, stock solutions, primers, PCR cycling conditions and media

Appendix 1: Buffers, Stock solutions

TE buffer (pH 8)
1 M Tris-HCl 1 mL
0.5 M EDTA 200 µL
Deionized water added up to 100 mL

Breaking Buffer
2 % Triton ×100
1 % [w/v] SDS
100 mM NaCl
10 mM Tris-HCl pH 8
1 mM EDTA

Lysis buffer
25 mM Tris-HCl pH 8
50 mM Glucose
10 mM EDTA
25 mg/ml lysozyme powder

Ampicillin stock
100 mg/ml Ampicillin in sterile dH_2O
Stored at -20° C

Cycloheximide stock
20 mg/ml in sterile water
stored at 4ºC

EDTA (0.5 M, pH 8) stock solution

EDTA salts 186.1 g
NaOH pellets approx. 20 g

CTAB/NaCl solution
10 % [w/v] CTAB
0.7 M NaCl

NaCl solution (5 M)
146.1 g
500 ml of sterile dH_2O

DNA loading buffer
20 % [v/v] Glycerol
1 % [w/v] SDS
0.1 M EDTA
0.25 % Bromophenol blue

3M Sodium Acetate

24.6 g sodium acetate anhydrous
100 mL

1× TAE buffer (pH8)
40 mM Tris acetate
1 mM EDTA
0.2 mM glacial acetic acid

Wolfe's vitamin solution

Pyridoxine hydrochloride – 10 mg
Calcium D-(+)-pantothenate – 5 mg
p-Aminobenzoic acid – 5 mg
Thioctic acid – 5 mg
Thiamine-HCl - 5 mg
Riboflavin – 5 mg
Nicotinic acid – 5 mg
Biotin – 2 mg
Folic acid – 2 mg
Vitamin B12 – 0.1 mg
dH_2O – 1 L

Wolfe's mineral solution

Nitrilotriacetic acid – 1.5 g
$MgSO_4$-$7H_2O$ – 3 g
$MnSO_4$-H_2O – 0.5 g
NaCl – 1 g
$FeSO_4$-$7H_2O$ – 0.1 g
$CaCl_2$ – 0.1 g
$CoCl_2$ – $6H_2O$ – 0.1 g
$ZnSO_4$-$7H_2O$ – 0.1 g
$CuSO_4$-$5H_2O$ – 0.01 g
$AIK(SO)_4$-$12H_2O$ – 0.01 g
Boric acid – 0.01 g
Na_2MoO4-$2H_2O$
dH_2O – 1 L

Appendix 2: General media for culturing marine sponge associated bacteria

1. Zobell Agar ¼ strength (ZBA)		1L
	yeast extract	1.25 g
	peptone	3.75 g
	NaCl	18 g
	$MgCl_2$	2 g
	KCl	0.525 g
	$CaCl_2$	0.075 g
	agar	15 g

2. Seawater Agar (SWA)		1L
	dH_2O	250 mL
	Sea Water	750 mL
	Wolfe's Mineral Solution	5 mL
	Wolfe's Vitamin Solution	10 mL
	Agar	15g

3. Complex Carbon Agar (CCA)	component in 1L	mass (g)
	Cellulose	0.5
	Xylan	0.5
	Chitin	0.5
	NH_4Cl	0.5
	NaCl	18
	$MgCl_2$	2
	KCl	0.525
	$CaCl_2$	0.075
	Agar	15

4. Medium A Agar (MAA)		mass (g)
	Glucose	10
	Peptone	5
	Yeast Extract	5
	KH_2PO_4	1
	$MgSO_4 * 7H_2O$	0.1
	NaCl	20
	Na_2CO_3	10
	Agar	15

5. Trypticase Soya Agar (TSA)		mass (g)
	TSB	3
	NaCl	18
	$MgCl_2$	2
	KCL	0.525
	$CaCl_2$	0.075
	agar	15

6. Sponge Extract Medium (SEM)	component in 1L	mass (g)
	Sponge Extract	10
	NaCl	18
	$MgCl_2$	2
	KCL	0.525
	$CaCl_2$	0.075
	Agar	15

