ISOLATION AND CHARACTERIZATION OF LOCAL STRAINS OF STREPTOMYCES FROM THE SOIL AND SCREENING FOR POSSIBLE ANTIBIOTIC ACTIVITY

BY

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DECLARATION

I, hereby solemnly declare that the work reported in this thesis was carried out by me in the Department of Microbiology, Faculty of Science, Ahmadu Bello University, Zaria under the supervision of Drs. J.B. Ameh and L.E. Odama (Mrs).

The work of other investigators in this area is duly acknowledged and referred to appropriately. No part of this thesis has previously been submitted for a degree or diploma.

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CERTIFICATION

This thesis titled: Isolation and Characterization of local strains of Streptomyces from the soil and screening for possible antibiotic activity by Ososanya Adebimpe D. meets the regulations governing the award of degree of Master of Science of Ahmadu Bello University, Zaria and is approved for its contribution to scientific knowledge and literary presentation.

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DEDICATION

This work is dedicated to my late father, Chief P.A. Ososanya for my educational development and to the Holy Spirit for being my senior partner throughout the working period.

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ABSTRACT

Fifteen soil samples obtained from the garden, refuse dump and grassland in Ahmadu Bello University, Zaria, were screened for Streptomyces spp. On the whole, 62 isolates of Streptomyces spp were obtained. Characterization of these isolates using the "ISP Criteria" gave 28 species of Streptomyces. 3.2% of the total isolates could not be characterized. Morphological and physiological characterization of the isolates based on the "ISP Criteria" showed that, the main coloration found in all the isolates were gray, yellow and white with the occurrence of 86.7%, 8.3% and 5.0%, respectively. The spore chain morphology was rectus-flexibilis (RF) for 22 isolates (36.7%) and spira (S) for 38 isolates (63.3%). All the isolates had smooth spore surfaces. Only 23.3% of the isolates were chromogenic. 76.7% of the isolates did not show any chromogenic reaction on any of the three test media. 70% of the isolates produced hydrogen sulphide, from either Peptone Iron Agar or Lead Acetate Agar. The carbon utilization test revealed that the isolates can utilize between 5-11 carbon sources. Based on the "ISP Criteria", 8 groups of organisms were obtained. The percentage of occurence of Group I to VIII are 6.4%, 24.2%, 16.1%, 37.0%, 1.6%, 4.8%, 1.6% and 4.8% respectively. Group IV had the highest percentage of occurrence. 32.3% of the total isolates were found to have antibiotic activity made up of 16.1% of isolates have antibacterial activity, 9.7% antifungal activity and 6.5% broad spectrum activity.

TABLE OF CONTENTS

		PAGE
Title Page		i
Declaration		ii
Certification		iii
Dedication		iv
Acknowledgement		v
Abstracts		viii
Table of Contents		ix
List of Tables		xii
List of Figures		xiii
List of Plates		xiii
CHAPTER ONE -	INTRODUCTION	1
CHAPTER TWO -	REVIEW OF LITERATURE	
2.1	The Actinomycetes	3
2.1.1	Historical Background	3
2.1.1.1	Classification	4
2.1.2	Taxonomy and Classification of the Actinomycetes	4
2.1.2.1	Classification	4
2.1.3	Actinomycetes as boundary micro-organisms	5
2.1.4	General Characteristics	6
2.1.4.1	Growth Characteristics	7
2.1.5	Habitat	8
2.1.6	Pathogenicity	8
2.1.7	Economic importance of Actinomycetes	9
2.2.0	Genus Streptomyces	10
2.2.1	General Characteristics	11
2.2.1.1	Growth Characteristics	11
2.2.2	Diagnostic Criteria for Streptomyces	12
2.2.2.1	Spore-Chain Arrangement	13
2.2.2.2	Spore Surface Ornamentation	13
2.2.2.3	Mass Colour of Aerial Mycelium	16
2.2.2.4	Production of Melanoid Pigments (Chromogenicity)	16
2.2.2.5	Vegetative Mycelium Pigments and Soluble Pigments	17
2.2.2.6	Carbon Utilization	17
2.3	The Antibiotic Concept	18
2.3.1	Antibiotic: A Metabolic Product	18
2.3.2	Antibiotic-Activity of Streptomyces	19
2.3.3	Antibiotic Production by Streptomyces	20

		PAGE
2.3.4	Types of Antibiotics produced by Streptomyces	20
2.3.4.1	Macrolide Antibiotic	20
2.3.4.2	Aminoglycoside Antibiotics	21
2.3.4.3	ß - Lactam Compounds	22
2.3.4.4	Polyene Antibiotics	22
2.3.4.5	Antitumour Agents	23
2.3.5	Sensitivity of Streptomyces to Antibiotics Produced by them	23
CHAPTER THREE -	MATERIALS AND METHOD	
3.1	Materials	25
3.1.1	Bacteriological Media used for Isolation	25
3.1.2	Materials used for Microscope Observation of the Isolates	25
3.1.3	Bacteriological Media used for Characterization	26
3.1.4	Chemical Reagents used for Characterization	26
3.1.5	Colour Standard used for Colour Determination	27
3.1.6	Carbon Sources used for Carbon Utilization Test	28
3.1.7	Materials used for Screening Possible Antibiotic Activity	28
3.1.8	Assay Organisms used for Screening possible Antibiotic Activity of the Isolates	29
3.2	Methods	30
3.2.1	Screening for Streptomyces Species	30
3.2.1.1	Sample Collection	30
3.2.1.2	Preparation of Medium	30
3.2.1.3	Preparation of Soil Inoculum	30
3.2.1.4	Culture and Isolation of Streptomyces spp	30
3.2.2	Characterization	31
3.2.2.1	Preparation of Streptomyces Inoculum	31
3.2.2.2	Preparation of Media used for Morphological Characterization	32
3.2.2.3	Colour Determination	32
3.2.3	Physiological Characterization	33
3.2.3.1	Melanin Production	33
3.2.3.2	Production of Hydrogen Sulphide	33
3.2.3.3	Carbon utilization	34
3.2.4	Screening for Antibiotic Activity	34
3.2.4.1	Preparation of Media	35
3.2.4.2	Preparation of the Inhibitory Activity of the by-product of the Isolates	35

		PAGE
CHAPTER FOUR	- RESULTS	37
CHAPTER FIVE		
5.0	Discussion and Conclusion	61
5.1	Discussion	61
5.2	Conclusion and Recommendation	67
	References	68

LIST OF TABLES

	TITLE	PAGE
Table 4.1	Source and number of isolates selected from each sample	38
Table 4.2	Streptomyces species found in each soil sample and the percentage of their occurrence	40
Table 4.3	Morphological and physiological characteristics of the isolates based on "ISP Criteria" expressed in percentages	42
Table 4.4	Grouping of the species by their spore chain morphology characteristics	44
Table 4.5	Chromogenicity of the species on the various ISP test media	46
Table 4.6	Groups of isolates using specific ISP characristics	49
Table 4.7	Results of carbon utilization: Showing the number of different carbon sources each species can use	50
Table 4.8	The carbon utilization pattern of the species within each group	54
Table 4.9	The antimicrobial activity of the isolated specie	56

LIST OF FIGURES

	TITLE	PAGE
Figure 1	Types of spore chain arrangement in *Streptomyces* Species	15

LIST OF PLATES

	TITLE	PAGE
Plate 4.1	The Various types of colour series found in the isolates	43
Plate 4.2	The various spore arrangement types	45
Plate 4.3	Chromogenic and non-chromogenic reactions	47
Plate 4.4	The various inhibiting activities of the metabolic products of the isolates using perpendicular streak method	58

CHAPTER ONE

1.0 INTRODUCTION

Streptomyces belong to the family Streptomycetacaea of the order Actinomycetales. The actinomycetes have always been a strange group to the bacterial taxonomists. Bacteriologists considered them as bacteria, while mycologists generally considered them as fungi. This confused taxonomy has been overtaken, and the actinomycetes are generally accepted as bacteria (Skinner and Skyes, 1973).

It is because of their ability to produce antibiotics that the actinomycetes are perhaps best known. A review of the microbial sources of antibiotics discovered in the U.S.A. and Japan between 1953 and 1970 reveal that 85% are produced by actinomycetes, 11% by fungi and 4.5% by bacteria (Skinner and Skyes, 1973).

The actinomycetes were formerly known as small and negligible group of organisms causing certain obscure diseases. They now have a comprehensive recognition as a large and highly important microbial group of universal distribution with numerous biochemical activities and great practical importance (Waksman, 1967).

Antibiotics are specific products of metabolism with high physiological activity against individual groups of micro-organisms (e.g. viruses, bacteria, streptomyces, fungi, algae, protozoa) or against malignant tumours that can selectively slow down or completely inhibit their growth (Waksman, 1944). Antibiotic substances are produced by many micro-organisms in various ecological conditions, most of which inhabit the soil (Egorov, 1985).

Presently, a great number of antibiotics (over 6,000) produced by bacteria, fungi, algae, higher plants and animals have been described. The most important of them are mainly produced by Streptomyces including those that are currently in use. Many antibiotics of the Streptomyces origin have been isolated and studied in recent years. These antibiotics have been used successfully in agriculture, food industry and medicine (Egorov, 1985).

There is relatively little information as to the study and use of Streptomyces in Nigeria. In the light of this, the study was carried out with the following objectives:

a. to examine local soil samples for Streptomyces strains;

b. to characterize the local strains obtained, and

c. to screen such strains for antibiotic-producing properties.

CHAPTER TWO

2.0 REVIEW OF LITERATURE

2.1 THE ACTINOMYCETES

2.1.1 Historical Background

In 1875, Ferdinand Cohn observed a filamentous organism in the tear duct of the human eye. He named it Streptothrix foersteri. Unfortunately, this name had been used years before to a group of true fungi and was not universally accepted. Years later (1877-1888), Harz described an organism found in "lumpy jaws" of cattle as Actinomyces bovis, because the masses of filament were arranged radially which suggested the name "actinomycetes" or "ray fungus". This was not universally accepted because the description of the organism was based on its etiology rather than its morphology and cultural characteristics, and furthermore, no pure culture was obtained (Waksman, 1959).

As far back as 1888, Globig drew attention to the occurrence of actinomycetes in the soil, from which he isolated a thermophilic form. Similar cultures were isolated from manure and hay. Gasperini (1870) and Lachner - Sandoval first used the name "actinomycetes" for this group of organisms. Rossdoria (1891) isolated from the air an organism that he designated Streptothrix alba. Sanfelice, first suggested a definite relationship between the actinomycetes and the true bacteria, notably the tuberculosis organism. Gasperini (1892) drew attention to the great variations in the form and in the behaviour of the actinomycetes, especially in their ability to produce aerial spores and soluble pigments; these depended on the conditions of culture and the composition of the medium (Waksman, 1967). Beijerinck established, in 1900, that actinomycetes occur in great abundance in the soil, to a depth of one meter in garden soil and two meters in sandy soil; they were also found in the bottom mud of a river bed.

2.1.1.1 Classification

The classification of the actinomycetes soon received attention. This was based either upon their manner of sporulation (Oerskov, 1923) or upon their cultural properties. The actinomycetes are recognised to be a heterogenous group of micro-organisms, comprising several genera and many species. These organisms vary greatly in their physiology and in their roles in natural processes (Waksman, 1967).

2.1.2 Taxonomy and Classification of the Actinomycetes

The taxonomy and classification of the actinomycetes are difficult and several symposia had been organized to arrive at the proper taxonomic methods used in classifying them. Skinner and Skyes, 1973). In 1962, Sylvestri et al. suggested a qualitative approach based on their overall similarities. The approach of Sylvestri et al. (1962) is based on the following criteria: macromorphology and coloration of the colonies; micromorphology of the sporophore; production of pigments; reaction to physical agents; capacity to attack certain substrates; sensitivity to a number of antibiotics; utilization of various carbon sources; utilization of various nitrogenous sources; sensitivity to actinophages and possession of some polysaccharide antigens.

Some of these methods are presently used for the classification of Streptomyces.

2.1.2.1 Classification

Actinomycetes are classified into 10 families, some of which are newly proposed (Skinner and Skyes, 1973). They are as follows:
ACTINOMYCETACEAE: Comprising the genera Actinomyces, Agromyces, Arachnia, Bacterionema, Bifidobacterium, Rothia.

ACTINOPLANACEAE: Comprising the genera Actinoplanes, Ampullariella,

Dactylosporangium, Planobispora, Planomonospora, Spirillospora and Streptosporangium.

DERMATOPHILACEAE: Comprising two genera Dermatophilus and Geodermatophilus.

FRANKIACEAE: There is a single genus Frankia.

MICROMONOSPORACEAE: There is a single genus, Micromonospora.

MYCOBACTERIACEAE: Comprising the genera Mycobacterium and Mycococcus.

NOCARDIACEAE: Comprising the genera Nocardia, Micropolyspora, "Mycobacterium" rhodochronous and Gordona.

STREPTOMYCETACEAE: Comprising the genera Chainia, Elytrosporangium, Kitasatoa, Microellobosporia, Streptomyces and Streptoverticillium.

THERMOACTINOMYCETACEAE: Only a single genus Thermoactinomyces.

THERMOMONOSPORACEAE: The family includes the genera Actinomadura, Microbispora, Microtetraspora, Saccharomonospora and Thermomonospora.

There is a great deal of morphological diversity among the genera of the actinomycetes and these have been used for the purpose of taxonomy at the family and genus levels (Cross and Goodfellow, 1973).

2.1.3 Actinomycetes as boundary Micro-organisms

Actinomycetes have frequently been looked upon as a separate group of micro-organisms occupying a position between the true fungi and the

true bacteria. It has even been suggested that they form the original prototypes from which both fungi and bacteria have been derived (Waksman, 1967). Certain genera among the actinomycetes, notably Nocardia, are closely related to the mycobacteria and corynebacteria. Other genera, notably Streptomyces and Micromonospora, have their counterparts among the fungi (Krassilnikov, 1941). A separate order was therefore created, the Actinomycetales, which is distinct from the Eubacteriales, or the true bacteria (Waksman, 1950).

