ISOLATION AND IDENTIFICATION OF ANTIBIOTIC PRODUCING MICROORGANISMS FROM NATURAL HABITATS IN THE KWAZULU-NATAL MIDLANDS

By

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ABSTRACT

The search for new antibiotics continues in a rather overlooked hunting ground. In the course of screening for new antibiotic-producing microorganisms, seventy-nine isolates showing antimicrobial activity were isolated from soil samples from various habitats in the KwaZulu-Natal midlands, South Africa. Existing methods of screening for antibiotic producers together with some novel procedures were reviewed. Both modified agar-streak and agar-plug methods were used in the primary screens. The use of selective isolation media, with or without antibiotic incorporation and/or heat pretreatment, enhanced the development of certain actinomycete colonies on the isolation plates. Winogradsky's nitrite medium (Winogradsky, 1949), M3 agar (Rowbotham and Cross, 1977), and Kosmachev's medium (Kosmachev, 1960), were found to be selective for actinomycetes. Statistical analysis showed highly significant interactions between isolates, assay media and the test organisms. The diameters of inhibition zones were found to be larger on Iso-sensitest agar (ISTA)[Oxoid, England] than in nutrient agar plates. Of the 79 isolates that showed antimicrobial activity, 44 isolates were selected for confirmatory screening. Of these, 13 were selected for secondary screening. Criteria for selection were based on significant inhibition of at least two test organisms and/or the inhibition of the specifically targeted organisms, *Pseudomonas* and *Xanthomonas* species. Following secondary screening eight isolates were considered for further investigation. The isolates were tentatively identified on the basis of morphological features, using both light microscopy and scanning electron microscopy(SEM); their ability to utilize various carbon sources; and selected physiological and staining tests. Suspected actinomycetes were further characterized on the basis of selected chemical properties using thin layer chromatography (TLC) and high pressure liquid chromatography (HPLC) techniques. High pressure liquid chromatography analysis (Beckman 6300 analyzer) detected the presence of diaminopimelic acid (DAP) in whole-cell hydrolysates

of six of the isolates while TLC analysis confirmed the type of DAP present. The isolates N2, N12, N16, N19 and N35 were tentatively identified as *Thermomonospora*, *Saccharopolyspora*, *Nocardiodes*, *Corynebacterium* and *Promicromonospora*, respectively. Isolate N30 was identified as belonging to the coryneform group of bacteria, possibly an *Arthrobacter* species. Isolate, N8, tentatively identified as *Actinosynnema*, was unique among the isolates tested as it showed good antimicrobial activity against all the Gram- positive and Gram-negative bacteria, and yeasts used as test organisms in the present investigation.

DECLARATION

I hereby declare that the work reported in this thesis, except where otherwise indicated, is entirely the result of my own research efforts.

Vincent Ifeanyi Okudoh

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Chapter1

Literature Review

1.1 Introduction

The diversity of soil microorganisms was of great significance as a factor promoting the early discovery of antibiotics (Woodruff,1996). Many types of microorganisms such as moulds, bacteria, protozoa and algae, all competing for limited nutrients in the soil, have to devise strategies to survive. Among these microbes are autotrophs, free living nitrogen fixers, thermophiles, acidophiles, pathogens and saprophytes (Woodruff,1996).

Support for the fact that antagonistic interrelationships occur among microorganisms can be traced back to Pasteur's observation that an injection of a mixed population of soil microbes, which included anthrax spores, was less infective for animals than injections of the anthrax organism alone (Woodruff,1996). Waksman and Foster (1937) noted that certain soil actinomycetes exert antagonistic effects against other soil microbes. Between 1939 and 1940, Howard Florey and his associates at Oxford University and Rene Dubos at the Rockefeller Institute reported that the antagonistic effect is based on certain chemical entities produced by antagonistic organisms (Dubos, 1939; Chain *et al.*, 1940). These reports initiated the search for antimicrobial chemicals and hence the initiation of antibiotic studies by Waksman and his associates. Thereafter, the study of interactions among soil inhabiting microbes ceased in favour of screening for new antibiotic producers (Woodruff, 1996).

Although the first commercially produced antibiotic, penicillin, was discovered by chance, most present day antibiotics are discovered by systematic searching. As the soil is a vast repository of microorganisms, many of which remain undetected, it is a potential source of many species with the ability to produce new antibiotics. Thus attention is most often turned to the soil whenever new antibiotic producers are being sought (Okafor, 1987).

The earliest concerted search for antibiotic producing microbes in the soil would appear to be that of

Dubos. He used essentially an enrichment method in which polysaccharides of Capsular III pneumococci were incubated in soil to bait soil organisms able to decompose them (Okafor,1987). Later Dubos introduced the actual pathogens into the soil. Although he obtained from *Bacillus brevis*, an antibiotic, Tyrothricin (later shown to consist of two antibiotics, Gramicidin and Tryocidin), his method presupposed that the antibiotic producing organism acts by attacking the cell wall material of sensitive strains. Therefore, it is not surprising that this method was later replaced by a more suitable method developed by Waksman and associates (Okafor,1987).

It was an appreciation of the diversity of soil micro-organisms that led Dubos, Waksman and those that followed to choose soil as the source for their experiments (Woodruff,1996). Thousands of antibiotics have already been discovered. However, only a very small proportion of known antibiotics are used clinically because the others are either too toxic, or show other undesirable properties. For example, by 1978, nearly 5000 antibiotics had been described, of which only 95 were being used for therapy (Okafor,1987).

In spite of the many antibiotics available, the need still exists for the discovery of new ones to solve urgent therapeutic problems, including:

- The continuing problem of development of drug resistance among pathogenic species;
- Organisms which were previously commensal are now becoming pathogens because of wide spread abuse of antibiotics. Examples are *Proteus spp*, *Staphylococcus spp* and several yeasts;
- Currently few satisfactory systemic antifungal antibiotics outside Amphotericin B seem to exist, and even Amphotericin B is not always effective;
- The growing needs for antibiotics for use in Agriculture, for combating plant diseases;
- Antiviral agents also need to be developed (Berdy,1980).

These problems cannot be solved by chemical synthesis of antibiotics alone. New types of antibiotics, exhibiting new profiles of activity and mechanism of action, may be found mainly by further systematic screening of microorganisms, performed according to fundamentally new principles.

With the advent of new techniques of screening, coupled with advances made in the field of genetic engineering, many microbiologists are taking up this challenge. This challenge is being brought to Africa because of lack of previous systematic searches on much of the continent. Thus the KwaZulu Natal midlands region of South Africa, with its abundance of nature was deemed a good hunting ground and was selected as the focus area of the present investigation.

Antibiotics and antibiotic-producing microorganisms indigenous to African soils may contribute significantly to keeping up with the spirit of this crusade.

1.2 What are Antibiotics ?

Antibiotics have been defined by Gottlieb and Shaw (1967) as " Organic substances that are produced by microorganisms and are harmful at low concentrations to growth and metabolic activities of other organisms". Lancini and Parenti (1982) limited the definition to special inhibitory products of low molecular weight and excluded enzymes, lactic acid, ethanol, and other similar substances that prevent growth of some microorganisms. Powerful antibiotics such as penicillin and cephalosporin are of such tremendous importance in medicine, that an ever increasing search is going on in the hope that agents superior to these and other antibiotics now in use might be isolated.

According to Berdy (1980), higher forms of life (algae, lichens, plants and animals) have been shown to produce anti-microbial substances of low molecular weight. These are secondary metabolites, just like conventional antibiotics. Because of this, over the past two decades, there has been a tendency to extend the term antibiotic to all secondary metabolites, irrespective of their origin, which are able to inhibit various growth processes at low concentration (Okafor,1987). It is not altogether an unreasonable redefining of terms, since the word antibiotic, derives from two origins, *anti* (against) and *bios* (life). Nothing in the word itself, either in origin or in use, restricts the term antibiotic to substances of microbial origin. Proponents of this view argue that any products from a living organism which, at low concentrations, kill any other living organism, be they microorganisms, higher plants or animals, should be described as antibiotics (Okafor,1987).

Perhaps most microbiologists, while accepting that the term antibiotic need not be limited to those

products with anti-microbial activity, may prefer to restrict the term to only those metabolites produced by microorganisms. After all, the microbiologist by his training feels more at home with the Petri dish than with zoological or botanical gardens (Okafor,1987).

1.3 Classification and Nomenclature of Antibiotics

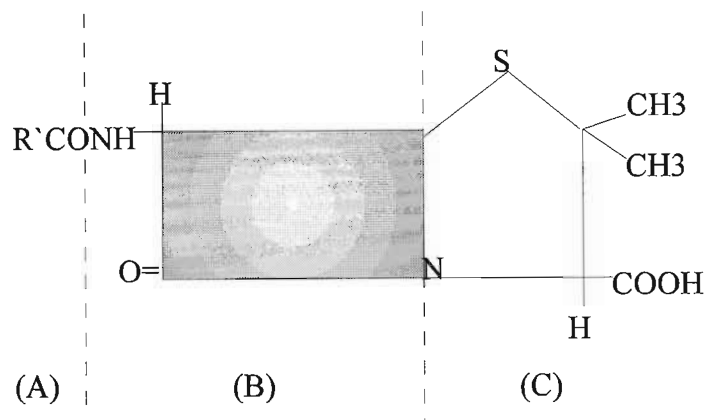
Several methods of antibiotic classification have been used by various authors. Some methods are based on the mode of action of compounds, e.g. whether they act on the cell wall or membrane or are inhibitors of protein or nucleic acid synthesis/functions, or interfere with the whole system of cellular metabolism. However, several mechanisms of action may operate simultaneously, making such classification difficult (Berdy, 1974). In some cases, antibiotics have been classified on the basis of the producing organism. However, some organisms may produce several antibiotics, e.g., the production of penicillin-N and cephalosporin C by *Cephalosporium acremonium* (Price, 1969 ; Berdy,1980) [Table1.1]. Alternatively, the same antibiotic may be produced by different organisms. For example, penicillin has now been shown to be produced by a wide range of organisms including species of the fungi *Aspergillus, Cephalosporium,* and *Trichophyton*, and some actinomycetes, such as *Streptomyces spp.* (Queener *et al.*, 1978).

Antibiotics have also been classified according to the route of biosynthesis. However, several different biosynthetic routes often have much commonality [section1.4]. The spectra of organisms killed/inhibited have also been used e.g. those affecting specifically bacteria, fungi, protozoa etc. However, some antibiotics belonging to a particular group may have a different spectrum from others in the same group. For instance, paromomycin is generally classified as antiprotozoal, when its entire spectrum of activity and chemical structure is almost identical with that of other amino-glycosides (viz. neomycin, kanamycin) which are regarded as antibacterial antibiotics (Berdy, 1974).

More recent classification methods are based on the chemical structure of the antibiotics. Queener *et al* (1978) divided antibiotics into thirteen groups [Table1.2], based strictly on their chemical structures. This enables the accommodation of new compounds as they are discovered. The question of nomenclature of antibiotics is highly confusing and the same antibiotic may have as many as thirteen

different trade names depending on the manufacturer. Antibiotics are identified by at least three names: viz.

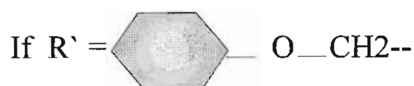
- The Chemical name - which is normally very long and is rarely used, except in scientific or medical literature. An example is shown in the structure of penicillin below.



(A) = side chain

(B) = B-lactam ring

(C) = Thiazolidine ring

If R` =  O__CH2--

Phenoxymethyl penicillin – Chemical name

Penicillin V – Generic name

Fig1.1 Structure and nomenclature of Penicillin

- The Generic Name - which is a more commonly used name, usually a shorter form of the chemical name or original name .
- The Brand name- given by the manufacturers to distinguish it from the product of other companies (Okafor, 1987).

The most obvious problems associated with nomenclature of antibiotics appear to be the following:

- There is a nearly complete lack of uniform denomination for antibiotics described by different scientists and found later to be identical e.g. desdanin, netropsin, neomycin etc.
- Designations used in patents constitute a problem in themselves. Frequently only numbers or letter-number combinations are used, which are not identical with names applied in later publications. e.g. (a) SF-733 antibiotic -- patent designation; or Ribostamycin -- generic name (non proprietary name); or Vistamycin -- brand (commercial name)/ trade name: (b) M-141, U-18409 -- patent designation; or Actinospectacin -- generic name; or Spectinomycin -- brand/trade name.
- Very often several names for one agent are in general use in various publications: (Paromomycin, aminosidin, monomycin); (Pentamycin, lagosin, fungichromin); (Nigericin, polyetherin A) etc.
- At present there are more than a thousand superfluous antibiotic names in current use (Berdy,1974; 1980).

Table 1.1 Antibiotics produced by various microorganisms

MICROORGANISM	ANTIBIOTIC PRODUCED	DISCOVERY DATE
Penicillium notatum	penicillin G	1929
Penicillium griseofulvum	griseofulvin	1939
Bacillus brevis	tyrothricin [tyrocidin,gramicidin]	1939 [1940, 1944]
Aspergillus fumigatus var.helvola	helvolic acid	1942
Streptomyces griseus	streptomycin	1944
Bacillus subtilis	bacitracin	1945
Streptomyces venezualae	chloramphenicol	1947
Streptomyces aureofaciens	*chlor* tetracycline	1948
Streptomyces fradiae	neomycin B	1949
Streptomyces rimosus	oxytetracycline	1950
Cephalosporium acremonium	cephalosporin C, penicillin-N	1950's
Streptomyces aureofaciens	tetracyclines	1952
Streptomyces antibioticus	oleandomycin	1954
Streptomyces orientalis	vancomycin	1956
Streptomyces kanamyceticus	kanamycin	1957
Norcadia(formally Streptomyces) mediterranei	rifamycin	1958
Streptomyces capreolus	capreomycin	1960
Fusidium coccineum	fusidic acid	1962
Streptomyces lincolnesis	lincomycin	1962
Micromonospora purpurea	gentamycin C1, C1a, C2	1963
Streptomyces sp	cephamycin	1971
Streptomyces spectabilis	spectinomycin	NA*
Streptomyces hygroscopicus	hygromycin B	"
Streptomyces rimofaciens	destomycin	"
Streptomyces nodosus	amphotericin B	"
Streptomyces erythreus	erythromycin[carbomycin]	"
Streptomyces verticillus	bleomycin	"
Streptomyces tenebrarius	tobramycin	"
Bacillus circulans	butirosin B	"

Source: Handbook of Antibiotics, edited by Baron (1950), with some additions.

*NA= not available.

Table 1.2 The thirteen named groups of antibiotics based on chemical structure

Group	Example
Aminoglycosides	Streptomycin
Ansamacrolides	Rifamycin
Beta - Lactams	Penicillins, Cephalosporins
Chloramphenicol and Analogues	Chloramphenicol
Lincosaminides	Lincomycin
Macrolides	Erythromycin
Nucleosides	Puromycin
Oligosaccharides	Curamycin
Peptides	Neomycin
Phenazymes	Myxin
Polyenes	Amphothericin B
Polyethers	Nigericin
Tetracyclines	Tetracyclines

Source: Queener *et al.* (1978).

1.4 Antibiotics from Microorganisms - A Review

The main sources of microbial antibiotics are the following groups of microorganisms;

(a) Actinomycetales

(b) True Bacteria

(c) Fungi

The cumulative totals of microbial antibiotics known to be produced by members of these groups are shown in Table1.3.

Table 1.3 Cumulative totals of antibiotic-producing microorganisms identified between 1945 and 1980 and their uses

Years					Total			Uses			
	Streptomyces	Other Actinomycetes	True Bacteria	Fungi		Biosynthetic Modified Derivatives	Minor Compounds	Anti-Tumor	Anti-Viral	Anti-Bacterial	Agricultural
1945	10	2	25	51	88	-	-	5	4	2	-
1950	72	10	94	140	316	1	3	18	21	9	-
1955	325	22	137	223	707	6	9	56	62	16	2
1960	760	40	181	294	1275	24	38	177	130	26	7
1965	1177	75	223	423	1898	37	77	367	175	38	13
1970	1745	136	328	680	2889	97	214	620	255	44	19
1975	2361	250	518	970	4099	187	451	830	339	50	29
1980	2769	396	657	1151	4973	225	621	1035	377	51	35
1985	-	-	-	-	-	-	-	-	-	-	-
1990	-	-	-	-	-	-	-	-	-	-	-
1995	-	-	-	-	-	-	-	-	-	-	-

source: Okafor, (1987) Industrial Microbiology. p337

Up to the end of the 1940's fungi, and to a lesser extent bacteria, furnished the greatest number of antibiotics discovered. Between 1955 and 1962, however, about 80% of the antibiotics found originated from Actinomycetales species (Berdy, 1974) [Fig1.2].

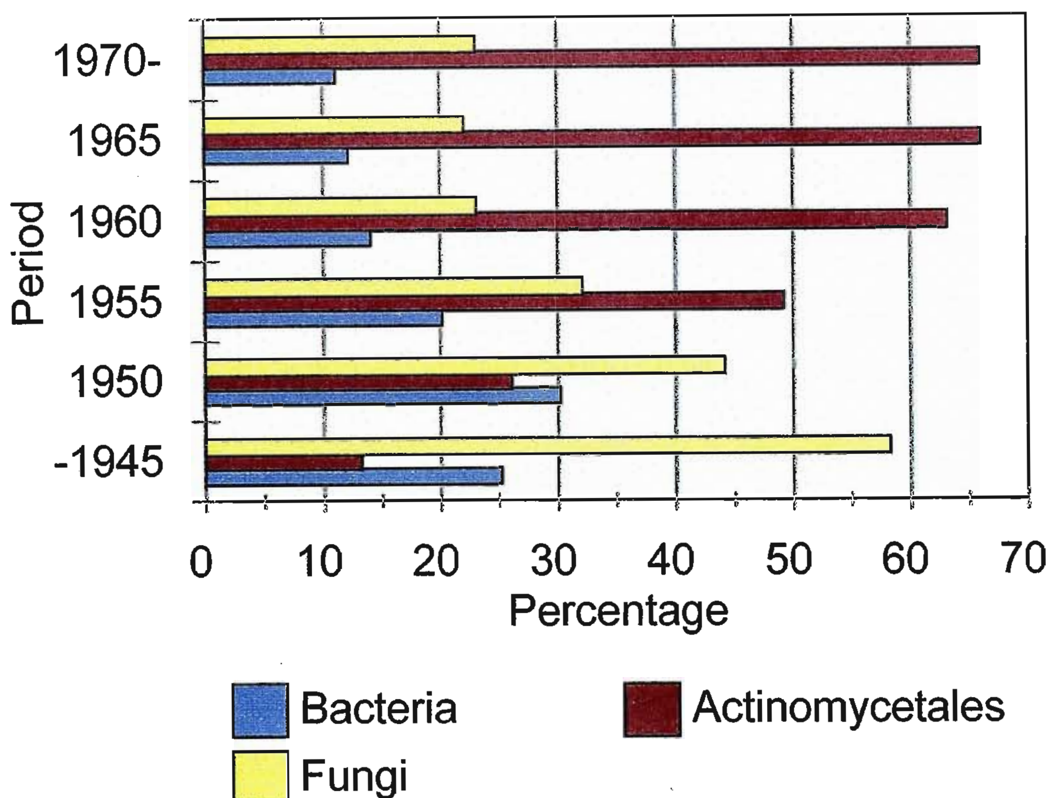


Fig 1.2 Percentage distribution of antibiotics according to type of producer microorganism

(a) Actinomycetales

Extensive screening programmes started after 1945, utilizing simple methods for isolating large numbers of strains from soil samples, resulted in the isolation and identification of hundreds of antibiotics of Actinomycetales origin. After reaching a peak in 1957, a slow decline in the number of strains isolated followed, and only after 1964, was further progress made, when the importance of non-streptomycetous-actinomycetales in antibiotic research was recognized (Berdy, 1974). According to various investigators (Lechevalier and Lechevalier, 1967; Rangaswami *et al.*, 1967) Streptomycetales species account for 90-95% of Actinomycetales isolated from soil samples. Consequently, about 92,5% of the antibiotics of Actinomycetales origin described to date, have been isolated from *Streptomyces spp* [Table1.3].

The ratio of antibiotics of non-streptomycetous origin has risen since 1965. Gentamicin, rifamycin and

[Table1.3].

The ratio of antibiotics of non-streptomycetous origin has risen since 1965. Gentamicin, rifamycin and ristocetin originate from *Micromonospora* and *Nocardia spp* although sometimes problems have arisen in identification of the microorganisms. For example, rifamycin-producing *Nocardia mediteranei* was for a long time thought to be a *Streptomyces sp* (Thiemann *et al.*, 1969).

Of the 25 new antibiotics introduced into clinical practice between 1960-1970, all except fusidic acid were produced by actinomycetales. Actinomycetales have furnished the greatest number of antibiotics in commercial use; altogether about 72 of them being utilized for various purposes (Berdy, 1974).

(b)True Bacteria.

Among this group, the organisms that deserve particular mention include *Pseudomonas* species and *Bacillus* species [Table1.4]. Members of these genera produce several useful compounds including: colistin, polymyxin B and M, gramicidin, tyrothricin, bacitracin, from *Bacillus* species; pyocyanin and pyrrolnitrin from *Pseudomonas* species (Berdy, 1974).

(c) Fungi.

Penicillium, *Cephalosporium*, *Aspergillus* and some members of the fungi imperfecti are of greatest importance here. The number of antibiotics produced by different fungi is shown in Table 1.5. Of these about ten have been commercialized, viz. penicillin G, V, O, cephalosporin, griseofulvin, fumagillin, variotin, fusidic acid, siccanin and xanthocillin (Berdy, 1974). It is interesting to note that most of the commonly used fungal antibiotics e.g. penicillins, cephalosporin C and fusidic acid are found among metabolites of different fungal species (Berdy, 1974).

Table1.4 Number of antibiotics produced by different schizomycetes

Pseudomonadales	87
Pseudomonadaceae	84
Pseudomonas	82
Aerobacter	2

Spirillaceae	3
Eubacteriales	274
Azobacteraceae	1
Rhizobiaceae	2
Achromobacteriaceae	9
Enterobacteriaceae	36
Brucellaceae	3
Micrococcaceae	16
Lactobacillaceae	28
Propionibacteriaceae	2
Brevibacteriaceae	1
Corynebacteriaceae	5
Bacillaceae	171
Bacillus	167
Clostridium	4
Actinomycetales	2078
Mycobacteriaceae	4
Actinoplanaceae	18
Actinoplanes	6
Spillospora	2
Streptosporangium	7
Microcellobosporia	3
Streptomycetaceae	1950
Streptomyces	1922
Streptoverticillium	19
Chainia	8
Kitasatoa	1
Micromonosporaceae	41
Micromonospora	41

Thermoactinomycetaceae	17
Thermoactinomyces	9
Microbispora	4
Thermomonospora	4
Nocardiaceae	48
Nocardia	45
Micropolyspora	2
Thermomonospora	1
Myxobacteriales(*Cytophaga,Myxococcus,*etc	9
Mycoplasmatales(Mycoplasma)	2

Source: Berdy (1974). *Advances in Applied Microbiology* 18: p328

Table1.5 Number of antibiotics produced by different fungi

Myxothallophytes (Myxomycotinia) *(Fuligo , Physarum, Lycologa* sp)	4
Eumycotinia (Mycophyta), ' true fungi'	768
Phycomycetes *(Phytophthora, Mucor , Rhizopus* sp.)	14
Ascomycetes	299
Protoascomycetes (Ascosporogenous yeasts)	8
Euascomycetes	291
Plectascales	248
Aspergillaceae	242
Penicillium	123
Aspergillus	115
Other orders of Euascomycetes	43
Basidiomycetes	140

Fungi imperfecti	315			
Moniliales		269		
Moniliaceae			102	
Cephalosporium				20
Tichoderma				13
Oospora				10
Acrostalagmus				7
Cylindrocladium				9
Demetaciae			69	
Helminthosporium (Ophiobolus)				23
Alternaria				10
Phoma				8
Tuberculariae			79	
Fusarium				46
Myrothecium				26
Stillbaceae (*Isaria , Metarrhyzium)*			10	
Melanconiales	1			
Mycelia sterilia	5			
Dermatophyta	9			
Asporogenous yeasts	19			
Unidentified fungi imperfecti	12			

Source: Berdy (1974). *Advances in applied microbiology* 18: p330

1.5 Biochemistry of Antibiotic Production

Antibiotics are considered generally as secondary metabolites, although some, for example, Nisin, have been considered by some workers to be primary metabolites (Hammond and Lambert, 1978). Secondary metabolism is characteristic of lower life forms and the metabolites are often species specific . In contrast with primary metabolites, from which they are created, secondary metabolites often accumulate

and are usually excreted into the medium. They have no obvious function in the growth of the cell and are usually produced late in the growth phase (Hammond and Lambert, 1978). The actual functions of antibiotics in the producing microorganism are still a matter of debate. There are three main schools of thought on this issue:

- They are simply waste products of metabolism - this view is no longer tenable, as many secondary metabolites have been found to have biological activity.
- They provide a competitive mechanism between organisms - this seems most likely. Unfortunately, very little is known about the synthesis of antibiotics in the natural environment. It could explain why actinomycetes, which as slow growers have to compete with fast growing microbes, produce many antibiotics.
- They serve as a metabolic regulation mechanism - this is unlikely to have general validity, as many antibiotics are not active against the producing organism. Some antibiotics have mechanisms of action that are difficult to reconcile with a regulatory role (Lancini and Parenti,1982).

Hammond and Lambert (1978), proposed that antibiotics, from their position as secondary metabolites, may function as a means of detoxification to protect the microbial cell from adverse conditions in the environment and may also inhibit macromolecule synthesis under hostile conditions, thereby preventing active growth. The enzyme reactions that lead to the synthesis of antibiotics are not fundamentally different from those that lead to the synthesis of primary metabolites. Only small variations occur in the principal biosynthetic pathways. Three major categories of antibiotics may be derived on the basis of their biosynthetic route.

- Antibiotics derived from a single primary metabolite .e.g. Chloramphenicol and nucleoside antibiotics.
- Antibiotics derived by condensation of several metabolites .e.g. Lincomycin and Novobiocin.
- Antibiotics derived by oligomer or polymer formation. There are about four sub-classes in this group.

(a) Polypeptide antibiotics:- from condensation of amino acids, e.g. Gramicidin S and Penicillins.

(b) Acetate/propionate polymerization antibiotics, e.g. Griseofulvin, Tetracyclines, and Erythromycin.

(c) Terpenoid antibiotics:- through isoprenoid synthesis, e.g. Fusidic acid.

(d) Aminoglycoside antibiotics: - made through a condensation reaction, similar to that of polysaccharides. e.g. Streptomycin and Neomycin.

The production of antibiotics is not rigorously species specific (Lancini and Parenti, 1982). The same antibiotic can be produced by different species of the same genera. And the reverse is also true; that is, the same species can produce different antibiotics. Most often microbial species or strains produce several biologically and chemically related antibiotics that constitute a 'family', which is to say that the same strain makes two or more antibiotics that resemble each other. The capacity to produce " families" of compounds is not unique to antibiotic biosynthesis but a general characteristic of secondary metabolites, mainly due to the large size of intermediate molecules (Lancini and Parenti,1982).

The concepts of 'metabolic trees' and 'metabolic nets' were used by Lancini and Parenti (1982) to explain the reason for these metabolic events. The same metabolite can be a substrate for two or more reactions generating different products which may be enzymatically transformed, giving rise to a metabolic tree. Or the same metabolite can be obtained through different pathways that vary only in the order of enzymatic reactions, giving rise to a metabolic net. They also noted, a combination pathway of these 'trees' and 'nets' was possible.

1.6 Mechanism of Antimicrobial Action

It is essential that a successful antibiotic must be selective in its mechanism of action, that is, it must have the ability to penetrate and concentrate in the microbial cell and should not interact with the cells of the host. They should show suitable absorption and distribution properties within the host body (Franklin and Snow, 1981).

Antimicrobial agents generally bring about microbial inhibition by interacting with specific cellular

components and disordering cell metabolism. They block the growth of sensitive microorganisms by inhibiting the action of a macromolecule, such as enzymes or a nucleic acid essential to the function of the cell. This means that the antibiotic molecule is able to bind to a specific site on the target macromolecule, forming a complex that is useless to the cell (Lancini and Parenti,1982).

Several authors have classified antibiotics based on the site of action in the microorganism (Hammond and Lambert, 1978; Franklin and Snow, 1981; Lancini and Parenti, 1982).

(a) Inhibitors of cell wall synthesis

Examples include Penicillin, Cephalosporin, Cycloserine and Vancomycin (Hammond and Lambert, 1978). About 50 - 70% of the Gram - positive bacterial cell wall mass and to a lesser extent (10 - 15%) in Gram-negatives is composed of peptidoglycan (sometimes referred to as Murein or Mucopeptide). Its cross-linked structure provides a tough, fibrous fabric, giving strength and shape to the cell and enabling it to withstand a high internal osmotic pressure (Franklin and Snow,1981).

Most of the antibiotics in this group affect peptidoglycan synthesis, while others interfere with the synthesis or assembly of other components of the wall, e.g. teichoic acid. β-lactam antibiotics (penicillins, cephalosporins), so-called because of the presence of a cyclic amide forming a four atom ring (β-lactam ring) in the molecule, all have a similar but not identical mechanism of action. They prevent peptidoglycan maturation by inhibiting the cross-linking of the linear peptidoglycan strands (Hammond and Lambert, 1978; Franklin and Snow,1981; Lancini and Parenti, 1982). Action of these antibiotics ultimately results in bacterial cell lysis.

Vancomycin will inhibit the transfer of muramyl pentapeptide-acetylglucosamide from the lipid carrier to the peptidoglycan chain being formed. Conversely, cycloserine, which is particularly active against mycobacteria, acts at the terminal position of the pentapeptide of muramic acid. All known inhibitors of cell wall synthesis are:

- Bactericidal in action;

- Inactive against resting cells;
- Inactive against bacteria that lack a conventional cell wall, e.g. Mycoplasmas, L- forms and protoplasts (Lancini and Parenti,1982).

(b) Inhibitors of cell membrane functions.

