EXPLORATION, ISOLATION, IDENTIFICATION AND SCREENING OF LOCAL ACTINOMYCETES STRAINS FOR INDUSTRIALLY USEFUL ANTIBIOTICS AND ENZYMES

THESIS TO BE SUBMITTED TO

GOA UNIVERSITY

FOR THE AWARD OF DEGREE OF

DOCTOR OF PHILOSOPHY

IN

BOTANY

579.37
PER-Vel EXP

BY

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CERTIFICATE

This is to certify that **MISS SONASHIA VELHO-PEREIRA** has satisfactorily completed the thesis entitled **"EXPLORATION, ISOLATION, IDENTIFICATION AND SCREENING OF LOCAL ACTINOMYCETES STRAINS FOR INDUSTRIALLY USEFUL ANTIBIOTICS AND ENZYMES"** submitted to Goa University for the award of the degree of **DOCTOR OF PHILOSOPHY IN BOTANY** is a record of original and independent work carried out during the period of February 2008 - 2014 in the **DEPARTMENT OF BOTANY, GOA UNIVERSITY** under my supervision and that it has not previously formed the basis for the award of any Degree, Diploma, Associate-ship or Fellowship or any other similar title to any candidate of this or any other University.

I affirm that the thesis submitted **by MISS SONASHIAVELHO-PEREIRA** is completely independent research work carried by her under my supervision.

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It is hereby declared that this thesis entitled **"EXPLORATION, ISOLATION, IDENTIFICATION AND SCREENING OF LOCAL ACTINOMYCETES STRAINS FOR INDUSTRIALLY USEFUL ANTIBIOTICS AND ENZYMES"** submitted to the Goa University, for the award of the degree of **DOCTOR OF PHILOSOPHY IN BOTANY** is a record of original and independent work carried out by me during February, 2008 - 2014, in the **DEPARTMENT OF BOTANY, GOA UNIVERSITY** under the supervision of **Dr. NANDKUMAR M. KAMAT**, Asst. Professor, Department of Botany, Goa University and that it has not previously formed the basis for the award of any Degree, Diploma, Associate-ship or Fellowship or any other similar title to any candidate of this or any other University.

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ACKNOWLEDGEMENT

'Sometimes our light goes out but is blown into a flame by another human being. Each of us owe our deepest thanks to those who have rekindled this light'

Albert Schweitzer

At the outset, I thank the Almighty for the wisdom, endurance and perseverance He has bestowed upon me. Without His innumerable blessings I would accomplish very little, as He is the one who gave me the strength in times of trials and difficulties.

I am fortunate and blessed to have the pleasure of working under the guidance of, Dr. Nandkumar M. Kamat, Asst. Professor in Botany at Goa University. I express my deepest gratitude to him. His untiring effort, constant encouragement, constructive criticism and inspiring guidance throughout the tenure of my research work have resulted in the successful and fruitful completion of a task. The trust, support and freedom which he provided, led me to implement ideas pertaining to the research summarized in this thesis.

I owe my thanks to the Head of the Department of Botany, Prof. M. K. Janarthanam for all his assistance, support and co-operation. My sincere thanks to the entire faculty of the Department of Botany namely Prof. P. K. Sharma, Prof. B. F. Rodrigues, Prof. S. Krishnan and Assoc. Prof. V. U. Kerkar for all their willingness to help whenever approached. I am also grateful to Prof. D.J Bhat, Former Head, Department of Botany, for all his help.

A special thanks to the members of the Faculty Research committee, Prof. G.N. Nayak (former Dean, Faculty of Life Science), Prof. S. Bhosale (Dean, Faculty of Life Science) and V.C.'s nominee, Dr. S Kerkar for their valuable suggestions.

My sincere gratitude goes out to Prof. S. Tilve, Head, Department of Chemistry, Goa University for his valuable suggestions and permitting me the use of FTIR instrument. Special thanks to Mr. Budhu for his assistance in operating the FTIR instrument.

Heartfelt thanks to Prof. I. J. Furtado, Department of Microbiology for allowing me to avail the facilities of her laboratory. Special thanks to Prof. S. Bhosale former Head, Department of Microbiology for permitting the use of UV-VIS spectrophotometer.

I am indebted to Dr. L. D'Sousa, Scientist and Head of the Bio-Organic Chemistry Lab, CSIR- National Institute of Oceanography, Goa for permitting the use of all the facilities in her Department. Words fall short to express my sincere gratitude to Dr. S. Tilvi, Scientist at the same Department for all her valuable guidance, assistance in chromatography and valuable insights in the structural elucidation of the compound. Special thanks to Dr. M. Majik for his timely help and cooperation whenever needed. A word of thanks to all the project assistants namely Harshada, Shweta, Deepika, Stacy and Cheryl for all their support and cooperation.

I express my sincere gratitude to Dr. K. Prasad, Scientist, Institute of Bioinformatics, Bangalore and Dr. A. Ozarkar, Scientist, Molecular Biology Research Laboratory, University of Pune for their timely help and assistance. Thanks to the Head, Department of Microbiology, Goa Medical College, Dr. S. Rodrigues for the clinical bacterial pathogens.

I thank the entire non teaching staff of Department of Botany. Special thanks to Mr. Vithal for assisting me with all the chemicals, glassware and other

miscellaneous laboratory items. A word of thanks goes out to Mr. Tari, Mrs Nutan, Mr. Goankar and Mr Dileep.

I also thank all the research scholars, project assistants and M.Sc. students past and present from the Department of Botany, for all the support given during this tenure and not forgetting the light moments we shared, which were real stress busters. Special thanks to my Ph.D. colleagues and project assistants of Mycolab namely Dr. Pratibha, Dr. Ashish, Dr Sarita, Mrs. Marina, Indira, Neysa, Priyanka, Harshal, Jenica and Sonia for their encouragement, timely help and friendly support.

A big thank you to research scholars, Ravikiran, for his assistance in the use and interpretation of MEGA v.5 software; Dr. Subhajit, for all his timely help and suggestions; Sanika, for her help in SDS-PAGE and Vera, for her help in the use of UV-VIS spectrophotometer. Special thanks to research scholars Dr. Baskaran, Dr. Prachi, Dr. Trelita, Kashinath and Durga for all their help whenever needed.

Special thanks to Dr. James for all his advice, insights, help, constant encouragement and most of all for having given me a patient listening ear when I needed it the most. A big thank you to Dr. Chandrashekar, Bombay College of Pharmacy for all his advice and help whenever needed. I owe my gratitude to Dr. S. Shetgoankar, Research Scientist, Syngenta-Goa for all his help. A word of thanks to Mr. Pai for all his help.

I duly acknowledge the monitory support by Goa University for the period January 2009 – March 2011 and UGC-JRF/SRF, Maulana Azad National Fellowship for the period April 2011 – February 2014.

Heartfelt thanks to my sisters Reelma, Sheryanne, Nellie and brother in law Rahul for their cooperation, patience, concern, affection which provided me a lot of encouragement when things were going weary.

Last but not the least I would like to thank my mother and father, as I owe everything I am today because of them. No words can eloquently describe my heartfelt gratitude, love and respect for them. I dedicate this Thesis work to them.

"You raised me up, so I can stand on mountains;

You raised me up, to walk on stormy seas;

I am strong, when I am on your shoulders;

You raised me up... To more than I can be"

Dedicated to my parents

Mr. Rafael Velho- Pereira

Mrs. Maria Fátima Velho-Pereira

With fondest love and affection

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Figure 4.22 Effect of different concentrations of $CaCO_3$ (%) as inorganic nitrogen source on antibiotic production

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Figure 4.26a&b Antimicrobial activity against *Shigella flexneri* where S1, Chloroform; S2, Methanol; S3, Butanol; S4, Propanol; S5, Aniline; S6, Acetone; S7, Ethyl acetate and S8, n-hexane and S1C-S8C represents the control (pure solvents)

Figure 4.27a&b Antimicrobial activity against *S. typhi* where S1, Chloroform; S2, Methanol; S3, Butanol; S4, Propanol; S5, Aniline; S6, Acetone; S7, Ethyl acetate and S8, n-hexane and S1C-S8C represents the control (pure solvents)

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Figure 4.29a&b Antimicrobial activity against *S. citreus* using various solvents **Figure 4.30** FTIR spectrum of the methanolic extract of CFCF of *Streptomyces parvulus* CFA-9

Figure 4.31a-d UV-Visible spectral data of four bioactive spots [**a)** SB, **b)** SE, **c)**, SH, and **d)** SI]

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4.34b The elucidated structure of Streptolydigin compound

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Brightfield and Phase Contrast Microscopy (PC) along with optical slicing revealed very clearly the appearance of actinomycetes fructifications, **a-d** -Branching, aerial hyphae with long spore chains that could be categorized as *spirales* on basis of their characteristic morphology, **e & f**- Actinomycetes spores

Chapter III

PLATE 3.1

Actinomycetes strains showing positive amylase activity

a-*Actinomadura* sp. MMA 1-06, **b**-*Streptomyces* sp. CFA-5, **c**-*Streptomyces* sp. BOA-2, **d**- *Streptomyces* sp. MMA 1-02, **e**-*Streptomyces* sp. PM 1-03, f-*Actinomadura* sp. MMA 1-03, **g**-*Actinomadura* sp. MMA 1-04, **h**-*Streptomyces* sp. CFA-1, **i**-*Streptomyces* sp. MMA 1-05

PLATE 3.2

Actinomycetes strains showing positive gelatinase activity

a-*Streptomyces* sp. CFA-3, **b**-*Streptomyces* sp. CFA-4, **c**-*Streptomyces* sp. CFA-6, **d**-*Streptomyces* sp. BOA-1, **e**-*Streptomyces* sp. BOA-5, **f**- *Actinomadura* sp. TA-3, **g**-*Streptomyces* sp. BOA-4, **h**-*Micromonospora* sp. MMA 2-02

PLATE 3.3

Control: Basal medium (AVA) including calcium penthatonate

a- Basal medium (AVA) excluding calcium penthatonate, **b**- Basal medium (AVA) excluding calcium penthatonate and addition of 0.01M $CaCl_2.H_2O$, **c**- Basal medium including calcium penthatonate and including 0.01M $CaCl_2$. H_2O, **d**- Basal medium (AVA) including calcium penthatonate and excluding 0.01M $CaCl_2$. H_2O

Chapter IV

PLATE 4.1

a-Test plate showing the central well, filled with inoculum of actinomycetes strain, surrounded by 8 wells of 0.01M $CaCl_2.2H_2O$ and corresponding 8 wells filled with inoculum of test pathogens, **b**-Control plate 1 showing the central well, filled with inoculum of actinomycetes strain , surrounded by 8 wells of 0.01M $CaCl_2.2H_2O$ and corresponding 8 wells filled with sterile distilled water, **c**-Control plate 2 showing the central well, with sterile distilled water, surrounded by 8 wells of 0.01M $CaCl_2.2H_2O$ and corresponding 8 wells filled with inoculum of test pathogens, **d**-Control plate 3 showing the central well, filled with inoculum of actinomycetes strain, surrounded by 8 wells of sterile distilled water and corresponding 8 wells filled inoculum of test pathogens

PLATE 4.2

a-y Calcium oxalate crystals in the shape of druzes. Magnification at 40 X

ab-pq Rhomboidal shaped calcium oxalate crystals

PLATE 4.3

a-Anti *S. citreus* activity using standard ICOO2 antibiotic discs (Hi Media, Mumbai, India)

b-Anti *S. citreus* activity using standard ICOO3 antibiotic discs (Hi Media, Mumbai, India)

PLATE 4.4

a-TLC plate with the marked spots (S_A - S_I), **b**-Bioautographic -TLC plate showing zone of clearance

CHAPTER I

Introduction and Literature Survey

Preface

1.1 The rationale for the study

The screening of microbial natural products continues to represent an important route to the discovery of novel chemicals for the development of new therapeutic agents (Bull *et al.*, 2000), primarily antibiotics (Singh *et al.*, 2006; Vasavada *et al.*, 2006), immunosuppressive agents, enzymes (Lam, 2006) and enzyme inhibitors (Pandhare *et al.*, 2002). Amongst prokaryotes, Actinomycetes, remain an unique source of natural products (Bérdy, 2005; Newman & Cragg, 2007; Goodfellow & Fiedler, 2010). Actinomycetes are an integral part of the indigenous soil microflora (Goodfellow & Williams, 1983; Ul-Hassan & Wellington, 2009; Goodfellow *et al.*, 2010) and account for about 45 % of all bioactive secondary metabolites with 7,600 of them (30 %) coming from *Streptomyces* strains (Bérdy, 2005). Novel antibiotics are continuously in demand due to the inevitable rise of antibiotic-resistant strains of pathogenic bacteria, reducing morbidity, mortality and life's expectancy (Fischbach & Walsh, 2009). However, antibiotic screening particularly from actinomycetes, had declined since 1990's, as culture extracts yielded unacceptably high numbers of previously described metabolites, necessitating development of methods to access rare or novel taxa and alternative antimicrobial screening strategies (Mincer *et al.*, 2002; Strobel & Daisy, 2003; Gavrish *et al.*, 2008). Consequently, actinomycetes are back in favour and the problem of rediscovery of known metabolites has being addressed by isolating actinomycetes from underexplored habitats (Bredholdt *et al.*, 2007; Hong *et al.*, 2009; Okoro *et al.*, 2009); by developing innovative strategies or methods to selectively isolate uncommon and rare actinomycetes from soil (Tan *et al.*, 2006; Gontang *et al.*, 2007); by exploration of new compounds from Genus S*treptomycetes*, with the fact that only a small range of their biosynthetic capacity been currently

exploited (Zahner & Fielder, 1995) and by screening for novel, broad-spectrum, partly anti resistant antimicrobial peptide antibiotics (Dubin *et al.*, 2005).

While most of the studies on actinomycetes have focused on antibiotic production, there are relatively few reports regarding their enzymatic potential (Metha *et al.*, 2006). In recent years, the possibility of obtaining high yields of commercially useful enzymes from actinomycetes has attracted attention. They are preferred over other microbial sources as they possess greater stability, activity at high temperatures and unusual substrate specificity (Prakash *et al.*, 2013). Hydrolytic enzymes account for 85 % of all industrial enzymes of which amylolytic enzymes represent about 25-33 % of the world enzyme market (Gangadharan *et al.*, 2006). These enzymes are been widely used in food, fermentation, starch sacccharification, pharmaceutical, detergent, textiles and paper industries and have also expanded their frontiers in the field of clinical, medical and analytical chemistry. The state of Goa has unique ecological niches and is rich in its biodiversity (Alvares & Gadgil, 2002). However its microbial diversity is unexplored, leaving a vast gap in our understanding of the actinomycetes diversity especially in various soil types. There is also no evidence in literature to suggest that these indigenous soil actinomycetes have been explored for their antibiotics and enzyme production potentials. Yet, the sustainable exploitation of actinomycetes for enzyme and antibiotic production holds considerable promise to the pharmaceutical industries and medicine to compact prevailing human pathogens in Goa-India and in the world at large.

1.2. General introduction on Actinomycetes

Actinomycetes are a diverse group of Gram-positive bacteria, with high guanine (g) plus cytosine (c) ratio in their DNA (>55 mol %), they are non-motile,

have non-sporing, non-capsulated filaments that break up into bacillary and coccoid elements (Goodfellow & Williams, 1983). A typical actinomycetes colony is shown in **Figure 1.1** .

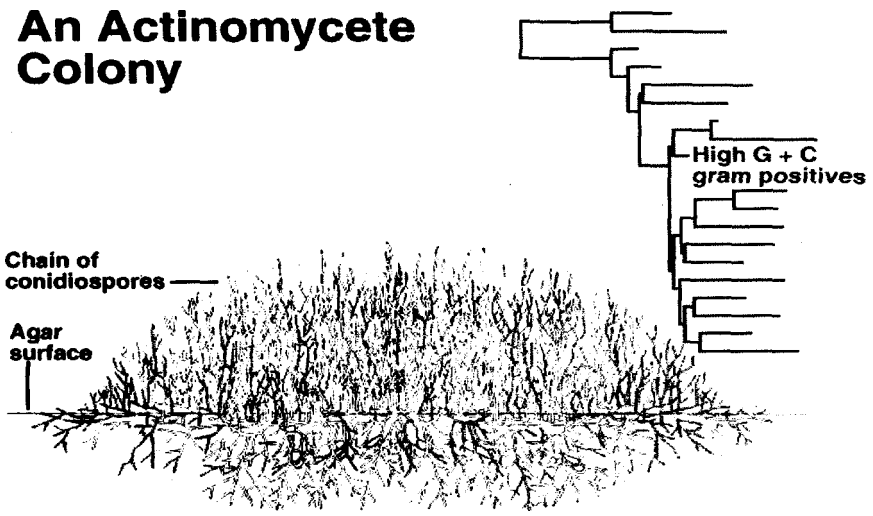

Figure 1.1 A typical actinomycetes colony (Source: Prescott *et al.*, 1999)

Due to high GC content in their DNA, they show various metabolic possibilities and been exploited in the pharmaceutical industry for their seemingly unlimited capacity to produce secondary metabolites with diverse chemical structures and biological activities (Shantikumar *et al.*, 2006).

The name "actinomycetes" was derived from Greek "atkis" (a ray) and "mykes" (fungus), and has features of both bacteria and fungi (Das *et al.*, 2008). The actinomycetes were placed in a separate phylogenetic line, believed to be more closely related to fungi than to bacteria. The spores of actinomycetes and its germination resemble those of fungi. The vegetative growth in surface and submerged culture is fungus like and colonies of actinomycetes and their mode of growth are more fungus like than bacteria (Waksman, 1963).

But now it has been proven that they comprise an ubiquitous order of bacteria which exhibit wide physiological and morphological diversity. The various bacterial properties of actinomycetes are: diameter of hyphae is about 1μ (Williams *et al.*, 1989); thin cell walls containing muramic acid; have prokaryotic nuclei (Ananthanarayana *et al.*, 2005); flagella when produced are of bacterial type, strict anaerobiosis (some forms), chemoautotrophy (some forms), sensitive to phages; sensitive to antibacterial antibiotics; lack of sterols and lysine synthesis through the diaminopimelic acid pathway (Gottlieb *et al.*, 1973).

1.2.1 *Actinomycete diversity in natural habitats*

Actinomycetes are widespread in nature and have been recovered from wide variety of sources, where they live as saprophytes, symbionts, parasites or even pathogens (Shirokikh & Merzaeva, 2005; Ward & Bora, 2006). Culture-dependent and culture-independent approaches are widely used to study prokaryotic communities in natural habitats (Bull *et al.*, 2000; 2005). Actinomycetes can be isolated from terrestrial or marine environments indicating their distribution all over the world (Fenical & Jensen, 2006). Actinomycetes populations have been identified as one of the major groups of the soil microbial community (Jayabarath *et al.*, 2010), where they play a crucial role in the recycling of refractory biomaterials by decomposing complex mixtures of polymers in dead plant, animal and fungal materials producing several volatile sesquiterpene substances namely, geosmins, responsible for the characteristic "wet earthy odor". Another role is its effect on the soil structure, wherein the clay particles are bound by the hyphal threads which imparts a granular viable structure that is conducive to crop production. Actinomycetes may grow on root fragments, soil crumbs and twigs, but they probably

exist mostly as spores. They remain dormant, sometimes being transported by the soil fauna, rainwater or air, until stimulated to grow and sporulate by suitable nutrients (Lacey, 1973). The common genera of actinomycetes in the order of abundance in soils are *Streptomyces* (nearly 70 %), *Micromonospora* and *Nocardia* although *Actinoplanes*, *Streptosporangium* and *Micromonospora* are also generally encountered. The abundance of actinomycetes in the soil microflora depends on many factors such as soil depth, water contamination, pH value, organic matter content, season and agricultural treatment and conditions in the microenvironment where actinomycetes are growing (Lacey, 1973).

The major population of actinomycetes (~80 %) is found in the surface layer (0-10 cm) and they gradually decrease as the depth increases. However sampling of lower layers may also result in isolating unique microorganisms (Yuzuru *et al.*, 1992). Actinomycetes usually prefer neutral or slightly alkaline soil however; although a few are able to grow in acidic soils of pH 5.

1.2.2 *Actinomycetes taxonomy*

The phylum *Actinobacteria* represents one of the largest taxonomic units among the 18 major lineages currently recognized within the domain bacteria (Ventura *et al.*, 2007). They belong to subclass *Actinobacteridae*, which includes the order *Actinomycetales* whose members are commonly referred to as actinomycetes. Thirty nine families have been recognized with the most common ones being *Actinomycetaceae*, *Actinoplanaceae*, *Dermatophilaceae*, *Frankiaceae*, *Mycobacteriaceae*, *Micromonosporaceae*, *Nocardiaceae*, and *Streptomycetaceae* comprising altogether 147 genera (Garrity *et al.*, 2004). Taxonomic ordering using Bergey's Manual of Systematic Bacteriology (Garrity *et al.*, 2004) showed that these

genera represent 14 different families and seven suborders under two subclasses **(Figure 1.2)**.

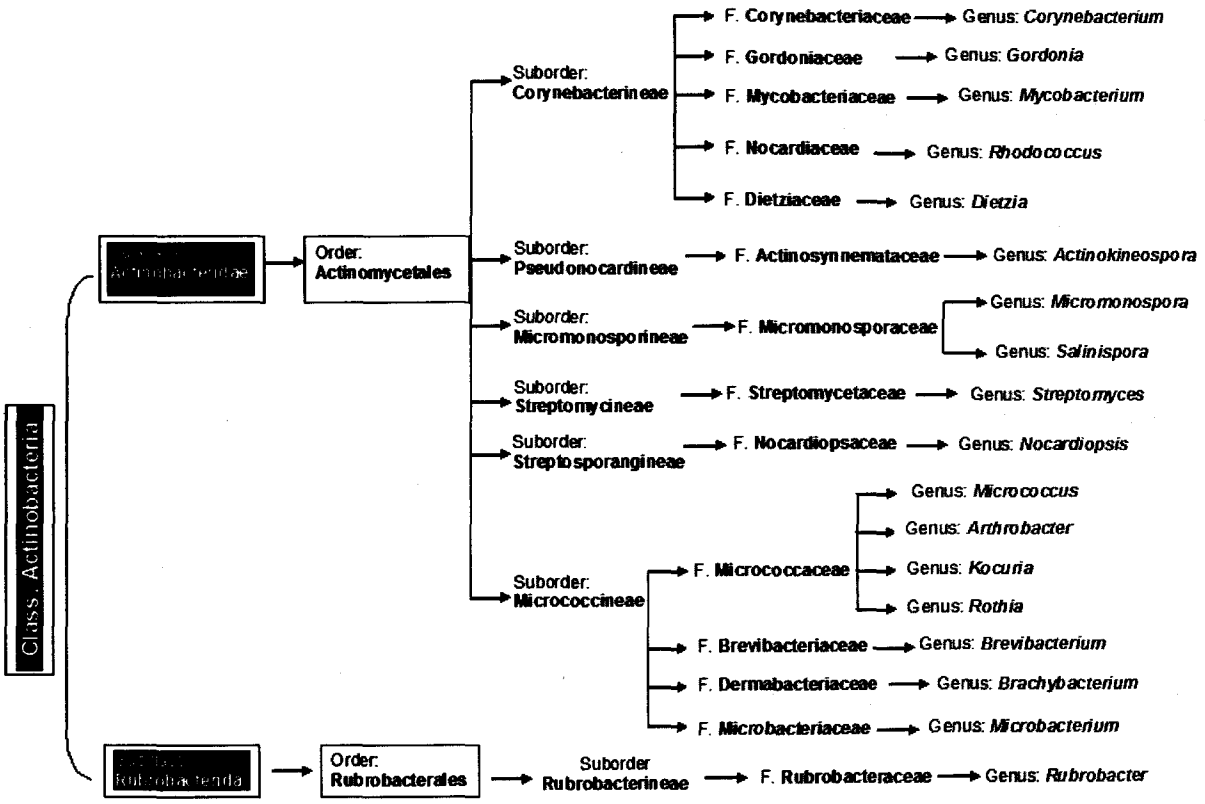

Figure 1.2 Taxonomic positioning of actinomycete strains (Source: Garittty *et al.*, 2004)

Though there have been attempts using taxonomic tools to map out the genera and the subgroups of actinomycetes (Goodfellow *et al.*, 1989), the most valuable information for the construction of a concise genera tree of actinomycetes came from the partial sequence analysis of 16S rRNA (Keiser *et al.*, 2000) **(Figure 1.3).**

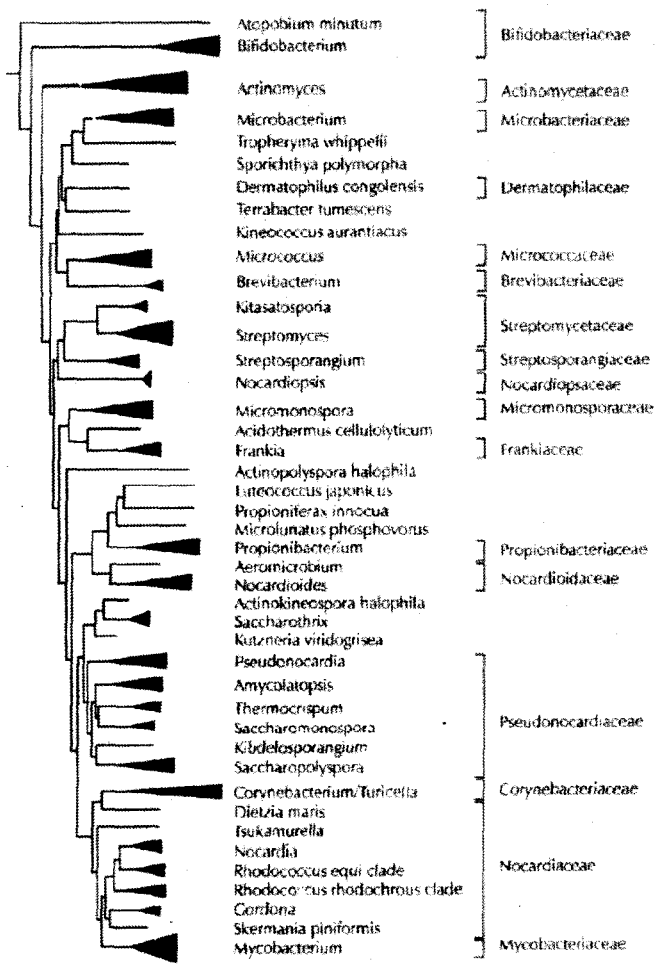

Figure 1.3 Phylogenetic tree of actinomycetes constructed by using 16S ribosomal RNA sequences (Keiser *et al.*, 2000)

Actinomycetes are metabolically and morphologically more complex than other bacteria. Genera of actinomycetes are distinguished on the basis of their morphology and marker chemical constituents of the cell wall, membranes and whole cell hydrolysates. They are characterized by their diverse shapes and colors, formation of a network of aerial and substrate hyphae as well as production of melanoid pigments of various colors (*i.e.*, greenish brown, brownish black or distinct brown) on the medium (Sykes & Skinner, 1973).

1.2.3 *Generic groups of Actinomycetes* (Syker & Skinner, 1973)

i) Nocardioform actinomycetes: This is a heterogenous group which forms filaments and fragment into shorter elements. Aerial growth is formed by some genera and may also produce chains of spores. Genera are distinguished primarily the presence or absence of mycolic acids and other chemical characters.

Subgroup 1: Mycolic acid-containing bacteria (Genus – *Gordona, Nocardia, Rhodococcus* and *Tsukamurella*); Subgroup 2: *Pseudonocardia* and related genera (Genus – *Actinobispora, Actinokineospora, Actinopolyspora, Amycolata, Pseudoamycolata, Kibdelosporangium, Saccharomonospora* and *Saccharopolyspora*). Subgroup 3: *Nocardioides* and *Terrabacter*; Subgroup 4: *Puomicromonospora* and related genera (Genus – *Jonesia, Oerskovia*)

ii) Genera with multilocular sporangia: These genera form filaments that divide by longitudinal and transverse septa. They produce large number of coccoid-like elements, which may be motile (*Dermatophilus, Geodermatophilus*) or non-motile (*Frankia*).

iii) Actinoplanes: Stable filaments are formed, with little or no aerial growth. Motile spores are produced in sporangia. (*Actinoplanes, Ampullariella, Dactylosporangium, Pilimelia*), non-motile spores are produced singly (*Micromonospora*) or in chains (*Catellatospora*). Cell wall contains meso-DAP while glycine, arabinose and xylose are found in whole cell hydrolysates.

iv) Streptomycetes and related genera: A heterogenous group, all of which have cell wall containing L-DAP and glycine. Stable filaments are formed and may produce extensive aerial growth with long spore chains (*Streptomyces, Streptoverticillium*). Other genera (*Intrasporangium, Kineospora Sporichthya*) produce little or no aerial growth and have a variety of spore forms.

v) Maduromycetes: Stable filaments are formed and produce spore bearing aerial growth. Short chains of non-motile arthrospores are produced by *Microbispora* (two spores), *Microtetraspora* (four spores), and *Actinomadura* (varying number). Other genera produce spores in sporangia which are motile (*Planobispora, Planomonospora, Spinillospora*) or non-motile (*Streptosporangium*). The cell walls contain meso-DAP, and cell hydrolysates contain madurose.

Subgroup 1: Streptosporangium and related group (Genus – *Microbispora, Microtetraspora, Planobispora, Planomonospora, Spirillospora, Streptosporangium*);

Subgroup 2: *Actinomadura*

vi) Thermomonospora and related genera: Stable filaments are formed and produce aerial growth bearing spores that are single (*Thermomonospora*), in chains (*Actinosynnema, Nocardiopsis*), or in sporangia-like structures (*Streptoalloteichus*). The cell wall contains meso-DAP, but have no characteristic amino acids or sugars in the whole cell hydrolysates.

vii) Thermoactinomycetes: This comprises only one genus, *Thermoactinomycetes*. The stable filaments produce aerial growth. Single spores (which are endospores) are formed on both aerial and vegetative filaments. All species are thermophilic. The cell wall contains meso-DAP but no characteristic amino acids or sugars.

viii) Other genera: This group comprises three genera (*Glycomyces, Kitasatosporia* and *Saccharothrix*) that cannot at present be assigned to other groups. They all produce aerial growth bearing chains of spores.

1.2.4 *Genus Streptomyces*

The genus *Streptomyces* was proposed by Waksman & Henrici (1943). They are well known by the presence of a linear chromosome with high GC content, complex morphological differentiation and an ability to produce many bioactive secondary metabolites of pharmaceutical importance (El-Gendy *et al.*, 2008) and are thus considered to be a rich biotechnological resource. Streptomycetes are classified within the Gram-positive bacteria and form together with the genera *Kitasatospora* (Ōmura *et al.*, 1982) and *Streptacidiphilus* (Kim *et al.*, 2003) the family *Streptomycetaceae*. The latter family constitutes a separate phylogenetic branch within the phylum *Firmacutes*, class *Actinobacteria*, order *Actinomycetales*, suborder *Streptomycineae* based on 16S rRNA gene sequence analysis (Stackebrandt *et al.*, 1997). There are more than 600 described species in these genera (http://www.bacterio.cict.fr/).

Streptomyces has a complex life cycle, which begins with the formation of branching hyphal filaments and ends with the formation of spores from the branching hypha of these mycelial structures. Aerial growth is achieved at the expense of the substrate mycelial biomass, undergoes extensive lysis and reuse to fuel the reproductive phase (Wildermuth & Hopwood, 1970; Migu´elez *et al.*, 1999). The few milligrams (wet weight) of the colony's substrate mycelium are thereby converted into ten million spores. The spores represent the semi-dormant stage of the life cycle.

There are three separate phases of the developmental life cycle of *Streptomyces* **(Figure 1.4)**. The cycle begins with a spore, also called arthrospore or conidium. Germination of these spores starts under favourable environmental conditions to give rise to an extensive branching network of multinucleoid hyphae known as the substrate (primary) mycelium. These are approximately 0.5-1.0 μm in

diameter, often lack cross-walls and grow at the apices. Differentiation starts with the formation of specialized aerial hyphae (secondary mycelium), which grow in air away from the surface of the colony. Later these aerial hyphae undergo septation into uninucleoid compartments and metamorphose into chains of spores (sporophores) that rarely fragment. The number of spores in a chain varies (3 to many). For some species e.g. former *Chainia* spp. (Goodfellow *et al.*, 1986), sclerotia may be formed, reaching up to 100 μm in diameter and consisting of dense masses of enlarged, branching hyphae. Finally, non-motile spores are released into the environment. The onset of differentiation is frequently associated with the production of pigments, antibiotics and other secondary metabolites.

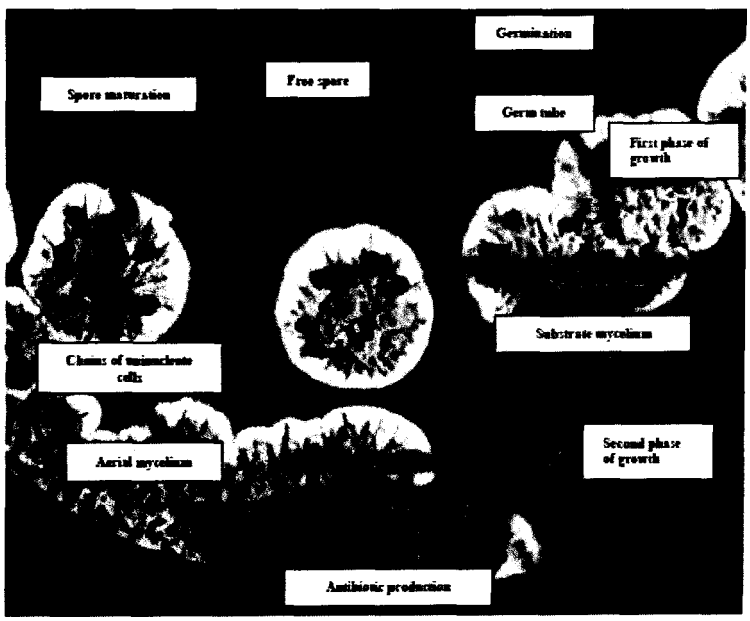

Figure 1.4 Genus *Streptomyces* life cycle (Source: http://homepage.smc. edu/ishida_carolyn)

Streptomyces, shows some distinctive properties when compared to other actinomycetes. The morphological heterogeneity of this genus is unique and has always been used for classification. Color of substrate and aerial mycelium, configuration of spore chains and spore ornamentation are used as taxonomic markers.

All of them are determined using cultivation on standard media and fixed incubation times (Shirling & Gottlieb, 1966). A system of color wheels has been devised for accurate determination of the color of mycelium (Tresner & Backus, 1963). Configuration of spore chains is determined by electron microscopy and is assigned to one of the following categories: straight, flexuous, spirales and verticilliate **(Figure 1.5)**. Spore ornamentation is either smooth, spiny, rugose or hairy.

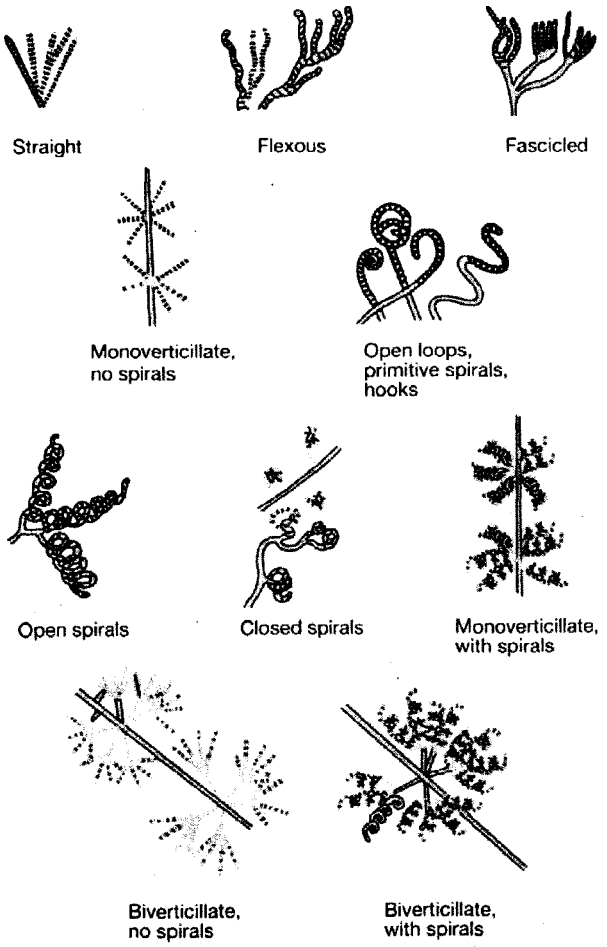

Figure 1.5 Different configuration of *Streptomyces* spore chains (Source: Gottlieb *et al.*, 1973)

1.3 Enzymological research

Enzymology is an important and oldest field of science. The global industrial enzyme market has evolved enormously with an estimated market of $7 billion in 2013 (http://www.reportlinker.com/p0747897-summary/). Highest growth is likely in developing countries of Asian, African and Mideast regions, along with Latin America and Eastern Europe, with great demand for diagnostic and therapeutic enzymes (Dewan, 2011).Technical enzymes such as leather and bioethanol markets and food and beverage industries are expected to have a turnover of $2.2 billion and $2.1 billion in 2016 respectively.

Hydrolases cover more than 75 % of commercially used enzymes of which proteases are extensively used in detergent industry followed by amylases (starch industry) in enzyme and textiles, baking, food and animal feed industries. Enzymes with unique properties like high productivity, specificity, stability at extreme temperature, pH or other physiological conditions, low cost of production, and tolerance to inhibitors are mostly sought to meet the industrial demand. A proper understanding of production biochemistry, the fermentation processes and recovery methods has lead to producing industrial enzymes affordably. Enzymes find great use in food, dairy, pharmaceutical, detergent, textile and cosmetic industries, due to different transformations and other enzyme mediated reactions that they catalyze which are alternatives to tedious and expensive chemical methods.

Research on industrial enzymes is progressing which is reflected through the number of ever increasing patents filed for various enzymes. Maximum number of patents has been issued for proteases followed by amylases and cellulases (Prakash *et al.*, 2013).

Novel strategies for selective isolation of potential microorganisms are being adopted. According to Kurtböke, (2012) a proper understanding of ecophysiological insight and subsequent habitat stimulation would be helpful in successful isolation of potent enzyme producers. Besides, an innovative way leading to development of high throughput screening programs in enzymological research would also be: exploring neglected or previously unexplored habitats such as extremobiosphere or microbes like extremophiles or endophytes; developing isolation procedures with design of habitat-based media; use of more efficient identification systems to determine novelty such as analysis of ribosomal proteins by use of MALDI-TOF Mass Spectrometry and rDNA technology; use of Flourescence activated cell sorting (FACS) and Gel MicroDrop technology for sorting of desired clones from a genomic library, wherein for FACS, fluorescent substrate specific for a particular enzyme is used and positive fluorescence indicates biocatalytic activity of the clone (Short & Keller, 2001). The he latter technique captures the fluorescence emitted due to catalytic breakdown of biotinylated substrate by the clone (Keller *et al.*, 2003); Adopting metagenomics for rapid screening of unculturable microbes which is achieved by use of substrate-induced gene expression screening (SIGEX), preamplification inverse-PCR (PAIPCR), metagenomic DNA shuffling; use of "directed evolution" approach, wherein mutants are screened from a library of genetic variants (Olsen *et al.*, 2000); inclusion of ultra high throughput FACS-based screening which aids in the rapid screening of the variant library (Yang & Withers, 2009); use of drop-based microfluidics to screen mutants (Agrestia *et al.*, 2010); use of reporter gene technology such as Green fluorescent protein (GFP) for detection and acquiescence with host systems (Paley *et al.*, 2013); use of ionization techniques include electrospray ionization mass spectrometry (ESI-MS) to measure femtomole quantities

of proteins (Smith *et al.*, 2013); use of *in silico* techniques to sort potent enzymes from database such as predictive 3DQSAR, CoMFA and CoMSIA which predict superior enzymes based on structural properties and microenvironment provided for *in silico* reaction with substrate (Murumkar *et al.*, 2009). Thus all these advanced technologies coupled with a sound understanding of the ecotaxonomical perspective can lead to isolation of superior strains producing enzymes with premium properties.

Although, India is rich in biodiversity and offers excellent human resource power, it imports 70 % of the total enzyme consumed by its market. Thus, there is a pressing need of indigenous manufacturers and technology, strong R&D in terms of investment and strong legislation and IPR regulations (http://ec.europa.eu/environment/archives/dansub/pdfs/enzymerepcomplete). The details of enzymological research in India are given in **section 1.5.2.3** of this Chapter.

1.3.1 *Actinomycetes as source of industrially usefully enzymes*

Microbes are currently employed for the production of various industrial enzymes due to their ease of growth, nutritional requirements and downstream processing. Majority of the industrial enzymes are of microbial origin. Among the microbes, actinomycetes hold a prominent position because of their diversity and proven ability to produce various industrial enzymes. A large number of enzymes produced by actinomycetes are of potential interest in areas of biotechnology such as enzyme processes, clinical chemistry and medical therapy. They are preferred over other sources as they possess greater stability, activity at high temperatures and unusual substrate specificity. In recent years, the possibility of obtaining high yields of commercially useful enzymes from actinomycetes has attracted attention and several screening methods have been described for detecting suitable strains

(Williams & Cross, 1971). Some industrially important enzymes from actinomycetes are discussed below.

1.3.1.1 *Xylanases*

Xylans are hydrolyzed mainly by β-1,4-endoxylanases (EC 3.2.1.8) and xylosidases (EC 3.2.1.37). Xylan degrading enzymes are of potential biotechnological importance in utilizing xylan containing wastes to produce xylose and other important industrial chemicals. They are also used for conditioning of dough, digestibility and bleach boosting in baking, animal feed and paper pulp industries. Their applications in the chlorine bleaching of kraft pulps, use in recovering fermentable sugars from hemicelluloses and their possible use in recovering cellulosic textile-fibers from ramie or flax has also been reported (Berens *et al.*, 1996).

Among the prokaryotes, certain actinomycetes have been shown to be highly efficient in xylan degradation (McCarthy & Cross, 1984) although, their xylan-degrading systems are clearly complex and non-uniform (Ball & Mc Carthy, 1989). Xylanases of actinomycetes are mainly reported from mesophilic strains, particularly *Streptomyces* (Holtz *et al.*, 1991) which are preferred in treatment of rice straw pulp to improve the pulp bleach ability. This preference is due to absence of cellulase contamination in the xylanase and also due to reduced usage of chemicals during bleaching and pulping (Rifaat *et al.*, 2006). Although, fungi are highly explored for xylanase production as compared to actinomycetes, they often cosecrete cellulases, which affect pulp quality adversely (Christov *et al.*, 1999). *Streptomyces* spp. were able to produce high levels of xylanase when untreated rice straw was utilized which resulted in significant biobleaching (Rifaat *et al.*, 2006). Similarly, *Streptomyces* sp.

could hydrolyze various agricultural residues like oil cake and straw waste which resulted in increased biogas production (Priya *et al.*, 2012).

Further, xylanases from actinomycetes are stable on kraft pulps and can be used in the crude form thereby making the process economical. Xylanases with high thermostability, specific activity and pH stability are being reported from *Streptomyces* sp. (Priya *et al.*, 2012), *Actinomadura* sp. FC7 and *Nonomuraea flexuosa* (Brzezinski *et al.*, 1999; Zhang *et al.*, 2011), *Thermomonospora* and *Saccharomonospora* (Ristroph & Humphrey, 1985) and recombinant strain (Fagerstrom *et al.*, 2008). Thermostability, optimum pH, enzyme dosage and dispersal, consistency and reaction time are desirable factors influencing for development of suitable xylanase as a pre-bleaching agent (Leskinen *et al.*, 2005). Besides alkaline xylanases may have the ability to convert lignocellulosic wastes to fermentable sugars, a desired feature for industries.

1.3.1.2 *Pectinases*

Pectinases are a heterogeneous group of related enzymes that hydrolyze pectin substances. According to the cleavage site, pectinases are divided into three groups: 1) hydrolases consisting of polygalacturonase, PG (EC 3.2.1.15); 2) lyase/trans-eliminases comprising pectinlyase, PNL (EC 4.2.2.10) and pectate lyase, PL (EC 4.2.2.2); 3) pectin esterase, PE (EC 3.1.1.11) (Yadav *et al.*, 2009). Pectinases are upcoming industrially important enzymes and are produced by variety of microorganisms.

Actinomycetes strains such as *Streptomyces* sp. (Khalil *et al.*, 2000; Chander *et al.*, 2004; Suneetha & Khan, 2011), *S. lydicus* (Jacob & Prema, 2006), *S. viridochromogenes* (Agate *et al.*, 1962) and *Thermomonospora flisca* (Stutzenberger,

1987) producing pectinases have been reported. Occurrence of pectin degrading genes in few actinomycetes suggests their characterization could perhaps yield pectinases with novel properties. Application of pectinolytic enzyme is varied and their usage in industries has promising applications. Food processing enzymes involving pectinases account for about 45 % of enzyme usage and are used particularly in clarification of fruit juices, in degumming of fibres, wine making, and retting of bast fibres. The polygalacturonase degrade pectins by the hydrolysis of α-1,4-glycosidic linkages and are extensively used in the clarification of fruit juice and the extraction of oils from vegetable and citrus peels. A potential application of pectinolytic enzyme systems is in the removal of xylan from kraft pulp fibers and in the processing of plant fibers such as flax, hemp and ramic. Pectinases in conjuction with other enzymes are used in textile processing and bioscouring of cotton fibers, purication of plant viruses. Using xylanases and pectinases for debarking and processing of wood (Khalil *et al.*, 2000) has also been reported. Other applications include waste water treatment, coffee and plant fermentation, paper and pulp industry and animal feed.

1.3.1.3 *Proteases*

Proteases account for upto 25 % of all commercial enzymes used in the world and are thus regarded as important class of industrial enzymes. It is estimated that two thirds of the industrially produced proteases are from a microbial source (Gerhartz, 1990). Proteases produced from actinomycetes particularly *Streptomyces* is advantageous as compared to others as they are easily secreted into the media for further extraction and purification (Phadatare *et al.*, 1993). Porto *et al.*, (1996) studied *Streptomyces clavuligerus* cultures for protease production.

Proteases find application in various food, pharmaceutical and detergent industries depending upon the type being produced. Alkaline proteases are used in detergent powders and in food processing. According to a study, most of the proteases reported from *Streptomyces* spp. are alkali-tolerant (Horikoshi, 1999). Acid proteases are used for meat tenderization, for production of fermented foods by moulds from soybean, rice and others cereals (Nout & Rombouts, 1990). They are also used in the baking industry for the modification of wheat proteins whereas in the dairy industry they are used in the manufacture of cheese. Proteases from *Nocardiopsis* spp. are employed as detergents additives (Moreira *et al.*, 2002) and for the depilation of hides and skins in the leather industry. In conjunction with other enzymes, proteases from actinomycetes (*Microbispora* sp.) are used for recovery of antioxidants from shellfish waste (Jaouadi *et al.*, 2010). Besides the end products of protein hydrolysis rich in amino acids and peptides serve as a low cost animal feed.

1.3.1.4 *Ligninase*

Lignin peroxidase (LiP, EC. 1.11.1.14.), commonly known as ligninase is the most important lignin degrading enzyme followed by laccase (EC.1.10.3.2.) and manganese peroxidase (MnP, EC.1.11.1.13.). Actinomycetes are biodegradative, terrigenous prokaryotes secreting a range of enzymes active against lignocelluloses. Since their primary carbon sources are insoluble and polymeric, they necessitate secretion of range of extracellular enzymes with their hyphae penetrating and colonizing the substrate. Some microorganisms secrete a range of enzymes that completely degrade all the components of lignocellulose (lignin, hemicelluloses and cellulose), while others secrete a narrower range of enzymes that only partially achieve this degradation. Actinomycetes as a preferred choice for ligninase

production is governed by its ease in strain improvement by genetic manipulation and large-scale cultivation, also their growth as branching hyphae is well adapted to the penetration and degradation of insoluble substrates such as lignocellulose. The biochemistry of lignin degradation by actinomycetes remains poorly understood and the enzymes involved have yet to be identified which can be achieved by the application of the recombinant DNA techniques (Niladevi & Prema, 2005). Crawford *et al.*, (1983) were among the first researchers to work with actinomycetes for extracellular LiP. MnP is least studied in actinomycetes, with the exception of few reports by Esposito *et al.*, 1998 in *Streptomyces* sp. Ligninolytic producing *Streptomyces* strains were reported by Ramachandra *et al.*, 1988; Adhi *et al.*, 1989; Lodha *et al.*, 1991. The enzymes involved in lignocellulose breakdown by thermophilic actinomycetes in particular, still remain elusive. Use of genetic manipulation studies and complete and well annotated genome sequencing of thermophilic actinomycetes followed by in-depth investigation of candidate genes, can help understand the lignin degradation processes. Screening for ligninolytic actinomycetes by using chemiluminescence analysis system has also been done (Mercer *et al.*, 1996).

Lignin degrading enzymes find wide application in paper industry due to the biodegradation of lignocellulose in the field of bioremediation (Mason *et al.*, 2001). Acinomycetes with proven ability to solubilise grass lignins may have a role in humification. Biotechnological applications of lignocellulose conversion have also been reported.

1.3.1.5 *Amylases*

Amylases are enzymes which hydrolyse starch molecules to give diverse products including dextrins and progressively smaller polymers composed of glucose units (Windish *et al.*, 1965). They are divided into α-amylase, β-amylase and glucoamylase (GA)] and are among the most important enzymes in present-day biotechnology and account for about 30 % of the world's enzyme production (van der Maarel *et al.*, 2002). Alpha amylases (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) are enzymes that catalyses the hydrolysis of internal α-1,4-glycosidic linkages in starch, glycogen, etc. and hydrolyze this bond to produce α-anomeric mono- or oligosaccharides (Prakash & Jaiswal, 2012). Most of the α-amylases are metalloenzymes, which require calcium ions (Ca^{2+}) for their activity, structural integrity and stability. In starch processing industry, enzymatic hydrolysis is preferred to acid hydrolysis due to specificity of the reaction, stability of the generated products, lower energy requirements and elimination of neutralization steps (Satyanarayana *et al.*, 2005). Due to the increasing demand for these enzymes in various industries, there is enormous interest in developing enzymes with better properties such as raw starch degrading amylases suitability for industrial applications and their cost effective production techniques (Burhan *et al.*, 2003).

Structure of α-Amylases

The structure consists of a single polypeptide chain folded into three domains called A, B, and C **(Figure 1.6)**.

