COMPARATIVE STUDY OF THE ANTIBIOTIC PRODUCING POTENTIALS OF THREE FRESHWATER ACTINOMYCETES BELONGING TO THE *SACCHAROPOLYSPORA* AND *ACTINOSYNNEMA* GENERA.

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DECLARATION

I, the undersigned, declare that this thesis and the work contained herein being submitted to the University of Fort Hare for the degree of Master of Science in Microbiology in the Faculty of Science and Agriculture, School of Science and Technology, is my original work with the exception of the citations. I also declare that this work has not been submitted to any other university in partial or entirety for the award of any degree.

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DATE

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Crude extracts of three actinomycetes species belonging to *Saccharopolyspora* (TR 046 and TR 039) and *Actinosynnema* (TR 024) genera were screened for antibacterial activities against a panel of several bacterial strains. The extracts showed antibacterial activities against both Gram-negative and Gram-positive test bacteria with inhibition zones ranging from 8 to 28 mm for extract obtained from TR 046; 8 and 15 mm for extract obtained from TR 039 and 10 to 13 mm for extract obtained from TR 024. The minimum inhibitory concentrations ranged from 0.078 to 10 $mgml^{-1}$ for extract obtained from TR 046; 5 and >10 $mgml^{-1}$ for extract obtained from TR 039 and 1.25 and 5 $mgml^{-1}$ for extract obtained from TR 024. The bactericidal activity of extract obtained from TR 046 was evaluated against 5 test bacteria with different susceptibilities to the extract by time-kill assay. The extract showed strong bactericidal activity against *Bacillus pumilus* (ATCC14884) reducing the bacterial load by 10^4 $cfuml^{-1}$ and 10^2 $cfuml^{-1}$ at 4 × MIC and 2 × MIC respectively after 6 hr of exposure. It also showed good bactericidal activity against *Proteus vulgaris* (CSIR 0030) achieving a $0.9\log_{10}$ and $0.13\log_{10}$ $cfuml^{-1}$ reduction at 5 $mgml^{-1}$ (4 × MIC) and 1.25 $mgml^{-1}$ (2 × MIC) respectively after 12 hr of exposure. The extract was however weakly bactericidal against two environmental bacterial strains *Klebsiella pneumoniae* and *Staphylococcus epidermidis*, and against *Pseudomonas aeruginosa* (ATCC 19582), the extract showed bacteriostatic activity across all MIC ranges used. The test actinomycetes appear to have immense potential as a source of new antibacterial compound(s).

CHAPTER I

INTRODUCTION

Actinomycetes are Gram positive bacteria with a high G+C (>55%) in their DNA (Lechevalier and Lechevalier, 1970). Most are free living, saprophytic bacteria found widely distributed in soil and water (Pandey *et al.,* 2008). Morphologically they resemble fungi but physiologically they resemble bacteria (Sultan *et al.,* 2002). Actinomycetes are noteworthy for their antibiotic production, producing more than 70% of all currently known antibiotics (Sathi *et al.,* 2001; Hongjuan *et al.,* 2006). Though they can grow on a wide variety of organic nutrients, synthetic media are more preferable for manipulative purposes (Sathi *et al.,* 2001). Their growth is characterised by small, compact, soft to leathery colonies tenaciously adhering to the medium, the surface being either raised or flat (Sathi *et al.,* 2001). Some actinomycetes colonies have smooth surfaces while others have folded surfaces (Muiru *et al.,* 2008). The colour of the mycelia can range from nearly colourless to white, chalky red, grey or olive (Mustafa *et al.,* 2004; Mutitu *et al.,* 2008) due to pigment production.

Actinomycetes have major socioeconomic importance. They include human pathogens such as *Actinomyces israelii*, a primary cause of tooth decay and other often serious, infections in humans (Beaman, 1981; Berkow and Fletcher, 1992). Non-pathogenic strains play essential roles as decomposers in terrestrial systems. These actinomycetes are vital to nutrient recycling and are among a small number of organisms capable of breaking down complex organic materials such as chitin (Lacey, 1973). Actinomycetes in the genus *Frankia* are also extremely important to

numerous plant genera, acting as root-nodulating, nitrogen-fixing plant symbionts (Huss-Danell, 1997). Many genera, notably *Streptomyces*, produce commercially important antibiotics (Goodfellow and O'Donnell, 1989) and an array of other secondary metabolites. These include aliphatic alcohols, lactones, biogenic sulphides, ketones, esters, thioesters, lactones, furanones and isoprenoids (Gerber, 1983; Wilkins and Parkallé, 1996; Jáchymová *et al*., 2002; Schöller *et al*., 2002). Early recognition of the potential application of many of these compounds has resulted in extensive research into their use as antibiotics, enzymes, enzyme inhibitors and other pharmaceutically useful compounds (Goodfellow and O'Donnell, 1989).

Antibiotics produced by actinomycetes are normally composed of heterogeneous and biologically active compounds (Mustafa *et al.,* 2004). To fully utilise them, it is necessary to characterise them by separating them in solvent systems, conducting bioassays and ascertaining their stability under different physical and chemical conditions (Mutitu *et al.,* 2008).

The success story that characterised the discovery and subsequent production of antibiotics by actinomycetes was however short lived as soon antibiotic resistant pathogens began to emerge. There has been an astronomic increase in the number of clinically important bacterial pathogens that are becoming resistant to the antibiotics that are currently in use (Parungawo *et al.,* 2007). According to the World Health Organisation, over prescription and improper use of antibiotics has led to the resistance of many pathogens. A survey carried out by the Centres for Disease Control and Prevention showed that between 1979 and 1987, only 0.02% of *Pneumococcus* strains infecting a large number of patients were penicillin resistant

but by June 1994, 6.6% of the *Pneumococcus* strains were penicillin resistant (CDCP, 2003). Such resistance might be related to the overuse of antibiotics.

In the opinion of McGowan (2001), the increased prevalence of antibiotic resistance is an outcome of evolution. The antibiotic does not technically cause the resistance but allows it to happen by creating a situation where an already existing variant can flourish, i.e., antibiotics apply selective pressure. Ruder, (2004) somewhat supports McGowan's view by suggesting that bacteria have always possessed the ability to protect themselves from naturally occurring antibiotics by acquiring resistance through the exchange of genetic material with other bacteria.

According to Lawson and Lawson (1998), antibiotic resistance in pathogens can be caused by indiscriminate use of antibiotics by humans. Common examples include erroneous antibiotic prescriptions for non-bacterial infections (Martin and Demain, 1980) and the addition of antibiotics to livestock feed and cleaning agents (Ruder, 2004) which creates a reservoir of antibiotic-resistant bacteria. However, the answer to the problem of antibiotic resistance lies partly on the potential of actinomycetes to produce antibiotics with novel properties which could see the problem of resistance being overcome. This research work focused on two freshwater actinomycete genera which were isolated from the Tyume River in the Eastern Cape Province by the Applied and Environmental Microbiology Research Group (AEMREG) at the University of Fort Hare. The two actinomycete genera were tentatively identified as *Saccharopolyspora* (TR046 and TR039) and *Actinosynnema* (TR024).

1.2 Freshwater actinomycetes

In recent years there has been a growing awareness of the potential value of freshwater habitat as source of actinomycetes that produce secondary metabolites of clinical importance (Rifaat, 2003). Some investigators emphasise that freshwater habitats are fruitful as those isolated organisms are from terrestrial locales (Cross, 1981). A review of literature reveals that little is known concerning the actinomycetes exhibiting antimicrobial properties from this habitat. The list of novel actinomycetes and products derived from poorly explored areas of the world stresses the importance of investigating new habitats (Nolan and Cross, 1988). New sources of actinomycetes would be welcome and as such, one useful approach adopted in this research was to isolate actinomycetes from the untapped waters of the Tyume River which is characterised by the presence of heterogeneous mass of soil, vegetation and organic matter in different stages of decay.

1.2.1 *Actinosynnema.*

Actinosynnema is an aerobic actinomycete that was first isolated from a grass (*Carex* species) blade in Shiga Prefecture, Japan, in September 1976 (Hasegawa *et al*., 1983). It was observed to form synnemata (synnemata, also called coremia, are compacted groups of erect hyphae which are often fused and which bear conidia at their apex only or at both their apex and on their sides. Sterile columnar hyphal structures are sometimes also called synnemata or coremia) with zoospores (Hasegawa *et al*., 1978). The new organism deferred significantly from previously described species of actinomycetes that it could not be accommodated in any of the

previously described genera of the actinomycetes. It was proposed to include it in a new genus, *Actinosynnema,* the type species of which is *A. mirum***.**

Actinosynnema has fine hyphae (about 0.5 mm in diameter) which form a mycelium differentiated into: (i) a substrate mycelium that penetrates the agar and also grows into and forms synnemata or domelike bodies on the surface of agar; and (ii) an aerial mycelium that arises from the synnemata, domelike bodies, or flat colonies. The aerial hyphae bear chains of conidia capable of forming flagella in an aqueous environment. The cell wall is of type 111**,** and the whole-cell sugar pattern is of type C (Higashide *et al*., 1977).

Its substrate mycelium penetrates the agar medium and form synnemata or domelike bodies that are at first whitish on most media and then become yellowish or yellowish orange (Arai *et al.,* 1979)**.** The aerial mycelium is made of hyphae that are white to pale yellow on most media. In young, growing cultures, the tips of the aerial hyphae often curl (Jones, 1949)**.** The cells have cell walls of type 111 containing meso-diaminopimelic acid in addition to alanine, glutamic acid, and glucosamine (Suput *et al*., 1967). Galactose and mannose are present in whole-cell hydrolysates. Madurose is absent. The organism can thus be considered to have a type C whole-cell sugar pattern.

Actinosynnema produces a pale yellowish-brown soluble pigment on tyrosine agar and a pale greenish one on oatmeal agar. It does not produce any diffusible pigments on other media. When antibiotic properties of *A. mirum* were investigated, zones of inhibition by cross-streak test were produced with Gram positive bacteria *Bacillus megaterium*, *Sarcina lutea*, *Mycobacterium smegmatis*, filamentous fungi *Aspergillus niger* (incomplete inhibition), *Penicillium notatum* (incomplete inhibition)

and yeasts *Candida tropicalis* and *Saccharomyces cerevisae*, (Hasegawa *et al*., 1978). This served as proof enough that *Actinosynnema* does produce some antimicrobials. Recent information about this actinomycete is however very scarce and more still needs to be done in terms of research in order to exploit this actinomycete to the maximum possible potential.

1.2.2. *Saccharopolyspora*

The genus *Saccharopolyspora* was created in 1975 by Lacey and Goodfellow (Lacey and Goodfellow, 1975) to describe isolates from sugar cane bagasse which grew at 40°C and produced white aerial mycelia with beadlike chains of spores enclosed in a characteristic hairy sheath. Species of this genus are known to have G+C contents of 77% (Lechevalier *et al.,* 1986). The bagasse isolates described as *Saccharopolyspora hirsuta* had fragmenting hyphae and wall chemotype IV and degraded casein, tyrosine, hypoxanthine, and xanthine. The chemical properties included a type A whole-cell sugar pattern (Potvin and Pringer, 1993), the absence of mycolic acids, the presence of saturated and branched fatty acids, a type PI11 phospholipid pattern, and MK-9(H4) as the major menaquinone. Currently, there over 21 described species belonging to the genus *Saccharopolyspora* though the most extensively studied is *S. erythraea* which is an industrial producer of the antibiotic erythromycin. Table 1.1 shows a list of some of the *Saccharopolyspora* species that have been positively identified to date. Fig 1.1 shows the characteristic growth pattern of *Saccharopolyspora* colonies on agar plates.

Source: genomenewsnetwork.org.

www.wnvis.kuenvbiotech.org/bacteria.htm

Fig1.1. Appearance of *Saccharopolyspora* colonies on Starch Casein Agar.