The similarities they bear with the true bacteria are; they are prokaryotic, their hyphae and spores are simple bacterial types, absence of true aerial mycelium (Gottlieb, 1973). They have bacterial type cell walls (Singleton and Sainsbury, 1978) and are inhibited by antibacterial agents (Waksman, 1967). On the other hand, the actinomycetes bear similarities with the true fungi in their growth characteristics on solid and liquid media, their manner of sporulation, the pleomorphism of certain species and the sensitivity of the spores to heat (Krassilnikov and Agre, 1964).

Because of these facts, the suggestion was made that the actinomycetes be placed in a taxonomic transition group between the Schizomycetes and the Hyphomycetes (Krassilnikov and Agre, 1964).

2.1.4 General Characteristics

All actinomycetes are Gram-positive, although certain thermophilic forms, according to Lieske (1921), may be Gram negative at temperatures above 50°C. Most actinomycetes are non acid-fast. Some of the Nocardia spp, however, especially many of the pathogenic forms are acid-fast (Gordon and Hagan, 1936, Umbreit, 1939). The mycelium stains uniformly except in older cultures. The presence of metachromatic granules has been observed by Drechsler (1919). Droplets of fat, often pigmented, can be seen frequently in the mycelium (Kriss, 1937). The actinomycetes are

generally aerobic except for the actinomycetaceae which contains aerobic or micro-aerophilic genera (Gottlieb, 1973).

2.1.4.1 Growth Characteristics

The growth structure of actinomycetes is made up of branching filaments producing a mycelium which may be either substrate or both substrate and aerial. The aerial mycelium vary in diameter from 0.2 to 0.8μm. Its colour ranges from white or virtually colourless to yellow, brown, red, pink, orange, green or black (Waksman, 1967).

It may form water soluble or water insoluble pigment depending on the nature of the organism and the composition of the medium (Jensen, 1932).

Various species of actinomycetes are characterized by the production of a typical aerial mycelium superimposed upon the substrate growth. The aerial mycelium may take the form of concentric rings of sporulating and spore-free zones upon the colonies growing on agar media and may reach a diameter of 1.0 to 1.4μm (Erikson, 1947). Pigmentation of the aerial mycelium is of great importance in characterizing the species. The pigmentation depends on the composition of the medium and the length of incubation period. The pigments range from white or gray to yellow, orange, red, rose, lavender, blue and green (Waksman, 1959).

On solid and liquid media, actinomycetes produce a mass of unicellular mycelium designated as a "colony". A colony in the bacteria, is used to describe an accumulation of a mass of branching filaments which originates from a spore or form a bit of mycelium (Waksman, 1967).

Reproduction is usually asexual, though sexual processes have been shown to occur by genetic analyses. In the non-hyphal forms, asexual reproduction is by fragmentation or by the usual fission of single cells. Vegetative reproduction is by well-formed spores resembling fungal anthrospores (Gottlieb, 1973).

2.1.5 Habitat

Actinomycetes are found in virtually every natural substrate, such as soils and composts, fresh water bodies, foodstuffs and the atmosphere. Deep seas, however, do not offer a favourable habitat (Waksman, 1967).

Alkaline and natural soils are more favourable habitats than acid soils. The sensitivity of Streptomyces spp to acidic P^H preclude high population in such soils (Gottlieb, 1973). Numerically, they are less dominant than the other bacteria and are more prominent than the fungi. Taber (1960) observed that it is common to find $5x10^6$ cells/g of soil in fertile soils from many different parts of the world. They occur in greatest numbers in the top few inches of the soil and decrease with depth (Waksman and Purvis, 1932).

High densities of spores can be found on plant materials such as Irish potato (Solanum tuberosum) and sweet potatoes Ipomoea batatas). Streptomyces spp are also common on most plants' parts. They can be isolated from all artifacts and natural materials (Grein and Meyers, 1958). Lacey and Lacey (1964) isolated Streptomyces from the air with the aid of Andersen sampler.

Streptomyces are found in marine environment in association with Micromonospora spp; especially in fresh water lakes and in bottom muds. Many actinomycetes can survive in a salty environment at low temperature for considerable periods of time (e.g. 3.5% of sodium chloride at 15°C), if proper nutrients and oxygen are supplied (Okazaki and Okami, 1976). Thermophilic forms are found commonly in compost manures. The actinomycetes are undoubtedly responsible in part for the earthy odour of soil (Gerber and Lechevalier, 1965).

2.1.6 Pathogenicity

In the order actinomycetales, majority of the species are saprophytes, but some species are pathogenic on man, animals or plants

(Singleton and Sainsbury, 1978). The pathogenic species are found mostly in the genera of Mycobacterium and Nocardia. Examples of those pathogenic for animals (birds and cows) are Mycobacterium bovis and M. avium, while M. tuberculosis and M. leprae are pathogenic for humans (Cheesbrough, 1987). The family Nocardiaceae contains a number of presumed species that are especially prevalent or pathogenic in the tropical regions. Among them are Nocardia asteroides and N, brasiliensis. Actinomyces bovis causes lumpy jaw of cattle (Thompson, 1950).

Despite the large number of species in the genus Streptomyces, only one specie has been shown to be pathogenic to animals (guinea pigs and rabbits); Stm. galtieri (Breed et al., 1957). Stm. scabies produces the common scab of potatoes (Solanum tuberosum) (Lambert and Rloria, 1989) and Stm. ipomoea, the scab of sweet potatoes (Labeda et al., 1992). Stm. somaliensis is known to cause mycetoma in man (Cheesbrough; 1987). Lu et al., (1991) reported that the thermophilic Stm. hygroscopicus is one of the pathogens of farmer's lung disease.

2.1.7 Economic importance of Actinomycetes

Actinomycetes, like fungi and bacteria are universal scavengers. They are able to decompose numerous organic compounds especially complex plant (McQueen et al., 1987) and animal residues that find their way into soils, water basins and composts. They bring about a much smaller increase in humus content (Waksman, 1967).

The isolation from lake muds of cultures of actinomycetes capable of reducing sulfate to hydrogen sulfide was believed to be a contributing factor to geological transformation. Thus they act as geological agents (Egorov, 1985).

The activities of actinomycetes on certain foodstuffs such as milk, cocoa, potable water and fish include decoloration, deterioration and impartation of earthy odour to such foods (Thaysen, 1936). They also stain and destroy fabrics and paper. This reduces the value of the

material.

Actinomycetes have found some application in the production of diastatic enzymes. A preparation named "super biolase" produced by an actinomycetes is used in desizing processes because of its ability to withstand higher temperatures (Mizusawa et al., 1966). The production by Stm. griseus of vitamin B12, a red crystalline material having all the properties of the compound isolated from liver led to considerable developments in this field (Waksman, 1967). The use of the vitamin as "animal protein factor" for the feeding of chicks at a level of 30μg/kg of diet has opened a new field of application (Moore et al., 1946, Maitra and Roy, 1960).

Actinomycetes are not known to cause great plagues that affect mankind and his domesticated animals. They only cause potato scab in plant, tuberculosis and leprosy in man (Cheesbrough, 1987) and certain other, rather minor diseases (Waksman, 1967).

Streptomyces species are of great importance in the pharmaceutical industries. They are the producers of about 60% of the antibiotics used today in agriculture, medicine and food industries (Egorov, 1985). Kuznetsov et al., (1992) isolated Stm. albiaxialis from highly mineralized stratum waters of the Perm-oblast oil field. He reported that this organism is capable of degrading crude oil and petroleum products and it can also be used to purify highly mineralized waste waters and substrates contaminated with petroleum products. They produce an extra cellular enzyme which is capable of attacking and modifying the polyethylene portion of heat-treated degradable plastics. Lee et al (1991) demonstrated this ability in Stm. badius, Stm. setonii and Stm. viridosporus. The modified polyethene portion is used in the composting of lawn, garden and shrub litter.

2.2.0 Genus Streptomyces

The name was derived from a Greek adjective "Streptos" which means

plaint or bent and a Greek noun "myces" meaning fungus. Therefore "Streptomyces" means "Pliant or bent fungus" (Pridham and Tresner, 1974).

Streptomyces are Gram-positive soil bacteria capable of synthesizing a large number of secondary metabolites with antibacterial or antifungal activities (Despande et al., 1988). They belong to the family Streptomycetaceae. Among all the genera in this family, Streptomyces is represented in nature by the largest number of species and varieties (Waksman, 1967). The name "Streptomyces" was introduced in 1943 for the aerial mycelium producing actinomycetes.

2.2.1 General characteristics

Streptomyces spp produce a slender, coenocytic hyphae of about 0.5-2.0μm in diameter (Waksman, 1959; Silvestri, 1962). The aerial mycelium at maturity forms chains of about 5 and often more than 50 spores. The cell wall contains LL-2, 6-diaminopimelic acid and glycine (Singleton and Sainsbury, 1978). Streptomyces lack a mucopolysaccharide cell wall which allows them to produce and secrete a variety of proteins directly into their environment (Strickler et al., 1992). Newton et al., (1993) has reported the presence of low-molecular weight thiols in Streptomyces spp.

2.2.1.1 Growth characteristics

They are heterotrophs and are highly oxidative (Benedict et al., 1955). They are able to degrade many macromolecules such as proteins, lipids, starch, chitin, pectin, xylan and cellulose (Moormann et al., 1993). They reduce nitrates to nitrites. They are aerobic and temperature of growth is between 25-35°C but the optimum is 28°C. Some species grow at temperatures within the thermophilic range (Cochrane and Cohn, 1947 and Corbaz et al., 1963). Optimum P^H range for growth is 6.5 - 8.0.

They produce a wide variety of pigments which are responsible for colours of vegetative mycelia. The colours produced range from yellow, red, blue, green to other soluble pigments which could be observed on appropriate synthetic media (Waksman, 1967, Cochrane and Cohn, 1947).

On isolation, colonies are small (1-10mm in diameter), discrete and lichenoid, leathery or butyrous. The colonies may appear smooth surfaced but later develop a weft of aerial mycelium that may be granular, powdery, velvety or floccose. They give off a distinctive musty odour characteristics of damp cellars and newly turned soil (Frobisher, 1978).

Species of Streptomyces are known to produce a variety of antimicrobial agents such as antibacterial, antifungal, anti-algal, anti-viral, antiprotozoal and anti-tumour antibiotics. Many strains are sensitive to antibacterial agents and to actinophages (Pridham and Tresner, 1974).

Although, there are isolated reports of definite cycle involving fusion of motile gametes, hybridization of wild-type strains has not been reported. Nevertheless, Bradely and Lederberg, (1956) reported the possibility of inducing hybridization among the auxotrophic mutant strains, while Nakazawa (1964) reported heterokaryosis among the Streptomyces.

2.2.2 Diagnostic Criteria for the Genus Streptomyces

Beyond these general characteristics, groups within the genus Streptomyces may be recognised using descriptive criteria adopted for the International Streptomyces Project (ISP). These criteria were carefully selected by two sub-committees (i.e. the Sub-committee on Systematic Bacteriology and the Sub-committee on Actinomycetales of the American Society for Microbiology). The criteria are often referred to as the "ISP criteria" (Shirling and Gottlieb, 1976). The ISP methods and criteria have now been used for characterization of more than 450 type strains of Streptomyces. The ISP criteria are as follows:

2.2.2.1 Spore-chain arrangement

The description of the spore-chain morphology in terms of ISP criteria are:

a. The spore arrangement could be described as rectus-flexibilis (RF). Under this arrangement, when the spores are in straight chains and branched, they are referred to as straight. When the chains are branched and twisted, they are referred to as flexuous and the third arrangement which is not common is referred to as fascicle (See Fig. 1a)

b. The spore chain arrangement could also be spira. There are two types of spira arrangement (i.e. retinaculum apertum (RA) and spira (S).

   i. the spore chain may form hook-like arrangement, open loops or greatly extended coils which are referred to as RA (See Fig. 1b).

   ii. the spore chain arrangement may also be in form of short, quarled and compact coils, or extended, long and open coils. These are referred to as spira. The type of the spira arrangement is determined by the diameter of the coil, whether the coil is closed or open or long or short (Pridham et al., 1958) (See Fig. 1c).

Approximately 206 ISP strains are potentially spira or RA strains. Of these potentially spira or RA strains, 42 or 20% produce short crooked, irregular spore chains terminating sometimes in small hooks or partial turns (Shirling and Gottlieb, 1976).

2.2.2.2 Spore surface ornamentation

Spore ornamentation was probably the least ambiguous of all ISP criteria (Shirling and Gottlieb, 1976). Frobisher (1978) showed that when the spores of Streptomyces is examined with the electron microscope at

magnification of around x8000, striking surface configurations or "ornamentation" were revealed. These ornamentations are of four types: glabrous (smooth), verrucose (warty), echinulate (spiny) and hirsute (hairy).

These ornamentations are found in the strains with short imperfect chains. Spiny and hairy spores are generally associated with spiral cultures, though smooth spores sometimes appear in spiny-spored strains (Shirling and Gottlieb, 1976).

Williams and Sharples, (1970) using scanning electron microscope observed that irregular folds or wrinkles on otherwise smooth-spored chains could sometimes be mistaken for warts when the transmission electron microscope silhouette is used.