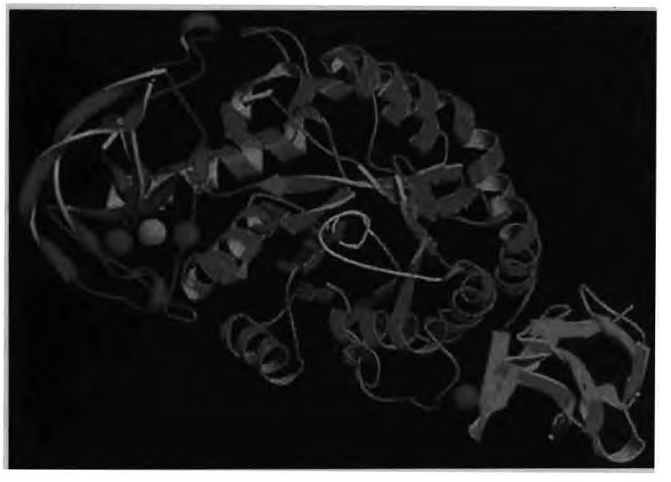

Figure 1.6 The domain organization of α-amylase. Domain A is shown in green, domain B is shown in magenta, and domain C is shown in cyan (Source: Prakash & Jaiswal, 2012).

The catalytic domain A consists of N-terminal (β/α)8 barrel which is made up of a highly symmetrical fold of eight parallel β-strands arranged in a barrel encircled by eight α-helices. This (β/α)8 barrel has first been observed in chicken muscle triose phosphate isomerase (TIM) (Banner *et al.*, 1975) and is therefore called the TIM barrel. Domain B is formed by a protrusion between the third β-strand and the third α-helix of the TIM barrel. It has a rather irregular β-rich structure and varies substantially in size and structure among the α-amylases (Svensson & Soggard, 1991). Domain C constitutes the C-terminal part of the sequence and is a β-sandwich domain containing a Greek key motif (MacGregor, 1988). Both domains B and C are located roughly at opposite sides of the TIM barrel.

Most of the microbial alpha amylases belong to the family 13 glycosyl hydrolases, group of enzymes and is the largest family of glycoside hydrolases, transferases and isomerases comprising of nearly 30 different enzyme specificities. Though they all catalyse the cleavage of the α-glucosidic bonds in the same substrate, structurally and mechanistically they are quite different. They have therefore been classified into different GH families: GH13 - α-amylases, GH14 - β-amylases, and GH15 - glucoamylases (Henrissat, 1991).

Glycoside hydrolases are able to metabolize a large variety of saccharides and most of starch converting enzymes belong to this family. GH-13 family can be classified based on a larger unit called clan, which is the three dimensional structure of catalytic module. A clan may consist of two or more families with the same three-dimensional structure of catalytic domain but with limited sequence similarities, indicating that protein structure is better preserved by evolution than amino acid sequence. Among the fourteen clans (A–N) defined for glycosidases and transglycosidases, α -amylase family (GH-13) belongs to the eighth clan, clan GH-H (MacGregor, 2005).

The common features of the α-amylase family (Reddy *et al.*, 2003; Janeček, 2009) are summarized below:

The enzymes act on the α- glycosidic bonds (not only the α-1,4- and α-1,6-linkages); and hydrolyse this bond to produce α-anomeric mono- or oligosaccharides or transglycosylation activity where α-1-4 or 1-6 glycosidic linkages are formed or a combination of both activities.

- They employ the retaining reaction mechanism.

- They contain from 4 up to 7 conserved sequence regions. Some of these conserved amino acids form the catalytic site and some are involved in the stability of the conserved TIM barrel topology.
- They possess a (β/α)8 or TIM barrel structure containing the catalytic site of the aspartate residue near the end of the strand β4 (catalytic nucleophile), glutamate residue near the end of the strand β5 (proton donor) and aspartate residue near the end of the strand β7 (transition-state stabiliser).

Sources of α-amylase

Alpha amylase can be derived from several sources such as plants, animals and microbes. Among the microbes, enzymes from fungal and bacterial sources have dominated applications in industrial sectors (Fukusumi *et al.*, 1988).The microbial enzyme meets the industrial demands a large number of them are available commercially and have almost replaced chemical hydrolysis of starch processing industry (Pandey *et al.*, 2000). The use of microorganisms for amylase production is advantageous as they are economical in bulk production, cost effective, consistent, less time and space is required and they also easy to manipulate so as to obtain enzymes of desired characteristics (Lonsane & Ramesh, 1990). Both fungal and bacterial systems are widely used for the production of α-amylases but bacterial systems are preferred because of several characteristic advantages such as survival in low moisture content and ease in isolation of thermostable microorganism (Pandey *et al.*, 2000). Although *Bacillus* sp. are widely exploited genus for production of α-amylase, actinomycetes as sources of α-amylase are of current interest. Several strains of actinomycetes belonging to *Streptomyces* sp. (Poornima *et al.*, 2008; Kar & Ray, 2008); *Micromonospora vulgaris* (Longinova *et al.*, 1970); *Nocardia asteroids*

(Stevens *et al.*, 1994); thermostable actinomycetes including *Thermonospora* and *Thermoactinomycetes* are versatile producers of amylases (De Souza & De Oliveira e Magalhães, 2010). Microbial amylases have completely replaced chemical hydrolysis of starch in starch processing industry (Pandey *et al.*, 2000) and have a broad spectrum of industrial applications as they are more stable as compared to plant and animal amylases (Tanyildizi *et al.*, 2005).

Applications of α -amylases

Alpha–amylases have several applications in industrial sectors such as food, fermentation, textile, paper, detergent, and pharmaceutical industries. With the advent of new frontiers in biotechnology, the spectrum of amylase application has also expanded into many other fields, such as clinical, medicinal and analytical chemistries (Pandey *et al.*, 2000).

a) *Starch liquefaction and saccharification*-The major market for α-amylases is in starch liquefaction process wherein starch hydrolysis converts starch into fructose and glucose syrups (Nielson & Borchert, 2000). The high sweetening property of fructose corn syrups (HFCS) is used in the beverage industry as sweeteners for soft drinks (Pandey *et al.*, 2000).

b) *Food industry*-Amylases are extensively employed in processed food industry such as baking, brewing, preparation of digestive aids, production of cakes, fruit juices and starch syrups (Couto & Sanroman, 2006). Microbial α-amylases have been widely used in the baking industry (Pintauro, 1979) and are beneficial as natural products free of chemical additives. Alpha-amylase supplementation in flour enhances the rate of fermentation, reduces the viscosity of dough, generates additional sugar in the dough, reduces the shelf life of these products thereby improving the taste, crust colour and

toasting qualities of the bread and also preventing it from staling (Van Dam & Hille, 1992)Amylases are also used for the clarification of beer or fruit juices, or for the pretreatment of animal feed to improve the digestibility of fiber (Gavrilescu & Chisti, 2005; Ghorai *et al.*, 2009; van der Maarel *et al.*, 2002). Alpha-amylase also carries out the reactions of alcoholysis by using methanol as a substrate (Santamaria *et al.*, 1999). In the chocolate industry, cocoa flavored syrups having a high cocoa content and excellent stability and flow properties at room temperature are produced by using an amylase.

Thermophilic and acidophilic amylases which can find applications in bakery, brewing and alcohol industries have been studied from *Streptomyces erumpens* (Kar & Ray, 2008). Thermostable amylases are reported from *Nocardiopsis* sp. which has important applications in bakery and paper industries (Stamphord *et al.*, 2001). The amylase from *Thermobifida* sp. produces maltotriose as the major end product from refined starch and raw sago starch (Yang & Lui, 2004).

c) *Textile industry*- Alpha-amylases are used in textile industry for desizing process which involves the removal of starch from the fabric which serves as the strengthening agent to prevent breaking of the warp thread during the weaving process. It also randomly cleaves the starch into dextrins that are water soluble and can be removed by washing (Hendricksen *et al.*, 1999).

d) *Paper industry*- Alpha-amylase has been used for the production of low viscosity; high molecular weight starch for coating of paper (Bruinenberg *et al.*, 1996).The coating treatment makes the surface of paper sufficiently smooth and strong, thus improving its writing quality. Examples of amylases obtained from microorganisms used in paper industry include Amizyme® (PMP Fermentation Products, Peoria,

USA), Termamyl®, Fungamyl, BAN® (Novozymes, Denmark) and α-amylase G9995® (Enzyme Biosystems, USA) (Saxena *et al.*, 2004).

e) *Detergent, building product and feed industries*-In detergent industries, α-amylases are widely used for laundry and automatic dishwashing to degrade the residues of starchy foods such as potatoes, gravies, custard, chocolate, etc. to dextrins and other smaller oligosaccharides (Olsen & Falholt, 1998; Mukherjee *et al.*, 2009). The main advantage of enzyme application in detergents is due to much milder conditions than with enzyme free detergents, it enhances the detergents ability to remove tough stains and also making the detergent environmentally safe (Van Ee *et al.*, 1992).

f) *Analysis in medicinal and clinical chemistry*-There are several processes in the medicinal and clinical areas that involve the application of amylases. The application of a liquid stable reagent, based on α-amylase for the Ciba Corning Express clinical chemistry system has been described (Beck *et al.*, 1995). Biosensors with an electrolyte isolator semiconductor capacitor (EIS-CAP) transducer for process monitoring are also been developed (Menzel *et al.*, 1998). The other applications of α-amylases include, use of amylase for serum evaluation (Sutton *et al.*, 1999); for developing a liquid-stable reagent containing α-amylase for the new Olympus AU6004 chemistry analyser (Lepp *et al.*, 1996); for detecting higher oligosaccharides, a method more efficient than the silver nitrate test (Giri *et al.*, 1990); for preparing a hybrid membrane using chitosan as a dispersant in the sol–gel process; as an enzyme thermistor for the biochemical analysis of cyclodextrins (Kolb *et al.*, 1996) and use of revised amylase method for the DuPont Dimension2 clinical chemistry system (Chiu & Chandler,1995).

1.4 Antibiotics

The term antibiotic is defined as "a chemical substance derived from microorganisms, which has the capacity of inhibiting growth and even destroying other organisms in dilute solutions" (Waksman *et al.*, 1942). Antibiotic drug discovery is a process to combat the emerging infectious diseases (Jose & Jebakumar, 2013). Since the inception of the first antibiotic, penicillin, in the 1940s, life expectancy has improved tremendously. Natural products play a major role in the development of new therapeutics such as: cholesterol lowering statin drugs (e.g., lovastin); antibiotics such as macrolides (erythromycin and its derivatives, including ketolides), b-lactams (including penicillins and cephalosporins), aminoglycosides (gentamicin, tobramycin and others), glycopeptides (vancomycin, teichoplanin and related molecules), lipopeptides (daptomycin), ansamycins (rifamycin), tetracyclines and many more; antimalarials (e.g., artemisinin, quinine) and anticancer agents (e.g,. paclitaxel, vinblastine, doxorubicin), and immunosuppressants (e.g. cyclosporine) (Baltz, 2006).

The natural products are mainly produced from primary and secondary metabolism of plants and microorganisms. Among all the natural products being reported, 20-30 % show biological activity, of which 10 % have been obtained from microbes. Microbes alone have yielded 22,500 compounds of which, 45 % are produced by actinomycetes, 38 % and 17 % are being produced by fungi and other bacteria respectively (Demain & Sanchez, 2009).

Drugs of natural origin have been classified as i) original natural products; ii) products derived or chemically synthesized from natural products or iii) synthetic products based on natural product structures (Demain & Sanchez, 2009). The major focus of current pharmaceutical research and development is on improvement of

natural product-based screening rather than relying on synthetic alternatives. This is mainly because, natural products inherently have higher molecular weights; more stereogenic centers; fewer rotatable bonds; larger and more complex ring systems; lower nitrogen, sulfur, and halogen content; higher oxygen content, more hydrogen bond donors and acceptors as compared to synthetic compounds. Using these characteristics and modifying them could potentially create analogues of bioactive compounds. Besides, natural products also yield higher bioactivity and have greater potency than synthetically produced small molecule libraries (Batlz, 2005).

1.4.1 *Different classes of antibiotics and their mechanisms of action*

There are several major classes of antibiotics that are categorized based on their mode of antibacterial action.

1.4.1.1 *Inhibitors of cell wall synthesis*

The β-lactams and the glycopeptides are two major groups of cell wall synthesis inhibitors.

The β-lactams include the penicillins, cephalosporins and the carbapenems. These drugs bind to the penicillin binding proteins (PBP's) that cross-link strands of peptidoglycan in the cell wall. Their action in Gram negative cells causes the spheroplasts to become fragile and get easily ruptured whereas in Gram positive cells, autolysis is triggered by the release of lipoteichoic acid (Greenwood, 2000).

The glycopeptides include vancomycin, avoparcin and others that bind to acyl-D-alanyl-D-alanine and prevent addition of new subunits to the growing peptidoglycan cell wall. Due to the large size of these molecules they are excluded

from gram negative cells by the outer membrane, thereby limiting their action to only gram positive organisms (Bryan, 1984).

1.4.1.2 *Inhibitors of protein synthesis*

There are many types of antibiotics that inhibit bacterial protein synthesis by taking advantage of the structural differences between bacterial and eukaryotic ribosomes. The three major groups of aminoglycosides are the streptomycins, neomycins and kanamycins. These drugs enter bacterial cells by an active transport that involves quinines. Streptomycins act by binding to the 30S ribosomal subunit whereas kanamycins and neomycins bind to both the 50S subunit and to a site on the 30S subunit different from that of streptomycin (Greenwood, 2000). Activity involving initiation complexes and cell membrane proteins that contribute to cell death plays a role in the action of these antibiotics, but this is poorly understood (Bryan, 1984; Greenwood, 2000). Chloramphenicol is a broad-spectrum antibiotic that, although naturally occurring, is produced by chemical synthesis. Chloramphenicol inhibits peptide bond formation on 70S ribosomes (Bryan, 1984). The tetracyclines are another group of broad-spectrum antibiotics that inhibit bacterial protein synthesis. They are brought into the cell by active transport and once they bind to the 30S subunit they prevent binding of aminoacyl tRNA (Roberts, 1996). The macrolides are a group of antibiotics commonly used to treat Gram positive and intracellular bacterial pathogens. Erythromycin was the first of these (Gaynor *et al.*, 2003), and several other important macrolides have been discovered since, including clarithromycin and azithromycin. It was originally believed that erythromycin inhibited protein synthesis by competing with amino acids for ribosomal binding sites, but newer research show that several mechanisms are involved (Garrod & O'Grady,

1971). The macrolides are now believed to promote dissociation of tRNA from the ribosome, inhibit peptide bond formation, inhibit ribosome assembly and prevent amino acid chain elongation (Gaynor *et al.*, 2003). The streptogramins are another class of antibiotic that inhibits bacterial protein synthesis, mostly in Gram positive organisms (due to decreased permeability of the gram negative outer membrane). These antibiotics are actually combinations of structurally different drugs; types A and B that act synergistically. These compounds bind to separate sites on the 50S subunit. Type A drugs block attachment of substrates at two sites on the 50S subunit, whereas type B drugs cause release of incomplete protein chains. The synergistic effect arises from a conformational change induced by the binding of a type A drug which significantly increases affinity of type B drugs. Streptogramins currently in use include virginiamycin, pristinamycin and quinupristin/dalfopristin (Gangle, 2005).

1.4.1.3 *Inhibitors of nucleic acid synthesis*

The sulfonamides and the diaminopyrimidines both indirectly inhibit nucleic acid synthesis by inhibiting folate synthesis. Folate is a coenzyme necessary for the synthesis of purines and pyrimidines. Although both types of drugs are useful on their own, they exhibit a synergistic effect when combined. Sulfonamides are currently not used commonly in medicine, but the combination drug trimethoprim-sulfamethoxazole is sometimes used in the treatment of urinary tract infections. Sulfonamides serve as an analogue of *p*-aminobenzoic acid; therefore, they competitively inhibit an early step in folate synthesis. Diaminopyrimidines, of which trimethoprim is the most common, inhibits dihydrofolate reductase, the enzyme that catalyzes the final step in folate synthesis (Greenwood, 2000).

1.4.1.4 *Nonribosomal peptides and polyketides*

Nonribosomal peptides and polyketides are very large classes of natural products which are assembled from simple acyl-coenzyme A or amino acid monomers (Marahiel *et al.*, 1997; Shin, 2003). They have a broad range of biological activities and pharmacological properties and are a source of novel antibiotics, cytostatics and immunosuppressants (Rascher *et al.*, 2003; Schwarzer *et al.*, 2003; Bergmann *et al.*, 2007). Non-ribosomal peptides and polyketides are synthesized by one or more large, specialized, multifunctional, modular enzymes, namely nonribosomal polypeptide (NRPS) and polyketide (PKS) synthases, respectively (Schwarzer *et al.*, 2003). NRPS and PKS catalyse chain elongation from simple building blocks to generate diverse natural products (Staunton & Weissman, 2001. Three PKS types have been described though only types I and II are of major interest (Shen, 2003). Type I PKS's are common in bacteria, including actinomycetes (Staunton & Weissman, 2001) and type II PKS's, which are multienzymes, carry a single set of iteratively acting activities, as illustrated by the biosynthesis of aromatic polyketides, tetracenomycin (Shin, 2003). Type III PKS types, known as chalcone synthase-like PKS's, are homodimeric enzymes which are mainly acting condensing enzymes (Watanabe *et al.*, 2003).

1.4.2 Antibiotic resistance and the urgent need for new drugs

Infectious diseases have been the leading cause of the global health burden accounting for a death toll of > 25 % in developing countries and over 40 % of deaths in developing countries. Need of antibiotics is on the rise with the pharmaceutical industry combating the double-edged sword facing drug development. On one side,

antibiotic discovery is on decline and on the other resistance is on the rise (Fischbach & Walsh, 2009).

Although antibiotic resistance has been a problem ever since their introduction to human therapy, it is the increase in number, diversity and range of resistant microbes which is the cause of the present situation. Three classes of antibiotic-resistant pathogens are emerging as major threats to public health. They are methicillin-resistant *Staphylococcus aureus* (MRSA) and the subsequent rise of of vancomycin-resistant *S. aureus* (VRSA); multidrug-resistant (MDR) and pan-drug-resistant (PDR) Gram-negative strains of bacteria namely *Acine-tobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* and MDR and extensively-drug-resistant (XDR) strains of *Mycobacterium tuberculosis* (MDR-TB and XDR-TB) (Fischbach & Walsh, 2009).

Pathogenic organisms counter the effects of antibiotics by use of various mechanisms. Some bacteria have an impermeable membrane or lack the target of the antibiotic (Brown & Balkwill, 2009; Allen *et al.*, 2010). Others have multidrug resistance efflux pumps which excrete antibiotics from the cell into the periplasm, while genetic mutations disable the antibiotic-binding sites (Nikaido, 1998; Poole, 2005; Reynolds & Courvalin, 2005). Besides, the resistance genes get transferred between members of distantly related taxonomic groups through horizontal gene transfer (Trieu-Cuot & Courvalin, 1986; Trieu-Cuot *et al.*, 1993; Mindlin *et al.*, 2006) and through plasmids or transposable elements (Davies, 1997; Poirel *et al.*, 2005; Mindlin *et al.*, 2006).

The increase in the current problems of growing resistance of numerous pathogens together with the new emerging infectious diseases necessitates the

searching for new anti-infective drugs by using promising approaches to scaffold discovery as mentioned earlier.

Some of the factors contributing to rise in resistance are: indiscriminate and inappropriate use of antibiotics in human medicine that provide selective pressure resulting in antibiotic resistant bacteria and resistance genes; release of pathogens and non pathogens into the environment thereby contributing to a web of resistance that includes human, animal, and the environment, essentially the biosphere (Gangle, 2005); inadequate use of non-therapeutic application of antibiotics (as growth promotants or for prophylactic purposes) in intensive animal husbandry and in agriculture.

1.4.3 *Strategies involved in production of antibiotics*

Due to the ever increasing rise in infectious diseases, several strategies/approaches have been suggested to facilitate antibiotic research. Eco-taxonomical approaches such as sampling of unexplored habitats for isolating rare or novel microbes and sound understanding of the microbe's biology so as to manipulate their potent ability to produce bioactive compounds are ways suggested to enhance discovery of novel metabolites. Besides, improvement over the conventional culturing approaches; High throughput whole cell screening; novel culturable methods such as ribosomal RNA (rRNA) sequencing for isolating unculturable bacteria with the potential of producing novel metabolites; heterologous DNA-based methods for identifying gene clusters by facilitating genetic manipulation; metagenomics by capturing the DNA from the environment and use it in heterologous expression systems, comparative genomics by way of whole genome sequencing to uncover cryptic pathways or biosynthetic pathways of undetected metabolites, isolation and

activation of silent clusters through the manipulation of pathway-specific regulatory genes, PCR-based genome screening approach for identifying coding sequences for putative halogenase enzymes (Hornung *et al.*, 2007); combinatorial biosynthesis and structural studies of compounds by use of efficient identification systems are also the latest strategies adopted for enhancing antibiotic research development. Hwang *et al.*, 2013 in their latest paper have highlighted the use of mass spectrometry-based proteomic approach, (PrISM) for competition-based adaptive evolution platform, synthetic biology-based plug-and-play scaffold and for the identification of novel secondary metabolites **(Figure 1.7)**.

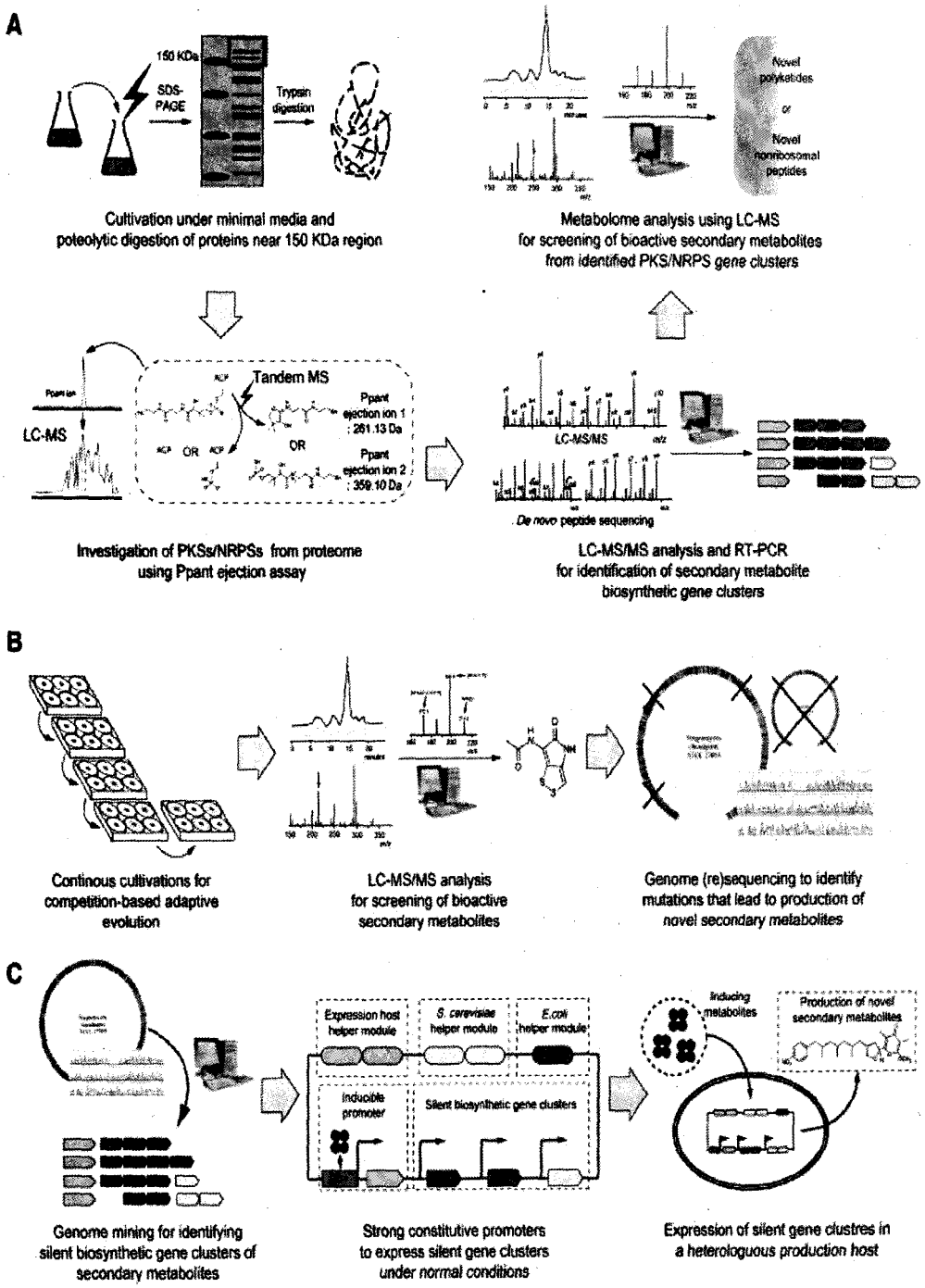

Figure 1.7 Use of mass spectrometry-based proteomic approach, PrISM, **a)** The Phosphopantetheinyl (Ppant) ejection assay detects ACPs bound to the PKSs and NRPSs, allowing them to separate from the rest of the proteins, **b)** Continuous cultivations for competition-based adaptive evolution, **c)** Genome mining to identify silent gene clusters of secondary metabolites (Source: Hwang *et al.*, 2013).

1.4.4 *Actinomycetes as a promising source of antibiotics*

Antibiotic are produced by prokaryotic (Prokaryotae, Monera) as well as eukaryotic organisms however, among all these groups, actinomycetes are the most versatile producers. Berdy, (2012) in his latest review, lists out the total number and distribution of natural products which includes synthetic antibiotics and antibiotic derivatives from various sources (**Table 1.1**)

Table 1.1 List of approximate numbers of known natural products till 2010 (Source: Modified from Berdy, 2012)

	Natural products			
Sources	**All known**	**All bioactive**	**Antibioti[a]**	**Prospects**
Actinobacteria	20,000	12000	10,000	++
Fungi[b]	30,000	15000	10,000	++
Unicellular bacteria[c]	20,000	7000	5000	+
Higher organisms: *Plant Kingdom*				
Lower plants (algae, etc.)	50-60,000	6000	100-200	Not known
Higher plants	300-350,000	25000	15,000	+
Animal Kingdom				
Marine invertebrates	25-30,000	10000	5000	++
Terrestrial animals	4-5000	200	100	Not known
Synthetic compounds				
Semisynthetic antibiotics	Over 200,000	Not Known	Almost all	++
Mimics, analogues	Over 50,000	Not Known	Few	+
Total synthetic products	millions?	Not Known	1000	±

[a]Antinfective, antitumour and antiviral compounds
[b]Microscopic and basidiomycetous fungi, myxomycetes, slime molds, yeasts
[c]Eukaryots including cyanobacteria and myxobacteria

Actinomycetes have produced over ~13,700 bioactive compounds of which ~10,400 are derived from *Streptomyces* and ~3,300 from other and rare actinomycetes such as *Actinokineospora, Acrocarpospora, Actinomadura, Actinosynnema, Amycolatopsis, Actinoplanes, Catenuloplanes, Cryptosporangium, Dactylosporangium, Kineosporia, Kutzneria, Micromonospora, Microbispora, Microtetraspora, Nocardia, Nonomuraea, Thermomonospora, Pseudonocardia, Thermobifida, Saccharomonospora, Spirilliplanes, Streptosporangium, Streptoverticillium*, and *Virgosporangium* species (Hayakawa, 2008; Berdy, 2012). The number of actinomycetes species producing bioactive metabolites are given in **Table 1.2.**

Table 1.2 Actinomycetes species producing bioactive metabolites (Source: Modified from Berdy, 2012)

Actinomycetes species			
Streptomycetaceae		**Pseudonocardiaceae**	
Streptomyces	8000	Saccharopolyspora	131
Streptoverticillium	258	Amycalotopsis/Nocardia	120/357
Kitasatosporia	37	Kibdellosporangium	34
Chainia	30	Pseudonocardia	27
Microellobosporia	11	Amycolata	12
Nocardioides	9	Saccharomonospora	2
		Actinopolyspora	1
Micromonosporaceae		**Streptosporangiaceae**	
Micromonospora	740	Streptosporangium	79
Actinoplanes	248	Streptoalloteichus	48
Dactylosporangium	58	Spirillospora	11
Ampullariella	9	Planobispora	10
Glycomyces	2	Kutzneria	4
Catenuliplanes	3	Planomonospora	2
Catelatospora	1		
Thermomonosporaceae		**Mycobacteriaceae**	
Actinomadura	345	Nocardia	357
Saccharothrix	68	Mycobacterium	57
Microbispora	54	Arthrobacter	25
Actinosynnema	51	Brevibacterium	17
Nocardiopsis	41	Proactinomyces	14
Microtetraspora/Nonomria	26/21	Rhodococcus	13
Thermomonospora	19		
Micropolyspora/Faenia	13/3		
Thermoactinomyces	14		
Thermopolyspora	1		
Thermoactinopolyspora	1		

Full genome sequences of model actinomycetes such as *Amycolatopsis mediterranei* U32 (Zhao *et al.*, 2010), *Saccharopolyspora erythraea* NRRL 23338 (Oliynyk *et al.*, 2007), *Salinispora tropica* CNB 440 (Udwary *et al.*, 2007), *Streptomyces avermitilis* (Ikeda *et al.*, 2003) and "*Streptomyces coelicolor*" A3(2) (Bentley *et al.*, 2002) have been found to contain over 20 natural product biosynthetic gene clusters for the production of known or predicted secondary metabolites. Despite this amazing productivity, only 10 % of the total number of antimicrobial compounds from actinomycetes has been discovered till date (Watve *et al.*, 2001; Goodfellow & Haynes, 1984).

Actinomycetes have shown to exclusively produce unique and structurally complex compounds such as polyene macrolides, other large-membered macrolides (oligomycin-type), anthracyclines (daunomycin-type), polyether antibiotics (nigericin-type), cyclopolylactones (nonactin-type), most aminoglycosides, anthracyclines, streptothricins, actinomycins and quinoxaline-peptides, macrocyclic lactones, lactams, specific cyclopeptides, depsipeptides and polycyclic quinone-based molecules. Glycopeptides and orthosomycins are mainly produced by non-*Streptomyces* actinomycetes. Other antibiotics are erythromycin produced by *Saccharopolyspora erythraea* (Oliynyk *et al.*, 2007), gentamicin by *Micromonopsora purpurea* (Weinstein *et al.*, 1963; Wagman, 1980), rifamycins by *Amycolatopsis mediterranei* (Jin *et al.*, 2002), teicoplanin by *Actinoplanes teichomyceticus* (Somma *et al.*, 1984; Jung *et al.*, 2009) and vancomycin by *Amycolatopsis orientalis* (Wink *et al.*, 2003). The ability to produce novel and diverse compounds in actinomycetes could be attributed to its biosynthetic capacity via extensive branching, and a series of alternative reactions by different enzymes, condensations, alkylations, oxidations and isomerizations. They also contain genes encoding PKS I, PKS II and NRPS (Metsa-

Ketela *et al.*, 1999; Schwarzer *et al.*, 2003; Ayuso & Genilloud, 2005; Ginolhac *et al.*, 2005). The antibiotics produced by actinomycetes launched since 2000 are shown in the **Table 1.3**

Table 1.3 Antibiotics launched since 2000 produced by actinomycetes species (Source: Modified from Butler *et al.*, 2013)

Year approved	Drug name	Class	Bacteria Type
2001	Telithromycin	Macrolide	Grm+ve/-ve
2002	Biapenem	Carbapenem	Grm+ve/-ve
2002	Ertapenem	Carbapenem	Grm+ve/-ve
2003	Daptomycin[b]	Lipopeptide	Grm+ve
2005	Doripenem	Carbapenem	Grm+ve/-ve
2005	Tigecycline	Tetracycline	Grm+ve/-ve
2009	Tebipenem pivoxil	Carbapenem	Grm+ve/-ve
2009	Telavancin	Glycopeptide	Grm+ve
2011	Fidaxomicin (1)[b]	Tiacumicin	Grm+ve

Legend: The superscript **b** indicates first member of the new antibiotic class approved for humans

1.4.4.1 *Bioactive compounds from Streptomyces spp.*

Streptomycetes, are the most prolific source of new antibiotics (Bérdy, 2005; Newman & Cragg, 2007; Goodfellow & Fiedler, 2010) with a diverse range of chemical structures (Bérdy, 1995, 2005). According to StreptomeDB (http://www.pharmaceuticalbioinformaticsde/streptomedb/) database >2400 unique and diverse compounds from >1900 different *Streptomyces* strains and substrains has been recorded. The secondary metabolites are produced during the shifting phase from the substrate mycelium to sporulation, accompanied by morphological differentiation (Dyson, 2011; Flardh & Buttner, 2009).

Although, many compounds have already been recovered from this group of microorganism, they still possess a lot more potential to produce secondary metabolites (Baltz, 2008, 2011; Craney *et al.*, 2013). This study has been supported

by the recent genome sequencing data which also indicates the ability of this genus to possess many more bioactive secondary metabolites, many yet to be elucidated.

The ability of this genus to produce various bioactive compounds is attributable to the unique features of its genome i.e the presence of biosynthetic gene clusters that encode enzymes contributing to the production of secondary metabolites with a variety of chemotypes, including polyketides, lactams, nonribosomal peptides, and terpenes **(Figure 1.8)** (Nett *et al.*, 2009). Specific structural types of antibiotics such as ansa-lactam rings, macrocyclic lactones, polyether and cyclopeptide skeletons are said to occur commonly within these groups (Bérdy, 1995, 2005). These bioactive compounds are not only used as antibiotics (e.g., daptomycin), but also as immunosuppressants (e.g., rapamycin), anticancers (e.g., doxorubicin), and antiparasitics (e.g., ivermectin) (Newman & Cragg, 2007).

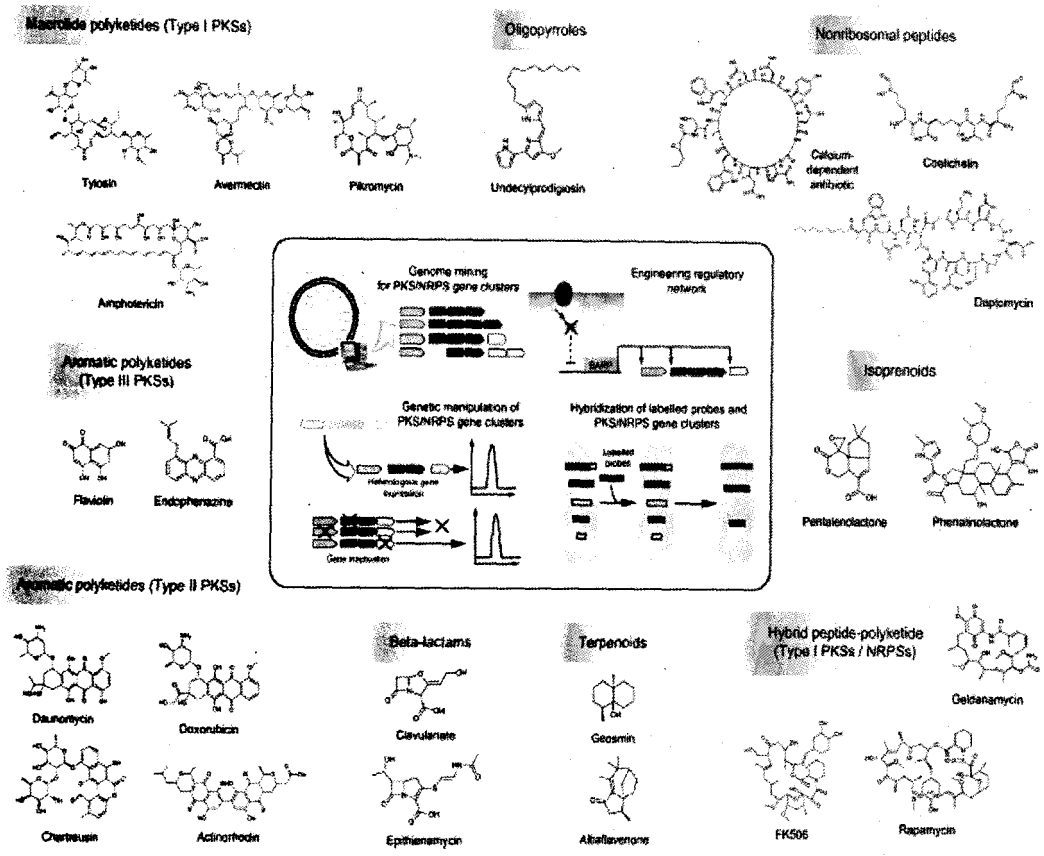

Figure 1.8 Some of the secondary metabolites produced by *Streptomyces* species with a typical *Streptomyces* colony shown in the middle. Parentheses next to each secondary metabolite type indicate the types of associated biosynthetic enzymes (Source: Nett *et al.*, 2009)

The *Streptomyces* species stands apart from other antibiotics producing microorganisms as they are better amenable to the application of high-throughput techniques (e.g., combination of genome mining and mass spectrometry) and gene manipulations to maximize their potential of producing potent antibiotics, compared to other (Nett *et al.*, 2009). They are also better for extraction and characterization of their bioactive compounds is much easier (Xu, 2011). Genetic manipulation of *Streptomyces* has been used to generate highly diverse chemical libraries by modification of synthesis routes (10–13).Thus, advanced technologies coupled with the species potentialities should be actively pursued to enrich options for combating antibiotic-resistant pathogens (Bush *et al.*, 2011).

1.5 Actinomycetological research in India

This review compiles the work done in last few years, with an emphasis on actinomycetes diversity and bioprospecting for pharmaceutically important compounds like antibiotics, enzymes and other important applications.

Actinomycetological research is still in its infancy in India, in view of a rapid decline in the rate of discovery of new genera and metabolites observed in the beginning of the 20th century and the period from 2011-2014, being the most productive, interms of actinomycetes diversity and bioprospecting **(Figure 1.9)**.

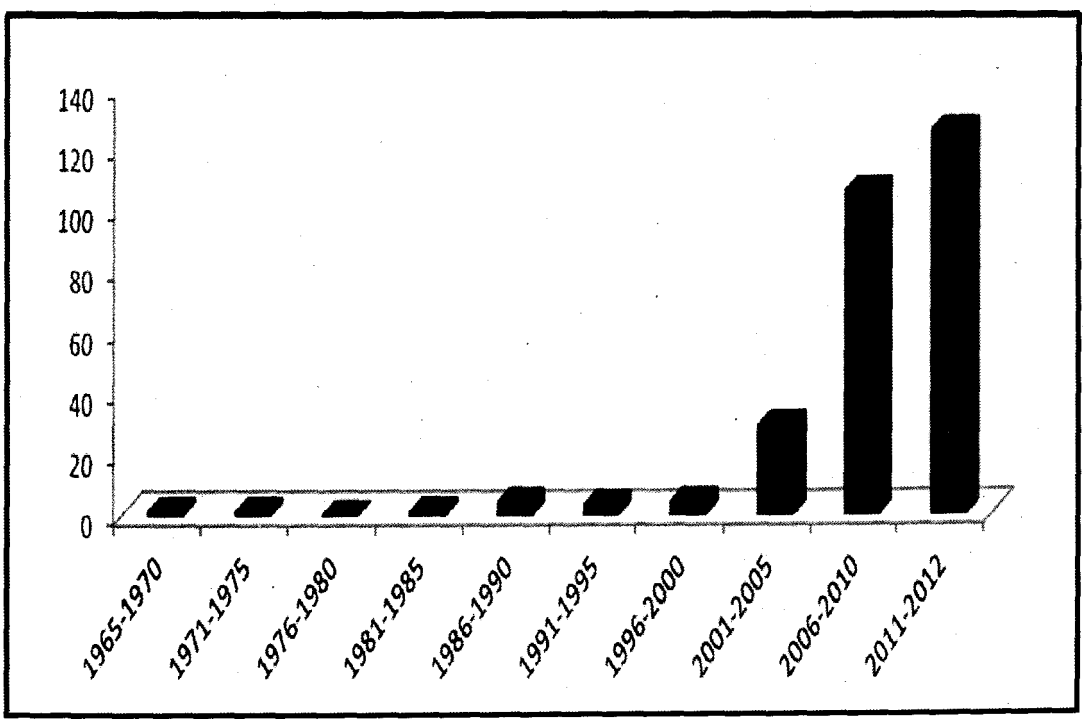

Figure 1.9 Indian publications on actinomycetes diversity and bioprospecting since 1965

1.5.1 *Biodiversity and taxonomy*

Baltz, (2007) opined that approaches to explore random and exotic soil, could accelerate the production of bioactive substances from actinomycetes. In view of this, in India diverse habitats and locations **(Figure 1.10)** have been screened for studying its diversity.

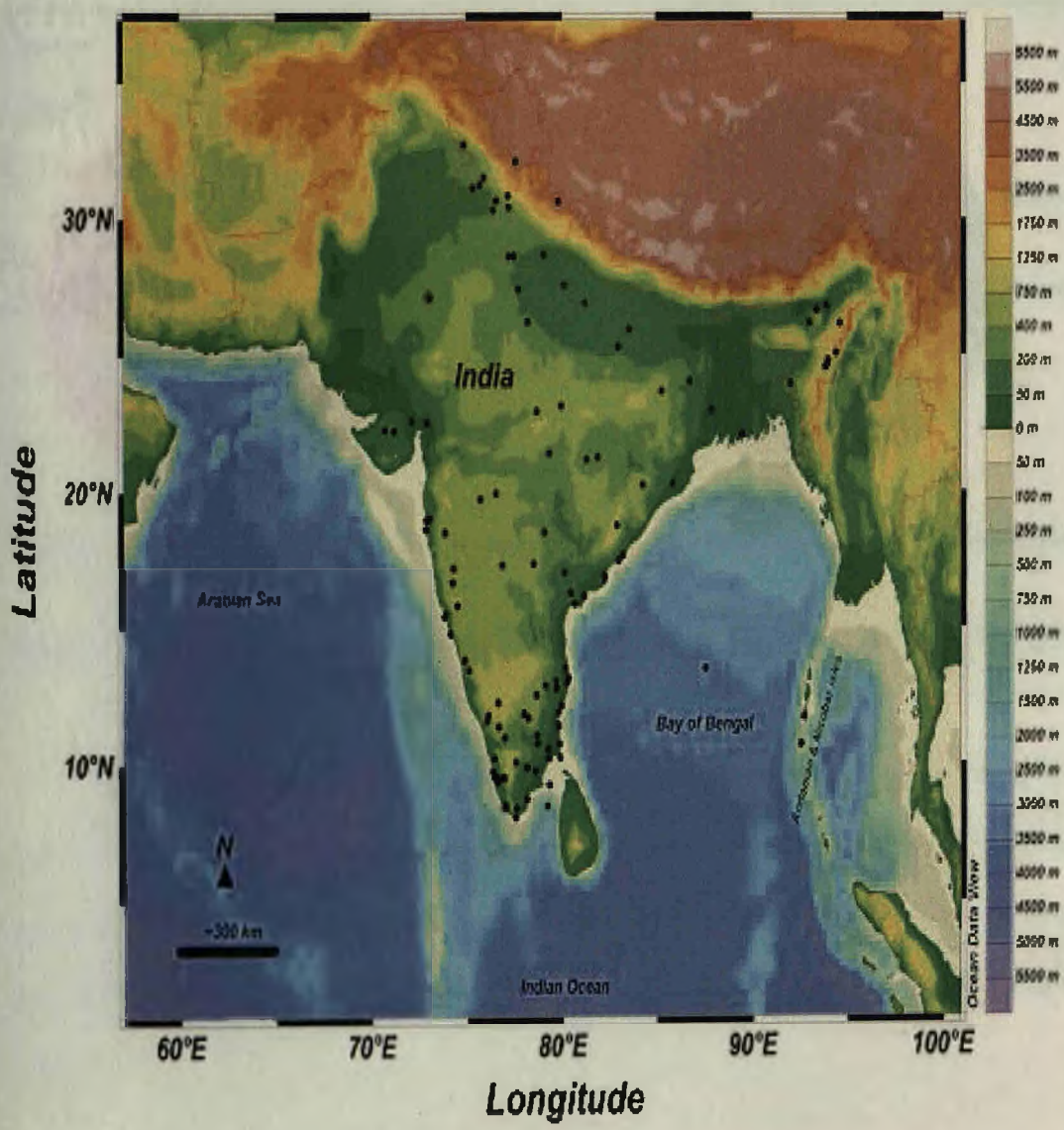

Figure 1.10 Distribution of habitats sampled in India for actinomycetes

Soil habitats (96 %) have been largely surveyed as compared to other resources like endophytic plants (3 %) and animal guts (1 %) **(Figure 1.11a)**. On the other hand, the percentage of actinomycetes isolations from marine resources (85 %) is much higher than freshwater resources (15 %) **(Figure 1.11b)**.

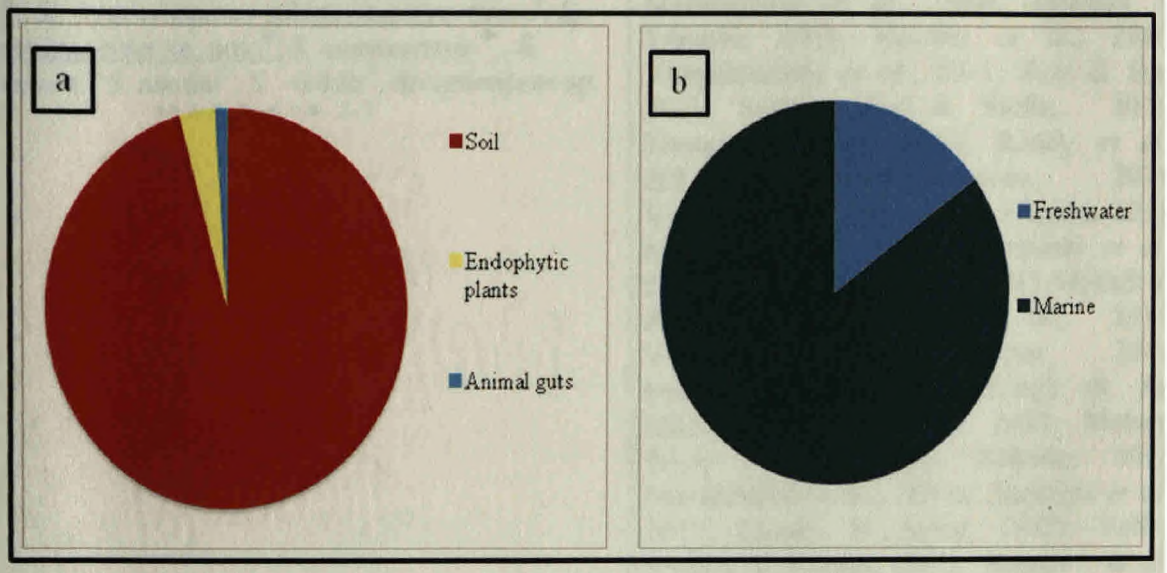

Figure 1.11a& b Ecosystem wise distribution of actinomycetes **(a)** Terrestrial and **(b)** Aquatic (Source: Table 2)

Research on actinomycetes diversity from underexplored ecological niches of terrestrial ecosystems from India, has led to the isolation of 26 actinomycetes genera, with Genus *Streptomyces* being the most dominant, followed by Genus *Micromonopora, Actinomadura, Rhodococcus, Microbispora, Nocardia* and others **(Table 1.4)**.