Examples include Polymyxin, tyrocidin, valinomycin and amphothericin B (Hammond and Lambert, 1978). Cell membranes have very similar constituents throughout the phylogenetic ladder from bacteria to mammalian cells. The only important difference is that there are no sterols in bacterial cell membranes, while zymosterol and ergosterol are present in the cell membranes of fungi and plants, and cholesterol in those of mammals. Some antibiotics in this group disorganize the super-molecular structure of the membrane, thus causing loss of cellular substance to the outside, while some act as carriers of specific ions (Ionophores) and cause an abnormal accumulation of ions inside the cell (Lancini and Parenti, 1982).

Polymixins and tryocidin are both cyclic polypeptide antibiotics. Their action disturbs membrane function by allowing leakage of cytoplasmic components (Ca^{2+} ions ; Mg^{2+} ions) and uncoupling of oxidative phosphorylation (prevention of ATP generation during sugar oxidation). Valinomycin specifically drains the cell of potassium ions (K^+) and growth ceases because of the requirement for potassium in protein biosynthesis (Franklin and Snow, 1981). Amphotericin B, a typical polyene antifungal antibiotic, creates instability in the membrane, by forming complexes with sterol components of the membrane that alter trans-membrane cation permeability. The majority of inhibitors of cell-membrane functions are non-selective and consequently are too toxic to be given systemically, and are therefore used exclusively as antiseptics or topical agents (Franklin and Snow,1981).

(c) Inhibitors of transcription and replication of genetic material (nucleic acids)

Examples include, rifamycin, actinomycin D and acridine dyes (Hammond and Lambert, 1978).

The synthesis of DNA and the various classes of RNA is an essential function of dividing and growing cells. Thus inhibition of DNA synthesis rapidly results in inhibition of cell division (Franklin and Snow, 1981). Rifamycins are enzyme inhibitors. They bind to, and inhibit the DNA dependant - RNA polymerase of sensitive bacteria, but the precise mechanism of inhibition is still uncertain (Hammond and Lambert,1978).

Actinomycin D molecules insert themselves into the smaller groove of the DNA double helix and form a reversible complex bound by hydrogen bonds. The complex does not permit RNA polymerase to travel along the DNA template thereby inhibiting synthesis of RNA (Hammond and Lambert, 1978). Acridines show antimicrobial activity by distorting the sugar phosphate backbone of the DNA helix created by the intercalated dye molecules (Franklin and Snow, 1981). Most of the inhibitors of genetic material are used in cancer chemotherapy rather than as antimicrobial agents. Unfortunately, many of these do not show sufficient selectivity against the tumour cells and are too toxic to be used effectively (Hammond and Lambert, 1978).

(d) Inhibitors of Protein Synthesis .

Examples include streptomycin, tetracycline and chloramphenicol (Hammond and Lambert, 1978). Some antibiotics in this group affect amino acid activation and transfer reactions, while others interfere with the functions of the 30S or 50S ribosomal subunit (Lancini and Parenti, 1982). Streptomycin and tetracycline distort the 30S ribosomal subunit enough to prevent normal bonding between the codon of mRNA and the anti-codon of tRNA. This causes mis-coding of the proteins, bringing normal protein synthesis to a halt (Hammond and Lambert, 1978).

Chloramphenicol acts on the 50S subunit by inhibiting peptide bond formation between the amino acids. Inhibitors of protein synthesis are bacteriostatic if they do not form irreversible bonds with some essential component of the synthetic system. If they do, they are bactericidal (Franklin and Snow, 1981).

1.7 Spectra of antimicrobial activity

Antibiotics may be grouped into five classes according to their spectra of activity; i.e. according to the classes of microorganisms they inhibit.

[1] Antiviral

[2] Antifungal

[3] Antiprotozoal

[4] Antibacterial

[5] Antitumour agents - justifiably classified as antibiotics because the compounds were originally isolated on the basis of their antimicrobial activities (Lancini and Parenti, 1982).

Occasionally an antibiotic is active against all microorganisms, but usually different antibiotics are active against selected organisms. The group of microorganisms whose growth is inhibited by an antibiotic constitutes its spectrum of activity. As an index of antimicrobial activity, microbiologists and clinicians use the Minimum Inhibitory Concentration (MIC), defined as the lowest antibiotic concentration that will inhibit the growth of a specific organism (Hammond and Lambert, 1978).

To determine MIC, small bottles of liquid growth medium, containing graded doses of antibiotic, are inoculated with the test organism. After suitable incubation, growth will occur in those bottles where the antibiotic is below the inhibitory level and the culture will become turbid (cloudy) from the large number of microorganisms present. Growth will not occur above the inhibitory level and the medium will remain clear. Samples are taken from the bottles and plated out on nutrient agar. The plate containing the lowest antibiotic concentration which shows no microbial colonies after incubation is the one with the MIC (Hammond and Lambert, 1978). Table1.6 shows the MIC of some important antibiotics against selected pathogenic microorganisms. Antibacterial antibiotics are said to have a narrow spectrum of activity if they are active against few species of either Gram - positive or Gram - negative bacteria only, and broad spectrum if they are active against majority of both Gram - positive and Gram -negative bacteria (Lancini and Parenti, 1982).

Table 1.6 MICs [μg/ml] of some antibiotics against representative pathogens

	Penicillin G	Amp	Cephalo	Erythro	Tetra	Chloram	Strept	Ampho B
Gram-positive cocci								
Diplococcus pneumoniae	0.010	0.04	0.003	0.01 - 2	0.05 - 1	1 - 3	0.2 -1	uns
Staphylococcus aureus	pnp 0.04 pp 50	0.1 Uns	0.05 13	0.01 - 2 0.01 - 2	0.1 0.1	4 - 8 4 - 8	1 - 5 1 - 5	uns
Gram-negative cocci								
Neisseria gonorrhoeae	0.05	0.1 - 0.6	0.1 - 4	0.02 - 1	0.1 - 3	1	2 - uns	uns
Gram-positive Bacilli								
Clostridium tetani	0.01 - 3	0.02	0.5	0.5 - 2	0.03 - 1	0.1 - uns	uns	uns
Corynebacterium diphtheriae	0.3 - 3	-	1	0.5 - 5	0.5 - 5	0.5 - 5	0.5 - uns	uns
Gram-negative bacilli								
Escherichia coli	uns	2 - uns	1 - uns	2 - 10	0.5 - 5	2 - 12	1 - uns	uns
Klebsiella spp	6	1.5	6 - uns	uns	0.5 - uns	0.3 - 1.5	8 - uns	uns
Pseudomonas aeruginosa	uns	uns	uns	uns	4 - 200	16 - uns	20 - 500	uns
Mycobacterium tuberculosis	uns	-	10	uns	uns	6 - 30	0.5 - 5	uns
Yeast and Fungi								
Candida albicans	uns	uns	uns	uns	uns	uns	uns	0.1 - 3
Dermatophilus spp	uns	uns	uns	uns	uns	uns	uns	0.2 - 40

uns = unsusceptible; Amp= Ampicillin; Ceph = Cephalosporin; Erythro= Erythromycin; Tetra = Tetracycline; Chloram= Chloramphenicol; Strept = Streptomycin; Ampho B = Amphotericin B; pnp= penicillase-non-producer; pp= penicillase producer; source: Lancini and Parenti, 1982. Antibiotics, An integrated View. pp24 - 25.

1.8 Review of screening methods

1.8.1 Primary Screening Methods

General screening methods have not changed fundamentally in the past 40 years. Millions of different microorganisms, mostly actinomycete species originating from soils, were screened all over the world by means of the classical methods of Waksman and Dubos.

[1] The crowded plate method

This method is used to isolate organisms able to produce antibiotics active against other soil organisms. A heavy aqueous suspension (1:10; 1:100) of soil is plated on agar in such a way as to ensure the development of confluent growth. Colonies surrounded by clear zones are purified for further study. By altering the media used, different groups of organisms can be encouraged to develop (Okafor, 1987).

[2] Dilution plate method

This method is applied when the aim is to isolate antibiotics against a known organism or organisms. It is based on the principle that 'Activity against a single test species/strain is a sufficient criterion for the selection of antagonistic organisms' (Okafor, 1987). The procedure involves mixing a sample of soil at a suitable dilution with an appropriate melted agar and the mixture poured into plates. After the agar has solidified, the plate is inverted and incubated until scattered colonies appear. The plate is then flooded with a suspension of the test organism and re-incubated. The test species forms a lawn on the surface of the agar interspersed with clear zones of inhibition. The clear zones indicate the presence of colonies from the soil sample which are capable of producing antibiotics active against the test bacterium. Those colonies surrounded by clear zones are purified and transferred to an agar slant to be held for further studies. By employing a series of soil agar plates each flooded with a different test species, it is possible to isolate forms which are antagonistic to a large number of organisms.

The essence of Waksman's method is that it facilitates preliminary screening of antibiotic producers

through development of an easily visualized clear zone in a confluent growth of the test organism (Waksman and Lechevalier, 1962).

1.8.2 Primary Testing of Antibiotic Production

Once an organism showing evidence of antibiotic action has been detected and isolated, it is then purified and several other steps are carried out to confirm antibiotic production. Some of the methods will be reviewed here.

[1] The cross streak method

This method is suitable for testing individual isolates. The purified isolate is streaked across the upper third of a plate containing a medium which supports its growth as well as that of the test organisms. A variety of media may be used for streaking the antibiotic producer. It is allowed to grow for up to 7 days, in which time, any antibiotic produced should have diffused a considerable distance from the streak. Test organisms are streaked at right angles to the producer isolate and the extent of inhibition of the various test organisms observed/measured (Okafor, 1987).

[2] The agar plug method

This method is particularly useful for testing fungal isolates for antibiotic production, especially when the test organism(s) grow(s) poorly in the medium that supports growth of the producer fungus. Plugs about 0.5 cm in diameter are made with a sterile cork borer from the fungus isolate plate. These plugs are then placed on plates seeded with different test organisms and incubated. Inhibition zones appear around those isolates producing antimicrobial compounds. This method may be used with actinomycetes (Okafor, 1987).

[3] The replica plating method

If a large number of organisms are to undergo primary screening, one rapid method is the use of replica plating. This is a well known method used in microbial genetics (Okafor, 1987). The method consists

of placing a sterile velveteen pad over the colonies growing on a soil agar plate, or on a plate containing separate, individually distinct, colonies to be tested for antibiotic properties. The pad is thereafter carefully touched onto the surface of four or five plates seeded with the test organism. Because an orientation mark is placed on the pad as well as on the plates, it is possible to tell which colonies are causing the cleared zones on the test plates (Okafor, 1987). There are various other screening techniques employed, e.g. ditch plates, gradient plates etc., that are not described here because of the remote possibility of using them in the present study.

1.8.3 Factors affecting antibiotic production

In all the procedures described above there are certain factors that will affect antibiotic production: (1) Nutritional conditions (2) Growth rate of the producing organism (3) The assay conditions.

Positive inhibition of test organisms by antibiotic-producing isolates, depends upon complex interactions between the antibiotic producer and its culture medium, and on cultural conditions such as temperature, pH and availability of oxygen (Lancini and Parenti, 1982). Several workers have reported that a strong relationship exists between nutritional factors and antibiotic production, for example, glutamate, as sole nitrogen source supported good growth of *Streptomyces griseus* but a very low yield of its antibiotic, streptomycin. Conversely, L- proline, supplied as sole nitrogen source, promoted both biomass and streptomycin yield (Dulaney, 1948). Also nitrogen, glucose and phosphate regulation of antibiotic production have been reported (Gallo and Katz :1972; Aharonowitz and Demain :1979; Martin and Demain :1976;1980; Hodgson:1982).

The growth rate of the producing organism is yet another factor affecting antibiotic production. Microorganisms seem to be programmed to produce antibiotics only when the specific growth rate decreases below a certain level (Martin and Demain, 1980). Expression of the genes coding for antibiotic biosynthesis usually does not occur at high growth rates, suggesting that during rapid growth, either antibiotic synthetases are not formed or, if formed, their activity is inhibited (Martin and Demain, 1980). The enzymes involved in the synthesis of antibiotics have been monitored by various workers (Liras *et al.*, 1977; Madry and Pape, 1982; Takeshima *et al.*, 1989).

ions are very important in determining the *in vitro* activity of an antibiotic. Lancini suggested that all test conditions must be specified precisely and if possible ishes to use inhibition of bacterial growth for quantitative determination of the and to have data that are reproducible in different laboratories. The assay d be standardized include:

organism/s .

sition [pH, ions, serum etc.]

sity [etherogeneticy of population etc.]

ns [time, temperature, aeration]

It must be noted that antibiotic activity may differ depending on whether the testing was done in a liquid or on a solid medium and also when the total number of bacteria in the inoculum is very high, there is a greater probability that some of the cells will be less susceptible to the antibiotic (Lancini and Parenti, 1982).

The effect of pH, temperature and alkali metal ions on antibiotic activity has also been studied by various investigators (James *et al.*, 1991; Kirpekar and Kirwan, 1991; Eckwall and Schottel, 1997; Paik *et al.*, 1997). Eckwall and Schottel (1997) observed that an antibiotic produced by the scab disease-suppressive *Streptomyces diastatochromogenes* strain Pon SS11 was stable for weeks at acidic pH but very labile at alkaline pH conditions. The pH instability of this compound may have some implications for the effectiveness of the organism as a bio-control agent in soils at neutral or alkaline pH. Some antibiotics, such as nitrofurantoin, are more active at acidic pH while aminoglycosides and sulfonamides are more active in alkaline medium (Jawetz *et al.*, 1984). Several antimicrobial agents lose their activity at commonly used incubation temperature (37°C). For example, chlortetracycline is inactivated rapidly and penicillin more slowly, whereas the aminoglycosides, chloramphenicol and polymixin B are quite stable for longer periods at this temperature (Jawetz *et al.*, 1984).

1.8.4 Extraction and purification methods

Over the years, various workers have used different methodologies to extract and/or purify antibiotics : solvent extraction (Arcamone *et al.*, 1969); a combination of molecular exclusion chromatography, ion exchange chromatography and sodium dodecyl sulphate polyacrilamide gel electrophoresis (Galvez *et al.*, 1986); adsorption to ion exchange resins (Horner *et al.*, 1989); flash chromatography (Stankovic *et al.*, 1990); HPLC (high performance liquid chromatography, Franco and Coutinho, 1991); a combination of thin layer chromatography and HPLC using three reagents Amberlite XAD -2, DEAE Sephadex A-50 and SP Sephadex C-25 (Eckwall and Schottel, 1997). Most of these methods are based on the affinity of different antibiotics for different eluting solvents and different matrices such as ion exchangers.

1.9 Some suggested newer methods of searching for antibiotic-producing microorganisms

Between the mid 1960s and mid 1970s, although the search for antibiotics continued, very few new antibiotics were in fact discovered. It was found that the majority of new isolates produced the well known antibiotics (Berdy, 1980). This was because the classical method of Waksman and Dubos described earlier was mainly based on inhibition/clear zones on the agar plate and also on the use of a few selected test organisms, usually of medical importance. Some suggested new methods have led to the discovery of new antibiotics.

[i] The use of super sensitive mutants

By using super sensitive strains of test organisms in primary screening procedures, organisms producing only small amounts of an antibiotic may be detected. Antibiotics so produced are useful because they seem to have a wider spectrum than those of the same class already in existence (Okafor,1987). Furthermore, they may provide better substrates for semi- or muta-synthesis. This method has led to the discovery of novel β-lactam antibiotics, thienamycin, olivanic acid, nocardicins, clavulanic acid, etc. The use of super sensitive mutants has shown that β- lactam antibiotics are produced by a wider spectrum of organisms - ascomycetes, fungi imperfecti and actinomycetes - than was previously thought

(Okafor, 1987).

[ii] The application of criteria other than death or inhibition

Reactions such as irregular growth of fungal mycelium and inhibition of sporulation, rather than death, may be used to follow an antibiotic's effect/s. When antifungal antibiotics are sought, the clear zone principle is employed using yeasts or fungal spores in the test plate. With this method, the existing antifungal antibiotics, namely, polyene as well as cycloheximide and actimycins were found. If criteria less drastic than death (e.g. abnormal growth of hyphae or inhibition of zygospore formation) are applied instead, a wide range of new antibiotics may be found. Using this method, some new actinomycete antibiotics have been found including boromycin, venturicidin and mikkomycin (Okafor, 1987).

[iii] The use of chemical assays

The use of chemical assays based on various chemical reactions (chemical screening) of the culture broth of an organism, using thin-layer chromatography with reagents specific for certain chemical groups, high pressure liquid chromatography etc., may lead to the discovery of new antibiotics, as was the case with dienomycins and arglecins (Okafor, 1987).

[iv] Search in novel environments

Systematic searches for antibiotics are usually from soil. Of course, other natural sources exist, although none can match the soil in the ubiquity and richness of flora. Nevertheless, searches in unusual habitats may provide novel organisms. One such habitat is the sea . It contains large amounts of salts and other mineral nutrients, has a fairly constant temperature, high hydrostatic pressure and less sunlight in the deep areas. The coastal area is constantly changing with the tides and such areas should be expected to have a wide variety of organisms, peculiar to the littoral environment. The search for antibiotics in the sea has indeed led to the discovery of new and unique compounds. These include: antibiotic SS-228R, from *Chainia sp.*, effective against gram-positive bacteria and tumours; bromo-pyrrol, from a marine *Pseudomonas*, and leptosphaenin from marine fungi (Okafor, 1987). The point is that novel environments, tree barks, leaf surfaces, sewage, all provide distinct environments, which may encourage the growth of microorganisms possessing a unique set of enzymes and, hence, the possible capacity to

synthesize new antibiotics (Okafor,1987).

(v) Changed culture conditions

The production of new antibiotics may be encouraged, even in known cultures, by subjecting them to new culture conditions such as a different carbon source, temperature, level of aeration etc. This is done in the hope that certain pathways may be blocked and others enhanced. Such carbon sources include monosaccharides, glycosides, substituted sugars, polyhydric alcohols, oligosaccharides, terpenes and hydrocarbons (Bunge *et al.*, 1978).

Isolation of microorganisms is done by sprinkling soil on plates containing the above carbon sources. A perfusion technique, in which soil is bathed constantly with a solution containing the chosen carbon source, may also be used. Isolates are grown in shake flasks using the various carbon sources and a variety of environmental variables, including pH, mineral composition and concentration, temperature, nitrogen source and aeration. Table1.7 illustrates new products formed when new culture conditions were provided to producers of well established antibiotics (Neijssel and Tempest, 1979).

Table1.7 New microbial products resulting from changed culture conditions

New conditions	Organisms	New Products
Increased PO_4^{2-}	*Pseudomonas sp*	Pyrrolnitrin
	Nocardia uniformis	Thiopeptin
	Streptomyces tatyamensis	Nocardin
	S . saptoronensis	Bicyclomycin
Lower Incubation Temp		
27°C - 12°C	*S . griseus*	Cryomycin
27°C - 15°C	*S . griseus*	Holomycin
Increased O_2 supply	*S. lavendulae*	Chlorocarcin

[vi] Strategy based on multiple antibiotic resistance

This strategy is based on the principle that antibiotics are not species specific, but rather strain specific. Usually one may regard resistance to own antibiotics as a specific phenotype conferred by a self resistant gene (Cundliffe, 1992). However, the resistance to specific antibiotics is not specific to the strains which produce them, because there are many strains with multiple- antibiotic resistance (Cundliffe, 1992). For example, there are many streptomycin-resistant actinomycetes other than streptomycin producers (Hotta and Okami, 1996). These methods involve random selection of many strains, from soil isolates with or without antibiotic resistance and examining them for antibiotic production by incubating them in a starch - soy bean meal medium (Hotta *et al.*, 1983). Strains with known resistance patterns are selected and screened for their ability to produce antibiotics. Two novel antibiotics, dopsisamine and bagougeramine (a backyard product) have been isolated using this method (Hotta and Okami, 1996).

Chapter 2

Isolation of Antibiotic-Producing Microorganisms

2.1 Introduction

According to a survey done in 1993, over 70% of the novel bio- active substances of microbial origin isolated in the decade 1984 - 1993 were derived from actinomycetes (Miyadoh, 1993). It is believed that actinomycetes will continue to play a major role in providing novel bio-active substances in the next decade (Hotta and Okami, 1996).

However, as the number of known secondary metabolites increases, the probability of finding new ones becomes increasingly remote. Innovative methods will be required in order to enhance the probability of finding new antibiotics. Accordingly, a new basis will need to be established for isolating and distinguishing strains with potential productivity of novel bio-active metabolites (Hotta and Okami, 1996).

Over the past two decades, taxonomists have shown increasing interests in rare actinomycetes as a potential source of bio-active secondary metabolites. Methods designed to isolate a wide variety of rare actinomycetes have been developed (Hayakawa and Nonomura, 1989; Shomura, 1993).

Five steps were observed in the modern isolation methods. These included the choice of substrate, composition of the isolation medium, pre-treatment and incubation conditions, colony selection and purification (Cross, 1982; Nolan and Cross, 1988). Of the five factors, composition of the isolation medium, pre-treatment and incubation conditions are the most important since they determine which organism will develop on the isolation plates.

Taxonomic characterization has many limitations since it still remains essentially at the species level, although the production of antibiotics and other secondary metabolites is strain specific (Hotta and Okami, 1996). Therefore a new basis will be required to distinguish isolates at the strain level in terms of potential productivity of novel antibiotics (Hotta and Okami, 1996).

Hotta and Okami (1996) also recommended primary metabolism as a key to establishing a new basis in the search for new antibiotics. Obviously, the supply of primary metabolites as precursors is necessary for biosynthesis of secondary metabolites. Therefore, it seems likely that the primary metabolism of antibiotic producers is appropriately regulated to supply precursors for biosynthesis of their specific secondary metabolites. For example, in the case of aminoglycoside producers, primary metabolism should be regulated to supply sugar precursors for aminoglycoside biosynthesis. Similarly, producers of amino acid-related antibiotics and polyketides, such as macrolides, will have specifically regulated primary metabolisms to supply precursors for their characteristic products. Therefore, any discovery of phenotypes relating to characteristic primary metabolism, will form a good basis for screening for novel antibiotics (Hotta and Okami, 1996).

In actinomycetes, however, primary metabolism has been studied rather poorly compared to secondary metabolism, and more studies on the genetics and biochemistry of primary metabolism will definitely be necessary. The present study incorporated some of the new ideas by employing changing culture conditions, such as different carbon sources, temperature and aeration during the growth cycle of the isolates investigated. This was done in the hope that certain metabolic pathways might be blocked and others enhanced.

Due to the slow growth rate of actinomycetes, numerous efforts have been made to develop selective procedures for their isolation from natural habitats. Such procedures include, use of selective media as well as mechanical and chemical separation of other unwanted organisms that might interfere with actinomycete isolation (Cross, 1982; Karwowski, 1986). The incorporation of antibiotics in actinomycete isolation media was also employed (Athalye and Lacey, 1981 ; Orchard and Goodfellow, 1974). Pretreatment methods used by some investigators (Cross, 1982; Ntuli, 1994) include : nutrient enrichment and physical and/ or chemical treatment applied individually, or in combination, to samples or sample suspensions, before plating out on suitable media.

Natal soils have been reported as a potential source of novel antibiotic producing microorganisms. For example, the organism, ***Streptomyces natalensis***, an actinomycete, first used in industrial production of pimaricin, was originally isolated from Natal soils (Baecker and Ryan, 1987).

The main aim of this study was to further the progress made in this area to isolate new antibiotic producers by applying some new ideas and methodologies. Members of the genus *Streptomyces* were excluded from the study, since they have been extensively studied in the past (Okami and Hotta, 1988; Ntuli, 1994).

2.2 Materials and Methods

2.2.1 Sample collection

All samples were collected from KwaZulu-Natal, Republic of South Africa. Samples were collected from: Charles Johnson Memorial Hospital; Nqutu compost dump, which consisted mainly of litter from cut trees and grass left to decompose with occasional burning; several Dundee farms, mainly cow manure and dung; Carnco Farm just outside Pietermaritzburg on the road to Richmond, comprised mainly of chicken litter and droppings. About 20 samples were collected from each site at depths of 3-5cm below the surface.

Additional samples were collected from the Mlangeni farm in Bisley, Pietermaritzburg, which consisted mainly of decayed chicken manure and droppings; from the banks of the Mzinyathi river at Rorkes Drift (northern KwaZulu-Natal); from uncultivated farmland in the Escourt area, and from the outskirts of Pietermaritzburg. See Table 2.1 for more details.

All samples, of which approximately 1500 were collected during the period of this investigation, were kept in a refrigerator(± 4°C) until required.

Table 2.1 Nature and location of samples collected and investigated over the period 1998-2000

Sample Type	Location	Description
Cow Manure	Dundee farms, Dundee, Northern Natal	3cm depth of soil covered with dung and decomposed cow feed.
Chicken Litter	Carnco farm, Pietermaritzburg	Poultry farm, litter covered with chicken droppings.
Chicken Manure	Mlangeni's Poultry Farm, Pietermaritzburg	3cm depth of soil; decomposed feed and droppings.
Chicken Manure	Family Poultry Farm, Sweet Waters	5cm depth of soil; decomposed feed and droppings.

Marshy soil	Mzinyathi River Bank, Rorke's Drift	5cm depth of marshy, muddy soil covered with decomposing grasses and algae.
Compost Soil	Charles Johnson Memorial Hospital,Nqutu	5cm depth of soil; Cut branches, leaves and refuse left to decompose with occassional burning
Creosote Contaminated Soil	Project Site, University of Natal	Phd Student's sample
Uncultivated Farm Land	N1 Freeway, Escourt Natal Midlands	3cm depth of soil.
Cane Sugar Field	Illovo Farms Ltd, Ellanspruit, Natal Midlands	3cm depth of soil; freshly cultivated sugar cane field.
Forest Soil	Sappi Farms Ltd, Seven Oaks, Natal Midlands	5cm depth of soil; decomposing leaves.

2.2.2 Isolation Media

Distilled water was used for all medium preparation. Attempts were made to use selective media in order to avoid the growth of *Streptomyces* species. Antibiotics were also added to autoclaved and cooled media (to prevent thermal destruction of the antibiotics) as filter-sterilized solutions, and after gentle swirling, the media were poured into petri dishes.

For chemical composition and preparation of the media used, refer to Appendix I.

Czapek's medium [Cross and Attwell,1974] referred to by Cross, 1981]. 25μg/ml of Novobiocin and 50μg/ml of cycloheximide were added when antibiotic supplementation was required.

Kosmachev's medium (Kosmachev, 1960)

M3 Agar (Rowbotham and Cross, 1977).

M G A (Nonomura and Ohara, 1971).

The medium was modified by omitting soil extract and vitamin solution but was made more selective by the addition of 10µg/ml polymixin B and 12µg/ml oxytetracycline.

Modified Czapek's Agar (Higgins and Lechevalier, 1969).

The medium was further modified by addition of 50µg/ml cycloheximide and 4µg/ml Thiamine-HCl.

Modified Nutrient Agar On many occasions half strength nutrient agar was used and made more selective by addition of 50µg/ml cycloheximide.

Oatmeal Agar [ISP medium 3] (Shirling and Gothlieb, 1966). Gentamicin (50µg/ml) was added when required.

Bacto Tryptic Soy Agar (Difco). Prepared as per manufacturers instructions. Gentamicin was sometimes added to the autoclaved and cooled medium to give a final concentration of 50µg/ml.

Winogradsky's Nitrite medium (Winogradsky, 1949)

Yeast Extract Agar [ISP medium 2] (Pridham *et al.*, 1956-1957).

2.2.3 Scope of the Isolation Programme

It is difficult to estimate the exact number of colonies screened in this programme. However, a rough idea of the scale of the isolation programme may be obtained by estimating the number of isolation plates used. For each of 220 samples investigated, three suspension dilutions (10^{-2} to 10^{-4}) were divided into four equal aliquots, and each aliquot was then treated differently before plating on triplicate plates of at least 8 appropriate isolation media. The treatments were as follows: (1) heat pretreatment followed by plating on an antibiotic(s) containing medium; (2) heat treatment followed by plating on an isolation medium with no antibiotics; (3) no heat pretreatment applied and no antibiotics added to the isolation

medium; (4) pretreatment omitted - suspensions plated on an isolation medium containing selective antibiotics. Thus a total of 220 x 3 x 4 x 3 x 8 = 63,360 isolation plates were used in this investigation. This figure is, however, an underestimation, since in some cases all 10 isolation media listed above were used.

2.2.4 Primary Isolation

As far as possible, aseptic procedures were used throughout the investigation. Each sample was prepared, firstly, by vigorous hand shaking in an autoclaved 1L beaker. All stones or debris present were subsequently removed. Samples were then placed in an autoclaved Waring blender mixing container fitted with four cutting blades and blended into a powder. About 1g of the sample was weighed out on a filter paper and transferred to an oven at 100°C for 1 hour after which it was sprinkled onto the surface of the isolation media (Pisano *et al.*, 1987).

Alternatively, a 1g portion of the sample was suspended in 10ml 25% (w/v) strength Ringer's solution containing 0,5g/l gelatin (Cross, 1981) and serially diluted to 10^{-4}. The 10^{-1} dilution was prepared by vortexing the suspension at maximum speed for 5 minutes. The 10^{-2} to 10^{-4} dilutions were separately plated. In some instances, samples were heat pre-treated in a water bath at 55^{0}C for 6 minutes (Rowbotham and Cross, 1977), or at 70^{0}C for 10 minutes (Sandrack, 1977, cited by Cross, 1981), and selective antibiotics added to the isolation media.

For the crowded-plate method, there was neither pretreatment of the samples nor antibiotic incorporation into the media used. All the isolation media mentioned earlier were employed during the primary isolation stage.

2.2.5 Primary screening

Primary screening of isolates for antimicrobial activity was carried out using a modified cross- streak method. Both plant and human pathogenic bacteria and yeasts were used as test organisms. The test organisms were *Staphylocccus aureus* (1), *Escherichia coli* (2), *Pseudomonas fluorescens* (3), *Serratia marcescens* (4), *Streptococcus faecalis* (5), *Candida utilis* (6), and *Xanthomonas campestris pv campestris* (7). Individual colonies were picked off with a sterile loop and streaked along the diameter

of Nutrient Agar (NA), Iso-sensitest agar (ISTA) [Oxoid] or Mueller-Hinton agar (Oxoid) plates. The plates were incubated for 4- 6 days at 30^{0}C, after which the test organisms were streaked randomly at right angles to the producer isolate growth. Standardized inoculum of the test organisms was achieved with the aid of a sterilized 4mm loop. Streaking was done outwards starting approximately 1mm from the edge of the producer isolate growth. The plates were then re-incubated for 24 hours at 30^{0}C before diameters of zones of inhibition were observed and measured.