Table 1.1. Positively identified species of the genus *Saccharopolyspora*

Species	Colour of substrate mycelium	Colour of aerial mycelium	Antibacterial produced	Reference
Saccharopolyspora hirsuta	Colourless to orange	white	ND	Korn-Wendisch *et al.* (1989).
Saccharopolyspora taberi	Yellowish to orange red	No aerial mycelium	ND	Korn-Wendisch *et al*. (1989).
Saccharopolyspora erythraea	Light brown to orange red	Pinkish red	erythromycin	Labeda (1987).
Saccharopolyspora rectivirgula	Yellow to orange	White to light pink	ND	Korn-Wendisch *et al*. (1989).
Saccharopolyspora gregorii	Brown to reddish-brown	Olive-grey	ND	Goodfellow *et al.* (1989).
Saccharopolyspora flava	Yellowish to-light brown	White to greyish	ND	Lu *et al*. (2001).
Saccharopolyspora cubuensis	Colourless to white	White to grey	ND	Pimentel-Elardo *et al*. (2008).
Saccharopolyspora antimicrobica	Reddish brown to orange	grey	ND	Yuan *et al.* (2008).
Saccharopolyspora jiangxiensis	Orange to yellowish orange	Pinkish white	ND	Zhang *et al*. (2009).
Saccharopolyspora gijiaojingesis	Light brown to darkish brown	Reddish brown	ND	Tang *et al.* (2009).
Saccharopolyspora rosea	White to grey	white	ND	Yassin (2009).
Saccharopolyspora shandongensis	colourless	White to grey	ND	Zhang *et al*. (2008).
Saccharopolyspora spinosa	Dark brown to yellowish	Yellowish orange	ND	Mertz and Yao, (1990).
Saccharopolyspora spinosporotrichia	Orange	Pinkish-red	ND	Zhou *et al*. (1998).
Saccharopolyspora thermophila	Brown to reddish brown	Grey to whitish-grey	ND	Lu *et al*. (2001).

Key: ND means not documented

1.3. Aims and objectives

The broader aim of this research was to investigate the antibiotic producing potentials of some indigenous freshwater actinomycetes belonging to the genera *Saccharopolyspora* and *Actinosynnema*, isolated from the Tyume River in the Eastern Cape Province of South Africa. The specific objectives of the study were;

- To prepare crude extracts of the Saccharopolyspora and Actinosynnema actinomycetes isolates
- To prepare the extracts for antibacterial activities against a group of bacterial strains
- To determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the extracts
- To determine the extracts' rate of kill using the time-kill assay
- To compare the activities of the extracts with that of standard antibiotics using the MIC and rate of kill values.

1.4. *Rationale of the study*

Nowadays, new resistant strains of pathogens are emerging more quickly while the rate of discovery of new antibiotics is slowing down (Parungawo *et al.,* 2007; Hongjuan *et al.,* 2006). While research work aimed at discovering new antibiotics has been going on for some time now, indigenous actinomycetes especially in the Eastern Cape Province of South Africa remain largely underexplored and therefore underutilised as a potential source of novel antibiotics. There is still need to speed up

research work especially with freshwater actinomycetes since terrestrial actinomycetes have been extensively explored and exploited and the chances of new antibiotics being discovered using them are growing slimmer. In Southern Africa, antibiotic producing actinomycetes have not been well investigated (Rifaat, 2003).

CHAPTER II

LITERATURE REVIEW

2.1. Antibiotic production by actinomycetes: a general overview

Natural products isolated from microorganisms have been the source of most of the antibiotics currently on market (Pelaez, 2006). In a broader sense, an antibiotic is defined as a chemotherapeutic agent that inhibits or abolishes the growth of microorganisms, such as bacteria, fungi or protozoa (Kümmerer, 2009). However, the classical definition of an antibiotic is a compound produced by a microorganism which inhibits the growth of another microorganism (Kümmerer and Henninger, 2003). One of the most basic questions to address in any discovery effort of new natural antibiotics is, “Which groups of organisms should be selected to improve the probability of success?”. The discovery of penicillin and its use in the clinic in the 1940s was soon followed by the discovery of a huge number of antibiotics from microbes, in particular members of the actinomycetes and fungi (Strohl *et al*., 2001).

Many antibiotics discovered until the early 1970s reached the market, and their chemical scaffolds were later used as leads to produce new generations of clinically useful antibiotics by chemical modification (Overbye and Barrett, 2005). Antibiotics can be grouped by either their chemical structure or mechanism of action. They are a diverse group of chemicals that can be divided into different sub-groups such as ß-lactams, quinolones, tetracyclines, macrolides, sulphonamides and others (Table 2.1). They are often complex molecules which may possess different functionalities within the same molecule. Therefore, under different pH conditions antibiotics can be neutral, cationic, anionic, or zwitterionic. Because of the different

functionalities within a single molecule, their physico-chemical and biological properties such as sorption behaviour, photo reactivity and antibiotic activity and toxicity may change with pH (Cunningham, 2008). Microorganisms are the most attractive source for the production of medically useful secondary metabolites. Many microorganisms can be grown to large scales in culture media, facilitating an unlimited and uninterrupted supply of the raw material needed for drug development.

Table 2.1-Important classes and groups of antibiotic compounds.

Class	Group	Subgroup	Example
β-lactams	Penicillins	Benzyl-penicillins	Phenoxypenicillin
		Isoxazolylpenicillins	Oxacillin
		Aminopenicillins	Amoxicillin
		Carboxypenicillins	carbenicillin
		Acylaminopenicillins	Piperacillin
	Cephalosporins	Cephazolin group	Cephazolin
		Cefuroxim group	Cefuroxim
		Cefotaxim group	Cefotaxim
		Cefalexin group	Cefprozil
	Carbpenems	-	Meropenem
Tetracyclines	-	-	Doxycycline
Aminoglycosides	-	-	Gentamycin
Macrolides	-	-	Erythromycin A
Glycopeptides	-	-	Vancomycin
Sulfonamides	-	-	Sulfamethoxazole
Quinolones	-	-	Ciprofloxacin

Source: Kümmerer (2009)

Actinomycetes are noteworthy for their antibiotic production, producing more than 70% of all currently known antibiotics (Sathi *et al.,* 2001; Hongjuan *et al.,* 2006). However it is critically important to find new antibiotic classes because of the increasing incidence of resistant pathogens, which, if we do not invest heavily in discovering and developing new antibiotic classes, we might well end up in a situation akin to the pre-antibiotic era (Pelaez, 2006). It is estimated that the number of antibiotics characterized to date represents less than 5% of the total. In other words, the chemical universe produced by actinomycetes remains largely underexploited (Singh and Pelaez, 2008). New compounds belonging to novel structural classes and possessing unprecedented biological properties can be discovered from microorganisms, whereas this is perhaps less probable with plants. In addition, the improvement of cultivation techniques together with the refinement of highly sensitive analytical methods should help the rapid dereplication of compounds (Lang *et al*., 2008; Koehn, 2008). The promises of structural novelty available only by exploring the world of secondary metabolites may well justify the investment of time and resources in the field of antibiotic discovery (Koehn and Carter, 2005). The failure of the paradigm based on synthetic compounds using target-based *in vitro* screens provides a window of opportunity for natural products as the source for the next generation of antibiotics (Pelaez, 2006). One could question why it has become so difficult to discover new antibiotics from natural products. It is generally accepted that the number of antibiotics in nature is vast (Watve *et al*., 2001). The percentage of actinomycete and fungal strains producing antimicrobial activities in standard agar diffusion assays ranges between 30 and 80%, depending on the ecological or taxonomic groups (Basilio *et al*., 2003; Pelaez and Genilloud, 2003). Fig 2.1 shows the process of antibiotic discovery from microbial natural products.

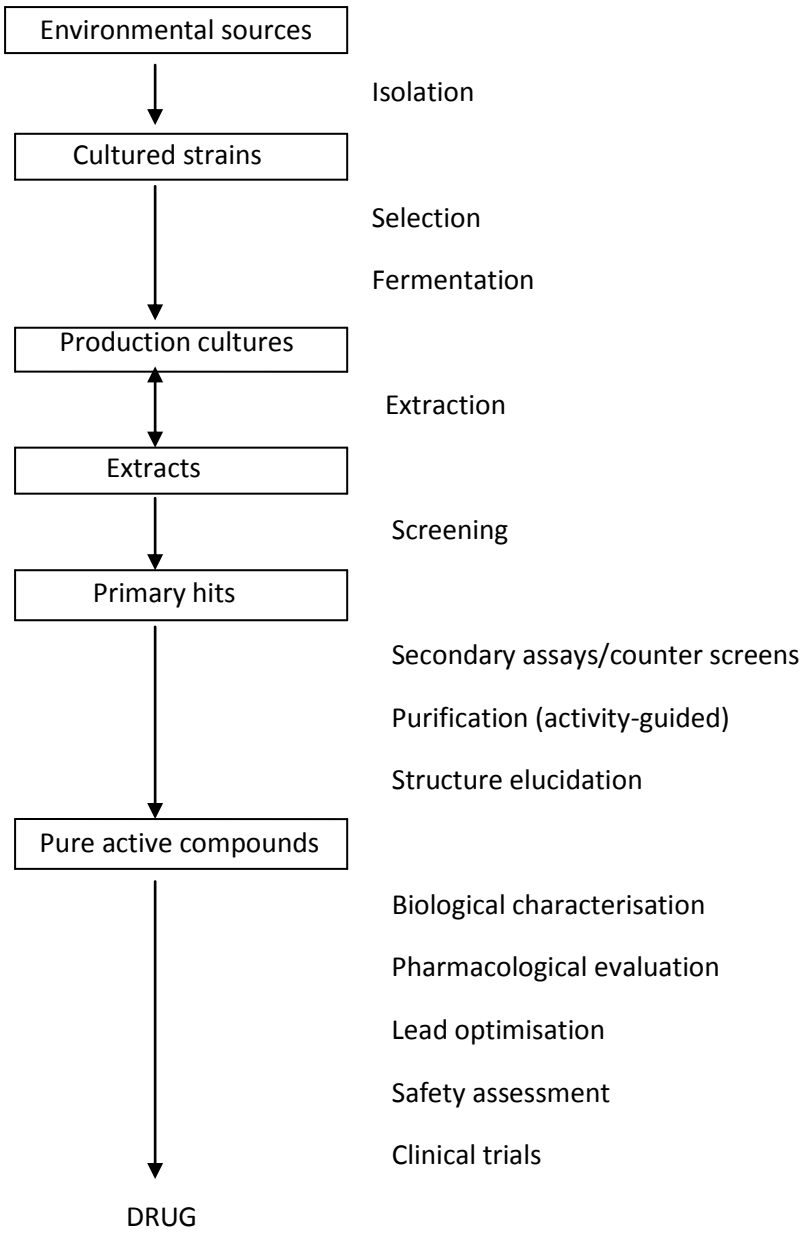

Fig 2.1 The process of antibiotic discovery from microbial natural products (Adapted from Pelaez, 2006).

Success in discovering new antibiotics from microbial natural products requires having a given microorganism grown in conditions appropriate to induce the production of the desired metabolite, which is then extracted and tested in a screen able to detect this as a hit (Fig 2.1). This compound has to be isolated from the original mixture and identified (Pelaez, 2006). Antibiotics produced by actinomycetes are normally composed of heterogeneous and biologically active compounds (Mustafa *et al.,* 2004). To fully utilise them, it is necessary to characterise them by separating them in solvent systems, conducting bioassays and ascertaining their stability under different physical and chemical conditions (Mutitu *et al.,* 2008). Actinomycetes are the strongest antagonists among microorganisms. The antibiotic substances they produce display antibacterial, antifungal, antitumor, antiprotozoic, and antiviral properties (Miyadoh, 1993). Actinomycetes have traditionally been the most prolific group in antibiotic production, and have been the origin of a good number of marketed antibiotics (Table 2.2).

Fungi are another rich source of antibiotics though only a few examples have reached the market. These two microbial groups have been the focus of most of the efforts by industrial and academic laboratories since the discovery of penicillin. It could be argued that since they have already been studied so extensively, the chances of finding anything new are too low to be worth the effort (Pelaez, 2006). However, the evidence is that only a minor fraction of all the species or genetically distinct strains of actinomycetes and fungi existing in nature have been grown in culture (Watve *et al*., 2001). New species and even major taxa of both fungi and actinomycetes are being discovered, opening windows of opportunity and proving that our knowledge of these microorganisms is far from exhaustive.