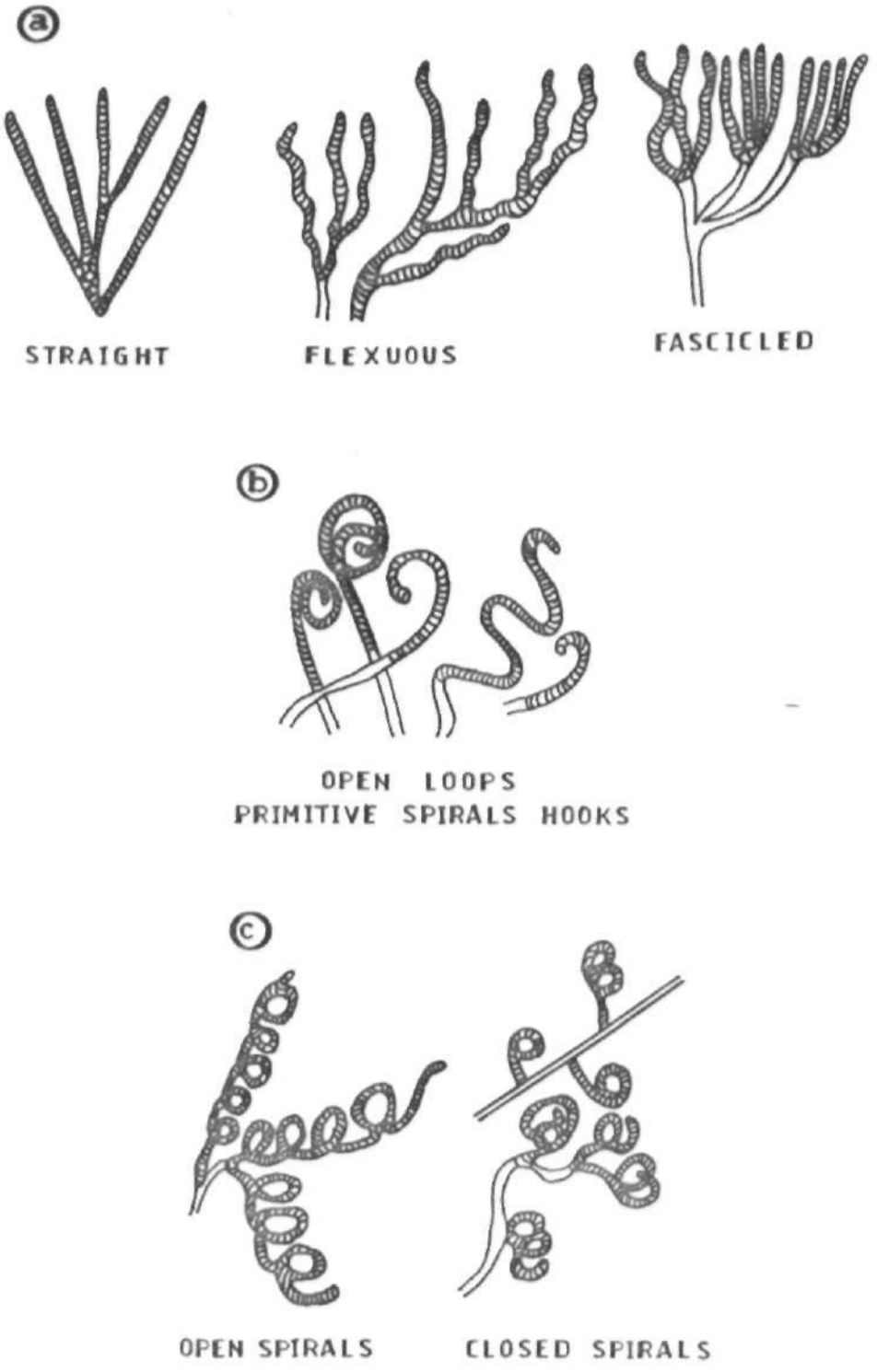


Fig. 1. Types of spore chain arrangement in Streptomyces species ; ⓐ Rectus-flexibilis ; ⓑ Retinaculum apertum ; ⓒ Spira .

2.2.2.3 Mass colour of aerial mycelium

Mass colour of the mature sporulating aerial mycelium, based on the Tresner-Backus Colour wheel has been a surprisingly good criterion for identification. But Tresner and Backus, (1963) observed that about 70% of the strains they studied were not easily identified using the colour wheels due to the small size of the aerial mycelium. The aerial mycelium, when large enough, shows sufficient differentiation and the colour may be white, gray (gray to brownish), yellow (yellowish to greenish yellow), red (tan, pink and rose shades), blue (bluish to grayish-blue shades), green (greenish to grayish green shades) or violet (Pridham and Tresner, 1974).

Before the use of the Tresner-Backus system, there was wide agreement in the assignment of Streptomyces to colour groups. ISP reports show excellent agreement among the collaborators when colour wheels were used (Shirling and Gottlieb, 1976).

2.2.2.4 Production of melanoid pigments (Chromogenicity)

Chromogenicity as a diagnostic criterion for Streptomyces has been the subject of studies by Arai and Mikami (1972). Production and non-production of melanin pigment is studied by ISP methods using complex and synthetic media. The complex media are Peptone Yeast extract Iron agar (PYEA) and Tryptone Yeast Broth (TYB), while the synthetic one is Tyrosine agar. These three media make up the ISP test media for chromogenicity (Shirling and Gottlieb, 1966).

Arai and Mikami (1972) have reported certain inconsistencies in ISP reports, such as the observation that a significant number of chromogenic Streptomyces producing melanoid pigment on complex organic media failed to produce the pigment in synthetic tyrosine agar, while some produce the pigment on synthetic tyrosine agar but failed to do so on complex organic media.

Shirling and Gottlieb, (1976) reported that 59% of 450 type strains studied were readily characterized as negative for chromogenicity, since

produce the pigment in synthetic tyrosine agar, while some produce the pigment on synthetic tyrosine agar but failed to do so on complex organic media.

Shirling and Gottlieb, (1976) reported that 59% of 450 type strains studied were readily characterized as negative for chromogenicity, since they did not produce darkening of any of the three ISP test media. Thus, more than half of the ISP strains are unequivocally melanoid-negative in terms of the ISP criterion. They also reported that 37% of the strains studied were chromogenic on complex organic media. Altogether, ISP workers were able to characterize 96% of the strains studied as melanoid-positive or melanoid-negative on organic media, though 4% could not be placed in any group (Arai and Mikami, 1972).

2.2.2.5 Vegetative mycelium pigments and soluble pigments

The colour of the reverse side of the mycelium and the presence of soluble pigment as defined in the ISP criteria and methods have been useful in only a limited number of strains. Most strains fall within a range of grayed-yellow, grayed greenish-yellow to olive or light grayish brown, but observers disagreed on exact placement within this range (Shirling and Gottlieb, 1976). This criterion is inconsistent because workers do not agree on the colour judgement (Pridham et al., 1974).

2.2.2.6 Carbon utilization

The ISP criteria include the determination of the utilization pattern among carbon compounds. The carbon compounds commonly used are D-glucose, D-xylose, L-arabinose, L-rhamnose, D-fructose, D-galactose, raffinose, D-mannitol, i-inositol and sucrose (Pridham and Tresner, 1974). These carbon compounds are supplied individually as sole carbon source in the synthetic agar medium. Synthetic medium plus a glucose and synthetic medium without added carbon source provide positive and negative controls, respectively (Shirling and Gottlieb, 1966).

The pattern of carbon utilization is useful in identifying existing similarities between strains. Within groups of type strains determined by other criteria, one finds sub-groups having carbon utilization patterns that are identical or that differ in only one or two carbon sources used for growth (Nonomura, 1974). Wang et al., (1992) obtained a stable mutant by treatment of Stm. mycarofaciens spores at high temperature. He observed that the physiological characteristics of both strains were very similar but they had slight differences in utilization of few carbon sources.

2.3 THE ANTIBIOTIC CONCEPT

In 1889, Vuillemin first used the word "antibiosis" to describe a type of association among living systems in which one organism destroys another in order to sustain its own life. Waksman in 1942 defined antibiotic as "a chemical substance, produced by micro-organisms, which has the capacity to inhibit the growth or even to destroy bacteria and other micro-organisms". This definition was later modified to comprise substances which act in very dilute solution, in order to avoid the inclusion of such products of microbial metabolism as organic acids and alcohol (Waksman, 1967).

2.3.1 Antibiotic: A metabolic product

The study of the role that antibiotics play in the life of Streptomyces spp producing these physiologically important substances has a great importance for general biology. Formation of a given antibiotic substance is a specific inherited property of one or several species of Streptomyces (Egorov, 1985).

Antibiotics are a product of secondary metabolism in micro-organisms. The outstanding factor common to all antibiotics is the fact that they are all secondary metabolites, that is, compounds which serve no obvious function in the cells which produce them (Turner, 1977). In the

case of antibiotics, it has been argued that their production confers upon the organism an advantage in its natural environment (Brian, 1957 and Pollock, 1967).

Secondary metabolic products are produced after the logarithmic phase i.e., stationary phase of growth (Ameh, 1990). Most of the known secondary metabolites of the Actinomycetales are, infact, antibiotics (Turner, 1977).

The Streptomyces spp produce an unrivalled range of chemically diverse secondary metabolites which are recognised as antibiotics, herbicides and pharmacologically active molecules and which presumably play an important role in the Streptomyces life cycle in nature (Hopwood; 1988).

2.3.2 Antibiotic - activity of Streptomyces

Among the various genera of Actinomycetes, the genus Streptomyces has yielded the largest number of antibiotic producing strains. As a result of intense search, many valuable preparations have been successfully used in agriculture, food industry and medicine (Egorov, 1985).

The nature of the organisms used in testing for antibiotic activity frequently influences the results obtained. Sensitive organisms, like Bacillus subtilis or Staphylococcus aureus are commonly employed as test organisms (Waksman, 1967).

Waksman et al. (1942) isolated and tested 244 cultures of actinomycetes. He observed that 43.4% of the isolates possessed antagonistic effect. He also showed that of these isolates, 20% were highly antagonistic and these were found to belong largely to the genus Streptomyces. Burkholder (1946) isolated 7,369 cultures of actinomycetes from soil, 1,869 of which inhibited Staph aureus, 261 inhibited E. coli and 514 showed an antagonistic effect against Candida albicans.

Lindner and Wallhäuser (1955) isolated 2,500 antagonistically active Streptomyces culture from numerous soil samples. Of these, 77% were active against Gram-positive bacteria (Staph aureus), 40% against Gram-negative bacteria (E. coli) and 18% against fungi (Aspergillus niger).

2.3.3 Antibiotic Production by Streptomyces

Antibiotic substances produced by Streptomyces and having antimetabolic properties have been discovered. Arai et al. (1976) reported that thermophilic Stm. macrosporus produces an antibiotic which inhibits the metabolic pathway of lysine, arginine and histidine.

In a study of 10,000 cultures of Streptomyces isolated from various substrates, Woodruff and McDaniel (1956) found that about 25% of the isolates produced antibiotics. Approximately 90% (2,250) of these cultures formed streptothricin or closely related compounds. Half of the remainder (125) yielded streptomycin; one-third of the other half (40), tetracyclines. Most of the remaining cultures were able to form substances that could be identified with one or another of the various antibiotics which had already been isolated from Streptomyces. Only 30 of the freshly isolated cultures yielded unknown compounds.

2.3.4 Types of antibiotics produced by Streptomyces

The antagonistic activity of antibiotics produced by Streptomyces could be antitumour, antifungal, antibacterial, antiprotozoan, antiviral or antirickettsia (Arai et al., 1976). But the antibiotics produced by them can be broadly classified into the following: macrolides, aminoglycosides, ß-lactam compounds, Polyene antibiotics and antitumour agents.

2.3.4.1 Macrolide antibiotics

Egorov (1957) defined macrolide antibiotics as antibacterial products of metabolism with high molecular weight but are weakly alkaline.

They are characterized by the presence in their molecules of the macrocyclic lactone ring connected with one or several carbohydrate residues which are usually amino sugars.

The group of the macrolide antibiotics is quite large. Examples include Pikromycin, erythromycin, magnamycin, methymycin, spiramycin and oleandomycin. They are normally active against Gram-positive bacteria but some of them are effective against fungi and Gram-negative form of bacteria. They are used as "reserve" antibiotics in the treatment of some diseases whose causative agents are resistant to other currently used antibiotics (Egorov, 1985).

By their biological potency the macrolides are divided into two groups. The substances belonging to one group (erythromycin, oleandomycin, magnamycin, etc) have marked bacteriostatic properties against most Gram-positive bacteria and are only slightly efficient against fungi. Levorin, filipin, pimaricin and many other compounds belonging to the other group contain conjugated polyene systems. These antibiotics have a pronounced antifungal activity and affect the growth of bacteria only weakly (Egorov, 1957).

2.3.4.2 Aminoglycoside antibiotics

The group of aminoglycoside antibiotics include biologically active polycationic compounds composed of amino sugars connected by glycosidic linkages or bonds in their molecules. They affect protein synthesis in bacterial cells, though they máy not all occupy the same active site on the ribosome or they may not have precisely the same mechanism of action (Odama, 1989).

This group includes streptomycin, neomycin, fortimycin, gentamycins, kanamycin, tetracyclines, chloramphenicol and some other substances. Antibiotics of this group are very important and many of them have been used extensively in medicine because they have a broad spectrum of activity (Egorov, 1985). At the present time, about a hundred

aminoglycoside antibiotics are known.

2.3.4.3 ß - Lactam compounds

Streptomyces clavuligerus produces a variety of ß-lactam compounds including penicillin N, cephamycin C and clavulanic acid (Duraire et al., 1992). In view of its diverse biosynthetic capabilities, this species has become one of the best characterized bacterial ß-lactam producers (Jensen, 1986). These compounds affect bacterial cells by inhibiting cell wall synthesis. This is done by interfering with mucopeptide synthesis thereby resulting in the production of deficient cell walls. Disulfide reductase plays a role in the biosynthesis of penicillin and cephalosporin in Streptomyces (Aharonowitz et al., 1993).

2.3.4.4 Polyene antibiotics

The antifungal antibiotics of Streptomyces can be classified into polyene and non-polyenic types. A survey made by Linderfelser et al (1964) of 500 strains showed nearly half (240) capable of forming active substances against Mucor ramanniacus. Two-thirds of these (166) were non-polyenic. Fifteen of the non-polyenic substances were heat-stable; 70, moderately stable and 81 unstable. Two of the promising antibiotics were identified as cycloheximide and musarin.

The production of polyené antifungal antibiotics represent a unique property of strains of Streptomyces and Streptoverticillia among the bacteria. The chemical nature of these polyene antifungal antibiotics may be triene, tetraene, pentaene, hexaene, heptaene, octaene or polyene (more than 10) (Pridham et al., 1976).