7. Trypticase Soya Agar pH 4.5 (TS4)		mass (g)
	TSB	3
	NaCl	18
	$MgCl_2$	2
	KCL	0.525
	$CaCl_2$	0.075
	Agar	20

8. Shivji Nutrient Agar ¼ strength (SNA)		mass (g)
	Peptone	5
	Beef Extract	3
	NaCl	18
	$MgCl_2$	2
	KCL	0.525
	$CaCl_2$	0.075
	Agar	15

9. ZoBell Agar + 0.0001% Crystal Violate (ZBV)	components	mass (g)
	yeast extract	1.25
	peptone	3.75
	NaCl	18
	$MgCl_2$	2
	KCl	0.525
	$CaCl_2$	0.075
	0.1% crystal violet	1
	agar	15

10. Ashby's Nitrogen Free Media (Liquid + Light) (ANFA)	components	mass (g)
	Mannitol	15
	KH_2PO_4	0.3
	$MgSO_3$ *7 H_2O	0.3
	10% Sodium Molybdate Solution	0.1
	10% $FeCl_3$ Solution	0.05
	NaCl	18
	$MgCl_2$	2
	KCL	0.525
	$CaCl_2$	0.3
	Agar	15

11. R2A Agar (R2A)		mass (g)
	R2A Agar	15.2

12. Oatmeal Agar (ISP Media 3)		mass (g)
(OMA)	Jungle Oats Oats Easy (No sugar)	40
	Agar	15

13. BG11 (BG11)	Chemical	g per liter
Stock Solution		
1	$NaNO_3$	15
2	K_2HPO_4	2
3	$MgSO_4 * 7H_2O$	3.75
4	$CaCl_2 * 2H_2O$	1.8
5	Citric Acid	0.3
6	Ammonium ferric citrate	0.3
7	Na_2 EDTA	0.05
8	Na_2CO3	1
	component	1L
	Solution 1	100 mL
	Solution 2	10 mL
	Solution 3	10 mL
	Solution 4	10 mL
	Solution 5	10 mL
	Solution 6	10 mL
	Solution 7	10 mL
	Solution 8	10 mL
	Wolf's mineral solution	1 mL
	NaCl	18 g
	$MgCl_2$	2 g
	KCl	0.525 g
	$CaCl_2$	0.075 g

14. Modified 172F medium (172)		mass (g)/L
	Glucose	10
	Yeast Extract	5
	Starch	10
	Tryptone	5
	$MgSO_4 * 7H_2O$	2
	$CaSO_4 * 2H_2O$	2
	NaCl	18
	$MgCl_2$	2
	KCL	0.525
	$CaCl_2$	0.075
	Agar	18

15. Activated Charcoal Medium (ACM) pH 7.0		mass (g)/L
	HEPES	2.38
	Sodium pyruvate	3
	Yeast Extract	0.1
	Soybean peptone	3
	$NaNO_3$	0.34
	$KHPO_4$	0.1
	$MgSO_4 * 7H_2O$	0.15
	Activated charcoal	3
	Agar	15

16. MagMin Semi-Solid Medium (MMM)	in 1L
KH_2PO_4	0.68 g
$NaNO_3$	0.12 g
Na acetate	0.07 g
Ascorbic Acid	0.035 g
Tartaric Acid	0.37 g
Succinic Acid	0.37 g
Sodium thiosulfate	0.05 g
Resazurine Solution	0.1 ml
Cysteine Solution	1 ml
Wolf's Mineral Solution	5 ml
FeQuinate Solution	1 ml
Agar	2 ml

17. Pringsheim's Cyanobacteria Specific Medium (PCS)	g/L
KNO3	0.2
$(NH_4)_2HPO_4$	0.02
$MgSO_4 * 7H_20$	0.01
$CaCl_2 * 2H_2O$	0.005
$FeCl_2$	0.5mg
NaCl	18
$MgCl_2$	2
KCl	0.525
$CaCl_2$	0.075
Agar	12