Table 2.2. Examples of marketed antibiotics originating from microbial natural products

Original metabolite	Commercial products	Producing organism
Penicillins	Penicillin G, V; ampicillin; Methicillin; Amoxicillin; Carbenicillin	*Penicillium spp., Aspergillus spp*
Cephalosporins	Mefoxin, Ceclor (Cefaclor)	*Acremonium*
	Claforan, Rocephin (Ceftriaxone)	*Amycolaptopsi lactamdurans*
	Ceftin (cefuroxime)	*Streptomyces clavurigerus*
Thienamycin	Primaxin, Invaz	*Streptomyces cattleya*
Erythromycin	Erythromycin, Zithromax, Blaxin, Ketek	*Saccharopolyspora erythraea*
Vancomycin	Vancolin	*Streptomyces arientalis*
Fosfomycin	Monuril	*Streptomyces fradiae*
Mupirocin	Bactroban	*Pseudomonas fluorenscens*
Fusidic Acid	Fusidin Leo	*Fusidium griseum*
Streptogramins	Synercid	*Streptomyces pristinaespiralis*
Daptomycin	Cubicin	*Streptomyces raseosporus*

Source: Pelaez (2006)

Aquatic microbes are particularly attractive because they have not been as extensively exploited as their terrestrial counterparts, and because of the high potency required for bioactive compounds to be effective in the aquatic environment, due to the diluting effect of water (Zhang *et al*., 2005). Furthermore there is compelling evidence that compounds formerly attributed to marine invertebrates are actually synthesised by bacterial symbionts (Piel *et al*., 2004). Overall, it is clear that expanding the diversity of actinomycetes and fungi is feasible and can be achieved by exploring little explored ecological niches and developing new ways of growing previously uncultivable strains (Harvey, 2000). Microbial natural products still appear as the most promising source of future antibiotics that society is expecting. Natural antibiotics have been shaped by evolution to make them effective in killing microorganisms as a competitive tool. Still, the field is huge and underexplored in terms of microbial diversity and the type of secondary metabolites that they can produce. In the areas of cancer and infectious disease, 60% and 70% of new drugs, respectively, originated from natural sources between 1981 and 2002 (Newman, 2003). Between 2001 and 2005, 23 new drugs derived from natural products were introduced for the treatment of disorders such as bacterial and fungal infections, cancer, diabetes, dyslipidemia, atopic dermatitis, Alzheimer's disease and genetic diseases such as tyrosinaemia and Gaucher disease (Lam, 2006).

Numerous microbial natural products have been identified that potentially represent new classes of antibiotics with unique modes of action and properties that are different from the currently used drugs, some examples are given in Table 2.3. Natural product databases contain many more scaffolds than synthetic ones; such unexploited scaffolds represent promising new starting points in drug discovery.

Recent research estimates that natural drug products have ~100-fold higher hit rate compared to synthetic compounds (Berdy, 2005). Whereas synthetic drugs are typically the result of numerous structural modifications over the course of an extensive drug discovery program, a natural product can go straight from 'hit' to drug (Lam, 2006). Microbial natural products are notable not only for their potential therapeutic activities, but also for the fact that they frequently have desirable pharmacokinetic properties required for clinical development. Antibacterial agents erythromycin A, vancomycin, penicillin G, streptomycin and tetracycline; antifungal agents amphotericin B and griseofulvin; the cholesterol-lowering agent lovastatin; anticancer agents daunorubicin, mitomycin C and bleomycin; and immunosuppressants rapamycin, mycophenolic acid and cyclosporine A are all examples of natural products that reached the market without requiring any chemical modifications (Lam, 2006).

2.2. Limitations to the presently used regime of antibiotics

The antibiotic era is threatened by a convergence of three adverse developments. The first is growing evidence of high levels of antibiotic resistance among important pathogens, including vancomycin resistant *Enterococci* (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-intermediate/resistant *S. aureus* (VISA/VRSA), and multidrug-resistant (MDR) *Pseudomonas aeruginosa*, which have emerged in the past 20 years (Wenzel, 2004). The second is the limited number and disparate availability of classes of effective antibiotics against diverse bacterial species. The final concern is a reduction in the number of pharmaceutical companies pursuing research and development of new anti-infectives since 1985 (Wenzel, 2004).

2.2.1. *Antibiotic resistance in important pathogens*

Ever since the discovery and subsequent clinical use of antibiotics, resistance to these agents have been observed, with a commensurate negative impact on the treatment of infectious diseases. Antibiotic resistance is now well recognized as a major problem in the treatment of infections in hospitals and, with increasing and alarming frequency, in the community. Resistance can be active (i.e., the result of a specific evolutionary pressure to adapt a counterattack mechanism against an antibiotic or class of antibiotics) or passive (where resistance is a consequence of general adaptive processes that are not necessarily linked to a given class of antibiotic; e.g., the nonspecific barrier afforded by the outer membrane of Gram-negative bacteria) (Wright, 2005). Bacteria achieve active drug resistance through three major mechanisms:

(1) Efflux of the antibiotic from the cell via a collection of membrane-associated pumping proteins; this is one of the ingenious mechanisms that microorganisms use to evade the toxic effects of antibiotics. Particularly intriguing are the so-called multidrug transporters, which have specificity for compounds with very different chemical structures and cellular targets. In a study carried out by Monique *et al*., (2000), they found out that a single ATP-dependent multidrug transporter of *Lactococcus lactis*, a bacterium used in dairy fermentations confers resistance to a record of eight classes of clinically relevant broad-spectrum antibiotics. Although most microbial multidrug efflux systems known to date mediate drug-proton exchange (Paulsen *et al*., 1996), the Lactococcal multidrug transporter LmrA uses the free energy of ATP hydrolysis to drive the export of toxic compounds from the inner leaflet of the cytoplasmic membrane (Bolhuis *et al*., 1996). LmrA, a 590-amino-

acid integral membrane protein, is a member of the ATP-binding Cassette (ABC) Superfamily (Higgins, 1992; van Veen *et al*., 1996). LmrA shares significant sequence identity with the hop resistance protein HorA of the beer-spoilage bacterium *Lactobacillus brevis*, and with ABC transporters in pathogenic microorganisms (van Veen and Konings, 1998). Furthermore, LmrA is a structural and functional homologue of the human multidrug transporter P-glycoprotein (van Veen *et al*., 1998).

(2) Modification of the antibiotic target (e.g., through mutation of key binding elements such as ribosomal RNA or even by reprogramming of biosynthetic pathways such as in resistance to the glycopeptide antibiotics); and

(3) Via the synthesis of modifying enzymes that selectively target and destroy the activity of antibiotics. All of these mechanisms require new genetic programming by the cell in response to the presence of antibiotics (Levy, 2002; Abraham and Chain, 1940). Table 2.3 shows some enzymatic strategies of antibiotic inactivation by pathogens.

Table 2.3. Enzymatic strategies of antibiotic inactivation.

Strategy	Type	Antibiotics affected
Hydrolysis		β-Lactams
		Macrolides
Group Transfer	Acyl	Aminoglycoside
		Chloramphenicol
		Type A streptogramin
	Phosphoryl	Aminoglycoside
		Macrolide
		Rifampicin
		Peptide
	Thiol	Fosfomycin
	Nucleotidyl	Aminoglycoside
		Lincosamide
	ADP-ribosyl	Rifampicin
	Glycosyl	Macrolide
		Rifamycin
Other	Redox	Tetracycline
		Rifamycin
		Type A streptogramin
	Lyase	Type B streptogramin

Source: Wright (2005).

2.3. The limited number and disparate availability of classes of effective antibiotics against diverse bacterial species.

If the development of antibiotics is reviewed it is clear that the current rate of discovery is far lower than in the golden age of antibiotics in the 1940s through to the 1960s when all the major families of compounds were identified (McDevitt and Rosenberg, 2001; Spellberg *et al*., 2004). There is no doubt that there is a need for new antibiotics, particularly in the hospital setting. The relentless rise of resistance in Gram-positive bacterial infections is creating everyday therapeutic challenges in managing these infections and much effort has been directed towards developing new compounds to meet this need (Abbanat *et al*., 2003). However, as the time required to bring an antibiotic from discovery to market generally is 8–12 years, research and development efforts must be focussed on compounds that will meet not just current needs but those that will be present 10 years in the future (Dimasi *et al*., 2003). Thus while there are a limited number of Gram-positive bacteria in late-stage development the majority of which stem from established classes of antibiotics, there is a lack of new antibiotics in development to tackle the return of multi-resistant Gram-negative pathogens (Poole, 2003).

2.4. The reduction in the number of pharmaceutical companies pursuing research and development of new anti-infectives.

In addition to the evidence for increasing levels of antibiotic resistance among diverse pathogens, few new antimicrobial agents are in development, probably due to relatively unfavourable returns on investment. Four new classes of antibiotics were introduced in the 1930s and 1940s, including sulphonamides, β-lactams,

aminoglycosides, and chloramphenicol (Wenzel, 2004). A further six classes were developed and approved in the 1950s and 1960s, including tetracycline, macrolides, glycopeptides, rifamycins, quinolones, and trimethoprim. However, from 1970 to the late 1990s, no new antimicrobial classes were approved and only a few new classes have been approved since 2000 for the treatment of Gram-positive bacterial infections; these are the oxazolidinones (linezolid), cyclic lipopeptides (daptomycin), and glycylcyclines (tigecycline). Pharmaceutical companies are discouraged from research and development of new antimicrobials due to high direct costs, risk, and the time associated with animal and *in vitro* studies (Wenzel, 2004; Rice, 2003).

Natural products were abandoned due to several factors, including the lack of success stories in the late 1980s and early 1990s (exceptions such as the echinocandin class of antifungal agents not withstanding); its association with antibiotics; and the introduction of two technologies that changed the paradigm of drug discovery in the industry: combinatorial chemistry, as a new way of generating the chemical diversity that was formerly expected from nature, and high throughput screening (HTS) (Koehn and Carter, 2005).

In addition, the structural complexity of many natural products has often been perceived as an obstacle, since it may impose serious challenges to chemical synthesis and derivatization during the lead optimization process (Tulp and Bohlin, 2004). The lack of truly novel new chemical entities (NCEs) emerging from antimicrobial drug discovery efforts overlooks two facts: (1) Highly effective agents have presumably provided competitive advantages for the microbes that produce them, resulting in the producers becoming more widespread. Therefore, by traditionally screening in a manner where the best agents are on an average the

most common, they, not surprisingly, have repeatedly been re-discovered. Ecopia BioSciences has successfully exploited genomics to discover NCEs by analyzing microbial isolates for genetic sequences that predict novel structures and then using this information to find conditions for their production (Ganesan, 2004);

(2) Microbes often make antimicrobial mixtures (Celmer and Sobin, 1956), containing antibacterials, antifungals, antiparasitics and other microbe-inhibitory agents and toxins, an example of nature indicating that mixtures are more effective than single NCEs and that the pursuit of NCEs active at multiple targets may be a more effective strategy by which to identify novel antibacterials. This is consistent with the hypothesis that resistant strains are less likely to occur when the multiple agents hitting multiple targets are used (Canu and Leclercq, 2001). As the emphasis of antibacterial research and development efforts has shifted away from many large pharmaceutical companies to a large contingent of biotechnology companies, the entrepreneur approach to discovery has led to an explosion of creativity in strategies, selection of targets, genomics and development paradigms. The output of this effort is a pipeline of primarily novel, but niche, antibacterials (Overbye and Barrett, 2005).

2.5. Non-conventional antibiotics

A different group of natural products that has been explored as a potential source of antimicrobial therapies are the so-called antimicrobial peptides. The term refers to peptides of variable length (typically below 25–30 kDa), synthesized in ribosomes and widespread in nature, from microorganisms to higher eukaryotes. In animals, these peptides (defensins, cathelicinds, protegrins, magainins and many others) play

a critical role in the innate immune response against pathogens. These peptides are composed mainly of cationic and hydrophobic amino acids, organized as an amphipatic structure. This structure is believed to confer to antimicrobial peptides their capacity to disrupt cell membranes, and their net positive charge is the reason why they are selective against bacterial cell membranes (Bulet *et al*., 2004; Reddy *et al*., 2004). Many of them show potent and broad spectrum *in vitro* activity against bacteria resistant to conventional antibiotics and, due to their particular mode of action, the development of resistance may be limited. However their use for systemic infections is much less obvious, given the inherent issues of instability, potential toxicity and the cost of large-scale production of these peptides (Bulet *et al*., 2004; Reddy *et al*., 2004).

The idea of using environmental DNA (or DNA from non-cultivable organisms) as a source for genes involved in secondary metabolite biosynthesis was heralded in the 1990s as the potential solution to the lack of productivity in natural products research (Osburne *et al*., 2000; Daniel, 2004). This approach was rooted in the notion that only a very small fraction of microorganisms in any environmental sample can be cultured by standard techniques (Ward *et al*., 1990), thus leaving a vast genetic pool unexplored by these conventional methods. In essence, the approach was based on the isolation of DNA from soil or other environments, followed by the generation of ‘‘metagenomic’’ libraries, using large DNA fragments cloned in *E. coli* or *Streptomyces* spp. (Osburne *et al*., 2000; Daniel, 2004). These libraries were subsequently screened for bioactive metabolites. Although proof of concept was obtained that this approach can indeed deliver the desired output (Wang *et al*., 2000; Gillespie *et al*., 2002), the truth is that it has so far failed to bring in the expected

leads useful for antibiotic development. Perhaps the main challenge is how to translate those early proof-of-concept experiments into a technology suitable for drug discovery at the industrial scale. Since the advent of recombinant DNA technology, genetic engineering of cells, particularly microorganisms, has been successfully practiced for the development of strains capable of overproducing recombinant proteins and small molecule chemicals. Metabolic engineering can be defined as purposeful modification of cellular metabolism using recombinant DNA and other molecular biological techniques (Bailey, 1991).