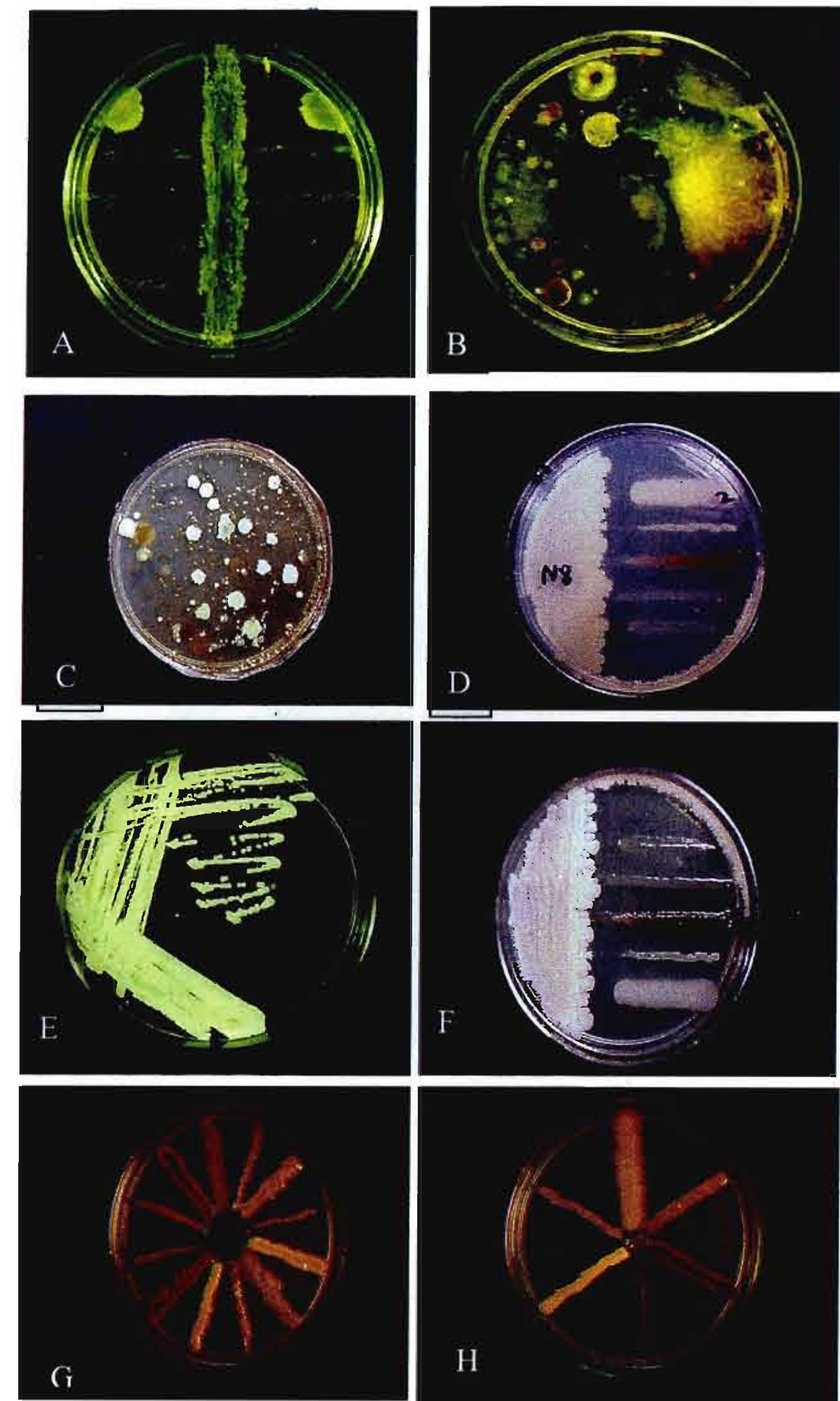
2.2.6 Purification of Isolates

Purification of isolates was carried out. Colonies of isolates showing antimicrobial activity were transferred separately to 40ml sterilised distilled water. Tween 60 (3-5 drops) were added as a wetting agent. Mycelial or spore suspensions were then homogenised using an ULTRA- TURRAX homogenizer fitted with a 18N shaft. A drop of the resultant suspension was placed on a haemocytometer, covered with a cover slip and mycelial fragments and /or spores counted under a bright-field microscope.

The original suspension was then diluted to give a concentration of 1 colony forming unit (cfu) per microlitre. A grid comprising ± 36 x 1cm^2 squares was drawn on the bottom of the agar plates and 1µl of each cell suspension pipetted onto the centre of each square. Plates were sealed with masking tape and incubated at 30^{0}C for 7 days to 3 weeks. In some cases the colonies were purified using the three way streak method before screening was carried out [Plate2.1E].

Plate2.1 Photographs of different plating methods used for the isolation and screening of microorganisms

A = Modified cross-streak method (10 test organisms per plate) for primary screening; B = Crowded plate method for primary isolation; C = Dilution plate method (10^{-4}) for primary isolation; D&F = Cross-streak method for confirmatory screening; E = Pure culture technique; G&H = Agar-well/radial-streak method for secondary screening



2.3 Results and Discussion

Plating Techniques

The crowded plate technique yielded profuse growth of many organisms [Plate2.1B]. However, the combined treatment of heat and antibiotic incorporation into the media greatly reduced the number of organisms present on the plates. The results were similar to those previously reported by several authors (Williams and Davies, 1965; Pisano *et al.*, 1986; Ntuli, 1994). The disadvantage of antibiotic addition is that a potent antibiotic producer, which is sensitive to the antibiotic added, may be inhibited and hence will not be detected. On the other hand, the antibiotic makes the medium more selective by limiting the number of organisms developing on the plates [Table 2.2].

Table 2.2 Percentage of the total number^ of isolates active against all or some of the test organisms* on primary screening

sample source	no. isolates per pre-treatment		total isolates screened	no. isolates active	%
Chicken Manure [CM]			354	12	3.39
	H +A	38			
	H + NA	46			
	NH +A	94			
	NH + NA	166			
Chicken litter [CL]			423	18	4.26
	H +A	34			
	H + NA	61			
	NH + A	123			
	NH +NA	205			
Cow Manure [COW]			313	11	3.51
	H + A	43			
	H + NA	55			
	NH + A	58			
	NH +NA	157			
Compost Soil [CS]			121	2	1.66
	NH +A	45			
	NH + NA	76			

sample source	no. isolates per pre-treatment	total isolates screened	no. isolates active	%
Uncultivated Farm Soil [US]		182	4	2.19
	NH + A 56			
	H + A 23			
	NH + NA 103			
River Bank Soil [RB]		94	0	0
	NH + A 13			
	NH + NA 45			
	H + A 36			
SAPPI Forest Soil [SF]		435	23	5.28
	NH + A 97			
	NH + NA 167			
	H + A 66			
	H + NA 105			
Creosate Contaminated Soil [CCS]		5	1	20
	NH + NA 5			
Sweet Waters Dry Soil [SW]		260	2	0.77
	NH + A 71			
	NH + NA 128			
	H + A 61			
Illovo Cane Sugar Field [CSF]		386	7	1.88
	NH + A 101			
	NH + NA 169			
	H + A 56			
	H + NA 60			
	Grand Total	**2573**	**79**	**2.72**

**Staphylococcus aureus, Escherichia coli, Pseudomonas fluorescens, Serratia marcescens, Streptococcus faecalis, Candida utilis* and *Xanthomonas campestris*; NH = Non - Heated; H = Heated; A = Antibiotics added; NA = No Antibiotics added; ^total number based on the average colony count of the 10^{-2} to 10^{-4} dilutions of the sample.

Also the heat pretreatment selected thermophilic organisms, which means that any novel strains which are heat sensitive may not have been detected. Sprinkling of heat pre-treated soil directly onto the media led to overcrowding of the colonies at particular places on the plates. Antimicrobial activity, if present, would be difficult to detect in such instances.

In contrast to the above procedure, the dilution plate method was more effective in isolating individual

colonies [Plate2.1C]. However, very few colonies showed antimicrobial activity, expressed as surrounding zones of clearing. Zones of inhibition were noticed on three plates; chicken manure plated on Czapek's agar; chicken litter, also on Czapek's agar, and cow manure on Kosmachev's medium. All other samples showed no noticeable zones of inhibition.

Although no direct comparisons of the media used in this investigation were made, significant observations were nonetheless possible. Some of the isolation media, namely, M3, Kosmachev's medium and Winogradsky nitrite medium were found to be highly selective for actinomycetes without the addition of any antibiotics to, or heat pretreatment of, the samples. Other authors have reported similar observations (Williams and Davies, 1965; Ntuli, 1994). A fairly wide variety of organisms grew on Czapek's agar which also enhanced pigment production in some of the organisms. MGA medium supported prolific fungal growth and Kosmachev's medium proved to be highly selective for actinomycetes, as most of the plates appeared to be colonized with almost pure cultures of actinomycetes, probably, polysporic actinomycetes (Kosmachev, 1960). Unamended YEA plates were overgrown with yeasts and fungi, but on antibiotic addition, growth of additional minute mycelial colonies was noted. The antibiotics used in this investigation seemed to inhibit the growth of fungi and yeasts while favouring the growth of small mycelial colonies, probably actinomycetes, or bacteria which were the main focus of this investigation.

The combination of selective pretreatment, selective media and incubation conditions, were helpful in eliminating the growth of unwanted *Streptomyces* species. Dry heat treatment of some samples, in an oven at 100°C for 1 hour before plating, meant that only actinomycetales having more heat resistant spores such as *Micromonospora, Microbispora* and *Streptosporangium* were selected (Labeda, 1990). The relatively mild heating regimens in a water bath (55°C for 6 minutes, Rowbotham and Cross, 1977) and 70°C for 10 minutes (Cross, 1981) eliminated the spreading bacterial colonies and enhanced the selective isolation of *Micromonospora* respectively (Labeda, 1990). Most of the media used were selective for specific genera. M3 agar (Rowbotham and Cross, 1977), modified Czapek's agar (Higgins and Lechevalier, 1969) and Winogradsky's nitrite medium (Winogradsky, 1949) were selective for nocardiae and rhodococci. The addition of antibacterial antibiotics such as gentamicin, oxytetracycline and novobiocin to the other isolation media used, also meant that non-streptomycetous actinomycetales

were selected for. Actinomycetales differ significantly in their sensitivity toward antibacterial antibiotics. Streptomycetes fortunately belong to the antibiotic sensitive group (Kutzner, 1981). It must be noted, however, that this author doubts the selectivity of some of the so-called selective media as a few colonies of other bacteria occasionally developed on the plates.

In the dilution plate method, there were remarkable variations in the numbers of organisms observed at different dilutions. A 10^{-4} dilution was found to be most suitable for isolating single colonies as it contained individual colonies of a large variety of organisms [Plate2.1C]. Although particular attention was directed in this study to organisms resembling actinomycetes, it was very important that each and every colony that developed on the isolation plates was treated as a potential antibiotic producer. The use of selective procedures minimized the laborious work involved in screening thousands of isolates.

Preliminary screening

The problem of screening each individual isolate with an overlay of a particular test organism was overcome, in part, by employing a modified cross-streak method [Plate2.1A]. Using this method facilitated testing a particular isolate against all the test organisms simultaneously. Only 79 of the approximately 2600 isolates screened, showed signs of antimicrobial activity [Table2.2]. All inactive isolates were discarded. The highest percentage of active isolates came from the SAPPI forest soil while the lowest percentage came from samples from the banks of the Mzinyati river. The composition of the medium is a very important factor affecting the production of active antibiotics (Lancini and Parenti, 1982). It is possible, therefore, that the nature of the media used was not conducive to antibiotic production by the riparian microbiota. Decolouration of *Serratia marcescens* was not regarded as a form of inhibition, as incubation at a lower temperature restored the red coloration.

Comparison of the number of antimicrobially-active isolates to the total number of isolates screened from the various samples is shown in Fig 2.2. It is clearly evident, that the number of culturable antibiotic-producing microorganisms constitute a small percentage of the total microbial population in the different samples studied.

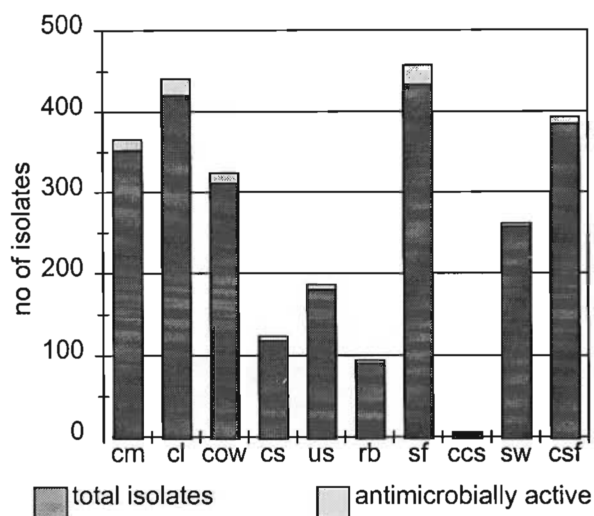


Fig 2.2 Comparison of antimicrobially-active isolates to total number of isolates following primary screening

Only those isolates that inhibited two or more test organisms were considered worthy of further analysis except in the case of *Pseudomonas* or *Xanthomonas*, which are notoriously difficult to control. Based on these criteria, only 44 isolates were selected for re-screening on NA and Mueller Hinton Agar to confirm the preliminary results [Chapter 3].

These preliminary findings indicate that KwaZulu-Natal soils do harbour antibiotic-producing microorganisms, confirming the results obtained by previous investigators (Baecker and Ryan, 1987; Ntuli, 1994).

CHAPTER 3

Screening of Isolates for Antimicrobial Activity

3.1 Introduction

Thousands of samples and millions of microbial colonies have been screened all over the world in the past 50 years, but compared to the screening rates, reports of success were minimal. At the beginning of the 1950's the screening of 10,000 microorganisms resulted in one single useful agent. Later the screening of 400,000 microorganisms over a ten year period also resulted in three utilizable drugs (Berdy, 1974).

The rediscovery of known substances has become more frequent. In order to maximize the chances of success, sampling and plating need to be a continuous exercise, so that screening itself becomes a continuous process. In the present study, about 220 samples and approximately 2600 colonies, from 10 different locations, were screened over a three year period. This involved plating, on average, two samples per week. Only 44 isolates showed any meaningful antimicrobial activity [Appendix 2].

Screening methods have metamorphosed over the years. From the Waksman and Dubos classical methods of agar-zone- diffusion (Linton, 1983), to discs impregnated with antibiotic substances (Pelczar and Reid, 1972), and to the more complex use of selected mutants (Paik *et al.*, 1997). Basically most of these methods were dependent on one criterion; - the production of growth inhibition zones on agar media. However, some authors have recommended the use of other criteria less drastic than death or inhibition of the test organism, such as the inhibition of sporulation, or abnormal growth of hyphae, as a novel way of screening for new antibiotics (Okafor, 1987).

Antibiotics diffuse through agar gel at different rates, .e.g. penicillin-G and chloramphenicol diffuse rapidly, while, polymyxin diffuses slowly, so that the zone sizes produced when testing specific isolates with different antibiotics are not directly comparable. In conclusion, it is wrong to assume that an organism is more susceptible to one agent than another on the basis of a larger inhibition zone size (Hammond and Lambert, 1978).

The size of the growth inhibition zone is a consequence of two dynamic systems proceeding simultaneously:

- the diffusion of the antibiotic from its source,
- the growth rate of the test organism.

The position of the growth-inhibition zone edge in an agar gel is fixed at a point in time when specific criteria in the two systems are fulfilled independently. An equation can be applied to the diffusion of antibiotics through gels (Linton, 1983).

$$X^2 = 4\ DT\ 2.3\ (\text{Log Mo} - \text{Log M}')$$

Where

X = The distance between the antibiotic source and the zone edge.

D = The diffusion coefficient of the antibiotic.

T = The time taken for the zone to be fixed.

Mo = The antibiotic concentration at the source.

M′ = The critical concentration of the antibiotic which inhibits the test organism.

Under standard conditions **X** and **Mo** are directly related . **D** is affected by temperature and viscosity of the solvent. A high **Mo** drives **M′** well out into the agar in **T**, forming large growth inhibition zones. Under normal assay conditions, **Mo** may be considered constant throughout the period **T**. However, in disc susceptibility tests, **Mo** is not known, since although the disc is impregnated with a known amount of the drug, this dissolves in an unknown amount of water in the assay agar. Hence the concentration cannot be determined. It is not possible to relate initial antibiotic concentration directly to the size of the inhibition zone produced (Linton, 1983).

However, the above problem may be minimized by determining the Minimum Inhibitory Concentration [MIC] of the antibiotic. The MIC, defined as the lowest antibiotic concentration that will inhibit the growth of a specific organism, has the advantage that it is performed in broth, thereby eliminating the disadvantage of slower diffusion rates in the case of large-molecular mass antibiotics (Okafor, 1987). A knowledge of MICs is very important to microbiologists and clinicians, as an index of antimicrobial activity (Hammond and Lambert, 1978). An attempt was made in this study to determine the MICs of

the antimicrobial compounds produced by the most promising isolates.

3.2 Materials and Methods

3.2.1 Isolates

Organisms screened for antimicrobial activity were isolated from samples from various habitats in KwaZulu-Natal midlands [section 2.2.1]. Stock cultures of pure isolates were kept on agar slants and on thick NA plates at ± 4^0C until required.

3.2.2 Test organisms

The test organisms, *Staphylococcus aureus* (1), *Escherichia coli* (2), *Pseudomonas fluorescens* (3), *Serratia marcescens* (4), *Streptococcus faecalis* (5), *Candida utilis* (6), and *Xanthomonas campestris pv campestris* (7) were provided by D. Fowlds of the Department of Microbiology & Plant Pathology Laboratory, University of Natal. They were all grown on thick Nutrient agar (NA) plates except for *Candida utilis*, which was grown on Yeast Extract agar (YEA), and kept as stock cultures in a refrigerator (± 4^0C). The test organisms were subcultured every 4 months throughout the period of this investigation.

3.2.3 Assay media

Nutrient Agar and Iso-sensitest agar (ISTA) [Oxoid] were used for antimicrobial-activity assay. Also Potato Dextrose agar (PDA) was used for assaying fungal isolates. The responses of the isolates to the different selective media were statistically analyzed using the SAS system.

3.2.4 Primary Screening

Individual purified isolates were streaked along the diameter of NA and ISTA (Oxoid) plates using a flamed sterilized loop. Each isolate was cultured for 6 - 7 days at 30°C, in which time any antibiotic produced would have diffused a considerable distance from the streak. Test organisms were then streaked at right angles to the original isolates starting from approximately 1mm distance from the edge of the producer organism's growth. Plates were then re-incubated for 24 hours at 30°C and the extent of the inhibition of the various test organisms observed. All platings were done in replicates of 3 to 5.

3.2.5 Secondary Testing of Antibiotic Production

3.2.5.1 Antibiotic-production media

Media used included Nutrient broth (NB) and M3 Broth (M3B) - an M3 medium without agar (Rowbotham and Cross, 1977). Other media used were Glucose-Yeast-Malt- Extract Broth (GYMB) and Oatmeal Broth [International Streptomyces Project media 3 and 2 respectively, in which agar had been omitted] (Shirling and Gottlieb, 1966).

3.2.5.2 Production of the antibiotic

Isolates which produced growth inhibition zones in the primary screens, were grown in nutrient broth or in one/several of the media mentioned above. A loopful of the isolate was introduced aseptically into 100 ml NB or in any of the production media contained in a 250 ml Erlenmeyer flask and cultured in a shaking incubator at 30°C for 5 to 8 days.

Attempts were made to produce large amounts of the antibiotics by using increased volumes of broth and culturing under static conditions. Since there were no significant increases in antibiotic production, static cultures were discontinued. After incubation, the cultures were centrifuged in a Heraeus Labofuge 200 at 9500 x *g* for 10-15 minutes. Aliquots of the supernatant (3-5ml) were filter sterilized (0.22μm) into sterile screw capped glass vials. In some instances the filtration procedure was repeated to ensure that cell-free supernatant (culture filtrate) was used for the antibiotic activity bioassay.

3.2.5.3 Bioassay for antibiotic activity

Samples of the culture filtrate were tested for antibiotic activity using a modification of the agar well-diffusion method of Paik and Glatz (1995). After pouring the agar (5 mm deep), plates were incubated for 24 hours at room temperature before wells were cut (7mm diameter). The plates were further incubated at 37°C for 2 hours to dry, thereby facilitating sample diffusion into the agar. Culture filtrate (50μl) obtained by filter sterilization (0.22μm) of the supernatant above, was pipetted into the agar well and the test organisms streaked radially outwards, starting 1mm from the edge of the well. The plates were then incubated at 30°C for 24 hours and observed for growth inhibition zones. All assays were carried out in triplicate (3 plates per test organism per isolate). Plates in which the wells were filled with

uninoculated culture media served as controls.

Also some samples were tested using the spot-on-lawn assay method of Eckwall and Schottel (1997). A 50μl aliquot of culture filtrate was spotted onto filter paper disks (6.5mm, Whatman No.2). These disks were allowed to dry before they were placed on Petri plates containing ISTA (Oxoid) medium which had been freshly seeded with the test organism. The plates were then incubated at 30°C for 3 days before the diameters of growth inhibition zones were measured.

The above method produced remarkable zones of growth inhibition but was discontinued due to its laborious and uneconomical nature. It involved testing each isolate with only a single test organism at a time, compared to the cross-streak method, which involved testing all the test organisms simultaneously, in a single Petri plate.

3.2.6 Determination of Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration (MIC) was determined using the broth micro-dilution method as recommended by the Swedish Reference Group for antibiotics and its sub committee on methodology [SRGA] (webite: www.SRGA) and as per standard protocol of Hancock's Doubling dilution method (website: www.interchg.ubc.ca/bobh/methods.html).

Preparation of Indicator test organisms

The organisms used as indicators were inhibited by the antimicrobially-active isolates on primary/secondary screens. The indicator test organisms were prepared by growing them in Nutrient Broth(NB) to an optical density (OD) of 0.5 -1.0 at 420nm. After which **X**μl cell suspension were added to 1ml NB. The value of **X** was calculated using the equation:

Xμl = 10/**OD**

Where **OD** = Optical Density

A 100μl of this cell suspension was re-suspended in 10ml Nutrient broth or 50μl to 5ml of the broth prior to testing.

Broth micro-dilution method

Dilutions of the antibiotic-producer culture filtrate (stock solution) were prepared in a micro-titre plate (10x10 wells). From the stock solution, nine dilutions were made using the growth medium. To each of the wells in micro-titre plate were added 100μl broth, except the first well. The first and second wells of the plate then received 100μl each, of the stock solution. The contents of the second well were thoroughly mixed, and 100μl transferred from this second well to the third. This process was continued until the dilution series was completed. A step-wise dilution factor of 2 (1.0, 0.5, 0.25, 0.125, 0.063) was maintained throughout the dilution procedure.

Finally, 100μl of the prepared suspension of the indicator test organism were added to each of the wells in the micro-titre plate and incubated at 30^{0}C. The plates were examined for growth after 12h, 24h and 48h incubation. The last well showing no visible growth, i.e. a clear medium, was regarded as the MIC. The results were confirmed by transferring 0.1ml aliquot, from each well, to nutrient agar plates, incubating at 30^{0}C for 24 hours and observing for colony formation. Practically, 99.9% reduction in colony forming units (cfu) was recorded as no growth (Spooner and Sykes, 1972).

3.3 Results and Discussion

Primary screening

Of the 79 isolates from various samples that showed antimicrobial activity against the test organisms on preliminary screening [Chapter2], 44 were selected for re-screening. The results of the primary screening programme are shown in Appendix 2. It should be noted that only isolates that inhibited two or more test organisms were considered except in the case of *Pseudomonas* or *Xanthomonas spp. Pseudomonas* infections still require very high doses of even the most active penicillins during treatment (Lancini and Parenti, 1982) and *Xanthomonas* infections remain a problem to plant pathologists.

The distribution of the isolates varied with the sample source and the isolation pre-treatment conditions. The chicken manure sample yielded isolate N8, that showed antimicrobial activity against the majority of test organisms used [Plate3.1A], while the SAPPI forest samples yielded isolates N19 [Plate3.1B] and N33 that showed good activity against *Pseudomonas fluorescens*, and isolate N35, which showed good activity against the plant pathogen, *Xanthomonas campestris pv campestris,* following isolation from an unheated sample of SAPPI forest soil [Table 3.1]. The forest soil, rich in organic content and acidic in nature may offer a good hunting ground for antibiotics judging from this result. Waksman *et al.* (1942) also reported that certain acid forest soils do harbour antibiotic producing microorganisms.

The majority of the isolates from the Sweet Waters sample showed no meaningful antimicrobial activity against the test organisms, with the exception of one isolate, N4a, which had a fairly good activity against *Pseudomonas fluorescens* [Plate3.1C&D]. Isolates from the banks of the Mzinyathi river at Rorke's Drift, showed negative results. The highly waterlogged soils devoid of oxygen may present an unfavorable environment for aerobic microorganisms, especially, actinomycetes. It may also be possible that the nature of the media used was not conducive to antibiotic production by the isolates screened.

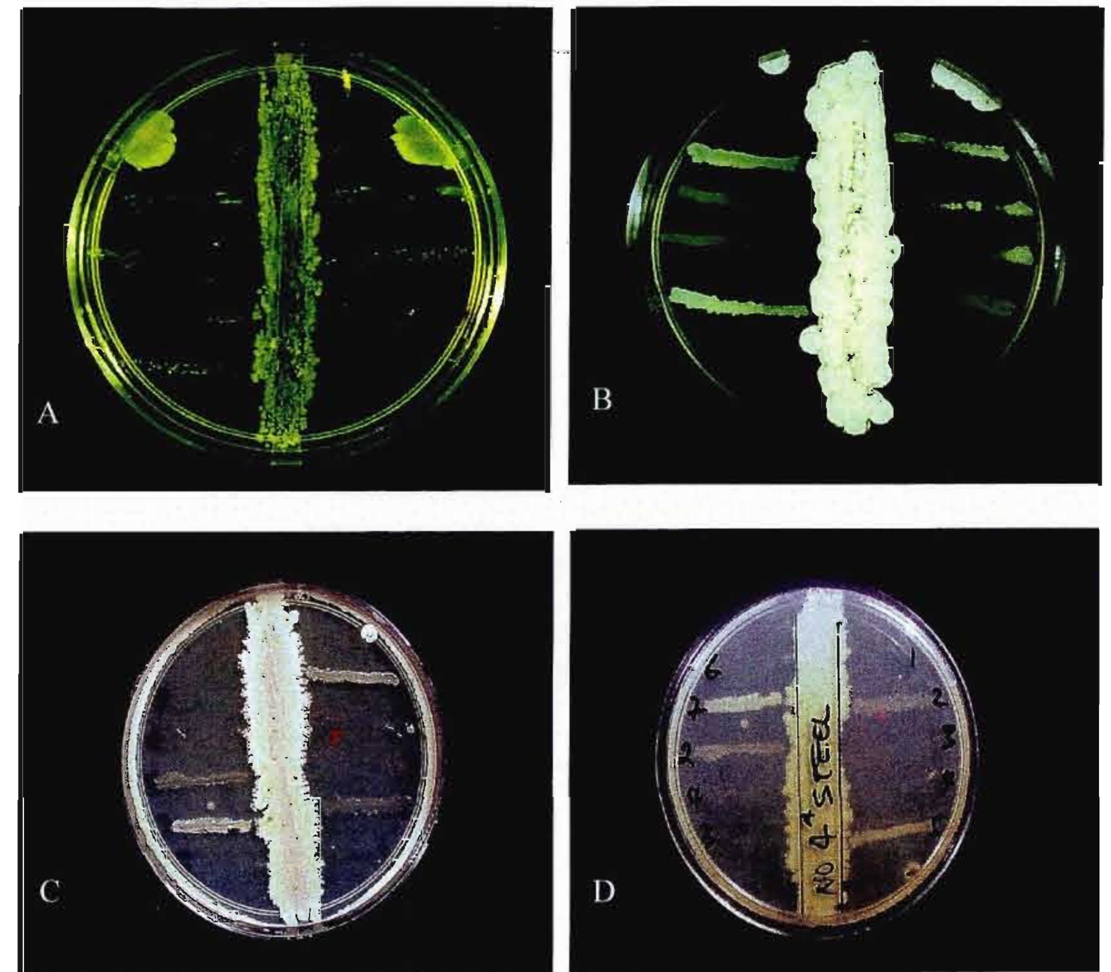


Plate 3.1 Preliminary screening of isolates showing activity against test organisms

A = Inhibition of test organisms by isolate N8; B= N19 inhibition of test organisms; C&D = Inhibition of test organisms (1,3,4,&6) by isolate N4a; test organisms include: *Staphylococcus aureus* (1); *Escherichia coli* (2); *Pseudomonas fluorescens* (3); *Serratia marcescens* (4); *Streptococcus faecalis* (5); *Candida utilis* (6); *Xanthomonas campestris pv campestris* (7)

The average size of the inhibition zone produced by isolates against the test organisms varied greatly, depending on the susceptibility of the test organism to the antibiotic produced. Isolate N2, produced inhibition zone sizes of 20mm, 21mm and 15mm against *Pseudomonas fluorescens*, *Serratia marcescens*, and *Xanthomonas campestris pv campestris* respectively [Table 3.1]. Isolate N8 produced average inhibition zones of 20 to 27mm against all the test organisms used. The test organisms, *Escherichia coli* and *Staphylococcus aureus* were sensitive to the largest number of antibiotic-producing isolates; of the 15 isolates selected for secondary screening, these organisms were susceptible to the antibiotics of 13 and 12 isolates respectively. Conversely, some of the isolates were inactive against these organisms; for example, Isolates N2 and N30b were inactive against *E .coli*, while N4b, N33 and N35 were inactive against *S .aureus* [Table 3.1]. Ten of the selected isolates were active against *Pseudomonas fluorescens*, while surprisingly, only two of the isolates showed antimicrobial activity against *Streptococcus faecalis*. Again the media used may not have been conducive to antibiotic production or the enzyme system of *Streptococcus faecalis* may confer resistance to the antibiotics produced. Further investigation may be necessary.