18. Planctomycetes Medium (PMM)	g/L
Glucose	1
$(NH_4)_2SO_4$	0.25
Peptone	0.15
Yeast Extract	0.15
NaCl	18
$MgCl_2$	2
KCl	0.525
$CaCl_2$	0.075
Agar	12

19. Actinomyces Isolation Medium (AIM)	g/L
Actinomyces Isolation Agar	22
NaCl	18
$MgCl_2$	2
KCl	0.525
$CaCl_2$	0.075

20. Starch Casein Nitrate Agar (SCN) pH 8		g/L
	Starch	10
	Casein	0.3
	KNO_3	2
	$MgSO_4.7H_2O$	0.05
	$CaCO_3$	0.3
	$FeSO_4.7H_2O$	0.01
	Agar	15

21. Yeast Extract - Malt Extract (YEME)		g/L
pH 7.3	Yeast Extract	4
	Malt Extract	10
	Glucose	4
	Agar	20

22. Glucose Yeast Media (GYM)		g/L
	D-Glucose	4
	Yeast Extract	4
	Malt Extract	10
	$CaCO_3$	2
	NaCl	24
	$MgCl_2$	5.3
	KCl	0.7
	$CaCl_2$	0.1
	Agar	15

23. Oatmeal Agar (ISP Media 3) + salt OMA		g/L
	Jungle Oats Oats Easy (No sugar)	40
	NaCl	18
	Agar	15

General purpose media

Luria-Bertani medium	**g/L**
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Agar	15 g

Soft agar	**g/L**
Tryptone	10
Yeast extract	5
NaCl	10
Agar	7.5

Luria-Bertani (LB)	**g/L**
Tryptone	10
Yeast extract	5
NaCl	10

Appendix 3 Matrix media

Group 1

Ammonium 0.1 Phosphate mannitol (A1M)	mass (g)/L
NH_4Cl_2	1.07
KH_2PO_4	0.068 in 1 L use 200 µL
Mannitol	1
NaCl	18
$MgCl_2$	2
KCl	0.525
$CaCl_2$	0.075
HEPES	2.38
	1L

Ammonium 0.5 Phosphate Mannitol (A5M)	mass (g)
NH_4Cl2	1.07
KH_2PO_4	0.068
Mannitol	1
NaCl	18
$MgCl_2$	2
KCl	0.525
$CaCl_2$	0.075
HEPES	2.38
	1L

Sodium Nitrate 0.1 Phosphate Mannitol (Na1M)	mass (g)
$NaNO_3$	0.2
KH_2PO4	0.068 in 1 L use 200 µL
Mannitol	1
NaCl	18
$MgCl_2$	2
KCl	0.525
$CaCl_2$	0.075
HEPES	2.38
	1L

Sodium Natrate 0.5 Phosphate Mannitol (Na5M)	mass (g)
$NaNO_3$	0.2
KH_2PO_4	0.068
Mannitol	1
NaCl	18
$MgCl_2$	2
KCl	0.525
$CaCl_2$	0.075
HEPES	2.38
	1L

Group 2

Ammonium 0.1 Phosphate Succinic acid (A1Sa)		mass (g)
	NH_4Cl_2	1.07
	KH_2PO_4	0.068 in 1 L use 200 µL
	S. acid	0.5
	NaCl	18
	$MgCl_2$	2
	KCl	0.525
	$CaCl_2$	0.075
	HEPES	2.38
		1L

Ammonium 0.5 Phosphate Succinic Acid (A5Sa)		mass (g)
	NH_4Cl_2	1.07
	KH_2PO_4	0.068
	S. acid	0.5
	NaCl	18
	$MgCl_2$	2
	KCl	0.525
	$CaCl_2$	0.075
	HEPES	2.38
		1L