Table 1.4 Diversity of actinomycetes from Indian terrestrial ecosystems

Species	References
Soil habitats	
Genus *Streptomyces* *S. aurantiacus*[•], *S. citricolor*[•], *S. clavuligerus*[•], *S. gulbargensis* sp. nov.[•], *S. kavamyceticus*[•], *S. platensis*[•], *S. spectabilis*[•], , *S. albidoflavus*[♦], *S. erumpens** *S. griseoruber*[□], *S. hyderabadensis* sp. nov.[▲], *S. hygroscopicus* subsp. *ossamyceticus*,[◊], *S. manipurensis* sp. nov.[■], *S. sannanensis* [▶], *S. sannanensis*,[♠] *S. tendae*[°], *S. viridis*[°], *Streptomyces* sp. [•,□,▲,■, ▶,♠,◊,■,♂,♀]	Franco *et al.*, 1987; Franco *et al.*, 1990a; Vasavada *et al.*, 2006; Dastager *et al.*, 2006; Mehta *et al.*, 2006; Kumari *et al.*, 2006; Srividya *et al.*, 2008; Kar & Ray, 2008; Ponmurugan & Nithya, 2008; Selvameenal *et al.*, 2009; Praveen & Tripathi, 2009; Kavitha *et al.*, 2010; Vijayabharathi *et al.*, 2011; Roy & Sen, 2011; Sathiyaseelan & Stella, 2011; Thumar & Singh 2011; Reddy *et al.*, 2011a; Kulkarni-Almeida, 2011; Vijayalakshmi & Raja Hima Bindhu, 2011; Malviya *et al.*, 2011; Tripathi *et al.*, 2011; Panigrahi *et al.*, 2011;Mythili & Ayyappa, 2011; Gautham *et al.*, 2011; Velho-Pereira & Kamat, 2011; Hemashenpagam, 2011; Singh & Rai 2011; Chaudhary *et al.*, 2011; Mohana Priya *et al.*, 2011; Nakade, 2012; Nimaichand *et al.*, 2012a; Malviya *et al.*, 2012; Quadri & Agsar, 2012; Velho-Pereira & Kamat, 2012; Kumar *et al.*, 2012
Genus *Micromonospora* *Micromonospora* sp. [▲,▶,♣]	Hemashenpagam, 2011; Velho-Pereira & Kamat, 2011; Nakade, 2012; Talukdar *et al.*, 2012
Genus *Actinomadura* *A. roseale*[•], *Actinomadura* sp. [□,♂,e]	Sathiyaseelan & Stella, 2011; Velho Pereira & Kamat, 2011; Velho-Pereira & Kamat, 2012; Kumar *et al.*, 2012
Genus *Rhodococcus* *R. kroppenstedtii* sp. nov.[Ψ], *R. canchipurensis* sp. nov.[■], *R. imtechensis*[Ω], *Rhodococcus* sp.[■]	Mayilraj *et al.*, 2006a; Nimaichand *et al.*, 2012; Vikram *et al.*, 2012; Ningthoujam *et al.*, 2012
Genus *Microbispora* *M. indica* sp. nov.[♠], *M. karnatakensis* sp. nov.[♠], *Microbispora sp.*[▶]	Rao *et al.*, 1987; Nakade, 2012
Genus *Nocardia* *Nocardia* sp. [▲,♀,♂]	Kavitha *et al.*, 2010; Hemashenpagam, 2011; Kumar *et al.*, 2012
Genus *Kitasatospora* *Kitasatospora sampliensis* sp. nov.[♠], *Kitasatospora* sp[▶]	Mayilraj *et al.*, 2006b; Nakade, 2012

Genus *Kocuria* *Kocuria himachalensis* sp. nov. [Ψ], *Kocuria* sp. [▶]	Mayilraj *et al.*, 2006c; Krishna *et al.*, 2008
Genus *Streptosporangium* *Streptosporangium* sp. [▪,♂]	Velho-Pereira & Kamat, 2012; Kumar *et al.*, 2012
Genus *Thermoactinomyces* *T. thalophilus* [▶]; *Thermoactinomyces* sp. [♂]	Kohli *et al.*, 2001; Kumar *et al.*, 2012
Genus *Actinoplanes* *Actinoplanes* sp. [▶]	Nakade, 2012
Genus *Actinoalloteichus* [54] *A. spitiensis* sp. nov. [Ψ]	Singla *et al.*, 2005
Genus Agrococcus [55, 56] *A. carbonis* sp. nov. [¢], *A. lahaulensis* sp. nov. [Ψ]	Mayilraj *et al.*, 2006d; Dhanjal *et al.*, 2011a
Genus *Arthrobacter* *Arthrobacter* sp. [§]	Chittpurna *et al.*, 2011
Genus *Brevibacterium* *Brevibacterium casei* [■]	Ningthoujam *et al.*, 2012
Genus *Microbacterium* *M. immunditiarum* sp. nov. [♦]	Krishnamurthi *et al.*, 2012
Genus *Micrococcus* *M. lactis* sp. nov. [§]	Chittpurna *et al.*, 2011
Genus *Norcardiopsis* *N. prasina* [■]	Ningthoujam *et al.*, 2005
Genus *Planomonospora* *Planomonospora* sp. [▶]	Nakade, 2012
Genus *Planococcus* *P. stackebrandtii* sp. nov. [Ψ]	Mayilraj *et al.*, 2005
Genus *Saccharomonospora* *S. saliphila* sp. nov. [◊]	Dastager *et al.*, 2008
Genus *Saccharopolyspora* *Saccharopolyspora* sp. [♂]	Kumar *et al.*, 2012
Genus *Streptoverticillium* *Streptoverticillium* sp. [▶]	Nakade, 2012
Genus *Thermomonospora* *Thermomonospora* sp. [Þ]	Ahmad *et al.*, 2003a
Genus *Yaniella* *Y. fodinae* sp. nov. [¢]	Dhanjal *et al.*, 2011b

Endophytic plants	
Genus *Streptomyces* *Azadirachta indica* stem [ω], leaves[ю] and roots [ψ], *S. aureus* [ю, ψ], *S. hydroscopicus* [ω, ψ], *S. greseofuscus* [ю, ψ], *S. albosporus* [ю, ψ], *S. cinereus* [ω, ю, ψ], *S. flavus*[ψ], *S. cyaneus* [ψ], *S. globisporus* [ю, ψ], *S. viridis*[ψ], *S. glaucus* [ω], *S. lavendulae*[ю, ω], *S. griseorubroviolaceus* [ω], *Streptomyces* sp. [*, ω, ю, ψ, ϫ, ◄, ☼, ϱ, ∂, d]	Rangarajan *et al.*, 1984; Verma *et al.*, 2009; Demosthenes & Chauhan, 2011
Genus *Nocardia* *Nocardia* sp. [ω, ю, ⊕, ‡, ñ, ≈]	Verma *et al.*, 2009; Demosthenes & Chauhan, 2011
Genus *Actinomadura* *Actinomadura* sp. [F, Θ, ¶]	Demosthenes & Chauhan, 2011
Genus *Microbispora* *Microbispora* sp. [ϫ, ω, ю, ψ]	Verma *et al.*, 2009; Demosthenes & Chauhan, 2011
Genus *Nocardiopsis* *Nocardiopsis* sp. [6]	Demosthenes & Chauhan, 2011
Genus *Sacchromonospora* *Sacchromonospora* sp. [ψ]	Verma *et al.*, 2009
Genus *Saccharopolyspora* *Saccharopolyspora* sp. [ч]	Demosthenes & Chauhan, 2011
Genus *Streptosporangium* *Streptosporangium* sp. [ω, ю, ψ]	Verma *et al.*, 2009
Genus *Streptoverticillium* *Streptoverticillium* sp. [ω, ψ]	Verma *et al.*, 2009
Animal Guts	
Genus *Streptomyces* *S. tritolerans* sp. nov. [♃], *S. noursei* [♂]	Aruna *et al.*, 2008; Subramanian *et al.*, 2009

Legend: Soils- Black soils [•], Surface soil of a landfill [♦], Brick-kiln soil [*], Rhizospheric soil [■], Red soil [▲], Desert soil, [■] Limestone quarry [◊], Alkaline soil [►], Agricultural soil [♦], Lateritic soil [◊], Bitumen (heavy crude oil) soil [■], Solitary wasp and swallow bird mud nest [♂], Tree Hollow [♀], Forest soil [♣], Termite mounds [o], Indian Himalayas [Ψ], Pesticide-contaminated soil [Ω], Coal mine soil [c], Dairy industry effluent treatment plant [ϱ], Self-heating compost [Þ].

The novel actinomycetes species reported from India have been underlined.

1.5.1.1 *Techniques used for isolation*

Isolation strategies like novel baiting bag (Singh *et al.*, 2009a), baiting slide (Velho-Pereira & Kamat, 2012) and enrichment culture (Bagewadi *et al.*, 2011) techniques to isolate uncommon and rare genera have also been reported. Exploration

of novel habitats has led to the discovery of 23 novel taxa from terrestrial as well as marine ecosystems. Among these novel strains, five belong to Genus *Steptomyces;* two to Genus *Agrococcus, Rhodococcus, Microbispora, Kocuria* and strains belonging to Genus *Actinoalloteichus, Planococcus, Kitasatospora, Micrococcus, Georgenia, Saccharomonospora, Microbacterium, Yaniella, Amycolatopsis* and *Agromyces*.

1.5.1.2 *Phylogenetic relationship of novel Indian actinomycetes taxa*

It is interesting to study the phylogenetic relationship of these taxa, so using MEGA5 software and 16S rDNA sequences available at NCBI (http://www.ncbi.nlm.nih.gov/genbank/), phylogenetic relationship of 21 Indian actinomycetes species was determined by neighbor joining method **(Figure 1.12)**.

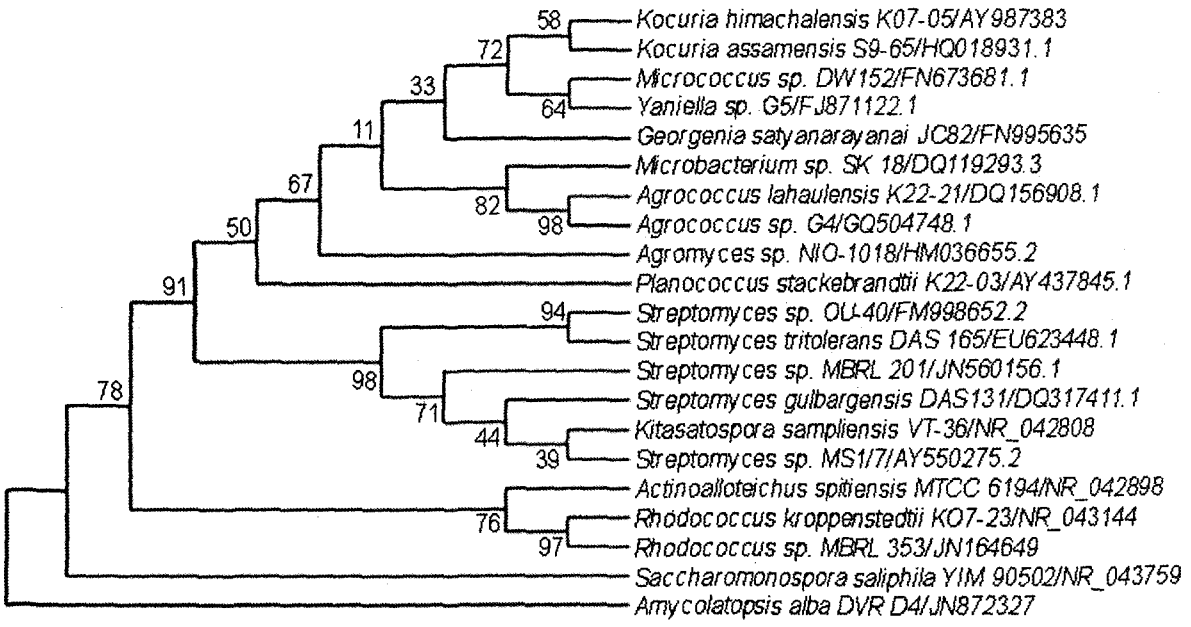

Figure 1.12 Phylogram indicating the placement and relatedness of 21 novel Indian actinomycetes strains within the order Actinomycetales under Actinobacteria. Numbers given at the branch nodes indicate (%) bootstrap values. Bar 0.01 substitutions per 100 nucleotide positions

1.5.2 *Bioprospecting for pharmaceutically important compounds*

1.5.2.1 *Antibotics*

Actinomycetes are noteworthy antibiotic producers and have yielded ~3,000 known antibiotics. Genus *Streptomyces* produces 75 % of all these (Watve *et al.*, 2001). Baltz, (2005), estimated that only 1-3 % of *Streptomycetes*antibiotics are so far discovered. Thus, there is an urgent need of producing novel antibiotics keeping in mind the ever increasing rise of resistant pathogens.

Strategies employed elsewhere in the world to obtain novel antibiotics from unexplored habitats have been used in India. Screening efforts made to obtain antibiotics against human pathogens is much higher than those against plant and animal pathogens. Since Indian research on antibiotic discovery, has focused mostly on preliminary screening and optimization of the various culture conditions, for antibacterial and antifungal compounds, further studies to reveal the identity of the bioactive molecules is on decline. A few reports however hold much promise for future. These include isolation of a cytotoxic compound 1, hydroxyl-1-norresistomycin from marine actinomycetes *Streptomyces chibaensis* (Gorajana *et al.*, 2005), Resistomycin an anticancer compound, from *S. aurantiacus* (Vijayabharathi *et al.*, 2011) and a broad spectrum antimicrobial compound; 7, demethoxyrapamycin produced by *Streptomyces hydroscopicus* (Parthasarathi *et al.*, 2012). Besides, four novel antibiotics include namely Swalpamycin, Butalactin, Alisamycin and 1(10-aminodecyl) Pyridinium salt **(Table 1.5)**.

Table 1.5 Novel metabolites from Indian actinomycetes strains

Strain	Novel metabolite	Antibiotic group	Reported activity	References
Streptomyces sp. Y-84,30967	Swalpamycin	macrolide	Against Gram positive bacteria including erythromycin-resistant strains	Franco *et al.*, 1987
Streptomyces sp. HIL Y-86,36923	Butalactin	Butanolide	Against Gram-positive and Gram-negative bacteria	Franco *et al.*, 1990a
Streptomyces sp. HIL Y-88,31582	Alisamycin	Manumycin	Against Gram-positive bacteria and fungi and weak antitumour activity	Franco *et al.*, 1991a
Amycolatopsis alba var. nov.	1(10-aminodecyl) Pyridinium salt	Pyridinium compound	potent cytotoxic and antibacterial activities	Dasaria *et al.*, 2012

1.5.2.1.1 *Process optimization*

Reports on optimization of physiological and biochemical parameters to increase the yield of the metabolites reveals that, different concentrations of Glucose (Gupte & Kulkarni, 2003; Pandey *et al.*, 2005; Jain *et al.*, 2011 and Pazhanimurugan *et al.*, 2012), Glycerol (Reddy *et al.*, 2011b; Gunda & Singara Charya, 2012), Starch (Vasanthabharathi *et al.*, 2011) Arabinose and sucrose (Thenmozhi & Kannabiran, 2012) as carbon sources and nitrogen sources such as Yeast extract (Nanjwade *et al.*, 2010), peptone (Reddy *et al.*, 2011b; Thenmozhi & Kannabiran, 2012), Soyabean (Gupte & Kulkarni, 2003; Singh *et al.*, 2009b; Vasanthabharathi *et al.*, 2011), Sodium nitrate and potassium nitrate (Gunda & Singara Charya, 2012), Ammonium dihydrogen phosphate (Pazhanimurugan *et al.*, 2012), Liver extract (Reddy *et al.*, 2011b) as optimal biochemical parameters and alkaline pH ranging from 7-8 (Ningthoujam *et al.*, 2005; Pandey *et al.*, 2005; Singh *et al.*, 2009b; Thenmozhi & Kannabiran, 2011; Roy & Sen, 2011; Jain *et al.*, 2011; Reddy *et al.*, 2011b; Vasanthabharathi *et al.*, 2011; Pazhanimurugan *et al.*, 2012; Thenmozhi &

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Kannabiran, 2012; Nanjwade *et al.*, 2012) and temperature ranging from 25 °C-35 °C (Roy & Sen, 2001; Ningthoujam *et al.*, 2005; Singh *et al.*, 2009a; Nanjwade *et al.*, 2010; Umasankar *et al.*, 2010; Thenmozhi & Kannabiran, 2011; Jain *et al.*, 2011; Reddy *et al.*, 2011b; Vasanthabharathi *et al.*, 2011; Pazhanimurugan *et al.*, 2012; Gunda & Singara Charya, 2012; Thenmozhi & Kannabiran, 2012) as optimal physiological parameters.

1.5.2.1.2 *Antimicrobials vis á vis humans pathogens*

Dawn of antibiotic era witnessed a decline of infectious diseases however, the new threat or multidrug resistant strains has created an urgency to promote novel antibiotic discoveries centered at antinomycetes (Baltz, 2007; Wenzel & Bandow, 2011).

Indian research, on harnessing antibiotics from actinomycetes resources has accelerated. However, the work is so far limited to mostly preliminary screening. Large number of actinomycetes strains have been screened against various human test pathogens, including the multidrug resistant strains as indicated in **Table 1.6 & 1.7.**

Table 1.6 Human bacterial pathogens used to screen actinomycetes lead molecules

Human bacterial pathogens used in screening	Symptoms/diseases	References
Genus *Acinetobacter* *A. baumanii, Acinetobacter sp.*	Severe pneumonia and Urinary Tract Infection	Ravikumar *et al.*, 2010; Demosthenes & Chauhan, 2011; Kumar *et al.*, 2012
Genus *Aeromonas* *A. formicans, A. hydrophila, A. veronii**	Gastroenteritis; Wound infections and diarrhoea*	Murugan *et al.*, 2007; Reddy *et al.*, 2011b; Nithyanand *et al.*, 2011;Chandra *et al.*, 2012
Genus *Alcaligenes* *A. faecalis*	Urinary Tract Infection	Franco *et al.*, 1987
Genus *Arthrobacter* *A. protophormiae*	Skin infection	Mitra *et al.*, 2011

Genus *Bacillus* *Bacillus sp., B. amyloliquefaciens, B. cereus, B. coagulans, B. megaterium, B. pumilis, B. saccharolyticum, B. sphaericus, B. subtilis*[∞] *B. thuringiensis*[#]	Foodborne illness; Skin infection[∞], Eye infection[∞]; Emetic syndrome and diarrhoea[#]	Franco *et al.*, 1987; Kokare *et al.*, 2004; Selvin *et al.*, 2004; Tripathi *et al.*, 2004; Kokare *et al.*, 2004; Gorajana *et al.*, 2005; Peela *et al.*, 2005; Singh *et al.*, 2006; Vasavada *et al.*, 2006; Thangapandian *et al.*, 2007; Anupama *et al.*, 2007; Thakur *et al.*, 2007; Ponmurugan & Nithya, 2008; Srividya *et al.*, 2008; Gandhimathi *et al.*, 2008; Malik *et al.*, 2008; Remya & Vijayakumar, 2008; Kaur *et al.*, 2008; Praveen & Tripathi, 2009; Subramanian *et al.*, 2009; Singh *et al.*, 2009; Ningthoujam *et al.*, 2009; Ramesh & Mathivanan, 2009; Yadav *et al.*, 2009; Suthindhiran & Kannabiran, 2009a; Suthindhiran & Kannabiran, 2009b; Kharat *et al.*, 2009; Dhanasekaran *et al.*, 2009; Vimal *et al.*, 2009; Kavitha *et al.*, 2009; Arumugam *et al.*, 2010; Nanjwade *et al.*, 2010; Ravikumar *et al.*, 2010; Radhakrishnan *et al.*, 2010; Kumar & Kannabiran, 2010; Duraipandiyan *et al.*, 2010; Dhananjeyan *et al.*, 2010; Basavaraj *et al.*, 2010; Kumar *et al.*, 2010; Vijayabharathi *et al.*, 2011; Sathiyaseelan & Stella, 2011; Vijayalakshmi & Raja, 2011; Panigrahi *et al.*, 2011; Mythili & Ayyappa, 2011; Singh & Rai, 2011; Hemashenpagam, 2011;Velho-Pereira & Kamat, 2011; Demosthenes & Chauhan 2011; Sanasam *et al.*, 2011; Vijayalakshmi *et al.*, 2011; Jose *et al.*, 2011; Satheeja & Jebakumar, 2011; Mitra *et al.*, 2011; Naikpatil & Rathod, 2011; Krishna *et al.*, 2011; Jain *et al.*, 2011; De *et al.*, 2011; Kavitha & Vijayalakshmi, 2011; Sivakumar *et al.*, 2011; Gopinath *et al.*, 2011; Usha Kiranmayi *et al.*, 2011; Baskaran *et al.*, 2011; Radhika *et al.*, 2011; Mohanraj *et al.*, 2011; Thangapandian *et al.*, 2011; Kumar *et al.*, 2012; Talukdar *et al.*, 2012; Kumari & Siddaiah *et al.*, 2012; Parthasarathi *et al.*, 2012; Pazhanimurugan *et al.*, 2012; Gunda

		& Singara, 2012; Thenmozhi& Kannabiran, 2012; Athoor & Janardhan, 2012; Nithya & Ponmurugan, 2012; Vijayakumar *et al.*, 2012; Bais *et al.*, 2012; Gulve & Deshmukh, 2012
Genus *Citrobacter* *C. freundii*	Nosocomial infections of the respiratory tract, urinary tract and blood	Franco *et al.*, 1987; Franco *et al.*, 1990a
Genus *Clostridium* *Clostridium sp.*, *C. botulinium*	Pseudomembranous colitis, food poisoining, tetanus, infections, Flaccid muscular paralysis	Selvin *et al.*, 2004; Ravikumar *et al.*, 2011
Genus *Corynebacterium* *C. diptheriae*	Diphtheria	Kavitha *et al.*, 2009
Genus *Enterobacter* *E. cloacae*♀, *E. aerogens*♠	Urinary and respiratory tract infections♀; Gut infections, opportunistic infections♠	Franco *et al.*,1987;Velho-Pereira & Kamat, 2011; Satheeja & Jebakumar, 2011; Gunda & Singara Charya, 2012
Genus *Enterococci* *Enterococci* sp., *E. faecalis*♦	Urinary Tract Infections, bacteremia, bacterial endocarditis, diverticulitis and meningitis; Gut infection, Meningitis, endocarditis and Urinary Tract Infections♦	Gandhimathi *et al.*, 2008; Vimal *et al.*, 2009; Kharat *et al.*, 2009; Duraipandiyan *et al.*, 2010; Umasankar *et al.*, 2010; Usha Kiranmayi *et al.*, 2011; Vijayabharathi *et al.*, 2011; Dwivedi *et al.*, 2011
Genus *Escherichia* *E. coli*	Gastrointestinal infection	Franco *et al.*, 1987; Franco *et al.*, 1990a; Kokare *et al.*, 2004; Selvin *et al.*, 2004;Tripathi *et al.*, 2004; Gorajana *et al.*, 2005; Peela *et al.*, 2005; Singh *et al.*, 2006; Kumari *et al.*, 2006; Thakur *et al.*, 2007; Thangapandian *et al.*, 2007; Anupama *et al.*, 2007; Srividya *et al.*, 2008; Gandhimathi *et al.*, 2008; Kaur *et al.*, 2008; Remya & Vijayakumar, 2008; Malik *et al.*, 2008; Verma *et al.*, 2009; Subramanian *et al.*, 2009; Singh *et al.*, 2009a; Ningthoujam *et al.*, 2009; Ramesh & Mathivanan, 2009; Yadav *et al.*, 2009; Lakshmipathy *et al.*, 2009 Suthindhiran & Kannabiran, 2009b; Vimal *et al.*,

		2009; Kavitha *et al.*, 2009; Dhanasekaran *et al.*, 2009; Manjula *et al.*, 2009; Selvin *et al.*, 2009; Arumugam *et al.*, 2010; George *et al.*, 2010; Nanjwade *et al.*, 2010; Ravikumar *et al.*, 2010; Radhakrishnan *et al.*, 2010; Kumar & Kannabiran, 2010; Duraipandiyan *et al.*, 2010; Dhananjeyan *et al.*, 2010; Umasankar *et al.*, 2010; Lakshmipathy & Kannabiran, 2010; Deepika & Kannabiran, 2010; Vijayabharathi *et al.*, 2011; Roy & Sen, 2011; Sathiyaseelan & Stella, 2011; Vijayalakshmi & Raja, 2011 Panigrahi *et al.*, 2011; Singh & Rai, 2011; Hemashenpagam, 2011; Sanasam *et al.*, 2011; Vijayalakshmi *et al.*, 2011; Naikpatil & Rathod, 2011; Dasari & Donthireddy, 2011; Vasanthabharathi *et al.*, 2011; Kavitha & Vijayalakshmi, 2011; Sivakumar *et al.*, 2011; Gopinath *et al.*, 2011; Usha Kiranmayi *et al.*, 2011; De *et al.*, 2011; Ravikumar *et al.*, 2011; Dwivedi *et al.*, 2011; Gayathri *et al.*, 2011; Radhika *et al.*, 2011; Mohanraj *et al.*, 2011; Nakade, 2012; Kumar *et al.*, 2012; Talukdar *et al.*, 2012; Kumari & Siddaiah, 2012; Parthasarathi *et al.*, 2012; Pazhanimurugan *et al.*, 2012; Gunda & Singara Charya, 2012; Thenmozhi & Kannabiran, 2012; Athoor & Janardhan, 2012; Chandra *et al.*, 2012; Nithya & Ponmurugan, 2012; Vijayakumar *et al.*, 2012; Bais *et al.*, 2012; Gulve & Deshmukh, 2012
Genus *Klebsiella*		Singh *et al.*, 2006; Selvin *et al.*, 2004; Tripathi *et al.*, 2004; Gandhimathi *et al.*, 2008; Kaur *et al.*, 2008; Dhanasekaran *et al.*, 2009; Vimal *et al.*, 2009; Singh *et al.*, 2009; Yadav *et al.*, 2009; Suthindhiran & Kannabiran, 2009a; Suthindhiran & Kannabiran, 2009b; Arumugam *et al.*, 2010; Nanjwade *et al.*, 2010; Ravikumar *et al.*, 2010; Kumar &

		Kannabiran, 2010; Umasankar *et al.*, 2010; Vijayabharathi *et al.*, 2011; Sathiyaseelan & Stella, 2011; Vijayalakshmi & Raja, 2011;Panigrahi *et al.*, 2011; Mythili & Ayyappa, 2011; Singh & Rai 2011; Hemashenpagam, 2011; Jose *et al.*, 2011; Satheeja & Jebakumar, 2011; Mitra *et al.*, 2011; Naikpatil & Rathod, 2011; Krishna *et al.*, 2011; Jain *et al.*, 2011; Vasanthabharathi *et al.*, 2011; Radhika *et al.*, 2011; Mohanraj *et al.*, 2011; Ravikumar *et al.*, 2011; Gayathri *et al.*, 2011; Baskaran *et al.*, 2011; Pazhanimurugan *et al.*, 2012; Gunda & Singara Charya, 2012; Thenmozhi & Kannabiran, 2012; Athoor & Janardhan, 2012; Vijayakumar *et al.*, 2012; Gulve & Deshmukh, 2012
Klebsiella sp., *K. aerogenes*[Ξ], *K.oxytoca*[Φ] *K. planticola*[γ], *K. pneumonia*[¤]	Nosocomial pneumonia septicaemia, Urinary Tract Infections [Ξ]; Colitis and sepsis[Φ]; Severe pancreatitis[γ]; Pneumonia thrombophlebitis, Urinary and upper respiratory tract infections, cholecystitis, diarrhea, wound infection, osteomyelitis, meningitis, bacteremia and septicemia[¤]	
Genus *Lactobacillus*		Arumugam *et al.*, 2010; Mitra *et al.*, 2011; Vasanthabharathi *et al.*, 2011; Usha Kiranmayi *et al.*, 2011

L. acidophilus[■], *L.casei*[§], *L. lactis*[€] *L. plantarum*[δ], *L. vulgaris*[ψ]	Vaginal infections, mild gastrointestinal discomfort or gas[■]; Opportunistic infections[§, ψ, €]; Mild gastrointestinal discomfort or gas[δ]	
Genus *Leuconostoc* *L. mesenteroides*	Bacteremias meningitis, breast abscess, abdominal abscess, peritonitis	Arumugam *et al.*, 2010
Genus *Listeria* *L. monocytogenes*	Listeriosis	Arumugam *et al.*, 2010
Genus *Micrococcus* *Micrococcus sp.*, *M. flavus*[▲], *M. luteus*[♂]	Skin infections, recurrent bacteremia, septic shock, septic arthritis, endocarditis, meningitis, and cavitating pneumonia[▲]; Intracranial abscesses, pneumonia, septic arthritis, endocarditis and meningitis[♂]	Franco *et al.*, 1987; Franco *et al.*, 1990a; Selvin *et al.*, 2004; Gandhimathi *et al.*, 2008; Malik *et al.*, 2008; Dhanasekaran *et al.*, 2009; Selvin *et al.*, 2009; Subramanian *et al.*, 2009; Singh *et al.*, 2009a; Ningthoujam *et al.*, 2009; Yadav *et al.*, 2009; Arumugam *et al.*, 2010; Sanasam *et al.*, 2011; Chandra *et al.*, 2012
Genus *Mycobacterium* *M. phlei, M. smegmatis, M. tuberculosis*[fi]	Tuberculosis[fi]	Praveen & Tripathi, 2009; Singh *et al.*, 2009a; Deepika & Kannabiran, 2010; Mitra *et al.*, 2011; Raja & Prabakaran, 2011 Kumar *et al.*, 2012

Genus *Pseudomonas* *Pseudomonas sp., P. aeruginosa, P. fluorescence*[≈], *P.luteola*[◊] *P. putida*[╬], *P. staetrolens*[Θ]; *P. solanacearum*[Θ]	Nosocomial infections, Gut Lung infections, Urinary tract infections; Infections in compromised immune systems[≈]; Peritonitis, cellulitis, and bacteremia[◊]; Bacteraemia, sepsis in neonatal, neutropenic and cancer patients, Urinary tract infections [╬]; Nosocomial infections[Θ]	Franco *et al.*, 1987; Franco *et al.*, 1990a; Kokare *et al.*, 2004a; Selvin *et al.*, 2004; Tripathi *et al.*, 2004; Gorajana *et al.*, 2005; Peela *et al.*, 2005; Thakur *et al.*, 2007; Thangapandian *et al.*, 2007; Anupama *et al.*, 2007; Srividya *et al.*, 2008; Gandhimathi *et al.*, 2008; Malik *et al.*, 2008; Kaur *et al.*, 2008; Subramanian *et al.*, 2009; Singh *et al.*, 2009b; Ningthoujam *et al.*, 2009;Ramesh & Mathivanan, 2009; Yadav *et al.*, 2009; Dhanasekaran *et al.*, 2009; Vimal *et al.*, 2009; Kavitha *et al.*, 2009; Manjula *et al.*, 2009; Selvin *et al.*, 2009; Kharat *et al.*, 2009; Nanjwade *et al.*, 2010; Ravikumar *et al.*, 2010; Deepika & Kannabiran, 2010; Radhakrishnan *et al.*, 2010; Duraipandiyan *et al.*, 2010; Umasankar *et al.*, 2010; Vijayabharathi *et al.*, 2011; Vijayalakshmi & Raja, 2011; Sathiyaseelan & Stella, 2011; Panigrahi *et al.*, 2011; Mythili & Ayyappa, 2011; Hemashenpagam, 2011; Demosthenes & Chauhan, 2011; Satheeja & Jebakumar 2011; Naikpatil & Rathod, 2011; Dasari & Donthireddy, 2011; Nithyanand *et al.*, 2011; Kavitha & Vijayalakshmi 2011; Sivakumar *et al.*, 2011; Gopinath *et al.*, 2011;Usha Kiranmayi *et al.*, 2011; Mohanraj *et al.*, 2011; Ravikumar *et al.*, 2011; Kumar *et al.*, 2012; Talukdar *et al.*, 2012; Gunda & Singara Charya; 2012; Thenmozhi & Kannabiran, 2012; Suguna & Rajendran, 2012; Chandra *et al.*, 2012; Athoor & Janardhan, 2012; Nithya & Ponmurugan, 2012; Bais *et al.*, 2012

Genus *Proteus* *Proteus sp., P. mirabilis, P.vulgaris*	Infections, septicemia, pneumonias - mostly in hospitalized patients, Urinary tract infections, wound infections	Franco *et al.*, 1987; Franco *et al.*, 1990a; Gorajana *et al.*, 2005; Thakur *et al.*, 2007; Gandhimathi *et al.*, 2008; Kavitha *et al.*, 2009; Singh *et al.*, 2009a; Yadav *et al.*, 2009; Arumugam *et al.*, 2010; Ravikumar *et al.*, 2010; Umasankar *et al.*, 2010; Vijayabharathi *et al.*, 2011; Singh & Rai, 2011; Velho-Pereira & Kamat, 2011; Hemashenpagam, 2011; Jose *et al.*, 2011; Mitra *et al.*, 2011; Naikpatil & Rathod, 2011; Vasanthabharathi *et al.*, 2011; Gopinath *et al.*, 2011; Usha Kiranmayi *et al.*, 2011 Ravikumar *et al.*, 2011; Talukdar *et al.*, 2012; Kumari & Siddaiah, 2012 Pazhanimurugan *et al.*, 2012; Gunda & Singara Charya, 2012; Vijayakumar *et al.*, 2012; Gulve & Deshmukh, 2012
Genus *Rhodococcus* *R. rhodochrous*	Infections in immunocompromised hosts	Selvin *et al.*, 2009; Arumugam *et al.*, 2010
Genus *Salmonella* *Salmonella sp., S. bovis, S. enteritidis, S. mgulani, S. paratyphi*$^{\Psi}$, *S. senftenberg, S. typhi*$^{\Psi}$, *S. typhimurium*$^{\Psi}$, *S. weltsverden*	Gut infection, salmonellosis; Typhoid$^{\Psi}$	Selvin *et al.*, 2004; Peela *et al.*, 2005; Kaur *et al.*, 2008; Remya & Vijayakumar, 2008; Singh *et al.*, 2009a; George *et al.*, 2010; Radhakrishnan *et al.*, 2010; Umasankar *et al.*, 2010; Vijayabharathi *et al.*, 2011; Sathiyaseelan & Stella, 2011; Vijayalakshmi & Raja, 2011; Singh & Rai, 2011; Velho-Pereira & Kamat, 2011; Demosthenes & Chauhan, 2011; Jose *et al.*, 2011; Satheeja & Jebakumar, 2011; Naikpatil & Rathod, 2011; Vasanthabharathi *et al.*, 2011; Parthasarathi *et al.*, 2012; Dasaria *et al.*, 2012; Gunda & Singara Charya, 2012; Vijayakumar *et al.*, 2012
Genus *Sarcina* *Sarcina lutea*	Skin infection	Gunda & Singara Charya, 2012

Genus *Serratia* *Serratia liquefaciens, S. marcescens*[đ]	Nosocomial infections, Gut infection[đ]	Franco *et al.*, 1987; Kumari *et al.*, 2006; Kavitha *et al.*, 2009; Arumugam *et al.*, 2010; Singh & Rai, 2011; Velho-Pereira & Kamat, 2011; Mitra *et al.*, 2011; De *et al.*, 2011; Nithya & Ponmurugan, 2012
Genus *Shigella* *Shigella* sp., *Shigella flexneri*	Dysentery, Gastrointestinal infection	Kaur *et al.*, 2008; Arumugam *et al.*, 2010; Vijayabharathi *et al.*, 2011; Vijayalakshmi & Raja, 2011; Velho-Pereira & Kamat, 2011; Usha Kiranmayi *et al.*, 2011; Gunda & Singara Charya, 2012
Genus *Staphylococcus* *Staphylococcus* sp., *S. aureus*[≠], *S. epidermidis*,*S. haemolyticus* (MDR)[‖]	Food poisoning, Skin infection[≠], Nosocomial infections, endocarditis, septicemia, peritonitis, urinary tract, wound, bone, joint infection[‖]	Franco *et al.*,1987; Franco *et al.*,1990a; Franco *et al.*, 1991a; Kokare *et al.*, 2004 Selvin *et al.*, 2004; Gorajana *et al.*, 2005; Vasavada *et al.*, 2006; Kumari *et al.*, 2006; Radhakrishnan *et al.*, 2007; Thangapandian *et al.*, 2007; Thakur *et al.*, 2007; Anupama *et al.*, 2007; Malik *et al.*, 2008; Ponmurugan & Nithya, 2008; Srividya *et al.*, 2008; Aruna *et al.*, 2008; Gandhimathi *et al.*, 2008; Dhanasekaran *et al.*, 2009; Vimal *et al.*, 2009; Kavitha *et al.*, 2009; Ningthoujam *et al.*, 2009; Ramesh & Mathivanan, 2009; Yadav *et al.*, 2009; Suthindhiran & Kannabiran, 2009a; Suthindhiran & Kannabiran, 2009b; Selvin *et al.*, 2009; Arumugam *et al.*, 2010 Lakshmipathy & Kannabiran, 2010a; Nanjwade *et al.*, 2010; Ravikumar *et al.*, 2010; Radhakrishnan *et al.*, 2010; Kumar & Kannabiran, 2010 Duraipandiyan *et al.*, 2010; Vijayabharathi *et al.*, 2011; Vijayalakshmi & Raja, 2011; Panigrahi *et al.*, 2011 Mythili & Ayyappa, 2011; Singh & Rai, 2011; Hemashenpagam, 2011; Velho-Pereira & Kamat, 2011; Demosthenes & Chauhan, 2011; Sanasam *et al.*, 2011; Vijayalakshmi *et al.*, 2011; Jose *et al.*, 2011; Satheeja & Jebakumar, 2011; Naikpatil & Rathod, 2011; Dasari & Donthireddy, 2011; Krishna *et al.*, 2011; Vasanthabharathi *et al.*, 2011;

		Nithyanand *et al.*, 2011; Usha Kiranmayi *et al.*, 2011; Baskaran *et al.*, 2011; Radhika *et al.*, 2011; Dwivedi *et al.*, 2011; Gayathri *et al.*, 2011 Sharma *et al.*, 2011; Singh & Shukla, 2011; Kavitha & Vijayalakshmi, 2011; Sivakumar *et al.*, 2011; Gopinath *et al.*, 2011; Nakade, 2012; Kumar *et al.*, 2012; Talukdar *et al.*, 2012; Kumari & Siddaiah, 2012; Parthasarathi *et al.*, 2012; Pazhanimurugan *et al.*, 2012;Gunda & Singara Charya, 2012; Thenmozhi & Kannabiran, 2012; Chandra *et al.*, 2012; Athoor & Janardhan, 2012; Nithya & Ponmurugan, 2012
Genus *Streptococcus* *Streptococcus* sp., *S. faecalis, S. mutans*[■], *S. oralis, S. pneumoniae*[♦], *S. pyogenes*[F], *S. viridians*[∂]	Streptococcal pharyngitis, meningitis, bacterial pneumonia, endocarditis, erysipelas, necrotizing fasciitis, Dental infections/decay[■], Pneumococcal infections[♦], Pharyngitis, skin infection, Erysipelas, cellulitis streptococcal pharyngitis Acute glomerulonephritis, inflammation of the renal glomerulus[F], Mouth or gingival infections[∂]	Franco *et al.*,1987; Franco *et al.*,1990a; Franco *et al.*,1991a; Thangapandian *et al.*, 2007; Gandhimathi *et al.*, 2008; Arumugam *et al.*, 2010; Raja *et al.*, 2010a; Ravikumar *et al.*, 2010; Kumar & Kannabiran, 2010; Krishna *et al.*, 2011; Panigrahi *et al.*, 2011; Jain *et al.* 2011; Usha Kiranmayi *et al.*, 2011; Mohanraj *et al.*, 2011
Genus *Streptomyces* *Streptomyces* sp., *S. griseus*	Mycetoma	Dasari & Donthireddy, 2011; Gayathri *et al.*, 2011
Genus *Vibrio* *Vibrio* sp., *V. alginolyticus*[‡], *V. cholerae*[•], *V. parahaemolyticus*[©], *V. vulnificus*[€]	Foodborne infection, Otitis and wound infection[‡], Cholera[•], Gastrointestinal illness[©], Cholera, cellulitis or septicemia[€]	Selvin *et al.*, 2009; Arumugam *et al.*, 2010 George *et al.*, 2010; Ravikumar *et al.*, 2010; Vasanthabharathi *et al.*, 2011; Nithyanand *et al.*, 2011; Jayasudha *et al.*, 2011; Sathiyaseelan & Stella, 2011; Vijayalakshmi & Raja, 2011
Genus *Yersinia* *Y. enterocolitica*	Yersiniosis, mild self-limiting entero-colitis or terminal ileitis	Arumugam *et al.*, 2010

Table 1.7 Human fungal pathogens used to screen actinomycetes lead molecules

Human fungal pathogens used in screening	Symptoms/ disease	References
Genus *Alternaria* *Alternaria* sp., *A. alternata, A. awamori*	Allergies like hay fever or asthma, opportunistic infections in immunocompromised patients, Lung disease	Kavitha *et al.*, 2009; Singh *et al.*, 2009a; Vijayalakshmi & Raja, 2011; Singh & Rai, 2011; Kumari & Siddaiah, 2012
Genus *Aspergillus* *Aspergillus flavus, A. fumigatus*[Ω], *A. niger*[∞], *A. terreus*[▲]	Aspergillosis of the lungs, corneal, otomycotic, and nasoorbital infections; Invasive fungal infection chronic pulmonary infections or allergic disease in immunosuppressed individuals, Skin infection[Ω], Serious lung disease, aspergillosis[∞], Opportunistic infection in immunocompromised patients[▲]	Kokare *et al.*, 2004a; Kokare *et al.*, 2004a; Srividya *et al.*, 2008; Gandhimathi *et al.*, 2008; Malik *et al.*, 2008; Suthindhiran & Kannabiran, 2009a; Suthindhiran & Kannabiran 2009b Vimal *et al.*, 2009; Kavitha *et al.*, 2009; Manjula *et al.*, 2009; Kavitha *et al.*, 2010; Nanjwade *et al.*, 2010 Kumar & Kannabiran, 2010; Deepika & Kannabiran, 2010; Bharti *et al.*, 2010; Valanarasu *et al.*, 2010; Thenmozhi & Kannabiran, 2011; Sivakumar *et al.*, 2011; Usha Kiranmayi *et al.*, 2011; Suguna & Rajendran, 2012; Thenmozhi & Kannabiran, 2012
Genus *Botrytis* *Botrytis cinerea*	Pneumonitis	Franco *et al.*, 1991a; Yadav *et al.*, 2009; Valanarasu *et al.*, 2010; Vijayalakshmi & Raja, 2011
Genus *Candida* *Candida albicans, C. glabrata, C. krusei*[F], *C. lipolytica*[IO], *C. neoformans, C. tropicalis*	Opportunistic oral and genital infections in humans, candidiasis, Candidemia; Nosocomial infections in immunocompromised and hematological malignancies patients[F]; Refractory oral candidiasis[IO]	Franco *et al.*,1987; Franco *et al.*, 1990a; Franco *et al.*, 1991; Mathew *et al.*, 1994; Tripathi *et al.*, 2004; Kokare *et al.*, 2004a; Jain & Jain, 2007; Thangapandian *et al.*, 2007; Thakur *et al.*, 2007; Srividya *et al.*,2008; Gandhimathi *et al.*, 2008; Remya & Vijayakumar, 2008; Verma *et al.*, 2009 Singh *et al.*, 2009a; Ningthoujam *et al.*, 2009; Suthindhiran & Kannabiran, 2009a; Ramesh & Mathivanan, 2009 Yadav *et al.*, 2009; Suthindhiran & Kannabiran, 2009b; Dhanasekaran *et al.*, 2009; Vimal *et al.*, 2009;Kavitha *et al.*, 2009; Selvin *et al.*, 2009; Kavitha *et al.*, 2010; Arumugam *et al.*, 2010; Nanjwade *et al.*, 2010; Ravikumar *et al.*, 2010; Kumar & Kannabiran, 2010; Bharti *et al.*, 2010; Valanarasu *et al.*, 2010; Sathiyaseelan & Stella, 2011; Vijayalakshmi & Raja, 2011; Singh &

		Rai, 2011; Hemashenpagam, 2011;Velho-Pereira & Kamat, 2011; Sanasam *et al.*, 2011; Jose *et al.*, 2011; Satheeja & Jebakumar, 2011; Mitra *et al.*, 2011;Naikpatil & Rathod, 2011; Kannan *et al.*, 2011; Shobha & Onkarappa, 2011; Jain *et al.*, 2011; Sivakumar *et al.*, 2011; Usha Kiranmayi *et al.*, 2011; De *et al.*, 2011; Nakade, 2012; Talukdar *et al.*, 2012; Kumari & Siddaiah, 2012; Parthasarathi *et al.*, 2012; Chandra *et al.*, 2012; Vijayakumar *et al.*, 2012; Gulve & Deshmukh, 2012; Usha & Masilamani, 2012
Genus *Cryptococcus* *Cryptococcus* sp., *C. neoformans*[Δ], *C. terreus*	Cryptococcosis, meningitis, in immunocompromised patients; Meningitis and meningo-encephalitis in HIV/AIDS patients[Δ]	Kokare *et al.*, 2004a; Thangapandian *et al.*, 2007; Remya & Vijayakumar, 2008; Singh *et al.*, 2009a; Nakade, 2012; Vijayakumar *et al.*, 2012
Genus *Epidermophyton* *E. floccosum*	Tinea pedis, tinea cruris, tinea corporis, onychomycosis	Augustine *et al.*, 2005; Lakshmipathy *et al.*, 2009; Kavitha *et al.*, 2009; Valanarasu *et al.*, 2010
Genus *Fusarium* *Fusarium* sp., *F. moniliforme*[‡], *F. oxysporum*[♠], *F. solani*[♂]	Opportunistic infections, onychomycosis, keratomycosis or mycotic keratitis; Skin infection[‡]; Opportunistic infections in immunocompromised patients[♠]; Neutropenia, aggressive fusarial infections[♂]	Kokare *et al.*, 2004a; Tripathi *et al.*, 2004; Thakur *et al.*, 2007; Singh *et al.*, 2009a; Kavitha *et al.*, 2009; Sathiyaseelan & Stella, 2011; Vijayalakshmi & Raja, 2011; Kavitha & Vijayalakshmi, 2011; Usha Kiranmayi *et al.*, 2011; Nakade, 2012; Talukdar *et al.*, 2012; Sinha *et al.*, 2012
Genus *Microsporum* *Microsporum* sp., *M. canis, M. gypseum, M. nanum*	Tinea capitis, tinea corpus, ringworm, and other dermatophytoses	Augustine *et al.*, 2005; Jain & Jain, 2007; Verma *et al.*, 2009; Lakshmipathy & Kannabiran, 2009; Bharti *et al.*, 2010; Jain *et al.*, 2011; Deepa *et al.*, 2011
Genus *Penicillium* *Penicillium* sp., *P. chrysogenum*[☼], *P. citrinum*[¥], *P. ochrochloron*	Penicilliosis, Keratitis, endophtalmitis, otomycosis, necrotizing esophagitis, pneumonia, endocarditis, peritonitis, UTI; Opportunistic infections in immunocompromised patients[☼]; Balkan nephropathy and yellow rice fever[¥]	Kokare *et al.*, 2004a; Kokare *et al.*, 2004b; Anupama *et al.*, 2007; Singh *et al.*, 2009a; Kavitha *et al.*, 2009; Sathiyaseelan & Stella, 2011; Singh & Rai, 2011; Usha Kiranmayi *et al.*, 2011; Sinha *et al.*, 2012

Genus *Scopulariopsis* *Scopulariopsis* sp.	Infections in immunocompromised patients	Valanarasu *et al.*, 2010
Genus *Trichoderma* *Trichoderma* sp.	Opportunistic infections in immunocompromised patients	Kokare *et al.*, 2004a
Genus *Trichophyton* *Trichophyton* sp., *T. mentagrophytes*[⊥], *T. rubrum*[u], *T. simii*[y]	Malabar itch, athlete's foot, ringworm, jock itch, infections of the nail, beard, skin and scalp; Fungal nail infections, tinea corporis, tinea cruris and tinea capiti[⊥]; Athlete's foot, jock itch and ringworm[u]; Tinea corporis, tinea cruris and tinea capiti[y]	Augustine *et al.*, 2005; Verma *et al.*, 2009; Singh *et al.*, 2009a; Lakshmipathy & Kannabiran, 2009; Bharti *et al.*, 2010; Valanarasu *et al.*, 2010

Several researches have reported Genus *Streptomyces* as the profilic producer of antimicrobials compounds, followed by *Micromonospora*, *Nocardia* and others **(Figure 1.13)**.

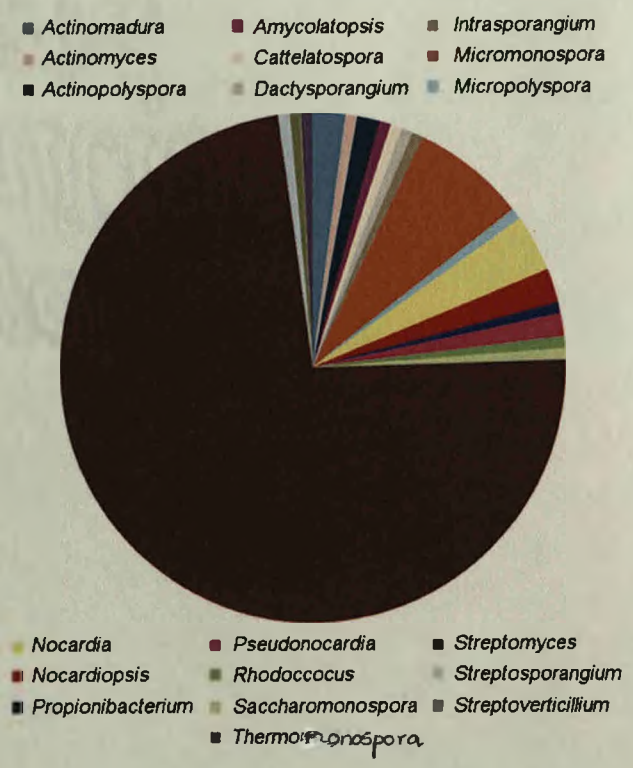


Figure 1.13 Precedence given to Indian actinomycetes taxa in antibiotic screening program

1.5.2.1.3 *Indian patent claims on Antibiotics*

As indicated in **Table 1.8**, patents have been obtained on varied aspects of antibiotic research.

Table 1.8 Antibiotic patents from India

Description/claims	Patent number	Claimants
Actinomycete strain SKF-CWI-785 producing novel glycopeptide antibiotics of the CWI-785 complex	US Pat 4742045	Verma *et al.*, 1988
Streptomyces species producing a novel Macrolide antibiotic Swalpamycin	US Pat 4988677	Franco *et al.*, 1991b
Actinomycete strain Y-86,21022 producing a novel Glycopeptide antibiotic Balhimycin	US Pat 5571701	Nadkarni *et al.*, 1996
Novel strain, *Streptomyces* sp. BICC 7522 producing macrolides	US Pat 7704725	Kulkarni *et al.*, 2010
Novel strain of *Streptomyces* sp., CIMAP A1 producing anti-microbial activity against phytopathogenic fungi	US Pat 6558940	Alam *et al.*, 2003
Streptomyces species (PM0626271/MTCC 5447) producing antibiotic compounds	US Pat 2012/0156295	Mishra & Mahajan, 2012
Actinoalloteichus spitiensis producing bipyridine compound bioactive molecule-Caerulomycin A, derivatives and analogs thereof as effective immunosuppressive agents	US Pat 8114895	Singla *et al.*, 2012
Actinomycete strain Y-86,36910 producing a novel glycopeptide antibiotic Decaplanin	EP0356894	Franco *et al.*, 1990b
Actinomycete strain Y-88,31582 producing a novel antibiotic Alisamycin	EP0436935	Franco *et al.*, 1991c
Actinomycete strain MTCC 5597 producing antibacterial and antifungal compound, (I) 5-(4-bromobutyl)-N-(but-3-enyl)dodec-11-enamide or a derivative thereof	WO/2012/104793	Kumar *et al.*, 2012

1.5.2.2 *Other bioactive compounds*

a) *Anticancer*—Since, cancer is recognized as one of the most dreaded diseases, there is a need for developing drugs to combat it. The most frequent cancers in men are of the lung, lip, oral cavity and in women these include those of the cervix, uteri, breast

and ovary (Behera, 2012). Anticancer drug research in India based on lead molecules from actinobacteria is highlighted in **Table 1.9.**

Table 1.9 Promising actinobacterial strains used in anticancer assays

Species	Human Cell lines (IC_{50}/ LC_{50} Values)					References
	A549 (lung)	HeLa (cervical)	HEP G2 (liver)	MCF-7(breast)	U87MG (brain)	
Streptomyces sp.	+ (NA)	NT	NT	NT	NT	Kumar *et al.*, 2010
Streptomyces sp.	NT	+ (IC_{50} 21.50µg/ ml)	NT	NT	NT	Rajan *et al.*, 2012
Streptomyces sp.	NT	NT	NT	+ (NA)	NT	Naikpatil & Rathod, 2011
Streptomyces aurantiacus	NT	+ (LC_{50} 0.013 µg/ml)	+ (LC_{50} 0.010 µg/ml)	NT	NT	Vijayabharathi *et al.*, 2011
Streptomyces avidinni	NT	NT	+ (IC_{50} 64.5 µg)	NT	NT	Sudha & Masilamani, 2012
Saccharopolyspora salina	NT	+ (IC_{50} 26.2 µg/ml)	NT	NT	NT	Suthindharan & Kannabiran, 2009b
Amycolatopsis alba var. nov.	NT	+ (NA)	NT	+ (NA)	+ (NA)	Dasaria *et al.*, 2012
Unidentified actinobacteria	NT	+ (IC_{50} 4.9 µg/ml)	NT	NT	NT	Athoor & Janardhan, 2012

Legend: +, positive activity; NT, not tested; NA, data not available; LC, lethal concentration; $I_{C\ 50}$, Half maximal inhibitory concentration. *Has also reported activity against VERO cell line (Ic_{50} 250 µg/ml).

b) *Antioxidants*—Thenmozhi *et al.*, 2010, evaluated the antioxidant activity of intracellular and extracellular metabolites of *Streptomyces* sp. VITTK3.

c) *Antiacaricidal/antilarvicidal/antifeedant*—Deepika *et al.*, (2012) studied the acaricidal and larvicidal property of marine actinobacterial compound (2S, 5R, 6R)-2-hydroxy-3,5,6-trimethyloctan-4-one isolated from *Streptomyces* sp. VITDDK3. The compound showed maximum efficacy against the larvae of *Rhipicephalus microplus*,

Anopheles subpictus and *Culex quinquefasciatus*. Vijayakumar *et al.* (2010) reported marine actinobacteria *Streptomyces* sp. and *Streptosporangium* sp. having notable larvicidal activity. Antifeedant activity was reported against *Helicoverpa armigera* and *Spodoptera litura* (Valanarasu *et al.*, 2010).