The average inhibition zone size also varied during the confirmatory screening stage [Plate3.2], but the spectra of activity of the respective isolates remained unchanged from those recorded in the primary screening phase. For example, Isolates N2, N8 and N16 retained their activity against the same test organisms previously recorded for them [Plate3.1]. However, the sizes of the inhibition zones were marginally reduced when compared to those determined in the preliminary screens. Such differences in sizes of inhibition zones have been reported by several authors (Linton, 1983, Ntuli, 1994). Since the size and position of the growth inhibition zone is a consequence of many dynamic systems proceeding simultaneously (Linton, 1983). It is doubtful to compare one antibiotic agent against another on the basis of inhibition zone size (Hammond and Lambert, 1978).

There was complete decolouration of *Serratia marcescens* by isolate, N48. However, on further incubation at a lower temperature (20°C), the pigment production was restored. This portrays the importance of choice of incubation temperature in screening for antibiotics (Lancini and Parenti, 1982).

Plate3.2 Primary screening of isolates confirming activity against test organisms

Photographs (A&B) = N8 activity against nos.1,2,3,4&7 test organisms; (C&D) = N16 activity against nos.1,2,3,&7 test organisms; (E, F&H) = N2 activity against nos.3&6; (G) = N19 showing inactivity against *Serratia marcescens* and *Streptococcus faecalis*. Test organisms include: *Staphylococcus aureus* (1); *Escherichia coli* (2); *Pseudomonas fluorescens* (3); *Serratia marcescens* (4); *Streptococcus faecalis* (5); *Candida utilis* (6); *Xanthomonas campestris pv campestris* (7)

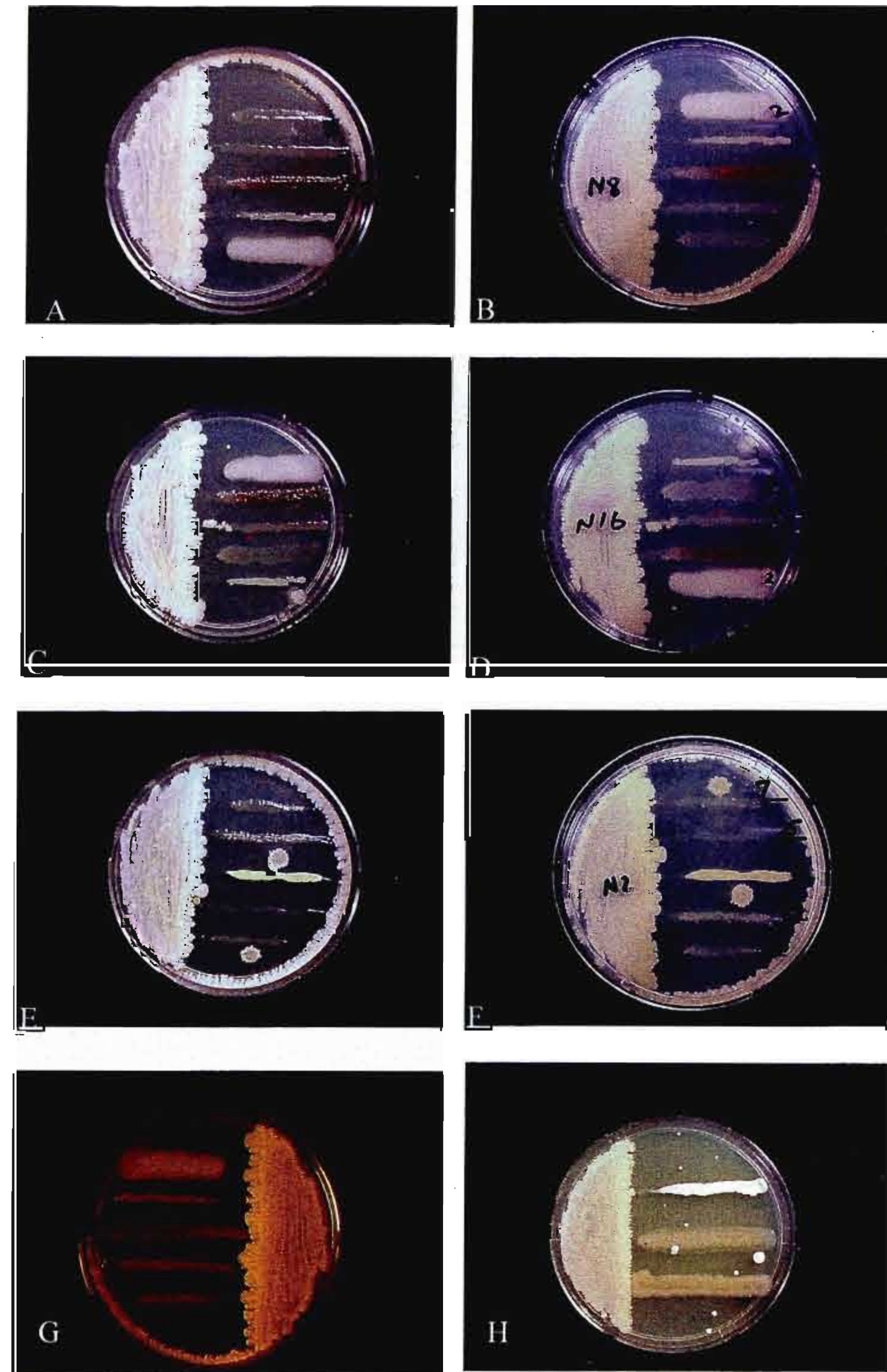


Table 3.1 Average size of inhibition zones produced by isolates against test organisms [recommended for secondary screening]*

Isolate	source	Test organisms/ Inhibition zone size (mm)						
		❶ Sa	❷ Ec	❸ Pf	❹ Sm	❺ Sf	❻ Cu	❼ Xc
N2	CL	13	10	20	21	–	20	15
N3	CM	25	20	25	–	–	nt	–
N4a	SW	15	-	30	20	-	nt	-
N4b	SW	-	30	15	-	-	-	-
N16	CCS	11	25	–	3	3	–	3
N8	CM	20	25	20	23	22	27	24
N15	CL	20	21	–	2	–	30	--
N16a	CL	25	5	14	–	–	Rs	–
N25a	CL	2	30	–	–	–	30	–
N48	CS	18	30	–	–dc	–	21	19
N12b	CSF(NH+A)	3	7	8	-	-	-	5
N19b	SF(NH+A)	3	4	10	-	-	-	5
N30b	CSF(H)	5	-	20	-	-	-	-
N33	SF(NH)	-	3	10	-	-	-	-
N35	SF(NH)	-	3	-	-	-	-	10

* Data generated from the results presented in Appendix 3; Sa = *Staphylococcus aureus*; Ec = *Escherichia coli*; Pf = *Pseudomonas fluorescens*; Sm = *Serratia marcescens*; Sf = *Streptococcus faecalis*; Cu = *Candida utilis*; Xc = *Xanthomonas campestris pv campestris;* Rs = Resistant strain; CL = Chicken litter; CM = Chicken manure; CS= Compost Soil; US = Uncultivated soil; CCS = Creosate Contaminated Soil; SW = Sweet Waters (Chicken manure); nt = not tested; -dc = decoloration; H=Heated; NH=Non-heated; A= Antibiotics added; SF=SAPPI forest; CSF=Cane sugar field.

The spectra of activity of isolates N2 and N8 were quite broad, although N2 was inactive against *Streptococcus faecalis* [Fig 3.3]. Morphological observations showed that the organisms may likely belong to the actinomycete group [Chapter4]. Isolate N48 was active against four of the test organisms; viz. *S.aureus, E.coli, C.utilis* and *X.campestris pv campestris*, with *E.coli* being the most susceptible. Although isolate N16 showed broad activity against the majority of the test organisms, their

susceptibilities were quite low, except for *E.coli*, which was highly susceptible to the antibiotic produced by the isolate [Fig3.3]. Only three of the test organisms used were susceptible to the antibiotic produced by isolate N3, however, this was quite remarkable considering the large size of the inhibition zones produced, especially against *P.fluorescens* [Fig3.3].

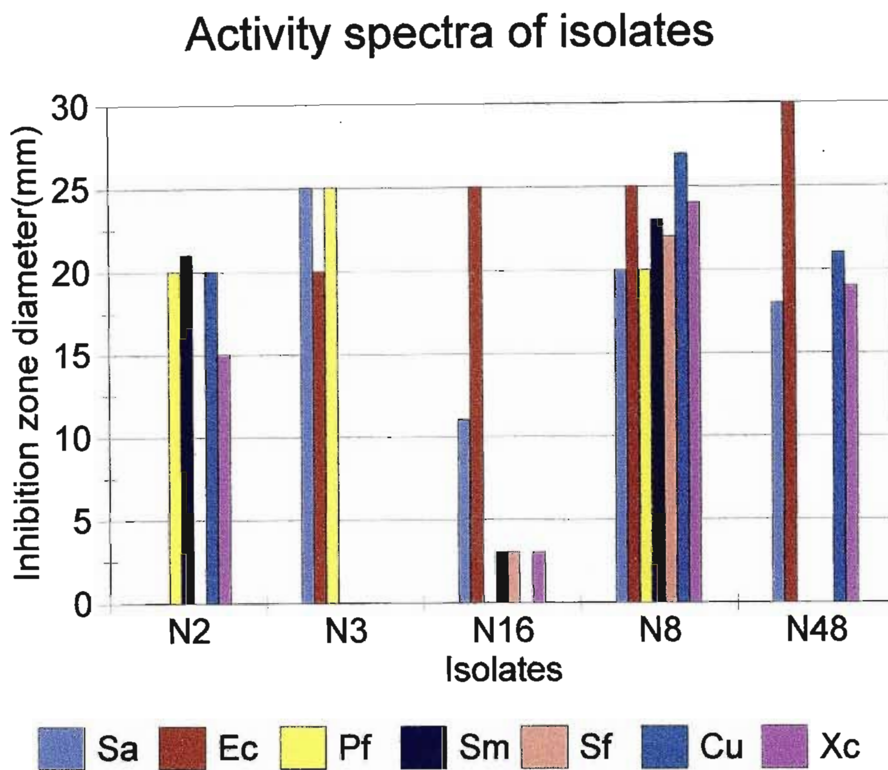


Fig 3.3 Comparison of antimicrobial spectra of some active isolates on primary screening.

Some of the isolates lost a substantial part of their activity on extended incubation . This may suggest that the antibiotic produced may have been inactivated, either by a substance released late into the medium or some resistant strains developed and grew in the inhibition zone. It is also possible that the initial inhibitory antibiotic concentration, have been altered as a result of antibiotic diffusion through the agar medium. Isolated colonies which grew within an inhibition zone were recorded as resistant strains. Similar findings have been reported by other authors (Linton,1983).

Some isolates spread over the plates when Mueller Hinton (Oxoid) and Nutrient agar were used making streaking of the test organisms difficult. In such instances, agar-plugs overgrown with the producer organism and placed on a lawn of the test organism were used for screening. The use of Iso-sensitest Agar (ISTA)[Oxoid] medium solved some of the problems of spreading, as the growth of the isolates was more restricted with clearly defined edges.

In some instances, the growth of the test organism, *Candida utilis*, was delayed on the media used with the result that some seemingly positive results were found to be fallacious on further incubation. The importance of the medium in screening of isolates for antimicrobial activity have been highlighted by several investigators (Lancini and Parenti,1982). Statistical analysis showed significant interaction between the isolates and the assay media [Table 3.2]. Variations in the interactions between the test organisms and the different isolates were also shown.

Table 3.2 Analysis of variance^ for inhibition zone size

Source of variation	DF	Sum of squares	Mean square	F value	P value
ISOLATE	7	606.7950055	86.6850008	100.81	0.0001**
MEDIUM	1	159.1105008	159.1105008	185.04	0.0001**
TORGSM	3	536.2431586	178.7477195	207.88	0.0001**
Isolate*Medium	7	19.3960055	2.7708579	3.22	0.0043*
Isolate*Torgsm	21	2396.3191102	114.1104338	132.71	0.0001**
Error	88	75.6675688	0.8598587		
Corrected Total	127	3793.5313492			

^ Table generated using The SAS system.

TORGSM= Test organism

* = Statistically significant interaction (P = 0.05).

** = Statistically significant interaction (P =0.01)

The age of the organisms became a serious factor over the course of the investigation. Repeated transfer of the cultures on fresh media over a three year period seems to have affected the characteristics

of some of the isolates. For example , isolate N8 lost the ability to change the colour of the NA medium to reddish brown, indicating the loss of pigment production ability. Possible contamination was ruled out as the culture growing on the test plate was a pure culture morphologically identical to the original organism. This loss of pigmentation may be due to a possible mutation of the isolate as a result of prolonged exposure to chemicals in the medium. According to Waksman (1937), the characteristic pigments produced by many actinomycetes species, may be lost or changed in kind, not only under the influence of changing environmental conditions but even on continued incubation under the same conditions.

Secondary screening

Fifteen isolates were selected for secondary screening. Of these, only eight isolates showed antimicrobial activity [Table 3.3]. The inhibition zones were more pronounced in Mueller Hinton (Oxoid) and ISTA (Oxoid) than on Nutrient agar plates.

Table 3.3 Antimicrobial activity spectra of isolates against test organisms on secondary screening*

Organisms were inoculated onto plates of one of NA, ISTA, or Mueller Hinton in the secondary screens. 50μl of the culture filtrate was pipetted into the agar-well of one of the media.

	Test organisms						
Isolate	**Sa**	**Ec**	**Pf**	**Sm**	**Sf**	**Cu**	**Xc**
N2	+	-	-	-	-	-	-
N8	-p	-p	++	+	-p	-p	+
N12	-	-	-	-	+	-	-
N16	+	-	-	-	-	-	-
N19	-	-	-	-	-	-	++
N30	-	++	-	-	-	-	-
N33	-	-	++	-	-	-	-
N35	-	-	-	-	-	-	++

* isolates were grown in Nutrient broth; -p =no inhibition, paper discs turned red; + = slightly positive inhibition; ++ = positive inhibition; - = no inhibition ; Sa = *Staphylococcus aureus*; Ec = *Escherichia coli*; Pf = *Pseudomonas fluorescens*; Sm = *Serratia marcescens*; Sf = *Streptococcus faecalis*; Cu = *Candida utilis*; Xc = *Xanthomonas campestris pv campestris*.

The paper discs used in the spot-on-lawn assay method turned red, when isolate N8 was tested against *Staphylococcus aureus*, but no noticeable inhibition zone was observed [Plate3.4D]. This may be due to the interaction between the antibiotics produced and the constituents of the media [Houang *et al.*, 1983].

Three isolates, namely N8, N19 and N35 were active against *Xanthomonas campestris pv campestris*, while *Pseudomonas fluorescen*s was inhibited by isolates N8 and N33. Isolates N2 and N16 inhibited only *S.aureus*, while isolate N30 was active against *E.coli* [Table3.3]. Most of the isolates were inactive against the majority of the other test organisms used.

A startling observation was made with isolate N8. It inhibited all the test organisms used in this investigation in the primary screens [Plate3.1A]. By contrast, minimal activity was observed against the test organisms on ISTA plates [Table3.3], and no activity on Nutrient agar plates during secondary screening [Plate3.4C]. It is possible that the isolate either did not produce significant amount of the antibiotic (s) in liquid culture (nutrient broth) or that the antibiotic was lost during the preparation of the culture filtrate and during incubation conditions. Another explanation may be that the antibiotic (s) was produced by the growing cells, and since cell-free preparations were used for the tests, a large proportion of the antibiotic produced may have been lost. Other investigators have reported similar observations (Ntuli, 1994). Jawetz *et al.* (1984) stated that, at certain temperatures and pH conditions, several antimicrobial agents lose their activity. For example, chlortetracycline is inactivated rapidly and penicillin more slowly at an incubation temperature of 37°C.

Unlike the MIC test results (Table 3.4), some of the secondary screening results did not correlate well with those obtained from the primary screens even after repeated screening. This may be due to the method used. Perhaps, a probable solution to the secondary screening procedure will be to collect samples during incubation, at every stage (12 hourly) of the isolate's life cycle in the liquid antibiotic production media, instead of waiting for some days for the antibiotic to be produced.

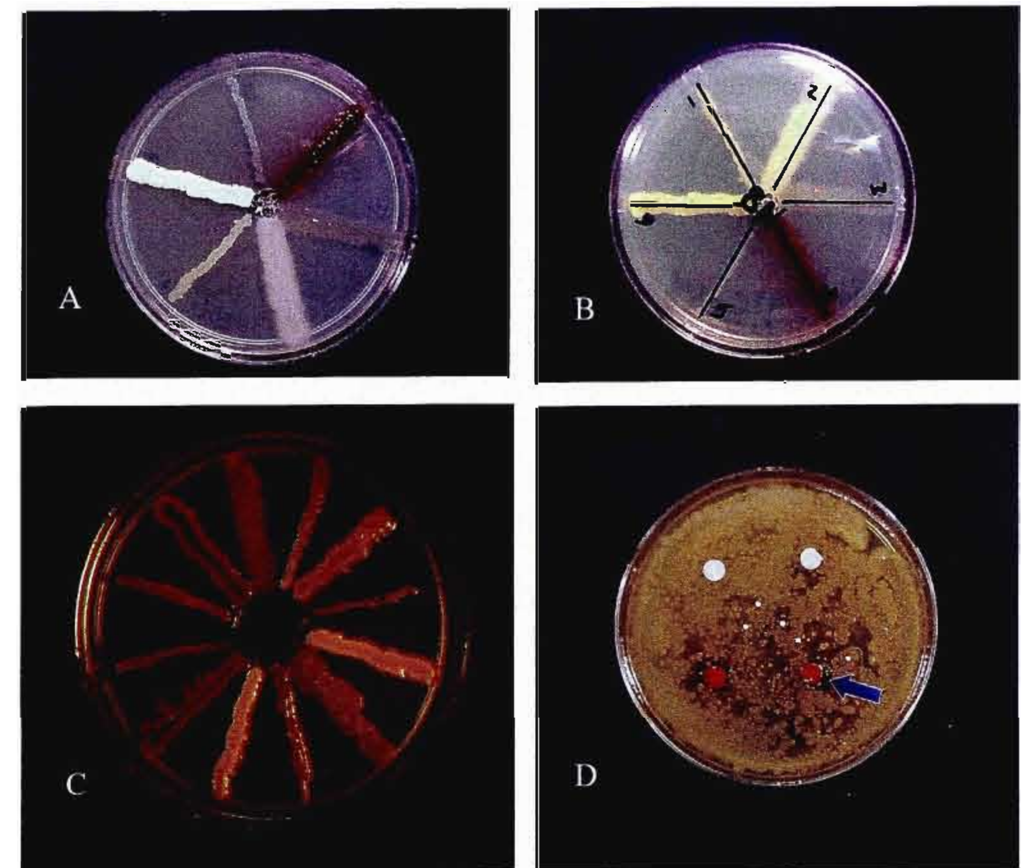


Plate3.4 Antimicrobial-activity of isolate N8 against the test organisms during secondary screening A, B&C = Agar-well showing activity loss of N8 against the test organisms; D = Isolate N8 as a spot-on-lawn of *Staphylococcus aureus* (arrow indicates paper disc turned red).

Minimal Inhibitory Concentration(MIC)

The method used for MIC determinations was appropriate but as a crude extract (culture filtrate) was used in the investigation, the concentration of the antibiotic in the crude filtrate could not be ascertained. Hence, the term, Arbitrary activity Unit, may be more suitable. The arbitrary activity unit (AU) of an isolate may be regarded as the reciprocal of the dilution of the last clear well in a micro-titre plate. The value is adjusted to obtain activity units per ml [AU/ml] by multiplying by ten (Papathanasopoulos, 1997). Isolate N2 showed activity against *Staphylococcus aureus*, *Escherichia coli, Pseudomonas fluorescens* and *Xanthomonas campestris pv campestris*. Its highest activity was against *P.fluorescens* with an MIC of 0,0039µg/ml, while the MIC for *X.campestris pv campestris* was 0,25µg/ml [Table 3.4].

Table 3.4 MIC's [µg/ml] of the selected antimicrobially-active isolates against the test organisms

Isolate No	Sa	Ec	Pf	Sm	Sf	Cu	Xc
N2	0,0078	0,0156	0,0039	uns	uns	nt	0,25
N8	0,0078	0,0039	0,0625	0,125	0,25	0,002	0,0025
N12	nt	nt	0,25	uns	uns	uns	nt
N16	0,125	0,0039	0,0039	uns	uns	uns	0,002
N19	nt	nt	0,125	uns	uns	0,25	0,25
N30	nt	uns	0,0625	uns	uns	uns	uns
N33	uns	nt	0,002	uns	uns	uns	uns
N35	uns	0,031	uns	uns	uns	uns	0,0039

*Uns= Unsusceptible; nt= not tested; Sa = *Staphylococcus aureus*; Ec = *Escherichia coli*; Pf = *Pseudomonas fluorescens*; Sm = *Serratia marcescens*; Sf = *Streptococcus faecalis*; Cu = *Candida utilis*; Xc = *Xanthomonas campestris pv campestris*.

The activity against *Pseudomonas fluorescens* was the main reason N2 was selected for further studies. N8 appears to be the isolate with the greatest chemotherapeutic value in the present investigation on the results obtained from screening and MIC determinations. It showed activity against all the test organisms used [Table 3.4], especially the *Pseudomonas* and *Xanthomonas* species, with MICs of 0,0625µg/ml and 0,0025µg/ml respectively. However, specificity of action of antibiotics is much valued

by medical practitioners. It may be that the substance (s) produced by isolate N8 is/are toxic to many more microorganisms. Therefore, further tests involving a set of different test organisms should be undertaken to determine this. It may also be that this isolate is capable of producing antibiotics active against major plant and animal pathogens. Occasionally, an antibiotic may be active against all organisms (Lancini and Parenti,1982).

The results for isolates N33 and N35 were interesting, with MIC values of 0,002μg/ml and 0,0039μg/ml against *P.fluorescens* and *X.campestris pv campestris*, respectively.

Table 3.5 Spectra of antimicrobial activity of cultures isolated from the Natal midlands

Isolate No	Type of organism*	Active against	Spectrum
N2	Actinomycete	Bacteria	Gram positive and Gram negative
N8	Actinomycete	Bacteria & Fungi	Gram positive and Gram negative ; yeast
N12	Actinomycete	Bacteria	Gram positive and Gram negative
N16	Actinomycete	Bacteria	Gram positive and Gram negative
N19	Coryneform bacterium	Bacteria & Fungi	Gram negative ; yeast
N30	Coryneform bacterium	*Pseudomonas*	Gram negative
N33	Actinomycete	*Pseudomonas*	Gram negative
N35	Actinomycete	Bacteria	Gram negative

*based on morphological, physiological and chemical characteristics. Refer to chapters 4 and 5.

The spectra of activity of the isolates showed that N2, N8 and N16 were active against both Gram positive and Gram negative bacteria. Isolate N8 produced a broad-spectrum antibiotic(s) active against both bacteria and fungi (yeasts), while N19 was active against both Gram negative bacteria and yeasts

[Table 3.5]. Isolates N30, N33 and N35 were active against the Gram negative bacteria.

The MIC values shown in Table 3.4, serve as an index of the antimicrobial-activity of the isolates investigated in this study.

Chapter 4

Identification of Antibiotic-Producing Microorganisms

I: DESCRIPTION OF ISOLATES

4.1 Introduction

Identification of cultures producing antibiotics is a necessary part of a patent application (Dietz, 1999). It is imperative for an industrial taxonomist to obtain as many type cultures as possible, study them under standardized conditions, and develop a practical in house identification scheme (Dietz, 1999).

Since the beginnings of microbiology, a legion of characterization tests has been developed and studied. Some of these tests have enjoyed popularity because they allowed categorization of strains, and as a means for communicating identifications. Many ultimately met with disfavor and were abandoned because study of additional strains led to detection of the inevitable 'different' strain or strains whose characteristics did not seem to correlate properly with other data or an overwhelming number of strains gave similar results and the hoped for separations could not be made (Pridham, 1999).

In early studies of actinomycetes, classification was based solely on morphological observations (Lechevalier *et al.*, 1961; Thiemann and Beretta, 1968). However, accurate morphological characterization of actinomycetes is dependent upon the use of a culture medium which supports good sporulation (Shirling and Gottlieb, 1966).

Morphological characteristics such as the nature of aerial mycelium and mode of spore formation are very important for generic identifications. The form of spores and sporophores are constant for every actinomycete species. For example, *Streptomyces* produces conidia (spores) that are spherical, oval or elongated while *Nocardia* oidiospores are cylindrical. However, morphology in itself is not adequate to differentiate many actinomycete genera . It has to be complemented with chemical and physiological investigations (Labeda,1987).

Lechevalier and Lechevalier (1965) pointed out that actinomycetes can be separated into broad groups on the basis of chemical and morphological characteristics. This approach is still the simplest and the

best (Lechevalier, 1989). Cell wall composition, whole-cell sugar patterns, mycolic acid structures, and more recently phospholipids, have been used as taxonomic markers in the actinomycetes (Lechevalier, 1999). It is possible by using one-way paper chromatography of whole-cell hydrolysates to determine the type of cell wall or diagnostic sugars present in the cell wall of an unknown organism. This simple method will reveal whether or not the cell wall of the unknown isolate contains diaminopimelic acid (DAP) and, if so, the isomers present, viz. the *meso*- or the L-form (Lechevalier,1999).

Identification to the species level requires physiological or biochemical characterization. There have been many attempts to utilise physiological reactions which represent multiple enzymatic activities, for characterization, classification and identification of actinomycetes. These attempts at applying conventional systematic criterion have been disappointing (Trejo,1970), Luedemann (1971), and Pridham (1976).

Characterization of progeny of strains is less reproducible, less understandable, and less communicable (Pridham, 1999). Major obstacles are the superfluity of tests and methodologies and their standardization. Beyond that, the reproducibility of conventional characterizations and their interpretation leaves much to be desired. From the examination of two volumes of Bergeys Manual it is apparent that little standardization of test methods prevails. The same is true for most other systematics treatises.

Gottlieb (1976) has addressed this problem to some extent in a letter to the editor of the *Newsletter of the U.S Federation for Culture Collections.* Even within the same genus of Actinomycetales, comparative data are lacking. For example, information on production of 20-β-keto reductase was presented for only one species of Streptomycetes (Pridham,1999).

In summary, conventional systematics of the Actinomycetales is still in an unresolved state because of reliance on imprecise methodology for determining physiological characteristics. This in turn affects the nature of the species concept derived therefrom (Pridham,1999).

An attempt was made to identify some of the antibiotic-producing actinomycetes isolated during this

investigation, up to the species level, using methods currently available in the literature.

4.2 Materials and Methods

4.2.1 Organisms

The organisms to be identified were isolated from soil samples collected from the KwaZulu-Natal midlands, South Africa. Eight isolates viz- N2, N8, N16, N12, N19, N30, N33, and N35 were selected from 13 isolates that showed good antimicrobial activity on both primary and secondary screening. The criteria for selection were indicated in Chapter 3.

Subcultures were inoculated on the same media used for their isolation and incubated at 30°C for 4 to 6 days. Stock cultures were grown at 30°C on slants and/or thick Nutrient Agar or Yeast Extract Agar (YEA) - ISP medium 2 (Shirling and Gottlieb,1966) plates and then stored at 4°C. In all the investigations, the inocula used were 7-14 day old stock cultures.

4.2.2 Morphological Characterization

The morphological features of each isolate grown on each of the ISP media 2, 3, 4 & 5 (Shirling and Gottlieb,1966) were studied under brightfield and phase contrast light microscopy. A more detailed study of the surfaces of spores and the spore chains was carried out with the aid of both the Environmental Scanning Electron microscope (ESEM) and the conventional Scanning Electron Microscope (SEM).

4.2.2.1 Light Microscopy

Micro-morphology of the actinomycete isolates was determined by either preparing Gram stained slides from 7 to 21 day-old cultures or by observing the organisms *in situ*. The presence of spore chains and aerial and/or substrate mycelium was observed *in situ* under bright field illumination using either a Nikkon (1000 X) or a Zeiss Axiophot light microscope which was used to take photographs at 400x and 1000x magnifications. Observations were made on cultures growing on all the media used [section 4.2.1].

4.2.2.2 Electron microscopy

Small agar plugs (2x2mm) were removed from the plate cultures, transferred to separate plastic -capped

glass vials, and fixed overnight in 3% cacodylate buffered gluteraldehyde. The fixed specimens were then washed (30 mins x 2 changes) in 0.05M cacodylate buffer and dehydrated in an alcohol series: once (10 mins each) in each of 30%, 50%, 70%, 80%, 90%, and thrice (10 mins each) in 100% alcohol. The dehydrated specimens were transferred to a Hitachi HCP-2 critical point dryer (CPD) under 100% alcohol and dried. These were then sputter coated with Gold palladium in a Polaron SEM coating unit E5100 (Polaron Equipment Ltd) and observed in a Hitachi S-570 SEM.

For Environmental Scanning Microscopy (ESEM), small agar plugs (2x2mm) covered with the actinomycete culture were cut and mounted on brass stubs using double sided cellotape. The specimens were then viewed in a Philips XL30 ESEM.

4.2.3 Physiological Characterization

Many physiological tests have been found unreliable and of little taxonomic value if a wide species concept is employed (Kutzner, 1981). Only two seem to have stood the test of time and have been used in the International *Streptomyces* Project (ISP) [Shirling and Gottlieb, 1966]. These include:

- Formation of pigments
- Utilization of nine carbon sources (mainly sugars and sugar alcohols).

4.2.3.1 Formation of melanin pigment

Melanin pigment production was tested on ISP medium 6 and ISP medium 7 as detailed by Shirling and Gottlieb (1966).

4.2.3.2 Carbohydrate utilization

Utilization of sugars and sugar alcohols by the isolates was determined using the Agar culture method of Nitsch and Kutzner (1973). The basal medium contained one-third agar (Difco AATCC) strength (Appendix1) while the carbon sources were filter sterilized and added as 10% solutions. Glucose was used as a positive control while the basal medium without any carbon source was used as a negative control. To prepare shake cultures, 0.2 ml carbon sources were added to sterile test tubes (10x100mm) and inoculated with 0.1 ml of a dense spore suspension of the isolate. Basal medium (1,7 ml; warmed to 70°C) was finally added and the test tubes incubated for 2 to 7 days at 30°C.