Development of structurally and functionally diverse antibiotics by metabolic engineering is of great importance to fight against emerging drug-resistant pathogens (Menzella and Reeves, 2007; Menzella *et al*., 2005). As these drugs are synthesized in only minute amounts, it is difficult to obtain them in suitable amounts. This is where metabolic engineering comes into play. Recent advances in understanding the metabolic pathways for the synthesis of these drugs together with the development of various genetic and analytical tools have enabled more systematic and rigorous engineering of microorganisms for enhanced drug production.

Several successful examples of applying metabolic engineering for the development of microbial strains producing drugs and drug precursors have recently been reported. Metabolic engineering of microbes for the production of artemisinic acid is one of the best examples that showcase the impact of intensive engineering of metabolic pathways on efficient drug production (Ro *et al*., 2006; Martin, 2003). Artemisinic acid is a precursor of artemisinin that is an effective drug against malaria-causing *Plasmodium* sp. Furthermore, metabolic engineering of microorganisms can

be performed more easily than mammalian and plant cells, which allows modification of metabolic pathways for the production of structurally more diverse analogs with potent biological activities, as in the cases of polyketides and non-ribosomal peptides (Nguyen, 2006). Metabolic engineering will play an increasingly important role in developing new drugs and drug precursors, including secondary metabolites found in living organisms that have complex structure and chirality that are difficult to synthesize chemically. It will also allow creation of libraries of compounds that can be screened for new drugs.

2.6. The need for new antibiotics

The rapid onset of resistance to most antibacterial drugs diminishes their effectiveness considerably and necessitates a constant supply of new antibiotics for effective treatment of infections. Although considerable progress is being made within the fields of chemical synthesis and engineered biosynthesis of antimicrobial compounds, nature still remains the richest and the most versatile source for new antibiotics (Baltz and Marcel, 2006; Koehn and Carter, 2005).

New antibacterial templates with novel mechanisms of action should have advantage over known antibacterial agents in the fight against multi-drug-resistant bacteria and emerging pathogens (Fenical and Jensen, 2006) owing to the high incidence of novel, bioactive metabolites detected in marine microbial fermentations (Pathom-aree, 2006; Lam, 2006). Marine environments contain taxonomically diverse bacterial groups exhibiting unique physiological and structural characteristics that enable them to survive in extremes of pressure, salinity and temperature, with

the potential production of novel secondary metabolites not observed in terrestrial microorganisms (Cross, 1981). Much interest on the screening of marine and aquatic microorganisms is focused on screening sediment derived microorganisms (Bredholt *et al*., 2008), and also on those that form highly specific symbiotic associations with marine plants and animals in response to the scarcity of nutrients in aquatic and marine environments, and thus produce compounds for defence and competition (Kurtboke, 2000; Williams *et al*., 2005). According to Williams *et al*. (2005), antibiotic production is often associated with sites of high nutrient content as in areas rich in decaying organic matter, with antibiotic production evolving in response to selective pressures created through increased competition. The production of inhibitory metabolites play an important ecological role in deterring free-living bacterial competitors, aiding particle attached bacteria during competition for resources and involvement in biogeochemical cycling. This might relate to increased biological activities among actinomycetes associated with detritus in aquatic environments as well as with sediments (Glen *et al*., 2008).

Further exploration of both marine and freshwater microorganisms would provide a steady supply of new drug leads. Natural products offer unmatched chemical diversity with structural complexity and biological potency (Clardy and Walsh, 2004). Natural drug product resources should be searched from relatively untapped sources, especially the aquatic environment, thus expanding our ability to find novel, potent and selective drug leads, given that only a tiny fraction of the microbial world has been explored and the exponential growth in the microbial genomic database, the outlook for discovering new biologically active natural products is promising (Lam, 2006). Researchers are finding new genera from

marine environments on a regular basis and discovering new metabolite producers never reported earlier. Actinomycete genera identified by cultural and molecular techniques from different marine ecological niches include *Actinomadura, Actinosynnema, Amycolatopsis, Arthrobacter, Blastococcus, Brachybacterium, Corynebacterium, Dietzia, Frankia, Frigoribacterium, Geodermatophilus, Gordonia, Kitasatospora, Micromonospora, Micrococcus, Microbacterium, Mycobacterium, Nocardioides, Nocardiopsis, Nonomurea, Psuedonocardia, Rhodococcus, Saccharopolyspora, Salinispora, Serinicoccus, Solwaraspora, Streptomyces, Streptosporangium, Tsukamurella, Turicella, Verrucosispora* and *Williamsia* (Ward and Bora, 2006). This study looked at two actinomycetes genera which were tentatively identified as *Saccharopolyspora* (TR 046 and TR 039) and *Actinosynnema* (TR 024).

2.6.1. *Actinosynnema*

Literature reveals that research work on the genus *Actinosynnema* is relatively in its early stages with less than 10 species having been discovered up to date. The most studied species of this genus to date is *Actinosynnema pretiosum* which is a commercially important organism due to its ability to produce ansamitocin P-3 (AP-3), a potent anti-tumor agent (Higashide *et al*., 1977; Tanida *et al*., 1980) though *Actinosynnema mirum* is the type species of the genus *Actinosynnema* (Hasegawa *et al*., 1978). They reported that this mutant (*A. pretiosum*), is capable of increased production of maytansinoid antibiotics. These are antibiotics which do not only target bacteria but cell proliferative diseases and conditions, and in particular, for use as antitumor drugs. When *Actinosynnema* was discovered it was originally classified as

Norcadia spp but subsequent characterisation demonstrated the absence of mycolic acids, cell wall type III, lack of sporangia and the formation of motile elements indicated that these strains are of the genus *Actinosynnema* (Hasegawa *et al*., 1983).

Most of the antibiotics produced by *Actinosynnema* species, generally called maytansinoids, were originally from African plants (Tanida *et al*., 1980). This isolation was very difficult because the maytansinoids from plant sources were present in very small amounts. A maytansinoids producing microorganism was subsequently isolated and classified as a new strain of the genus *Norcadia* before it was reclassified as *Actinosynnema* (Tanida *et al*., 1980). Isolation of antibiotics from the organisms proved much easier than from the plants. Because of the many therapeutic uses of maytansinoids, there exists a need for the discovery of new strains of bacteria that are capable of producing ansamitocins in improved yield and sufficient quantities to facilitate commercial production (Hasegawa *et al*., 1983).

Investigation of the antibiotic properties of *Actinosynnema* produced zones of inhibition by cross-streak test with Gram-positive bacteria *Bacillus megaterium* LL-CW 4A, 10 mm; *Sarcina lutea* IMRU 14, 23 mm, a mycobacterium *M. smegmatis* ATCC 607, 8 mm, filamentous fungi *Aspergillus niger* LL- 13, 25 mm (incomplete inhibition); *Penicillium notatum* LL-40, 25 mm (incomplete inhibition) and yeasts *Candida tropicalis* LL-206, 10mm; *Saccharomyces cereviszae* LL-216, 10 mm, (Hasegawa *et al*., 1978). The cytotoxicity of ansamitocin has prompted its use as a toxic "warhead" in immuno-toxin conjugates (Liu *et al*., 1996). There is interest in generating strains of *A. pretiosum* that produce greater concentrations of AP-3 to meet increasing industrial demands, particularly as the yield from wild type *A.*

pretiosum is low (~18 – 83 mg/l) (Tanida *et al*., 1980; 1981). Previously, a random mutagenesis approach (Chung and Byng, 2003), has been used to generate strains which produce 5- to 10-fold more AP-3 than the parental strain. Recently, deletion of a putative transcriptional repressor, asm2, has also been reported to increase AP-3 yield (Srinivasulu *et al*., 2006). The maytansinoids are extraordinarily potent antitumor agents that were originally isolated from members of the higher plant families Celastraceae, Rhamnaceae, and Euphorbiaceae, as well as some mosses, under the auspices of the U.S. National Cancer Institute (Tanida et al., 1980; Srinivasulu *et al*., 2006). They are 19-membered macrocyclic lactams related to ansamycin antibiotics of microbial origin, such as rifamycin B and geldanamycin (Liu *et al*., 1996). The similarity stimulated a search for maytansinoid-producing microorganisms, leading to the isolation of the ansamitocins from the actinomycete *Actinosynnema pretiosum ssp*. *pretiosum* and a mutant strain *Actinosynnema pretiosum ssp. auranticum* (Higashide *et al*., 1977). Both the structures and antitumor activity of the ansamitocins are similar to those of the maytansinoids from plant sources. A number of structural variations are encountered naturally, including different ester side chains at C-3, and the presence or absence of the 4,5-epoxide, the N-methyl group, the halogen, and oxygens at C-15 and at the C-14 methyl group (Reider and Roland, 1984). Efforts to develop maytansine into a clinically useful anticancer drug proved disappointing in phase II clinical trials (Reider and Roland, 1984), probably because of dose-limiting toxicity in humans, but there is continuing interest in the development of conjugates of maytansinoids for targeted delivery. Extensive work by Tanida et al, (1980) and at the Takeda Company (Kalakoutskii and Agre, 1974), to define structure-activity relationships among maytansinoids relied entirely on semi synthesis from the available natural products.

2.6.2. *Saccharopolyspora*

Intensive study has been carried out on *Saccharopolyspora erythraea* which many scientists refer to as *Streptomyces erythreus* (Escalante *et al*., 2007; Flores and Sanchez, 1989; Potvin and Poringer, 1993). Up to date, about 21 species of *Saccharopolyspora* have been isolated and studied over a period ranging from 1968 to 2008. *Saccharopolyspora erythraea* is used for the industrial production of erythromycin by either submerged culture system of either free cells or immobilised cells (Heydarian *et al*., 1999). Of all the 21 known species of *Saccharopolyspora*, it seems the species *erythreus* has been receiving the greatest attention over the rest. It is therefore this researcher's hope that a new species of *Saccharopolyspora* may be discovered here in the Eastern Cape which might make a significant contribution to the standing pool of available knowledge.

2.7. Perspective

There is increasing concern particularly over the increasing and relentless resistance of nosocomial pathogens such as *Staphylococcus aureus* to mainline antibiotics and the emergence of multi-drug resistant Gram-negative bacteria. The pace of drug resistance has outstripped the discovery of new antimicrobial agents and there is an urgent need for new antibiotic drugs with novel mechanisms of action. The question is about how to tackle the problem more effectively in the future, particularly given the fact that since 1970, only three new classes of antibiotics have been marketed. There are two main sources for antibiotic leads—natural products and synthetic compounds.

Natural products have been the mainstay in providing novel chemical scaffolds for many drugs (Newman *et al*., 2003), as well as leads that were chemically modified and developed as antibacterial agents. In fact, natural products account for all but three antibiotic classes. Antibiotics from natural sources range from small molecular weight compounds e.g., penicillins, to large peptides e.g., teicoplanin. They generally possess complex architectural scaffolds and densely deployed functional groups, affording the maximal number of interactions with molecular targets, often leading to exquisite selectivity for pathogens versus the host (Singh and Barrett, 2006). Synthetic chemical collections have played a minimal role as sources of leads for antibiotics. The synthetic sulfonamides, discovered in the 1930s, were the first class of antibiotics used in clinical practice before the discovery of penicillins and continue to be used today. The second and highly successful class of synthetic antibiotics were the quinolones discovered in 1962 by empirical screening of the by-product of chloroquine synthesis. This led to the discovery and development of nalidixic acid as the first quinolone antibiotic which eventually evolved to quinolones such as ciprofloxacin and others (Bull *et al*., 2000).

Oxazolidinones represent the third class of synthetically derived antibiotic leads discovered in 1979 that led to the clinical development and approval of linezolid in 1999. While quinolones were originally discovered as antibiotics from total synthesis, natural quinolones e.g. aurachins C and D (Kunze *et al*., 1987), were subsequently discovered and could have served as a scaffold. Scaffolds, from which medicinal chemists can create a structure–activity relationship, are key in drug discovery and in optimizing antibiotics for human use. The abundant scaffold diversity in natural products is coupled with ‘purposeful design’ – most microbes

make by-products with a purpose – usually to afford an advantage for survival in environments threatening their growth and/or survival (Cundliffe, 1989).