Polyenic antifungals continue to elicit the interest of antibiotic researchers because of their high activity against yeasts and molds and because there is continuing need for non-toxic, synthetic antifungals for

medical or non-medical purposes.

Conover (1971) listed eight useful antifungal agents. Of these, six are polyene and they are amphotericin B, candicidin, hamycin, nystatin, pimaricin and trichomycin. Conover (1971) observed that microbial metabolites used as antifungal drugs play a major role in chemotherapy. New uses of these polyene antibiotics include treatment of acne, reduction of sperm levels in a male host, virucidal activity, alteration of lipid metabolism, control of prostrate hypertrophy and effect on absorption of sterols (Lechevalier et al., 1961; Muftic, 1968; Mechlinski, 1973).

2.3.4.5 Antitumour agents

Recently, Streptomyces hygroscopicus which produced a new antitumour substance (himastatin) was isolated in Japan from a soil sample collected in India (Lam et al., 1990). Other antitumour substances include puromycin, bruneomycin, rubomycin, etc (Arai et al., 1976).

2.3.5 Sensitivity of Streptomyces to antibiotics produced by them

Antibiotics are quite varied from the point of their chemical structure and the mechanism of physiological action. Antibiotics serve a protective function and in addition, may affect separate stages of the metabolic processes of their own producers by activating or inhibiting some enzymatic system (Pollock, 1967). They also aid sporulation in some species of Streptomyces (Szabo et al., 1985).

As micro-organisms grow, they become gradually adapted to the action of certain amounts of the antibiotics produced by them. But if their concentration in the medium is increased artificially, the micro-organisms producing these antibiotics will respond to this change. For example, some Streptomyces are inhibited when the concentration of the produced antibiotics is increased (Egorov; 1985).

Waksman et al., (1946) showed that various strains of Stm. griseus

differ in their sensitivity to streptomycin. The strains capable of forming higher quantities of the antibiotic can stand higher concentrations of streptomycin in the medium, while less active strains will not grow in these conditions. Strains that do not form streptomycin will not develop in the medium containing the antibiotics.

This property of Streptomyces was used for selection of most productive strain of Stm. griseus because adding certain amounts of streptomycin to the media, promotes the growth of most active forms of Streptomyces.

Gella and Vining (1975) established that Stm. griseus is sensitive to streptomycin during the exponential phase of its growth. But the Streptomyces is stable to the action of the antibiotic when its synthesis begins.

Micro-organisms isolated from natural sources ("wild" strains, rather than mutants with a high biosynthetic activity) are resistant to their own antibiotic at the membrane level. Highly productive mutants of micro-organisms protect themselves from their own antibiotics by enzymatic inactivation (Egorov; 1985). It follows that antibiotics influence their own producers. They upset vital functions of the micro-organisms and thus inhibit their growth.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Bacteriological Media used for Isolation

Czapek -Dox Agar	Oxoid Ltd. Basingstoke,
Code CM 97	England.
Bacteriological Agar	Oxoid Ltd. Basingstoke,
Code L11	England.

3.1.2 Materials used for Microscopic Observation of the Isolates

Crystal Violet	Hopkin and Williams Ltd.
Product No. 355250	Chadwell Heath Essex,
Batch No. 52224	England.
Gram's Iodine	Microscopical Stains,
Product No. 9621	Hopkin and Williams Ltd.
Batch No. 20772B011413	Chadwell Heath Essex,
	England.
Acetone	GPR, BDH Chemicals Ltd.,
Product No. 27023	Poole, England.
Safranin	BDH Stains, BDH Chemicals
Product No. 34067	Ltd., Poole, England.
Xylene	GRP, BDH Chemicals Ltd.,
Product No. 30575	Poole, England.
Batch No. 4923400H	
Oil Immersion	Hughes and Hughes Ltd.,
	Romford, Essex.

3.1.3 Bacteriological media used for Characterization

Tryptone	Oxoid Ltd., Basinstoke
Code L42	England.
Batch No. 645763	
Peptone	Biotec Laboratories
Batch No. 061001	Ligut Water Surrey
	GU 185TA, U.K.
Proteose Peptone	Oxoid Ltd., Basinstoke
Code L46	England.
Batch No. 346 4201	
L-tyrosine	Biochemicals, Hopkin and
Product No. 885400	Williams Ltd., Chadwell
Batch No. 51725	Heath, Essex, England
Yeast Extract	Oxoid Ltd., Basingstoke
Code L21	England.
Batch No. 145 7477	
Bacteriological Agar	Oxoid Ltd., Basingstoke, Code L11
England.	
Czapek-Dox Agar	Oxoid Ltd., Basingstoke,
Code CM97	England.
Oatmeal	Quaker Oats B.V.
	Rotterdam - Holland.

3.1.4 Chemical Reagents used for Characterization

Dipotassiumphosphate (K_2HPO_2)	AnalaR, BDH Chemicals Ltd.,
	Poole, England.
Potassium dihydrogen	May and Baker Laboratory
Phosphate (KH_2PO_4)	Chemicals, Dagenham,
Product No. P251/18/66	England.
Manganous Chloride	AnalaR, BDH Chemicals Ltd.,
($Mucl_2$. $4H_2O$)	Poole, England.

Product No. 10152	
Magnesium Sulphate	AnalaR, BDH Chemicals Ltd.,
($MgSO_4$. $7H_2O$)	England.
Product No. 10151	
Ferric Sulphate	AnalaR, BDH Chemicals Ltd.,
($FeSO_4$. $7H_2O$)	England.
Product No. 26094	
Copper (II) Sulphate	AnalaR, BDH Chemicals Ltd.,
($CuSO_4$. $5H_2O$)	England.
Product No. 27849	
Zinc Sulphate	AnalaR, BDH Chemicals Ltd.,
($ZnSO_4$. $7H_2O$)	England.
Product No. 30621	
Sodium thiosulphate	AnalaR, BDH Chemicals Ltd.,
($Na_2S_2O_3$. $5H_2O$)	England.
Product No. 30235	
Ferric ammonium citrate	AnalaR, BDH Chemicals Ltd.,
Product No. 27163	England.
Ammonium sulphate	May and Baker Laboratory
$(NH_4)_2SO_4$)	Chemicals, Dagenham, England.
Product No. A224/18/66-1	
Lead Acetate	AnalaR, BDH Chemicals Ltd.,
Product No. 10142	Poole, England.
Concentrated hydrochloric	GPR, BDH Chemicals Ltd.,
acid (Hcl)	Poole, England.
Product No. 10725	

3.1.5 Colour Standard used for Colour Determination

Colour Identification	Royal Botanical Garden,
Chart	Edinburgh.

3.1.6 Carbon sources used for Carbon Utilization Test

D-Glucose	May and Baker Laboratory
Product No. 915/18/67	Chemicals, Dagenham,
	England.
Sucrose	Fisher Scientific Company
	Fairlawn, New Jersey.
D-Galactose	Hopkin and Williams Ltd.,
Product No. 436200E	Essex, England.
D-Xylose	Hopkin and Williams Ltd.,
Product No. 904800G	Essex, England.
D-Mannitol	AnalaR, BDH Chemicals Ltd.,
Product No. 10330	Poole, England.
i-Inositol	Biochemical, BDH Chemicals
Product No. 38044	Ltd., Poole, England.
Raffinose	Biochemical, BDH Chemicals
Product No. 38056	Ltd., Poole, England.
Salicin	Biochemical, BDH Chemicals
Product No. 38060	Ltd., Poole, England.
Rhamnose	AnalaR, BDH Chemicals Ltd.,
Product No. 38057	Poole, England.
D-Fructose	AnalaR, BDH Chemicals Ltd.,
Product No. 10367	Poole, England.
Arabinose	Calbiochem, Sandiego,
	California, 92132.

3.1.7 Materials used for screening possible antibiotic Activity

Sodium Chloride	AnalaR, J.T. Baker Chemical
	Co. Phillipsburg, N.J. 08865.
Calcium carbonate	AnalaR, BDH Chemicals Ltd.,
Product No. 27579	Poole, England.
D-glucose	May and Baker Laboratory
Product No. 915/18/67	Chemicals, Dagenham,
	England.

Soya bean flour	Samaru Market, Samaru, Zaria, Nigeria.
Bacteriological agar Code L11	Oxoid Ltd., Bassingstoke, England.

3.1.8 Assay organisms used for screening possible antibiotic activity of the isolates

Staphylococcus aureaus	Medical Microbiology Laboratory, Department of Microbiology, A.B.U., Zaria.
Escherichia coli	Medical Microbiology Laboratory, Department of Microbiology, A.B.U., Zaria.
Candida albicans	Medical Microbiology Laboratory, Department of Microbiology, A.B.U., Zaria.

3.2 METHODS

3.2.1 Screening for Streptomyces Species

3.2.1.1 Sample Collection

Fifteen samples of garden, refuse dump and grassland soil were randomly obtained and transported in clean polyethene bags to the laboratory. The soil samples were taken from a depth of 10-15 centimeters (cm) below ground level using an augar. The samples were collected on five different occasions between the month of April and June.

3.2.1.2 Preparation of medium

The medium used for isolation of Streptomyces spp from their natural habitat (soil) was Czapek-Dox agar (CDA) and it was prepared according to the manufacturer's (OXOID) instructions with the addition of 1% bacteriological agar to ensure that the medium gels properly (Egorov, 1985). The P^H was adjusted to 6.8 and autoclaved at 121°C for 15 minutes (mins). After sterilization, the medium was cooled to 45°-40°C before pouring into sterile petri dishes. The medium was allowed to solidify and sterility was checked by incubating overnight at 37°C. Contaminated plates were discarded.

3.2.1.3 Preparation of soil inoculum

Ten grammes (g) of each soil sample were weighed into 90ml of sterile normal saline contained in a flask to give approximately 1:10 dilution. The flask's content was shaken thoroughly and 10ml of the suspension was placed in a sterile centrifuge tube and was centrifuged at 3,000rpm for three minutes. Five millilitres of the supernatant was transferred into a flask containing 45ml of sterile 0.9% normal saline with a sterile pipette to make 1:100 dilution. This was serially diluted further until 1:10,000 dilution was obtained.

3.2.1.4 Culture and Isolation of Streptomyces spp

Isolation of Streptomyces spp was carried out by the surface plating

technique. One millilitre of the above dilutions were inoculated in duplicates on CDA. A sterile bent glass rod was used to spread the inoculum over the entire surface of the plate. The plates were allowed to dry, then inverted to prevent condensation of moisture on the surface of the agar. The plates were then incubated at 28°C for 7-10 days (Waksman, 1967).

At the end of incubation period, flat, rough, strongly adherent colonies whose surface was thrown into radial folds or ridges were picked from CDA plates and were Gram-stained.

Confirmation of isolates was done by direct microscopic observation. Gram-positive, twig-like rods were regarded as Streptomyces spp (Stevenson, 1970). The colonies of such cells were purified by streaking on CDA plates, incubated for another 7-10 days at 28°C and stored on CDA slants for further physiological and morphological characterization.

3.2.2 CHARACTERIZATION

The morphological and physiological characterization of the isolates were carried out as described by Pridham et al., (1958), Gottlieb (1961), Kuster et al., (1963), Shirling and Gottlieb, (1966, 1976) and Pridham and Tressner, (1974). Brief description of the tests are presented below.

3.2.2.1 Preparation of Streptomyces Inoculum

The inoculum was grown in Tryptone Yeast Extract Broth (TYB) of the following composition: tryptoné, 5g; yeast extract (OXOID), 3g; distilled water, 1000ml. Aliquots of 100ml were dispensed into 500ml Erlenmeyer flasks and autoclaved at 121°C for 15mins. One flask was used for each isolate studied. Sterile broth was seeded with one loopful of inoculum from a stock culture slant. The culture was incubated at 28°C for 48 hours in a reciprocal shaker (94 strokes per min). The mature culture was dispersed by a vigorous agitation of the flask. Slants were inoculated with 0.2ml of fragmented mycelium, liquid media with 0.1ml and plates were streaked with 0.1ml of the suspension.

3.2.2.2 Preparation of media used for Morphological Characterization

The media used for morphological studies for all isolates were oatmeal agar and CDA. The oatmeal agar contained oatmeal, 20g; agar, 18g; trace salts solution ($CuSO_4.3H_2O$; 0.102g, $FeSO_4.7H_2O$; 0.176g, $Mncl_2.4H_2O$; 0.126g, $ZnSO_4.7H_2O$; 0.024g and Distilled water; 100ml), 1ml; distilled water, 1,000ml. The oatmeal was boiled in 1,000ml distilled water for 20mins and was then filtered through a muslin cloth. More distilled water was added to restore the volume to 1,000ml. 1ml aliquot of the trace salts solution was added, P^H was adjusted to 7.2 with sodium hydroxide (10%w/v). The agar was added, the mixture was boiled for 15-20 mins. CDA (OXOID) was prepared according to the manufacturer's instructions with the addition of 1% bacteriological agar. The components of oatmeal and Czapek-Dox's agar media were dissolved and then autoclaved at 121°C for 15mins.

Each medium was inoculated with 0.1ml of fragmented mycelium which was streaked in a cross-hatched pattern. The inoculated media were incubated at 28°C for up to 14 days.

At the end of the incubation period, the characteristics of the spore bearing hyphae and spore chains were determined by direct microscopic examination of the culture. Mature cultures of each isolate was Gram-stained and observed on a minimum of 10 microscopic fields under 100x objective. The spore chain morphology was described in terms of the morphological groups of Pridham et al., (1958). Spore ornamentation was described according to the system of Cross et al., (1966).

3.2.2.3 Colour Determination

The colours of mature cultures were observed on days 7 and 14 for mass colour of mature sporulating aerial surface growth, while the colour of the substrate mycelium as viewed from the reverse side, and for diffusible soluble pigments other than melanin. Colour of the aerial mycelium was compared to a colour standard (colour identification chart) under bright daylight according to (Shirling and Gottlieb, 1966). The colour of the soluble pigment was recorded without a reference to a colour standard.

3.2.3 Physiological Characterization

3.2.3.1 Melanin Production

Production of melanoid pigments was determined on agar plates of peptone iron yeast extract agar (PIYEA), tyrosine agar (TA) and tryptone yeast extract broth (TYB).