4.2.3..3 Other physiological tests

All the isolates were Gram stained using Hucker's modification of the Gram stain procedure (Conn *et al.*, 1960) while acid fastness was determined using the Ziehl-Neelsen staining method (modified by adding 50:50 volumes of 3% HCl and alcohol for decolorisation).

The catalase and oxidase tests were carried out on 7-day old cultures of the isolates. Kovac's method was used for the oxidase test. Examination of the isolate's spores was by Conklin's modification of the Wirtz spore stain. All tests were performed according to the Manual of Microbiological Methods, McGraw-Hill, New York (1957) and the examples from Bergey's Manual of Determinative Bacteriology (8th ed., 1974) and Bergey's Manual of Systematic Bacteriology (Vol.4., 1989).

Motility was determined using two procedures; the hanging drop method ; and an *in situ* motility determination method. The latter method involved placing a drop of sterile distilled water directly on the bacterial growth(single colonies) on the plates, covering with a coverslip and gentle pressing down to spread the water evenly. Motility determination were made after 18 hrs, three days and seven days incubation, under a light microscope (oil lens, 1000x magnification; Zeiss Axiophot).

4.2.4 Biochemical Characterization

For the actinomycete isolates, the first stage in the chemical procedure was the examination of whole-cell and/or cell wall hydrolysates for diagnostic sugars and diaminopimelic acid (DAP) isomers (Becker *et al.*, 1964; Lechevalier, 1968; Berd, 1973; Staneck and Roberts, 1974). Different solvent systems, or combinations thereof, were used.

4.2.4.1 Preparation of the whole-cell hydrolysates

The isolates were grown in modified Santon's medium as detailed by Mordaska *et al.* (1972). The organisms were aseptically inoculated by transferring growth from stock cultures with an inoculating loop, into 50ml medium in 250ml Erlenmeyer flasks and incubated at 30°C in a rotary shaker [New Brunswick, Edison, USA] at 300rpm for seven days. The biomass was harvested by centrifugation at 3000 x *g* for 10 mins in a Heraeus Labofuge 200. The organisms were then washed once with 0,5M NaCl in distilled water, twice with distilled water and freeze-dried under vacuum in a GAMMA 1 freeze

dryer.

Diagnostic cell wall sugars were extracted from freeze-dried biomass using a combination of two methods: organisms were hydrolyzed by the method of Murray and Procter (1965) ; and the hydrolysate then evaporated using the method of Staneck and Roberts (1974).

Freeze-dried cells (50mg) of each isolate were suspended separately in 3ml of 5% H_2SO_4in 20ml screw-cap bottles and autoclaved at 5lb/sq in. for 20 minutes. After cooling, 1g $Ba(OH)_2$ was added to the hydrolysates. The mixtures were adjusted to pH5.0 - 5.5 with 5%H_2SO4, centrifuged at 6000 x *g* in a Heraeus Labofuge 200, the supernatants removed by Pasteur pipette and the remaining liquid evaporated over boiling water. The residues were re-suspended in 1ml dH_2O and centrifuged to remove insoluble material before the solutions were used for chromatography.

4.2.4.2 Determination of sugars occurring in whole-cell hydrolysates

4.2.4.2.1 One-way paper chromatography for sugar determination

The sugar standards: Galactose , Arabinose and Xylose (1st standard) and Rhamnose, Mannose, Glucose and Ribose (2nd standard) were prepared separately as 1% (w/v) solutions. In all cases 20x20 cm sheets of Whatman's chromatographic paper were used and the sample and standards (10μl each) applied separately, one centimeter from the bottom edge of the chromatogram. The chromatograms were developed in the solvent system of Becker *et al.* (1964). Completed chromatograms were air-dried with a hair-dryer and visualized by spraying with 0,2% ninhydrin in acetone followed by heating at 105°C for two to five minutes.

4.2.4.3 Determination of Diaminopimelic acid (DAP) Isomers

The presence and form of DAP was determined by the method of Becker *et al.* (1964) as described by Ntuli (1994). For each isolate, fifty milligrams of freeze-dried biomass were mixed with 5ml 6M HCl in a small screw-cap bottles. The mixture was hydrolyzed at 100°C for two hours in a hot air oven. After cooling to room temperature the hydrolysate was filtered through Whatman No.2 paper. The filtrate was collected in a 50ml glass beaker and evaporated to dryness over boiling water. The residue was re-dissolved in 5ml distilled water and re-evaporated and the final residue re-suspended in 1ml distilled

water before being used for chromatography.

4.2.4.3.1 HPLC analysis of whole-cell hydrolysates for DAP detection

The freeze-dried biomass (50mg) of each isolate was hydrolyzed separately in 6N HCl at 110°C for 24 hours according to the protein hydrolysis procedure of Moore and Stein (1948). An internal standard was added to correct for any inaccuracies that may have occurred during sample preparation.

The hydrolysates were transferred separately to a Beckman 6300 analyzer. The analyzer comprised a single-column ion-exchange chromatography utilizing HPLC techniques involving; sodium elution buffers, a regenerating reagent (ninhydrin), and a sodium high performance column. The standard detection system featured the classical Moore and Stein (1948) ninhydrin reagent. The combined column effluent/reagent mixture was heated at 135°C and ran at a flow rate of 14.5ml/min for a period, prior to photometric visualisation at wavelengths of 440 and 520 nanometers. These analyses were carried out in the Animal Science Laboratory of the University of Natal.

4.2.4.3.2 Two-Dimensional Thin Layer Chromatography for DAP Determination

For the actinomycete isolates, the DAP isomers were determined by the method described by Harper and Davis (1979). Commercial cellulose-coated aluminum sheets were used. Isopropanol-acetic acid-water (75:10:15) and methanol-pyridine-10N HCl-water (64:8:2:14) were used as first and second solvents respectively. To ensure saturation of the tank atmosphere with the solvents, two walls of each tank (Shandon TLC tanks 30x22x13cmX2) were lined internally with absorbent paper and 100ml of solvent poured into the tank 24hrs before chromatograms were run.

Cellulose-coated aluminum sheets (20x20cm, Merck, cat. No.5552) were marked with origin points, 1,5 cm from two sheet edges and pencil lines made 15,5cm from, and parallel to, the sheet edges. Each two-dimensional analysis was made with 2 to 4μl of hydrolysate with the sample spot size kept as minimal as possible. The strips, 4,5cm wide, marked on the sheet were used to make one-dimensional runs of the sample and control mixtures of standard amino acids in each solvent. The first dimension solvent mentioned above took approximately 3h to reach the 15,5cm mark. The chromatogram was then dried overnight in a ventilated hood at room temperature or in a ventilated oven at 105°C for 2 hours.

The second solvent mentioned above took approximately 1,25h to reach the 15,5 mark. Completed chromatograms were then air-dried and visualized with 0,2% ninhydrin in acetone by spraying and heating at 105°C.

4.2.4.3.3 One-way paper/thin-layer chromatography for DAP determination.

Diaminopimelic acid (DAP) isomers were also determined by one-way paper/thin-layer chromatography. Whole-cell hydrolysis was carried out by the modified method of Becker *et al.* (1964) as described earlier. The following solvent mixture was used for separation of the amino acids: methanol-water-10N HCl-pyridine (80: 17.5: 2.5: 10, by volume).

4.3 Results and Discussion

Selective media were used for isolation of the organisms to be identified . Lechevalier (1989) practical guide to generic identification of Actinomycetes was adhered to, and the results compared with descriptions of similar and/or related organisms in Bergey's Manual of Systematic Bacteriology (Vol.4, 1989).

Isolate N2

Morphological properties

The colonies were curled and irregularly branched in young cultures, forming a mycelium resembling a contour map [Plate4.1B]. Colonies were white to pale orange [Plate4.1D]. The young hyphae penetrate the agar [Plate4.1C], and after 14 days incubation, fuse together at the surface of the colonies [Plate4.1D]. Environmental Scanning Electron Microscopy (ESEM) revealed the spores silhouette contained in sheaths of extracellular material [Plate4.1E]. The surface of the oval shaped spores is smooth and the spores are packed in dense clusters [Plate4.1F] as seen under the Scanning Electron Microscope (SEM).

Physiological tests

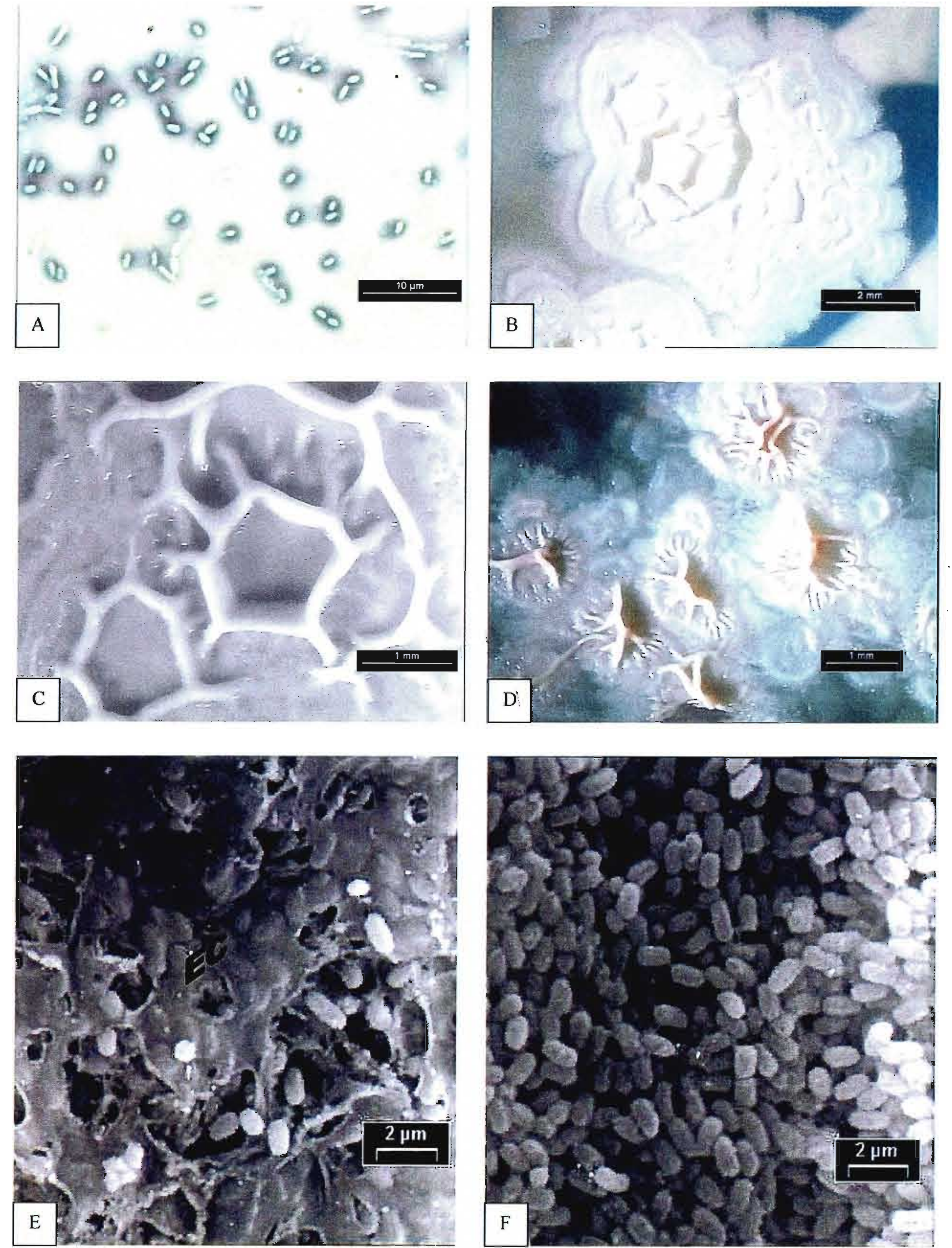
Cells were Gram-positive, non-acid fast [Plate4.1A], catalase positive and formed moderate filiform growth on nutrient agar slants. A pellicle was formed in test tube cultures which later detached and fell to the bottom of the tube. The broth remained clear afterwards. A reddish brown soluble pigment was produced on ISP medium 7 and in nutrient broth shake cultures incubated for seven days. No diffusible pigment was produced on the other ISP media used. The oxidase test was negative.

Carbohydrate utilization

On ISP medium 9, the organism utilized L-arabinose, fructose, glucose, inositol, rhamnose and sucrose and to a lesser extent raffinose and D-xylose as carbon sources [Table 4.1]. Pink-brown pigment was produced on all the carbon sources except raffinose and D-xylose. There was no noticeable growth in the control which contained only the basal medium without any carbon source.

Plate 4.1 Morphological features of isolate N2 as revealed by the Zeiss Axiophot light microscope and scanning electron microscopy

A = Non-acid fast cells; B = colony on Nutrient agar; C = young substrate hyphae (penetrates the medium); D = mature hyphae fused together (pale white to orange colour); E = scanning electron micrograph showing clusters of single spores covered by a sheath of extracellular material; F = scanning electron micrograph showing spores in dense clusters.



Whole-cell hydrolysate analysis

The whole-cell hydrolysate contained *meso*-Diaminopimelic acid (DAP) revealed by two dimensional thin-layer chromatography and one-way paper chromatography [Plate5.2]. High Pressure Liquid Chromatography (HPLC) analysis of whole-cell hydrolysates using a Beckman 6300 amino acid analyser (Moore and Stein,1948) showed high concentrations of DAP [Appendices5&6], aspartic acid and glutamic acid with relatively low concentrations of glycine, and phenylalanine. Traces of tyrosine and methionine were also present.

Table 4.1 Utilization of carbon sources and production of acid, gas and pigment by antimicrobially-active isolates

	carbon sources									
Isolate	**L-Ara***	**Fructose**	**Glucose**	**Mannitol**	**Inositol**	**Raffinose**	**Rhamnose**	**Sucrose**	**D-xylose**	**Control**
N2	+++	+++	+++	++	+++	++	+++	+++	++	-
	pp	pp	pp	pp	pp		pp	pp		
N8	+++	++	++	++	++	+	+	++	+++	-
	pp			pp	pp					
N12	+++	±	+++	+++	++	++	+++	+++	+++	-
	a									
N16	+++	+++	+++	+++	+++	+++	+++	++	+++	-
	pp	pp		pp	a	pp	pp		pp	
N19	++	±	+++	+++	±	±	±	++	±	-
	bp									
N30	+++	+++	+++	+++	++	+++	±	++	±	-
N33	+++	++	++	+	++	++	++	±	++	-
	pp	pp		gas	pp		pp		pp	
N35	+++	+++	+++	+++	±	++	+	+	+++	-
								gas		

* L- arabinose; bp = brown pigment; a = acid produced (medium turned yellow); pp = pink-brown pigment; gas= gas produced; +++ = Good (thick surface growth); ++ = Poor (Initial submerged growth, progresses to the surface); + = Weak (submerged growth); - = no detectable growth.

Diagnostic sugar analysis

Separation of sugars in whole-cell hydrolysates by one-way paper chromatography showed that isolate N2 contained none of the characteristic actinomycete sugars [Appendix4]. Thus, the culture can be considered to have a type III cell wall according to the scheme of Becker *et al.* (1965) and a type C whole-cell sugar pattern (WCSP) according to the scheme of Lechevalier (1968).

Isolate N8

The physiological and chemical characteristics were similar to those of isolate N2 described above. However, there are variations in morphology and utilization of carbon sources. Unlike N2, N8 could not utilize raffinose and rhamnose as carbon sources [Table 4.1]. Isolate N2 produced pink-brown pigment on fructose, glucose, rhamnose and sucrose carbon sources, whereas no pigment was produced when grown on any of these sugars by isolate N8 [Table 4.1]. The cells of isolate N8 were non-acid fast [Plate4.2A].

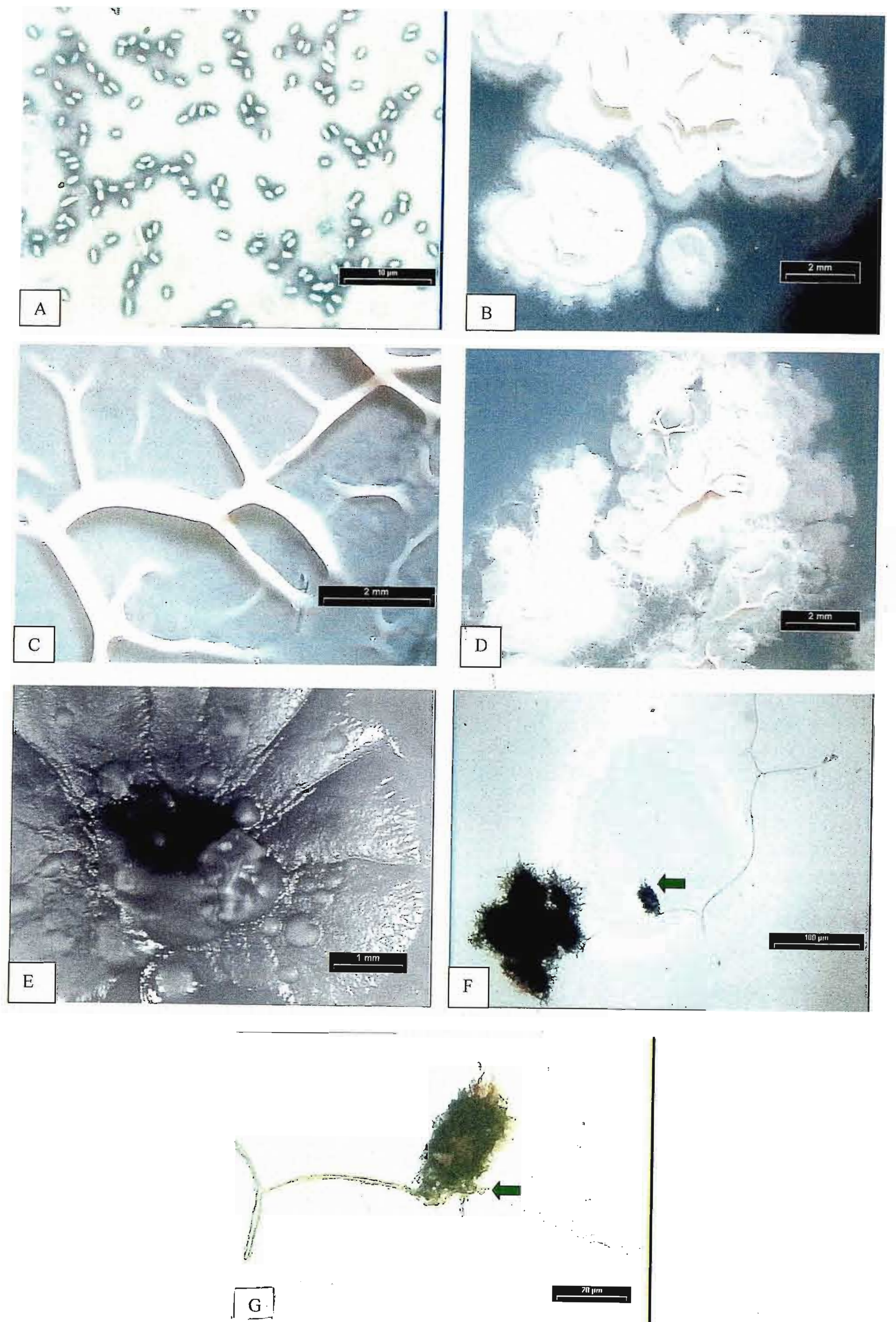
Morphological Properties

On nutrient agar plates, N8 appeared as pale white to orange colonies that resembles a contour map [Plate4.2B]. The young hyphae were fleshy, intertwined, and penetrated the medium [Plate 4.2C], while the mature hyphae fused together [Plate4.2D] to form dark brown to gray coloured dome-like bodies that contained the spores [Plate 4.2E]. In broth the long branching hyphae arose from a synnemata (which is a collection of hyphae fused together) that bears spores [Plate 4.2 F&G].

Environmental scanning electron microscopy (ESEM) revealed that the spores are enclosed in a thick slimy layer/sheath [Plate4.3A&B], which when broken exposes the spore mass [Plate4.3D]. Scanning electron microscopy (SEM) showed the spores as smooth surfaced, oval to rod shaped [Plate 4.3C], and packed in dense clusters. Growth on agar slants was moderate and filiform in pattern. The pellicle formed by broth cultures in test tubes later detached and fell to the bottom of the test tube leaving the broth clear.

Plate 4.2 Isolate N8 morphological features as revealed by the Zeiss Axiophot light microscope

A = Non-acid fast cells; B = colony on nutrient agar; C =branched substrate hyphae on colony surface; D = fused hyphae on nutrient agar; E = mature mycelium with dome-like body; F = hyphae with synnemata (arrow) in broth; G = synnemata (arrow) as a collection of hyphae.



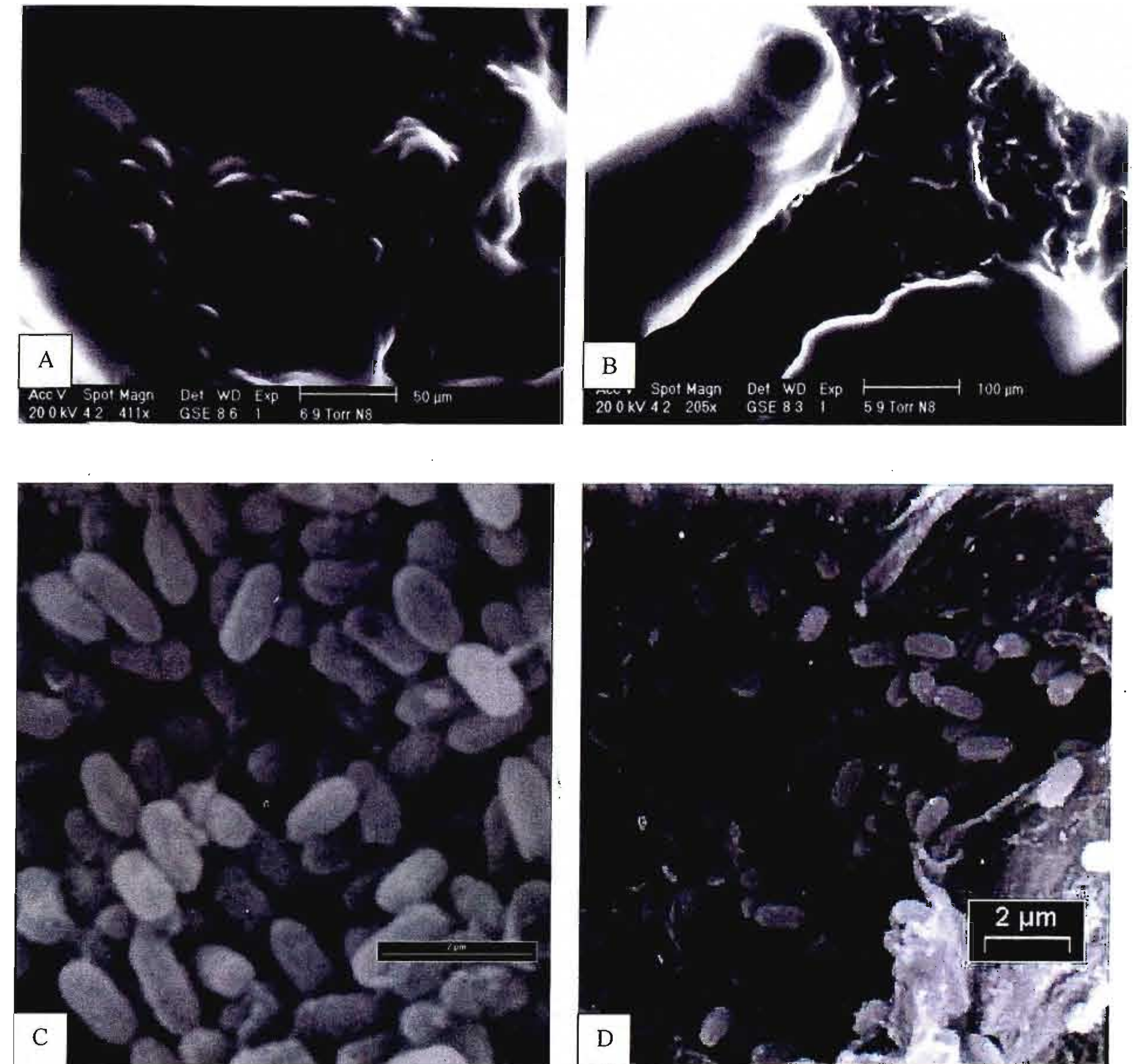


Plate 4.3 Scanning electron micrographs of isolate N8

A = sheath of extracellular material overlying spore mass; B = broken dome-like body with underlying spores becoming exposed; C = spores in dense clusters; D = exposed spores.

Whole-cell hydrolysate analysis

High performance amino acid analysis (Beckman 6300 amino acid analyser) showed that N8 contained major amounts of L- or *meso*-DAP and glycine (cell wall chemotype I) of Becker *et al.* (1965) [Appendices5&6]. There was little or no separation ofDAP isomers on cellulose TLC plates [Plate5.1B]. None of the diagnostic sugars was present [Appendix 4] on one-way paper chromatograms suggesting a type C whole-cell sugar pattern (WCSP) based on the Lechevalier (1968) scheme.

Isolate N12

Morphological Properties

Colonies on Nutrient agar plates were off-white, smooth and flat with entire margins [Plate 4.4B]. Aerial mycelium was absent or not visible. The vegetative mycelium fragmented into rods or bacillary elements. The spore chains were of indeterminate length and the spores were characteristically separated by short lengths of apparently 'empty' hyphae, giving a beadlike appearance [Plate4.4A]. Scanning electron microscopy (SEM) revealed long chains of mycelial fragments or rod-like structures [Plate4.4E]. The lateral surfaces of the fragments were rough and indented [Plate4.4F].

Physiological tests

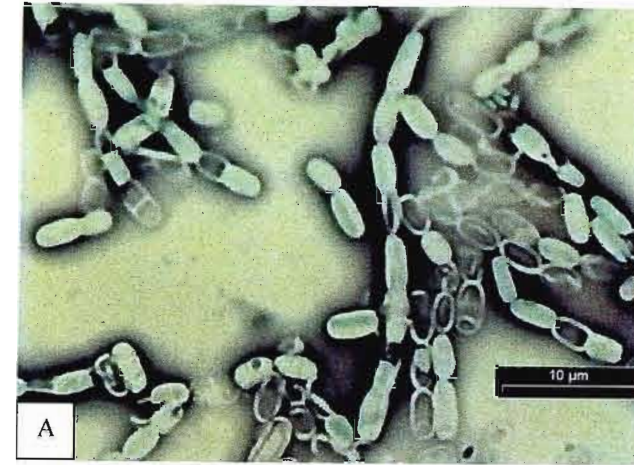
Cells were Gram-variable [Plate4.4D], and the hyphae fragmented on Gram stain treatment [Plate4.4C]. The cells were also non acid fast, catalase negative and formed moderate beaded growth on Nutrient agar slants. No pellicle was formed in test tube broth cultures. The oxidase test was positive. A yellow water soluble pigment was produced on ISP medium 7 and in nutrient broth shake cultures incubated for 7 days. No diffusible pigment was observed on ISP medium 6.

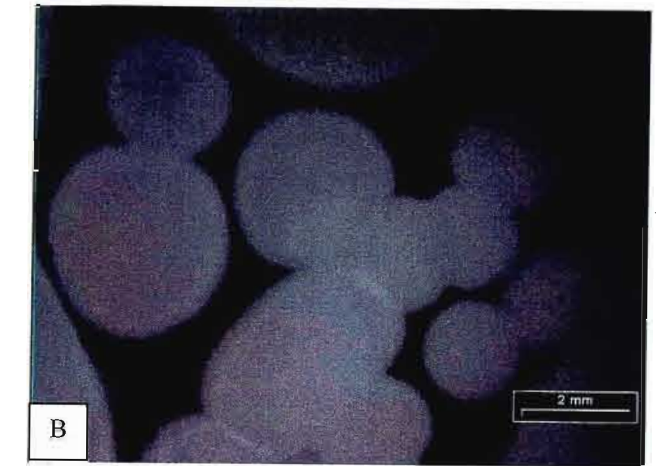
Carbohydrate utilization

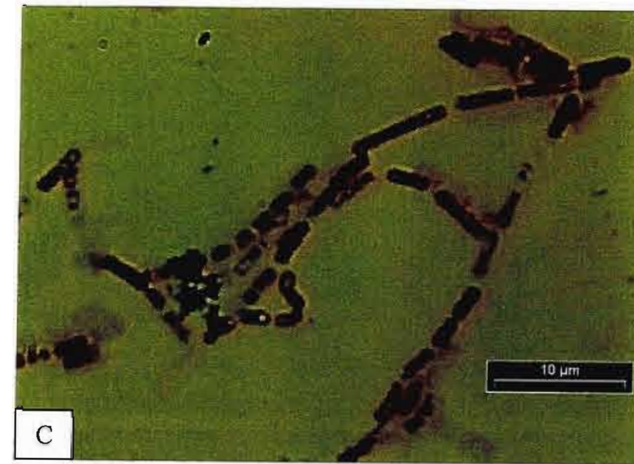
The isolate utilized all the carbon sources tested on ISP medium 9, except fructose [Table 4.1]. A yellow water soluble pigment was formed in the medium with L-arabinose as carbon source. Growth was poor on raffinose and *i*-inositol and no pigment was produced on the other carbon sources tested [Table 4.1].