It is reasoned that these ecological defence systems, produced to combat competing microbial life forms, would have some antimicrobial activity that gives the producer organism an advantage and as such, are antimicrobials to begin with (Cundliffe, 1984). In the search for novel antibiotics, it would be difficult to imagine a more specific source of naturally occurring antimicrobials than in nature itself. With new targets and advances in natural product-based technologies, the time is ripe to refocus efforts on natural products, to revitalize antibiotics discovery. The use of bacterial genomic data together with innovative natural products approaches, will create a second ‘‘Golden Age’’ of antibacterial agents, both addressing bacterial resistance and commercial limitations of current approaches (Overbye and Barrett, 2005; Nathan and Goldberg, 2005). Natural products are a logical starting point for discovering new drugs to treat infectious diseases. Significant advances in compound separation technology and structure elucidation mean that these former bottlenecks in the discovery process no longer exist (Butler, 2004; Koehn and Carter, 2005) The major challenge that remains for natural product based drug discovery is the willingness of medicinal chemists to take up the task of optimising relatively complex, often chiral chemical scaffolds with arrays of diverse functional groups.

Most organisms found in the environment cannot easily be grown (Bull *et al*., 2000), nor can all of the NCEs genetically predicted be produced. Transfecting DNA from soil to an industrialized strain may allow these new NCEs to be made (Handelsman *et al*., 1998). Sequencing of actinomycete strains has revealed the existence of multiple clustered secondary metabolite genes (Bentley *et al*., 2002;

Donadio *et al*., 2002). By determining the growth conditions for these cultures using genomic analysis, and then establishing the conditions required to induce expression of their full biosynthetic potential is a necessary approach to natural product drug discovery. The need for agents effective against antibiotic-resistant pathogens led many biotech/small pharma-companies to reinvestigate previously identified NCEs that had failed early tests for various side effect and pharmacokinetic reasons. Among these were daptomycin (Cubist), Telavancin (Theravance) and ramoplanin (Oscient) (Farver *et al*., 2005). Acer (2000), suggested that synergistic combinations of antibiotics have generally been developed in response to difficult therapeutic challenges and that the time has come to apply current knowledge of resistance and synergistic mechanisms to develop viable commercial combinations. Two successful synergistic combinations are the β-lactam with a β-lactamase inhibitor that attacks a resistance determinant (β-lactamase) and the newer semi-synthetic natural product combination of the synergistic streptogramins, quinupristin and dalfopristin, introduced in the late 1990s for the treatment of MRSA (methicillin resistant *Staphylococcus aureus*) (Canu and Leclercq, 2001). Agents that inhibit antibiotic efflux in combination with an otherwise effective agent that is eliminated from the cell may also lead to synergistic combinations (Canu and Leclercq, 2001). The potential for this type of synergy includes increased sensitivity to antimicrobials in *Pseudomonas* strains mutated to eliminate efflux genes (Duncan *et al*., 2004).

Extensive work by Microcide and Daiichi have provided proof-of-concept that efflux can be reversed in clinically relevant *P. aeruginosa* strains of bacteria by the addition of an efflux inhibitor (Kriengkauykiat *et al*., 2005). Johnson & Johnson researchers have reported that an agent that targets virulence can be synergistic

with ciprofloxacin in a *Pseudomonas* lung infection model (Fernandez *et al*., 2004). Although genomics-based drug discovery might offer an alternative strategy in the future, the true tools of genomics (i.e. full bacterial genome sequences) have only been available since 1995, which makes the expectations of genomic based target classes rather remote in the short-term (Forsyth *et al*., 2002; Ji, 2002). There is an absolute need for new antibacterial agents, especially now that resistance emergence has been unequivocally documented by the medical need (based on morbidity/ mortality) (Avorn *et al*., 2002), microbiology data obtained through surveillance (Mathai *et al*., 2001), and basic research studies of resistance (Barlow and Hall, 2003; Barrett, 2001). The underlying assumption is that microbial genomics will provide several essential, validated targets as possible candidates for therapeutic intervention through the discovery of new antimicrobials. The ideal new antibacterial target should have two key properties: it should have a broad spectrum of activity and the gene should encode an essential function, the inhibition of which would have a bacteriostatic or bactericidal effect on the pathogen. Discovery will come from the genomics research efforts still waiting to demonstrate success. Greater chance of success can be realised if research returns to the successes of the past in mining natural products, where nature has empirically engineered the starting scaffolds for antimicrobial agents that allow the producer organism to survive in a hostile environment.

The manufacture of secondary metabolites by ‘producer organisms’ in nature is believed to support multiple functions, including the ability to communicate with other microorganisms and to protect the organism from attack (Demain, 2002; Topliss, 2002). If the microbial strategy is to protect the producer organism, it is

logical that antimicrobials would be identified in nature under adverse conditions because their production would assure and/or enhance the survival of the producer organism from environmental assault. Thus, the rich source of antibiotic activities in nature (some that have selectivity over eukaryotes and others that do not), is understandable in terms of structural design from an evolutionary survival standpoint and represents a great sourcing pool for novel chemotypes (Wu, 2004). Because of the oceans' vastness, the rich diversity of microbes in them and their extremes in pH, temperature and pressure, it is not surprising that most focus has fallen on marine actinomycetes in the search for novel antimicrobials since the reasoning is that their harsh environment has evolutionary contributed in them being better antibiotic producers than those from other environments. This has largely caused freshwater actinomycetes to be overlooked as potential sources of novel antibiotics, which is one reason why this present research was undertaken.

CHAPTER III

METHODOLOGY

3.1. Test Actinomycetes

Three actinomycetes strains belonging to the genera *Saccharopolyspora* (TR 046 and TR 039) and *Actinosynnema* (TR 024) were obtained from the culture collections of the Applied & Environmental Microbiology Research Group (AEMREG), University of Fort Hare. The strains were isolated from the Tyume River in the Eastern Cape Province of South Africa and confirmed to possess antimicrobial potential (Ogunmwonyi, 2008). The organisms were maintained on agar slants and in 20% glycerol.

3.2. Preparation of actinomycetes inocula

The stock culture of the test actinomycetes used in this experiment was prepared by streaking actinomycetes from the agar slants onto starch casein agar (SCA) which was prepared as follows per litre of freshwater: soluble starch, 10 g; potassium phosphate dibasic, 2 g; potassium nitrate, 2 g; sodium chloride, 2 g; casein, 0.3 g; magnesium sulphate.$7H_2O$, 0.05 g; calcium carbonate, 0.002 g; ferrous sulphate.$7H_2O$, 0.01 g and agar agar 16 g. Each of these compounds was added to the diluents and allowed to dissolve completely using a magnetic stirrer. The medium was then autoclaved at 121^oC and 15 mm Hg for 15 minutes and allowed to cool down to 50^oC before being poured into 90 mm Petri dishes. The test actinomycetes were streaked on the prepared medium and incubated at 28^oC for between 7 and 14 days under aerobic conditions. Actinomycetes inocula were then prepared by

transferring several colonies into sterile normal saline (10 ml) and the suspensions vortexed for 20 seconds to ensure homogeneity.

3.3. Preparation and inoculation of fermentation broth

The fermentation broth medium was prepared following a method outlined by Muiru *et al*. (2004) as follows per litre of distilled water: 10 g starch, 4 g yeast extract, 2 g peptone, 5 ml potassium bromide (20 g/L) and 5 ml iron (iii) sulphate tetrahydrate (4.76 g/L). The medium was aliquoted in 500 ml aliquots into 1L Erlenmeyer flasks and sterilized by autoclaving at 121^{o}C and 15 mm Hg for 15 minutes and allowed to cool after which 100 µL volumes of the standardized actinomycetes suspensions were used to inoculate the flasks. The flasks were then incubated at 27^{o}C on a shaker at 300 rpm for 10 days.

3.3.1. Quality control.

Confirmation of purity was done by streaking the fermentation cultures onto nutrient agar (NA), potato dextrose agar (PDA) and starch casein agar (SCA) plates. In the absence of any contaminant(s), only the actinomycete strain contained in the fermentation broth would grow on NA and SCA but nothing would grow on PDA. This would later help us to rightfully conclude that whatever results would be obtained after antimicrobial assays were carried out would be purely a result of the antimicrobial activity of the actinomycete(s) extract(s).

3.4. Extraction of the crude-antibiotic extracts from fermentation cultures

Crude antibiotic extracts were recovered from the culture filtrate by solvent extraction using ethyl acetate in accordance with the description of Liu *et al.* (1986). Ethyl acetate was added to the filtrate in the ratio 1:1(v/v) and shaken vigorously for 1hr for complete extraction. The ethyl acetate phase that contains the antibiotic was separated from the aqueous phase and concentrated in vacuum at 60^{o}C using a rotary evaporator. The residue obtained was weighed and reconstituted in 50% methanol to make a working concentration of 10 mg.ml^{-1} for the antibacterial assays.

3.5. Test bacteria and inocula preparation

The test microorganisms used in this study were obtained from the culture collection of the Applied and Environmental Microbiology Research Group (AEMREG) laboratory at the University of Fort Hare, and include the following bacteria:

Referenced strains: *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 19582), *Staphylococcus aureus* (ATCC 6538), *Enterococcus faecalis* (ATCC 29212), *Bacillus cereus* (ATCC 10702), *Bacillus pumilus* (ATCC 14884), *Pseudomonas aeruginosa* (ATCC 7700), *Enterobacter cloacae* (ATCC 13047), *Klebsiella pneumoniae* (ATCC 10031), *Klebsiella pneumoniae* (ATCC 4352), *Proteus vulgaris* (ATCC 6830), *Proteus vulgaris* (CSIR 0030), *Serratia marcescens* (ATCC 9986), *Acinetobacter calcoaceticus* (UP), *Acinetobacter calcoaceticus anitratus* (CSIR), *Escherichia coli (25922).*

Environmental strains: *Klebsiella pneumoniae, Bacillus subtilis, Shigella dysenteriae, Staphylococcus epidermidis, Pseudomonas aeruginosa, Proteus*

vulgaris, Enterococcus faecalis, Staphylococcus aureus, Micrococcus kristinae and *Micrococcus luteus, Shigella flexineri, Salmonella sp*

Clinical isolates: *Staphylococcus aureus* OKOH 1, *Staphylococcus aureus* OKOH 2A, *Staphylococcus aureus* OKOH 3, and *Staphylococcus sciuri* OKOH 2B.

The test bacteria were confirmed for purity by streaking onto nutrient agar plates. These pure bacterial isolates were then inoculated into nutrient broth and incubated at 37^{o}C for 24 hours. The turbid broths were later centrifuged at 7000 rpm and the supernatant discarded. The pellets of cells were resuspended and double washed in sterile normal saline and standardized to OD_{600nm} 0.1. The washed and standardized cells were subsequently used for various experiments described below.

3.6. Antibacterial assays

3.6.1. Antibacterial susceptibility assay

Antibacterial activities of the crude extracts were determined using agar well diffusion technique. This was done following a method described by Pandey *et al*., (2008). Test organism cultures were grown overnight (18 hrs) in nutrient broth and standardised to OD_{600nm} 0.1. Test organisms were then spread-plated onto Muller Hinton agar (MHA) plates using sterile cotton swabs. A flame sterilised cork borer with a diameter of 6 mm was used to bore wells into the agar and 100 µL of the extract (10 mg/ml) loaded into the wells. Control wells were loaded with 100 µL of 50% methanol. The extract was allowed to diffuse into the agar before the plates were incubated under aerobic conditions at 37^{o}C for 24 hours. At the end of the

incubation period, the plates were observed for zones of inhibition around the wells. Inhibition zone is defined as the area free of growth in a bacterial lawn which results from the effect of antibiotic that has diffused into the medium from its applied source (EUCAST Definitive Document, 2000). A pour plate technique was also carried out as a confirmatory test to the agar well diffusion tests. Here 100 µL of the standardised test organism suspensions were pipetted into molten (45^{o}C) double strength MHA to which was added an equal volume of the crude extract to yield 10 mg.ml^{-1} of the extract and the mixture was then poured onto sterile 90 mm Petri dishes. A control containing same volume of 50% methanol as the crude extract was also set up. The agar was left to set after which the plates were incubated under the same conditions with the swab plates. The development of inhibition zone diameters on the swab plates compared with the absence of growth on the pour plates was used to indicate the inhibitory activity of the extracts.

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

The MICs were determined using test organisms that showed susceptibility to the crude extracts using the broth microdilution method as outlined by the EUCAST DISCUSSION DOCUMENT (2003). Sterile plastic, disposable microtitre plates with 96 flat-bottom wells were used. The medium used in the plates was prepared at double the final strength to allow for a 50% dilution once the inoculum and solvents/ or antimicrobial were added. A 100 µL volume of double strength Muller Hinton broth was introduced into all the 96 wells. Wells in columns 1, 2 and 12 were used as control wells. Wells in columns 3-11 were then loaded with 50 µL of the extract

starting with the highest concentration of 10 $mg.ml^{-1}$ in column 3 to the lowest concentration of 0.034 $mg.ml^{-1}$ in column 11 by serial dilution and 50 µL of test organism suspension added to the wells. Wells in column 1 were used as the positive control and contained 50 µL of extract and 50 µL of sterile distilled water. Wells in column 2 were used as the negative control and contained 50 µL of 50% methanol (v/v) and 50 µL of test organism suspension. Wells in column 12 were used as the growth controls and contained 50 µL of test organism and 50 µL of sterile distilled water. The microtiter plate was then incubated at $37^{o}C$ for 18- 24 hours. Results were read using a microtitre plate reader (BIO-RAD model 680) at 490 nm. Visual reading of results was done by first adding resazurin dye into all the wells. Wells with no growth turned blue in colour while those with growth turned pink, this helped to give a clear visual demarcation of the MIC wells. The MIC was determined by the well with the lowest concentration of the extract that inhibited growth of the test organism.