Sodium Nitrate 0.1 Phosphate Succinic acid (Na1Sa)		mass (g)
	$NaNO_3$	0.2
	KH_2PO_4	0.068 in 1 L use 200 µL
	S. acid	0.5
	NaCl	18
	$MgCl_2$	2
	KCl	0.525
	$CaCl_2$	0.075
	HEPES	2.38
		1L

Sodium Nitrate 0.5 Phosphate Succinic acid (Na5Sa)		mass (g)
	$NaNO_3$	0.2
	KH_2PO_4	0.068
	S. acid	0.5
	NaCl	18
	$MgCl_2$	2
	KCl	0.525
	$CaCl_2$	0.075
	HEPES	2.38
		1L

Group 3

Ammonium 0.1 Phosphate Starch (A1S)		mass (g)
	NH_4Cl_2	1.07
	KH_2PO_4	0.068 in 1 L use 200 µL
	Starch	1.71
	NaCl	18
	$MgCl_2$	2
	KCl	0.525
	$CaCl_2$	0.075
	HEPES	2.38
		1L

Ammonium 0.5 Phosphate Starch (A5S)		mass (g)
	NH_4Cl_2	1.07
	KH_2PO_4	0.068
	Starch	1.71
	NaCl	18
	$MgCl_2$	2
	KCl	0.525
	$CaCl_2$	0.075
	HEPES	2.38
		1L

Sodium Nitrate 0.1 Phosphate Starch (Na1S)		mass (g)
	$NaNO_3$	0.2
	KH_2PO_4	0.068in 1L use 200uL
	Starch	1.71
	NaCl	18
	$MgCl_2$	2
	KCl	0.525
	$CaCl_2$	0.075
	HEPES	2.38
		1L

Sodium Nitrate 0.5 Phosphate Starch (Na5S)		mass (g)
	$NaNO_3$	0.2
	KH_2PO_4	0.068
	Starch	1.71
	NaCl	18
	$MgCl_2$	2
	KCl	0.525
	$CaCl_2$	0.075
	HEPES	2.38
		1L

Appendix 4: Primers and PCR cycling conditions

Table 5.1: PCR cycling conditions

Polymerase	Target gene	Primer Set	Sequence (5' to 3')	Cycling conditions	Reference
Phusion	16S rRNA gene/ T-RFLP PCR	E9F_FAM	GAGTTTGATCCTGGCTCAG	initial denaturation 98 ºC for 3 min, 35 cycles of 98 ºCfor 20 sec, 54 ºCfor 30 sec, 72 ºCfor 1 min, final elongation step of 72 ºC for 5 min	(Ramond *et al*. 2013)
		U1510R	GGTTACCTTGTTGTTACACTT		
Dream Taq	16S rRNA gene	E9F_FAM	GAGTTTGATCCTGGCTCAG	initial denaturation 95 ºC for 5 min, 35 cycles of 95 ºC for 45 sec, 55 ºC for 45 sec, 72 ºC for 1.30 min and final elongation of 72 ºC for 10 min	(Ramond *et al*. 2013)
KAPA2G Robust ReadyMix				initial denaturation 95 ºC for 3min, followed by 35 cycles of 95 ºC 15 sec, 60 ºC for 15 sec, 72 ºC for 1.30 min, and final extension of 72 ºC for 5min	
KAPA2G Fast		U1510R	GGTTACCTTGTTGTTACACTT	initial denaturation 95 ºC for 3min, followed by 35 cycles of 95 ºC for 15 sec, 60 ºC for 15 sec, 72 ºC for 5 sec, and final elongation of 72 ºC for 2 min	(Ramond *et al*. 2013)
Dream Taq	Vectors	pJET 1.2 forw.	CGACTCACTATAGGGAGAGCGGC	initial denaturation 95 ºC for 5 min, 35 cycles of 95 ºC for 45 sec, 55 ºC for 45 sec, 72 ºC for 1.30 min and final elongation of 72 ºC for 10 min	Fermentas
		pJET1.2 rev.	AAGAACATCGATTTTCCATGGCAG		Fermentas