1.5.2.3 *Enzymological research-enzymes and enzyme inhibitors*

Enzymes

Enzymes of actinomycetes origin have triggered scientific interest due to their wide range of applicability in textile, beverage, food, feed and other industries. As shown in **Figure 1.14** the Indian efforts of screening useful enzymes have been encouraging. Genus *Streptomyces* has been largely studied, followed by few reports on genus *Nocardiopsis, Micromonospora* and *Thermoactinomyces*.

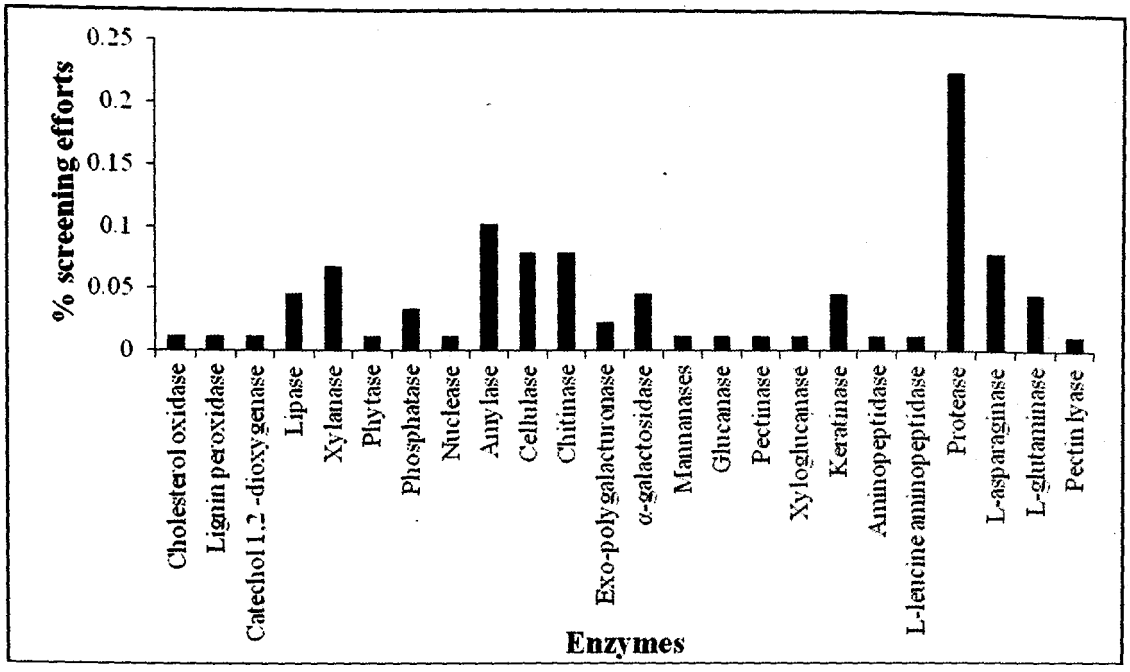


Figure 1.14 Preliminary enzyme screening activity reported in India [**Cholesterol oxidase** (Chauhan *et al.*, 2009), **Lignin peroxidase** (Niladevi & Prema, 2005), **Catechol 1,2-dioxygenase** (Nadaf & Ghosh, 2011), **Lipase** (Selvin *et al.*, 2009; Vishnupriya *et al.*, 2010; Malviya *et al.*, 2011; Selvam *et al.*, 2011), Xylanase (Keskar *et al.*, 1989; Kohli *et al.*, 2001;Kumari *et al.*, 2006; Krishna *et al.*, 2008; Bhosale *et al.*, 2011; Boroujeni *et al.*, 2012), **Phytase** (Bajaj & Wani, 2011), **Phosphatase** (Sahu *et al.*, 2007; Sabarathnam *et al.*, 2010; Kaviyarasi *et al.*, 2011), **Nuclease** (Deshmukh & Shankar, 2004), **Amylase** (Ellaiah *et al.*, 2002; Ellaiah *et al.*, 2004; Deshmukh & Shankar, 2004; Sahu *et al.*, 2007a; Kar & Ray, 2008; Selvin *et al.*, 2009; Haritha *et al.*, 2010; Chakrabortya *et al.*, 2011; Salahuddin *et al.*, 2011), **Cellulase** (Murugan *et al.*, 2007; Chellapandi & Jani, 2008; Selvin *et al.*, 2009; Arunachalam *et al.*, 2010; Boroujeni *et al.*, 2012; Sinha *et al.*, 2012; Salahuddin *et al.*, 2012), **Chitinase** (Nawani *et al.*, 2002; Mane & Deshmukh, 2009; Deepika & Kannabiran, 2010; Malviya *et al.*, 2011; Kavitha & Vijayalakshmi 2011; Subramaniam *et al.*, 2012; Sudha & Masilamani, 2012), **Exo-polygalacturonase** (Jacob & Prema 2006; Kar & Ray, 2011), **α-galactosidase** (Anisha & Prema, 2006; Anisha & Prema 2007; Anisha *et al.*, 2008a; Anisha *et al.*, 2008b), **Mannanases** (Bhoria *et al.*, 2012), **Glucanase** (Malviya *et al.*, 2011), **Pectinase** (Boroujeni *et al.*, 2012), **Xyloglucanase** (Pol *et al.*, 2012), **Keratinase** (Chitte *et al.*, 1999; Saha & Dhanasekaran, 2010; Jayalakshmi *et al.*, 2011; Yadav *et al.*, 2011), Aminopeptidase (Rahulan *et al.*, 2012), **L-leucine aminopeptidase** (Nagy *et al.*, 2008), **Protease** (Dessai & Dhala, 1969; Patke & Dey, 1998; Ellaiah *et al.*, 2002; Ellaiah *et al.*, 2004; Kathiresan *et al.*, 2005; Ningthoujam *et al.*, 2005; Vasavada *et al.*, 2006; Mehta *et al.*, 2006; Ponmurugan & Nithya, 2008; Dastager *et al.*, 2008; Kaur *et al.*, 2008; Vonothini *et al.*, 2008; Selvin *et al.*, 2009; Haritha *et al.*, 2010; Haritha *et al.*, 2011; Thumar & Singh, 2011; Gohel & Singh, 2012; Sudhir *et al.*, (in press); Jani *et al.*, 2012; Malviya *et al.*, 2012), **L-asparaginase**

(Dhevagi & Poorani, 2006; Sahu *et al.*, 2007b; Sahu *et al.*, 2007c; Poorani *et al.*, 2009; Basha *et al.*, 2009; Dharmaraj, 2011; Sudhir *et al.*, (in press)), **L-glutaminase** (Sivakumar *et al.*, 2006; Sahu *et al.*, 2007d; Balagurunathan *et al.*, 2010; Krishnakumar *et al.*, 2011), **Pectin lyase** (Kar & Ray, 2011)].

Thus, this data lays an emphasis on the need to screen other Genus of actinomycetes origin like *Acinetobacter, Actinobispora, Kocuria, Microbispora, Microtetrospora* and *Thermomonospora,* for obtaining industrially important enzymes. As indicated in **Table 1.10** the the studies on enzymes have prioritized lipases, amylases, proteases, endoglucanases, α-galactosidases, pectin lyases, xylanases, L-asparaginases, L-glutaminases and cellulases.

Table 1.10 Indian research on enzymes from actinomycetes

Enzyme	Sources strain	References
Cholesterol oxidase	*Streptomyces lavendulae*	Chauhan *et al.*, 2009
Lignin peroxidase	*Streptomyces psammoticus*	Niladevi & Prema, 2005
Catechol 1,2-dioxygenase	*Rhodococcus* sp.	Nadaf & Ghosh, 2011
Lipase	*Nocardia dassonvillei*, *Streptomyces griseus*	Selvin *et al.*, 2009; Vishnupriya *et al.*, 2010
Xylanase	*Kocuria* sp., *Thermoactinomyces thalophilus*, *Steptomyces* sp., *Steptomyces rameus*	Keskar *et al.*, 1989; Krishna *et al.*, 2008; Kohli *et al.*, 2001; Boroujeni *et al.*, 2012
Phytase	*Nocardia* sp.	Bajaj & Wani, 2011
Phosphatase	*Actinobispora yunnanensis*, *Microtetraspora fastidiosa*, *Micromonospora echinospora*, *Saccharopolyspora hirsute*, *Saccharopolyspora viridis*, *Thermoactinomyces mesophila*,, *Streptomyces albus*, *Streptomyces cyaneus*, *Acinetobacter* sp., *Streptomyces* sp., *Nocardiopsis* sp.	Sahu *et al.*, 2007b; Kaviyarasi *et al.*, 2011
Nuclease	*Streptomyces thermonitrificans*	Deshmukh & Shankar, 2004
Amylase	*Streptomyces erumpens*, *Saccharopolyspora* sp. *Streptomyces* sp.	Kar & Ray, 2008; Haritha *et al.*, 2010; Chakrabortya *et al.*, 2011

Cellulase	*Streptomyces actuosus, Streptomyces* sp., *Nocardiopsis dassonvillei,Streptomyces noboritoensis*, *Microbispora* sp.	Murugan *et al.*, 2007; Selvin *et al.*, 2009; Haritha *et al.*, 2010; Arunachalam *et al.*,2010; Boroujeni *et al.*, 2012; Salahuddin *et al.*, 2012
Chitinase	*Streptomyces canus, Streptomyces pseudogriseolus*, *Streptomyces* sp., *Micromonospora brevicatiana*	Nawani *et al.*, 2002; Mane & Deshmukh, 2009;
Exo-polygalacturonase	*Streptomyces erumpens, Streptomyces lydicus*	Kar & Ray, 2011 Jacob & Prema, 2006
α -galactosidase	*S. griseoloalbus*	Anisha & Prema, 2007; Anisha *et al.*, 2008a
Mannanases	*Streptomyces* sp.	Bhoria *et al.*, 2012
Pectinase	*Streptomyces* sp.	Boroujeni *et al.*, 2012
Xyloglucanase	*Thermomonospora* sp.	Pol *et al.*, 2012
Keratinase	*Streptomyces thermoviolaceus, Streptomyces sclerotialus*	Chitte *et al.*, 1999 Saha & Dhanasekaran, 2010
Aminopeptidase	*Streptomyces gedanensis*	Rahulan *et al.*, 2012
L-leucine aminopeptidase	*Streptomyces mobaraensis, Streptomyces gedanensis, Streptomyces platensis*	Nagy *et al.*, 2008
Protease	*Streptomyces clavuligerus, Nocardiopsis prasina, Streptomyces carpaticus, Streptomyces moderatus, Streptomyces megasporus, Streptomyces gulbargensis, Nocardiopsis alba, Streptomyces roseiscleroticus*	Patke & Dey, 1998; Ningthoujam *et al.*, 2005; Dastager *et al.*, 2008b; Vonothini *et al.*, 2008; Haritha *et al.*, 2010; Kumar & Kannabiran, 2010; Thumar & Singh, 2011; Haritha *et al.*, 2011; Gohel & Singh, 2012
L-asparaginase	*Streptomyces canus, Streptomyces noursei, Streptomyces griseoloalbus, Streptomyces ceolicolor, Streptomyces* sp., *Streptomyces aureofasciculus, Streptomyces chattanoogenesis, Streptomyces hawaiiensis, Streptomyces orientalis, Streptomyces olivoviridis*	Sivakumar *et al.*, 2006; Sahu *et al.*, 2007a; Sahu *et al.*, 2007b; Vijayakumar *et al.*, 2007; Dharmaraj, 2011; Sudhir *et al.*, (in press)
L-glutaminase	*Streptomyces rimosus, Streptomyces* sp.	Sivakumar *et al.*, 2006; Krishnakumar *et al.*, 2011
Pectin lyase	*Streptomyces lydicus*	Kar & Ray, 2011

Enzyme inhibitors

Enzyme inhibitors are gaining importance due to wide applications in chemotherapeutic drugs, metabolic control, pesticides, herbicides and natural poisons. Indian research in this area is still in infancy, with more interest in amylases, α-glucosidases and proteases inhibitors. Raja *et al.*, (2010b) studied amylase inhibitors producing actinomycetesl strains SSR-10 and strain SSR-2 using *Bacillus subtilis* and *Aspergillus niger* as test organisms. Ganesan *et al.*, (2011) tested marine actinomycetes strains for their ability to produce yeast and rat α-glucosidase inhibitors while actinomycetes strains producing alkaline protease inhibitors was reported by Pandhare *et al.*, (2002).

1.5.2.4 *Research on other useful products and processes*

Besides the normal areas of interest reviewed earlier, Indian actinomycetes research has attempted to move in novel directions for assessment of actinomycetes processes such as bioemulsification, biodegradation, biosorption; biosurfactants, osmolytes, nanoparticles production, mineral biosynthesis which are still in the experimental stage (**Table 1.11**).

Table 1.11 Other useful actinomycetes products and processes

Other uses	Strains	References
Biodegradation	*Streptomyces rochei*[♠], *S. krainskii*[♦], *Rhodococcus* sp.[Ψ]; *Rhodococcus* sp.*, *Nocardia* sp.*, *Gordonia* sp.*, *Dietzia* sp.*, *Brevibacterium casei*[¤], *Rhodococcus* sp.[¤], *Slackia exigua*[IO], *Corynebacteium liquefaciens*[⚭]	Saini *et al.*, 1998; Mane *et al.*, 2008; Chaudhary *et al.*, 2011;Mohanraj *et al.*, 2011; Mereena *et al.*, 2011; Raut & Bajekal, 2011; Ningthoujam *et al.*, 2012
Biosurfactants	*Streptomyces* spp., *S. gedanensis*, *Nocardiopsis alba*	Gandhimathi *et al.*, 2009; Lakshmipathy *et al.*, 2010; Lakshmipathy & Kannabiran, 2010b; Karthik *et al.*, 2010
Biosorption of heavy metals	*Streptomyces* sp.[ϰ]	Saurav & Kannabiran, 2011
Osmolytes	*Actinopolyspora* sp., *Streptomyces* sp., *Nocardiopsis* sp.	Kundu *et al.*, 2008; Ameur *et al.*, 2011
Biominerals	*Thermomonospora* sp.	Rautaray *et al.*, 2004
Pigments	*Streptomyces* sp.[◊]; *Streptomyces* spp.[♂]	Dharmaraj *et al.*, 2009; Quadri & Agsar, 2012
Nanoparticles	*Thermomonospora* sp.[•], *Rhodococcus* sp.[•], *Streptomyces* sp.[▪]	Ahmad *et al.*, 2003a; Ahmad *et al.*, 2003b; Usha *et al.*, 2010

Legend: **Biodegradation**- 3-4 ring PAH compounds degraders (anthracene, fluorene, phenanthrene and pyrene)[♠], textile dye reactive blue-59 degraders[♦], aliphatic and aromatic crude oils degraders[Ψ], hydrocarbon degraders*, p-nitrophenol degraders[¤], agar degraders[IO], chlorobenzoates degraders[⚭]; **Biominerals**-produced extra and intracellular formation of $CaCO_3$; **Pigments**- melanin[◊]; carotenoids mainly phytoene[♂]; **Nanoparticles**- synthesis of gold nanoparticles[•]; synthesis of metal oxide nanoparticles[▪].

Other applications based genomic studies include the first putative PHA synthase gene from a *Streptomyces* sp. with serine as the active nucleophile in the conserved lipase box (Ramachander & Rawal, 2005), the sequence of the bacteriophage (phage) PIS136 isolated from a strain of *Saccharomonospora* (Bajpai *et al.*, 2012) and investigation of 8.231-Mb genome sequence of *Rhodococcus imtechensis* (Vikram *et al.*, 2012). Besides this studies, co-production of caffeic acid and p-hydroxybenzoic acid by *Streptomyces caeruleus* (Sachan *et al.*, 2006), production of poly-e-lysine by *Streptomyces noursei* (Bankar & Singhal, 2011) and

biotransformation of the anti-inflammatory compound meloxicam by *Streptomyces griseus* NCIM 2622 and *S. griseus* NCIM 2623 (Gurram *et al.*, 2009) as also been reported.

1.5.2.5 *Problems and Challenges related to study of actinomycetes*

Early work on actinomycetes in India started in the 20th century, made slow and steady progress despite lack of success in discovery of new genera and metabolites. According to Berdy (2012), the major cause of such declining trends in microbial metabolite research is human responsibility, scientific failure mainly due to limitations of use of modernised techniques like high-throughput screening and combinatorial synthesis and problematic economic-regulatory environment. Such limitations may be prime cause of declining productive actinomycetes research in India. Many institutions working in India might not be well equipped with all the facilities required to expedite the research. Besides other research limitations like extremely slow doubling time of the actinomycetes in comparison to other microorganisms, making the isolation programmes to take several months is a major issue. Also, despite high frequency sampling and isolation efforts, one cannot ensure isolation of novel genera which could lead to a novel antibiotic lead molecule. But all these problems could be reduced through a national strategy to boost actinomycetes research aiming at using more advanced natural isolation and screening strategies. Besides providing new avenues, facilities and funds is a must in all leading research institutions so as to upgrade the standards of research which will lead to positive outcomes.

Actinomycetes have proven the potential as chemically creative species which could be sustainably utilized for human welfare. In India, actinomycetes research has

not accelerated despite the country having interesting and fertile habitats waiting to be exhaustively explored. The Indian research so far has progressed in a few areas and needs more thrust through a national strategy and action plan for biodiversity and biosprospecting of actinomycetes. Such strategy would advance knowledge, build indigenous capacities, throw up new techniques, spawn novel technologies and continue to sustain wealth generation and national development. The present century offers promising challenges and opportunities for Indian actinomycetological research.

1.5.2.6 Scenario post Nagoya protocol

A vast area is opened by Nagoya protocol (www.cbd.int; Laird & Wynberg, 2012) for biodiversity and bioprospecting of microbial resources. India can reap rich dividends through a national strategy and action plan to explore and exploit actinomycetes resources. Considering the diverse physiological and climatic conditions, various regions in mainland India and the islands offer huge scope for sampling and isolation of many interesting and novel actinobacterial strains. Besides modern metagenomic tools can also be employed for mapping its unculturable diversity. Promising strains of culturable actinomycetes can be subjected to national bioprospecting efforts in which the corporate biotech and pharma R&D sector could play a major role. Biotechnological applications of culturable actinomycetes especially in bioremediation and nanobiotechnological processes and products are another promising area of research.

There is further scope to generate nationally important and useful intellectual property from industrially useful strains considering the positive trends emerging out of almost a century of actinomycetes research. An attempt has been made to suggest an outline of such plan to benefit from Nagoya protocol **(Figure 1.15)**.

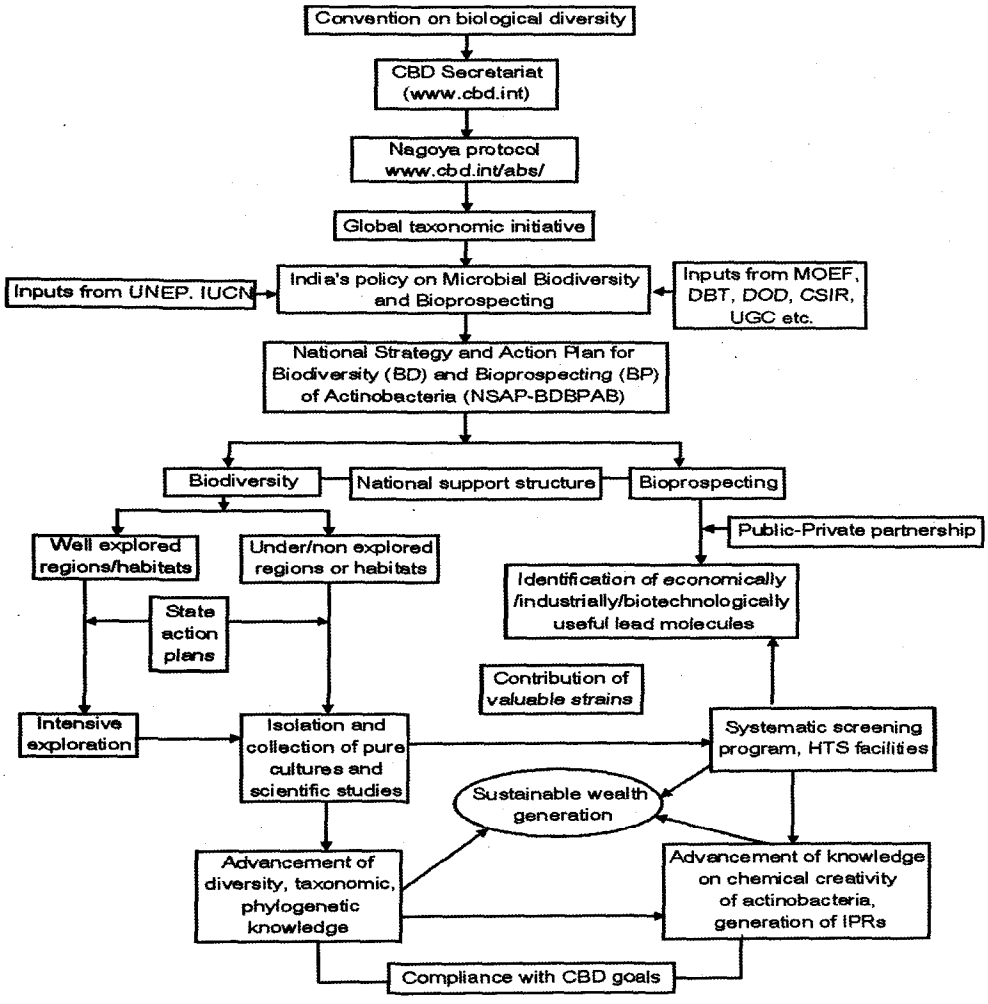


Figure 1.15 Schematic representation of basic elements of a National Strategy and action plan for boosting actinomycetological research

Since India has already ratified this protocol, it would be advantageous to make an early start. If this strategy or action plan is launched systematically and successfully, it could make India a global leading player in identification of useful strains, novel products and processes from actinomycetes diversity, both, from pure academic and practical technological angles. The future scope for advancement of actinomycetes research in India appears bright.

OBJECTIVES OF THE PRESENT STUDY

There is tremendous potential in Goa for biodiversity cataloguing of actinomycetes and assessment of their biotechnological potential. In view of the huge gaps left in the understanding its biodiversity and bioprospecting the present study was formulated under the following objectives:

- Exploration of habitats for isolation of soil dwelling actinomycetes
- Selection of representative strains and their systematic screening for an industrially promising enzyme and a potentially new antibiotic compound
- Identification of chemically creative superior strains
- Standardization of benchtop bioprocess parameters for enhancing the enzyme (α-amylase) and antibiotic production
- Purification and characterization of the enzyme (α-amylase) and antibiotic compound

The experimental set up, the results obtained, inferences and conclusions drawn from the content of the thesis is organized into four chapters, followed by the summary, bibliography, appendices and annexures.

CHAPTER II

Ecology and biological aspects of soil dwelling actinomycetes

2.1 INTRODUCTION

Ecologically, actinomycetes are ubiquitous soil-dwelling organisms, performing significant biogeochemical roles in the soil (Williams *et al.*, 1984; Singh *et al.*, 2006). They hold a prominent position as targets in screening programmes due to their diversity.

Terrestrial sources are known to be valuable for the isolation of potentially useful strains of actinomycetes. According to Baltz, (2007), less than one part of the Earth's soil surface has been screened for actinomycetes, exploring only a fraction of the world's biodiversity. Ecological studies of actinomycetes in soils have been conducted in various habitats across the globe including grasslands (Lee & Hwang, 2002), beach sands (Suzuki *et al.*, 1991), underground caves (Groth *et al.*, 1999), rice-paddies (Hayakawa *et al.*, 1988), orchards (Lee & Hwang, 2002) and sub-glacial ice of Antarctica (Priscu *et al.*, 1999). Studies on actinomycetes diversity from various ecological niches of terrestrial ecosystems in India, has led to isolation of 26 actinomycetes genera, with genus *Streptomyces* being the most dominant, followed by *Micromonopora, Actinomadura, Rhodococcus, Microbispora, Nocardia* and others (Velho-Pereira & Kamat, 2013).

The pharmaceutical importance of such microorganisms (Basilio *et al.*, 2003; Takahashi & Omura, 2003; Berdy, 2005) has necessitated development of novel methods to access new representatives of this group (Gavrish *et al.*, 2008). Microbiologists, universally have often expressed constraints to undertake work on actinomycetes as differential and selective media are required for isolation, a cocktail of antimicrobials need to be incorporated in the media and the incubation period is prolonged lasting upto four weeks (Williams & Cross, 1971).

In India, only few isolation strategies like novel baiting bag (Singh *et al.*, 2009), enrichment culture (Bagewadi *et al.*, 2011) techniques have been employed for isolating soil actinomycetes. As compared to the other countries contributing to the actinomycetes research, Indian research on actinomycetes has not accelerated despite the country having interesting and fertile habitats waiting to be exhaustively explored.

Goa is the smallest state of India, encompassing an area of 3,702 km^2 (1,429 sq mi) in the Western Ghats of India; rich and varied in biodiversity (Alvares & Gadgil, 2002). However its microbial diversity is unexplored, leaving a vast gap in our understanding of the actinomycetes diversity especially in various soil types (Singh *et al.*, 1998). Till date, studies on actinomycetes of this area refer to marine species by Kerkar, (1990); Chandramohan & Nair, (1992); D'Souza *et al.*, (2000); Kokare *et al.*, (2004); Chakraborty *et al.*, (2012), Vijayakumar *et al.*, (2012), Koli & Kulkarni, (2012) and Dastager & Damare, (2013).

Inorder to bridge this gap in knowledge, a modest attempt was made to understand actinomycetes diversity in the state of Goa. The retrieved actinomycetes morphotypes were characterized upto generic level and subsequently evaluated for their dominance and commonality with respect to different sites through PRIMER.v.6.

2.2 METHODOLOGY

2.2.1 Studying the ecological aspects of soil actinomycetes (Williams *et al.*, 1972)

The present study was undertaken to identify a particular humus rich soil fraction which would lead to high frequency actinomycetes isolation.

2.2.1.1 *Location and processing of soil samples*

Based on the success of the previous studies (Velho-Pereira, 2007) for testing the technique, humus rich soils from the lateritic plateau Taleigao, Goa University campus, Goa, India from 5 x 5 m quadrat below the canopy of two indigenous keystone plant species namely *Ficus benghalensis* L., (FB) (a sacred tree of 400 years age as per local knowledge, Lat. 15°27'-36.77''N and Long. 73°49'-59.44''E and *Bombax ceiba* L. (BC), Lat. 15°27'24''N and Long. 73°49'54''E were selected **(Figure 2.1a&b)**. The soils were collected at a depth of 10-20 cm in clean polythene bags, transported to the laboratory and stored at 22 °C before use.

For recording the pH and redox potential measurement at room temperature, soil extracts were prepared by taking 1gm of air dried soil sample stirred with 25 ml distilled water and filtered.

2.2.1.2 *Determination of the percent dry weight of soil samples and its micromorphological studies*

50 grams of each of the air dried soil samples was passed through surface sterilized brass sieves (Kumar Test sieves, Bombay) having mesh of sizes 1000, 710, 355, 250, 150, 105 & 53 μ. The sieved fraction was collected carefully to avoid minimum retention of soil particles on the respected sieves. These sieved fractions were subsequently labeled and weighted to obtain the percent dry weight.

Each of the sieved fraction of soil was mounted in DPX mountant and used as permanent soil mount for detail micromorphological studies.

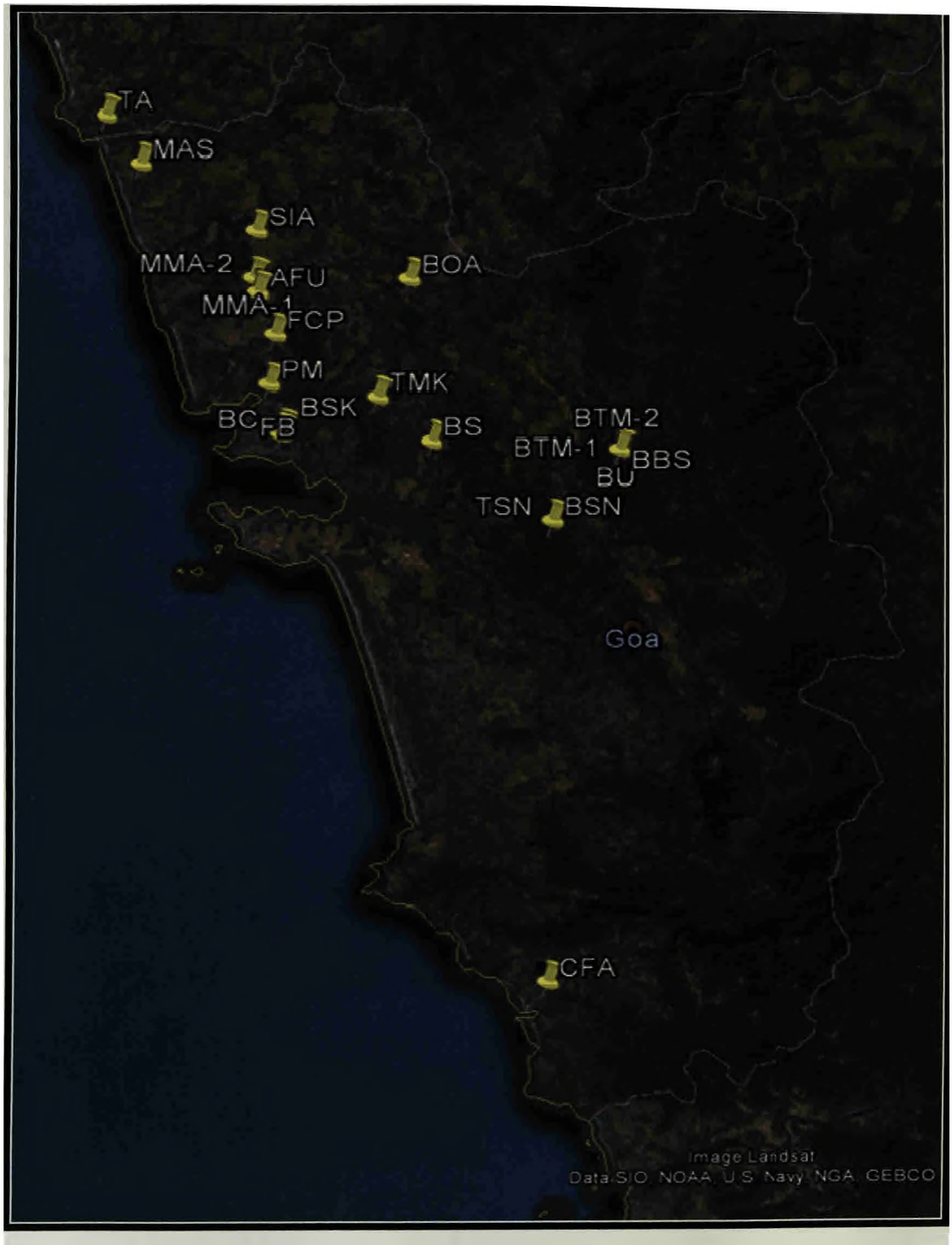


Figure 2.1a Map of Goa showing the various sampling sites (Source: Google earth)

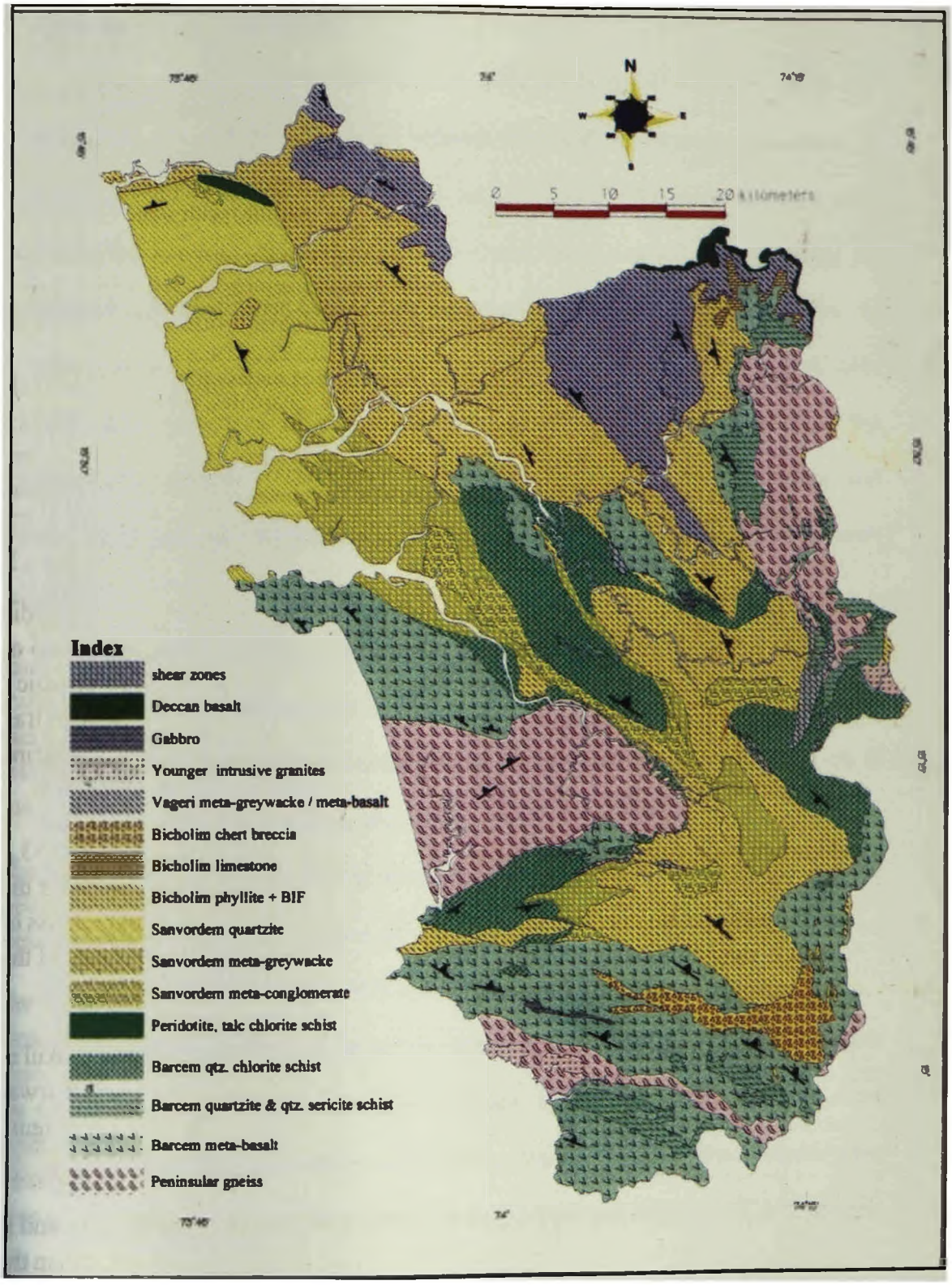


Figure 2.1b Geological map of Goa compiled from various GSI maps (Mascarenhas & Kalavampara, 2009)

2.2.1.3 *Isolation of actinomycetes*

Actinomycetes were isolated by pour plate (Mehrotra & Sumbali, 2009) and soil imprint technique using Arginine Vitamin Agar (AVA) medium (Nonumura & Ohara, 1969). Selected sieved soil fraction ranging from 354-52 μ and less were used for isolating actinomycetes. The AVA medium was prepared by modifying its components to specifically capture actinomycetes diversity. The composition of modified AVA medium is given in **Table 2.1**. The basal medium and the stock trace element solution were sterilized by autoclaving at 121 °C for 20 min and pH of the medium was adjusted to 6.8 by using 0.1N NaOH/HCl. The stock vitamin and antibiotic solution was membrane sterilized and added later to the presterilised basal medium.

Pour plate technique (PPT) is a standard technique which gives the distribution of the colonies as a function of depth of the medium. Soil imprint technique (SIT) is a modification of the adhesive tape profile technique (Fenwick & Knapp, 1982) which was used to specifically study the origin of soil actinomycetes and subsequently isolate them. The selected sieved soil fraction was allowed to come in contact with the sticky surface of the tape which was subsequently rested on previously poured and solidified AVA medium.

For both the techniques the culture plates were incubated at 28 °C and growth was recorded every alternate day. The colonies with different morphologies were selected, purified and transferred to AVA slants. One set of pure cultures/strains were transferred in cryovials containing sterile 20 % glycerol and kept at 4 °C for ensuring long term preservation.

Table 2.1 AVA medium composition (Velho-Pereira & Kamat, 2011)

Basal medium	
Glycerol	0.08 ml
Glucose	0.1 g
K_2HPO_4	0.03 g
L-arginine	0.03 g
NaOH	0.03 g
$MgSO_4.7H_2O$	0.02 g
Agar	1.8 g
Distilled water	100 ml
Stock trace element solution (Add 0.1 ml to the basal medium)	
$Fe_2(SO_4)_3$	50 mg
$CuSO_4.5H_2O$	50 mg
$MnSO_4.4H_2O$	50 mg
$ZnSO_4.7H_2O$	50 mg
Distilled water	50 ml
Stock vitamin solution (Add 0.1 ml to the basal medium)	
Thiamine	100 mg
Calcium penthatonate	25 mg
Meso-inositol	25 mg
Nicotinic acid	25 mg
P-aminobenzoic acid	25 mg
Pyridoxine	25 mg
Riboflavin	25 mg

Biotin	12.5 mg
Distilled water	50 ml
Antibacterials	
Nalidixic acid	0.0176 mg/ml
Neomycin and polymyxin b sulphate	0.01 mg/ml
Antifungals	
Nystatin	0.004 mg/ml
Actidione	0.00192 mg/ml

2.2.2 Novel baiting technique for diversity assessment of soil Actinomycetes (Velho-Pereira & Kamat, 2012)

The present study describes a simple, inexpensive, slide based technique with defined coating of AVA medium to capture actinomycetes diversity.

2.2.2.1 *Study site and sampling*

For testing the technique initially, soil samples were collected from the same sites as mentioned in **section 2.2.1.1**.

2.2.2.2 *Preparation of the coated slides*

AVA medium as mentioned in **section 2.2.1.3** was used as 'Slide Coating Medium' (SCM). Standard plain microscope glass slides (HiMedia, Mumbai, India) 75 x 25 mm and 1.0 mm thick were used as inert coating surface. To permit direct microscopic observations in a marked pre-defined area, a quadrat of the size of a standard cover slip, app. 324 mm^2 (Corning Glass, USA, Type n No.1, 18 x 18 mm, thickness 0.16 mm) was chosen at the centre of the reverse side of the slide area to be

coated. The slides were then sterilized by autoclaving at 121 °C for 20 min and transferred to an 11 cm diameter sterile petri plate (Himedia, Mumbai, India) for coating of the medium to the marked central area. Sterile homogenous molten AVA medium cooled to 40 °C was carefully poured in a single thin stream from a 500 ml Erlenmeyer flask on each slide to get a uniform coating. Two sets of slides in triplicates one with the coating medium incorporated with antibacterial and antifungals and other without were prepared. The coated slides were then transferred back to the sterile petri plate for solidifying and excess medium crossing the edges of the marked area was removed with a sterile scarpel (Hi Media, India). The solidified coated slides were designated as per a preset scheme and used within 15-30 min. All the operations were performed on a laminar air flow bench.

2.2.2.3 *'Insitu' baiting technique from the soil microcosm under natural/outdoor conditions*

For '*Insitu*' baiting technique, random spots under the canopy of the *F. benghalensis* and *B. ceiba* trees was selected **(Figure 2.2a-c)**. Vertical slits of upto 10-12 cm depth were made radially with a surface sterilized knife blade to create identical 'Slide Insertion Slots' (SIS). The SIS was marked using flags for spot identification so as to enable periodic removal of colonized slides for observations. Preselected and labeled pair of coated test slides (one with and another without antibiotics, in triplicates) was then gently inserted in SIS, taking care to bring the medium in contact with the surrounding soil without disturbing the coated surface. The strategy used in novel baiting slide technique is represented in the flowchart below:

A representative flowchart of the strategy used in actinomycetes isolation

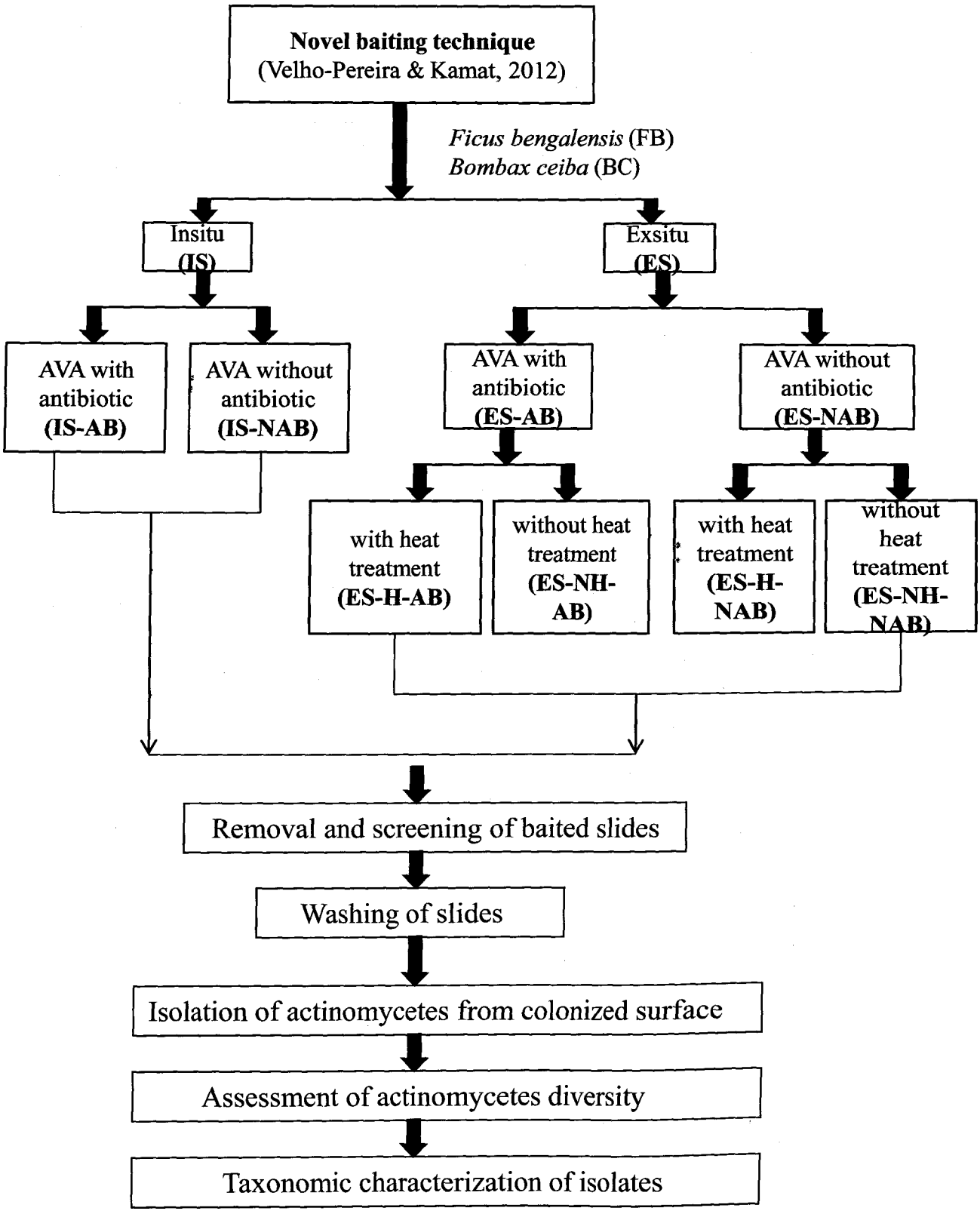


Figure 2.2 '*Insitu*' baiting technique from the soil microcosm under natural/outdoor conditions; **a)** *Ficus benghalensis* L. tree; **b)** *Bombax ceiba* L. tree; **c)** Coated slides inserted into the soil

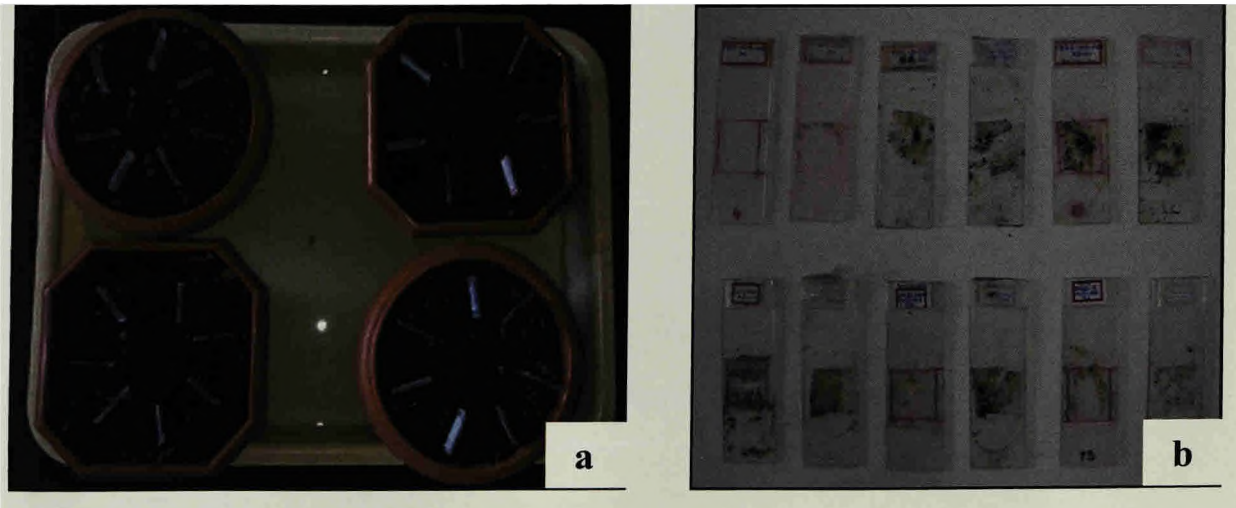


Figure 2.3a) '*Exsitu*' baiting technique from Laboratory Soil Microcosm (LSM) **b)** Freshly extricated AVA baited slides

2.2.2.4 *'Exsitu' baiting technique from Laboratory Soil Microcosm (LSM).*

The LSM was prepared in triplicates in brand new Garden PVC pots (length x width x depth; 13.5 x 13.5 x 10.5 cm, 1.2 kg capacity) which were filled with the moistened soil samples **(Figure 2.3a)**. Two sets of LSM were prepared, wherein in one set the soil was provided with heat treatment (70 °C) and other without. Equidistant (1 cm) vertical slits of upto 5-8 cm depth were made radially with a surface sterilized knife blade to create seven identical SIS to enable periodic removal of colonized slides for observations.

Preselected and labeled pair of coated test slides (with and without antibiotics) was then gently inserted in SIS in the LSM, taking care to bring the medium in the contact with the surrounding soil without disturbing coated surface. The LSM was then covered with aluminum foil and incubated at 28 °C on a clean laboratory platform.

2.2.2.5 *Removal and screening of slides from 'Insitu' and 'Exsitu' soil microcosm*

A pair of slides **(Figure 2.3b)** was sequentially extricated from respective SIS on 4, 7, 12, 15, 22, 26 and 28 days. After removing loose extraneous soil particles, these were observed under Olympus BX41TF microscope (Tokyo, Japan) with digital photomicrographic attachment.

The extent of microbial colonization was scored by noting the relative area under Actinomycetes (AM), Non Actinomycetes' (NAM) and Fungal (F) in each of the 200+ random optical fields and expressed as average percentage colonization.

2.2.2.6 *Actinomycetes isolation from colonized surface*

For sampling AM colony forming units (CFUs) from colonized surface, jets of 2 ml of sterile distilled water were directed towards the extricated slides using a sterile accupipet model T1000 (Tarsons Products Pvt. Ltd., Kolkata, India). Washings (combined of triplicate sets of slides) were collected in a sterile glass beaker and an aliquot (0.1 ml) was then plated on AVA medium with antibiotics (as described previously) and incubated at 28°C for upto 30 days.

2.2.2.7 *Assessment of actinomycetes diversity*

Plates were observed daily under Olympus SZ2-ILST stereomicroscope (Tokyo, Japan) and morphologically dissimilar colonies were identified by recording growth, aerial spores-mass colour, texture, elevation; substrate mycelium colour; pigment production and presence or absence of exudates (Cross & Goodfellow, 1973). Isolates representing dissimilar morphotypes were then identified, selected and purified on AVA plates. Pure colonies were transferred to AVA slants. Isolated pure strains were designated and maintained at temperature of 28 °C. One set of pure strains were transferred in cryovials containing sterile 20 % glycerol and kept at 4 °C for ensuring long term preservation.

2.2.2.8 *Taxonomic characterization of isolates*

For recording micromorphological details, air dried smears of actinomycetes culture were stained in 1 % (w/v) crystal violet. Besides, cover slip culture technique was also adopted (Pridham *et al.*, 1958) wherein the spore bearing hyphae were identified with entire spore chain along with other microstructures (Locci, 1989).

Using the keys described by (Locci, 1989) the isolates were tentatively classified as Genus *Streptomyces* (spore chain with coiling, spiral and looped), Genus *Micromonospora* (formed a single spore at the tip of sporophore on substrate mycelium), Genus *Actinomadura* (short chains of straight and open hooked chains), Genus *Streptosporangium* (Branched aerial mycelium spores are spherical, rod shaped, or oval), Genus *Nocardia* (Fragmented mycelium both in substrate and aerial), Genus *Saccharomonospora* (single spore on unbranched aerial mycelium).