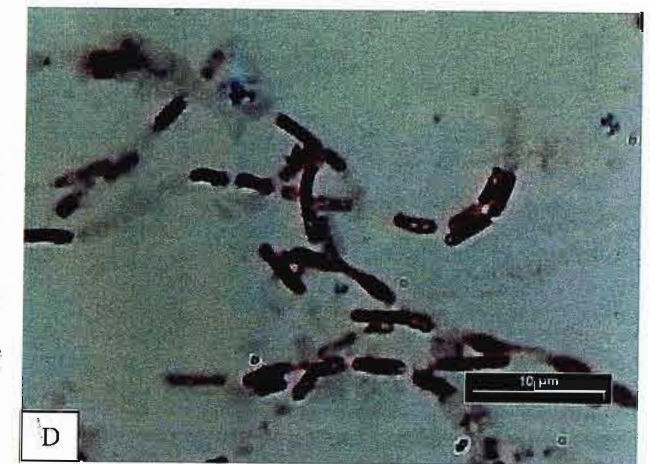
Whole-cell hydrolysate analysis

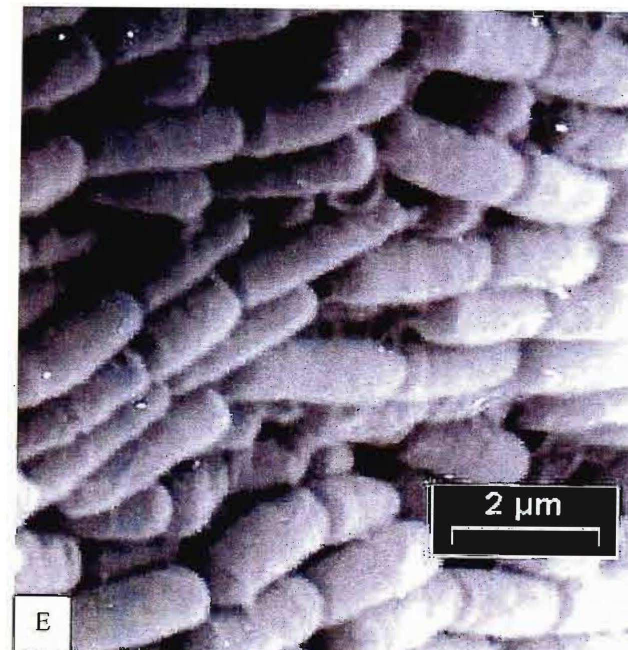
The whole-cell hydrolysate contained *meso*-DAP on one dimensional TLC analysis [Plate5.2]. Arabinose and galactose were the diagnostic sugars present. Thus, N12 can be considered to have a cell wall of type IV according to the scheme of Becker *et al.* (1965) and a type A WCSP based on Lechevalier (1968) scheme.











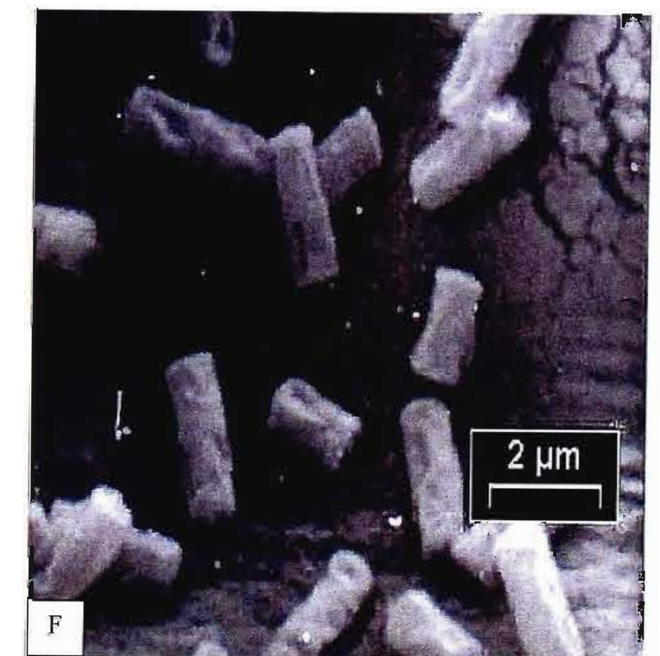


Plate 4.4 Isolate N12 morphological features as revealed by the Zeiss Axiophot light microscope and scanning electron microscopy

A = Non-acid fast cells; B = colony on Nutrient agar plate; C = fragments on gram stain treatment; D = Gram variable cells; E = scanning electron micrograph showing long chains of fragmented hyphae; F = scanning electron micrograph showing bacillary elements with lateral indentations.

Isolate N16

Morphological properties

The primary mycelium were branched and irregularly wrinkled [Plate4.5C], cream colored with some tinges of pink/orange [Plate 4.5D]. And in the older cultures, the mycelium fragmented into short rods or coccoid elements. No aerial hyphae or synnemata was observed. Scanning electron micrograph (SEM) revealed that the surfaces of spores were covered by a few strands [Plate4.5F] to several sheaths of slimy extracellular material (Williams *et al.*, 1976). SEM revealed oval to rod-shaped cells packed in clusters [Plate 4.5E].

Physiological characteristics

Cells were Gram-positive [Plate4.5B], non acid fast [Plate4.5A] and oxidase negative. Catalase was produced. A pellicle was developed on the surface of test tube broth cultures. The broth was clear as the pellicle remained at the top, indicative of strong aerobic growth. In agar shake cultures incubated for seven days, a reddish-brown water soluble pigment was produced. Moderate arborescent growth was observed on agar slants.

Carbohydrate utilization

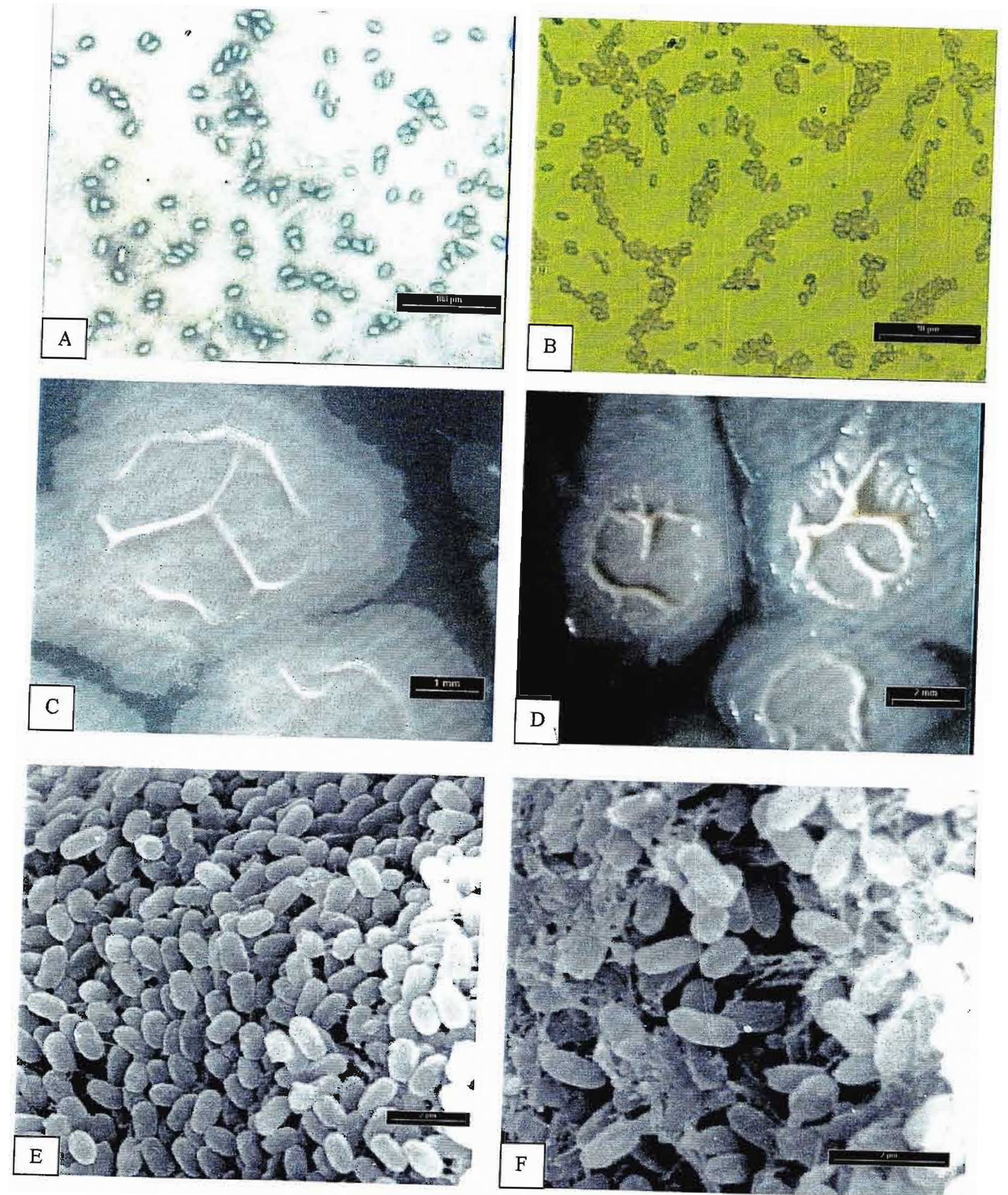
Isolate N16 utilized all nine carbon sources tested on ISP medium 9. Pink to brown pigment was produced on all the sugars except glucose and sucrose [Table 4.1]. Acid was produced in *i*-inositol and no gas was produced on any of the carbon sources tested.

Chemical properties

The whole-cell hydrolysates contained low amounts of L-DAP [Plate5.2&Appendix6] with arabinose and galactose as the diagnostic sugars present. Thus, N16 can be considered to have a type I cell wall according to the scheme of Becker *et al.* (1965) and a WCSP type A sensu based on Lechevalier and Lechevalier (1970) scheme.

Plate 4.5 Isolate N16 morphological features as revealed by the Zeiss Axiophot light microscope and scanning electron microscopy

A = non-acid fast cells; B = Gram positive cells; C=young colony surface; D = mature colony on nutrient agar (pale white to yellow to orange colour); E = scanning electron micrograph (SEM) showing spores in dense clusters; F = SEM showing a few strands of extracellular material.



Isolate N19

Morphological properties

N19 was isolated on Czapek's agar as tiny punctiform, flat, translucent colonies. On agar slants, growth was abundant and spreading. Two distinct growth forms were observed on nutrient agar. A yellow to orange, oval to lemon shaped colony, surrounded at the edges or covered entirely, by a milky flat spreading growth [Plate4.6D], possibly a hyphal outgrowth. In liquid culture, mycelium was lacking except for sparse club-shaped cell doublets, joined at the tapered ends [Plate 4.6C]. No spores were formed. Both acid-fast and Gram-stained preparations revealed an angular arrangement of cells, sometimes in V-formations [Plate4.6A&B]. Individual cells were rod-shaped [Plate4.6F]and densely packed [Plate 4.6E] when viewed under the Scanning electron microscope.

Physiological characteristics

Gram reaction, catalase and oxidase tests were positive for isolate N19. Cells were non acid-fast but tended to retain carbol fuschin [Plate4.6B]. Motility was not observed.

Carbohydrate utilisation

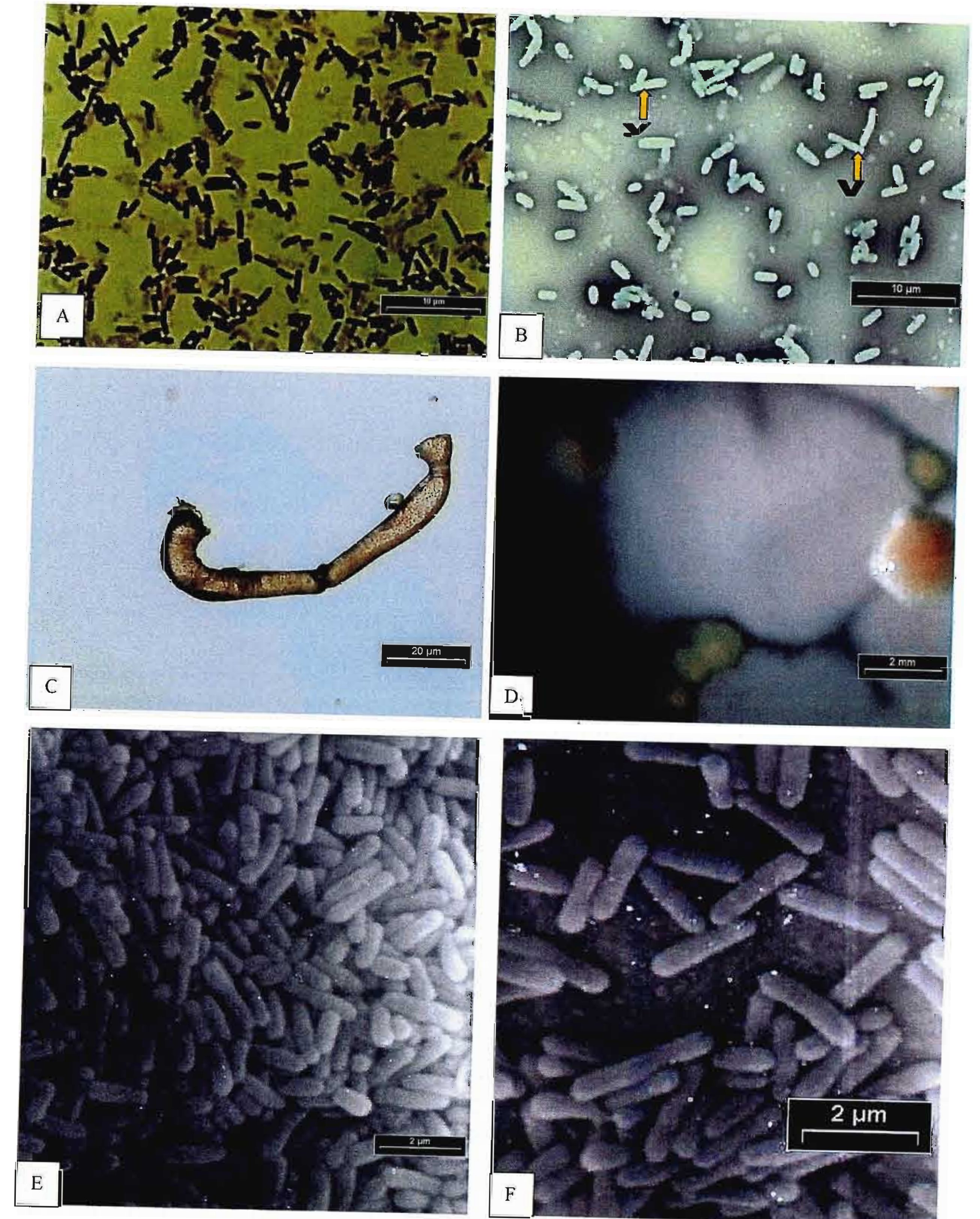
Only glucose and mannitol was well utilized in ISP medium 9 [Table 4.1]. Sucrose and L-arabinose yielded poor growth, with brown pigment production in the case of the latter sugar. There was little or no utilization of fructose, inositol, raffinose, rhamnose and D-xylose. Gas and/or acid production was absent in all cases.

Whole-cell hydrolysate analysis

High performance amino acid analysis revealed that N19 contained only a trace amount of DAP [Appendix 5] and very high glycine concentration [Appendix 6]. Separation of DAP on silica and cellulose TLC plates was doubtful. However, with paper chromatography, L-DAP was evident from the R_f value calculated. The presence of L-DAP and glycine is characteristic of type I cell wall chemotype of Becker *et al.* (1965). Arabinose and galactose were the diagnostic sugars present. Thus, N19 can be considered to have a cell wall chemotype I and a type A WCSP based on the scheme of Lechevalier (1968).

Plate 4.6 Isolate N19 morphological features as revealed by the Zeiss Axiophot light microscope and scanning electron microscopy

A = Gram positive cells; B = non-acid fast cells arranged in Y and V-formations (arrows); C = club-shaped doublets joined at tapered ends; D = two colony forms on Nutrient agar; E = scanning electron micrograph (SEM) irregularly shaped rods packed closely together; F = SEM showing irregular arrangement of cells.



Isolate N30

Morphological properties

On Nutrient Agar plates, N30 appeared as tiny, punctiform, raised, smooth, milky translucent colonies. No definite mycelium was formed. Aerial hyphae were lacking and the angular spread of colonies formed a bracelet-like structure with multiple pendants [Plate4.7E]. No pellicle was formed in test tube broth cultures, a few clump of cells was observed [Plate4.7F]. On agar slants, growth was moderate and filiform. No spores were formed [Plate 4.7D]. Both acid-fast and Gram-stained preparations [Plate4.7A,B&C] showed the presence of irregular rods which varied considerably in size and shape, including bent, curved, wedge-shaped, drumstick, hammer and club-shaped forms [Plate 4.7A&B]. Some of the rods were arranged at an angle to each other leading to V-formations [Plate4.7B,C&D]. Other angular arrangements were seen under the Scanning Electron Microscope (SEM) [Plate 4.8A,B&C].

Physiological characteristics

Cells were Gram-variable [Plate4.7A]. They were acid-alcohol fast, catalase negative and oxidase positive. Motility was not observed in any of the cells.

Carbohydrate utilization

Good thick surface growth was observed on L-arabinose, fructose, glucose, mannitol and raffinose. Utilization of sucrose and inositol was weak. Rhamnose and D-xylose were not utilized [Table 4.1]. No pigment, acid or gas was produced on ISP medium 9.

Whole-cell hydrolysate analysis

The whole-cell hydrolysate contained none of the DAP isomers (cell wall chemotype VI) as revealed on paper chromatograms [Plate5.2]. However, doubtful results were obtained in the DAP separation and identification on silica and cellulose chromatograms. The only diagnostic sugar present was galactose [Appendix 4].

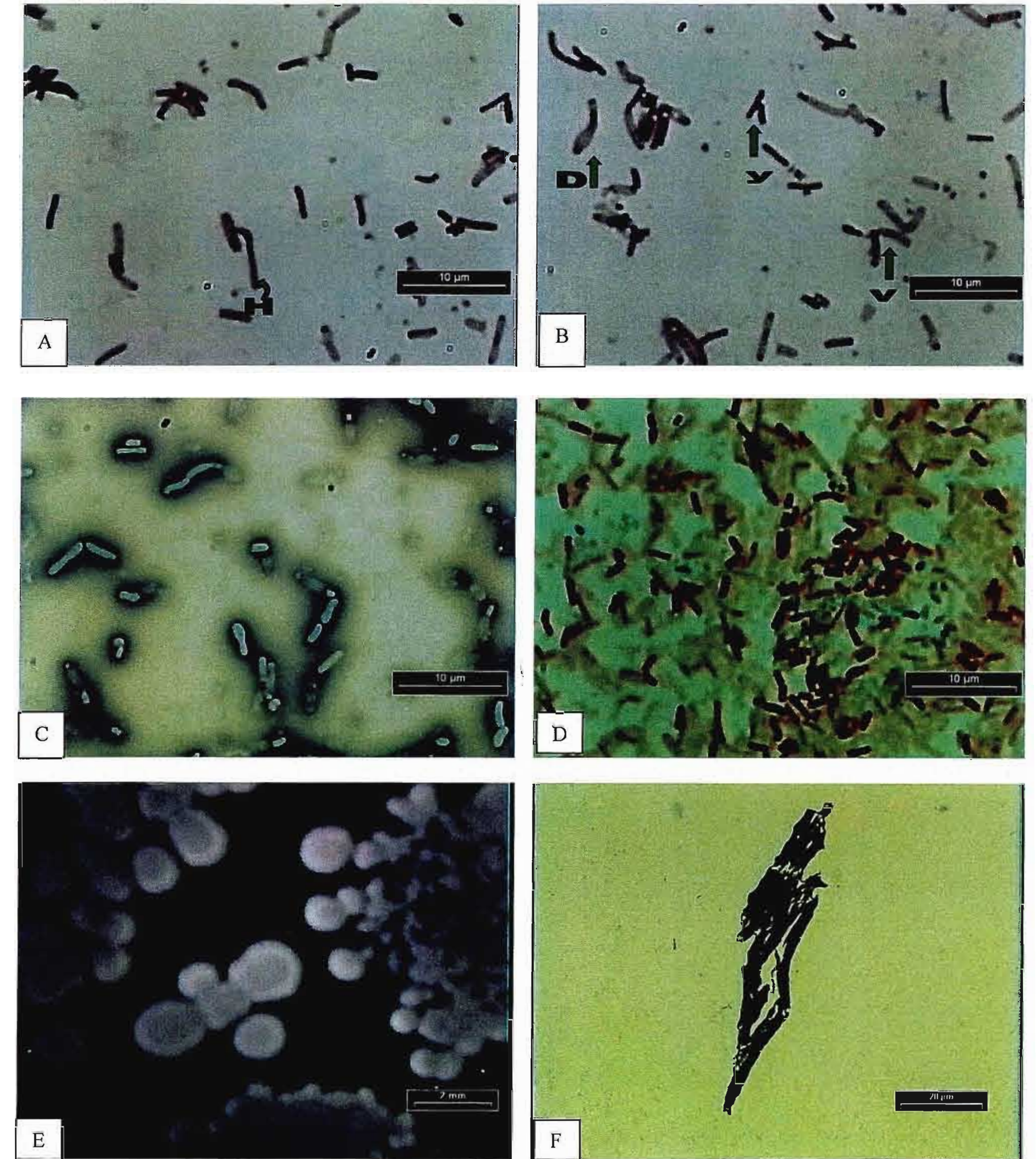


Plate 4.7 Isolate N30 morphological features as revealed by Zeiss Axiophot light microscope

A = Gram variable cells; B = drumsticks (D), hammer (H), club-shaped, bent, curved and V-formations (arrows); C = non-acid fast cells; D = mainly vegetative cells on Ziehl-Neelsen spore stain; E = angular spread of colonies on agar plate; F = clump of cells in Nutrient broth.

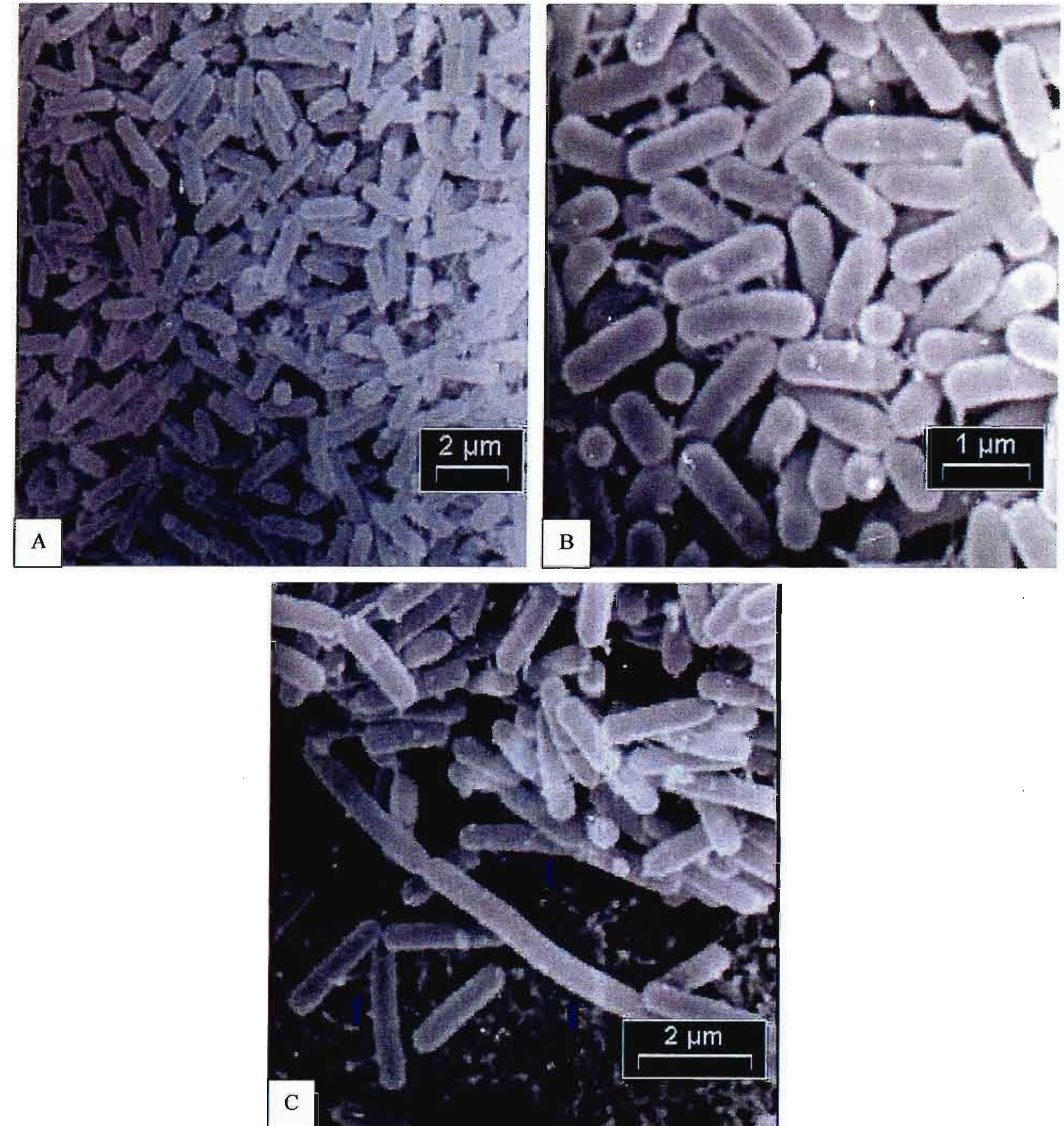
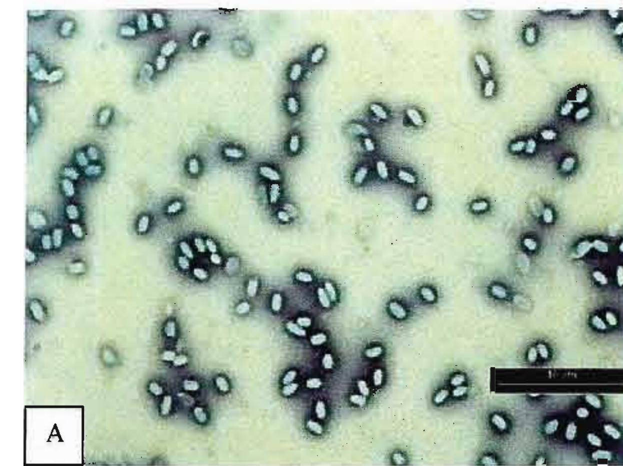


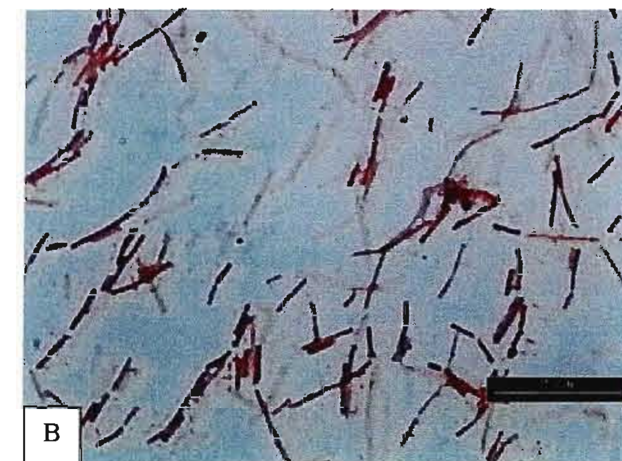
Plate 4.8 Scanning electron micrographs of Isolate N30

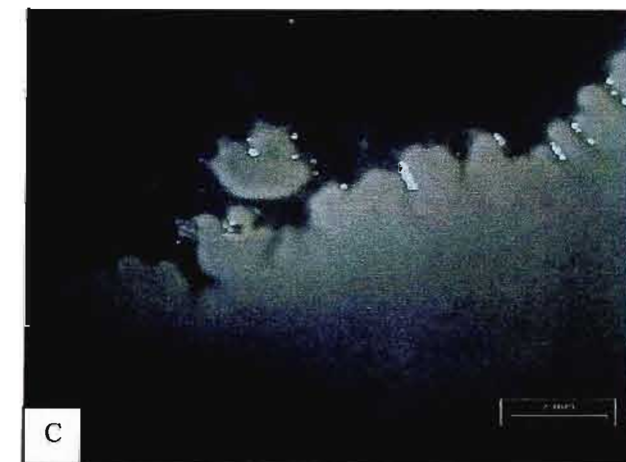
A = rods in dense clusters; B = irregularly packed rods; C = straight chain (SC) and Y or V-arrangement of cells (arrows).

Plate 4.9 Isolate N33 morphological features as revealed by the Zeiss Axiophot light microscope

A = non-acid fast cells; B = vegetative cells on Ziehl-Neelsen spore stain (no spores); C = shiny glistening spreading colony on Nutrient agar.







Isolate N33

The whole-cell hydrolysate contained arabinose and galactose as diagnostic sugars (WCSP type A). However, doubtful results were obtained in the separation and identification of DAP isomers [Plate5.2]. The isolate was able to utilise L-arabinose and to a lesser extent fructose, glucose, inositol, raffinose, rhamnose and D-xylose as carbon sources [Table 4.1]. Sucrose was not utilized. Gas was produced in the medium containing mannitol. The isolate was non acid-fast [Plate4.9A], catalase negative, oxidase positive and lacked the ability to form spores [Plate4.9B]. On Nutrient agar plates, the colonies were shiny, glistening and spreading [Plate4.9C].

Isolate N35

Morphological properties

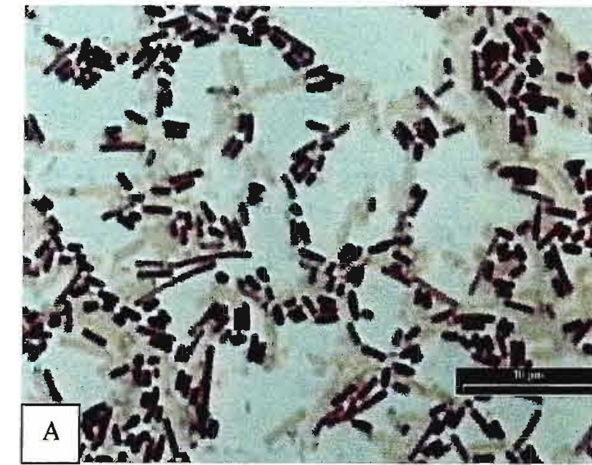
Colonies on Nutrient agar plates were convex with a ribbed appearance and lobate margins. The surface is smooth and leathery [Plate 4.10D]. In nutrient broth, hyphae were straight to curved and sparsely branched [Plate4.10E]. On ageing the hyphae breaks up into fragments of various shapes and sizes [Plate 4.10F]. Fragmentation in older cultures resulted in rod-like or elongated coccoid elements, the majority of which stained as spores with only a few vegetative structures observed [Plate 4.10B]. Because of their staining reaction, these elements were regarded as spores by several authors (Krasil'nikov *et al.*, 1961; Luedemann, 1974). Angular arrangements of bacillary elements were evident [Plate 4.11B] under the scanning electron microscope. SEM also showed occasional rod-like [Plate4.11A], enlarged cells (up to 4μm in diameter) [Plate 4.11C].