The MBC was determined from the MIC plate following a method outlined by the CLSI (2002), and is defined as the lowest concentration of an antibiotic, expressed in mg/L, that under defined *in vitro* conditions reduces by 99.9% the number of organisms in a medium containing a defined inoculum of bacteria, within a defined period of time (EUCAST Definitive Document, 2000). It was determined by inoculating the broths in the MIC range into drug-free nutrient agar medium. The MBC was determined by the plate in which no growth was observed after incubation for 48 hours.

3.6.3. Determination of the rate of kill of the crude extract

The rate of kill assay was done only for TR 046 extract which appeared to exhibit more antibacterial potency than the other actinomycetes. This was done by monitoring bacterial cell death over time in accordance with the description of Okoli and Iroegbu (2005). Also, five test bacteria were selected for this assay based on their susceptibility and Gram's reaction. The inocula were prepared following the described guidelines of EUCAST Discussion Document (2003). The resultant cell suspension was diluted 1:100 with fresh sterile broth and used to inoculate 50 ml volume of nutrient broth incorporated with the extract at multiples of the MIC to a final cell density of 5×10^5 cfu/ml (Aiyegoro *et al*. 2008; Sibanda and Okoh, 2008). The flasks were then incubated with shaking at 37^oC on an orbital shaker at 120 rpm and samples of 100 µL were then withdrawn at 6hr and 12 hour intervals and diluted appropriately. Approximately 100 µL volumes of the diluted samples were then plated out in triplicate on nutrient agar. Plates were incubated at 37^oC for 24 hours after which the numbers of surviving cells were enumerated (Sibanda and Okoh, 2008). Controls consisted of extract free nutrient broth inoculated with test organism.

3.7. Statistical analysis

Results were analysed under Minitab Release 14.2 using a 2-sample T-test and One-way Analysis Of Variance (ANOVA) as the statistical packages. Mean zone diameters were compared for all the three actinomycetes extracts. Analysis was carried out at 95% confidence interval (see appendix).

CHAPTER IV

RESULTS

When the crude extracts of each of the test actinomycetes were tested against a total of 32 test bacteria (7 gram-positive and 25 gram-negative) at a concentration of 10 mg/ml, extract obtained from *Saccharopolyspora* (TR 046) was observed to be active against 9 test bacteria with zones of inhibition ranging from 8-28 mm. Extract obtained from *Saccharopolyspora* (TR 039) was active against 8 of the test bacteria with zones of inhibition varying from 8-15 mm; while extract obtained from *Actinosynnema* (TR 024) was active against four test bacteria with zones of inhibition ranging from 10-13 mm (Table 4.1). Extracts from *Actinosynnema* and *Saccharopolyspora* (TR 039) appeared to be active against only Gram-negative bacteria while extract from *Saccharopolyspora* (TR 046) had a broad spectrum antibacterial activity. *Proteus vulgaris* (CSIR 0030) was most susceptible to the extract obtained from *Saccharopolyspora* (TR 046) while the environmental *Staphylococcus epidermidis* was least susceptible. However, with extract from *Saccharopolyspora* (TR039), *Enterobacter cloacae* (ATCC 13047) were least susceptible, while *Proteus vulgaris* (ATCC 6830) was most susceptible. There does not appear to be significant dynamics in the susceptibilities of the bacteria for which extract from *Actinosynnema* was active (Table 4.2).

Table 4.1. Antibacterial activities of crude extracts obtained from *Saccharopolyspora* (TR 046 and TR 039) and *Actinosynnema* (TR024) isolates.

Test organism	**Gram's reaction**	**Antibacterial activity [inhibition zone diameter (mm)]**		
		Extract from TR 046 (10 mg/ml)	**Extract from TR 039 (10 mg/ml)**	**Extract from TR 024 (10 mg/ml)**
Enterococcus faecalis (ATCC 29212)*	+	-	-	-
Bacillus cereus (ATCC 10702)*	+	-	-	-
Bacillus pumilus (ATCC 14884)*	+	+ (27)	-	-
Micrococcus kristinae™	+	-	-	-
Bacillus subtilis KZN™	+	-	-	-
Micrococcus luteus™	+	-	-	-
Staphylococcus epidermidis KZN™	+	+ (8)	-	-
Pseudomonas aeruginosa (ATCC 7700)*	-	+ (17)	+ (10)	+ (13)
Enterobacter cloacae (ATCC 13047)*	-	-	+ (8)	-
Klebsiella pneumoniae (ATCC 10031)*	-	-	-	-
K. Pneumoniae (ATCC 4352)*	-	-	-	-
Proteus vulgaris (ATCC 6830)*	-	-	+ (15)	+ (13)
Proteus vulgaris (CSIR 0030)*	-	+ (28)	+ (12)	+ (12)
Serratia marcescens (ATCC 9986)*	-	+ (20)	+ (14)	-
Staphylococcus aureus (ATCC 6538)*	-	-	-	-
Acinetobacter calcoaceticus (UP)*	-	+ (17)	-	-
Acinetobacter calcoaceticus anitratus (CSIR)*	-	-	-	-
Klebsiella pneumoniae KZN™	-	+ (20)	+ (12)	+ (10)
Escherichia coli (ATCC 8739)*	-	-	-	-
Shigella flexineri KZN™	-	-	-	-
Escherichia coli (ATCC 25922)*	-	-	-	-
Salmonella sp KZN™	-	-	+ (13)	-

Pseudomonas aeruginosa (ATCC 9582)*	-	+ (16)	-	-
Pseudomonas aeruginosa KZN™	-	+ (12)	-	-
Proteus vulgaris KZN™	-	-	-	-
Enterobacter faecalis KZN™	-	-	-	-
Escherichia coli KZN™	-	-	-	-
Staphylococcus aureus KZN™	-	-	-	-
Staphylococcus aureus OKOH 1®	-	-	+ (13)	-
Staphylococcus aureus OKOH 2a®	-	-	-	-
Staphylococcus aureus OKOH 2b®	-	-	-	-
Staphylococcus aureus OKOH 3®	-	-	-	-

Diameter of zones of inhibition exclude the diameter of the well; (-) denotes no activity; (+) denotes activity; * denotes referenced strains; ™ denotes environmental strains; ® denotes clinical isolates

It was also observed that referenced strains *Pseudomonas aeruginosa* (ATCC 7700) and *Proteus vulgaris* (CSIR 0030), and environmental strain *Klebsiella pneumoniae* (KZN) were reactive to all three extracts. Results of the MIC and MBC of the test actinomycetes antibiotics are as shown in Table 4.2. The MIC for extract from *Saccharopolyspora* (TR 046) against the nine susceptible bacteria ranged from 0.078 $mg.ml^{-1}$ to 10 $mg.ml^{-1}$ while its MBC varied between 1.25 $mg.ml^{-1}$ and >10 $mg.ml^{-1}$. For the extract from *Saccharopolyspora* (TR 039), MIC ranged between 5 and >10 $mg.ml^{-1}$ while its MBC was generally >10 $mg.ml^{-1}$. The extract from *Actinosynnema* on the other hand had MICs ranging between 1.25 and 5 $mg.ml^{-1}$ and an MBC of >10 $mg.ml^{-1}$ (Table 4.2).

Table 4.2. MIC and MBC results for extracts obtained from *Saccharopolyspora* (TR 046 and TR 039) and *Actinosynnema* (TR 024)

Extract	Test organism	Gram reaction	MIC (mg/ml)	MBC (mg/ml)
TR 046	*Staphylococcus epidermidis (KZN)™*	+	0.078	1.25
	Bacillus pumilus (ATCC 14884)*	+	0.078	10
	Acinetobacter calcaoceticus (UP)*	-	1.25	>10
	*Pseudomonas aeruginosa (ATCC 7700)**	-	0.625	5
	*Pseudomonas aeruginosa (ATCC 19582)**	-	0.625	2.5
	*Proteus vulgaris (CSIR 0030)**	-	0.625	5
	*Serratia marcescens (*ATCC 9986)*	-	0312	10
	Klebsiella pneumoniae (KZN)™	-	1.25	5
	Pseudomonas aeruginosa (KZN)™	-	10	>10
TR 039	*Pseudomonas aeruginosa (ATCC 7700)**	-	>10	>10
	*Enterobacter cloacae (ATCC 13047)**	-	>10	>10
	Proteus vulgaris (ATCC 6830)*	-	10	>10
	Proteus vulgaris (CSIR 0030)*	-	5	>10
	Serratia marscens (ATCC 9986)*	-	5	>10
	Klebsiella pneumoniae (KZN)™	-	>10	>10
	Salmonella spp (KZN)™	-	5	>10
	Staphylococcus aureus OKOH1®	-	5	>10
TR 024	*Pseudomonas aeruginosa (*ATCC 7700)*	-	5	>10
	Proteus vulgaris (ATCC 6830)*	-	5	>10
	Proteus vulgaris (CSIR 0030)*	-	1.25	>10
	Klebsiella pneumoniae™	-	1.25	>10

* denotes referenced strain; ™ denotes environmental strain; ® denotes clinical isolate

Time-kill studies revealed that the extract obtained from *Saccharopolyspora* (TR 046) had bacteriostatic effects on referenced bacterial strain *Pseudomonas aeruginosa* (ATCC19582) with no major changes in the bacterial load with time. The extract was however, strongly bactericidal against another referenced bacterial strain *Bacillus pumilus* (ATCC 14884) at 4 × MIC resulting in the killing of approximately 10^4 cfu.ml^{-1} in 6 to 12 hrs and 10^2 cfu.ml^{-1} at 2 × MIC after 6 hrs of exposure to the extract suspension (Table 4.3). It was however, weakly bactericidal at MIC (0.078 mg.ml^{-1}).

Table 4.3. Time kill results for extract obtained from *Saccharopolyspora* (TR 046)

Susceptible isolate	**MIC (mg/ml)**	**log_{10}Kill 4×MIC**		**Log_{10}Kill 2×MIC**		**Log_{10}Kill MIC**	
		6hrs	12hr	6hrs	12hr	6hrs	12hr
Bacillus pumilus (ATCC 14884)*	0.078	1.26	0.31	1.13	0.12	0.20	-2.41
Klebsiella pneumoniae (KZN)™	1.25	0.26	-0.05	0.25	-1.00	0.77	-0.27
*Pseudomonas aeruginosa (ATCC 19582)**	0.625	-0.01	-0.30	-0.09	-0.12	-0.92	-1.31
Staphylococcus epidermidis (KZN)™	0.078	0.74	-0.19	0.54	-0.27	0.02	-0.45
Proteus vulgaris (CSIR 0030)*	0.625	0.09	0.90	0.08	0.13	-0.11	-0.04

*denotes referenced strain; ™ denotes environmental strain; (-) denotes bacteriostatic effect.

The extract also showed good bactericidal activity against referenced strain *Proteus vulgaris* (CSIR 0030) achieving reductions of $0.9\log_{10}$ and $0.13\log_{10}$ cfu.ml^{-1} at 4 × MIC (2.5 mg.ml^{-1}) and 2 × MIC (1.25 mg.ml^{-1}) respectively after 12 hrs of exposure. The extract showed limited bactericidal activity against both *Klebsiella pneumoniae* (environmental strain) and *Staphylococcus epidermidis* (environmental strain) at all MIC levels after 6 hrs of exposure but showed bacteriostatic effects at all MIC levels after 12 hrs of exposure.

CHAPTER V

DISCUSSION

Actinomycetes are widely distributed in nature and they occupy diverse and mild to extreme environments (Gershon, 2002). They have been isolated from habitats ranging from farming soils, mine dumps, oceans to freshwater habitats like streams, rivers and lakes, from lake mud and river sediments (Demain, 2006).