2.2.3 Use of baiting technique for studying soil actinomycetes diversity from various other unexplored habitats

Based on the success of the previous experiment, twenty one soil samples from unexplored habitats **(Figure 2.1a&b, Table 2.2)** were studied for actinomycetes diversity using the novel baiting technique. Selection of candidate test sites were done on the basis of mainly exploring the untapped habitats with the following factors like a) sparsely populated areas /areas lacking human interference b) areas with thick vegetation. All the soil samples were subjected to '*Ex situ*' baiting technique without heat treatment (on the basis of the previous results). All the procedures from baiting the soil actinomycetes from the respective samples **(section 2.2.2.2, 2.2.2.4)** to assessing its diversity as mentioned in **sections 2.2.2.5 - 2.2.2.8** was followed.

Table 2.2 Various sites selected for sample collection.

Type of soils	Sample code	Location	Latitude and Longitude
Forest soils	BT	Bondla forest, Sanguem	Lat. 15°26'36.54"N Long.74°06'31.34"E
	BU	Bondla forest, Sanguem	Lat. 15°26'36.54"N Long.74°06'31.34"E
	KFA	Karmali forest, Tiswadi	Lat. 15°29'16"N Long. 73°54'40"E

	CFA	Delem forest, Canacona	Lat. 14°59'45.76"N Long. 74°03'02.17"E
Hollow of tree cavities	BSK	Karmali, Tiswadi	Lat.15°29'16"N Long. 73°54'40"E
	BBS	Bondla forest, Sanguem	Lat. 15°26'36.54"N Long.74°06'31.34"E
	MAS	Arambol, Pernem	Lat. 15° 41'02.28"N Long. 73°43'05.17"E
Soils from Termite mound	TMK	Karmali, Tiswadi	Lat. 15°29'16"N Long. 73°54'40"E
	TSN	Nirankal, Ponda	Lat. 15°23'1"N Long. 74°3'10"E
	BTM-1	Bondla forest, Sanguem	Lat. 15°26'36.54"N Long.74°06'31.34"E
	BTM-2	Bondla forest, Sanguem	Lat. 15°26'36.54"N Long. 74°06'31.34"E
Orchard soils	VSN	Nirankal, Ponda	Lat. 15°23'1"N Long. 74°3'10"E
	BOA	Bicholim orchard, Bicholim	Lat. 15°35'13.96"N Long. 73°56'11.06"E
Soils from Market drains	MMA-1	Mapusa market, Bardez	Lat. 15°35'16.73"N Long. 73°48'32.66"E
	MMA-2	Mapusa market, Bardez	Lat. 15°35'16.73"N Long. 73°48'32.66"E
	PM	Panjim market, Tiswadi	Lat. 15°29'55.64"N Long. 73°49'18.40"E
Other random soils	AFU	Guirim, Bardez	Lat. 15°34'33"N Long. 73°48'44"E
	FCP	Porvorim, Tiswadi	Lat. 15°32'24"N Long. 73°49'35"E
	BS	Kundaim, NH 4A, Bardez	Lat. 15°27'01.90"N Long. 73°57'17.65"E
	TA	Terekhol, Pernem	Lat. 15°43'29.36"N Long. 73°41'17.92"E
	SIA	Siolim, Bardez	Lat. 15°37'39.35"N Long. 73°48'38.79"E

2.2.3.1 *Statistical measures of diversity*

Diversity studies were conducted for each site separately inorder to arrive at univariate measures of the retrieved actinomycetes community. The data of FB and BC sampling sites (without heat treatment) were also statistically analyzed. Isolation Frequency (%) of each taxa from the different sites was calculated as (*si/S*) 100, where *si* is the number of soil samples containing actinomycetes of the ith taxa and S is the total number of soil samples examined.

Statistical anaylsis was also done by calculating Margalef's species richness (*d*), Pielou's eveness (*J'*) and Shannon-Wiener's diversity index (*H'*) using PRIMER v.6. Margalef's species richness (*d*), is given by d = (S-1) / ln N, where S is the number of genera and N is the number of individuals. The evenness of a community is represented by Pielou's evenness index: *J'=H'/H'*max where H' is the number derived from the Shannon diversity index and H'_{max} is the maximum value of H' given by the equation: $H_{max} = -\sum_{i=1}^{S} \frac{1}{S} \ln \frac{1}{S} = \ln S.$

Shannon diversity index (*H'*) is commonly used to characterize species diversity in a community, accounting for both abundance and evenness of the species present, H= −Σ (*Pi* ln (*Pi*) (Shannon and Weaver, 1949), where *Pi=ni/N* (ni, the relative abundance (RA) of the taxa calculated as the proportion of individuals of a given taxa to the total number of individuals in a community, *N*).

2.2.4 Actinomycete morphology in shaken culture

Actinomycetes produce some interesting morphologies in submerged conditions. Very meager information about this phenomenon has been reported, although these groups of microorganisms are producers of many potential antibiotics of current use (Lawton *et al.*, 1989). Thus in view of the above, the present study was

undertaken to bridge this gap in knowledge of the various gross morphologies of actinomycetes strains grown in shaken liquid culture medium.

Forty two well growing cultures isolated from various sampling sites were randomly selected for studying its gross morphological forms in shaken culture conditions. Kuster's broth (Kuster & Williams, 1964) with pH adjusted to 7.2 was used as the production medium. A loopful of the culture was inoculated into 50 ml of Kuster's broth. The culture flasks were fixed on a rotary shaker (Orbitek^R, Scigenics Biotech, Pvt. Ltd., India) 250 rpm; rotation diameter: 2.0 cm, placed in a thermostated cabinet at 28 °C for 16-20 days fermentation process. The morphological characterization of pellets was done using the key provided by Lawton *et al.*, (1989) and photomicrographs were taken under the Olympus SZ2-ILST stereomicroscope (Tokyo, Japan).

2.3 RESULTS

2.3.1 Ecological aspects of soil actinomycetes

The soil grain size distribution of soil samples from both plant species shows that as the mesh size decreases, the percentage weight of the soils also decreases. The soil fractions size ranging from 709-355 μ and 1000 μ was not selected for further detailed micromorphological studies as fractions were coarse containing pebbles and stones thus, considered not desirable for microbial colonization. In case of BC soils the particles of sizes 354-250 μ showed distinct presence of large sized humus and mineral particles as compared to others. 150 μ and 105 μ fraction showed presence of medium sized humus and mineral particles. 52 μ and less than 52 μ fraction showed particles not very distinct and this could be attributed to the complete disintegration of soil particles. There was not much difference in micromorphology of soil particle

sizes of FB and BC. 250 μ and 150 μ fraction sizes consisted of distinct presence of large humus, some mineral particles and presence of more long, medium sized root like fragments. 105 μ size consisted of root and soil humus particles of comparatively smaller size. 53 μ and less consisted of small to very fine minute particles which were not distinct.

Nighty two isolates were obtained from the humus fraction of the soil sample of *F. benghalensis* using PPT **(Figure 2.4a)** and 57 isolates using SIT **(Figure 2.4b)**. Similarly, 108 and 69 isolates were obtained from the humus fraction of the soil sample of *B. ceiba* using PPT **(Figure 2.4c)** and SIT **(Figure 2.4d)** respectively.
Soil sieved fraction of 354-250 μ and 52 μ and less served as a humus rich soil fraction/s for high frequency actinomycetes isolation.

2.3.2 Novel baiting technique for diversity assessment of soil Actinomycetes (Velho-Pereira & Kamat, 2012)

Slide coated technique showed evidence of a succession of microorganisms predominating the slides at different time interval. Diversity was seen in the form of bacterial and actinomycetes spores and fungal filaments. Actinomycetes dominated majority of the slides. Fructifications such as the formation of branching, stable substrate and aerial hyphae with long spore chains which could be categorized as *rectiflexibiles, rectinaculiaperti* or *spirales* on basis of their characteristic morphology was observed **(Plate 2.1a-f)**. Genus *Streptomyces* was dominant as compared to other actinomycetes genera. It was observed that colonization was not only restricted where agar medium was present but also extended on the surface free of medium.

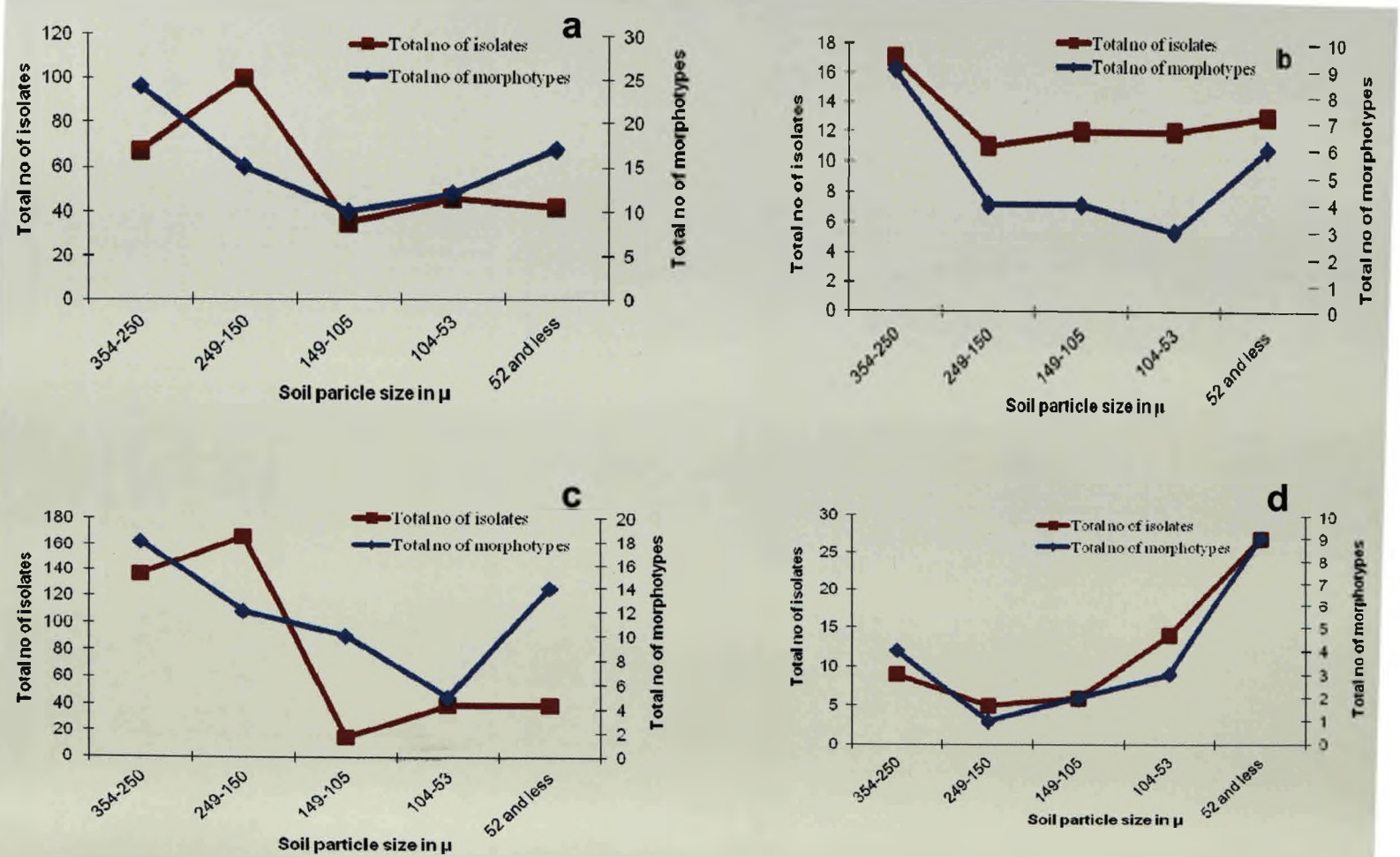


Figure 2. 4 Relationship of morphotypic diversity to soil fractions **a)** *F. benghalensis* using PPT **b)** *F. benghalensis* using SIT **c)** *B. ceiba* using PPT **d)** *B. ceiba* using SIT

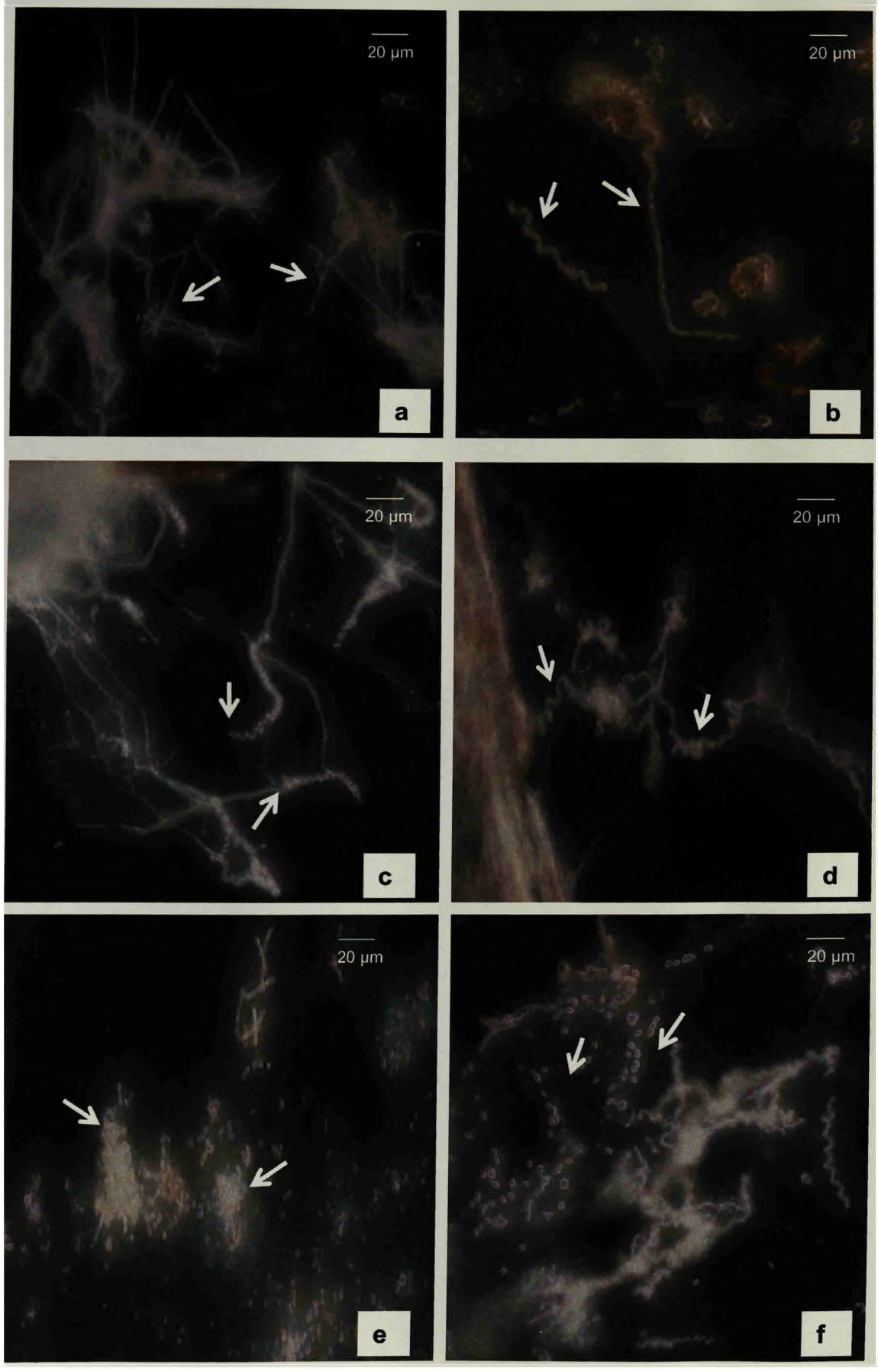
PLATE 2.1

Brightfield and Phase Contrast Microscopy (PC) along with optical slicing revealed very clearly the appearance of actinomycetes fructifications

a-d -Branching, aerial hyphae with long spore chains that could be categorized as *spirales* on basis of their characteristic morphology

e & f -Actinomycetes spores

PLATE 2.1



2.3.2.1 *'Insitu' baiting technique*

The technique showed successful colonization of actinomycetes in all the baited slides. It was observed that FBIS-AB and FBIS-NAB baited slides showed presence of *Streptomyces* sp. dominating most of the slides. The colonization was mostly near the humus particles. A great majority of the baited slides showed the presence of profuse well developed vegetative mycelium. FBIS-AB showed less presence of unwanted fungal and bacterial colonization as compared to FBIS-NAB baited slides. Colonization was seen fluctuating with the varying days of incubation with maximum average colonization seen on the 4th day, in both the cases **(Figure 2.5a&b)**. In FBIS-NAB baited slides, actinomycetes attacking fungal cell walls were observed wherein the lysing of the fungal walls was seen and the fungus appeared vacuolated. Also in some slides dominance of bacterial spores were less as compared to actinomycetes.

On the other hand, BCIS-AB and BCIS-NAB baited slides **(Figure 2.5c&d)** showed less actinomycetes colonization as compared to FBIS baited slides. But the dominance of *Streptomyces* sp. and its interaction with other non actinomycetes microbes was the same as observed in FBIS baited slides.

2.3.2.2 *'Exsitu' baiting technique*

Around 60-70 % colonization by actinomycetes was seen in all the slides extricated from the soil of *F. benghalensis* and *B. ceiba*. FBES-H-AB and BCES-H-AB baited slides showed that heat treatment not only reduced the fungal growth but also partially suppressed the growth of certain actinomycetes. Heat treatment also killed soil arthropods which were seen in slides without treatment. Maximum percentage of colonization was observed on the 7th day in case of FBES-H-AB

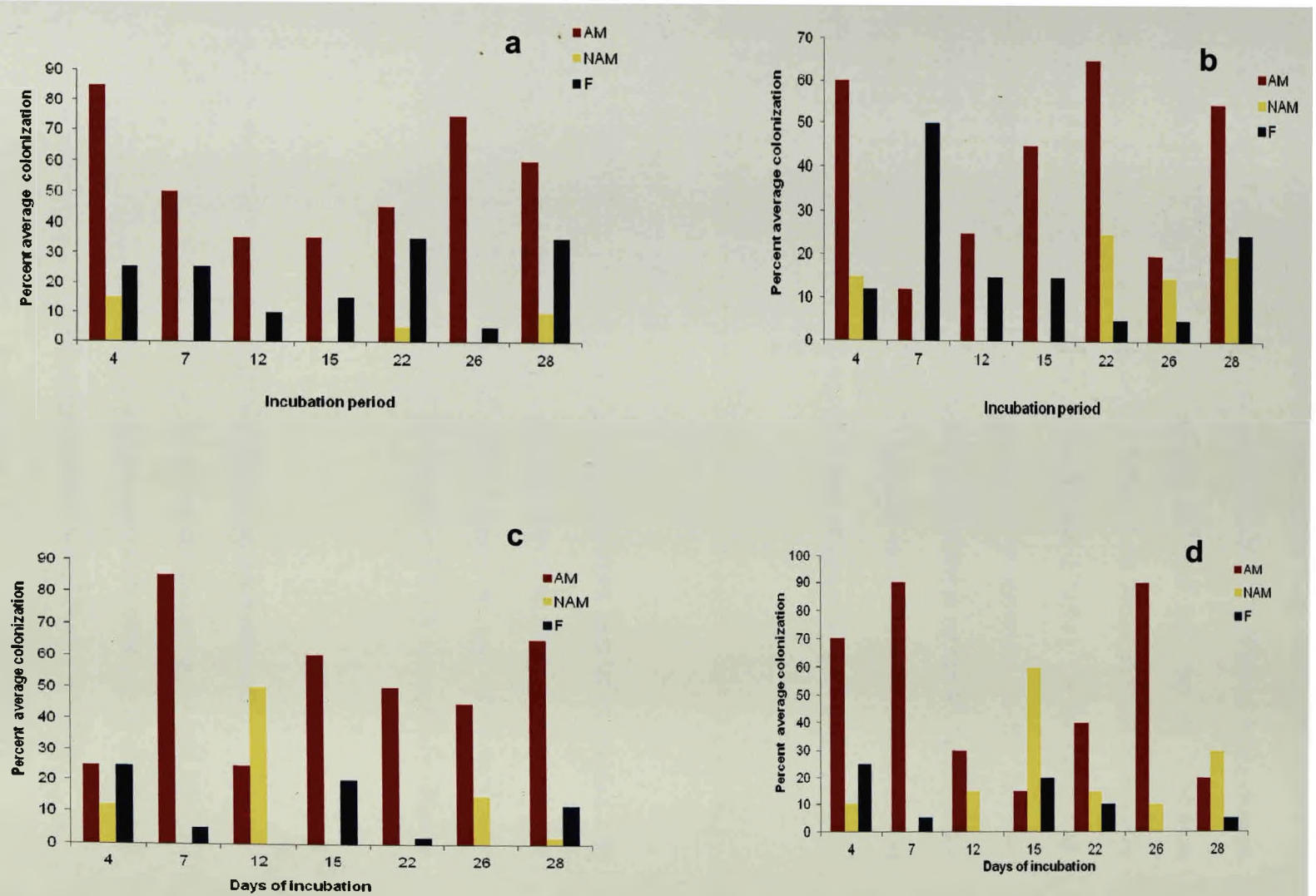


Figure 2.5 Microbial colonization (%) of baited slides **a)** FBIS-AB **b)** FBIS-NAB, **c)** BCIS-AB, **d)** BCIS-NAB

(Figure 2.6a) and BCES-H-AB **(Figure 2.6b)** baited slides. Besides BCES-H-AB baited slides also showed the dominance of non septate fungi despite the addition of antifungals. FBES-H-NAB **(Figure 2.6c)** and BCES-H-NAB **(Figure 2.6d)** showed actinomycetes diversity predominating on the 28th and 26th day of incubation respectively. In case of FBES-NH-AB and BCES-NH-AB baited slides actinomycetes dominated on the 4th and 7th day respectively **(Figure 2.7a&b)**. On the other hand, FBES-NH-NAB **(Figure 2.7c)** showed maximum colonization on the 28th day whereas BCES-NH-NAB **(Figure 2.7d)** showed colonization on the 4th day. Majority of actinomycetes colonies formed well developed microscopically distinguishable vegetative mycelia. Spiral spore chains with spores of Genus *Streptomyces* approx 1-1.5 μm in length dominated the slides.

2.3.2.3 *Assessment of actinomycetes diversity*

Fifty four and 39 isolates were obtained from FBIS and BCIS **(Figure 2.8)** respectively. Eighty two and 62 isolates were obtained from FBES-H-AB/NAB and BCES-H-AB/NAB respectively **(Figure 2.9a)** whereas 36 and 57 isolates were obtained from FBES-NH-AB/NAB and BCES-NH-AB/NAB respectively **(Figure 2.9b)**.

2.3.3 Soil actinomycetes diversity from various unexplored habitats

All soil samples collected were organic in nature excepting TA and SIA soil samples which were silty and clayey in nature respectively. The pH of all the soil samples ranged from 6.4 - 7.4 and the redox potential ranged from 198 - 365.

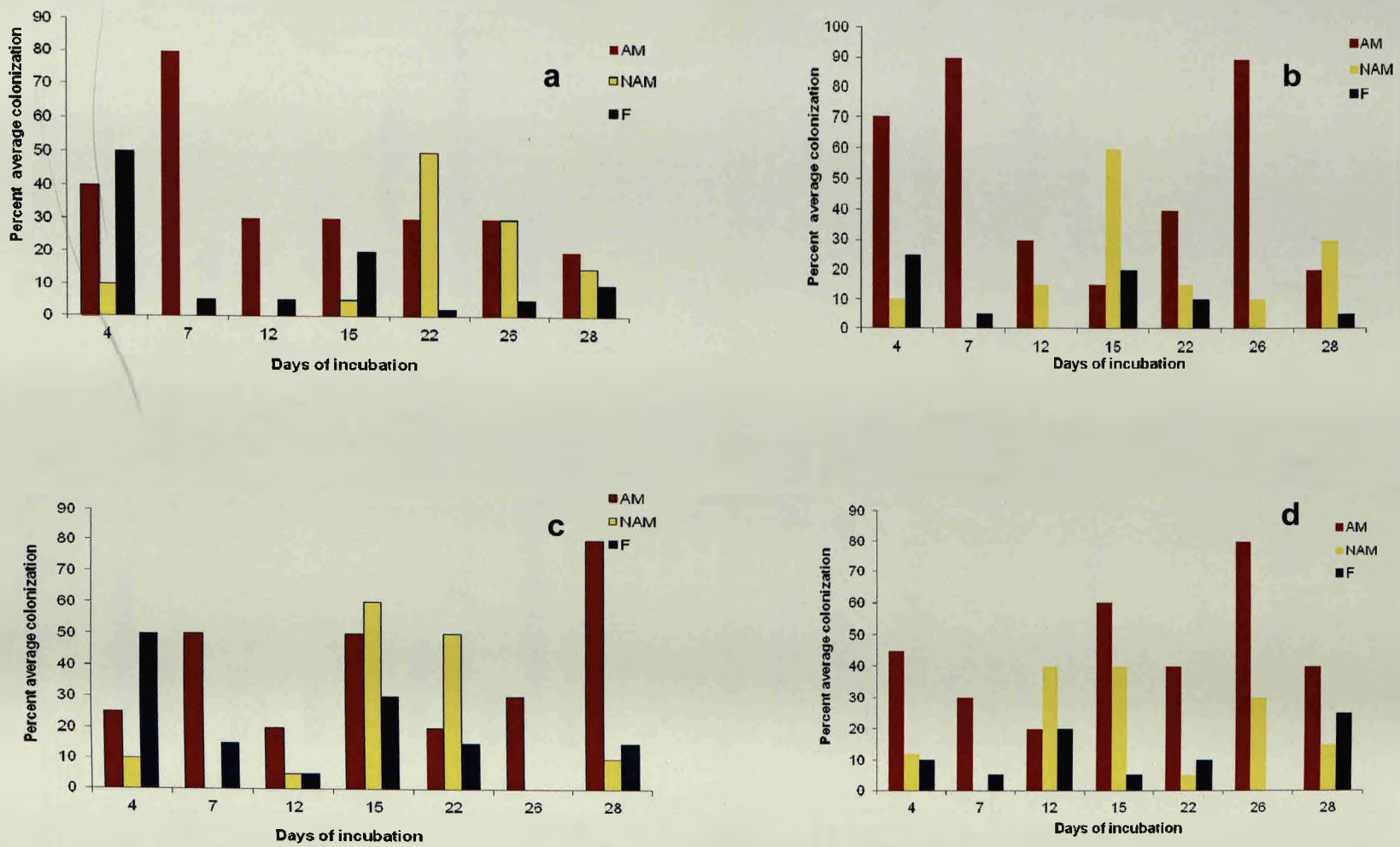


Figure 2.6 Microbial colonization (%) of baited slides **a)** FBES-H-AB **b)** BCES-H-AB, **c)** FBES-H-NAB, **d)** BCES-H-NAB

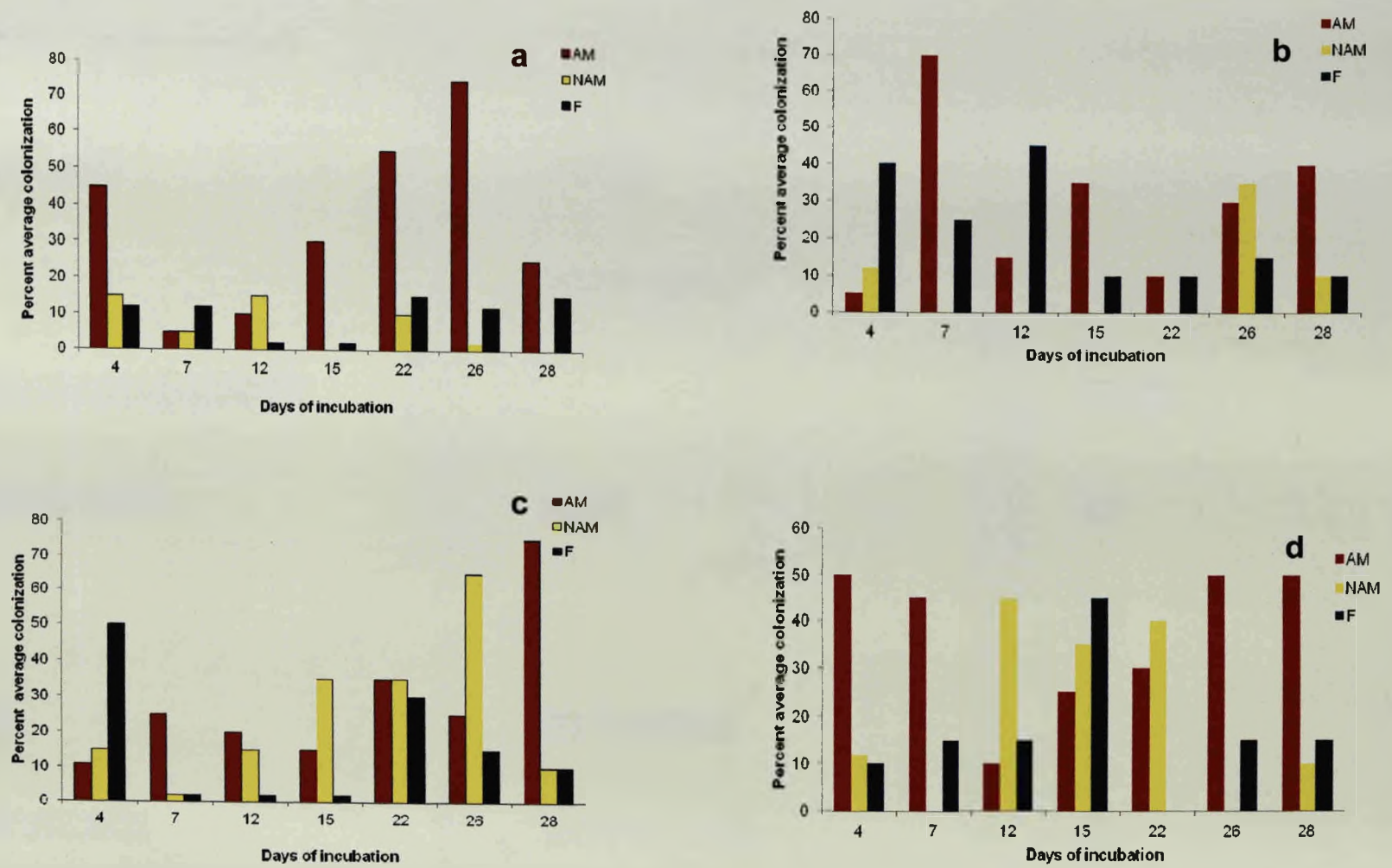


Figure 2.7 Microbial colonization (%) of baited slides **a)** FBES-NH-AB **b)** BCES-NH-AB, **c)** FBES-NH-NAB, **d)** BCES-NH-NAB

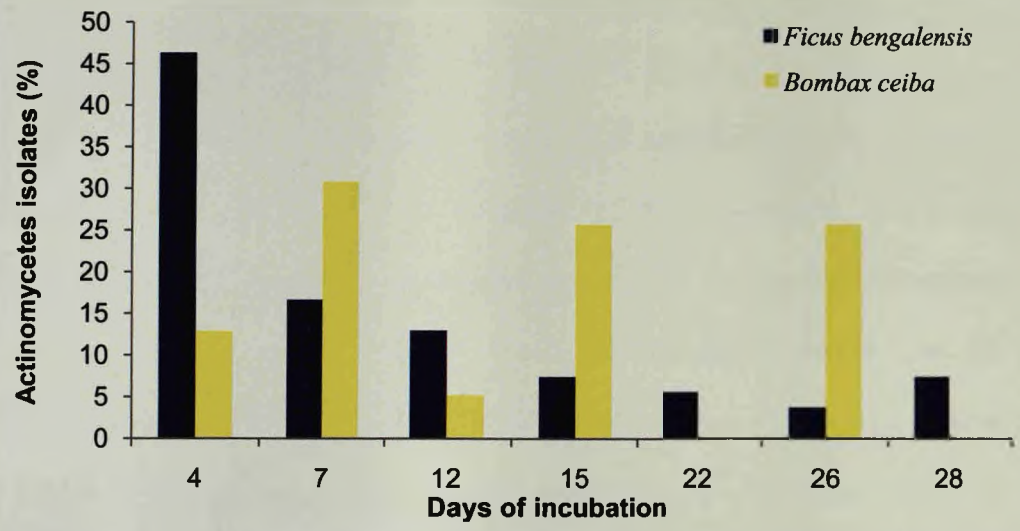


Figure 2.8 Total number of actinomycetes (%) isolates obtained from FBIS and BCIS

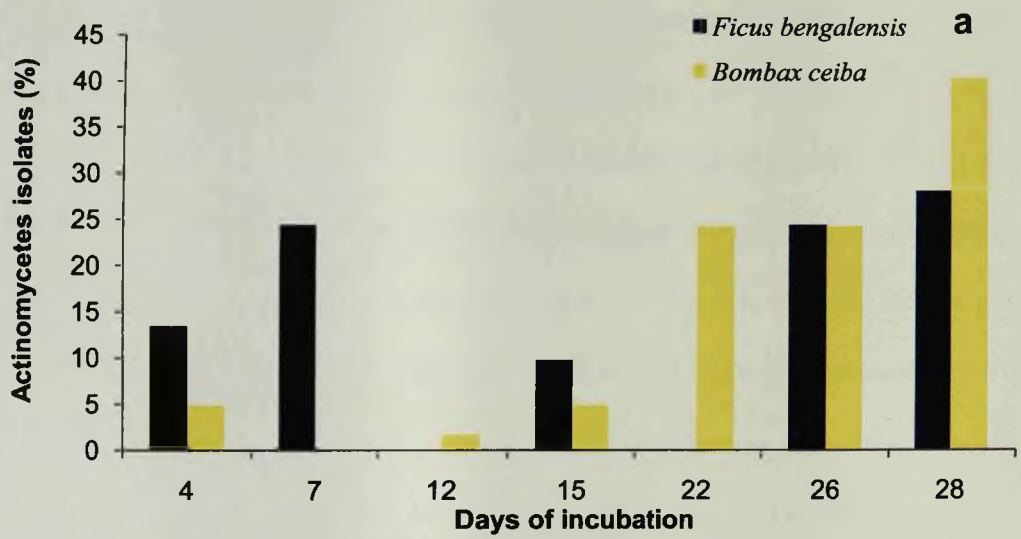


Figure 2.9a) Total number of actinomycetes (%) isolates obtained from FBES-H-AB/NAB and BCES-H-AB/NAB

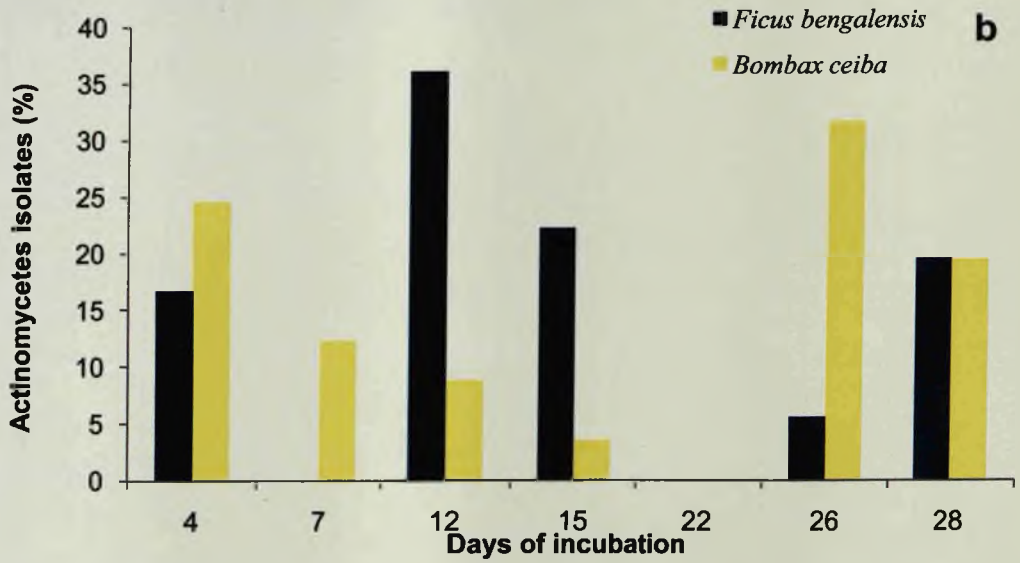


Figure 2.9b) Total number of actinomycetes isolates obtained from FBES-NH-AB/NAB and BCES-NH-AB/NAB

2.3.3.1 *Statistical measures of diversity*

Out of 255 isolates of actinomycetes from all 23 sampling sites, the frequency of the genus *Streptomyces* was 53 %, followed by *Micromonospora* (25 %) and *Actinomadura* (10 %). The taxa *Streptosporangium* (3.9 %), *Saccharomonospora* (2.4 %) and *Nocardia* (1.2 %) showed low percentage of frequency **(Figure 2.10)**. All the isolates belonged to five families namely Streptomycetaceae, Micromonosporacea, Nocardiaceae, Streptosporangiaceae and Thermonosporaceae. The occurrence and distribution of these taxa are presented in **(Table 2.3)**. Among the taxa recorded, *Streptomyces* was prevalent in all the sites with MAS sampling site recording the highest frequency percentage (83 %). Beside *Streptomyces*, the frequency of genus *Micromonospora* varied from 0 to 60 %. The genus *Actinomadura* recorded a range of frequency from 0 to 29 %. The genus *Streptosporangium, Saccharomonospora* and *Nocardia* were recorded in frequencies ranging from (0 to 40 %), (0 to 22 %) and (0 to 25 %) respectively **(Figure 2.11)**. Highest generic richness was observed in BT sampling site, followed by VSN and BTM-1 site. Actinomycetes taxa were evenly distributed at BU sampling site followed by TSN, MMA-2 and TA. Diversity was observed highest in BT site followed by FCP and BBS. Lowest diversity was observed in MAS sampling site **(Figure 2.12)**.

Table 2.3 Occurrence and distribution of actinomycetes at various sampling sites

Sampling sites code	Total no. of colonies/actinomycetes isolates	Actinomycetes isolates grouped in different tentatively identified taxa						
		Streptomyces	*Micromonospora*	*Actinomadura*	*Nocardia*	*Saccharomonospora*	*Streptosporangium*	Unidentified genera
FB	36/24	17	3	4	0	0	0	0
BC	57/15	8	5	2	0	0	0	0
BT	87/34	15	5	2	1	2	4	5
BU	12/7	3	2	2	0	0	0	0
KFA	14/8	6	2	0	0	0	0	0
CFA	26/14	10	4	0	0	0	0	0
BSK	30/10	5	4	0	0	0	1	0
BBS	50/22	9	7	4	0	0	0	2
MAS	8/6	5	0	1	0	0	0	0
TMK	54/10	6	3	1	0	0	0	0
TSN	19/5	3	0	0	0	2	0	0
BTM-1	51/17	8	4	3	0	0	2	0
BTM-2	3/3	2	1	0	0	0	0	0
VSN	22/6	2	3	1	0	0	0	0
BOA	21/9	5	2	0	0	0	2	0
MMA-1	16/8	4	0	2	2	0	0	0
MMA-2	9/5	3	2	0	0	0	0	0
PM	12/6	4	2	0	0	0	0	0
AFU	8/3	2	1	0	0	0	0	0
FCP	41/20	6	8	4	0	0	0	2
BS	10/8	4	0	0	0	2	0	2
TA	27/5	2	3	0	0	0	0	0
SIA	18/10	6	3	0	0	0	1	0

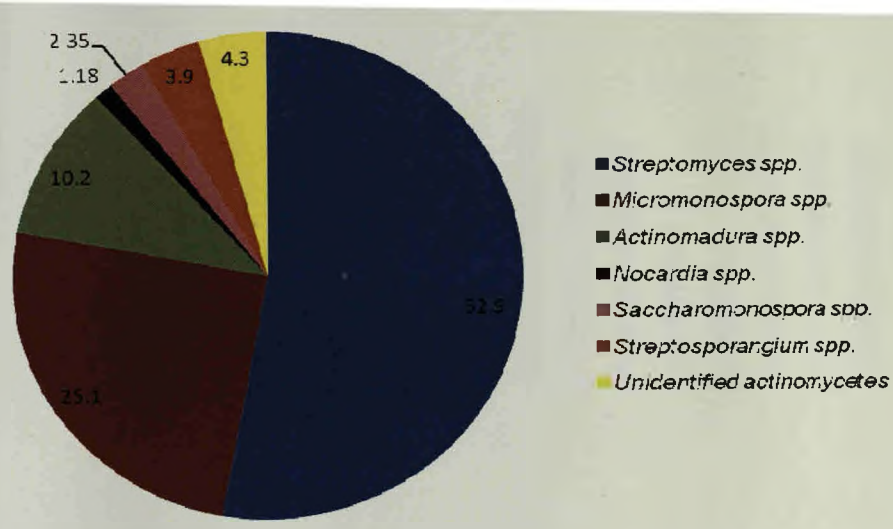


Figure 2.10 Percentage frequency of isolated actinomycetes genera

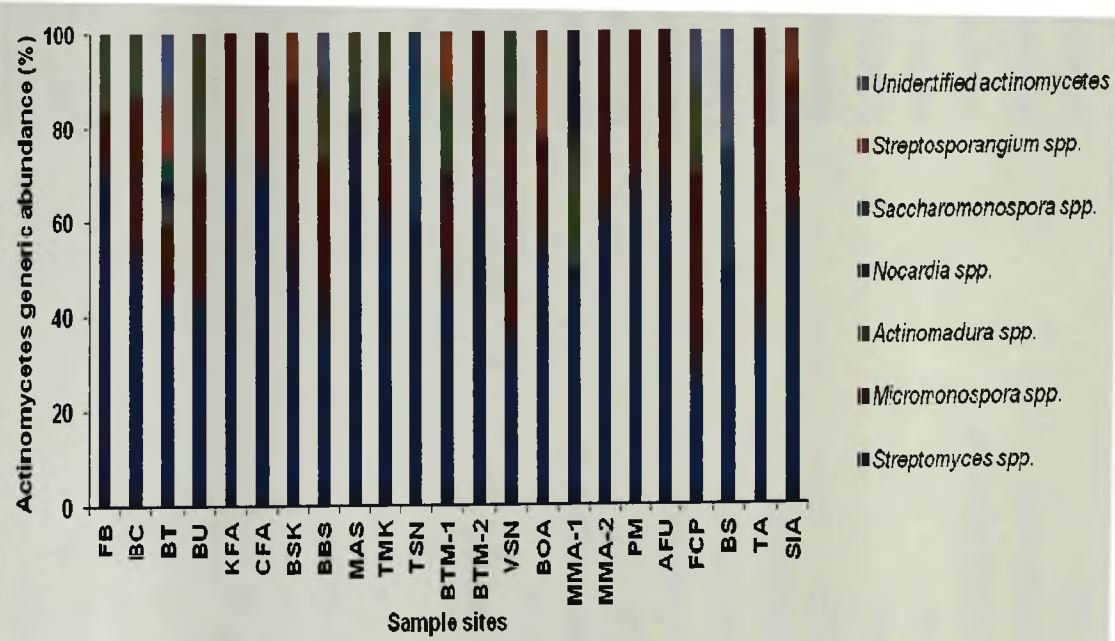


Figure 2.11 Dominant actinomycetes genera from various sampling sites

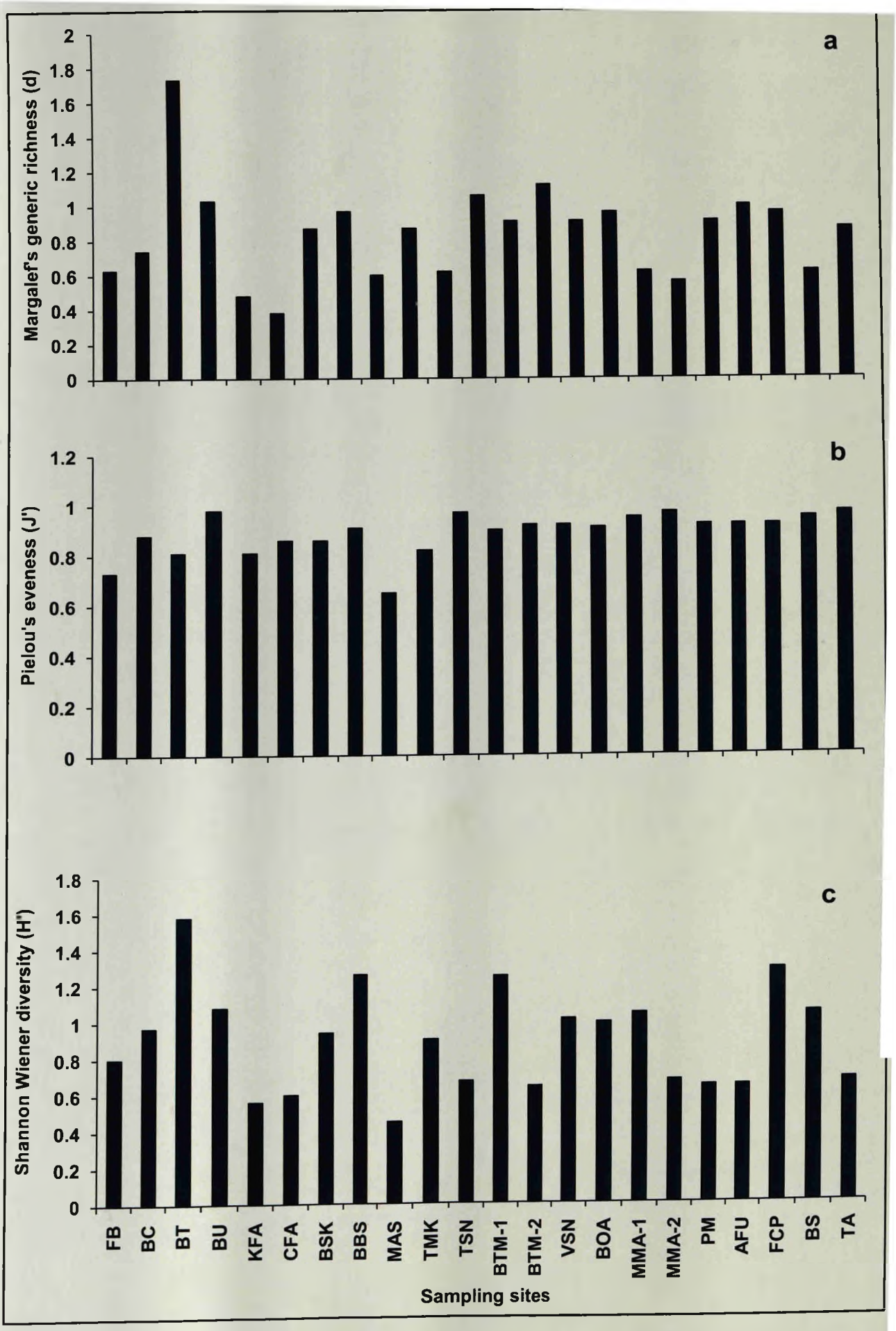


Figure 2.12 a) Margalef's Generic richness (d), **b)** Pielou's evenness (J'), **c)** Shannon-Wiener (H') diversity of actinomycetes community from different sampling sites

2.3.4 Actinomycete morphology in shaken culture

The gross morphological forms of actinomycetes differed from compact pellets, spiky/fluffy pellets, oblong pellets, micropellets, loose aggregates, hydrophobic rafts and fragmented mycelium. These differed from one strain to another as illustrated in **Table 2.4**.

Table 2.4 Actinomycetes morphologies in shaken culture

Actinomycetes strains	**Gross morphological form** (+, present; -, absent)								
Genus *Streptomyces*	**Compact pellets**	**Fluffy pellets**	**Oblong pellets**	**Spiky pellets**	**Micro-pellets**	**Hydrop hobic rafts**	**Dotted pellets**	**Dispersed mycelium**	**Loose aggreg ates**
Streptomyces sp. BC 36	-	-	-	-	+	-	-	-	-
Streptomyces sp. BTM 1-44	-	+	-	+	-	-	-	-	-
Streptomyces sp. BBS 35	+	-	-	+	+	-	-	+	-
Streptomyces sp. BS1	-	+	-	-	-	-	-	+	-
Streptomyces sp. FB 8	+	-	-	-	-	-	-	-	-
Streptomyces sp. BTM 1-16	-	+	-	-	-	-	-	-	-
Streptomyces sp. BTM1-43	+	+	-	-	-	-	-	+	-
Streptomyces sp. BTM 1-38	-	+	-	+	-	-	-	-	-
Streptomyces sp. BBS 39	+	-	+	-	-	+	-	-	-
Streptomyces sp. BTM2-2	-	-	-	-	+	-	-	-	-
Streptomyces sp. BTM1-29	+	+	-	-	-	-	-	+	-
Streptomyces sp. BTM 19	-	-	-	+	-	-	-	+	-
Streptomyces sp. BBS 3	-	-	-	-	+	-	-	-	-
Streptomyces sp. BBS 34	-	-	+	-	+	-	-	-	-
Streptomyces sp. BC 16	+	-	-	-	-	-	-	-	+
Streptomyces sp. BC 37	-	-	+	-	+	-	-	-	-
Streptomyces sp. TA-4	+	-	-	-	-	-	-	-	+
Streptomyces sp. FCP 27	-	-	-	-	+	-	-	+	-
Streptomyces sp. BSK 3	-	-	-	-	+	-	-	-	+
Streptomyces sp. MAS 11	-	-	-	-	-	-	-	+	-
Streptomyces sp. BSK 19	+	-	-	-	-	-	+	+	-

Actinomycetes strains	Gross morphological form (+, present; -, absent) (Table 2.4 cont'd)								
Streptomyces sp. MMA 1-5	-	+	-	-	-	-	-	+	-
Streptomyces sp. MMA 1-7	-	+	-	-	-	-	-	-	-
Streptomyces sp. KFA 11	-	-	-	-	-	-	-	+	-
Streptomyces sp. BBS 9	-	-	-	-	+	+	-	-	-
Streptomyces sp. BTM 14	-	+	-	-	-	-	-	-	-
Genus *Micromonospora*									
Micromonospora sp. BSK 12	+	-	+	-	-	-	-	+	-
Micromonospora sp. CFA 8	+	-	-	-	-	-	-	-	-
Micromonospora sp. BC 18	+	-	+	-	-	-	+	+	-
Micromonospora sp. FB 22	+	-	+	-	-	-	-	-	-
Micromonospora sp. VSN 12	-	-	-	-	+	-	-	+	-
Micromonospora sp. BSK 25	-	-	-	-	-	-	-	+	-
Micromonospora sp. FB 8	-	-	-	-	-	+	-	+	-
Genus *Actinomadura*									
Actinomadura sp. BBS 40	+	+	-	-	-	-	-	-	-
Actinomadura sp. BBS 22	-	-	-	-	+	+	-	-	-
Unidentified actinomycetes									
Strain BTM 1-36	+	-	-	-	-	-	-	-	-
Strain BTM 1-27	-	-	-	-	-	-	-	-	-
Strain FB 15	-	-	-	-	+	-	-	-	-
Strain FCP 3	+	-	-	-	-	-	-	+	-
Strain SIA 12	-	-	+	-	-	-	-	+	-
Strain BS 7	-	-	+	-	-	+	-	-	-

2.4 DISCUSSION

2.4.1 Ecological aspects of soil actinomycetes

Soil serves as a reservoir for many microbes as well as myriads of other lower plants and animals which play a part in the soil economy. Fungi and actinomycetes play an important role in soil decomposition, its conservation wherein its mycelium aid as soil-binding agents transforming the organic matter to the soil (Waksman & Martin, 1939; Waksman, 1945; Went & Stark, 1968). Although humus is fairly resistant to decomposition, it is subject to slow and gradual attack by a variety of microorganisms. Approximately 35 % of the total amount of microbial biomass found in soil below a depth of 25 cm, comprise of Gram positive bacteria and actinomycetes which increase in relative abundance with depth (Eijsackers & Zehnder, 1990). A definite correlation between number of actinomycetes in soils and the level of organic matter and moisture content has been reported (Hayakawa *et al.*, 1988; Paul & Clark, 1996; DeAngelis *et al.*, 2011). Rangaswami *et al.*, (1967) had established a correlation between organic matter content and number of actinomycetes in the soils from South India. The present study was focused on identifying the particular humus rich soil fraction/s which would lead to high frequency actinomycetes isolation.