PIYEA was prepared by supplementing peptone iron agar (PIA) with 0.1% yeast extract. The medium was composed of peptone, 15g; proteose peptone, 5g; ferric ammonium citrate, 0.5g; dipotassium phosphate, 1g; sodium thiosulphate, 0.08g; yeast extract, 1g; agar, 15g; distilled water, 1,000ml. TA was composed of L(-) -tyrosine, 1g; bacteriological agar, 15g; distilled water, 1,000ml. TYB was composed of tryptone, 5g; yeast extract, 3g; distilled water, 1,000ml. Components of PIYEA, TA and TYB were dissolved by boiling for 15-20 mins. P" of all the media was between 7.0-7.2 before autoclaving. They were autoclaved at 121°C for 15 mins. PIYEA and TA were allowed to cool to 45-40°C, then were dispensed into sterile petri dishes. About 4ml of TYB was dispensed in tubes before autoclaving.

All the media were inoculated in duplicates with a heavy inoculum of spores and fragmented pieces of mycelium picked upon a standard wireloop. The inoculum was streaked on the surface of the sterile agar plate. Uninoculated media were used as controls. The cultures were incubated at 28°C for 14 days. Readings were made on days 2, 4, 7, for PIYEA and TA, while for TYB, readings were made on 4th, 7th and 14th day of incubation.

Melanin pigments produced on TA and PIYEA were observed, and these plates were compared with uninoculated controls. Cultures forming brown to black or a distinct brown pigment modified by other colours were recorded as positive (+). For TYB, browning of the medium was recorded as positive (Shirling and Gottlieb, 1966).

3.2.3.2 Production of Hydrogen Sulphide

In this study, media containing salts of two metals were used in conjuction with lead acetate strips (LAS). Media used were Peptone Iron

agar (PIA) made of peptone, 15g; proteose peptone, 5g; ferric ammonium citrate . 0.5g; K_2HPO_4, 1g; sodium thiosulfate, 0.08g; agar, 15g; and distilled water, 1,000ml. Lead Acetate Agar (LAA) was prepared according to the formula of the Difco Product, Peptone, 15g; proteose peptone, 5g; dextrose, 1g; lead acetate, 0.2g; sodium thiosulphate, 0.08g; agar, 15g; and distilled water, 1,000ml. The components of these media were boiled and then the P^H was adjusted to 7.0. They were dispensed into tubes, autoclaved at 121°C for 15 mins. and allowed to solidify as slants.

The fragmented mycelia were inoculated onto the agar slants and a strip of lead acetate paper was inserted into each culture. The tubes were then incubated at 28°C for 7 days. Positive control tubes were prepared by passing chemically produced hydrogen sulfide from Kipp's apparatus into a tube of each medium until the metal sulfide was formed.

After incubation period, formation of pigment in the medium was noted as positive for melanin production and darkening of LAS was noted as positive for hydrogen sulphide production (Kuster et al., 1964).

3.2.3.3 Carbon utilization

Chemically pure carbon sources certified to be free of impurities were used. Carbon sources used in this test were; L-arabinose, Sucrose, D-xylose, *i*-inositol, D-mannitol, D-fructose, rhamnose, raffinose and salicin. D-glucose is positive control while the negative control contained no carbon source. Solution (10%w/v) of each carbon source was filter-sterilized using 0.22μm membrane filter. Each carbon source was aseptically dispensed into sterile tubes in 5ml aliquot and were stored at 4-5°C in the refrigerator until required.

The basal medium (Pridham and Gottlieb, 1948) was composed of potassium dihydrogen phosphate, 2.38g; K_2HPO_4, 5.65g; magnesium sulphate, 1g; ammonium sulphate, 2.64g; trace salt solution, ($CuSO_4.3H_2O$; 0.102g, $FeSO_4.7H_2O$; 0.176g, $Mncl_2.4H_2O$; 0.126g, $ZnSO_4.7H_2O$; 0.024g and Distilled water; 100ml), 6.25ml; agar, 15g; and distilled water, 1,000ml. The components of the medium were dissolved, dispensed into tubes in 9ml aliquots and autoclaved at 121°C for 10 minutes.

carbon source solution aseptically to the sterile basal agar medium which had been cooled to about 60°C. The final concentration was approximately 1%. The mixture was agitated and the medium was allowed to solidify as slants.

Duplicate tubes of each carbon source were each inoculated with 0.1ml aliquot of the inoculum. Each isolate was also inoculated on each control tube. The tubes were incubated at 28°C for 10-16 days. Growth on a given carbon source was compared with the two controls which were basal medium above (negative control) and basal medium plus glucose (positive control).

Carbon utilization is regarded as strongly positive (++) when the growth on tested carbon in the basal medium is equal to or greater than that of the positive control. When the growth on tested carbon is less than that of the positive control, the utilization is regarded as being positive (+). The carbon utilization is regarded as being doubtful (±) when the growth on the tested carbon is significantly less than that of the positive control but slightly better than that of the negative control. Negative utilization (-) is when the growth on the tested carbon is equal or less than that of the negative control.

3.2.4 Screening for Antibiotic Activity

3.2.4.1 Preparation of Medium

The medium recommended by Golyakov and Tsygankov (1961) (G&T medium) was used for the verification of the antibiotic-producing properties of the isolates. The medium was composed of 10g of soya bean flour, 10g of glucose, 5g of Nacl, 1g of calcium carbonate and 15g of bacteriological agar which were dissolved in 1,000ml distilled water by boiling. P^{H} was adjusted to 6.8 and the medium was autoclaved at 121°C for 15 minutes.

3.2.4.2 Determination of the inhibiting activity of the isolates

Perpendicular streak method was used to verify the inhibitory activity of the by-product of the isolates and surface culture technique

was employed for this method (Egorov, 1985). Duplicate plates of the medium were inoculated by streaking with each of the Streptomyces isolate. After 18 - 20 hours of incubation at 28°C, various assay organisms which are Staphylococcus aureus, Escherichia coli and Candida albicans were seeded perpendicularly to the previously inoculated Streptomyces culture. The control was only inoculated with the assay organisms alone. The petri dishes were incubated at a temperature of 37°C for 24-36 hours. The ability of the test organism to produce anti-microbial substances is seen by the inhibitory effect observed on the assay organisms.

CHAPTER FOUR

4.0 RESULTS

4.1 ISOLATION OF STREPTOMYCES SPECIES

The study revealed high prevalence of Streptomyces spp in the soil. Table 4.1 shows that out of the 15 soil samples collected, two of the samples taken from refuse dump did not yield Streptomyces spp. The remaining 13 soil samples from garden and grassland soils yielded 62 Streptomyces isolates as shown by direct microscopic observation, morphological and physiological characterization. Thirty-two of the isolates were from the grassland soil samples, while the remaining 30 isolates were obtained from the garden soil samples.

Table 4.1: Source and number of isolates selected from each sample.

Sample No.	Source	No. of Isolates
1	Grassland soil	6
2	Refuse dump soil	-
3	Garden soil	6
4	Grassland soil	4
5	Refuse dump soil	-
6	Garden soil	4
7	Grassland soil	5
8	Grassland soil	5
9	Grassland soil	5
10	Garden soil	5
11	Garden soil	4
12	Garden soil	5
13	Grassland soil	5
14	Grassland soil	2
15	Garden soil	6

Only 96.8% of the 62 isolates selected were speciated using the "ISP criteria" and methods. The remaining 3.2% of the isolates could not be characterized under any species name.

Twenty-eight species of Streptomyces were obtained after characterization using the "ISP criteria". Table 4.2 shows the names of the species, source and the percentage of occurrence of each species. Majority of the samples (46.7%) had Stm. nigrifaciens. This species was the most prevalent being 16.7% of all the isolates. Stm. albogriseolus was next in prevalence with an occurrence of 11.7%, followed by Stm. misionensis, Stm. recifensis and Stm. griseolus being 6.7% respectively of all the isolates. Sixteen out of all the 28 species obtained in the study had an occurrence of 1.7%. Stm. noboritoensis, Stm. fulvoviolaceus, Stm. olivochromogenes, Stm. griseolosuffuscus, Stm. galbus and Stm. griseoluteus had an occurrence of 3.3% respectively while Stm. luteogriseus had 5.0% occurrence.

Table 4.2: Streptomyces species found in each soil sample and the percentage of their occurrence.

No.	Names of Species	Source	No. of Isolates	Percentage of Occurrence (%)
1	Stm. nigrifaciens	G1,S2,G2,G3,G4,S4,G5	10	16.7
2	Stm. albogriseolus	S3,S5,G3,G6	7	11.7
3	Stm. misionenis	G6,S6,G5	4	6.7
4	Stm. recifensis	G4,S6	4	6.7
5	Stm. griseolus	S1	4	6.7
6	Stm. luteogriseus	S2,S3,G5	3	5.0
7	Stm. noboritoensis	S1,G5	2	3.3
8	Stm. fulvoviolaceus	G2,G6	2	3.3
9	Stm. olivochromogenes	S2,G6	2	3.3
10	Stm. griseoluteus	G1,S3	2	3.3
11	Stm. galbus	G3,G4	2	3.3
12	Stm. griseolosuffuscus	S5	2	3.3
13	Stm. humidus	G5	1	1.7
14	Stm. erumpens	G6	1	1.7
15	Stm. cirratus	S7	1	1.7
16	Stm. chibaensis	G3	1	1.7
17	Stm. canarius	S4	1	1.7
18	Stm. ochraceisclerotícus	G1	1	1.7
19	Stm. microflavus	S6	1	1.7
20	Stm. fimicarius	G2	1	1.7
21	Stm. spiroverticillatus	S4	1	1.7
22	Stm. cacaoi	S4	1	1.7
23	Stm. gelaticus	S3	1	1.7
24	Stm. rameus	S7	1	1.7
25	Stm. aureofaciens	G1	1	1.7
26	Stm. anandii	S5	1	1.7
27	Stm. intermedius	S5	1	1.7
28	Stm. alboflavus	S4	1	1.7
29	Unclassified Isolates	S1, S6	2	3.3

KEY: Stm. - Streptomyces
S1-S7 - Grassland soil samples
G1-G6 - Garden soil samples.

Morphological and physiological characterization of isolates based on "ISP criteria" and methods (Table 4.3) showed 3 major colour types which are gray, yellow and white (see Plate 4.1) and the percentage of isolates with gray, yellow and white represented 86.7%, 8.3% and 5.0%, respectively. The spore chain morphology was rectus-flexibilis for 22 isolates (36.7%); retinaculum-apertum for 12 isolates (20%); spira for 26 isolates (43.3%) (see Plate 4.2). All the isolates had smooth spore wall surface.

Physiological characterization of the isolates revealed that the isolates were predominantly melanin negative with only 23.3% (14 isolates) being melanin positive. Among the melanin positive isolates, 15% produced melanin on TA, 3.3% produced on both TA and TYB, while 3.3% produced on PIYEA, TA and TYB. Hydrogen sulphide was produced on either LAA or PIAS by 70% of the isolates, while 30% could not produce H_2S either media (neither PIA nor LAA).

Table 4.3: Morphological and Physiological characteristics of the isolates based on "ISP Criteria" expressed in percentages.

Tests		Observations (%)	
Morphological Characterization Mass colour of aerial mycelium:			
	Gray	86.7	
	Yellow	8.3	
	White	5.0	
Spore Chain Morphology			
Rectus-Flexibilis		36.7	
Retinaculum-Apertum		20.0	
Spira		43.3	
Spore Wall Ornamentation:			
	Smooth	100.0	
	Hairy	-	
	Spiny	-	
	Warty	-	
Physiological Characterization			
Chromogenicity (C)		C+ 23.3	C- 76.7
Pigmentation on TA		15.0	
Pigmentation on TA and TYB		3.3	
Pigmentation on PIYEA and TA		1.7	
Pigmentation on PIYEA, TA and TYB		3.3	
Production of H_2S - H_2S Positive		70%	
H_2S Negative		30%	

KEY:

C+	-	Chromogenic
C-	-	Non-Chromogenic
TA	-	Tyrosine agar
TYB	-	Tryptone yeast broth
PIYEA	-	Peptone-iron yeast-extract agar
H_2S	-	Hydrogen sulphide
H_2S Positive	-	Hydrogen sulphide produced
H_2S Negative	-	Hydrogen sulphide not produced

Gray, white and yellow colour were the types of colour series found in all the isolates. Gray series had the highest occurrence of 86.7% followed by yellow (8.3%) and then white (5%). It was observed that the isolate which belongs to the white colour series in the plate below produced diffusible pigment which masked the white colour.



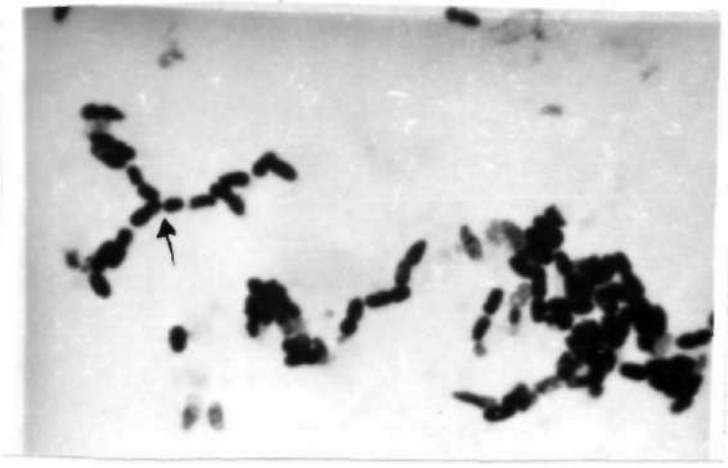
Plate 4.1: The various types of colour series found in the isolates.

KEY: GY - Gray, Y - Yellow, W - White.