The antibacterial activity of three freshwater actinomycetes extracts was observed against a series of bacterial isolates. The Gram-positive test bacteria showed limited susceptibility to the extracts while the Gram-negatives were more susceptible. Results from this research confirm that both *Saccharopolyspora* and *Actinosynnema* isolated from a freshwater habitat possess antibacterial properties. However, results indicate that *Saccharopolyspora* (both TR 046 and TR 039) possess consistently greater antibacterial activity against test bacteria as compared to *Actinosynnema* (TR 024) as evidenced by the fact that the extract from *Saccharopolyspora* (TR 046) was active against 9 test organisms from a total of 32 indicating approximately 30% activity: extract from *Saccharopolyspora* (TR 039) was active against 8 of the 32 test organisms indicating 25% activity while extract from *Actinosynnema* was active against 4 from a possible total of 32 test organisms indicating a 12.5% activity. The limited antibacterial activity of *Actinosynnema* extract is in agreement with the findings of Hasegawa *et al*. (1983) who reported that actinomycetes belonging to the genus *Actinosynnem*a mainly produce maytansinoid antibiotics (potent anti-tumor agents) although it is worth noting that they still do produce antibacterials, howbeit against a limited range of bacteria.

These results show that there is no much difference in the antibiotic producing potentials of the two *Saccharopolyspora* strains (TR 046 and TR 039) but there is appreciable difference in the antibiotic producing potentials of *Saccharopolyspora* and *Actinosynnema* genera. This might be explained by the fact that *Actinosynnema* is well known for producing ansamitocins rather than antibacterials (Higashide *et al*., 1977; Hasegawa *et al*., 1978) whereas *Saccharopolyspora* is a well documented producer of antibiotics (Bhattacharyya *et al*., 1998), with *S. erythraea* well known for the industrial production of the antibiotic erythromycin.

Out of the two *Saccharopolyspora* strains (TR 046 and TR 039), extract obtained from TR 046 produced consistently larger inhibition zone diameters than that of TR 039. This result suggests that the extract from TR 046 has greater efficacy compared to the extract from TR 039. This result is further proven by the MIC and MBC results which show that for the extract from TR 039 MIC values ranged from 5 $mg.ml^{-1}$ to >10 $mg.ml^{-1}$ while the MBC values were all >10 $mg.ml^{-1}$. The MIC values for the extract from TR 046 ranged between 0.078 $mg.ml^{-1}$ and 10 $mg.ml^{-1}$ and MBC values ranged from 1.25 $mg.ml^{-1}$ to 10 $mg.ml^{-1}$ with the exception of MBC for *Acinetobacter calcoaceticus* and *Pseudomonas aeruginosa* whose MBC values were >10 $mg.ml^{-1}$. The observed differences in the antibiotic producing potentials of the two *Saccharopolyspora* strains, TR 046 and TR 039, can possibly be explained in terms of the ecological conditions from where they were isolated. Actinomycetes isolated from a place richer in microorganism (competing bacterial sp) tend to produce more antagonistic substances as a competitive tool for survival compared to those isolated from places with low numbers of competing microorganisms (Parungawo *et al*., 2007). In this regard, it can be suggested that *Saccharopolyspora*

TR 046 was isolated from a place richer in competing species of microorganisms compared to *Saccharopolyspora* TR 039.

Extracts obtained from both *Saccharopolyspora* TR046 and TR039 were active against five referenced bacterial strains each and whereas the extract from TR 046 was active against three environmental strains and showed no activity against clinical isolates, the extract from TR 039 showed activity against two environmental strains and one clinical isolate, a result which emphasises the similarities in the genotypic origin of the two organisms. Their limited activity against clinical isolates confirms that these clinical strains of bacteria may possess characteristics that differ or are absent from the non-pathogenic strains (Hiramitsu *et al*., 1997). Pathogenic strains may possess specific virulence determinants (toxins and adhesions, etc.) encoded by monocistronic genes, plasmids, or pathogenicity islands as well as plasmids that code for drug resistance, which may partially account for the ineffectiveness of the extracts against clinical strains (Todar, 2002; Pupo *et al*., 1997).

While the results of the research suggest limited activity against Gram-positive bacteria by all the actinomycetes extracts used, it is worth noting that seven Gram-positive bacteria were tested as compared to twenty-five Gram-negative test bacteria and it can therefore be deduced that it may not be necessarily correct to suggest that the extracts lacked broad spectrum activity. Besides, Gram-negative bacteria are inherently more resistant to antimicrobials than Gram-positive bacteria due to the combined exclusion of the antimicrobial compounds by the double membrane barrier and transmembrane efflux present in this group of organisms (Zgurskaya and Nikaido, 2000), so sourcing actinomycetes that produce effective antimicrobials

against Gram-negative bacteria is a step in the right direction for this war against antibiotic resistance.

The rate of kill of the test organisms by the extract from *Saccharopolyspora* TR 046 appears to be both concentration and time dependent. Results indicate that the extract was mostly bactericidal at 2 × MIC and 4 × MIC up to 6 hrs of exposure with the effect waning off after 6 hrs. This might suggest that dosing frequency needs to be increased to once after every 6 hrs to maintain the bactericidal effect of the extract at its optimum. Complete eradication of the test organisms was not achieved. In view of the knowledge that actinomycetes produce secondary metabolites, especially antibiotics, in order to overcome other competing microorganisms by killing them (Parungawo *et al*., 2007), this could be attributed to the fact that the sediments from which the actinomycetes isolates were obtained have a small bacterial population which competes with them and hence rely less on antibiotic production as a competitive tool against other bacteria.

However, the extract exhibited a strong bactericidal efficacy against *Bacillus pumilus* (ATCC 14884) achieving a 1.26$\log_{10}$ reduction in counts of the test organism after 6 hrs of exposure at 0.312 mg.ml^{-1} (4 ×MIC) and moderate bactericidal efficacy against *Proteus vulgaris* (CSIR 0030) achieving a 0.9$\log_{10}$ reduction in counts of the test organism after 12 hrs of exposure at 2.5 mg.ml^{-1} (4 × MIC). The extract exhibited bacteriostatic effects on two test bacteria (*Klebsiella pneumoniae* {KZN} and *Staphylococcus epidermidis* {KZN}) with no major changes on the bacterial load with time. *Pseudomonas aeruginosa* showed persistive response to the extract, with a marked delay in its growth at 2.5 mg.ml^{-1} (4 × MIC) after 6 hrs of exposure increasing by 0.01$\log_{10}$ counts as compared to an increase of 1.02$\log_{10}$ counts after 6 hrs of

exposure at 0.625 $mg.ml^{-1}$ (MIC). A $3log_{10}$ or 99.9% reduction in viable bacterial density in an 18-24 hr period is the generally accepted definition of bactericidal activity in antibiotics (Pankey and Sabath, 2004).

In conclusion, this study has shown that freshwaters could serve as potential reservoirs of actinomycetes of antimicrobial importance with varying spectra of activities. It is probable that more interesting results could have been obtained had this study been carried out using a larger number of test bacteria than was used in this study, with approximately the same number of Gram-negative and Gram-positive test bacteria. A detailed characterisation of the active principles of the extract is suggested for future studies.

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APPENDIX

MIC results for extract obtained from *Saccharopolyspora* TR 039, wavelength 490 nm

			Concentration mg/ml									
	positive control	**methanol control**	**10**	**5**	**2.5**	**1.25**	**0.625**	**0.3125**	**0.156**	**0.078**	**0.039**	**Growth control**
Pse 2	0.164	0.461	0.497	0.67	0.568	0.814	0.991	1.118	1.269	1.496	1.575	1.632
	0.163	0.457	0.443	0.675	0.517	0.734	1.143	1.489	1.488	1.617	1.595	1.601
	0.169	0.363	0.5	0.536	0.782	0.898	1.04	1.305	1.412	1.485	1.578	1.539
average	**0.165333**	**0.427**	**0.48**	**0.627**	**0.622333**	**0.815333**	**1.058**	**1.304**	**1.389667**	**1.532667**	**1.582667**	**1.590667**
Ent 1	0.166	0.596	0.611	0.809	1.114	1.303	1.434	1.378	1.418	1.487	1.408	1.425
	0.181	0.591	0.719	0.961	1.289	1.296	1.44	1.319	1.322	1.455	1.429	1.321
	0.159	0.628	0.769	0.987	1.287	1.206	1.406	1.385	1.354	1.487	1.423	1.466
average	**0.168667**	**0.605**	**0.699667**	**0.919**	**1.23**	**1.268333**	**1.426667**	**1.360667**	**1.364667**	**1.476333**	**1.42**	**1.404**
Pre 8	0.207	1.149	1.039	1.167	1.19	1.197	1.169	1.23	1.318	1.376	1.395	1.392
	0.149	1.055	1.091	1.124	1.145	1.153	1.183	1.185	1.269	1.419	1.386	1.472
	0.148	1.02	1.02	1.076	1.118	1.128	1.253	1.172	1.175	1.238	1.238	1.473
average	**0.168**	**1.074667**	**1.05**	**1.122333**	**1.151**	**1.159333**	**1.201667**	**1.195667**	**1.254**	**1.344333**	**1.339667**	**1.445667**
Pre 6	0.162	1.079	1.037	1.055	1.138	1.172	1.27	1.243	1.318	1.356	1.337	1.339
	0.142	1.048	1.04	1.042	1.214	1.203	1.182	1.442	1.369	1.349	1.288	1.384
	0.201	1.193	1.07	1.04	1.228	1.273	1.269	1.402	1.415	1.416	1.421	1.42
average	**0.168333**	**1.106667**	**1.049**	**1.045667**	**1.193333**	**1.216**	**1.240333**	**1.362333**	**1.367333**	**1.373667**	**1.348667**	**1.381**
Ser 12	0.159	1.163	1.161	1.142	1.207	1.2	1.182	1.259	1.213	1.368	1.414	1.476
	0.18	1.118	1.088	1.115	1.174	1.226	1.234	1.297	1.327	1.314	1.306	1.328
	0.168	1.115	1.043	1.114	1.218	1.235	1.218	1.27	1.313	1.334	1.333	1.396
average	**0.169**	**1.132**	**1.097333**	**1.123667**	**1.199667**	**1.220333**	**1.211333**	**1.275333**	**1.284333**	**1.338667**	**1.351**	**1.4**
KZN 18	0.243	0.926	1.045	1.043	1.264	1.092	1.166	1.201	1.212	1.309	1.349	1.235
	0.233	1.025	1.032	0.978	1.116	1.139	1.213	1.288	1.301	1.337	1.32	1.397

	0.269	0.937	1.044	1.058	1.02	1.116	1.207	1.27	1.202	1.255	1.309	1.399
average	**0.248333**	**0.962667**	**1.040333**	**1.026333**	**1.133333**	**1.115667**	**1.195333**	**1.253**	**1.238333**	**1.300333**	**1.326**	**1.343667**
KZN 21	0.105	0.716	0.619	0.648	0.74	0.822	0.791	0.994	0.93	0.966	0.951	0.964
	0.124	0.622	0.501	0.594	0.685	0.825	0.863	0.945	0.903	0.965	0.982	0.928
	0.108	0.588	0.469	0.529	0.639	0.773	0.843	0.934	0.912	1.042	0.913	0.924
average	**0.112333**	**0.642**	**0.529667**	**0.590333**	**0.688**	**0.806667**	**0.832333**	**0.957667**	**0.915**	**0.991**	**0.948667**	**0.938667**
OK 1	0.103	0.443	0.166	0.212	0.474	0.562	0.655	0.788	1.062	1.146	1.059	1.209
	0.117	0.445	0.147	0.198	0.506	0.631	0.81	1.138	1.047	1	1.06	1.212
	0.103	0.487	0.287	0.285	0.576	0.639	0.745	0.915	0.854	1.045	1.083	1.168
average	**0.107667**	**0.458333**	**0.2**	**0.231667**	**0.518667**	**0.610667**	**0.736667**	**0.947**	**0.987667**	**1.111**	**1.067333**	**1.196333**

Pse 2 = *Pseudomonas aeruginosa* (ATCC 7700); Ent 1 = *Enterobacter cloacae* (ATCC 13047); Pre 8 = *Proteus vulgaris* (ATCC 6830); Pre 6 = *Proteus vulgaris* (CSIR 0030); Ser 12 = *Serratia marscens* (ATCC 9986); KZN 18 = *Klebsiella pneumoniae* (KZN); KZN 21 = *Salmonella* sp (KZN) and OK1

MBC results for extract obtained from *Saccharopolyspora* TR 039

Test Organism	CONCENTRATION (mg/ml)							
	10	5	2.5	1.25	0.625	0.312	0.156	0.078
Pseudomonas aeruginosa (Pse2)	+	+	+	+	+	+	+	+
Enterobacter cloacae (Ent 1)	+	+	+	+	+	+	+	+
Proteus vulgaris (Pre 8)	+	+	+	+	+	+	+	+
Proteus vulgaris (Pre 6)	+	+	+	+	+	+	+	+
Serratia marscens (Ser 12)	+	+	+	+	+	+	+	+
Klebsiella pneumoniae (KZN)	+	+	+	+	+	+	+	+
Salmonella sp (KZN)	+	+	+	+	+	+	+	+
OK 1	+	+	+	+	+	+	+	+