Among the two techniques tested for this study, pour plate technique (PPT) was a model representative of a particular soil fraction which yielded information of its actinomycetes diversity. On the other hand, soil imprint technique (SIT) led to an equal distribution of soil particles on the surface agar plate due to the sticky nature of the tape. This further facilitated better and clearer visualization of emerging actinomycetes colonies from different sized soil particles. 150 μ sieved fraction showed presence of medium sized soil particles whereas 105 μ showed particles not very distinct. High actinomycetes counts could also be attributed to microcolonies

emerging from spore masses or single spores possibly released from humus particles. It was noticed that only a particular fraction size of particles of 354-250 μ and 52 μ and less yielded more actinomycetes morphotypes, although the colony counts were less. Thus, this size fraction of soil sample could be considered as the best or optimal to capture maximum actinomycetes diversity. However, it was easier to recover pure intact colonies from larger humus particles than from smaller particles. Fifty three micron and less than 53 μ fraction showed particles not very distinct and this could be attributed to the complete disintegration of soil particles. The possible variables influencing presence of actinomycetes on plates could be attributed to placement of soil sample in the plates, moisture content of soil particles in each fraction, adhesion of soil particles to the sticky surface, release of particles from the surface, its contact interface and contact time with each sector, moisture content of the medium.

2.4.2 Novel baiting technique for diversity assessment of soil actinomycetes

Difficulties encountered using older techniques for specific isolation, have led to development of baiting techniques which uses addition of natural substrates to soil as baits to study the population and succession of microbes in different habitats (Warcup, 1965; Tribe, 1957; Warnes & Randles, 1980). Although, there are novel technologies like high throughput screening, whole genome sequencing which make targeted isolation possible, but being expensive these are beyond the reach of large culture collections in Third World Countries.

Rossi-Cholodny technique was limited by the inability of microbes to attach to the slide and the difficulty in further isolation, as the mycelia growing over the slides were usually sterile (Garrett, 1951; Alexander & Jackson, 1954). The present study

describes a technique with slides specifically coated with an actinomycetes isolation medium combining direct visualization with isolation techniques.

The technique demonstrated the usefulness of AVA as a coating medium for selective growth of actinomycetes. AVA is fairly clear medium, yields a large number of actively growing actinomycetes colonies (Nonomura & Ohara, 1969), supports good sporulation and permits microscopic detection of the individual colonies including spores/cells diversity with relative ease and had emerged in our routine isolation work as a suitable candidate for slide coating medium (Velho-Pereira & Kamat, 2011).

Selection of a marked quadrat on the baiting slides allowed focused, prolonged, reproducible observations and quick visual estimates of colonization. Placement of coated slides inside defined SIS within LSM was also an improvement over previous techniques. The present technique also permitted real time still image and video documentation of the growing actinomycetes colonies (www.youtube.com/ watch?v=bJ8pDnXamPw).

Instead of glass slides, previous modifications had employed, nylon mesh (Warcup, 1965), pieces of glass, or cover slips (Warcup, 1965; Linford, 1942; Takahashi & Omura, 2003), or strips of cellophane attached to cover slips (Tribe, 1957; 1961) and hair (Griffin, 1960) which were buried in soil to study the succession of microbes by direct examination. These techniques however did not permit isolation of morphological dissimilar colonies on plate. Gavrish *et al.*, (2008) had developed semi-permeable membrane trap for specifically capturing and cultivating actinomycetes *in situ,* but it employed a relatively expensive polycarbonate membrane filters and diffusion chamber. Lewis *et al.*, (2010) on the other hand, introduced isolation chips (ichips) which are more advanced than diffusion chambers for *in vitro*

isolation of actinomycetes but in the same year, Nicholas *et al.*, (2010) showed the limitations of the technique for successful isolation. All these reports further emphasize the need of an effective equipment/technique for specific actinomycetes isolation (Hame-Kocaba & Uzel, 2012).

The novel baiting technique facilitated specific capturing of actinomycetes diversity and also gave a quick insight into community structure from mixed populations of microorganisms from different soil samples. In tropical countries, it may be found useful for high frequency exploration and isolation of actinomycetes.

According to Durbin, the buried slide technique could also be used to obtain selective isolation of a particular organism rather than to study general microbial colonization by using many natural substrates as baits (Warcup, 1965). Incorporation of specific enzyme substrates in the baiting medium so as to isolate enzyme producing actinomycetes has also been carried out using this technique (as described in **Chapter III**).

2.4.2.1 *'Insitu' and 'Exsitu' baiting technique*

There was not much difference in actinomycetes colonization in both the approaches. However, '*Ex situ*' was a much easier and practical approach as compared to '*Insitu*'. The *'Insitu'* baited slides showed decrease in colonization after 26th day and sudden increase on 28th day which could be attributed to medium depletion caused by presumptive grazing of soil arthropods/nematodes. Besides, distribution of actinomycetes colonies at different time intervals could be due to the appearance of late colonizers. The grazing effect was thereafter avoided by preheating the soils samples in '*Exsitu*' approach. According to Seong *et al.*, (2001), the aerial spores of most actinomycete genera resist desiccation and show a slightly higher

resistance to wet or dry heat than do the corresponding vegetative hyphae (Subramani & Aalbersberg, 2013).Use of antibiotics in '*Ex situ*' technique helped get rid of unwanted fungal and bacterial contaminants. On the other hand, examination of the baited slides without the use of antibiotics showed sterile fungal hyphae observed in colony margins adjacent to the antagonistic actinomycete colonies, indicating production of diffusible antifungal and antibacterial compounds. Lysing of fungal hyphae and aborted hyphae of germinating spores was also observed. Previous studies related to antibiotic substances which induced malfunctions such as stunting, distortion, swelling, hyphal protuberances or highly branched appearance of fungal germ tubes (Getha & Vikineswary, 2002) has been reported. According to a study by Wohl & McArthur, (2001) another explanation of the suppression of fungal growth could be attributed to the initial colonization of organic substrates by some actinomycetes.

With regard to the different actinomycetes encountered in both '*Insitu*' and '*Exsitu*' experiments, the genus *Streptomyces* was dominant. The study corroborates with the findings of Lee & Hwang, (2002), Davies & Williams, (1970). In the soil, *Streptomyces* colonies degrading recalcitrant biopolymers such as cellulose, hemicelluloses or lignocellulose found in wood has been reported (Gottschalk *et al.*, 1999; Bruce *et al.*, 2010; Adams *et al.*, 2011; Amore *et al.*, 2012 and Li *et al.*, 2012).

2.4.3 Soil actinomycetes diversity from various unexplored habitats

Soils are considered important source of actinomycetes, screening such organisms raises the prospect of discovering new natural products of pharmaceutical and industrial interest (Hong *et al.*, 2009). According to Baltz, 2007 only a miniscule

fraction of globe soil surface has been sampled with only a small fraction of actinomycetes taxa been discovered.

Studies by Waksman, (1959) had revealed factors like the nature and abundance of the organic matter, soil pH, relative moisture content, temperature, soil aeration and vegetation type affecting abundance of actinomycetes in soil. The results of the present study revealed, actinomycetes survival and propagation in neutral to alkaline soils (Elamvazhuthi & Malarvannan, 2013). Previous studies has shown the preference of actinomycetes to neutral or slightly alkaline soil and a few are also able to grow in acidic soils with pH 5 (Gottlieb *et al.*, 1973 and Lacey, 1973). According to a study by Simon & Sivasithambaram, (1990) and Ownley *et al.*, (1992), soil pH influences nutrient availability and microbial activity in general.

2.4.3.1 *Statistical measures of diversity*

Two hundred and fifty five actinomycetes isolates, belonging to six known taxa of actinomycetes were isolated from twenty three unexplored terrestrial habitats of Goa. Many researchers have underlined the significance of microbial diversity for search and discovery of novel metabolites (Bull *et al.*, 2000; Langer *et al.*, 2006; Tripathi *et al.*, 2007; Beloqui *et al.*, 2008; Singh & Pelaez, 2008). Consequently, our study was to search in untapped habitats actinomycetes that may produce enzymes as well as antimicrobial agents.

The result of the statistical analysis indicated BT site (Forest soils) with highest actinomycetes diversity and richness. Previous studies have reported actinomycete communities in forest soils (a cool temperate pine forest in Lancashire (Davies & Williams, 1970), a tropical rain forest in Singapore (Wang *et al.*, 1999),

mountain forests of Japan (Hayakawa *et al.*, 1988), a pine forest in north east India (Das & Mishra, 1989).

In this study, the isolated strains based on the colony gross morphological characteristics, were tentatively identified to generic level. There was difficulty to identify different species of actinomycetes which could be attributed to great variability of these organisms (Waksman, 1957). Results thus revealed, actinomycetes generic distribution of different sampling sites exhibiting not much significant differences. This could be explained due to the edaphic factors in combination with other environmental conditions such as prevailing winds and community patterns (Whitaker *et al.*, 2003; Papke & Ward, 2004) responsible for creating specific niches for survival, distribution and preservation of actinomycetes (Khandan & Janardhana, 2013). With regard to the genera encountered in all sampling sites, the genus *Streptomyces* was dominant and exhibited the highest relative abundance accounting for 53 % of all actinomycetes isolated. This dominance of Genus *Streptomyces* corroborates with the findings of Davies & Williams, (1970); Lee & Hwang, (2002); Mansour, (2003); Vijayakumar *et al.*, (2007); Wang *et al.*, (1999); Xu *et al.*, (1996); Williams & Wellington, (1982) and Takizawa *et al.*, (1993).

2.4.4 Actinomycete morphology in shaken culture

The present study showed actinomycetes exhibiting diverse morphological forms when grown in shaken cultures. The study corroborates with the findings of Braun & Vechtlifshitz, (1991); Dobson *et al.*, (2008); O'Cleirigh *et al.*, (2005) and Whitaker, (1992) who have attributed the phenomenon to environmental factors within the fermentor which includes medium composition and fluid mechanical forces. Earlier studies by Waksman (1950, 1959) had reported small bead-like colonies,

granules, or flakes of actinomycetes grown in submerged cultures. Later Tresner *et al.*, (1967) reported large macroscopic mycelial pellets several millimeters in diameter by some *Streptomyces* spp., to profusely fragmented particles, which sometimes produced a bacterial-like turbidity in the medium. A range of morphologies which included loose open colonies, free hyphal colonies, with dense central regions, and radiating hyphae as well as hyphal ropes in *Streptomyces* spp. was reported by Williams *et al.*, (1974). Lawton *et al.*, (1989) did an extensive study on gross actinomycetes morphology in shaken cultures. According to his study, pellets have originated from spores coagulating together, freshly germinating spores aggregating, or by mycelial entanglement. Whittaker, (1992) also studied the some actinomycetes strains in submerged conditions and reported growth forms ranging from compact pellets of mycelium of 5mm diameter to suspensions of fragmented hyphae that have a bacillary forms.

Several workers have reported that depending on the genotype of the strain, the morphological forms prevailing within a submerged culture are correlated to the amount of antibiotic produced. Pellets of small size and high density promoted avermenctin and nystatin production by *Streptomyces averminitilis* (Yin *et al.*, 2008) and *Streptomyces noursei* NG7.19 respectively (Jonsbu *et al.*, 2002). Several works by Sanchez and coworkers have reported the production of antibiotics, undecylprodigiosin and actinorhodin, in submerged cultures of *Streptomyces coelicolor* A3(2) and correlated its production with the development of a second, multinucleated, mycelium (Manteca *et al.*, 2008)

Thus, a good understanding of the factors that influence their morphology could lead to important new ways of optimizing industrial fermentations which could further boost the pharmaceutical industry (Lawton *et al.*, 1989).

CHAPTER III

Screening, purification and characterization of an α-amylase enzyme

3.1 INTRODUCTION

Hydrolytic enzymes account for 75 % of all industrial enzymes of which amylolytic enzymes represent about 25-33 % of the world market (Gangadharan *et al.*, 2006). These enzymes have a wide spectrum of biotechnological applications ranging from food, fermentation, starch saccharification, pharmaceutical, detergent, textiles and paper industries. Due to the various applications, these enzymes expand their frontiers in the field of clinical, medical and analytical chemistry (Prakash *et al.*, 2013).

Microbes from terrestrial sources are currently been employed for production of various industrial enzymes. Soil dwelling actinomycetes have a profound role in recycling carbon by degrading naturally occurring complex nutrients and their produced industrial enzymes have been reported by many researchers (Mohamedin, 1999; Azeredo *et al.*, 2001; Pandhare *et al.*, 2002; Stamford *et al.*, 2002; Goshev *et al.*, 2005; Sharma *et al.*, 2005). Microbial amylases in particular, are of potential use in pharmaceutical and fine-chemical industries replacing the chemical catalysts due to their productivity and thermostability (Burhan *et al.*, 2003).

Among the actinomycetes genera, *Streptomyces* are known to produce various hydrolytic cell wall-degrading enzymes. *Streptomyces* species being heterotrophic feeders utilize both simple and complex molecules as nutrients. Thus, their ability to hydrolyse starch raw starch granules is exploited for various industrial purposes (Syed *et al.*, 2009). Few strains of genus *Streptomyces* spp. have been reported to have maltase, α-amylase, pullulanase and glucosyltranferase activities (Salahuddin *et al.*, 2011). Other actinomycetes genera reported as versatile producers of thermostable amylases are *Thermomonospora curvata, Thermomonospora* and *Thermoactinomycetes* (Ammar *et al.*, 2002).

While most of the studies on actinomycetes have focused on antibiotic production, there are relatively few reports on their enzymatic potential (Schippers *et al.*, 2002; Metha *et al.*, 2006). Due to the ever increasing demand for amylolytic enzymes in various industries, there is enormous interest in developing such enzymes with better properties (Sivaramakrishnan *et al.*, 2006).

This chapter deals with screening of soil actinomycetes for various industrially useful enzymes. The study led to the identification of *Streptomyces* sp. CFA-5 which showed maximum α-amylase activity having unique properties with respect to thermostability and calcium ion independency. The study also reports standardization of parameters required for maximizing amylase production, its partial purification and characterization.

3.2 METHODOLOGY

3.2.1 Enzyme substrate specific slide coated technique (Velho-Pereira & Kamat, *unpublished*)

This is a modified enrichment technique, which relies upon baiting actinomycetes on a specific isolation medium incorporated with enzyme substrates so as to isolate potential enzyme producers.

3.2.1.1 *Identification of nutrient loaded habitats for sample collection*

According to Kurtböke, 2003 and Goodfellow, 2010, an efficient way to recover bioactive species is by having knowledge of microbial ecology and an ecophysiological insight of the characteristic environmental substrate that would influence microbial growth. In view of the above, soil habitats loaded with presumptive carbohydrate, protein and lignin substrates were explored and samples

collected, for the purpose of isolating enzyme substrate specific actinomycetes. The criterion used in identification of these nutrient enriched habitats was based on local information and observations. Sampling sites selected are enlisted in **Table 3.1**.

3.2.1.2 *Setting up of soil microcosm*

Soil samples presumed to be loaded with the particular substrate were baited with slides coated with modified Arginine Vitamin Agar (AVA) incorporated with that specific substrate **(Table 3.1).** For example, starch loaded habitats were baited with starch incorporated AVA medium slides.

Table 3.1 Various sites selected for soil sample collection and setting up of soil microcosm.

Sample designation	Location	Presumptive source and nature of enrichment	Incorporated enzyme substrate					
			Starch	Xylan	Poly-R-478	Pectin	Casein	Gelatine
KFA	Forest Karmali, Tiswadi	Animal wastes(Protein), Litter (Carbohydrate, Lignin)	+	+	+	+	+	+
CFA	Forest Canacona	Animal wastes(Protein), Litter (Carbohydrate, Lignin)	+	+	+	+	+	+
MMA 1	Mapusa market, Bardez	Animal, fish, crustacean wastes (Protein) Plant wastes (Carbohydrate, Lignin)	+	+	+	+	+	+
MMA 2	Mapusa market, Bardez	Animal, fish, crustacean wastes (Protein) Plant wastes (Carbohydrate, Lignin)	+	+	+	+	+	+
PM	Panjim market, Tiswadi	Animal, fish, crustacean wastes (Protein) Plant wastes (Carbohydrate, Lignin)	+	+	+	+	+	+
BOA	Bicholim Orchard, Bicholim	Animal wastes (Protein) Plant wastes (Carbohydrate, Lignin)	+	+	+	+	+	+
TA	Terekhol, Pernem	Animal, fish, crustacean wastes (Protein)	-	-	-	-	+	+
SIA	Siolim, Bardez	Animal, fish, crustacean wastes (Protein)	-	-	-	-	+	+

Legend: (+) enzyme baited slides used, (-) enzyme baited slides not use.

3.2.1.3 *Preparation of the coated slides*

A modification of the isolation procedure described in the **chapter II section 2.2.2** was used to prepare standard coated glass slides. Synthetic opaque medium, Arginine Vitamin Agar (AVA), pH 6.8 a selective medium normally employed for actinomycetes isolation (Nonomura and Ohara, 1969) was modified by eliminating the major carbon sources and supplementing the same with 0.1 % (w/v) enzyme

substrates such as starch (HiMedia, India), xylan from oat spelts (Sigma, US), pectin (HiMedia, India), Poly-R-478 (Sigma, US), casein (Hi Media, India) and gelatin (HiMedia, India). The solidified coated slides were numbered as per a preset scheme and used immediately on the same day while taking care to maintain their sterile nature.

3.2.1.4 *Enzyme producing actinomycetes (EPA) recovery from soil microcosm*

The slides were extricated after 7 and 14 days of incubation, this being optimal period for colonization as mentioned in previous chapter **(Figure 3.1)**. Isolation of actinomycetes from colonized surface was done as described in **chapter II section 2.2.2.6.**

3.2.1.5 *Assessment of EPA diversity*

The same methodology as described in **chapter II section 2.2.2.7 and 2.2.2.8** was followed.

Figure 3.1 Freshly extricated slides for EPA recovery from soil microcosm

3.2.1.6 *Screening of EPA for confirmation of enzyme activity*

EPA morphotypes were screened for amylase, xylanase, ligninase and protease (caseinase and gelatinase) enzyme activity. The cultures were either spot inoculated or streaked and incubated at 28 °C for a period of 7-14 days.

a. Amylase activity was carried out on starch agar media (Aneja, 1993), in petriplates containing soluble starch (0.1 %). The positive test was a zone of clearance seen after flooding plates with iodine.

b. Xylanase activity was carried out on nutrient agar plates (Ninawe *et al.*, 2006) containing xylan from oat spelts (0.1 %). The positive test was a zone of clearance seen after flooding plates with congo red solution and destaining with 1M NaCl.

c. Pectinase activity was carried out on nutrient agar plates (Ninawe *et al.*, 2006) containing pectin (0.1 %). The positive test was a zone of clearance seen after flooding plates with iodine-potasssium iodide solution (Fernandes-Salomão, 1996)

d. Ligninase activity was carried out on nutrient agar plates (Niladevi *et al.*, 2005) in petriplates containing Poly-R-478 (0.1 %). The positive test was seen as decolourization of the media.

e. Protease

i. Caseinase activity was carried out on nutrient agar plates (Aneja, 1993) containing casein (0.1 %). The positive test was seen as decolourization of the media.

ii. Gelatinase activity (Aneja, 1993), was carried out with tubes containing nutrient broth incorporated with gelatin (15 %). The positive test was

hydrolysis of gelatin after refrigeration. The degree of viscosity of the medium was considered as the measure of total gelatinase activity.

3.2.2 Selection of hyper amylase producing strain for further studies

Based on the results of preliminary screening efforts, a single hyper amylase producing strain CFA-5 was selected.

3.2.2.1 *Taxonomic and molecular identification of the producer organism*

The morphological and cultural characteristics of the strain CFA-5 were studied by using traditional criteria of classification (Locci, 1989; Cross & Goodfellow, 1973). The micromorphological studies were done using light and scanning electron microscopy (Williams & Davies, 1967). The 16S rRNA gene sequence obtained from the strain CFA-5 was compared with other bacterial sequences using NCBI mega BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for their pairwise identities. The sequencing work was done by Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala (as shown in **Annexure IV**). The sequence was deposited in Genebank database (http://www.ncbi.nlm.nih.gov/genbank/submit) with Gene bank accession number of KF799976.

Phylogenetic analysis were conducted using MEGA v.5 (Tamura *et al.*, 2011) and the phylogenetic tree was inferred by the maximum-likelihood method (Felsenstein, 1985) based on the Kimura 2-parameter (K2) (Tamura *et al.*, 2011) model. Tree topologies were evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings.

3.2.3 Effect of calcium penthatonate on amylase production

The aim of this experiment was to mainly rule out the calcium penthatonate effect/interference present in the basal medium (vitamin solution) on amylase production. AVA medium was poured in plates and *Streptomyces* sp. CFA-5 was streaked on each set of plates and incubated for 7 days at 28 °C. The experiment was performed in triplicates.

Four sets of medium were prepared i.e.

a. Basal medium (AVA) excluding calcium penthatonate
b. Basal medium (AVA) excluding calcium penthatonate and addition of 0.01M $CaCl_2 . H_2O$
c. Basal medium including calcium penthatonate and including 0.01M $CaCl_2 . H_2O$
d. Basal medium (AVA) including calcium penthatonate and excluding 0.01M $CaCl_2 . H_2O$

Control: Basal medium (AVA) including calcium penthatonate

3.2.4 Impact of process parameters on amylase production (Kar & Ray, 2008a)

Amylase activity was optimized at laboratory scale by using different nutritional and physiological parameters *viz.*, carbon and nitrogen sources, temperature, pH and days of incubation. The effect of activators on amylase production was also studied. The optimum conditions identified for one parameter were used for optimizing the other parameters sequentially. All experiments were performed in triplicates (n=3) and the characterization parameters were subjected to DNS (Plummer, 1971) and Folin Lowry method (Lowry *et al.*, 1951). The controls for each of the parameters were based on previous findings (i.e before optimization).

3.2.4.1 *Amylase production medium*

Modified Arginine Vitamin broth (AVA medium devoid of agar) (Velho-Pereira & Kamat, 2011), pH 7 was used as the basic fermentation medium. All chemicals were procured from HiMedia, Mumbai, India. Pre-inoculum was prepared in Erlenmeyer flasks, containing 100 ml of modified AV broth, previously sterilized at 121 °C for 15 min, inoculated with 10 day year old culture of *Streptomyces* sp. CFA-5 and cultivated under agitation at 250 rpm on a shaker (Orbitek^R, Scigenics Biotech, Pvt. Ltd., India) for four days at 28 °C.

The fermentation medium was inoculated with 5 % v/v pre-inoculum under sterile conditions. The culture flasks were kept stationary at 28 °C for seven days fermentation process. The cells were harvested by centrifugation at 7,000 g (Remi, India) for 30 min, at 4°C. The cell free centrifugate (CFC) containing amylase was used as the starting material to evaluate enzymatic activity.

3.2.4.2 *Analytical procedures*

Quantitative assays such as DNS method (Plummer, 1971) was used for estimating reducing sugars on a UV-VIS spectrophotometer (UV-1800, Shimadzu) wherein glucose was used as standard. Protein was determined by the method of Lowry *et al.*, (1951), using bovine serum albumin, w/v (Hi Media, India) as the standard.

Units calculation (ml) = μg of glucose released x volume of reaction x dilution factor/molecular weight of glucose x incubation time x volume of enzyme.

3.2.4.3 *Optimizing parameters*

Carbon and nitrogen source

To determine the effect of carbon and nitrogen sources on amylase production, the carbon source i.e glucose and glycerol of the basal medium (AV broth) was replaced by different concentrations of starch i.e. 0.5 %, 1 % and1.5 % and the nitrogen sources of the medium was replaced by 1 % of different other nitrogen sources such as arginine, L-asparagine, beef extract and peptone, while the other components of the medium remained constant. The temperature was maintained at 28 °C and pH at 6.8 for seven days. Altogether twelve combinations of both carbon and nitrogen sources were used in the study as shown in **Table 3.2**. Two sets of flasks one kept under shaken (250 rpm) and other at stationary conditions were maintained.

Table 3.2 Different combinations of carbon and nitrogen sources

Sr. no	Carbon and nitrogen source (w/v)
1	0.5 % starch and 1 % arginine
2	1 % starch and 1 % arginine
3	1.5 % starch and 1 % arginine
4	0.5 % starch and 1 % asparagine
5	1 % starch and 1 % asparagine
6	1.5 % starch and 1 % asparagine
7	0.5 % starch and 1 % yeast extract
8	1 % starch and 1 % yeast extract
9	1.5 % starch and 1 % yeast extract
10	0.5 % starch and 1 % peptone
11	1 % starch and 1 % peptone
12	1.5 % starch and 1 % peptone

Incubation period

To study the effect of incubation period on amylase production, cultures were grown for a period of 2, 4, 6, 9, 10, 12, 14, 16, 18, 20, 23, 24 days. Growth for 7 days was used as the control.

pH

Influence of pH on amylase production of the strain was determined by initially adjusting the pH of production medium ranging from 2-12 in the multiples of 0.5 with 0.1 N NaOH/0.1 N HCl. pH 6.8 was used as the control. Since the values of pH 5, 5.5, 6 and 6.5 showed close similarity. The experiment was further refined by adjusting the pH of production medium ranging from 5-6.5 in the multiples of 0.1.

Temperature

The optimum temperature for amylase production was assayed by incubating the production medium at 40, 50, 60 and 70°C. The control was maintained at 28 °C.

Effect of activators (inorganic salts)

Culture was grown, using 10^{-2} M of Barium chloride ($BaCl_2.2H_2O$, Merck, India) **(A)**; Calcium chloride ($CaCl_2.2H_2O$, Merck, India) **(B)**; Cobalt chloride ($CoCl_2.6H_2O$, Merck, India) **(C)**; Lithium chloride ($LiCl_2$, Merck, India) **(D)**; Mercuric chloride ($HgCl_2$, Qualigens, India) **(E)**; Manganese chloride ($MnCl_2.4H_2O$, Hi Media, India) **(F)** and Sodium chloride (NaCl, Hi Media, India) **(G)** incorporated individually in the basal medium.

3.2.4.4 *Statistical analysis*

All the optimization parameters were subjected to statistical analysis where in the results are expressed as a mean of three experiments. Statistical analysis were performed using WASP (Web Based Agricultural Statistics Software Package 1.0, (http://www.icargoa.res.in/wasp/index.php) and differences were considered significant if $p \leq 0.05$.

3.2.5 Enzyme purification

Based on the results of the previous experiments, 1L of the culture *Streptomyces* sp. CFA-5 was grown under optimum conditions. The culture filtrate was centrifuged at 7000 g for 30 mins to obtain CFC. The CFC was subjected to purification. Alpha-amylase was purified by the method described by Rosenberg, (2009). The strategy adopted included fractionation by ammonium sulphate followed by dialysis.

Ammonium sulphate fractionation

Finely powdered ammonium sulphate was added slowly into CFC and bought to 70 % ammonium sulphate saturation at 4 °C in an ice bath. The precipitated protein was removed by centrifugation at 7000 g at 4 °C and dissolved in a minimum volume of phosphate buffer (0.2 M; pH 6.4). The protein content of the fractions were determined by Lowry's method.

Dialysis

The precipitate obtained after ammonium sulphate fractionation was dialyzed at 4 °C against phosphate buffer (0.2 M; pH 6.4) for 24 h with continuous stirring and three changes of the same buffer. Dialysis membrane-50 tubes (Hi Media, India) with Av. Flat width, 24.26 mm; Av. Diameter, 14.3 mm and Capacity approx. 1.61 ml/cm were used for dialysis. The dialysate was collected the following day in sterile tubes and analyzed for protein content and amylase activity as described earlier under **section 3.2.4.2.**

The following calculations were carried out:

Total activity (Units): Activity (Units/ml) x volume (ml)

Specific activity (U/mg): (Units/ml) / protein content (mg/ml)

Yield (%): Total amount of enzyme after a purification step/ Total amount of enzyme before that step

Purification factor: Specific activity of enzyme after a purification step/Specific activity of enzyme before that step.

SDS-PAGE electrophoresis

SDS-PAGE electrophoresis was carried out to separate the proteins on the basis of its size and determine the molecular weight. Electrophoresis was performed according to the method described by Laemmli, (1970). Separating gel (10 % T and 2.5 % C) was prepared as mentioned in **appendix section** and poured in the chamber between the glass plates. Water saturated butanol was layered on top of the gel and kept to set for 20-30 min. After polymerization, the water-saturated butanol was decanted from the top of the gel and washed 2-3 times with distilled water. The stacking gel (4 % T and 2.5 % C) was poured on top of the separating gel. Gel comb was placed and allowed to set for 15-20 min. After polymerization of stacking gel, comb was removed without distorting the shape of the wells.

Tank buffer was filled in electrophoretic unit and connected to a power pack. The protein sample was injected into sample well using micro syringe through tank buffer. Similarly, one well was loaded with standard α-amylase marker (Diastase 41 kDa, Hi media, India) in the sample buffer. Electrophoresis was performed in Mini Gel electrophoretic system (Bangalore Genei Pvt Ltd. Bangalore, India) at constant voltage of 50V till the proteins entered the separating gel and then the run was continued at 100V voltage until the dye front (bromophenol blue) reaches the bottom of the gel. After electrophoresis, the gel between the plates was removed carefully and

immersed in staining solution for at least 2 h with uniform shaking on a rocking table. Subsequent to the staining, the gel was transferred to suitable container with at least 200-300 ml of destaining solution and kept on a rocking table for 30 min, the destainer was changed frequently until the background of the gel is colorless. Analysis of the gel was done using Alpha digiDoc gel documentation system(BioRad, Italy).

3.2.6 Characterization of partially purified enzyme

Effect of pH and temperature on enzyme activity and stability

The optimal production and stability of partially purified enzyme at various pH (acetate buffer-3.6, 4.4, 5.2; phosphate buffer-5.8, 6.4, 6.8, 7.0, 7.4, 7.8; Tris buffer- 8.6, 9.0) and temperatures (4, 28, 35, 42, 55, 60 °C) were studied. All experiments were carried out in triplicates.

3.3 RESULTS

3.3.1 Enzyme substrate specific slide baiting technique

3.3.1.1 *EPA recovery from soil microcosm*

The technique showed evidence of a succession of actinomycetes dominating the slides. Most of the strains belonged to Genus *Streptomyces*. The percentage of colonization **(Figure 3.2a&b)** was more on the slides extricated after 14 days of incubation as compared to those extricated after 7 days.

3.3.1.2 *Assessment of EPA diversity*

EPA morphotypic diversity in all sites was high with respect to xylan utilizers (24 %) as shown in **Figure 3.3a-d** followed by pectin (19 %) **(Fig. 3.4a-d)**, casein (18 %) **(Figure 3.5a-d)**, Poly-R-478 (16 %) **(Figure 3.6a-e)**, gelatin (15 %) **(Figure**

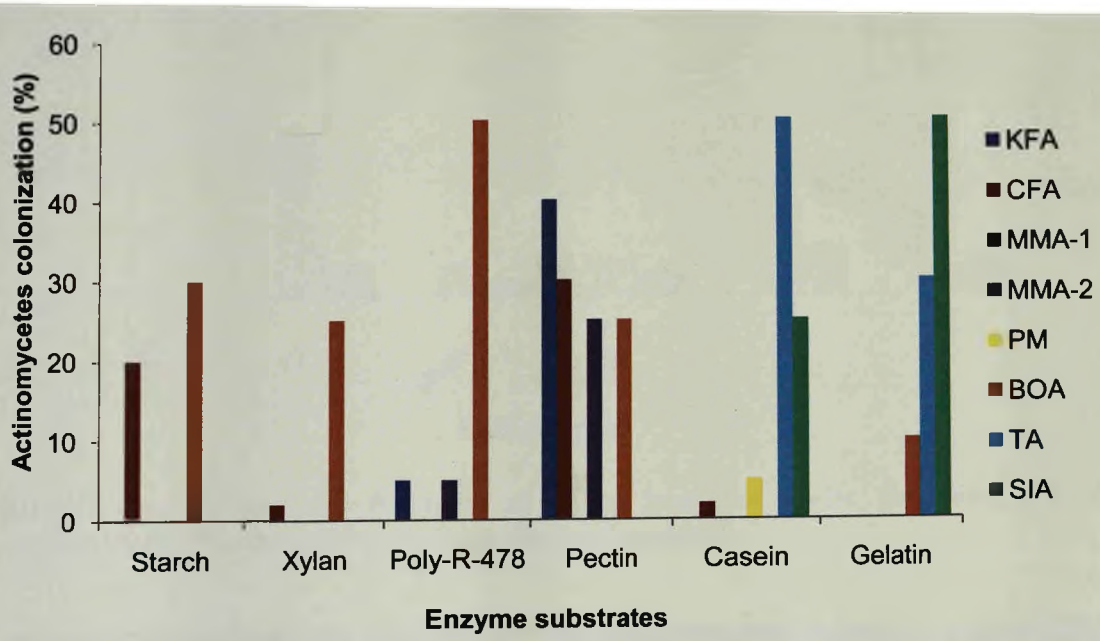


Figure 3.2a Actinomycetes colonization (%) on the 7th day of incubation

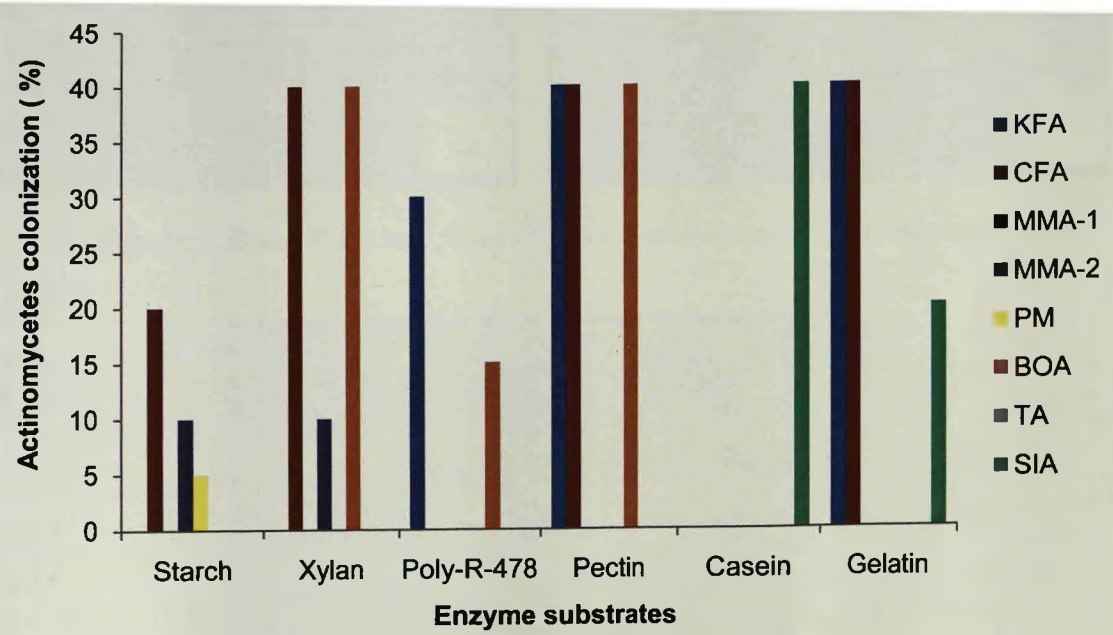


Figure 3.2b Actinomycetes colonization (%) on the 14th day of incubation

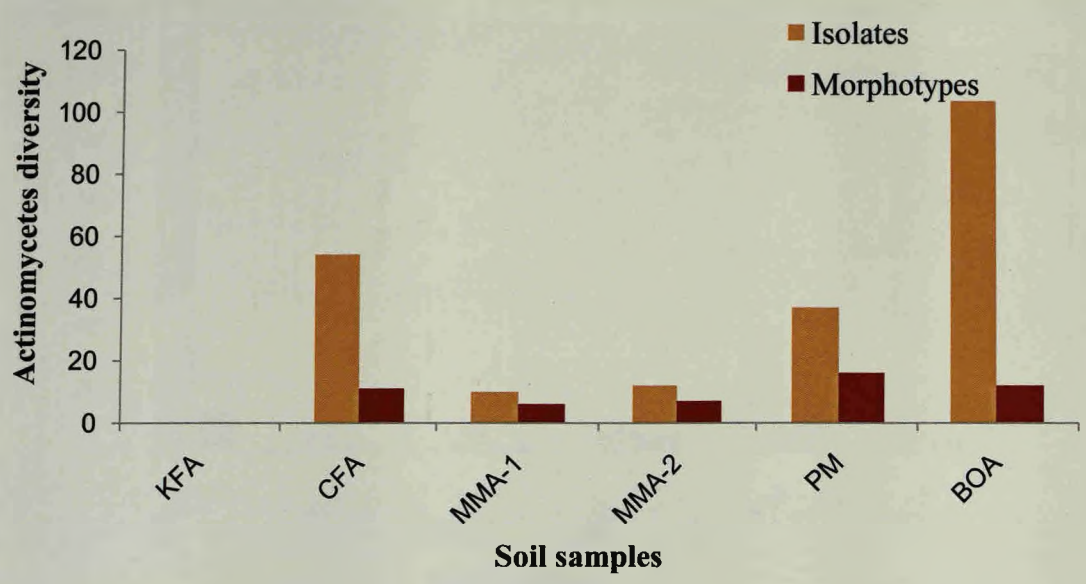


Figure 3.3a Actinomycetes diversity of Xylan from oat spelts, degraders (Model compound for xylanase activity) from various soil samples

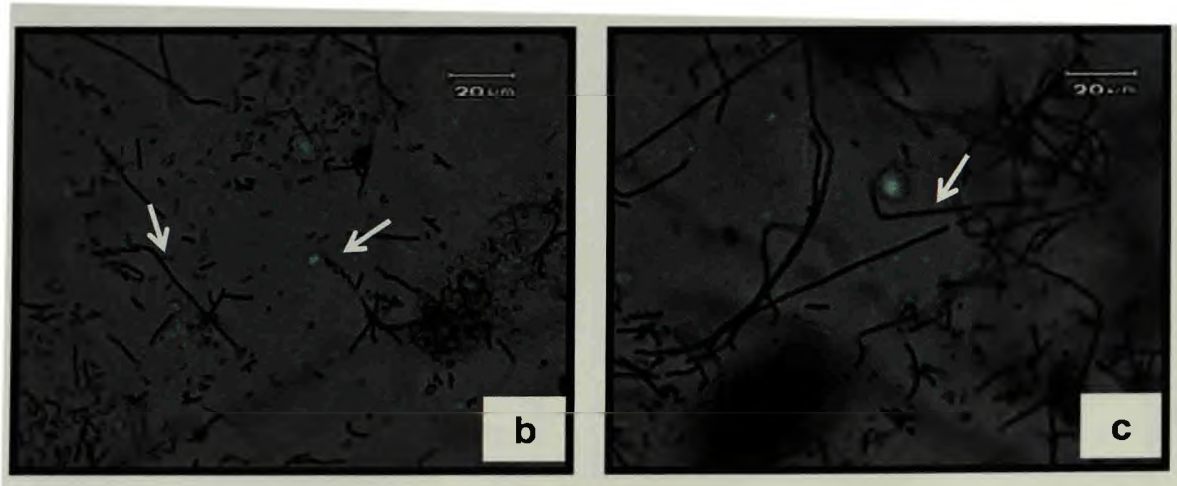


Figure 3.3b&c Short and long chains of spores resembling *Streptomyces* sp.

Figure 3.3d Short chains of actinomycetes spores

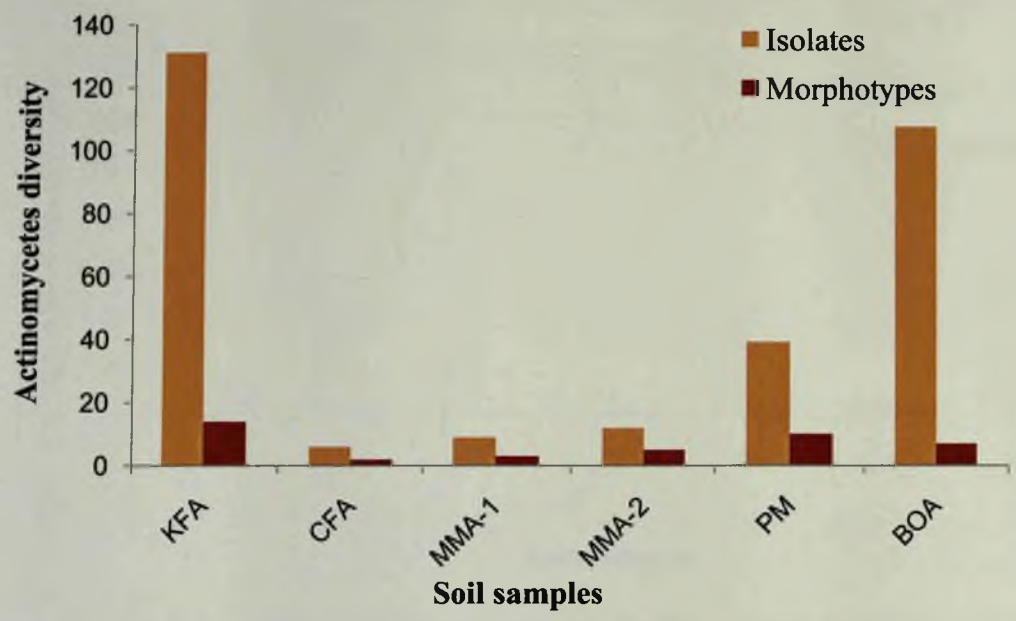
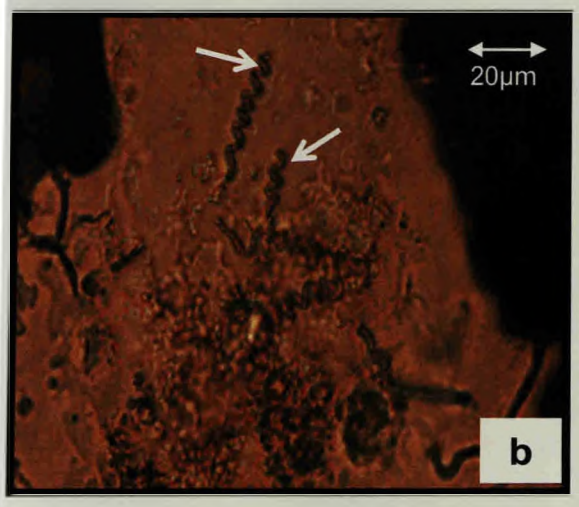


Figure 3.4a Actinomycetes diversity of Pectin, degraders (Model compound for pectinase activity) from various soil samples



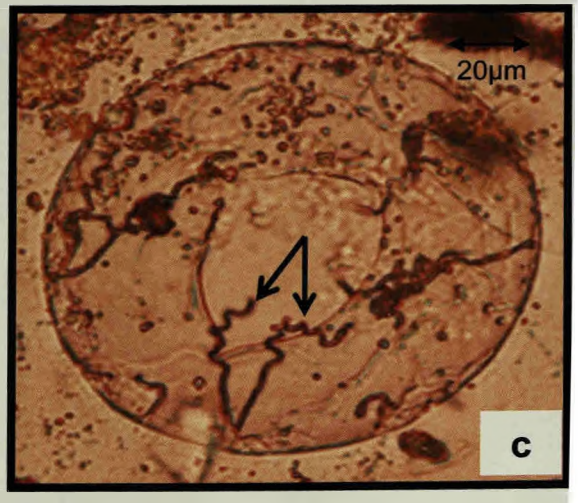


Figure 3.4b&c Spiral chains of spores emerging from a plant tissue resembling cock-screw *Streptomyces* sp.

Figure 3.4d Long linear chains of spores

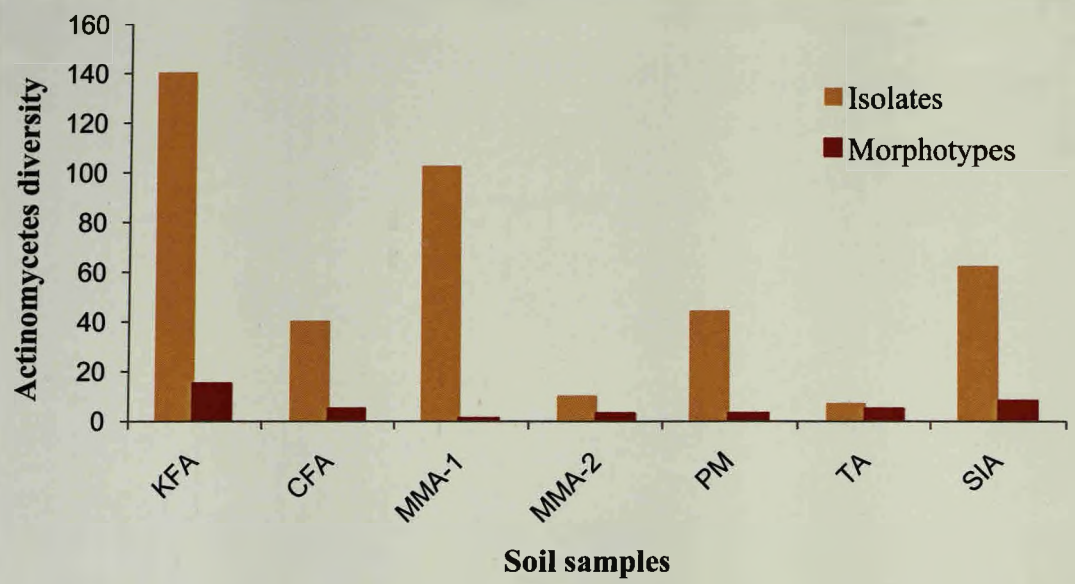
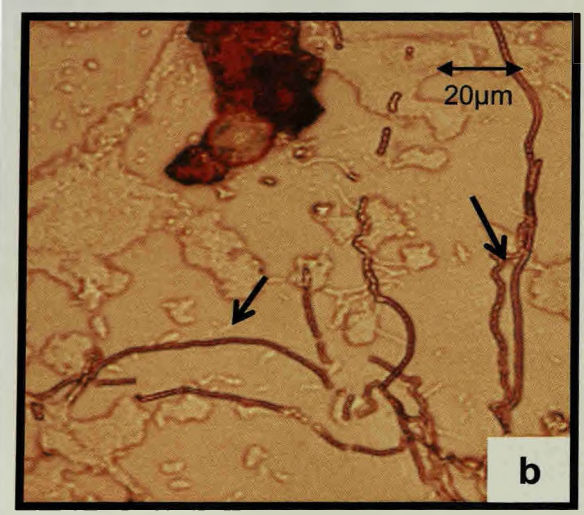


Figure 3.5a Actinomycetes diversity of Casein, degraders (Model compound for caseinase activity) from various soil samples



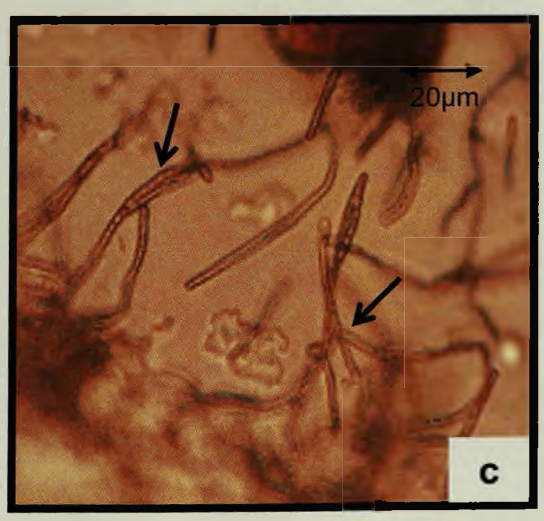


Figure 3.5b&c Actinomycetes spore chains

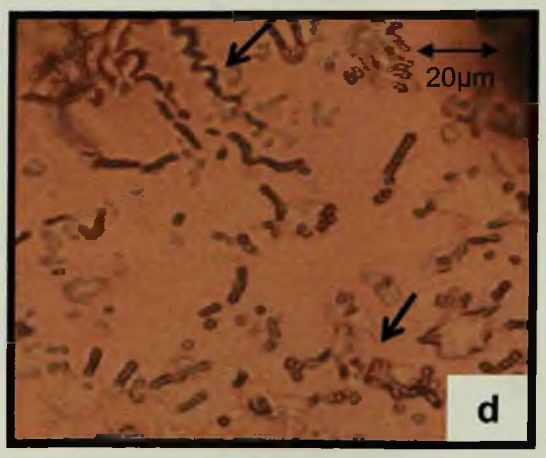


Figure 3.5d Broken spore chains & spiral chains of *Streptomyces* sp.