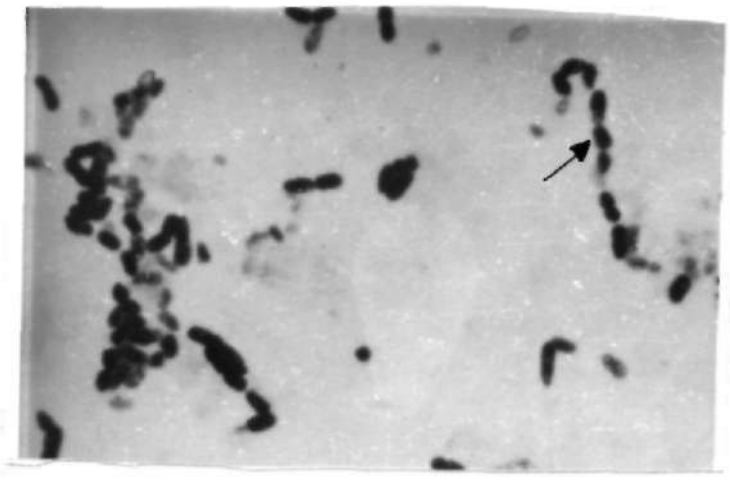
The isolates with the spira type of spore chain morphology had the highest occurrence of 43.3%, while those with the rectus flexibilis type of arrangement were 36.7%. Retinaculum-apertum had the least occurrence which was 20%. The names of the species and the type of spore chain arrangement they bear are shown in Table 4.4 while plates 4.2(a), 4.2 (b) and 4.2 (c) shows the various arrangements.

Table 4.4: Grouping of the Species by their Spore Chain Morphology Characteristics.

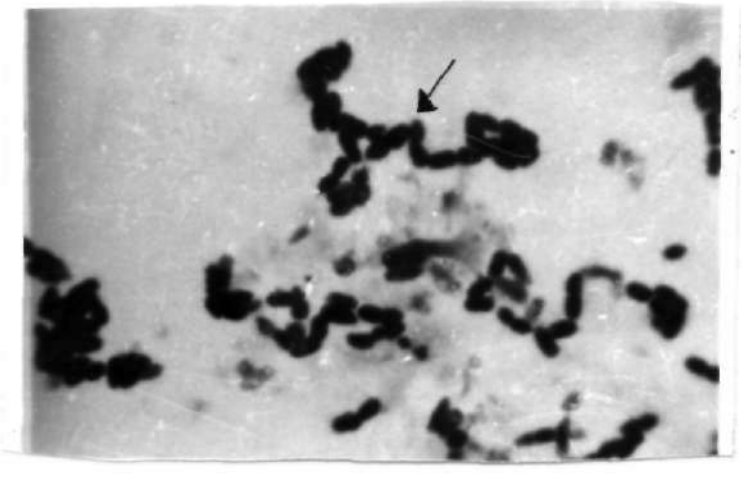
NAMES OF SPECIES	TYPES OF SPORE CHAIN MORPHOLOGY		
		SPIRA	
	Rectus-Flexibilis	Retinaculum-Apertum	Spira
	Stm. fimicarius	Stm. alboflavus	Stm. albogriseolus
	Stm. fulvoviolaceus	Stm. aureofaciens	Stm. anandii
	Stm. gelaticus	Stm. cacaoi	Stm. canarius
	Stm. griseolus	Stm. cirratus	Stm. chibaensis
	Stm. intermedius	Stm. griseoluteus	Stm. erumpens
	Stm. microflavus	Stm. humidus	Stm. galbus
	Stm. nigrifaciens	Stm. recifensis	Stm. griseolosuffuscus
	Stm. noboritoensis	Stm. spiroverticillatus	Stm. luteogriseus
			Stm. misionensis
			Stm. ochraceiscleroticus
			Stm. olivochromogenes
			Stm. rameus



(a)



(b)



(c)

Plate 4.2: The various spore arrangement types (a) Rectus flexibilis isolate (b) Retinaculum apertum isolate (c) spira isolate.

All the chromogenic species produced melanin on at least one medium. They all produced melanin pigment on TA. Stm. cirratus produced melanin pigment on both PIYEA and TA while Stm. noboritoensis produced on both TA and TYB. Stm. olivochromogenes was found to be highly chromogenic by producing melanin pigment on PIYEA, TA and TYB. The colours of melanin pigment produced on PIYEA, TA and TYB were blue-black, reddish-brown and brown respectively.

Table 4.5: Chromogenicity of the species on the various ISP Test Media.

NAMES OF SPECIES	PIGMENT PRODUCTION		
	PIYEA	TA	TYB
Stm. fulvoviolaceus	-	+	-
Stm. galbus	-	+	-
Stm. cirratus	+	+	-
Stm. luteogriseus	-	+	-
Stm. olivochromogenes	+	+	+
Stm. anandii	-	+	-
Stm. alboflavus	-	+	-
Stm. noboritoensis	-	+	+

KEY: + - Melanin pigment production
- - No Melanin pigment production.

The ability to produce melanin was observed by the browning of the medium. The plate shows the production of melanin on CDA by Stm. olivochromogenes. The non-chromogenic species did not produce any pigment as seen in Plate 4.3.



Plate 4.3: Chromogenic and Non-chromogenic reactions.

KEY: C+ - Chromogenic species

C- - Non-Chromogenic species.

Morphological and physiological characteristics of the isolates obtained in this study revealed that they could be placed into 8 groups. Most of the species belong to group IV. The species in this group are 37% of the total isolates. Group V and VII had the least number of species with a frequency of 1.6%, respectively.

Although, morphological and physiological characteristics of the species within a group are similar, they were differentiated by their pattern of carbon utilization. The characteristics of each group are shown in Table 4.6.

Carbon utilization pattern was variable for all the species as shown in Table 4.7. Some were found to have a wide sugar spectrum with the ability to utilize between 10-11 carbon sources. 8 species were found within this range. 9 species utilized between 8-9 carbon sources while the remaining 11 species were only able to utilize between 5-7 carbon sources.

Table 4.6: Groups of Isolates using specific ISP Characteristics.

Group	ISP Characteristics				Number of Species
	Colour	Spore Chain arrangement	Chromogenicity	Spore wall appearance	
I	Gray	Rectus flexibilis	Positive	Smooth	2
II	Gray	Rectus flexibilis	Negative	Smooth	3
III	Gray	Spira	Positive	Smooth	6
IV	Gray	Spira	Negative	Smooth	9
V	Yellow	Rectus flexibilis	Positive	Smooth	1
VI	Yellow	Rectus flexibilis	Negative	Smooth	3
VII	Yellow	Spira	Negative	Smooth	1
VIII	White	Spira	Negative	Smooth	3

Table 4.7: Results of Carbon Utilization: Showing the number of different carbon sources each specie can use.

NAMES OF SPECIES		The Number of Carbon Sources		
	No.	5 - 7	8 - 9	10 - 11
	1	Stm. alboflavus	Stm. anandii	Stm. albogriseolus
	2	Stm. aureofaciens	Stm. fimicarius	Stm. canarius
	3	Stm. cacaoi	Stm. galbus	Stm. chibaensis
	4	Stm. cirratus	Stm. humidus	Stm. fulvoviolaceus
	5	Stm. erumpens	Stm. intermedius	Stm. luteogriseus
	6	Stm. gelaticus	Stm. misionensis	Stm. nigrifaciens
	7	Stm. griselosuffuscus	Stm. noboritoensis	Stm. ochraceiscleroticus
	8	Stm. griseolus	Stm. rameus	Stm. olivochromogenes
	9	Stm. griseoluteus	Stm. recifensis	
	10	Stm. microflavus		
	11	Stm. spiroverticillatus		

Species in Group I belong to the gray series, their spore chain arrangement is rectus-flexibilis, they are chromogenic and their spore wall surface is smooth (Table 4.6). Stm. fulvoviolaceus and Stm. noboritoensis belong to this group (see Table 4.8). Stm. fulvoviolaceus had a wide sugar utilization spectrum with the ability to utilize between 10-11 carbon sources amongst the 11 tested. Stm. noboritoensis was not able to utilize rhamnose and sucrose, it utilized nine carbon sources out of the 11 tested. Isolates that belong to this group form 6.4% of the total isolates.

Group II organisms belong to the gray series, their spore chain arrangement is rectus-flexibilis, they are non-chromogenic and their spore wall surface is smooth. Stm. gelaticus, Stm. griseolus and Stm. nigrifaciens belong to this group. They utilized seven, seven and ten carbon sources respectively. Stm. gelaticus was not able to utilize L-arabinose and inositol. It had a doubtful utilization of D-fructose and D-mannitol. Stm. griseolus was not able to utilize rhamnose, D-mannitol, *i*-inositol and salicin while Stm. nigrifaciens was not able to utilize only salicin and strongly utilized both D-fructose and sucrose. The species in this group form 24.2% of the total isolates.

The species in Group III belong to the gray series, their spore chain morphology is spira, they are chromogenic and their spore wall surface is smooth. Stm. anandii, Stm. cirratus, Stm. galbus, Stm. luteogriseus, Stm. olivochromogenes and Stm. rameus belong to this group. Stm. anandii was able to utilize all the carbon sources except rhamnose and salicin, its utilization of L-arabinose was doubtful. Stm. cirratus was not able to utilize rhamnose, raffinose, inositol and salicin. Its utilization of D-fructose and D-mannitol was doubtful. Stm. galbus was not able to utilize rhamnose, raffinose and sucrose. Stm. luteogriseus and Stm. olivochromogenes had a wide sugar spectrum, they were both able to utilize all the carbon sources, although, Stm. olivochromogenes's use of *i*-inositol was doubtful. Stm. rameus was not able to utilize rhamnose

and inositol. The species that belong to this group formed 16.1% of the total isolates.

The species in Group IV belong to the gray series, their spore chain arrangement is spira, they are non-chromogenic and their spore wall surface is smooth. Species within this group are Stm. albogriseolus, Stm. aureofaciens, Stm. chibaensis, Stm. erumpens, Stm. griseolosuffuscus, Stm. griseoluteus, Stm. humidus, Stm. misionensis and Stm. recifensis. Stm. albogriseolus was able to utilize most of the carbon sources well but it had doubtful utilization of D-galactose. Stm. aureofaciens had doubtful utilization of D-mannitol and D-xylose, negative utilization of rhamnose, raffinose, inositol and salicin, and it had a positive utilization for the remaining five carbon sources. Stm. chibaensis was able to utilize all the carbon sources except salicin. Stm. erumpens had doubtful utilization of sucrose and fructose and was not able to use D-xylose, rhamnose and salicin. Stm. griseolosuffuscus had doubtful utilization of raffinose, D-mannitol, inositol and sucrose and was not able to utilize rhamnose and salicin. Stm. griseoluteus strongly utilized fructose and sucrose, was not able to utilize L-Arabinose, rhamnose, raffinose and salicin and had a doubtful utilization of inositol. Stm. humidus was not able to use raffinose and has a doubtful utilization of sucrose. Stm. misionensis was not able to use rhamnose and salicin and doubtfully utilized sucrose. Stm. recifensis was not able to use rhamnose and inositol. 37% of the total isolates belong to this group.

The only species in Group V is Stm. alboflavus and it belongs to the yellow series, has rectus-flexibilis spore chains, is chromogenic and it's spore wall surface is smooth. It was not able to utilize rhamnose and salicin and it's utilization of raffinose and sucrose was doubtful. 1.6% of the total isolates belong to this group.

The species in Group VI belong to the yellow series, their spore chain morphology is rectus-flexibilis, they are non-chromogenic and have smooth spore wall surface. Species within this group are Stm. finicarius,

Stm. intermedius and Stm. microflavus. Stm. fimicarius was not able to use inositol and salicin, while Stm. intermedius was not able to utilize rhamnose and inositol. They both had doubtful growth on raffinose and salicin. Stm. microflavus was not able to utilize L-arabinose, raffinose, i-inositol and salicin. The species that belong to this group form 4.8% of the total isolates.

The only species in Group VII is Stm. canarius. It belongs to the yellow series, the spore chain morphology is spira, it is non-chromogenic and has smooth spore surface. It has a wide sugar-spectrum by utilizing all the carbon sources except salicin which it utilizes doubtfully. Only 1.6% of all the total isolate belong to this group.

The species in Group VIII belong to the white series, their spore chain morphology is spira, they are non-chromogenic and have smooth spore surface. Stm. cacaoi, Stm. ochraceiscleroticus and Stm. spiroverticillatus belong to this group. Stm. cacaoi was not able to utilize rhamnose and inositol and had doubtful utilization of L-arabinose and sucrose. Stm. ochraceiscleroticus strongly utilized fructose and sucrose but had a doubtful use of inositol. Stm. spiroverticillatus was not able to utilize rhamnose, raffinose, galactose, mannitol and inositol. It had a doubtful use of salicin. This group formed 4.8% of the total isolates.