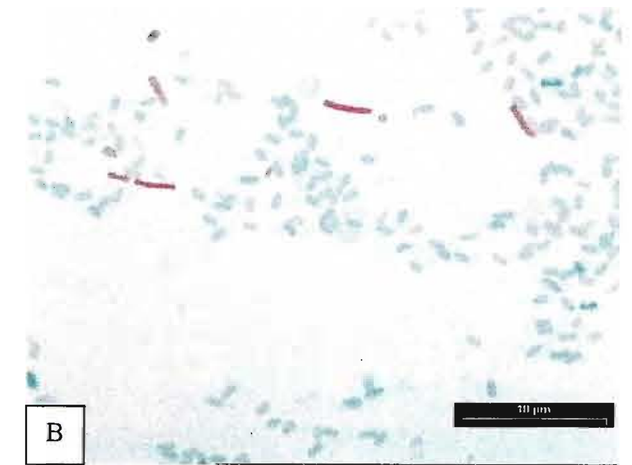
Physiological characteristics

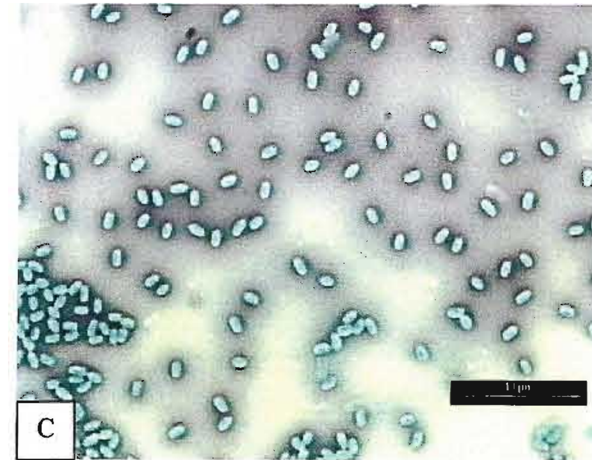
Gram reaction [Plate4.10A], catalase and oxidase tests were positive. Cells were partially acid-fast but tended to bind and retain carbol fuschin [Plate 4.10C]. Motility was not detected.

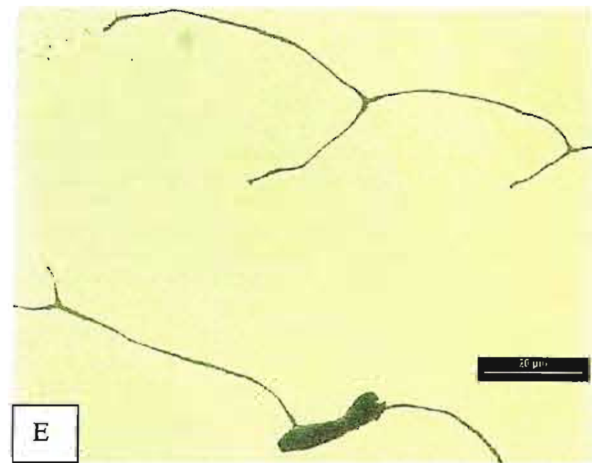
Plate 4.10 Isolate N35 morphological features as revealed by the Zeiss Axiophot light microscope

A = Gram positive cells; B = spores with few vegetative forms on Ziehl-Neelsen stain; C = partially acid fast cells; D = ribbed, smooth, leathery surface colony on Nutrient agar plate; E = branched hyphae in broth; F = hyphal fragmentation in broth.









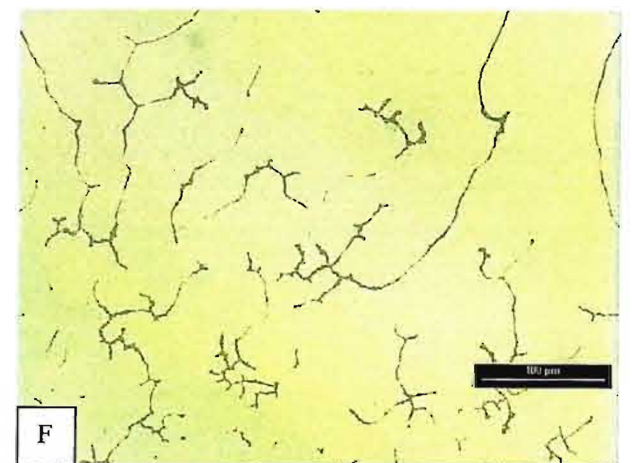
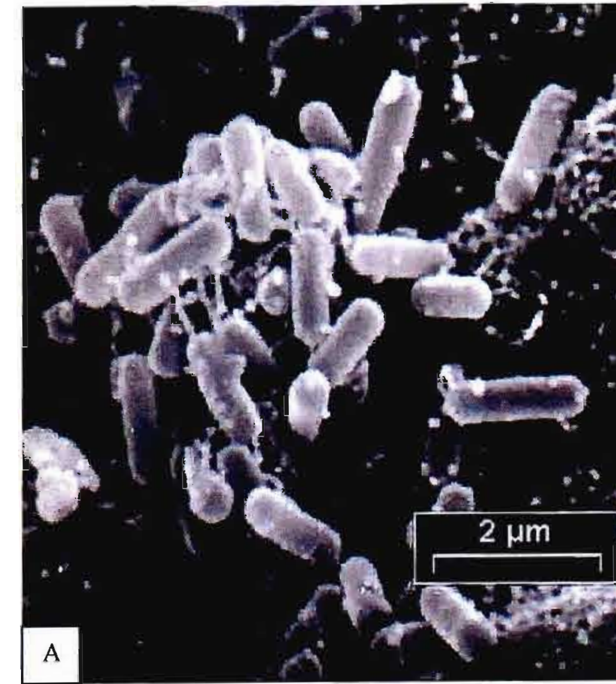
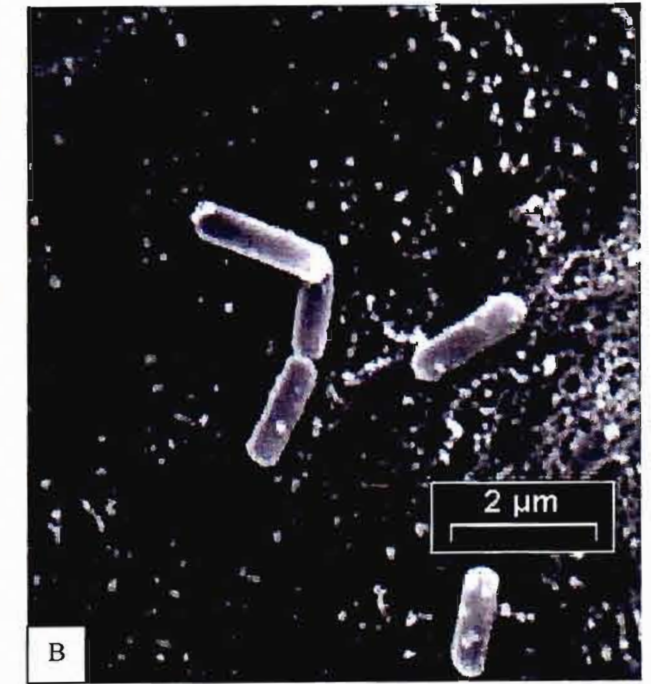
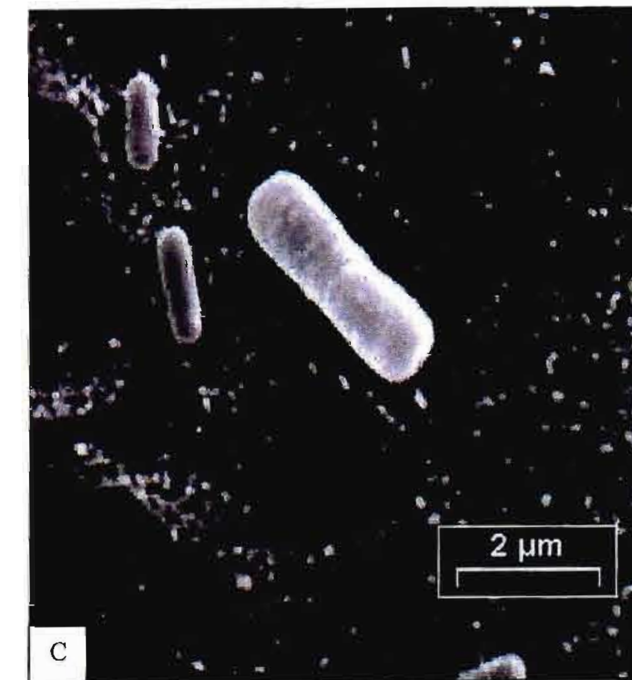


Plate 4.11 Scanning electron micrographs of Isolate N35

A = rod-shaped cells; B= angular arrangement of bacillary elements; C = enlarged cells (up to 4μm in diameter).







Carbohydrate utilization

On ISP medium 9, the isolate utilised L-arabinose, fructose, glucose, mannitol and D-xylose as carbon sources. Raffinose was poorly utilised, while very weak growth was observed in sucrose, rhamnose and inositol [Table 4.1]. Gas pockets were produced in the ISP medium containing sucrose as carbon source. No pigment was observed in any ISP media used.

Whole-cell hydrolysate analysis

Diaminopimelic acid (DAP) was not detected on paper [Plate5.2], cellulose and silica chromatograms. Galactose was present as the only diagnostic sugar. Thus, the culture can be considered to have cell wall chemotype VI based on the scheme of Lechevalier and Lechevalier (1970).

The morphological, physiological and chemical characteristics of the antimicrobially-active isolates investigated, are summarised in Table 4.2.

Table 4.2 Morphological, physiological and chemical characteristics of antimicrobially-active isolates

	Isolate Number							
	N2	**N8**	**N12**	**N16**	**N19**	**N30**	**N33**	**N35**
Physiological characteristics								
Gram reaction	+	+	variable	+	+	variable	nt	+
Catalase	+	+	–	+	+	–	–	+
Oxidase	–	–	+	–	+	+	+	+
Acid-fastness	–	–	–	–	–[a]	–	–	–
Spore production	+	+	+[b]	+	–	-	–	+
V-formation	–	–	–	–	+	+	–	–
Motility	-	-	-	–	-	–	-	-
Morphological characteristic								
Aerial mycelium	Pale white-Orange	Pale white-range	primary	primary	NA	NA	NA	NA
Substrate mycelium	YBR	YBR	–	YBR	extensive	–	–	–
Cells/spores Shape	oval spores	oval spores	rod shaped cells	oval spores	rod shaped cells [c]	drumsticks, hammer	rods	rod shaped cells
Spore surfaces	smooth	smooth	-	smooth[d]	–	–	–	–
Pellicle Formation	+	+	–	+	nt	nt	–	nt
Chemical characteristics								
Diaminopimelic acid (DAP) isomer	*meso*-DAP	L- or meso-DAP	meso-DAP	L - DAP	L - or meso-DAP	None[f]	L- / Meso-DAP	None
Diagnostic sugar	NC	NC	Ara +Gal	Ara +Gal	Ara +Gal	Gal	Ara +Gal	Gal
Cell wall chemotype	I , III	I, III	IV	I	VI sensu	VI	NA	VI
WCSP type	C	C	A	A sensu	A	NA	A	NA
Glycine	+	+ +	nt	+	+ + +	nt	+ +	nt
Melanin Pigment	nt	+	nt	+	nt	–	–	–

+ = positive; - = negative; nt = not tested ; NA = Not applicable; WCSP = Whole-cell sugar pattern of Lechevalier and Lechevalier (1965);[a] binds and retains fuschin; Ara = Arabinose ; Gal = Galactose ; NC = No Characteristic sugar;[c] appear as doublets with tapered ends; *YBR=Yellow-Brown-Red [color groups according Szabo and Marton (1964)]; [d] spores linked by threadlike structures; [f] doubtful.

Chapter 5

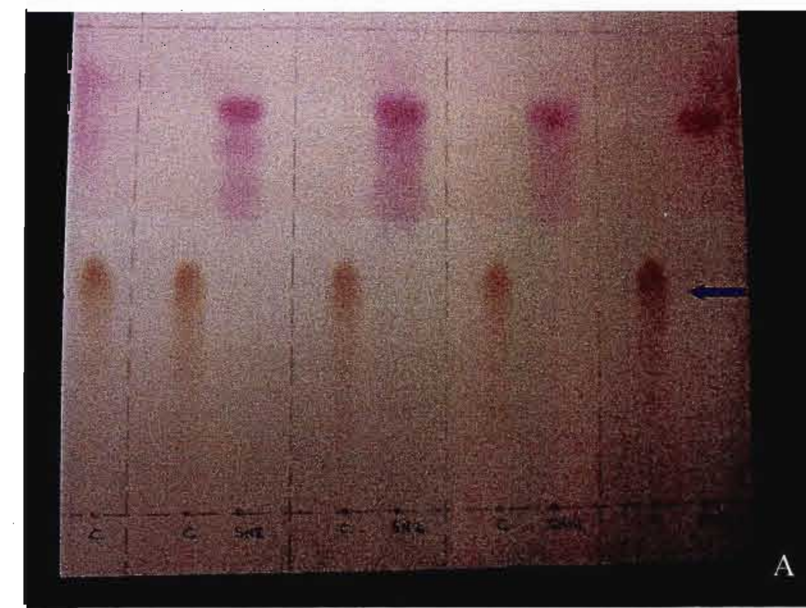
Identification of Antibiotic-Producing Microorganisms

II: TENTATIVE IDENTITY OF ISOLATES

5.1 Identity of Isolate N2

The production of spores borne on curled and irregularly branched hyphae [Plate4.1C], the cultural characteristics [Table 4.2], the presence of *meso*-DAP [Plate5.2], and absence of diagnostic sugars in the whole-cell hydrolysates (wall chemotype III) [Appendix4] place N2 possibly in the genus *Thermomonospora* or a related genus (Hasegawa *et al.*, 1978). When compared with descriptions of other genera, the isolate most closely resembled *Actinosynnema, Nocardiopsis and Streptoalloteichus.* All of these have a common wall chemotype III but are morphologically diverse. The genus *Actinosynnema* produces spores on aerial hyphae, and in some cases, substrate hyphae that fuse to form synnemata on the surface of the agar. Isolate N2 did not form synnemata and hence is unlikely to belong to this genus.

The genus *Nocardiopsis* has its origin in *Actinomadura* and was originally created to accommodate strains of the latter that lacked the characteristic whole-cell sugar, madurose (Meyer, 1976). Unlike Isolate N2, *Nocardiopsis* is characterized by the production of chains of arthrospores on the aerial mycelium that exhibit a tendency to fragment. In *Streptoalloteichus,* the spores originate from vesicles or sporangia on the substrate hyphae whereas in isolate N2, sporangia-like structures are lacking. Isolate N2 produced smooth single spores on aerial hyphae . The irregularly branched hyphae later fused together, bearing large numbers of spores in dense clusters [Plate4.1D]. Thus, based on its morphological, physiological and chemical similarities to *Thermomonospora* (McCarthy and Cross,1984), isolate N2 may be tentatively identified as a member of this genus. N2 was isolated from a decomposing chicken litter [Appendix2]. Members of the genus *Thermomonospora* have an important role in the primary degradation of organic material, and are one of the most active groups of lignocellulose-degrading procaryotes, the main reason they are mostly isolated from composts and overheated fodders [McCarthy, 1989].



1 2 3 4 5 6 7 8 9

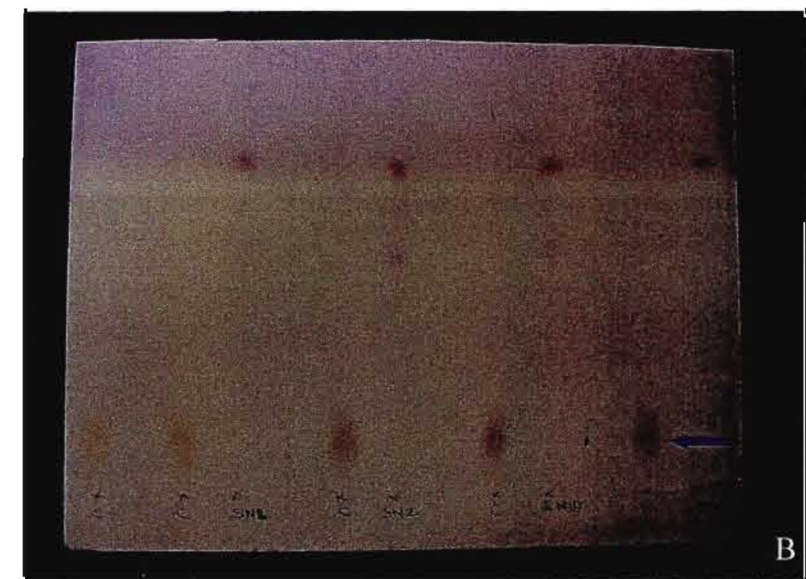


Plate 5.1 Thin-layer chromatograms for detection of diaminopimelic acid (DAP)

A = silica chromatogram (Harper and Davis solvent system I and II); B = cellulose chromatogram; Reading from right to left, nos 1-9 in both chromatograms (A and B)represent different isolates alternating with a standard DAP marker as control.

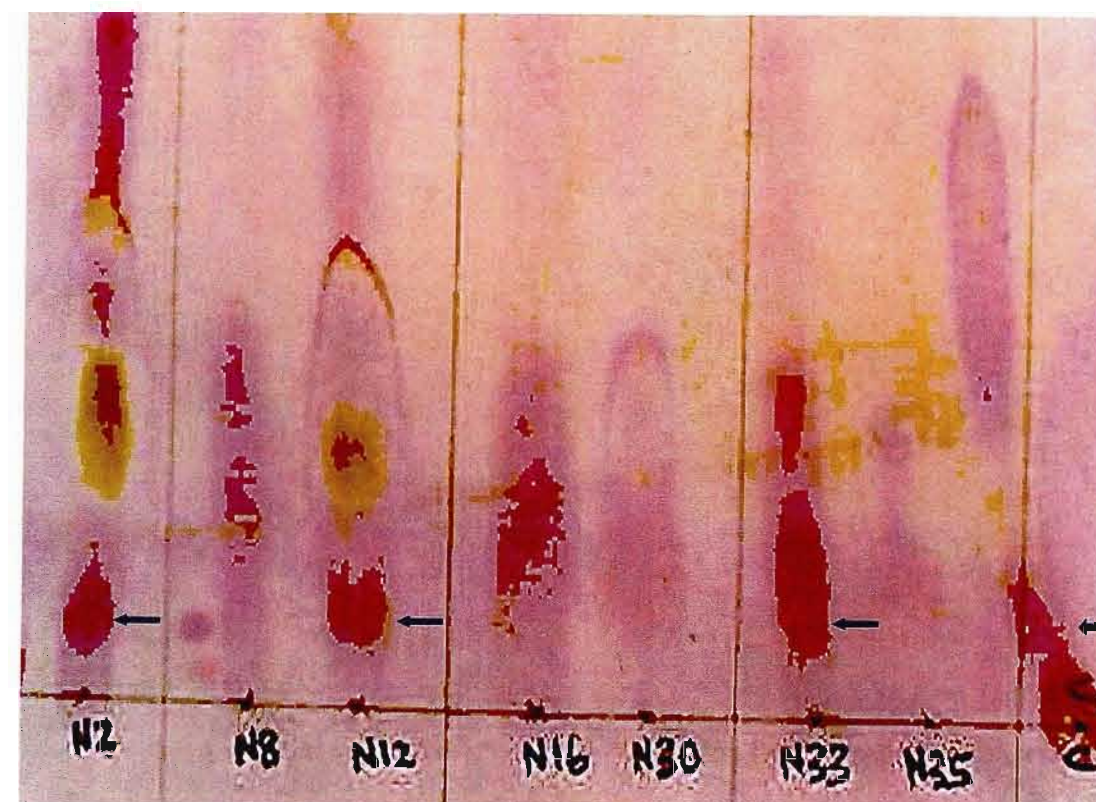


Plate 5.2 Detection of diaminopimelic acid (DAP) on paper chromatogram

Long arrows indicate *meso*-DAP (dark pink colour); short arrow indicate L-DAP (light pink); C=control (DAP (SIGMA)standard, mixture of LL-, DD-, and *meso*-DAP); No distinction is made between the *meso* and D forms of this acid.

5.2 Identity of Isolate N8

The absence of diagnostic sugars in whole-cell hydrolysates (wall chemotype III) [Appendix4], the cultural characteristics [Table 4.2] and the branched filaments that penetrate the agar [Plate4.2C] and later fuse together to form dome-like bodies [Plate4.2E] and synnemata [Plate4.2F&G], place isolate N8 possibly in the genus *Actinosynnema* or a related genus.

Comparisons with descriptions of other genera in Bergey's Manual of Systematic Bacteriology (Vol.4,1989) showed isolate N8 to closely resemble members of the genera *Sporichthya, Planomonospora, Planobispora, Spirillospora, Dermatophilus,* and *Geodermatophilus*. All members of the above, except *Sporichthya,* have a type III cell wall, but in contrast to isolate N8, they have a type B WCSP (Lechevalier and Lechevalier, (1965). Though *Geodermatophilus* species do have type C WCSP, similar to N8, the absence of spore bearing aerial mycelium indicates that isolate N8 does not belong to this genus. The presence of substrate mycelium [Plate4.2B] and synnemata [Plate4.2F&G], in isolate N8 and their absence in the genus *Sporichthya* presents evidence for non-inclusion of isolate N8 in this genus.

The genus *Actinosynnema* (Hasegawa *et al.*, 1978; 1983), apart from the formation of synnemata, also called coremia, possesses similar morphological, physiological and chemical properties to isolate N8. Thus, N8 may be identified possibly as an *Actinosynnema sp.*

5.3 Identity of Isolate N12

The production of a primary mycelium that fragments into bacillary or rod-shaped elements [Plate4.4F], the presence of major amounts of *meso*-DAP [Plate5.2], and the occurrence of L-arabinose, and galactose in the whole-cell hydrolysates (wall chemotype IV) [Appendix4] place isolate N12 possibly in the genus *Nocardia* or related genera. The above description is, however, also applicable to *Rhodococcus, Actinopolyspora, Bacterionema, Corynebacterium, Micropolyspora, Mycobacterium, Saccharomonospora, Saccharopolyspora* and *Pseudonocardia* (Gochnauer *et al.*, 1975; Goodfellow and Minnikin, 1977) and by the "*aurantica*" taxon, which contains organisms previously classified as *Gordona aurantiaca* (Goodfellow *et al.*, 1978). Unfortunately, lipid analysis, useful in distinguishing

representatives of *Nocardia* and *Rhodococcus* from actinomycetes that share cell wall chemotype IV, was not carried out. However, other morphological, physiological and chemical properties [Table 4.2] correlate closely with those in published descriptions of *Nocardia* and *Saccharopolyspora.* The disintegration of the mycelium into rod-shaped elements [Plate4.4D&F], together with the beadlike chains of spores [Plate4.4A], suggest *Saccharopolyspora* (Lacey and Goodfellow, 1975) is the more likely.

Thus, isolate N12 may be identified tentatively as *Saccharopolyspora* species. Isolate N12, designated as N12b [Appendix2], was isolated from cane sugar bagasse, the same natural habitat where the original isolate described by Lacey and Goodfellow (1975) was isolated.

5.4 Identity of Isolate N16

The presence of major amounts of L-DAP [Plate5.2], detection of glycine (cell wall chemotype I *sensu* Becker *et al.*, 1965)[Appendix6], the occurrence of arabinose and galactose in the whole-cell hydrolysates [Appendix4], together with other morphological, chemical and physiological properties [Table 4.2] place isolate N16 in the genus *Nocardiodes* or closely related genera (Prauser, 1976). When compared with descriptions of other genera, the isolate most closely resembled *Pimelobacter, Intrasporangium, Mycobacterium, Nocardia, Spirochthya, Arachnia, Streptomycetaceae, Rhodococcus, Oerskovia,* and *Promicromonospora.* However, unlike N16, *Pimelobacter*, *Spirochthya* and *Mycobacterium,* do not produce primary mycelium on agar media. Hence, the isolate is unlikely to belong to these genera. The primary mycelium of isolate N16 breaks up completely into smooth surface spore-like elements [Plate4.5E], not normally observed in *Mycobacterium* and *Streptomycetaceae.* *Nocardia* are partially acid-alcohol fast, whereas N16 was not and, hence, may be excluded from this genus. Most species of the genera, *Intrasporangium*, *Arachnia, Rhodococcus,* and *Oerskovia* do not produce aerial mycelium, which was observed in isolateN16 [Plate4.5D]. *Promicromonospora* has a cell wall chemotype VI, whereas, N16 has a chemotype I cell wall, and hence is unlikely to belong to this genus. The presence of a few to several layers of slimy extracellular material, with a pasty consistency, overlying the colonies [Plate4.5D], in isolate N16, point strongly to the genus *Nocardiodes* (Prauser, 1976).

Thus, based on its morphological, physiological and chemical similarities to *Nocardiodes* (Prauser,

1976), isolate N16 may be tentatively identified as a member of this genus.

5.5 Identity of N19

The presence of major amounts of glycine [Appendix6], the detection of L- or *meso*-DAP [Appendix5], the formation of an extensive mycelium [Plate4.6D], the angular arrangement of cells in Y or V formations [Plate4.6B], and other morphological and chemical properties [Table 4.2], place isolate N19 possibly in the genus *Corynebacterium* or related genera (Barksdale, 1970). When compared with published descriptions of various genera, isolate N19 most closely resembled genera in the aerobic nocardioform, *Mycobacterium* and coryneform groups (O'Donnell *et al.*, 1982; Colliins *et al.*, 1983; Suzuki and Komagata, 1983). Unfortunately, mycolic acid analysis necessary to separate these genera effectively was not carried out.

Most of the nocardioforms have a type IV cell wall composition, as do the coryneform bacteria (Lechevalier, 1981), whereas N19 has a chemotype IV *sensu* of Lechevalier and Lechevalier (1970). This, together with other morphological, physiological and chemical properties, points strongly to the genus *Corynebacterium* (Barksdale, 1970). Thus, isolate N19 was tentatively identified as a member of this genus.

5.6 Identity of N30

The presence of galactose and total absence of arabinose [Appendix4], doubt as to the absence of L- or *meso*-DAP [Plate 5.2], and the cultural and morphological characteristics displayed [Table 4.2] suggest N30 possibly belongs in the genus *Arthrobacter* or related genera. When compared with published descriptions of the genera within this group, isolate N30 most closely resembled the Cure and Keddie (1973) description of the coryneform group of bacteria. The organisms originally placed in this group, included, *Corynebacterium, Arthrobacter and Cellulomonas*, with *Brevibacterium* and *Microbacterium* as *genera incertae sedis* and 'tentatively' *Kurthia* (Keddie, 1978). Barksdale (1970) had earlier suggested that the word 'coryneform' be dropped because of the large, complex and broad morphological and chemical differences among members of this group (Keddie, 1978).

Although, N30 appeared to have a close phenotypic relationship [Plate4.7A,B&C] with *Arthrobacter*, doubt concerning the presence of meso- DAP, disallowed confident assignment to this genus. Additional chemotaxonomic tests are necessary before this can be done.

In *Bergey's Manual of Systematic Bacteriology* (Vol.4, 1989), the genus *Arthrobacter* was described as catalase positive whereas isolate N30 did not produce catalase. Keddie (1978) noted that *Arthrobacter tumescens* was wrongly described in the 7th edition of Bergey's Manual as catalase negative. To the present author's knowledge, the problem of catalase activity in taxonomic identification of *Arthrobacter* species has not yet been adequately addressed. Further investigations are necessary to clarify the situation.

5.7 Identity of N33

Although fragmentation into bacillary to coccoid elements (a property isolate N33 shares with *Nocardioform* actinomycetes) was observed [Plate4.9A&B], insufficient information was obtained to assign this organism to any specific genus. Additional chemotaxonomic tests are necessary before this can be done. This isolate was included in the investigation since it showed strong bactericidal activity against *Pseudomonas fluorescens* (Chapter 3) and to the author's knowledge, treatment of *Pseudomonas* infections still poses a problem in medicine.

5.8 Identity of N35

The absence of DAP in the whole-cell hydrolysate [Plate5.2], and galactose being the only diagnostic sugar (cell wall chemotype VI) present [Appendix6], and other physiological and morphological characteristics [Table 4.2], suggest isolate N35 possibly belongs in the genus *Promicromonospora* or related genera (Krasil'nikov *et al.*, 1961). Comparisons with published descriptions showed close resemblance of N35 to *Cellulomonas*, *Oerskovia* and the so-called non-motile organisms (NMOs) of Lechevalier and Lechevalier (1981), and also to *Nocardia cellulans*, *Brevibacterium fermentans*, and '*Corynebacterium manihot*'. The inability of N35 to form aerial hyphae, which are characteristic of the abovementioned organisms, warrants its exclusion from these genera. *Promicromonospora* is distinguished from all these genera by its inability to form aerial hyphae. Thus, based on its

morphological, physiological and chemical similarities to *Promicromonospora* (Krasil'nikov *et al.*, 1961), isolate N35 may be tentatively identified as a member of this genus.

Chapter 6

GENERAL DISCUSSION

The results of the screening program carried out in this study (Chapters 2, 3 , 4 and 5) have shown that KwaZulu-Natal soils do harbour microorganisms (both coryneform and non-streptomycetous actinomycetes), capable of producing antibiotics against some major plant and human pathogens. The numbers and types of actinomycetes present in a particular soil are strongly influenced by geographical location, soil temperature, soil type, soil pH, organic matter content, cultivation practices, aeration and moisture content (Williams *et al.*, 1971; 1972). All these factors were taken into consideration during sample collection.