Key: + indicates growth, - indicates absence of growth

MIC results for extract obtained from *Saccharopolyspora* TR 046, wavelength 490 nm

	CONCENTRATION mg/ml											
	Positive control	**Methanol control**	**10**	**5**	**2.5**	**1.25**	**0.625**	**0.312**	**0.156**	**0.078**	**0.039**	**Growth control**
Pse 2	0.132	1.208	0.131	0.139	0.147	0.494	0.818	0.991	1.33	1.352	1.304	1.36
	0.128	1.104	0.129	0.14	0.142	0.492	0.681	1.113	1.223	1.215	1.27	1.333
	0.126	1.121	0.128	0.136	0.152	0.482	0.644	0.981	1.132	1.275	1.255	1.319
average	**0.128667**	**1.144333**	**0.129333**	**0.138333**	**0.147**	**0.489333**	**0.714333**	**1.028333**	**1.228333**	**1.280667**	**1.276333**	**1.337333**
Pre 6	0.13	1.119	0.213	0.26	0.378	0.764	1.111	1.153	1.245	1.265	1.262	1.307
	0.129	1.142	0.204	0.249	0.684	0.781	1.083	1.145	1.186	1.224	1.236	1.319
	0.126	1.169	0.218	0.254	0.496	0.752	1.138	1.241	1.266	1.232	1.278	1.378
average	**0.128333**	**1.143333**	**0.211667**	**0.254333**	**0.519333**	**0.765667**	**1.110667**	**1.179667**	**1.232333**	**1.240333**	**1.258667**	**1.334667**
Aci 1	0.127	1.1	0.324	0.541	0.658	1.043	1.177	1.34	1.352	1.329	1.333	1.355
	0.129	1.117	0.306	0.498	0.634	0.908	1.164	1.284	1.316	1.334	1.351	1.362
	0.134	1.132	0.32	0.562	0.644	0.856	1.202	1.224	1.329	1.306	1.336	1.387
average	**0.13**	**1.116333**	**0.316667**	**0.533667**	**0.645333**	**0.935667**	**1.181**	**1.282667**	**1.332333**	**1.323**	**1.34**	**1.368**
KZN 22	0.13	0.802	0.134	0.163	0.2	0.208	0.287	0.45	0.654	0.696	0.767	1.497
	0.126	0.741	0.136	0.172	0.18	0.209	0.304	0.496	0.598	0.694	0.998	1.549
	0.128	0.857	0.132	0.166	0.198	0.203	0.284	0.377	0.64	0.69	0.987	1.456
average	**0.128**	**0.8**	**0.134**	**0.167**	**0.192667**	**0.206667**	**0.291667**	**0.441**	**0.630667**	**0.693333**	**0.917333**	**1.500667**
Pse 3	0.127	1.001	0.129	0.137	0.147	0.245	0.445	1.432	1.567	1.879	2.117	2.12
	0.129	1.078	0.134	0.136	0.152	0.283	0.438	1.442	1.528	1.72	1.772	1.935
	0.131	1.089	0.127	0.138	0.156	0.246	0.39	1.368	1.759	1.886	1.792	1.986
average	**0.129**	**1.056**	**0.13**	**0.137**	**0.151667**	**0.258**	**0.424333**	**1.414**	**1.618**	**1.828333**	**1.893667**	**2.013667**
Bac 42	0.133	1.096	0.12	0.124	0.13	0.136	0.199	0.244	0.284	1.048	1.23	1.331
	0.123	1.053	0.114	0.127	0.129	0.139	0.189	0.219	0.297	0.984	1.033	1.249
	0.132	1.1	0.11	0.123	0.132	0.143	0.213	0.235	0.291	1.176	1.07	1.277
average	**0.129333**	**1.083**	**0.114667**	**0.124667**	**0.130333**	**0.139333**	**0.200333**	**0.232667**	**0.290667**	**1.069333**	**1.111**	**1.285667**

Ser 12	0.123	1.414	0.234	0.285	0.398	1.203	1.28	1.396	1.481	1.48	1.498	1.513
	0.111	1.461	0.22	0.281	0.521	1.234	1.269	1.417	1.463	1.463	1.507	1.498
	0.113	1.506	0.228	0.328	0.434	1.262	1.289	1.36	1.447	1.487	1.493	1.5
average	**0.115667**	**1.460333**	**0.227333**	**0.298**	**0.451**	**1.233**	**1.279333**	**1.391**	**1.463667**	**1.476667**	**1.499333**	**1.503667**
KZN 18	0.125	1.302	0.226	0.333	0.456	1.295	1.34	1.493	1.575	1.577	1.632	1.821
	0.134	1.331	0.219	0.345	0.547	1.274	1.434	1.439	1.546	1.598	1.599	1.856
	0.132	1.35	0.198	0.234	0.532	1.263	1.482	1.504	1.567	1.572	1.635	1.783
average	**0.130333**	**1.327667**	**0.214333**	**0.304**	**0.511667**	**1.277333**	**1.418667**	**1.478667**	**1.562667**	**1.582333**	**1.622**	**1.82**
KZN 23	0.25	0.671	0.382	0.682	0.727	1.025	1.153	1.45	1.69	1.73	1.723	1.752
	0.35	0.742	0.401	0.642	1.106	1.226	1.467	1.511	1.587	1.631	1.668	1.773
	0.334	0.561	0.426	0.649	1.009	1.184	1.35	1.394	1.497	1.613	1.623	1.698
average	**0.311333**	**0.658**	**0.403**	**0.657667**	**0.947333**	**1.145**	**1.323333**	**1.451667**	**1.591333**	**1.658**	**1.671333**	**1.741**

Pse 2 = *Pseudomonas aeruginosa* (ATCC 7700); Pre 6 = *Proteus vulgaris* (CSIR 0030); Aci 1 = *Acinetobacter calcaoceticus* (UP); KZN 22 = *Staphylococcus epidermidis* (KZN); Pse 3 = *Pseudomonas aeruginosa* (ATCC 19582); Bac 42 = *Bacillus pumilus* (ATCC 14884); Ser 12 = *Serratia marscens* (ATCC 9986); KZN 18 = *Klebsiella pneumoniae* (KZN) and KZN 23 = *Pseudomonas aeruginosa* (KZN)

MBC results for extract obtained from *Saccharopolyspora* TR 046

Test organism	Concentration (mg/ml)						
	10	5	2.5	1.25	0.625	0.312	0.156
P aeruginosa (Pse 2)	-	-	+	+	+	+	+
P vulgaris (Pre 6)	-	-	+	+	+	+	+
A calcaoceticus (Aci 1)	+	+	+	+	+	+	+
S epidermidis (KZN)	-	-	-	-	+	+	+
P aeruginosa (Pse 3)	-	-	-	+	+	+	+
B pumilus (Bac 42)	-	+	+	+	+	+	+
S marscens (Ser 12)	-	+	+	+	+	+	+
K pneumoniae (KZN)	-	-	+	+	+	+	+
P aeruginosa (KZN)	+	+	+	+	+	+	+

Key: + indicates growth, - indicates absence of growth

MIC results for extract obtained from *Actinosynnema* TR 024, wavelength 490 nm

	Concentration (mg/ml)											
	positive control	**methanol control**	**10**	**5**	**2.5**	**1.25**	**0.625**	**0.312**	**0.156**	**0.078**	**0.039**	**growth control**
Pse 2	0.485	1.174	0.649	0.724	1.283	1.561	1.573	1.649	1.581	1.707	1.624	1.569
	0.495	1.199	0.593	0.766	1.276	1.549	1.549	1.565	1.564	1.6	1.544	1.643
	0.494	1.156	0.644	0.779	1.202	1.589	1.597	1.672	1.557	1.609	1.604	1.659
average	**0.491333**	**1.176333**	**0.628667**	**0.756333**	**1.253667**	**1.566333**	**1.573**	**1.628667**	**1.567333**	**1.638667**	**1.590667**	**1.623667**
Pre 8	0.274	0.911	0.63	1.038	1.049	1.066	1.308	1.45	1.423	1.485	1.31	1.611
	0.389	0.907	0.607	0.93	1.095	1.12	1.413	1.355	1.54	1.409	1.477	1.573
	0.39	1.215	0.615	0.966	0.966	1.098	1.306	1.289	1.47	1.404	1.487	1.602
average	**0.351**	**1.011**	**0.617333**	**0.978**	**1.036667**	**1.094667**	**1.342333**	**1.364667**	**1.477667**	**1.432667**	**1.424667**	**1.595333**
Pre 6	0.19	1.265	1.055	1.168	1.217	1.257	1.309	1.342	1.354	1.386	1.438	1.496
	0.213	1.301	1.031	1.125	1.249	1.303	1.357	1.389	1.423	1.402	1.425	1.473
	0.198	1.318	1.05	1.148	1.231	1.295	1.302	1.304	1.447	1.407	1.484	1.41
average	**0.200333**	**1.294667**	**1.045333**	**1.147**	**1.232333**	**1.285**	**1.322667**	**1.345**	**1.408**	**1.398333**	**1.449**	**1.459667**
KZN 18	0.412	1.305	0.848	1.044	1.141	1.243	1.299	1.42	1.452	1.462	1.462	1.497
	0.462	1.31	0.853	1.004	1.162	1.25	1.257	1.445	1.46	1.479	1.459	1.483
	0.428	1.278	0.879	1.027	1.208	1.255	1.316	1.431	1.472	1.481	1.509	1.5
average	**0.434**	**1.297667**	**0.86**	**1.025**	**1.170333**	**1.249333**	**1.290667**	**1.432**	**1.461333**	**1.474**	**1.476667**	**1.493333**

Pse 2 = *Pseudomonas aeruginosa* (ATCC 7700); Pre 8 = *Proteus vulgaris* (ATCC 6830); Pre 6 = *Proteus vulgaris* (CSIR 0030) and KZN 18 = *Klebsiella pneumoniae* (KZN)

MBC results for extract obtained from *Actinosynnema* TR 024

Test organism	CONCENTRATION (mg/ml)							
	10	5	2.5	1.25	0.625	0.312	0.156	0.078
Pseudomonas aeruginosa (Pse2)	+	+	+	+	+	+	+	+
Proteus vulgaris (Pre 8)	+	+	+	+	+	+	+	+
Proteus vulgaris (Pre 6)	+	+	+	+	+	+	+	+
Klebsiella pneumoniae (KZN)	+	+	+	+	+	+	+	+

Key: (+) indicates growth; (-) indicates absence of growth

Statistical analysis, Minitab output

```
Two-Sample T-Test and CI: TR046, TR024

Two-sample T for TR046 vs TR024

       N   Mean  StDev  SE Mean
TR046  9  18.33   6.42      2.1
TR024  4  12.00   1.41     0.71


Difference = mu (TR046) - mu (TR024)
Estimate for difference:  6.33333
95% CI for difference:  (1.23302, 11.43365)
T-Test of difference = 0 (vs not =): T-Value = 2.81  P-Value = 0.020  DF = 9


Two-Sample T-Test and CI: TR039, TR024

Two-sample T for TR039 vs TR024

       N   Mean  StDev  SE Mean
TR039  8  12.13   2.23     0.79
TR024  4  12.00   1.41     0.71


Difference = mu (TR039) - mu (TR024)
Estimate for difference:  0.125000
95% CI for difference:  (-2.271998, 2.521998)
T-Test of difference = 0 (vs not =): T-Value = 0.12  P-Value = 0.909  DF = 9
```

```
Two-Sample T-Test and CI: TR046, TR039

Two-sample T for TR046 vs TR039

       N   Mean  StDev  SE Mean
TR046  9  18.33   6.42      2.1
TR039  8  12.13   2.23     0.79


Difference = mu (TR046) - mu (TR039)
Estimate for difference:  6.20833
95% CI for difference:  (1.12442, 11.29225)
T-Test of difference = 0 (vs not =): T-Value = 2.72  P-Value = 0.022  DF = 10


One-way ANOVA: TR046, TR039, TR024

Source  DF     SS     MS     F      P
Factor   2  200.9  100.5  4.88  0.020
Error   18  370.9   20.6
Total   20  571.8

S = 4.539   R-Sq = 35.14%   R-Sq(adj) = 27.93%


                        Individual 95% CIs For Mean Based on
                        Pooled StDev
Level  N    Mean  StDev  --+---------+---------+---------+-------
TR046  9  18.333  6.423                     (-------*-------)
TR039  8  12.125  2.232      (-------*--------)
TR024  4  12.000  1.414  (-----------*-----------)
                        --+---------+---------+---------+-------
                        8.0      12.0      16.0      20.0

Pooled StDev = 4.539
```