Table 4.8 The Carbon Utilisation Pattern of the Species within each group

			Utilization of Carbon Compound (P & G basal)											
Group	No.	Names of Species	No Carbon Source	D-Glu	D-Xyl	L-Ara	Rham	D-fruc	D-Gal	Raff	D-Man	Inositol	Salicin	Suc
I	1	Stm. fulvoviolaceus	-	+	+	+	+	+	±	+	+	+	+	+
	2	Stm. noboritoensis	-	+	+	+	-	+	+	+	+	+	+	±
II	3	Stm. gelaticus	-	+	+	-	+	±	+	+	±	-	+	+
	4	Stm. griseolus	-	+	+	+	-	+	+	+	-	-	-	+
	5	Stm. nigrifaciens	-	+	+	+	+	++	+	+	+	+	-	++
III	6	Stm. anandii	-	+	+	±	-	+	+	+	+	+	-	++
	7	Stm. cirratus	-	+	+	+	-	±	+	-	±	-	-	+
	8	Stm. galbus	-	+	+	+	-	+	+	-	+	+	+	-
	9	Stm. luteogriseus	-	+	+	+	+	+	+	+	+	+	+	+
	10	Stm. olivochromogenes	-	+	+	+	+	+	+	+	+	±	+	+
	11	Stm. rameus	-	+	+	+	-	+	+	+	+	-	+	+
IV	12	Stm. alboqriseolus	-	+	+	+	+	+	±	+	+	+	+	++
	13	Stm. aureofaciens	-	+	±	+	-	+	+	-	±	-	-	++
	14	Stm. chibaensis	-	+	+	+	+	+	+	+	+	+	-	+
	15	Stm. erumpens	-	+	-	+	-	±	+	+	+	+	-	±
	16	Stm. griseolosuffuscus	-	+	+	+	-	+	+	±	±	±	-	±
	17	Stm. griseoluteus	-	+	+	-	-	++	+	-	+	±	-	++
	18	Stm. humidus	-	+	+	+	+	+	+	-	+	+	+	±
	19	Stm. misionensis	-	+	+	+	-	+	+	+	+	+	-	±
	20	Stm. recifensis	-	+	+	+	-	+	+	+	+	-	+	+
V	21	Stm.alboflavus	-	+	+	+	-	+	+	±	+	+	-	±
VI	22	Stm. fimicarius	-	+	+	+	+	+	+	±	+	-	-	+
	23	Stm. intermedius	-	+	+	+	-	+	+	+	+	-	±	+
	24	Stm. microflavus	-	+	+	-	+	+	+	-	+	-	+	-
VII	25	Stm. canarius	-	+	+	+	+	+	+	+	+	+	±	+
VIII	26	Stm. cacaoi	-	+	+	±	-	+	+	+	+	-	+	±
	27	Stm. ochraceiscleroticus	-	+	+	+	+	++	+	+	+	±	+	++
	28	Stm. spiroverticillatus	-	+	+	+	-	+	-	-	-	-	±	+

KEY:

++	-	Strong utilization of carbon source
+	-	Utilization of Carbon source
±	-	Doubtful utilization of Carbon source
-	-	Non-utilization of Carbon source
D-Glu	-	D-gluctose
D-Fru	-	D-fructose
D-Xyl	-	D-xylose
Raff	-	Raffinose
Suc	-	Sucrose
L-Ara	-	L-Arabinose
D-Man	-	D-Mannitol
D-Gal	-	D-galactose
Rham	-	Rhamnose.

Table 4.9 shows the types of antimicrobial activities exhibited by the metabolic product of each species against the assay organisms. Inhibitory activities were observed in only 32.3% of the isolates. 67.7% of the isolates did not have any inhibitory activity on the assay organisms. Antibacterial and antifungal activities were exhibited by 16.1% and 9.7% of the isolates, respectively. Only 6.5% of the isolates exhibited broad spectrum of activity. 11.3% were active against both S. aureus and E. coli while only 4.8% were active on only S. aureus.

Organisms found to have broad spectrum activity were Stm. humidus, Stm. noboriteonsis and Stm. erumpens. Antibacterial activity was detected in Stm. fulvoviolaceus, Stm. griseolutens, Stm. cirratus, Stm. chibaensis, Stm. microflavus, Stm. ochraceiscleroticus and Stm. olivochromogenes. Stm. anandii, Stm. misionensis and Stm. intermedius were found to have antifungal activity. No antibiotic activity was detected for the remaining species.

4.3 Assay for possible Antibiotic activities

Table 4.9: The antimicrobial activities of the isolates species

Species	Inhibitory Activity		
	S. aureus	E. coli	C. albicans
Stm. humidus	+	+	+
Stm. noboritoensis	+	-	+
Stm. erumpens	+	+	+
Stm. fulvoviolaceus	+	+	-
Stm. griseoluteus	+	+	-
Stm. cirratus	+	-	-
Stm. chibaensis	+	-	-
Stm. olivochromogenes	+	+	-
Stm. microflavus	+	+	-
Stm. ochraceisclerotigus	+	-	-
Stm. misionensis	-	-	+
Stm. anandii	-	-	+
Stm. intermedius	-	-	+
Stm. griseolosuffuscus	-	-	-
Stm. alboflavus	-	-	-
Stm. canarius	-	-	-
Stm. recifensis	-	-	-
Stm. albogriseolus	-	-	-
Stm. luteogriseus	-	-	-
Stm. aureofaciens	-	-	-
Stm. rameus	-	-	-
Stm. galbus	-	-	-
Stm. spiroverticillatus	-	-	-
Stm. nigrifaciens	-	-	-
Stm. griseolus	-	-	-
Stm. fimicarius	-	-	-
Stm. cacaoi	-	-	-
Stm. gelaticus	-	-	-

KEY: + - Inhibition
- - No inhibition.

Plate 4.4 shows the various inhibitory activities of the metabolic product of the isolates using perpendicular streak method. In Plate 4.4(a), the metabolic product of the test organism which diffused into the agar inhibited the growth of the three assay organisms used in this study. This shows that the metabolic product has a broad spectrum of activity.

The metabolic product which diffused into the medium shown in Plate 4.4(b) was only active against the bacteria used in this study (E. coli and S. aureus). This means that the test organism in Plate 4.4(b) produce a metabolic product which has an antibacterial activity.

Both antibacterial and antifungal activities were observed on Plate 4.4(c). The test organism produced a metabolic product into the medium which inhibited Gram positive bacteria (S. aureus) and fungi (C. albicans). No inhibitory activity was observed against Gram negative bacteria (E. coli).

The metabolic product of the test organism in Plate 4.4(d) showed antifungal activity. No inhibitory activity was observed against both the Gram negative and Gram positive bacteria.

All the assay organism were found to grow well without any inhibitory activity from the metabolic product of the test organism. This shows that the metabolic product of the test organism had no inhibitory activity against the assay organisms as shown in Plate 4.4(e).

Plate 4.4(f) is the control and it shows that all the assay organisms grew well in the absence of the test organism.

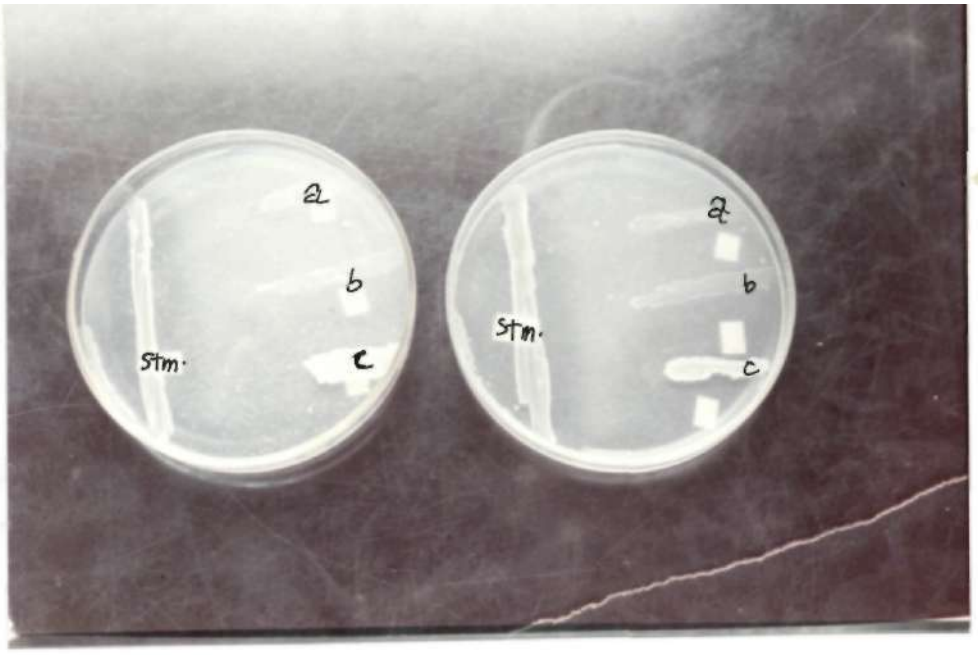


Plate 4.4(a) Broad Spectrum Activity.

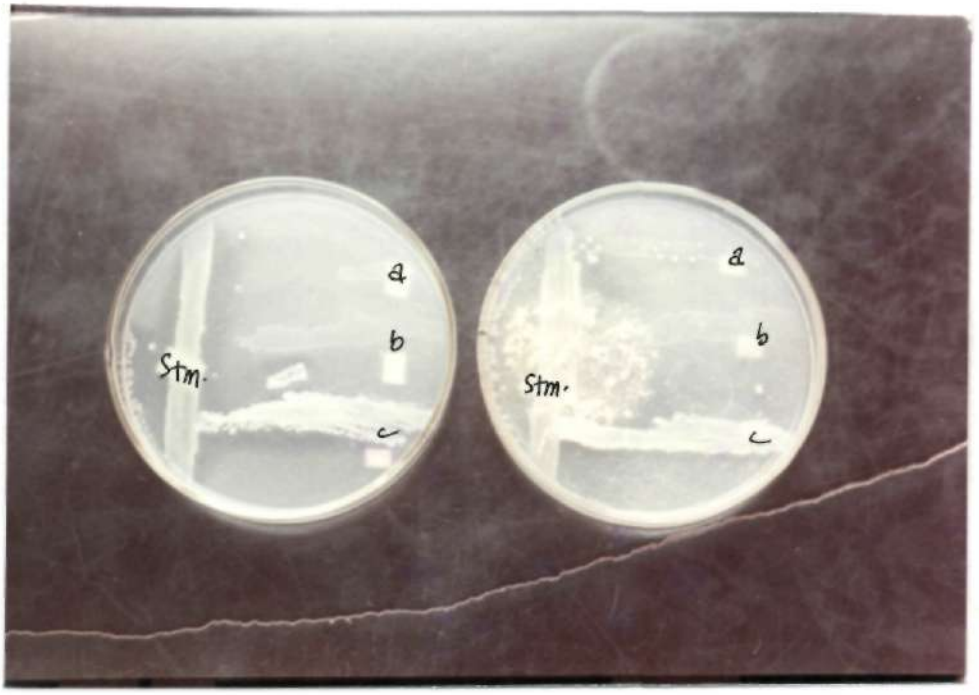


Plate 4.4(b) Antibacterial Activity

KEY: Stm. - Streptomyces

(a) - S. aureus

(b) - E. coli

(c) - C. albicans

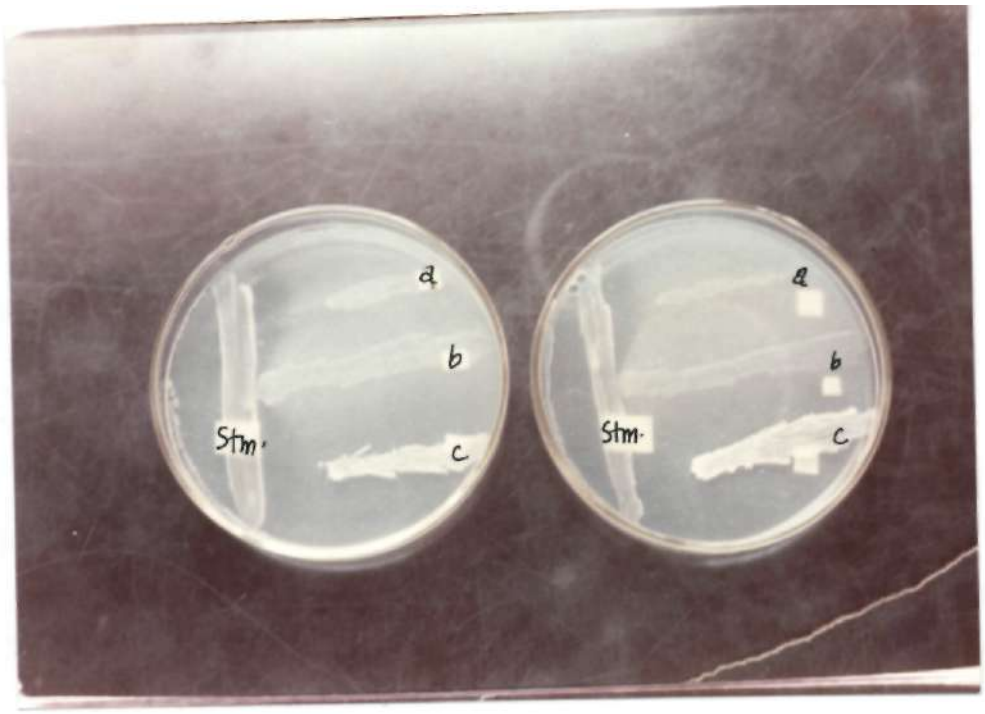


Plate 4.4(c) Both antibacterial (Gram positive bacteria) and antifungal activities.



Plate 4.4(d) Antifungal Activity.

KEY: Stm. - Streptomyces

(a) - S. aureus

(b) - E. coli

(d) - C. albicans

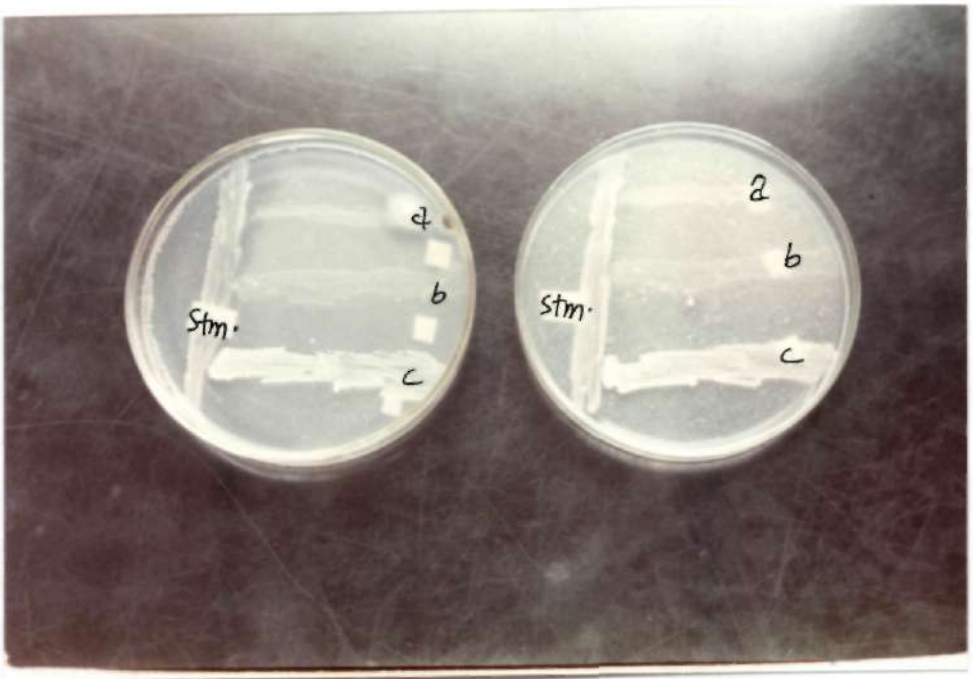


Plate 4.4(e) No inhibitory activity.