Some of the isolation media used, were found to be selective for actinomycetes. For example, Winogradsky's Nitrite medium (Winogradsky, 1949), M3 medium (Rowbotham and Cross, 1977), and Kosmachev's medium (Kosmachev, 1960) were selective without heat pretreatment and/or antibiotic incorporation, although a few colonies of other bacteria did occasionally develop on the plates. Although no direct comparisons of the media used in this investigation were made, some differences between them were noted. Modified Czapek's agar (Higgins and Lechevalier,1969) appeared to enhance pigment production, or to select for pigment-producing strains of actinomycetes, most probably *Nocardioform* group. MGA (Nonomura and Ohara, 1971) supported growth of mainly filamentous organisms, possibly actinomycetes with aerial hyphae, such as *Actinomadura* as reported by Athalye and Lacey (1981). Of all the media used, Kosmachev's medium (Kosmachev, 1960) appeared to be the most selective, as most of the plates contained virtually pure cultures of actinomycetes. This medium has been reported to be selective for *Excellospora* and related genera (Kosmachev, 1960). Some doubt has been expressed regarding the selectivity of some of the other so-called selective media used in this study, as portrayed in literature (Goodfellow and Minnikin, 1981; Rowbotham and Cross, 1977).

Heat pretreatment of the samples was beneficial as it eliminated most of the unwanted Gram-negative bacteria that produce mucoid, spreading colonies on soil dilution plates (Williams and Wellington, 1982), thereby facilitating the isolation of actinomycetous organisms. It is conceded that such treatment may have concomitantly eliminated other heat sensitive producers of novel antibiotics. A 10^{-4} dilution

was found to be optional for isolation as it resulted in reasonably well separated individual colonies of a variety of organisms. Antibiotic incorporation into the media, together with heat pretreatment, eliminated most of the unwanted fungal and yeast growth. The present study did not target fungi and yeasts as part of the screening programme. Non-streptomycetous actinomycetes, coryneform bacteria and other related organisms were the main targets. *Streptomyces* species were also disregarded mainly because the large number of such organisms would have increased the scope of the investigation beyond the capacity of the facilities available, and many of the isolates would be found to produce already well documented antibiotics.

The simplest and least time consuming screening methods (modified cross-streak and agar-plug) were used. The methods employed were suitable for the kind of routine, high-rate screening programme (approximately 63,000 isolation plates used) embarked on in this study, and produced acceptably reproducible results. The highest percentage of antimicrobially-active isolates came from the SAPPI forest soil, while the lowest percentage came from the banks of the Mzinyathi river. Forest soils usually have a relatively low pH and contain a predominance of actinomycetes that are tolerant of acid conditions (Davies and Williams, 1970; Williams *et al.*, 1971). Actinomycete populations are usually small in water-logged soils with low oxygen tension (Labeda, 1990), and the nature of the isolation media used may not have been conducive to antibiotic production by the riparian microbiota.

Many of the other samples screened, such as, compost soil, cow and chicken manure, and soil sugar-cane fields, were rich in organic matter and contained a large variety of actinomycetes. The dry, uncultivated soil sample contained fewer actinomycete isolates than the other samples examined. However, arid soils usually have an alkaline pH and have been reported to contain more of the so-called 'rare' actinomycetes (Labeda, 1990). The results of a study on the distribution of actinomycetes in South African soils were recently reported by Kizuka *et al.* (1997). Apart from this report, current quantitative data on the geographical and ecological distribution of actinomycetes appears to be scarce in the general scientific literature (Goodfellow and Williams, 1983; Williams and Vickers, 1986).

Antibiotic production by organisms is a complex process and assessment thereof consideration of many interacting factors. The mere observation of an apparent inhibition zone on agar plates may be deceptive at times. For example, the complete decolouration of *Serratia marcesens* observed on plates

inoculated with isolate N 48 and incubated at 30°C was thought to indicate an inhibitory effect. However, on further incubation at a lower temperature (20°C), pigment production was fully restored, indicating that no antibiotic action had in fact occurred. In some instances the growth of the test organism was delayed in/on the media used, with the result that some of the early, seemingly positive, results were found to be negative after longer incubation.

The use of Iso-SensiTest Agar (ISTA) [Oxoid] is recommended for primary screening as the zone edges of the isolate/test organism were better defined. Some isolates tended to spread profusely on Mueller Hinton medium and, sometimes, on nutrient agar making streaking and reading of the plates very difficult. This reiterates the importance of the culture medium, in any screening programme. Other factors that need to considered are the test organisms, the inoculum size, pH and incubation conditions, oxygen availability, which must be standardized if one wishes to have data that is reproducible and comparable under standard laboratory conditions.

No attempt was made in this study to isolate or purify the antibiotic substance(s) produced. This is a complicated task, constituting a project in itself. Time and economic constraints were also considered. Hopefully, future attempts to isolate and purify the antibiotics produced, especially by isolate N8, will prove to be worthwhile.

The actinomycetes are a large, heterogenous and complex group of microorganisms. Their identification requires detailed morphological, physiological and biochemical investigation. An attempt was made in this study to identify the isolated antibiotic producers to the species level, using as many as possible of the methods described in literature. Despite this the amount of data obtained (Chapter 4), was not sufficient to allow incontrovertible identification of the species. To simultaneously identify several potential antibiotic producers to species level proved very difficult, mainly due to time, and financial constraints and lack of facilities. This was not too serious however since detailed identification of antibiotic-producing organisms is only required when a patent application is sought (Dietz, 1999).

Much time was spent on standardising the numerous tests and methodologies used in the investigation. This was felt to be necessary since an examination of both the 8th and 9th edition of Bergey's Manual revealed that little standardization of the many methods used in the identification of actinomycetes has

been achieved.

Separation of diaminopimelic acid isomers (L- or *meso*-DAP) during chemical analysis of the whole cell hydrolysates was successfully carried out in most instances, although dubious results were obtained. Sometimes different results were obtained depending on whether paper, silica or cellulose thin layer chromatography plates were used. Other problems were also encountered with this test. Firstly, the DAP (SIGMA) standards obtained from the manufacturers failed to separate in the prescribed solvent system. Secondly, migration of the sample spots sometimes failed in the recommended solvents. This led to the author having to conduct extensive trials on many solvent systems and combinations of plates and paper before acceptable results were obtained. Two dimensional thin layer chromatography based on the method of Harper and Davis (1979) proved helpful in some cases where separation could not be obtained using the conventional one-dimensional paper chromatography technique. However, it should be recommended only as a second option, i.e. where one-way paper chromatography does not yield satisfactory results, since it is time consuming and expensive, especially when large numbers of samples are to be analysed.

The techniques employed in the present investigation yielded reproducible results and were thus considered reliable. Isolate N8 should be further investigated since it showed strong inhibitory action against all the test organisms used, including Gram positive and Gram negative bacteria and Eukaryotes. Further testing of the spectrum of the antibiotic produced by isolate N8 should be undertaken in clinical trials, perhaps by a multinational pharmaceutical company, to determine its efficacy against the many human and animal pathogenic microorganisms that have developed resistance to the currently widely used antibiotics. The substance might prove to very useful if it could be shown to have broad antifungal activity. The activity of the compound should also be tested against a range of phytopathogenic bacteria and fungi since a major issue at present is the development of biocontrol agents to replace chemical treatments. Whether or not this substance is novel, only the future will tell.

APPENDICES

Appendix 1

Media Composition and Preparation:

Distilled water was used for all media preparation. Except where contra-indicated, all media were autoclaved at 121°C for 15 minutes before use.

1.1 **Czapek's medium** (Cross and Attwell, 1974., referred to by Cross, 1981). (g/l): Sucrose, 30.0 ; $NaNO_2$, 2.0; $MgSO_4.7H_20$, 0.5; KCl, 0.5; $FeSO_4.7H_2O$, 0.01; KH_2PO_4, 1.0; Yeast extract, 2.0; Vitamin-free casamino acids, 6.0; Agar, 13.0.

1.2 **Kosmachev's medium** (Kosmachev, 1960) (g/l): KNO_3, 1.0; $(NH_4)_2SO_4$, 1.0; $Na_2HPO_4.12H_2O$, 1.0; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.01; $CaCO_3$, 4.0; Yeast extract, 3.0; Agar, 15.0.

1.3 **M3 Agar** (Rowbotham and Cross, 1977). (g/l): KH_2PO_4, 0.466; $Na_2HPO_4.12H_2O$, 0.732; KNO_3, 0.10; NaCl, 0.29; $MgSO_4.7H_2O$, 0.10; $CaCO_3$, 0.02; Sodium propionate, 0.20; $FeSO_4.H_2O$, 200µg; $ZnSO_4.7H_2O$, 180µg; $MnSO_4.7H_2O$, 20µg; Agar, 18.0; Cycloheximide and Thiamine-HCl, 4.0 mg each; pH 7.0. Cycloheximide and thiamine-HCl were dissolved in 70% (v/v) alcohol, filter- sterilised and added to the autoclaved and cooled medium.

1.4 **MGA** (Nonomura and Ohara, 1971). (g/l): Glucose, 2.0; L-asparagine, 1.0; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.5; Trace salts solution, 1.0ml; Agar, 20.0. Trace salts solution consisted of mg/ml: $FeSO_4.7H_2O$, 10.0; $MnSO_4.7H_2O$, 1.0; $CuSO_4.5H_2O$, 1.0; $ZnSO_4.7H_2O$, 1.0. Sufficient polymixin B and oxyteracycline were dissolved in 70% alcohol, filter-sterilised and added to the medium to give a final concentration of 10µg/ml and 12µg/ml respectively.

1.5 **Modified Czapek's Agar** (Higgins and Lechevalier ,1969). (g/l): $NaNO_3$, 2.0; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4$, 0.01; Sucrose, 30.0; Yeast extract, 2.0; Agar, 15.0; pH 7.2.

1.6 **Ringer's solution** (¼ strength) (g/l): NaCl, 2.25; KCl, 0.105; $CaCl_2$, 0.06; $NaHCO_3$, 0.05; gelatin, 0.5.

1.7 Trace salts solution (Shirling and Gottlieb, 1966) (g/l): $FeSO_4.7H_2O$, 0.1; $MnCl_2.4H_2O$, 0.1; $ZnSO_4.7H_2O$, 0.1.

1.8 Bacto Tryptic Soy Agar (Difco). This medium was prepared as per the manufacture's instructions. Gentamicin sulphate was added to the autoclaved and cooled medium to give a final concentration of 50μg/ml.

1.9 Winogradsky's nitrite medium (Winogradsky, 1949). (g/l): $NaNO_2$, 2.0; Anhydrous Na_2CO_3, 1.0; KH_2PO_4, 0.5; Agar, 15.

1.10 Yeast extract agar [ISP medium 2] (Pridham *et al.*, 1956-1957). (g/l): Yeast extract, 4.0; Malt extract, 10.0; Glucose, 4.0; Agar, 15.0; pH 7.0.

International Streptomyces Project (ISP) media used in these investigations (Shirling and Gottlieb, 1966)

ISP medium 1 : Tryptone- yeast extract broth (g/l) Bacto-tryptone (Difco), 5.0; Bacto-yeast extract (Difco), 3.0; pH 7.0 - 7.2.

ISP medium 2 : Yeast extract agar (see 1.10 above)

ISP medium 3 : Oatmeal agar (Shirling and Gottlieb, 1966). (g/l) :

Oatmeal, 20.0; Agar, 18.0; Trace salts solution, 1 ml. 1L of distilled water was poured into a 2 L conical flask, and 20.0g of pinhead oatmeal (Tiger Oats Co.,S.A) were added and boiled for 20 minutes. The medium was then filtered through cheese cloth. The volume of filtrate was restored to 1L by addition of de-ionized water. 1.0 ml trace salts solution was then added and the pH adjusted to 7.2. 18g agar were added and heated until completely dissolved. The complete medium was then dispensed into 500ml flasks in 250ml quantities and autoclaved at 121°C for 15 minutes. Gentamicin sulphate was added to the cooled medium to give a final concentration of 50μg/ml.

ISP medium 4: Inorganic salts-starch agar

Solution 1: 10.0g of soluble starch was made to a paste, and then brought to 500ml with distilled water.

Solution II : (g/500ml): K_2HPO_4 (anhydrous basis), 1.0; $MgSO_4.7H_2O$, 1.0; NaCl, 1.0; $(NH4)_2SO4$, 2.0; CaCO3, 2.0; trace salts solution, 1.0ml.

Solutions I and II were mixed and 18g agar added and the medium boiled until the agar dissolved, the medium was then autoclaved at 121°C for 15 minutes.

ISP medium 5: Glycerol-asparagine agar

(g/l): L-asparagine (anhydrous basis), 1.0; glycerol, 10.0; K_2HPO_4 (anhydrous basis), 1.0; trace salts solution, 1.0ml; agar, 18.

ISP medium 6: peptone - yeast extract iron agar

(g/l) : Bacto-peptone, 15; protease peptone, 5.0; Ferric ammonium citrate, 0.5; dipotassium phosphate, 1.0; sodium thiosulphate, 0.08; yeast extract , 1.0; agar, 15.

ISP medium 7: tyrosine agar

(g/l): Glycerol, 15.0; L-tyrosine, 0.5; L-asparagine, 1.0; K_2HPO_4 (anhydrous), 0.5; $MgSO_4.7H_2O$, 0.5; NaCl, 0.5; $FeSO_4.7H_2O$, 0.01; trace salts solution, 1.0ml; agar, 18.

ISP medium 9: carbon utilization medium

Carbon sources were added as filter sterilized 10% solutions (w/v) in basal mineral salts agar to give a final concentration of 1% (10ml of a 10% solution to 100ml basal medium).

D-glucose was added as a positive control, whereas the basal mineral salts agar without any carbon source , served as a negative control.

A. Sterile carbon sources

L- arabinose	D- fructose
Sucrose	Rhamnose
D-xylose	Glucose
I - inositol	Raffinose
D- mannitol	Cellulose

B. Pridham and Gottlieb trace salts

(g/ 100ml): $CuSO_4.5H_2O$, 0.64; $FeSO_4.7H_2O$, 0.11; $MnCl_2.4H_2O$, 0.79; $ZnSO_4.7H_2O$, 0.15.

C. Basal mineral salts agar

(g/L): $(NH4)_2SO4$, 2.64; KH_2PO_4 anhydrous, 2.38; $K_2HPO_4.3H_2O$, 5.65; $MgSO_4.7H_2O$, 1.0; Pridham and Gottlieb trace salts(9B), 1.0ml. Adjust pH to 6.8-7.0; Agar, 15.0

D. Complete medium

Autoclaved basal medium (C) was cooled to 60°C and then the sterile carbon sources (A) added separately, to give a final concentration of approximately 1%. The mixture was agitated and 25ml medium transferred into 9cm petri dishes.

Appendix 2

Antimicrobial-activity of selected isolates against test organisms on primary screening medium, NA

		Test organisms						
Isolate number	**sample source**	**❶ Sa**	**❷ Ec**	**❸ Pf**	**❹ Sm**	**❺ Sf**	**❻ Cu**	**❼ Xc**
N1	CM	–	–	–	+	–	δ+	–
N2*	CL	δ+	++	++	++	–	++	++
N3*	CM	+++	++	+++	–	–	nt	–
N4a*	SW	++	–	+++	++	-	nt	-
N4b*	SW	-	+++	++	–	–	–	–
N5	"	+	–	nt	–	δ+	–	–
N7	"	nt	nt	–	–	–	+	+
N8	"	++	+++	++	+++	++++	++++	+++
N12a	CL	–	δ+	–	–	–	+	–
N13	"	+++	Rs	–	–	–	–	–
N14	"	+	δ+	–	++	–	–	–
N15	"	++Rs	++	–	δ+	–	++++	–
N16a*	"	+++Rs	+Rs	++	–	δ+	δ+	–
N17a	"	+	++++	–	–	–	–	–
N20	COW	–	+	–	–	–	–	–
N22	CL	+++	+++Rs	–	–	–	–	–
N23	"	–	+	δ+	–	–	Rs	–
N24	"	+	–	–	δ+	–	–	–
N25a*	"	δ+	++++rs	–	–	–	++++	–
N26	"	δ+	–	–	–	–	+	–
N27	COW	+	+	–	δ+	–	+	–
N28a	"	+	–	–	–	–	+	–
N30a	CL	+	–	–	–	–	+	–
N37	CL	+Rs	–	–	–	–	+	–
N41	"	++Rs	++++	–	–	–	+	–
N45	CS	+++	δ+	–	–	–	δ+	–

N48	"	+ +	+ + + +	–	–dis	–	+ + +	+ +
N17b	US	+	+ + + +	–	–	–	–	–
N16b	"	+Rs	–	–	–	–	+ + + +	–
N19a	"	+Rs	+ +	–	–	–	–	–
N10	"	–	+ +	–	–	–	–	–
N6*	CCS	+	+ + +	–	+	+	–	+
N4c	SF(NH)	+	–	–	–	–	–	–
N12b*	CSF(NH+A)	+ +	+ +	+ +	δ+	–	+	+ +
N17c	SF(NH)	+	+ +	–	-	–	–	–
N19b*	SF(NH+A	+ + +	+ +	+ + +	-	+	+ +	+ +
N25b	SF(NH)	+ +	–	+Rs	-	–	–	+
N28b	"	–	+	–	-	–	–	–
N30b*	CSF(H)	+	–	+ + +Rs	-	–	–	–
N31	"	+	–	–	-	–	–	Rs
N33	SF(NH)	+	+ +	+ + +	-	–	–	nt
N34	CSF(H)	+	–	+ +	-	–	nt	–
N35	SF(NH)	+ +	+	–	-	–	–	+ + +
N36	"	+	–	–	-	–	–	+ +
N38	SF(H)	–	–	–	δ+	–	–	+ +

+ positive; --negative; nt = not tested; δ+ =partially positive Rs = Resistant strains; SF = sappi forest; CSF = cane sugar field; CM = chicken manure; CL = chicken litter; CS = compost soil; CCS = creosate contaminated soil; COW = cow manure; SW = chicken manure from sweet waters; US = uncultivated soil; A = antibiotics added; NA = no antibiotics added; H = heated; NH = non-heated. Sa = *Staphylococcus aureus*; Ec = *Escherichia coli*; Pf = *Pseudomonas fluorescens*; Sm = *Serratia marcescens*; Sf = *Streptococcus faecalis*; Cu = *Candida utilis*; Xc = *Xanthomonas campestris pv campestris*

Appendix 3

Average diameters of inhibition zones produced by selected active isolates against test organisms on primary screening medium, NA

Test organisms/ Inhibition zones (mm)

Isolate number	sample source	❶ Sa	❷ Ec	❸ Pf	❹ Sm	❺ Sf	❻ Cu	❼ Xc
N1	CM	-	-	-	5	-	2	-
N2*	CL	13	10	20	21	-	20	15
N3*	CM	25	20	25	-	-	nt	-
N4a*	SW	15	-	30	20	-	nt	-
N4b*	SW	-	30	15	-	-	-	-
N5	"	5	-	nt	-	2	-	-
N7	"	nt	nt	-	-	-	6	nt
N8*	"	20	25	20	23	22	27	24
N12a	CL	-	3	-	-	-	5	-
N13	"	30	-	-	-	-	-	-
N14	"	9	5	-	15	-	-	-
N15*	"	20	21	-	2	-	30	-
N16a*	"	25	5	14	-	-	Rs	-
N17a	"	2	26	-	-	-	-	-
N20	COW	-	8	-	-	-	-	-
N22	CL	25	30	-	-	-	-	-
N23	"	-	7	3	-	-	-	4
N24	"	5	-	-	3	-	-	-
N25a*	"	2	30	-	-	-	30	-
N26	"	3	-	-	-	-	5	-
N27	COW	5	6	-	3	-	14	-
N28a	"	7	-	-	-	-	5	-
N30a	CL	6	-	-	-	-	5	-
N37	CL	5	-	-	-	-	4	-
N41	"	20	30	-	-	-	2	-

N45	CS	24	2	-	-	-	2	-
N48*	"	18	30	-	-dis	-	21	19
N17b	US	2	26	-	-	-	-	-
N16b	"	4Rs	-	-	-	-	26	-
N19a	"	5	19	-	-	-	-	-
N10	"	-	20	–	-	-	–	-
N6	CCS	11	25	-	3	3	-	3
N4c	SF(NH)	5	-	-	-	-	-	-
N12b*	CSF(NH+A)	3	7	8	-	-	-	5
N17c	SF(NH)	3	10	-	-	-	-	-
N19b*	SF(NH+A	3	4	10	-	-	-	5
N25b	SF(NH)	5	-	3	-	-	–	3
N28b	"	-	5	-	-	-	-	-
N30b*	CSF(H)	5	-	20	-	-	-	-
N31	"	6	-	-	-	-	-	-
N33*	SF(NH)	-	3	10	-	-	-	-
N34	CSF(H)	3	-	5	-	-	nt	-
N35*	SF(NH)	-	3	-	-	-	-	5
N36	"	5	-	-	-	-	-	5
N38*	SF(H)	-	-	-	4	-	-	6

* isolates selected for secondary screening; nt = not tested; Rs = Resistant strains; Sa = *Staphylococcus aureus*; Ec = *Escherichia coli*; Pf = *Pseudomonas fluorescens*; Sm = *Serratia marcescens*; Sf = *Streptococcus faecalis*; Cu = *Candida utilis*; Xc = *Xanthomonas campestris pv campestris*; CL = Chicken Litter; CM = Chicken Manure; SW = chicken manure from Sweet Waters; CS = Compost Soil; COW = cow manure; US = Uncultivated Soil; CCS = Creosate Contaminated Soil; CSF = Cane-sugar field; SF = SAPPI forest; NH=Non-Heated; H=Heated; A= Antibiotics added.

Appendix 4

R_f values of Diagnostic sugars in whole-cell hydrolysates of isolates in Solvent I(Isopropanol)* separated by Thin Layer Chromatography

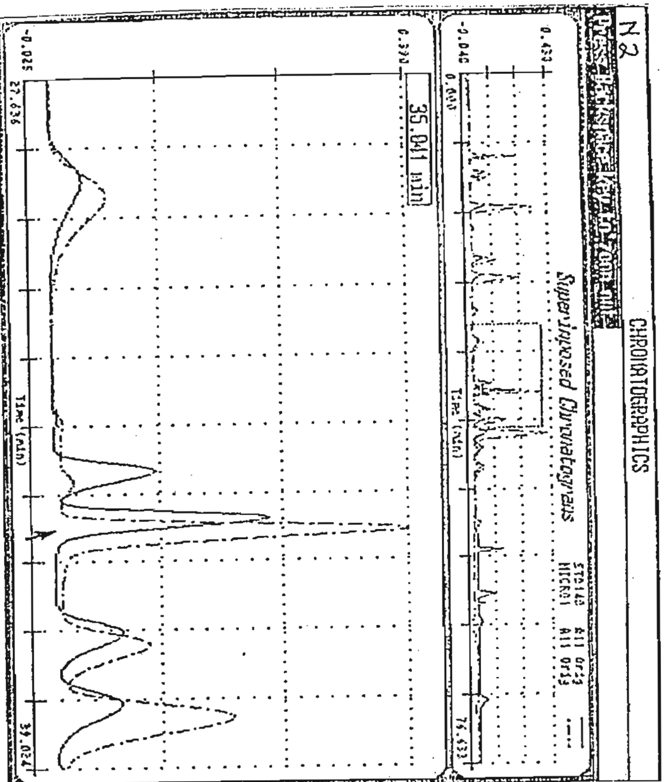
Isolate	Sugar standard	Rf (standard)	Rf (Isolate)	Main Diagnostic sugar
-	Arabinose	30	-	-
-	Fucose	47	-	-
-	Galactose	26	-	-
-	Rhamnose	49	-	-
-	Xylose	36	-	-
N2	-	-	NA	None
N8	-	-	NA	None
N12	-	-	26, 31	Arabinose, Galactose
N16	-	-	27, 29	Arabinose, Galactose
N19	-	-	27, 30	Arabinose, Galactose
N30	-	-	27	Galactose
N33	-	-	26, 30	Arabinose, Galactose
N35	-	-	26	Galactose

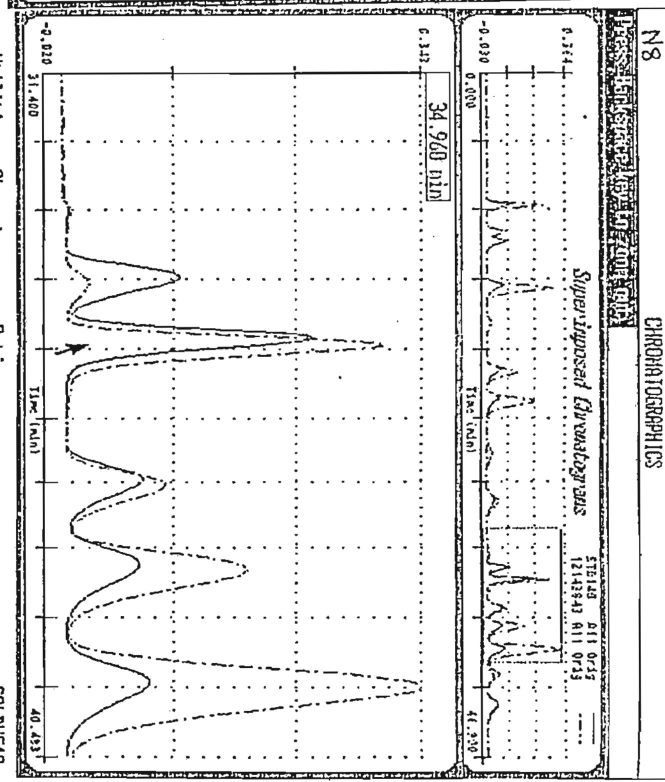
* Solvent system of Harper and Davis (1979); NA = Not applicable

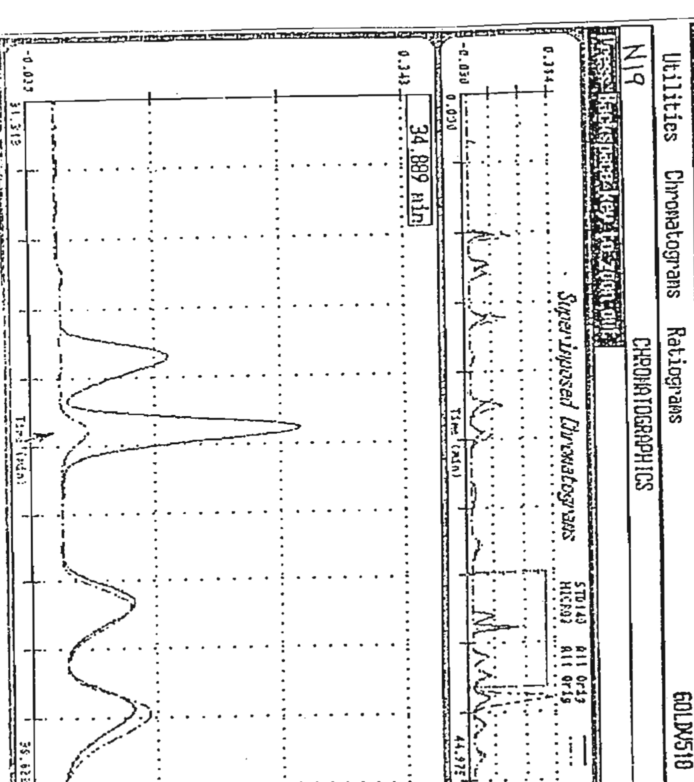
Appendix 5

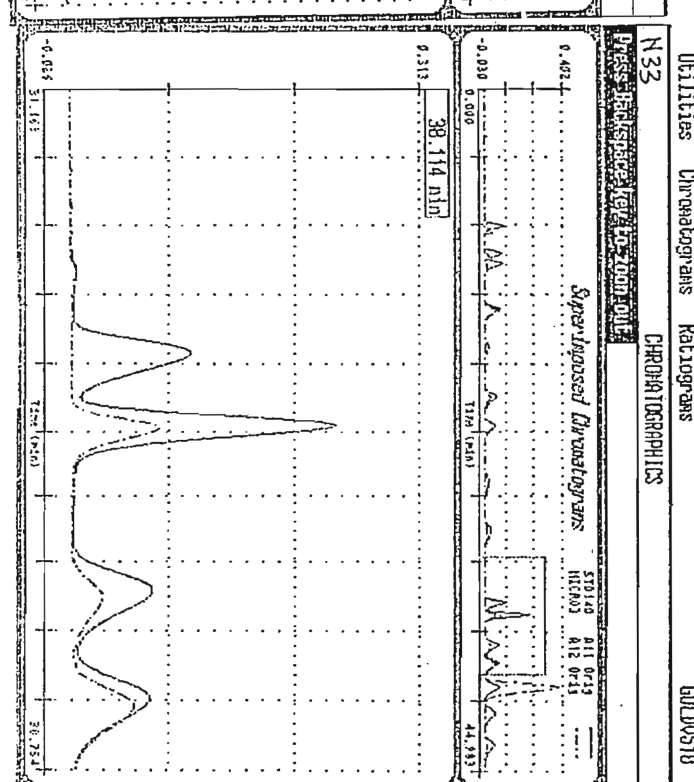
Superimposed chromatograms of whole-cell hydrolysates* from selected antimicrobially-active isolates

Arrows indicate superimposed diaminopimelic acid (DAP) peaks of sample and control (standard); *as determined by Beckman 6300 amino acid analyser









Appendix 6

Percentage amino acid concentration by weight of whole-cell hydrolysates* from selected antimicrobially-active isolates.

	% sample by weight (10mg) per isolate				
Amino acid	**N2**	**N8**	**N16**	**N19**	**N33**
Aspartic	5.710	6.533	4.091	15.652	7.334
Threonine	2.290	2.560	1.538	10.227	2.747
Serine	1.423	1.524	0.912	4.880	1.715
Glutamic	11.693	11.099	6.934	20.007	13.682
Proline	1.377	1.598	0.000	7.374	1.944
Glycine	2.739	3.114	1.781	11.240	3.289
Alanine	7.742	7.079	4.403	11.473	8.491
Valine	3.585	3.613	2.404	10.669	5.063
Methionine	0.323	0.530	0.000	0.000	0.000
Diaminopimelic	8.355	5.657	1.475	2.092	8.592
Isoleucine	3.094	3.291	2.140	8.683	4.846
Leucine	6.346	6.255	4.128	11.084	9.926
Tyrosine	1.178	1.710	0.000	2.329	0.175
Phenylalanine	2.753	3.215	1.998	8.604	2.979

* As determined by Beckman 6300 amino acid analyzer. All the whole-cell preparations contain major amounts of alanine and glutamic acid.

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As per standard protocol of Hancock's Doubling Dilution Method for determination of MIC using the micro titre plate. website: www.interchg.ubc.ca/bobh/methods.html.

As per SRGA (Swedish Reference Group for Antibiotics and its sub committee of methodology) Method for MIC determination. website: www.SRGA.

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