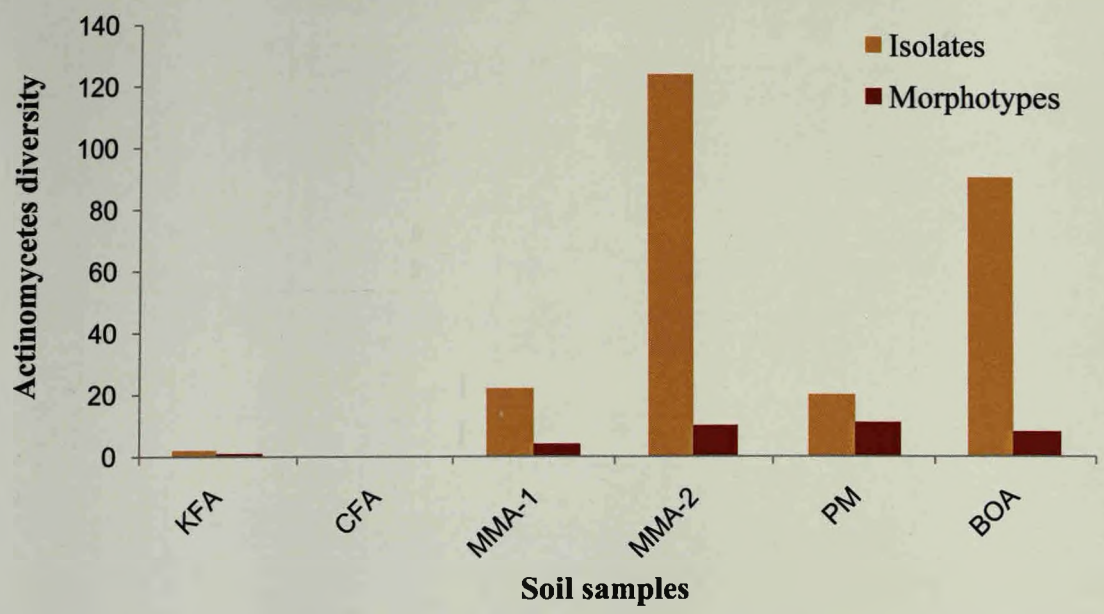
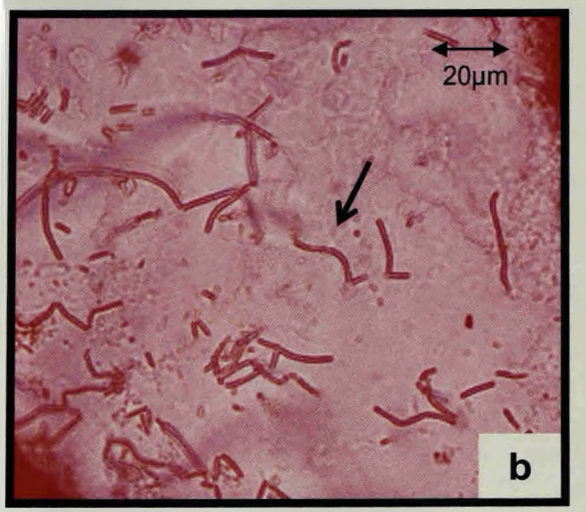


Figure 3.6a Actinomycetes diversity of Poly-R-478, degraders (Model compound for Ligninase activity) from various soil samples



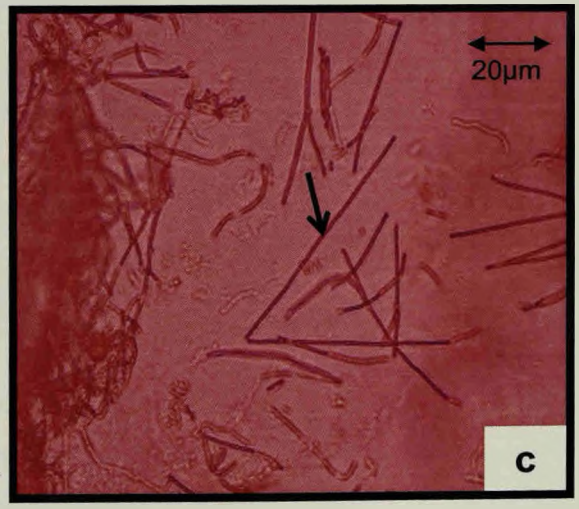


Figure 3.6b&c Short and long chains of actinomycetes spores

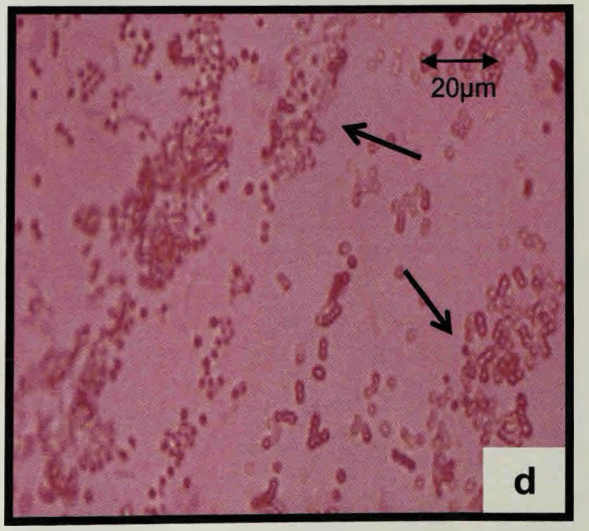


Figure 3.6d Spores of *Micromonospora* sp.

Figure 3.6e Coiled spore chains of *Streptomyces* sp.

3.7a-e) and starch (9 %) **(Figure 3.8a-d)** utilizers. High EPA diversity was observed at PM (21 %) site, followed by MMA-2 (16 %), BOA (16 %), KFA (15 %) and CFA (14 %). KFA, TA and SIA soil samples showed high morphotypic diversity of casein utilizers whereas CFA, PM and BOA soil samples showed high morphotypic diversity with respect to xylan utilizers. MMA-1 soil sample showed high morphotypic diversity of xylan and gelatin utilizers and MMA 2 showed high morphotypic diversity of Poly-R-478 utilizers. The data also showed negative EPA diversity in some sites, although these areas were presumptively assumed to be loaded with the respective enzyme substrate **(Table 3.3)**.

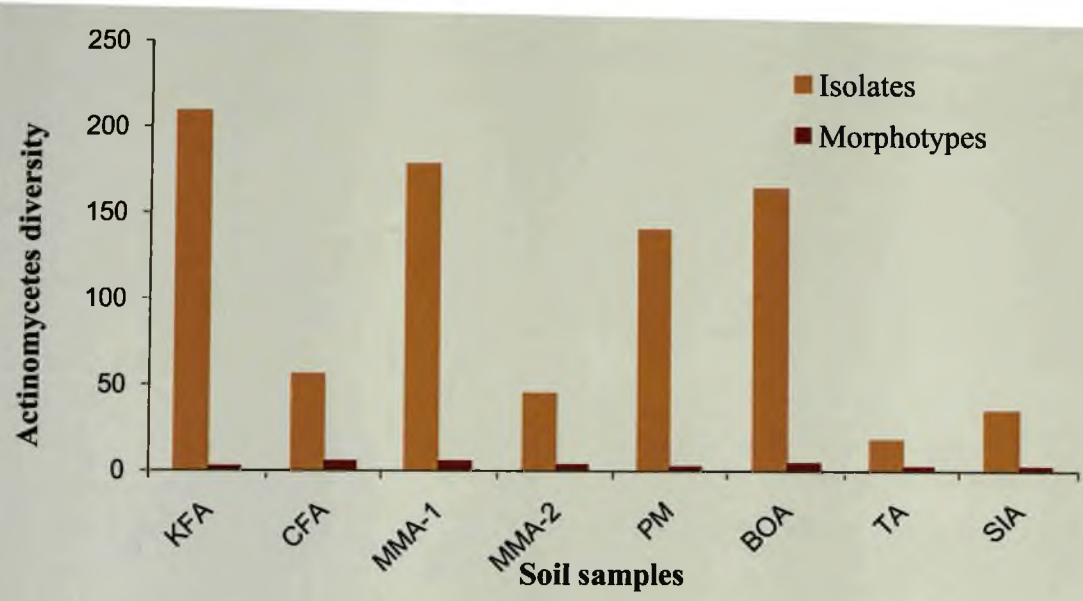


Figure 3.7a Actinomycetes diversity of Gelatin, degraders (Model compound for Gelatinase activity) from various soil samples

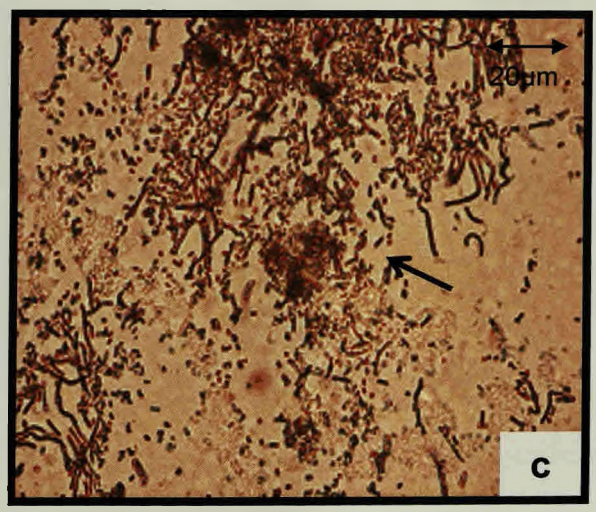


Figure 3.7b&c Different morphological forms of unidentified actinomycetes genera

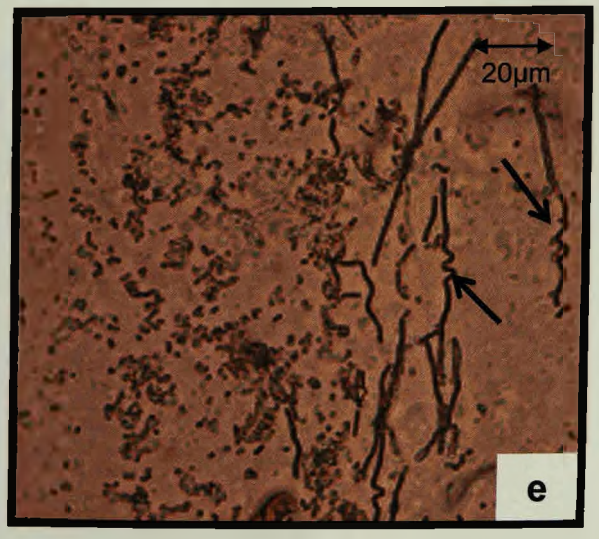


Figure 3.7d Broken chains of actinomycetes spores

Figure 3.7e Spiral chains of *Streptomyces* sp.

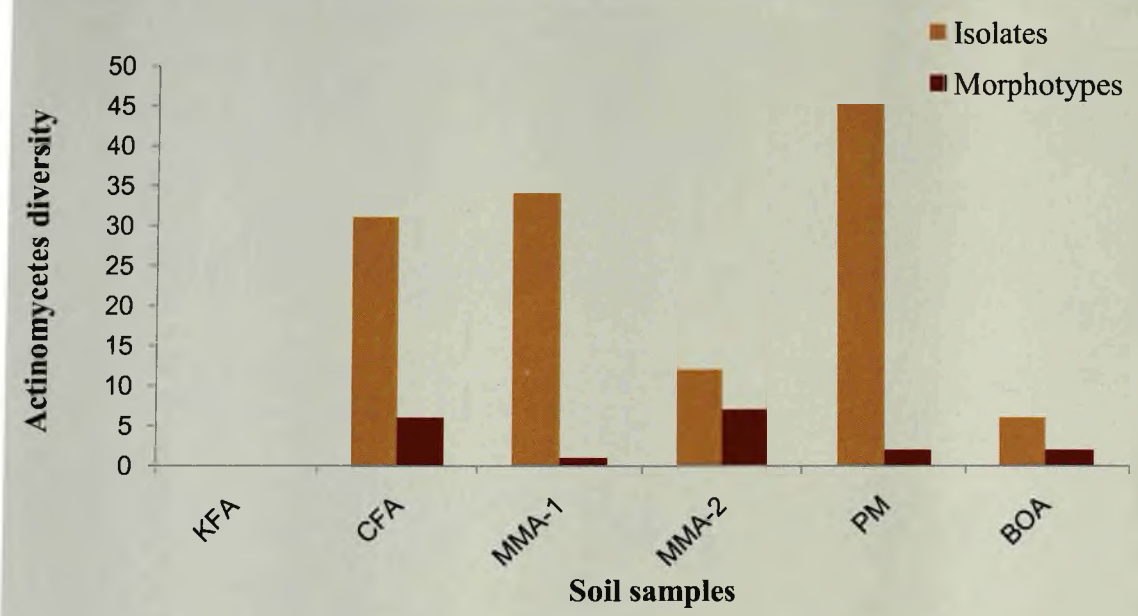


Figure 3.8a Actinomycetes diversity of Starch, degraders (Model compound for Amylase activity) from various soil samples

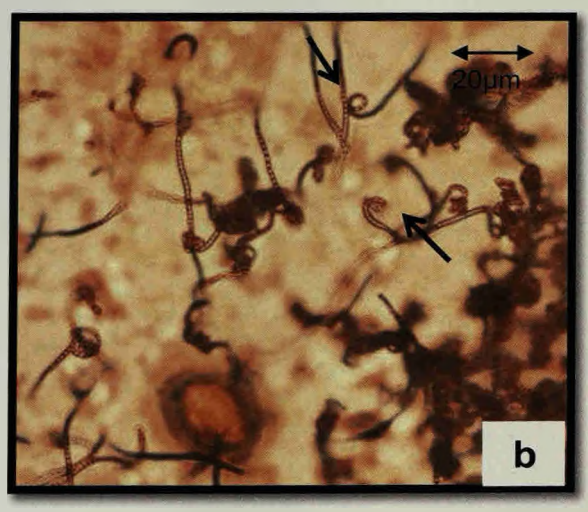


Figure 3.8b Hooked shaped spore chains resembling *Chainia* sp.

Figure 3.8c Spore chains of *Streptoverticillium* sp.

Figure 3.8d Long linear chains of actinomycetes spores

Table 3.3 EPA diversity from various soil samples

Sample designation	EPA diversity											
	Starch		Xylan		Poly-R-478		Pectin		Casein		Gelatin	
	I	M	I	M	I	M	I	M	I	M	I	M
KFA	0	0	0	0	2	1	131	14	140	15	209	3
CFA	31	6	54	11	0	0	6	2	40	5	56	6
MMA 1	34	1	10	6	22	4	9	3	102	1	178	6
MMA 2	12	7	12	7	123	10	12	5	10	3	45	4
PM	45	2	37	16	20	11	39	10	44	3	140	3
BOA	6	3	103	12	90	8	107	7	0	0	164	5
TA	0	0	0	0	0	0	0	0	7	5	18	3
SIA	0	0	0	0	0	0	0	0	62	8	35	3

Legend: I, Isolates; M, Morphotypes

3.3.1.3 *Screening of EPA for confirmation of enzyme activity*

Nine cultures out of 19 showed amylase activity **(Table 3.4, Plate 3.1a-j)** with the highest zone of clearance of 4.9 cm exhibited by strain *Streptomyces* sp. CFA-5 **(Plate 3.1b)**. Out of 33 cultures screened for gelatinase activity, 8 cultures showed positive gelatin hydrolysis **(Plate 3.2a-h)** of which three cultures belonging to Genus *Streptomyces* exhibited high gelatinase activity **(Table 3.5)**.

The technique showed negative xylanase (52), pectinase (41), ligninase (40) and protease (40) activity respectively on confirmation.

PLATE 3.1

Actinomycetes strains showing positive amylase activity

a- *Actinomadura* sp. MMA 1-06

b- *Streptomyces* sp. CFA-5

c- *Streptomyces* sp. BOA-2

d- *Streptomyces* sp. MMA 1-02

e- *Streptomyces* sp. PM 1-03

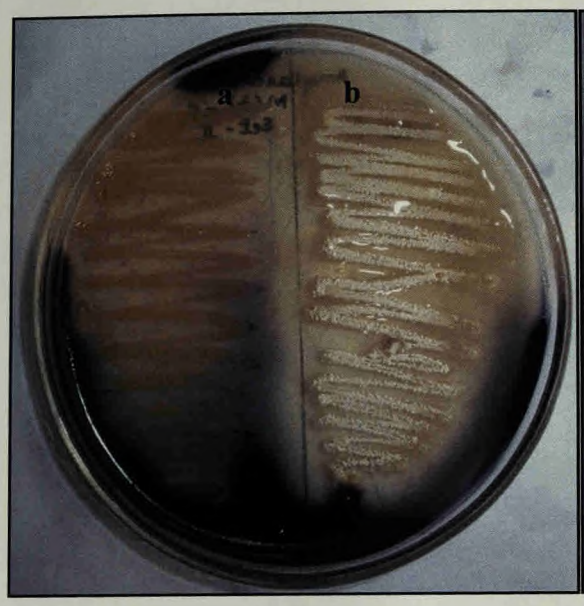
f- *Actinomadura* sp. MMA 1-03

g-*Actinomadura* sp. MMA 1-04

h-*Streptomyces* sp. CFA-1

i- *Streptomyces* sp. MMA 1-05

PLATE 3.1



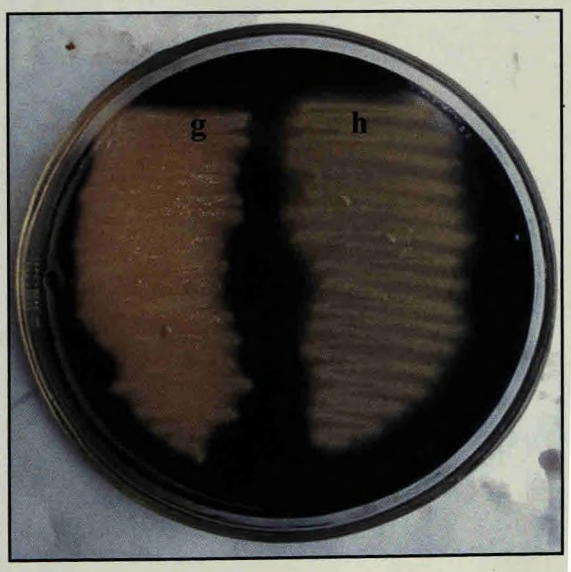


PLATE 3.2

Actinomycetes strains showing positive gelatinase activity

a- *Streptomyces* sp. CFA-3

b- *Streptomyces* sp. CFA-4

c- *Streptomyces* sp. CFA-6

d- *Streptomyces* sp. BOA-1

e- *Streptomyces* sp. BOA-5

f- *Actinomadura* sp. TA-3

g- *Streptomyces* sp. BOA-4

h- *Micromonospora* sp. MMA 2-02

PLATE 3.2

Control
21/7/09
For-2
Set-I
21/7/09
a
Control
21/7/09
For-4
Set-I
21/7/09
b
Control
21/7/09
For-5
Set-I
21/7/09
c
Control
21/7/09
Orch-1
Set-I
21/7/09
d
Control
21/7/09
Orch-5
Set-I
21/7/09
e
Control
21/7/09
Tea-2
Set-I
21/7/09
f
Control
21/7/09
Orch-4
Set-II
21/7/09
g
Control
21/7/09
Mm-2
Set-I
21/7/09
h

Table 3.4 Amylase activity of actinomycetes strains

Actinomycetes strains	Amylase production
Actinomadura sp. MMA 1-06	++
Streptomyces sp. CFA-5	+++
Streptomyces sp. BOA-2	++
Streptomyces sp. MMA 1-02	++
Streptomyces sp. PM-2	++
Actinomadura sp. MMA 1-03	++
Actinomadura sp. MMA 1-04	++
Streptomyces sp. CFA-1	++
Actinomadura sp. MMA 1-05	+

Legend: -, No clearance; +, Clearance was medium; ++, Good zone of clearance; +++ The entire area has been cleared

Table 3.5 Gelatinase activity of actinomycetes strains

Actinomycetes strains	Gelatinase production
Streptomyces sp. CFA-3	++
Streptomyces sp. CFA-4	++
Streptomyces sp. CFA-6	++
Streptomyces sp. BOA-1	+
Streptomyces sp. BOA-5	+
Actinomadura sp. TA-3	+
Streptomyces sp. BOA-4	+
Micromonospora sp. MMA 2-02	+

Legend: +, low viscosity; ++, high viscosity

3.3.2 Selection of a hyper amylase producing strain

Among all the strains tested for amylase activity, *Streptomyces* sp. CFA-5 emerged as the best candidate strain showing high amylase activity.

3.3.2.1 *Taxonomic and molecular identification of the producer organism*

The strain which was labeled as CFA-5 exhibited white aerial mycelium, with spiral spore chains. Sporophores monopodially branched, with open, regular spirals. The culture was identified as *Streptomyces* sp. (GenBank Accession Number: KF799976) based on nucleotide homology and phylogenetic analysis **(Figure 3.9a-d)**.

3.3.3 Effect of calcium penthatonate on amylase production

Distinct growth was observed on all the plates of different combinations with no much growth variations with respect to the control. The results showed that calcium penthatonate has no significant effect on the amylase production **(Plate 3.3a-e)**.

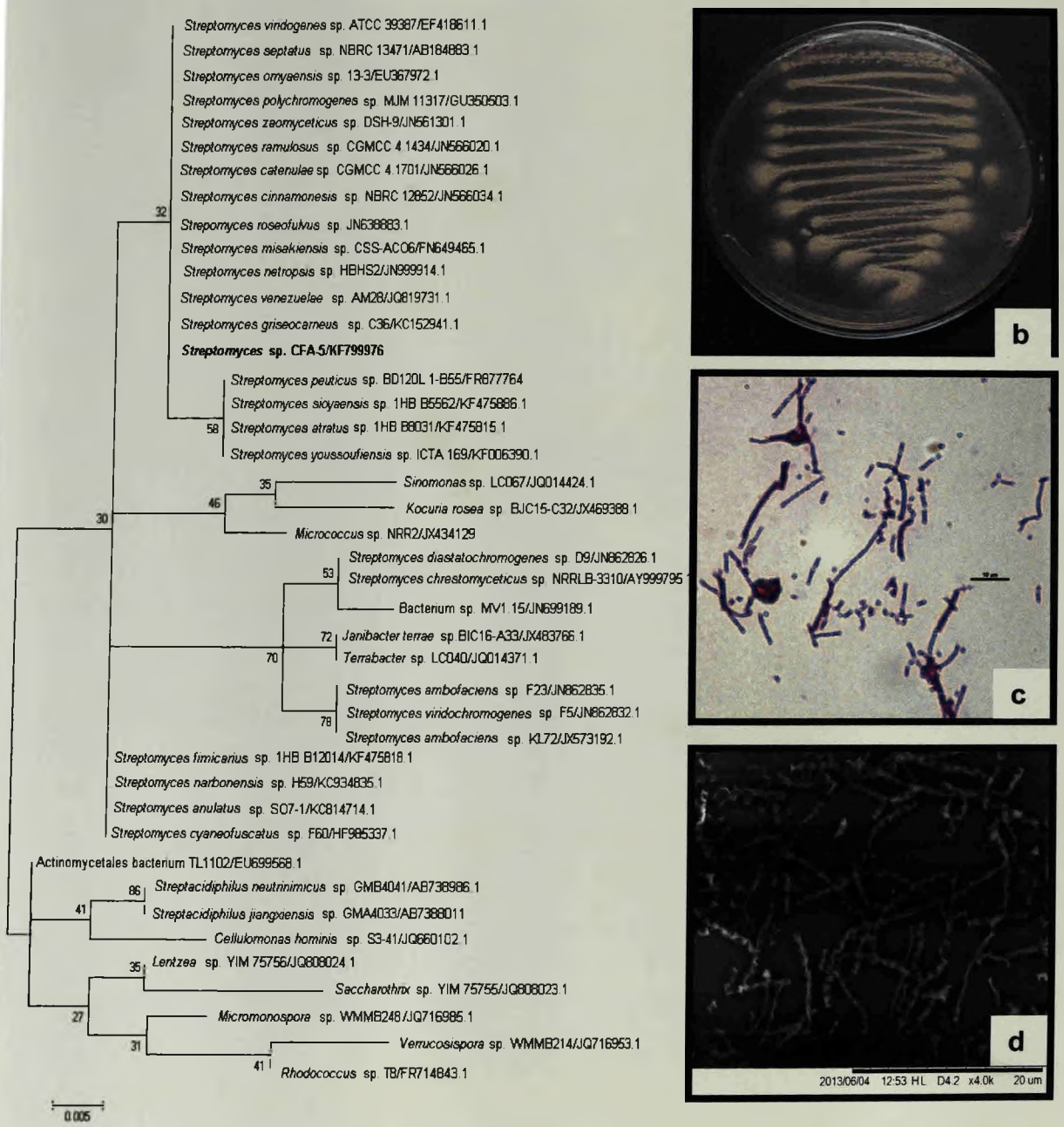


Figure 3.9a) Maximum-likelihood tree based on 16S rRNA gene sequence showing the relations between amylase producer strain *Streptomyces* sp. CFA-5 and type species of the genus *Streptomyces* within the order actinomycetales under actinobacteria. The numbers at the nodes indicate the levels of bootstrap support based on maximum-likelihood analyses of 1000 resampled data sets (only values >50% are shown). The scale bar indicates 0.002 substitutions per nucleotide positions, **b)** *Streptomyces* sp. CFA-5 strain on AVA medium, **c)** *Streptomyces* sp. CFA-5 strain smeared in crystal violet stain as seen under compound light microscope (Olympus, BX 41, Japan) and **d)** SEM photographic image of *Streptomyces* sp. CFA-5

PLATE 3.3

Control: Basal medium (AVA) including calcium penthatonate

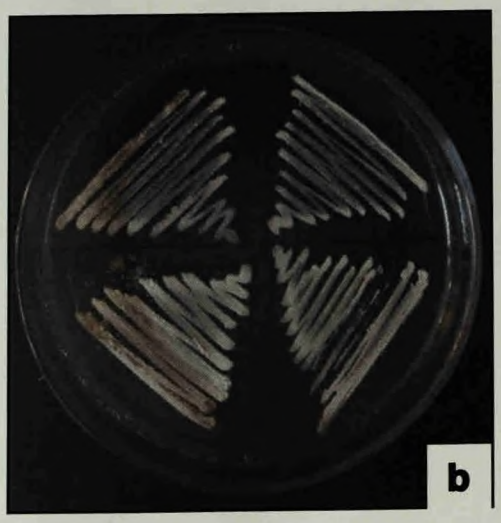
a - Basal medium (AVA) excluding calcium penthatonate

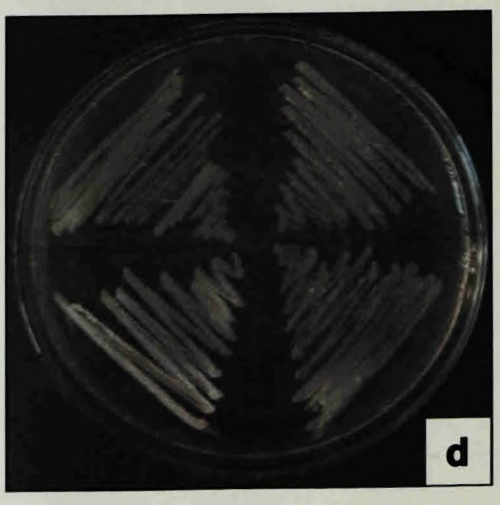
b - Basal medium (AVA) excluding calcium penthatonate and addition of 0.01M $CaCl_2.H_2O$

c - Basal medium including calcium penthatonate and including 0.01M $CaCl_2.\ H_2O$

d - Basal medium (AVA) including calcium penthatonate and excluding 0.01M $CaCl_2.\ H_2O$

PLATE 3.3





3.3.4 Impact of process parameters on α-amylase production

Carbon and nitrogen source

Optimum amylase production was observed under stationary conditions with the cells grown in a minimal medium containing starch and yeast extract at a concentration of 1:1 % (w/v). The yield was 2403.8 U/L with a protein content of 0.8 mg/ml and showed significant variation ($p \leq 0.05$) as shown in **Figure 3.10**. This was followed by 1.5 % starch and 1 % yeast extract and 1.5 % starch and 1 % peptone with a yield of 1846.99 U/L and 1820.23 U/L respectively.

Incubation period

The optimum incubation period for α-amylase production was observed on the 9th day with a yield of 10350.6 U/L showing significant variation ($p \leq 0.05$) as shown in **Figure 3.11** with protein content of 0.27 mg/ml.

pH

The data indicated that, production of the enzyme required neutral conditions. Maximum yield of 5760.5 U/L with a protein content of 2.2 mg/ml was observed at pH 6.4 which was significant ($p \leq 0.05$) as shown in **Figure 3.12**. Thereafter there was decline in enzyme production with increase in pH values.

Temperature

As seen in the **Figure 3.13**, optimum amylase activity was observed at 60 °C (15747.6 U/L) with a protein content of 9.8 mg/ml which was significant ($p \leq 0.05$). Thereafter the incremental temperature rise led to a decrease in the enzyme production reflected through low production yield of 2827.9 U/L.

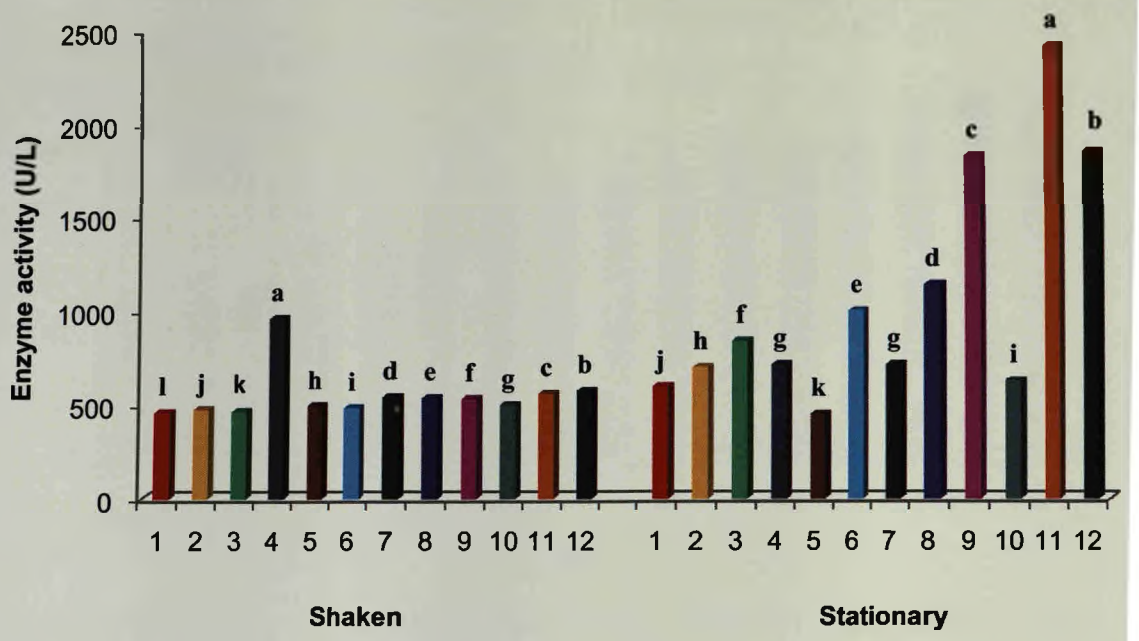


Figure 3.10 Effect of carbon and nitrogen sources on α-amylase activity by *Streptomyces* sp. CFA-5. Data are the average of three experiments (**1**, 0.5 % starch and 1 % Arginine; **2**, 1 % starch and 1 % Arginine; **3**, 1.5 % starch and 1 % Arginine; **4**, 0.5 % starch and 1 % Asparagine; **5**, 1% starch and 1 % Asparagine; **6**, 1.5 % starch and 1 % Asparagine; **7**, 0.5 % starch and 1 % Peptone; **8**, 1% starch and 1 % Peptone; **9**, 1.5 % starch and 1 % Peptone; **10**, 0.5 % starch and 1 % Yeast extract; **11**, 1% starch and 1 % Yeast extract; **12**, 1.5 % starch and 1 % Yeast extract. The superscripts indicate significant differences ($p \leq 0.05$)

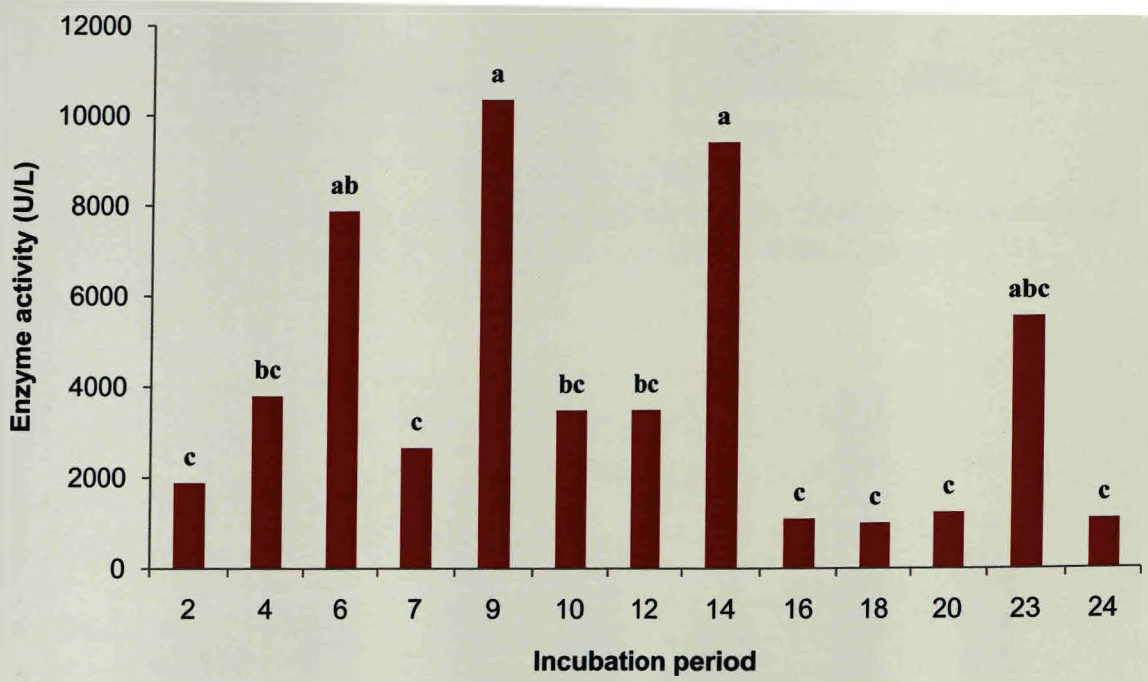


Figure 3.11 Effect of incubation period on α-amylase activity. Data are the average of three experiments . The superscripts indicate significant differences ($p \leq 0.05$)

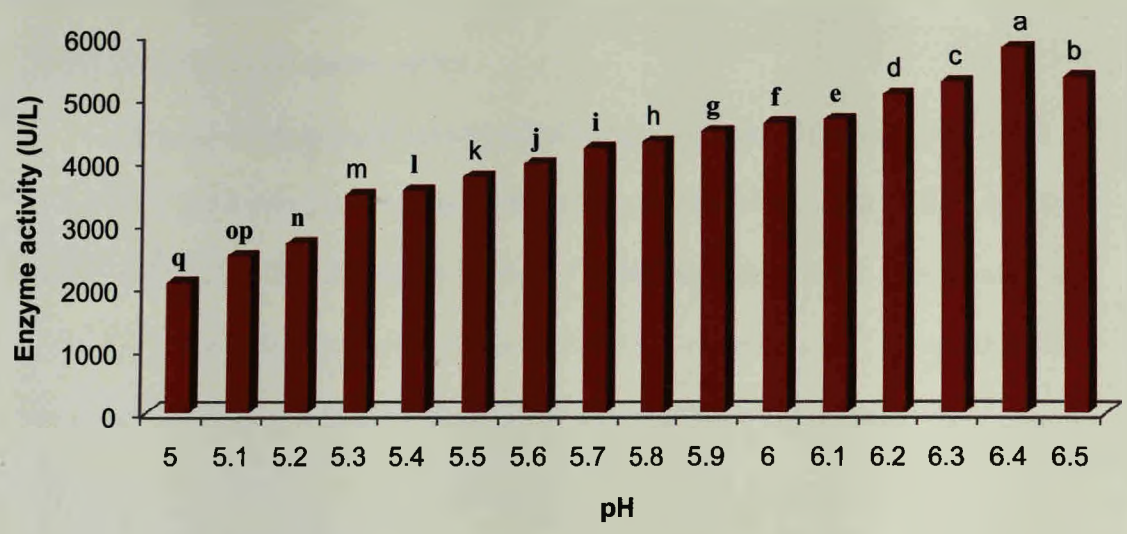


Figure 3.12 Effect of pH on α-amylase activity. Data are the average of three experiments. The superscripts indicate significant differences ($p \leq 0.05$)

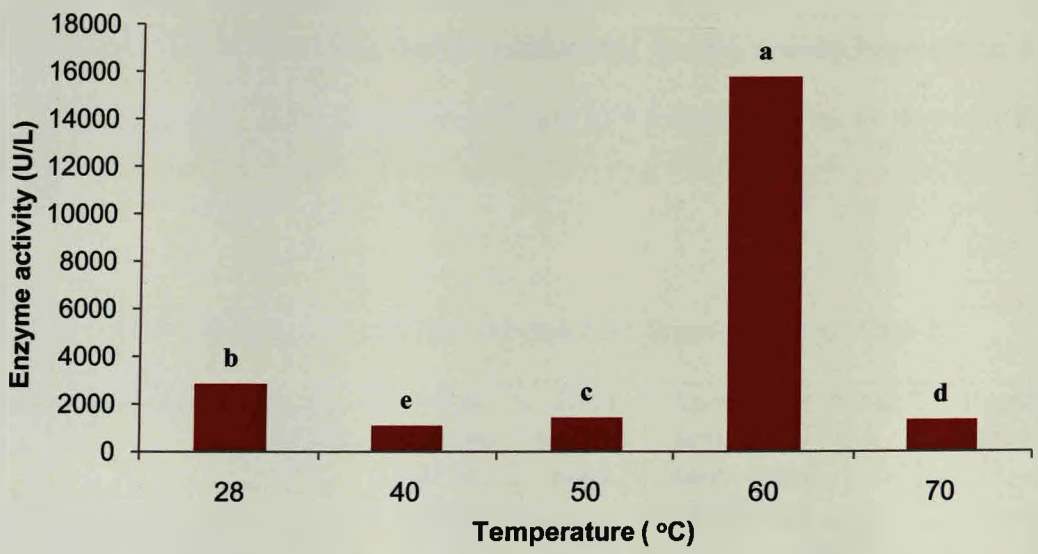


Figure 3.13 Effect of temperature on α-amylase activity. Data are the average of three experiments. The superscripts indicate significant differences ($p \leq 0.05$)

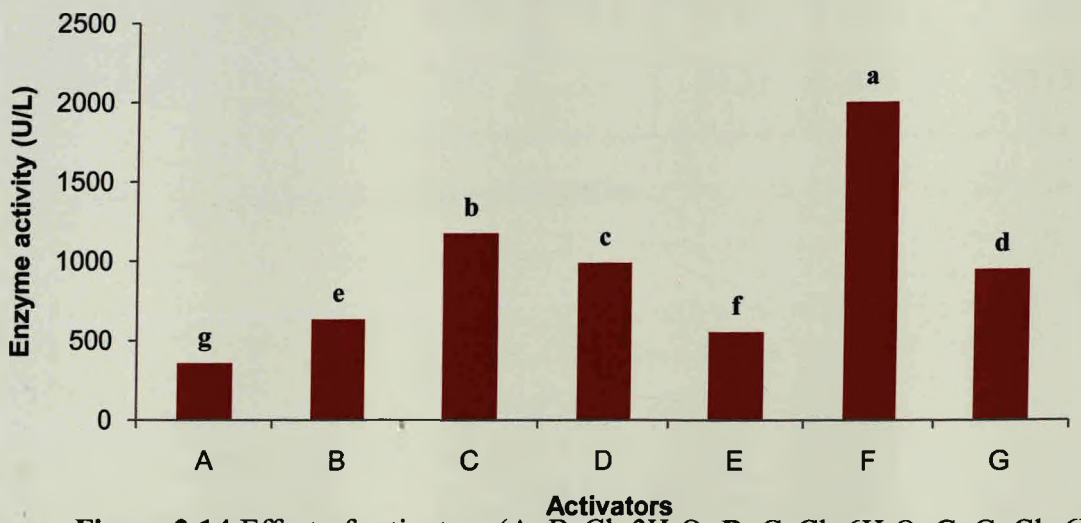


Figure 3.14 Effect of activators (**A,** $BaCl_2.2H_2O$; **B,** $CaCl_2.6H_2O$; **C,** $CoCl_2.6H_2O$; **D,** $LiCl_2$; **E,** $HgCl_2$; **F,** $MnCl_2.4H_2O$ and **G,** NaCl)on α-amylase activity. Data are the average of three experiments. The superscripts indicate significant differences ($p \leq 0.05$)

Effect of activators (inorganic salts)

The data **(Figure 3.14)** revealed that Manganese chloride as an activator had a comparable good effect on amylase activity (1996.5 U/L) with protein content of 2.15 mg/ml which was significant ($p \leq 0.05$). Other inorganic salts also showed a comparable effect on amylase activity with Cobalt chloride and Lithium chloride showing a maximum enzyme yield of 1171.5 U/L and 984.5 U/L respectively.

3.3.5 Enzyme purification

Crude extract contained 195.2 mg protein and showed a specific activity of 1.02 units/mg protein. After partial purification, specific activity increased to 24.22 units/mg protein with a yield of 40.1 % and 23.8 fold purification as shown in **Table 3.6.**

Table 3.6 Purification of α-amylase enzyme from *Streptomyces* sp. CFA-5

Purification step	Total volume (ml)	Total enzyme activity (Units)	Total protein (mg)	Specific activity (Units/mg protein)	Yield %	Purification factor or fold
Culture filtrate before ASP	312	432.95	561.6	0.77	100	0
After ASP and centrifugation	160	199.36	195.2	1.02	46.1	1.33
After dialysis	50	79.93	3.3	24.22	40.1	23.8

Legend: ASP; Ammonium sulphate precipitation

Molecular weight determination

Electrophoretic studies of the partially purified enzyme revealed a dark band that matched the diastase marker with a molecular mass of approx. 41 KDa **(Figure 3.15)**.

3.3.6 Characterization of the partially purified enzyme

Effect of pH and temperature on enzyme activity and stability

Partially purified enzyme showed similar pH (6.4) and temperature (60 °C) optima and stability as in crude enzyme.

Figure 3.15 Protein bands of α-amylase from *Streptomyces* sp. CFA-5 on SDS-polyacrylamide gel electrophoresis. Lane1 (Left), Diastase (α-amylase), Hi Media, 41 KDa as marker; Lane 2 (Right), Alpha-amylase from *Streptomyces* sp.CFA-5, approx. 41 KDa

3.4 DISCUSSION

3.4.1 Enzyme substrate specific slide coated technique

Based on the success of the previously employed baiting technique as mentioned in **chapter I** (Velho-Pereira & Kamat, 2012) for rapid isolation of actinomycete strains, a proposed modified technique, was used to bait specific enzyme producers (Goodfellow & Williams, 1986). Beijerinck in the late 1800's demonstrated "enrichment culture" technique wherein taxonomic groups were isolated using a medium baited with appropriate carbon source such as cellulose, sodium benzoate, creosote, paraffin, and Tween 80 (Durbin, 1961). According to Durbin, (1961) some taxonomic groups possess specialized enzyme systems for utilizing the carbon source compound. The use of buried slide technique along with many natural substrates as baits for selective isolation of a particular organism rather than to study general microbial colonization was thus suggested (Warcup, 1965).

Several researchers have adopted different enrichment techniques and shown the success of recovering organisms using pollen and hair (Couch, 1954); skin, wool, or hair was used for kerotinophilic chytrids (Karling, 1946) and dermatophytes (Ajello, 1954); chitin for chitinophilic chytrids (Karling, 1945); panels of different kinds of wood, rope, or cordage for marine fungi (Barghoorn & Linder, 1944) or wood rotters; autoclaved wool or cotton for fabric-decomposing organisms (White *et al.*, 1950); rice grains for *Chromobacterium* spp. (Corpe, 1951); accumulating zoospores in glass capillaries containing chemoattractants (Palleroni, 1980).

The present enrichment technique is a modification of the above techniques and relies upon baiting actinomycetes on a specific isolation medium with enzyme substrates so as to isolate potential enzyme producers. Actinomycetes as a source of industrial enzymes is known and their products have been applied in various

biotechnological industries and biomedical fields (Prakash *et al.*, 2013). *Streptomycetes* in particular, are known to obtain nutrients from macromolecular organic debris by using a wide range of hydrolytic enzymes which include proteases, nucleases and lipases (Suneetha & Khan, 2011). Use of AVA (a specific actinomycetes isolation medium) as the basal medium for enzyme production, further facilitated the recovery of actinomycetes as enzyme producers. Thus, highlighting that this technique could be extended to isolation of any micro-organisms capable of producing potential enzyme/s, provided the choice of basal medium favors its growth. The technique also finds itself useful especially in tropical countries for high frequency isolation of specific actinomycetes enzyme producers.

3.4.1.1 *EPA recovery from soil microcosm*

All soil samples collected for the study were rich in organic matter and were from pristine unexplored environments suggesting the possibility of obtaining actinomycetes producing novel metabolites (Batlz, 2006; Prakash *et al.*, 2013). Actinomycetes are well adapted in soil. Their hyphae penetrate and colonize various substrates, as their primary carbon sources are insoluble and polymeric, necessitating the secretion of a range of extracellular enzymes. According to Kurtboke, (2012) there is a need of designing a strategy for effective isolation of bioactive actinomycetes. This is possible if there is a proper understanding of the functional diversity and physiological characteristics of these organisms that inhabit indigenous locations. Based on this information, isolation strategies such as designing a habitat-based media or simulation of natural environment needs to be adopted. Considering that specific actinomycetes would prefer to colonize such nutrient enriched habitats and display a range of enzyme activities (Kizhekkedathu & Prema, 2005). In the present study,

nutrient rich habitats were explored which were presumptively considered to be loaded with protein, carbohydrate and lignin substrates. The identification of such habitats was based on local information and observations.

The EPA recovery from soil microcosms showed evidence of Genus *Streptomyces* dominating the slides. *Streptomyces* as a dominant genus in various soil habitats with the ability to decompose organic material through their large repertoire of cellulases, xylanases, lignocellulase and other enzymes has been studied (Schrempf, 2007). Schlatter *et al.*, (2009) reported that addition of specific type and amount of carbon sources alone or in combination with native soil microcosms has resulted in *Streptomyces* showing potential enzyme systems to that particular carbon source. The study also showed prolonged incubation periods correlating with percentage of colonization. This is because actinomycetes are slow growing in nature and it is this property which enables them to produce various secondary metabolites (Williams & Cross, 1971).

3.4.1.2 *Assessment of EPA diversity*

The present study reports recovery of EPA morphotypic diversity from most of the sample sites. However, in some sites negative EPA diversity has also been reported, although these areas were presumptively assumed to be loaded with the respective enzyme substrate. Previous studies, have explained the presence of hydrolytic enzymes in the soil that provide a favorable source of nutrients for bacterial growth in addition to providing a suitable environment for their replication and survival (Selvin *et al.*, 2009). The negative record of EPA diversity in some substrate loaded sites is justifiable as generally organisms capable of producing all these extracellular enzymes are infrequent in the environment (Dixit & Pant, 2000).

3.4.1.3 *Screening of EPA for confirmation of the enzyme activity*

During the present study, EPA strains recovered from baiting technique were screened for confirming their respective enzyme activity (Vihinen & Mantsala, 1989). Results of screening efforts showed only few strains capable of producing enzymatic activity. Although, the baiting technique had resulted in high frequency isolation of actinomycetes strains, presumed to be potential enzyme producers. According to a study by Chamberlain & Crawford, (2000) the ability of isolates to metabolize a particular enzyme or only break it up or feed on other natural substrates is not clear (Bontemps *et al.*, 2013).

In case of lignin producing actinomycetes, Poly R was used as its dye based model compound for screening (Kiiskinen *et al.*, 2004) and although Poly R supported growth of actinomycetes, the phenomenon could not be correlated to the ability of these strains to produce such enzymes. The study contradicts with that of Pasti & Crawford, (1991) but corroborates with the findings of Niladevi & Prema, (2005).

Another explanation could be, soil actinomycetes processing some biopolymers partially but not exhibiting presence of full hydrolytic system *eg.* in case of ligninolysis, xylanolysis or pectinolysis which involve participation of a more than single enzyme (Suneetha & Khan, 2011).

3.4.2 *Streptomyces* sp. as a potential α-amylase (EC 3.2.1.1) producer

Streptomyces sp. CFA-5 was selected as a potential α-amylase producer strain for further analysis. Exploiting actinomycetes in particular, *Streptomyces* for amylase production has recently been investigated (Cotarlet *et al.*, 2011). In terrestrial habitats, Streptomycetes act as saprophytes, solubilizing organic material in the soil by their

large repertoire of extracellular hydrolytic enzymes that include amylases, xylananses, cellulases and proteases and inturn obtaining nutrients and energy (McCarthy & Williams, 1992; Schrempf, 2007). Among all the enzymes, starch hydrolyzing activity is widely distributed in genus *Streptomyces* with several species such as *Streptomyces limosus* and *Thermomonospora curvata* have been found to be potent source of α-amylase enzyme (Salahuddin *et al.*, 2011).

In the present study, the α-amylase producer *Streptomyces* sp. CFA-5 was isolated from forest soil in Canacona, Goa and as mentioned in Chapter II, the soil sample is from a lateritic profile on the basement rock known as Peninsular gneiss which is more than 3 billion years old. Amore *et al.*, (2012) and Li *et al.*, (2012) have reported Genus *Streptomyces* playing a potential role in carbon cycling due to their carbohydrate-active enzyme reservoirs which help them degrade recalcitrant biopolymers especially in forest ecosystems. Thus, from all the above observations it could be inferred that, although the forest environs harbour a good number of actinomycete strains, a few have the potential of producing novel enzymes which have very good industrial and commercial applications.

3.4.3 Effect of calcium penthatonate on amylase production

The results of the present study showed that calcium ions had no potentiating role in amylase production. Thus indicating that the enzyme produced was calcium independent.

Calcium ion independency is considered as a desirable feature for industrial applications in some cases as its presence could lead to the formation of inorganic precipitates which have deleterious effects on fermentation and downstream processing of the enzyme (Kelly *et al.*, 2009). Besides, calcium ion independency also

eliminates the use of calcium in starch liquefaction thereby, avoiding its subsequent removal by ion exchange (Shafiei *et al.*, 2010). Removal of these metal ions is both cost and time consuming to the overall industrial process (Kelly *et al.*, 2009) thus, favoring the use of stable and functional α-amylases in the absence of Ca^{2+} ions at high temperatures (El-Fallal *et al.*, 2012). Calcium ion independent α-amylase from *Streptomyces* strains has been reported (Ray *et al.*, 2011; Chakraborty *et al.*, 2012).