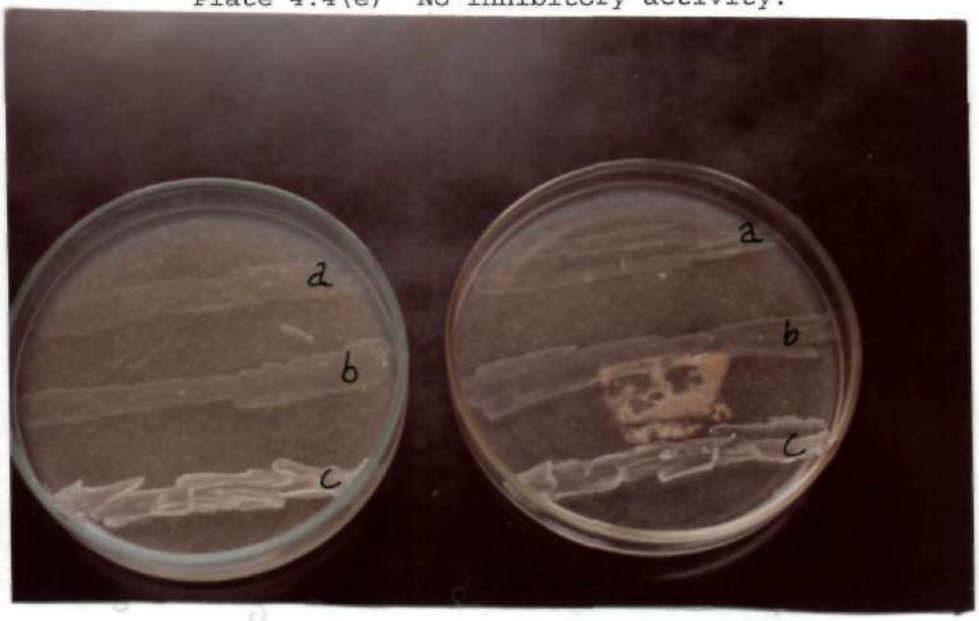


Plate 4.4(f) Control.

KEY: Stm. - Streptomyces

(a) - S. aureus

(b) - E. coli

(c) - C. albicans.

CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.1 DISCUSSION

It has now become important in the light of the economic situation worldwide that each nation starts to look inward, and identify what internal resources they have, develop then and make them viable source of revenue, so that it could be less dependant on other nations.

Micro-organisms have their own economic values. While some of them are very harmful, some are known to be very useful economically in the industries (such as food processing, fermentation, pharmaceuticals and oil industries etc).

Streptomyces spp are a large and highly important microbial group with numerous biochemical activities and great practical importance (Waksman, 1967). These organisms were first isolated in 1943 by Waksman and co-workers and were found to possess the ability to produce substances that are of great therapeutic values. Previous to the advent of drug synthesis, most of the aminoglycosides, polyene and macrolide antibiotics were obtained as by - products of the metabolic activities of Streptomyces (Egorov, 1957).

Streptomyces spp have been found to be predominant in the soil (Kuster, 1976). Thus soil samples were obtained in locations in Kaduna State and examined for Streptomyces. Isolates obtained were also tested for possible production of antimicrobial substances.

Table 4.1 shows the sources of the soil samples and the number of isolates obtained from each sample. It is important to note the predominance of this group of organisms in soil. Out of the 62 isolates obtained from the 15 soil samples, over 50% of them were isolated from the grassland soil while the remaining were isolated from the garden soil. This shows the richness of our soil for Streptomyces. It should be noted that, no isolate was obtained from the refuse dump sample. This might be due to the fact that, soils from such locations are too rich for the survival of this group of organisms (Kuster, 1976). When the

morphological and physiological characteristics of each isolate were compared with the description of the species of Streptomyces in Bergey's manual of Determinative bacteriology, 28 species of Streptomyces were obtained. The percentage of occurrence of each of the specie isolated is evenly distributed between the two major soil types (Table 4.2).

Table 4.2 also shows the various species of Streptomyces isolated in the order of their prevalence. It should be noted that most of these species occur in both soil types examined. Amongst the prevalent species, Stm. griseolus, Stm olivochromogenes and Stm. griseolosuffuscus were isolated solely from grassland while Stm. fulvoviolaceus and Stm. galbus were isolated from garden soils. Worthy of note is Stm. cacaoi which was isolated by Waksman (1961) from musty cocoa beans in Nigeria, but in this study it was isolated from a soil sample obtained from garden. Cocoa is grown in the western part of this country having a different vegetation from the northern part where this work is being done. Although it has been observed that occurrence of particular species of Streptomyces could be influenced by soil types (Kuster, 1976), this factor has been found not to play any role in the occurrence of Stm. cacaoi.

The ISP criteria used for characterization of the isolates was found very appropriate in speciating the isolates. It was also observed that 86.7% of the isolates predominantly belong to the gray colour series (Table 4.3). Gupta et al. (1991) also observed that the predominant colour series of all the species they isolated from the soil belong to the gray colour series.

In the determination of the ability of these organisms to produce melanin pigment, three types of media were used. These are tyrosine agar, Tryptone yeast broth and Peptone iron yeast extract agar. Only 23.3% of the isolates (8 species) were found to possess the ability to produce melanin pigment. It was also observed that using these three types of media, their pattern of chromogenicity varied. Some of the species produced pigments on only one of the media e.g Stm. fulvoviolaceus, Stm.

galbus, Stm. luteogriseus, Stm. anandii and Stm. alboflavus produced pigment only on tyrosine agar. Stm. cirratus and Stm. noboritoensis were found to be chromogenic on two of the media. Although these two different species of Streptomyces are capable of producing pigment on two of the test media, it is important to note that the chromogenicity characterization could be a useful tool of differentiating between Stm. cirratus and Stm. noboritoensis. This is because Stm. cirratus produced pigment on tyrosine agar and PIYEA while Stm. noboritoensis produces pigment on tyrosine agar and TYB. So the ability to produce pigment on either TYB or PIYEA could differentiate these two species. Table 4.5 shows the pattern of their chromogenicity. Since all of the chromogenic species produce pigment on tyrosine agar it is advisable to always use the other two media in addition to tyrosine agar so as to facilitate identification of some of the species of this group of organisms.

A reddish brown pigment was produced by all the chromogenic species on tyrosine agar. This indicates tyrosinase activity due to the formation of dihydroxyphenylalanine - melanin (red indole quinone) from tyrosine (Pometto III et al., 1985). This means that all the chromogenic species obtained in this study have the enzyme tyrosinase.

Using specifically, the ISP criteria in determining the colour series; spore chain arrangement, chromogenicity and the spore wall appearance (i.e the physiological and morphological characterization) it is possible to put all the isolated species into eight groups (Table 4.6). Actually in this method of grouping, the most important criteria to observe are the colour series, that is whether it is gray, yellow or white; the spore chain arrangement (Plate 4.2), that is, whether the arrangement is spira or RF; and the chromogenicity, that is, whether there is melanin production or not. The fourth criterion, that is, the spore wall appearance is the same in all the species. Although, Streptomyces spp are known to have either spiny, warty, hairy or smooth spore surface, all the isolates obtained in this study had smooth spore wall. This means

that the sheath covering the spore surface is smooth (Cross and Goodfellow, 1973).

Majority of the isolates in this work belong to group IV which represents 37% of the total isolates. These are the species which are gray, having spira spore chain arrangement and non-chromogenic. This group is followed by group III also gray in colour, spira in spore chain arrangement but are chromogenic. In groups I and II the difference also resides in their ability to produce melanin, but the two groups have the same colour and spore chain arrangement. Chromogenicity also differentiates group V from VI, but colour series differentiates group VII from VIII. These two groups are spira in their spore chain arrangement and are non-chromogenic but group VII is yellow while group VIII is white.

Although, grouping of species of Streptomyces has not been done by any of those who worked on this group of organisms, but in the course of this work it was observed that these morphological and physiological characteristics could serve as useful tools in breaking this large group of organisms into smaller easily manageable units.

Table 4.8 shows that speciation is even easier amongst each group when carbon utilization ability is determined. For example, group I consists of two species of Streptomyces and each of these species is seen to have its own specific carbon utilization pattern. Even amongst the species in the largest group (i.e group III) there is no two species with identical pattern of carbon utilization. This kind of grouping should be encouraged as done for the Enterobacteriaceae, since it will aid quick identification of the existing species as well as new ones. It is important that any work done on Streptomyces must incorporate as many carbon sources as possible, because in this work, it has helped to differentiate species. The differentiation was more pronounced in the utilization of salicin and rhamnose by species within the same group. It is therefore recommended for further works that salicin, rhamnose and inositol should be individually incorporated into P and G basal medium in

testing for their carbon utilisation pattern.

It was observed by Davies and Williams (1970) that the most frequently occuring Streptomyces in the soil are those that possess the ability to utilise a large number of different carbohydrates for their carbon sources. This is because their ability to utilise large number of carbon sources makes it possible for them to survive in their ecological environment. In this study, it was observed that Stm. nigrifaciens which had the highest percentage of occurence (16.7%) (Table 4.2) was able to utilise 10 of the 11 carbon sources tested (Table 4.8).

Many workers (Kuster, 1976) have used few number of carbon sources to test for carbon utilisation pattern of Streptomyces, but following the recommendation of Pridham et al., (1974), large number of carbon sources was used in this work (Table 4.8). Eight species were found to have the ability to utilise 10 - 11 carbon sources, nine utilised eight to nine carbon sources while the remaining 11 were only able to utilise five to seven carbon sources. According to Davies et al., (1970) all the eight species that are capable of utilising a great number of carbon sources are expected to have a high percentage of occurence in the soil, but in this work, it was observed that Stm. canarius, Stm. chibaensis and Stm. ochraceisclerotícus are exception to the rule. Even though these species of Streptomyces are capable of utilising up to 11 carbon sources, their percentage of occurence was found to be only 1.7% (Table 4.2).

It was not surprising to find that two of the isolates could not be placed under any species name. This is because it had been reported by Lessel et al., (1970) that taxonomically difficult strains, lacking certain characters or displaying unusual characters are often encountered in nature.

The screening of the metabolic products of the isolated species for their antimicrobial properties showed that 32.3% of the isolates are capable of producing substances that can inhibit other micro-organisms. Kuster (1976) reported that the highest antibiotic activity were exhibited

possess the highest antibiotic activity in this study were obtained from garden soils. Stm. humidus and Stm. erumpens were the only two species amongst the whole isolates that possessed wide spectrum antibiotic activity (Table 4.9). This two species of Streptomyces have been found to produce aminoglycoside and polyene antibiotics (Arai et al., 1976) but the group of antimicrobial substances produced in this study was not determined. The degree of the inhibitory action and the spectrum of activities of the metabolic products are shown in Plate 4.4(a).

The metabolic products that diffused from Stm. fulvoviolaceus, Stm. griseoluteus, Stm. olivochromogenes and Stm. microflavus into the medium are inhibitory to both Gram-positive and Gram-negative organisms (Plate 4.4(b). The activity of the metabolic product of Stm. noboriteonsis is mainly against Gram-positive and fungi (Plate 4.4(c) while the activity of products from Stm. cirratus, Stm. chibaensis and Stm. ochraceiscleroticus is mainly against Gram-positive organisms. Those from Stm. misionensis, Stm. anandii and Stm. intermedius are specific for the fungi (Plate 4.4(d).

Although greater percentage of these isolated species were found not to possess any antimicrobial activity, but in the literature, some of them were found to produce some antimicrobial substances (Pridham et al., 1974, Arai et al., 1976 and Egorov, 1985). For example, Stm. griseolosuffuscus was found to produce antimycobacterium agent (Arai et al., 1976), Stm. canarius was found to produce antiviral agent (Pridham et al., 1985). The inability to detect the antimicrobial activities of Stm. griseolosuffuscus and Stm. canarius in this study was because the organisms mycobacterium and virus that could have shown whether the metabolic products present in the medium have antimicrobial activity were not available for testing. Also neomycin was not detected in Stm. albogriseolus because it needs zinc to produce the antibiotic (Egorov, 1985) which was not available in the medium. The antimicrobial activity of Stm. aureofaciens which has been

reported to be a potent producer of chlortetracycline was not detected in this study, however, Waksman (1967) has reported that it also produces vitamin B_{12} which has been exploited in the industries to manufacture vitamin supplement.

5.2 CONCLUSION AND RECOMMENDATION

This study clearly shows that Streptomyces spp is prevalent in our soil as shown by the fact that Streptomyces spp were isolated from 86.7% of all the soil samples examined. The study also shows that 28 species of Streptomyces are available in our soil. The morphological and physiological characterisation of the isolates obtained in the study revealed that peculiar characteristics exist among Streptomyces spp. The grouping system used in this study was found to quicken the identification of Streptomyces spp and is therefore recommended for further works.

Although, only 32.3% of the total isolates were found to be antibiotically active, all the antibiotically active isolates were found to have either antifungal activity, antibacterial activity or broad spectrum activity.

In the light of the advantages that can be derived from this group of micro-organisms, it is recommended that the ability of these isolates of Streptomyces spp to produce antibiotics should be further investigated and exploited in the pharmaceutical industries, since some of the active antibiotic producing strains are available in Nigerian soil. This will reduce some of the problems faced by our pharmaceutical industries. It will also help to reduce the amount of money used to import drugs and the problem of bringing expired drugs to the country will be minimized. However, further work has to be done to quantity the amount of antibiotics produced, to modify the isolates genetically to produce more antibiotics, to maximise its production, to identify the antibiotics and test for the potency of the product.

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