3.4.4 Impact of process parameters on α-amylase production

Medium composition significantly affects the product yield. Thus designing a proper fermentation medium is critical in fermentation industry (Silva & Roberto, 2001). Besides optimization of fermentation conditions such as pH, temperature, agitation, dissolved oxygen, inoculum density, medium components and minerals have a great impact on the economy and practicability of the fermentation process (Francis *et al.*, 2003). Extracellular α-amylase production by actinomycetes, expected to improve with optimization of cultivation conditions has been well documented (Sivakumar *et al.*, 2007). It has also been observed that the physio-chemical and nutritional requirements for extracellular amylase production, differs from species to species (Babu & Satyanarayana, 1993; Dey *et al.*, 2001; Gigras *et al.*, 2002), with no defined medium for its production (Pandey *et al.*, 2000).

The present study reports high amylase activity with stationary culture conditions. This was in contrary to the results recorded by Loginova *et al.*, (1970) and Perlman, (1969) who have reported high improvement in yields of amylase production in shaken cultures.

Carbon and nitrogen source

Production of α-amylase enzyme is greatly influenced by nutrition factors (Kar & Ray, 2008). The results of this study revealed that maximum amylase activity was observed with 1 % (w/v) starch and yeast extract. Both carbon as well as nitrogen sources are necessary for the growth and metabolism of organisms and the carbon–nitrogen balance plays an important role in optimal production of the enzyme (Subramanian *et al.*, 2012).

Starch has been reported as the better carbon source for enhancing amylase production wherein it acts as an inducer for the expression of gene required for its production (Melasniemi, 1987; Brown *et al.*, 1990; El-Assar *et al.*, 1992, Narayana & Vijayalakshmi, 2008). A similar observation was reported by Kuo & Hartman, (1966) in *Thermoactinomyces vulgaris* strain and Syed *et al.*, (2009) in *Streptomyces gulbargensis.* On the other hand, enzymes degrading starch in particular are of great importance to biotechnological industries (Sivakumar *et al.*, 2012).

Nitrogen source as a basal component of the medium is a major factor affecting α-amylase production as its acts as a metal ion source and a pH controller as well (El-Fallal *et al.*, 2012). It has been reviewed that among all organic nitrogen sources yeast extract was found to be essential for α-amylase production, serving as potential sources of amino acids and vitamins (Mc Mahon *et al.*, 1999; Yassien & Asfour, 2012; El-Fallal *et al.*, 2012).

Incubation period

Period of incubation had a profound effect on amylase production with maximum activity exhibited on the 9th day. A study by Shatta *et al.*, 1990, has shown *Streptomyces aureofaciens* with optimum amylase production recorded on the 7th day

of incubation. Since most actinomycetes species are slow growing, its enzyme production usually starts in early log phase with increase in production of enzyme at late growth phases (Chakraborty *et al.*, 2009). However, in the present study, amylase production by *Streptomyces* sp. CFA-5 occurred during all the incubation days and the data obtained varied which clearly indicates the kinetics of enzyme production in this regard was non-growth associated type (Chakraborty *et al.*, 2009).

pH

pH is one of the important factors affecting the synthesis and secretion of α-amylase. Besides, growth and morphology of microorganisms are sensitive to the concentration of hydrogen ions present in the medium which inturn regulate enzyme production (Kuddus *et al.*, 2011). pH values also serve as an indicator of the initiation and termination of enzyme synthesis (Friedrich *et al.*, 1989) and change in pH affects α-amylase stability in the medium (Calamai *et al.*, 2005).

This study found pH 6.4 as the optimum for amylase production indicating the strain prefers neutral conditions for better enzyme production. Neutral to alkaline α-amylases produced by *Streptomyces megasporus* (Dey & Agarwal, 1999), *S. rimosus* (Yang & Wang, 1999) and *Thermoactinomyces vulgaris* (Heese *et al.*, 1991 and Kuo & Hartman, 1966) has been reported. The present data is also in accordance to the reports by Hamilton *et al.*, (1999) and Kar & Ray, (2008) who had observed maximum amylase activity at pH 6 while, amylase production at pH 4.8 and 6.5 by *Streptomyces aureofaciens* 77 had been reported by Shatta *et al.*, (1990).

α-Amylases are generally stable over a wide range of pH from 4 to 11 i.e. acidic to alkaline (Pandey *et al.*, 2000; Bozic *et al.*, 2011; Sun *et al.*, 2010), this is mainly because the α-amylase active site consists of a large number of charged groups

(Lawson *et al.*, 1994; Strokopytov *et al.*, 1996). However, α-amylases with stability in a narrow range have also been reported (El-Fallal *et al.*, 2012).

Temperature

Temperature is also a significant parameter affecting the production of α-amylase activity. This study showed optimum enzyme production at 60 °C. The data corroborates with the findings of McMahon *et al.*, (1997) (*Streptomyces* sp. IMD 267), Yang & Wang, (1999) (*S. rimosus*), Goldberg & Edwards, (1990) (*Streptomyces thermoviolaceus*), Ammar *et al.*, (2002) (*Streptomyces* sp.), Kar & Ray, (2008) (*S. erumpens* MTCC 7317) and Chakraborty *et al.*, (2009) (*Streptomyces* sp. D1) who have reported α-amylase production at the 45-70 °C temperature range. Other actinomycetes genera reported as versatile producers of thermostable amylases are *Thermomonospora curvata,* (Collins *et al.*, 1993; Busch & Stutzenberger, 1997), *Thermomonospora*, (Kuo & Hartman 1966; Ammar *et al.*, 2002) *Thermoactinomyces vulgaris* (Abou Dobara *et al.*, 2011), *Thermomonospora fusca* (Busch & Stutzenberger, 1997), *Thermoactinomyces* sp. 15 (Obi & Odibo, 1984) and *Nocardiopsis* sp. (Stamford *et al.*, 2001; Kundu, 2006). Thus, the above data revealed that optimum conditions for enzyme production differ from species to species (Poornima *et al.*, 2008).

Thermostable microbial amylases are mostly used in industries (Burhan *et al.*, 2003) especially in starch processing industry as it helps increase reaction rate and decrease contamination risk through the use of high temperatures (Kelly *et al.*, 1986; Kaur & Satyanarayana, 2004). According to Fitter, (2005) various structural strategies, many ionic interactions and increased hydrophobic surface burial, disulphide bridges,

prolines, improved core packing, shorter surface loops and higher states of oligomerization are partly proven to increase thermostability.

Effect of activators (inorganic salts)

Activators are substances that combine with an enzyme to increase its catalytic activity (Koaze *et al.*, 1975). In the present study, $MnCl_2$ as an activator had a comparable effect on amylase activity. Earlier studies have reported most of amylases known to be metal ion-dependent enzymes, namely divalent ions like Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, *etc.* (Pandey *et al.*, 2000). Similar to the present study, Mn^{2+} ions promoted α-amylase production in *Bacillus* sp. I-3 (Goyal *et al.*, 2005), *Vibrio* sp. (Najafi & Kembhavi, 2005) and *Bacillus* sp. L1711 (Bernharsdotter *et al.*, 2005), respectively. Shatta *et al.*, (1990), has shown *Streptomyces aureofaciens* having high stimulatory effect on amylase production on addition of $MnCl_2$.

Use of $CaCl_2$ showed neither inhibitory nor potentiating activity on amylase production. This clearly confirms that α-amylase produced is calcium independent and is in conformity with the findings of Chakraborty *et al.*,(2012) who reported calcium independency in amylase producing marine *Streptomyces* sp. A3. A similar finding was also reported by Malhotra *et al.*, (2000) for amylase production from *Bacillus thermooleovrans.*

3.4.5 Enzyme purification

The partially purified enzyme when subjected to SDS-PAGE for molecular weight determination showed a relative molecular weight of ~ 41 kDa. The result was supported by the fact that despite wide difference of microbial α-amylases characters, their molecular weights are usually in the range 40-70 kDa (Gupta *et al.*, 2003).

Previous results have shown wide variations in molecular mass of different Actinomycetes spp., *Thermoactinomyces vulgaris* (53, 000 Da) (Heese *et al.*, 1991), *S. rimosus* (43,000 Da) (Vukelic *et al.*, 1992) *S. megasporus* SD12 (97 kDa) (Dey & Agarwal, 1999); *Streptomyces erumpens* MTCC 7317 (54, 500 Da) (Kar & Ray, 2008), *Saccharopolyspora* sp. A9 (66 kDa) (Chakraborty *et al.*, 2011) and *Streptomyces* sp. A3 (45 kDa) (Chakraborty *et al.*, 2012).

According to a study by Chen *et al.*,(2012) who studied the evolutionary scenarios for the GH 13 enzymes opines that the α-amylase produced by actinomycetes is ancestral and the subsequent gene flow has led to its present distribution in bacteria and fungi. This finding also explains the recovery of a low molecular weight α-amylase of 41 kDa in the present study from *Streptomyces* sp. CFA-5 and indicates that it is an ancestral molecule.

3.4.6 Characterization of the partially purified enzyme

The present study has shown that the partially crude enzyme had similar optimum pH (6.4) and temperature (60 °C) as in crude enzyme. The data is in accordance to previous studies by Kar and Ray, (2008). Besides, α-amylases stable over a wide range of pH from 4 to 11 has also been reported (Gupta *et al.*, 2003).

CHAPTER IV

Screening, purification and characterization of a hybrid polyketide peptide antibiotic

4.1 INTRODUCTION

Novel antibiotics are continuously in demand due to the inevitable rise of antibiotic-resistant strains of pathogenic bacteria, reducing morbidity, mortality and life expectancy (Fischbach & Walsh, 2009). Nature still remains the most versatile source of such novel antibiotics, despite considerable progress made in the fields of chemical synthesis and engineered biosynthesis of antibacterial compounds (Kpehn & Carter, 2005; Baltz, 2006; Pelaez, 2006).

Among all microorganisms, actinomycetes are the most prolific producer of antibiotics (Berdy, 2005). The discovery of new molecules from actinomycetes has marked an epoch in antibiotic research, as most of its antibiotics are too complex to be synthesized by combinatorial chemistry (Lazzarini *et al.*, 2000; Baltz, 2007). Out of the eight thousand antimicrobial products described in antibiotic literature database (ABL database), 45.6 % are produced by Streptomycetes and 16 % by strains belonging to rare genera of actinomycetes (Lazzarini *et al.*, 2000). Most of the strains form an important component of the soil microflora (Singh *et al.*, 2006). Baltz, (2007) has estimated that less than one part in 10^{12} of the earth's soil surface has been screened for actinomycetes (Fischbach & Walsh, 2009). Only 10 % of natural products from screened strains and just 1 % of molecules from the global consortium of microbial producers may have been discovered by researchers (Clardy *et al.*, 2006).

According to Watve *et al.*, (2001), predictive modeling of genus *Streptomyces* suggests that over 1,50,000 bioactive metabolites from the soil ecosystem still remains undiscovered. Besides, the rate of discovery of new compounds from terrestrial actinomycetes has decreased, whereas the rate of re-isolation of known compounds has increased (Sanasam & Ningthoujam, 2010).

Search of underexplored ecological niches has revealed new molecules which stresses the need to explore new groups of actinomycetes from such habitats as source of novel bioactive secondary metabolites. This is preceeded by selection of novel media and/or improvement of isolation methods (Singh *et al.*, 2006; Joseph *et al.*, 2009; Sanasam & Ningthoujam, 2010). With diverse physiographic conditions prevailing in India, various unexplored habitats offer vast opportunities for isolation of chemically creative strains of soil dwelling actinomycetes (Velho-Pereira & Kamat, 2013).

Antibiotic biosynthesis in actinomycetes particularly *Streptomycetes* has been reported to be highly dependent on the nutritional and physiological factors prevailing during its growth since it helps in cell proliferation which in turn expresses genetic information favoring secondary metabolism (Abbanat *et al.*, 1999). These metabolic processes are species specific and can be enhanced or minimized under different physiological conditions (Yarbrough *et al.*, 1993; Abbanat *et al.*, 1999; Selvin *et al.*, 2009; Visalakchi & Muthumary, 2009; Rezuanul *et al.*, 2009; Elleuch *et al.*, 2010; Nanjwade *et al.*, 2010; Panda *et al.*, 2011; Chhabra & Keasling, 2011; Darabpour *et al.*, 2012; Mangamuri e*t al.*, 2012; Singh & Rai, 2012; Luthra & Dubey, 2012; Gunda & Charya, 2013). Thus, it is essential to standardize growth conditions of the producer strain, for maximum synthesis of its bioactive molecules (Sujatha *et al.*, 2005; Olmos *et al.*, 2013). Although, classical strain improvement techniques is laborious and time consuming, it is still being widely used due to their high success rate yielding improved production titers of antibiotics such as penicillin, cephalosporin C, tylosin, salinomycin, chlortetracycline and tetracycline (Chhabra & Keasling, 2011).

This chapter deals with the screening of actinomycetes strains from tropical soil for antimicrobial activity. The study led to the identification of *Streptomyces*

parvulus strain which showed broad spectrum activity against five human pathogenic bacteria with maximum activity against *Staphylococcus citreus*. The current study also reports standardization of parameters required for maximizing antibiotic production, purification of the compound and the structure elucidation of the purified compound. The various methodologies and results dealing with the above are delved in this chapter.

4.2 METHODOLOGY

4.2.1 Preliminary screening of pigmented strains for antimicrobial activity

Thirteen pigmented actinomycetes strains as shown in **Table 4.1** were primarily screened by perpendicular streak method (Badji *et al.*, 2007) using Mueller Hinton Agar (MHA) medium, HiMedia, India, (Selvin *et al.*, 2009) against eight test organisms which included human pathogenic Gram positive bacteria such as *Staphylococcus aureus, Bacillus subtilis, Staphylococcus citreus;* Gram negative bacteria such as *Escherichia coli* (MTCC 739), *Enterobacter aerogens;* Dimorphic yeast such as *Candida albicans* (MTCC183); pathogenic microfungi such as *Aspergillus niger* (MTCC 282) and *Alternaria brassicola* (MTCC 2102). The test organisms *S. aureus, Bacillus subtilis*, *Enterobacter aerogens* and *S. citreus* were procured from Department of Microbiology, Goa Medical College, Goa and maintained on nutrient agar slants (Himedia, Mumbai, India). The other test pathogens were procured from Microbial Type Culture Collection (MTCC) and Gene bank, Chandigarh. The fungal pathogens were maintained on malt extract agar slants (Himedia, Mumbai, India).

Table 4.1 Selected pigmented actinomycetes strains from various sites for antimicrobial activity

Sampling site designation	Total no of actinomycetes isolates	Actinomycetes isolates grouped in different taxa			
		Streptomyces	*Actinomadura*	*Micromonospora*	Unidentified Genera
KFA	1	1	0	0	0
CFA	1	1	0	0	0
TMK	1	0	0	0	1
BSK	2	0	0	2	0
MAS	6	6	0	0	0
FCP	1	0	1	0	0
MMA	1	0	0	0	1

4.2.2 Selection of strains for antibiotic screening by modified cross-streak method (MCSM) (Velho-Pereira & Kamat, 2011)

Despite the addition of antibiotics in the slide coating medium (SCM) to inhibit growth of unwanted microbes; bacterial and fungal spores were observed. Microscopic observations of colonized slides revealed the absence of bacterial and fungal competitors in a few. The selection of strains for antibiotic screening was guided by this lead as these were assumed to be potential producers of antibiotics. The occurrence and distribution of the selected isolates of actinomycetes from different soil samples are presented in **Table 4.2.**

Table 4.2 Selected pigmented strains from various sites for antibiotic screening

Sampling site designation	Total no of actinomycetes isolates	Actinomycetes isolates grouped in different taxa			
		Streptomyces	*Actinomadura*	*Micromonospora*	Unidentified Genera
KFA	7	3	0	2	2
CFA	4	2	0	0	2
TMK	1	0	1	0	0
MAS	6	5	1	0	0
MMA	6	3	1	2	0
PM	1	1	0	0	0
BOA	1	0	0	0	1
SIA	4	2	0	0	2

4.2.2.1 *Screening cultures for potential antimicrobial activity by MCSM*

Preliminary screening using perpendicular streak method, showed difficulty in quantifying the results, besides mutual antibiosis due to use of multiple cultures in the test panel could not be ruled out. This prompted the use of cross streak method (CSM) and its further modification allowed better applicability of the technique.

The cross streak method (CSM) (Fernando, 1947) was modified by keeping the distance between the test pathogen streak fixed i.e. 1 cm; length of the test

pathogen streak line, 3 cm; streak width, 0.5 cm; length of actinomycetes streak, 7 cm and width of the actinomycetes streak, 0.5 cm. The markings were drawn on a transparency sheet, which acted as a template and was attached below the plates to facilitate streaking and thereafter in quantifying the results. The test organisms were streaked perpendicular to the line of actinomycetes growth as shown in **Figure 4.1a&b**.

MHA medium (Himedia, India) plates were prepared and inoculated individually with 30 actinomycetes cultures by a single streak in the centre of the petri dish and incubated at 28 °C for 7 days (Selvin *et al.*, 2009). Care was taken to create an uniform streak covering the entire marked surface area. Antagonism was observed based on the inhibitory interaction between the actinomycetes and test strains.

4.2.2.2 *Test organisms*

The actinomycetes strains were screened against a panel of pathogens which included human pathogenic Gram negative bacteria such as *S. typhi, S. flexneri, Proteus* sp., *E. aerogenes, S. marcescens,* Gram-positive bacteria such as *S. aureus, B. subtilis* and dimorphic yeast such as *Candida albicans* (MTCC183). The test organisms *S. typhi, S. flexneri, Proteus* sp., *E. aerogenes, S. marcescens* and *B. subtilis* were procured from Department of Microbiology, Goa Medical College, Goa and maintained on nutrient agar slants (Himedia, Mumbai, India). The test pathogen *Candida albicans* was procured from Microbial Type Culture Collections (MTCC), Chandigarh and maintained on malt extract agar slants (Himedia, Mumbai, India).

Figure 4.1 Representative MH plate showing modified cross streak method [**a)** Front and **b)** Reverse view]

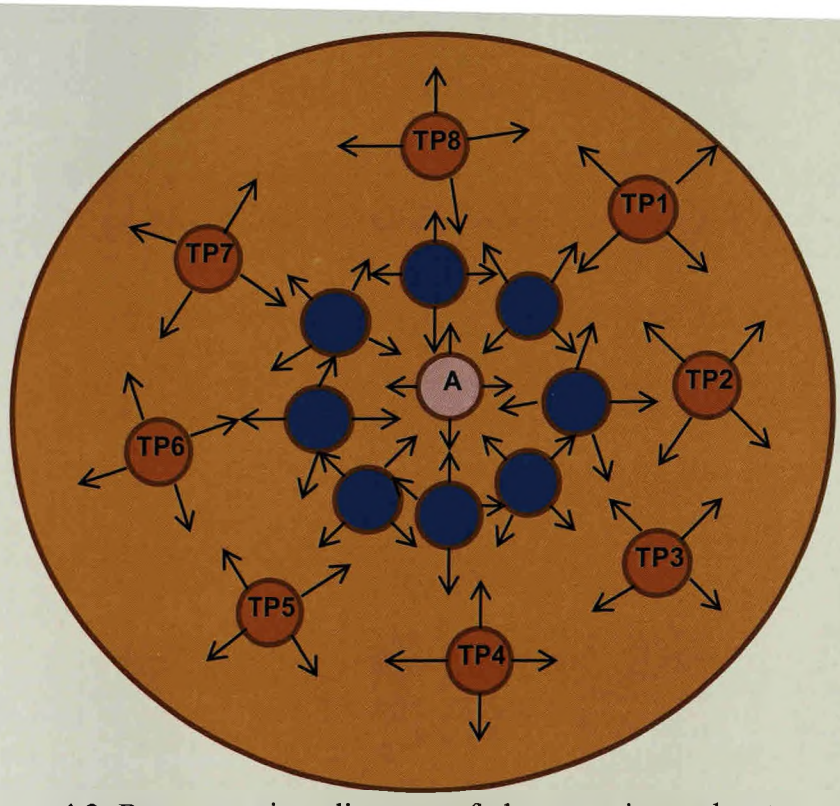


Figure 4.2 Representative diagram of the experimental set up with arrows indicating the expected direction of diffusion resulting in formation of crystals. Well at the centre (marked in white) was filled with inoculum of actinomycete culture (A), wells (marked in blue) were filled with sterile 0.01M $CaCl_2.2H_2O$, wells (marked in orange) were filled with inocula of test pathogen cultures (TP1-TP8)

4.2.2.3 *Preparation of Quantitative Antibiotic Activity Score Matrices (QASM)* (Velho-Pereira & Kamat, 2011)

Since the area of each test streak was fixed at 150 mm^2 it was possible to quantify regions of growth and measure the clear/inhibited areas using a graph paper template. The clear areas for different test species were indicators of antibiotic activity and were proportional to antibiotic concentration and diffusion.

To express these results quantitatively and to prepare quantitative antibiotic activity score matrices, the following Eqns. were used,

Percent area specific differential antibiotic activity score (PASDAAS)= (AWG/TSA)×100....(1), where, AWG is the area on the plate without growth for each test panel streak, TSA is the total streak area (150 mm^2) scored for each test panel streak.

Percent multispecific antibiosis efficiency score (PMSAES), gives the efficiency of tested actinomycetes strain to inhibit more than a single test pathogen. The ideal score would be 100. This is computed using the Eqn. PMSAES=(ΣPASDAASTP1-8/ TPS)×100.....(2), where $\Sigma PASDAASTP_{1\text{-}8}$ is the percent area specific differential antibiotic activity score of test pathogens 1-8 and TPS is total possible score for all test pathogens (i.e. 100×8=800).

Since multiple microbial species were used in the test panel it was possible to calculate a scoring total or percent overall inhibition efficiency score (POIES) for each actinomycetes using the following Eqn, POIES = (TNIS/TNTS)×100.....(3), where, TNIS is total number of inhibited species and TNTS is total number of test species. The ideal score for multi-specific inhibition would be 100.

Having calculated the total number of positive results of each test pathogen against 30 actinomycetes strains, it was possible calculate percent overall screening

efficiency score (POSES). This is computed by the equation, POSES= (TPR/TAS)×100.....(4), where TPR is total no of positive results of each test pathogen and TAS is total no of actinomycetes strains.

4.2.3 Detection of Calcium Oxalate Crystals (COC's)

A week after recording the antibiosis on MHA plates, COC's were detected under Olympus SZ2-ILST stereomicroscope (Tokyo, Japan). The crystals were identified on the basis of standard calcium oxalate crystal morphology (Arnott & Wells, 2002; Khan, 1995) and solubility test. The culture plates were studied stereomicroscopically on a daily basis and the COC's were counted, their location marked so as to distinguish them from newly formed crystals and a cumulative count of all the crystals were recorded after three weeks. The COC's counts were recorded separately along the actinomycetes streaked line and the corresponding test pathogens for all the thirty actinomycetes strains.

4.2.3.1 *Studying the production of COC's*

The major aim of this study was to answer some basic questions like whether actinomycetes strain have the ability of producing extracellular calcium oxalate crystals; if yes, then whether these produce or get triggered in presence of test pathogens or vice versa or whether external source of calcium chloride is required to trigger the formation of calcium oxalate crystals or whether the MHA medium is producing the same.

In order, to understand the above phenomenon well diffusion precipitation experiments, using the principles of well diffusion and immunoprecipitation assay were set up. The principle was based on detection of an extracellular biogenic flux of

oxalic acid triggered by the presence of pure calcium chloride. The flux that radiates from the growing colony would interact with the soluble calcium gradient and at this point, contact precipitation would take place that could be detected stereomicroscopically.

COC's production assay (Velho-Pereira & Kamat, *unpublished*)

Medium for bioassay was MHA (Himedia Veg, Mumbai, India). A central well, surrounded by 8 wells which were further surrounded by 8 wells representing the 8 test pathogens were excised from the MHA plates **(Figure 4.2)**. All wells were excised 6 mm diameter deep and were kept at a distance of 3 cm apart.

A representative actinomycetes strain *Streptomyces* sp. MAS-11 was selected for this assay based on the previous results of antibiosis and detection of COC's formation. The inoculum (5 %) was prepared by inoculating a loopful of the actinomycetes strain in Arginine Vitamin (AV) broth and kept shaking at 250 rpm, 28 °C for four days, whereas the test pathogens were grown on Nutrient broth (Hi Media, Mumbai, India) for 24-48hrs. The test pathogens (TP) used for the assay were TP1-*Salmonella typhi,* TP2-*Shigella flexneri,* TP3-*Proteus,* TP4-*Enterobacter aerogens,* TP5-*Serratia marcescens,* TP6-*Staphylococcus aureus*, TP7-*Bacillus subtilis* and TP8-*Candida albicans*. The culture plates were studied stereomicroscopically under Olympus SZ2-ILST stereomicroscope (Tokyo, Japan) and the experiment was performed in triplicates.

The experimental setup was as follows:

Test 1: The central well, filled with inoculum of actinomycetes strain, surrounded by 8 wells of sterile 0.01M $CaCl_2.2H_2O$ and corresponding 8 wells filled with inoculum of test pathogens.

Three controls were maintained i.e.

Control 1: The central well, filled with inoculum of actinomycetes strain, surrounded by 8 wells of sterile 0.01M $CaCl_2.2H_2O$ and corresponding 8 wells filled with sterile distilled water.

Control 2: The central well, with sterile distilled water, surrounded by 8 wells ofsterile 0.01M $CaCl_2.2H_2O$ and corresponding 8 wells filled with inoculum of test pathogens.

Control 3: The central well, filled with inoculum of actinomycetes strain, surrounded by 8 wells of sterile distilled water and corresponding 8 wells filled with inoculum of test pathogens.

4.2.4 Selection of a hyper producer antibiotic strain

Based on the results of the preliminary screening efforts, a single actinomycete strain CFA-9 which showed broad spectrum chemical creativity was selected for further studies.

4.2.4.1 *Taxonomic and molecular identification of the hyper producer strain*

The morphological and cultural characteristics of the actinomycetes strain CFA-9 were studied by using traditional criteria of classification (Locci, 1989; Cross & Goodfellow, 1973). The micromorphological studies were done using light and scanning electron microscopy (Williams & Davies, 1967).

For molecular identification, genomic DNA of the strain was extracted and its quality was evaluated as a single distinct band on 1.2 % agarose gel. The fragment 16S rRNA gene was amplified by PCR and the amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was

carried out with 8F and 1492R primers using BDT v3.1 cycle sequencing kit on ABI 3730xl Genetic analyser. Consensus sequence of 1347 bp 16S rRNA gene was generated from forward and reverse sequence data using aligner software. This sequencing work was done by Xcelris Labs Ltd. (www.xcelerislabs.com) and is given as **Annexure V**. The sequence was deposited at Genebank database (http://www.ncbi.nlm.nih.gov/genbank/submit) with Gene Bank accession number of KC904376.

Phylogenetic analyses were conducted using MEGA v5 (Tamura *et al.*, 2011). The 16S rRNA gene sequence of strain CFA-9 was aligned using the Clustal W program against corresponding nucleotide sequences of representatives of *Streptomyces* genus retrieved from Genbank (http://www.ncbi.nlm.nih.gov/genbank). Phylogenetic tree was constructed by the maximum-likelihood method (Felsenstein, 1985) based on the Hasegawa–Kishino–Yano (HKY) (Hasegawa *et al.*, 1985) model. Tree topologies were evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings.

4.2.5 Secondary screening

4.2.5.1 *Antibiotic production medium*

Kuster's broth (Kuster & William, 1964) was used as the basic fermentation medium. All chemicals were procured from HiMedia, Mumbai, India.

4.2.5.2 *Antibiotic bioassay*

The fermentation medium was inoculated with a seven day old culture (5 % inoculum, determined at OD 520 nm) of *Streptomyces parvulus* CFA-9 under sterile conditions. The culture flasks were fixed on a rotary shaker (Orbitek^{R}, Scigenics

Biotech, Pvt. Ltd., India) 250 rpm; rotation diameter: 2.0 cm, placed in a thermostated cabinet at 28 °C for 2, 7, 12, 14, 16, 18 and 20 days fermentation process. The culture broth was subjected to centrifugation at 4000 *g* for 20 min to obtain a CFC (Cell Free Centrifugate). Cell Free Culture Filtrate (CFCF) was thereafter obtained by subjecting the CFC to filtration using a 0.22 μm membrane filter on a membrane filtration unit (Tarsons, Products Pvt. Ltd., Kolkata, India)

Agar well diffusion method (Devillers *et al.*, 1989) was used for detection of antimicrobial activity. Medium for bioassay was MHA (Himedia Veg, Mumbai, India). Three cores of 6 mm diameter were excised from the MH agar plates, pre-seeded with the 24/48 hrs test organism namely *S. flexneri*, *B. subtilis*, *E. aerogens*, *S. typhi* and *S. citreus* using sterile swabs. The wells were filled with CFCF (50-70 μl) using Accupipet model T1000 (Tarsons Products Pvt. Ltd., Kolkata, India). Uninoculated Kuster's broth was used as a negative control. The plates were incubated at 28 °C for 48 h and inhibition zones (ZOI) were visualized and measured in millimeters (mm).

4.2.5.3 *Spectroscopic measurements* (Malekil & Mashinchian, 2011)

The absorption spectrum of each active extract (CFCF) on varying days of incubation was recorded in the UV-VIS region (190-800 nm) on a UV-VIS spectrophotometer (UV-1800, Shimadzu). Uninoculated Kusters broth was kept as control. Similarly infrared spectrum was determined using Shimadzu FT-IR System spectrophotometer in KBr pellet scanned at wavelength 400 to 4600 cm^{-1}.

4.2.6 Impact of different process parameters on antibiotic production

Antibiotic production was optimized at bench top scale by using different physiological and nutritional parameters *viz.*, days of incubation, temperature, pH, sodium chloride, organic and inorganic carbon and nitrogen sources and phosphate. The optimum conditions identified for one parameter were used for optimizing the other parameters sequentially. The efficiency of the optimized parameters was established on the basis of zone of inhibition (ZOI) in mm. All experiments were performed in triplicates (n=3).

4.2.6.1 *Antibiotic bioassay and test pathogens*

The antibiotic bioassay was carried out as mentioned in **section 4.2.5.2**. Since, *Streptomyces parvulus* CFA-9 exhibited highest ZOI against test pathogenic strain *Staphylococcus citreus* (31 mm), it was decided to utilize only this particular test strain for subsequent characterization of antimicrobial compound.

4.2.6.2 *Physiological parameters*

Incubation period

To study the effect of incubation period on antibiotic production, 10 ml aliquot of the culture broth was collected aseptically at regular intervals of 4, 7, 12, 14, 16, 18 and 20th days and the CFCF was subjected to bioassay.

Temperature

The optimum temperature for antibiotic production was assayed by incubating the production medium at 25, 28, 32 and 37 °C. The control was maintained at 28 °C.

pH

Influence of pH on antibiotic production of the strain was determined by adjusting the pH of production medium ranging from 3 - 11 with sterile 0.1 N NaOH / 0.1 N HCl. pH 7 was used as the control.

Effect of light

To study the effect of light on antibiotic production, two sets of flasks (F 1 (light) and F 2 (dark) were kept under shaken condition at 250 rpm under the lamp power (100 W) switched on for 14 days at 28 °C. The flask F 2 was kept in dark by covering the flask with aluminium foil on all sides to block the exposure to light.

Effect of autoclaving on antimicrobial activity

The CFCF was autoclaved at 121 psi for 15-20 min along with, uninoculated Kuster's broth and distilled water as controls. The CFCF was later subjected to bioassay.

4.2.6.3 *Nutritional parameters*

NaCl concentration (ppm)

The effect of sodium chloride on antibiotic production was studied using different concentrations of 0; 10,000; 15,000; 20,000; 25,000; 30,000 and 35,000 ppm. NaCl concentration of 20,000 ppm was kept as control.

Organic carbon and nitrogen source (%)

The carbon and nitrogen sources in the production medium were varied or manipulated to study its influence on antibiotic production.

Glycerol being the sole organic carbon source of the Kuster's broth medium, its concentration was varied from 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.2 and 1.3 % (v/v). Glycerol concentration of 0.8 % (v/v) served as control. Casein being the sole organic nitrogen source of the medium was studied by varying its concentration from 0.01-0.09 % (w/v). Casein concentration of 0.03 % (w/v) was kept as control.

Phosphate concentration (%)

To study the effect of phosphate mineral (K_2HPO_4) on antibiotic production different concentrations viz., 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45 and 0.5 % (w/v) were tested. Phosphate concentration of 0.2 % (w/v) was kept as control.

Inorganic nitrogen and carbon source (%)

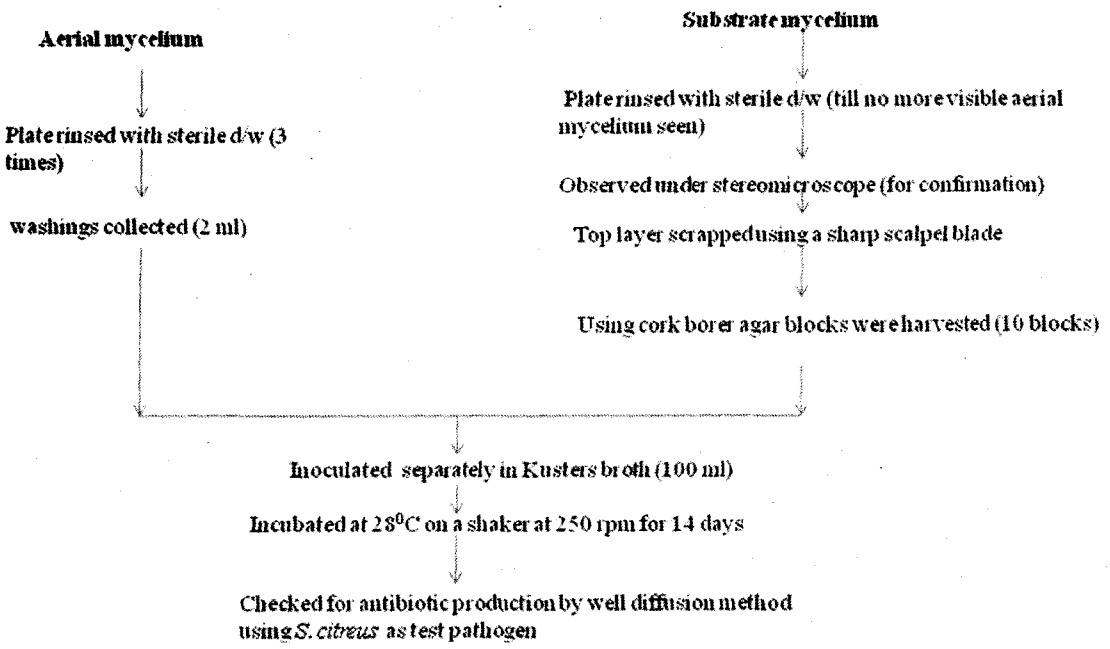
The influence of inorganic nitrogen (KNO_3) and carbon source ($CaCO_3$) present in the antibiotic production medium was studied using varied KNO_3 concentration of 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, 0.5 and 0.55 % (w/v). KNO_3 concentration of 0.1 % (w/v) was kept as control and eight varied concentration of $CaCO_3$ i.e. 0, 0.0005, 0.001, 0.0015, 0.002, 0.0025, 0.003, 0.0035 % (w/v) was studied. $CaCO_3$ concentration of 0.001 % (w/v) was kept as control.

Effect of culture inoculum (%)

To evaluate the effect of inoculum size on antibiotic production, varied inoculums volumes (v/v) i.e 3.5, 4, 4.5, 5, 5.5, 6, 6.5 and 7 % was added to antibiotic production medium and the CFCF was subjected to bioassay. The cell concentration of 5 % v/v was kept as the control.

Effect on antibiotic production efficacy by differential sampling of its aerial (AM) and substrate mycelium (SM)

The experiment was setup to study the specific cellular localization of antibiotic production with respect to AM and SM (Manteca & Sanchez, 2010). A seven day old culture was used to harvest the aerial and substrate mycelium.The schematic scheme of its separation and production is represented in the flowchart below.



4.2.6.4 *Statistical analysis*

All the optimization parameters were subjected to statistical analysis wherein the results are expressed as a mean of three experiments ± standard deviations (SDs). Statistical analysis were performed using WASP (Web Based Agricultural Statistics Software Package 1.0, (http://www.icargoa.res.in/wasp/index.php) and differences were considered significant if $p \leq 0.05$.

4.2.7 Testing the relative efficacy of CFCF with standard antibiotics

To compare the relative efficacy of CFCF with standard antibiotics, 36 different broad as well as narrow spectrum standard antibiotics (Himedia ICOSA Disc™) were tested against test pathogen *S. citreus* using Kirby-Bauer antibiotic testing technique (Bauer *et al.*, 1966). The antibiotic susceptibility discs provided the following antibiotics: Amikacin (30 mcg), Amoxyclav (30 mcg), Ampicillin (10 mcg), Augmentin (30 mcg), Azithromycin (15 mcg), Cefpodoxime (30 mcg), Ceftriaxone (30 mcg), Cephalothin (30 mcg), Chloramphenicol (30 mcg), Ciprofloxacin (5 mcg), Clarithromycin (15 mcg), Clindamycin (2 mcg), Colistin (10 mcg), Co-Trimoxazole (25 mcg), Erythromycin (15 mcg), Gatifloxacin (5 mcg), Gentamicin (10 mcg), Imipenem (10 mcg), Kanamycin (30 mcg), Levofloxacin (5 mcg), Linezolid (30 mcg), Methicillin (5 mcg), Moxifloxacin (5 mcg), Nalidixic acid (10 mcg), Norfloxacin (10 mcg), Novobiocin (5 mcg), Ofloxacin (5 mcg), Oxacillin (1 mcg), Penicillin (10 units), Sparfloxacin (5 mcg), Streptomycin (10 mcg), Teicoplanin (10 mcg), Tetraycline (30 mcg), Ticarcillin (75 mcg), Tobramycin (10 mcg) and Vancomycin (30 mcg).

4.2.8 Extraction of antibiotic compounds from CFCF using various solvents (Selvin *et al.*, 2009)

4.2.8.1 *Solvent extraction*

Various solvents (polar, dipolar and nonpolar) namely chloroform (GR grade, Merck, India); methanol (GR grade, Merck, India); butanol (GR grade, Merck, India); propanol (GR grade, Qualigens, India) ; acetone (GR grade, Merck, India), ethyl acetate (GR grade, Merck, India); n-hexane (GR grade, Qualigens, India) and aniline (GR grade, Merck, India) were used for extraction of the metabolites. The solvent was added to the CFCF in 1:1 proportion in a separating funnel which was shaken

vigorously for 5 mins and allowed to settle. The organic layer was thereafter collected in a sterile flask and was concentrated using rotary evaporator (Buchi, Germany) at 40 °C. Uninoculated Kuster's broth extracted in the respective solvents was used as controls.

4.2.8.2 *Antimicrobial bioassay*

Antibiotic bioassay using various solvent extracts along with the appropriate controls was tested following the methodology mentioned in **section 4.2.5.2**. On the basis of previous findings, the selected test pathogens used for the bioassay were namely *S. flexneri*, *B. subtilis*, *E. aerogens*, *S. typhi* and *S. citreus*. Uninoculated Kuster's broth extracted in the respective solvents and the respective solvents were used as negative and positive controls of the experiment. The experiment was performed in triplicates.

4.2.8.3 *Spectroscopic measurements* (Malekil & Mashinchian, 2011)

The absorption spectrum of each active extract was recorded in the UV-Visible region (190-800 nm) on a UV-VIS spectrophotometer (UV-1800, Shimadzu). Uninoculated Kuster's broth extracted with the respective solvents was kept as control.

Infrared spectrum of only the active methanolic extract (based on the UV-Visible spectral analysis) was recorded on a Shimadzu FT-IR spectrophotometer in KBr pellet scanned at wavelength 400 to 4600cm^{-1}.

4.2.9 Testing of the crude methanolic extract for number of components by TLC

Crude concentrated methanolic extract (10 µl) was subjected to thin-layer chromatography (TLC). The TLC was performed on precoated 20×20 cm and 0.25

mm thick plates (Merck 60 F 254; Merck, Darmstadt, Germany). These plates were activated by hot air oven at 100 °C for 1hr. Inorder to select an appropriate TLC solvent system on basis of distinct resolution of spots, two pure solvents and 51 standard combinations (Heftmann, 1983) were used as shown in **Table 4.3**. The plates were run in the above solvent systems and dried at room temperature. The spots were examined with the naked eye and visualized under UV at 254 and 365 nm and by spraying iodine vapours. Different bands/spots were observed and corresponding R_f values are determined. R_f value of each spot was calculated as:-

R_f= Distance travelled by the solute / Distance travelled by the solvent.

Table 4.3 Solvent system for TLC analysis

Solvent System (mobile phase)	**Standard Combinations (v/v)**
chloroform	100 %
methanol	100 %
chloroform: methanol	9:1
	10:1
	5:1
	1:1
	95:5
	98:2
	87:13
	20:1
	97:3
	50:1
	100:15
	96:4
	4:1
	65:1
	92:8
	3:1
	6:1
	9.5:0.5
ethyl acetate: methanol	4:1
	1:1
	3:1
	5:1
	10:1

n-butanol: methanol: water	4:1:2
	2:1:1
	2:1:3
	3:1:2
n-butanol	0.07
benzene: methanol	1:1
	10:1
	5:1
	55:45
	4:1
	80:20
	50:50
	97:3
	6:1
chloroform: methanol: water	65:35:10
	2:2:1
	5:4:2
	1:1:1
chloroform: methanol: benzene	7:3:2
chloroform: acetone: methanol: water	200:20:50::50
chloroform: methanol: acetone	78:20:2
chloroform: ethyl acetate: methanol	2:4:1
acetone: methanol	4:1
	9:1
methanol: water	17:3
	1:1
benzene: ethyl acetate: methanol	10:2.5:1
	6:4:1

4.2.10 Bioautography and chemical identity of antibiotics

The crude concentrated methanolic extract (10 µl) was subjected to TLC silica gel plate (Merck 60 F 254; Merck, Darmstadt, Germany) and developed in a mixed solvent system of chloroform: methanol (5:1, v/v) as mobile phase. The plates were run in duplicate; one of which was used for bioautography and the other was used as reference plate (Alekhova & Novozhilova, 2001). For bioautography, semisolid MHA medium (HiMedia, Mumbai, India) was poured onto TLC plates. The plates were thereafter seeded with *S. citreus* as the test pathogen using sterile swabs. After 1 hr of refrigeration, the plates were incubated for 24 hrs at 28 °C. Clear halos due to growth

inhibition of the test pathogen indicated the location of antimicrobial compounds on the TLC plates. Inhibition zones and R_f values of the active compound were compared with the reference TLC plate.

Each of the bioactive TLC spots were eluted in methanol and its UV absorption spectra was measured using a UV-Visible spectrophotometer (UV-1800 Shimadzu) to determine the λ_{max} of the spot. The FTIR spectra were recorded on a Shimadzu FT-IR spectrophotometer scanned at 400 to 4600 cm^{-1} using a KBr pellet.

4.2.11 Purification of crude antibiotic compounds

The bioactive TLC spots when subjected to LC-MS (Agilent technologies, USA) showed presence of multiple or mixture of compounds which was difficult to interprete, indicating that it needed further purification.

Thus, inorder to further purify the compounds, CFCF was bulk produced from 5 L of culture broth. The CFCF was then extracted with ethyl acetate in a ratio of 1:1 (v/v) and the organic layer was concentrated on a rotary evaporator (Heidolf, Germany) at 30 °C. The residue (9.9565 g) dry weight was dissolved in methanol to extract all methanol soluble compounds (polar compounds) labeled as (EAM) and subjected to column chromatography on Sephadex LH-20 (Methanol:Chloroform; 1:1v/v). The fractions thus obtained as EAM-1 - EAM-4 **(Figure 4.3a)** were collected and concentrated on a rotary evaporator at 30 °C and weighed. The ninhydrin positive and bioactive fraction EAM-3 was further subjected to column chromatography on Sephadex LH-20 (Methanol; v/v) of which nine fractions namely EAM (S) 1 - EAM (S) 9 were obtained **(Figure 4.3b&c)**. The fraction EAM (S) 4 and EAM (S) 5 were combined on the basis of TLC similarity to give fraction EAM (SC) 2. The bioactive fraction EAM (SC) 2 was further chromatographed on silica gel

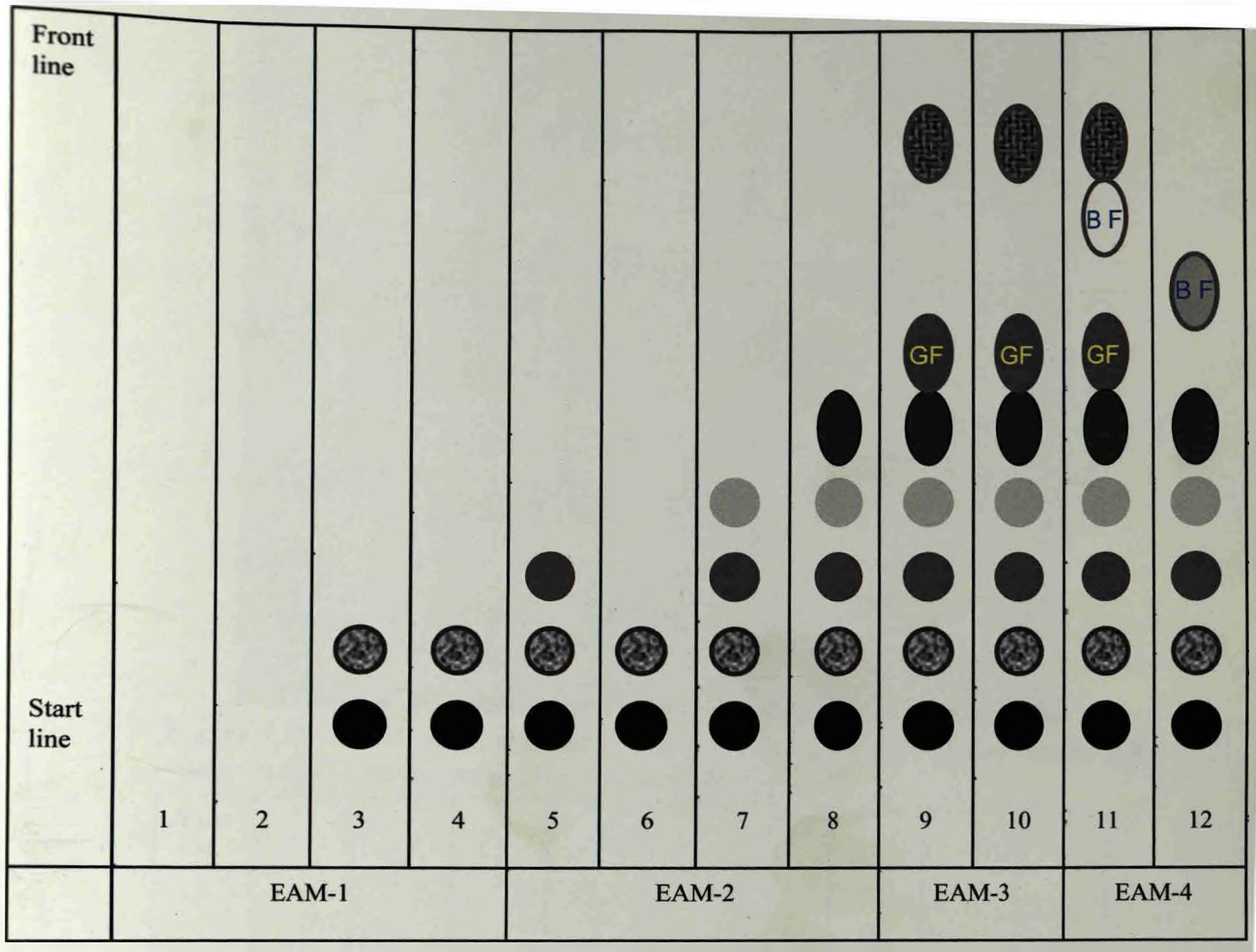


Figure 4.3a A representative diagram of a chromatogram of ethyl acetate extracted methanolic fraction (EAM) (Sephadex LH 20 column , 1: 1 v/v Methanol: Chloroform fraction) run in Butanol: Acetic acid: Water (BAW) and visualized in UV and with ninhydrin. All spots are ninhydrin active spots with BF representing blue fluorescence and GF, green fluorescence under UV

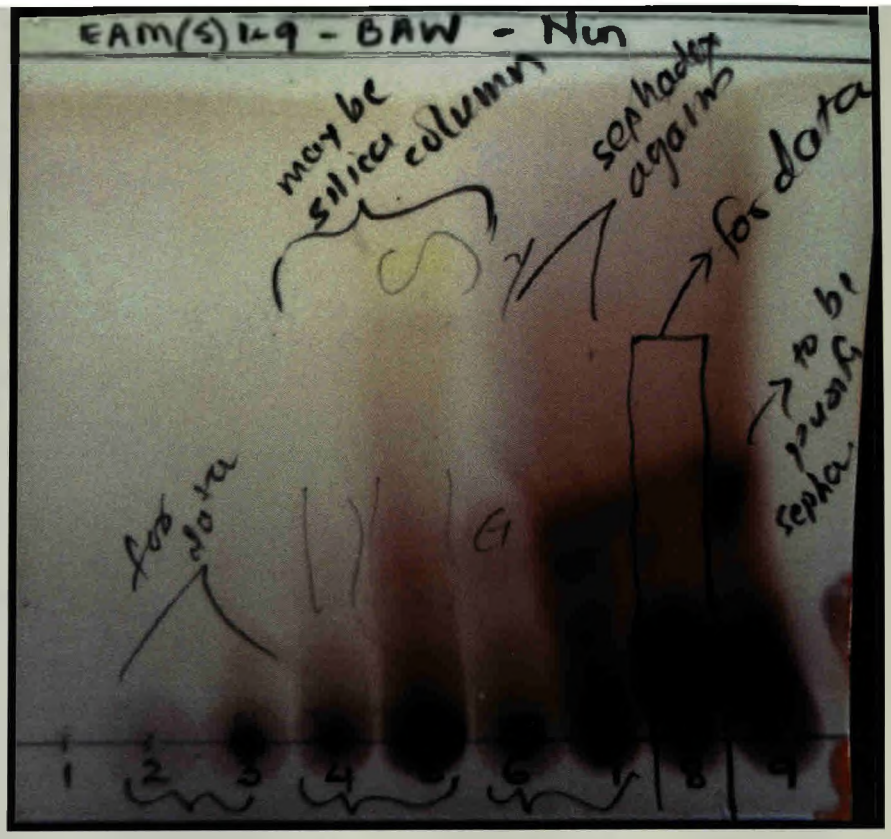


Figure 4.3b Chromatogram of EAM (S)-1-9 fraction run in Butanol: Acetic acid: Water (BAW) and visualized in UV and with ninhydrin

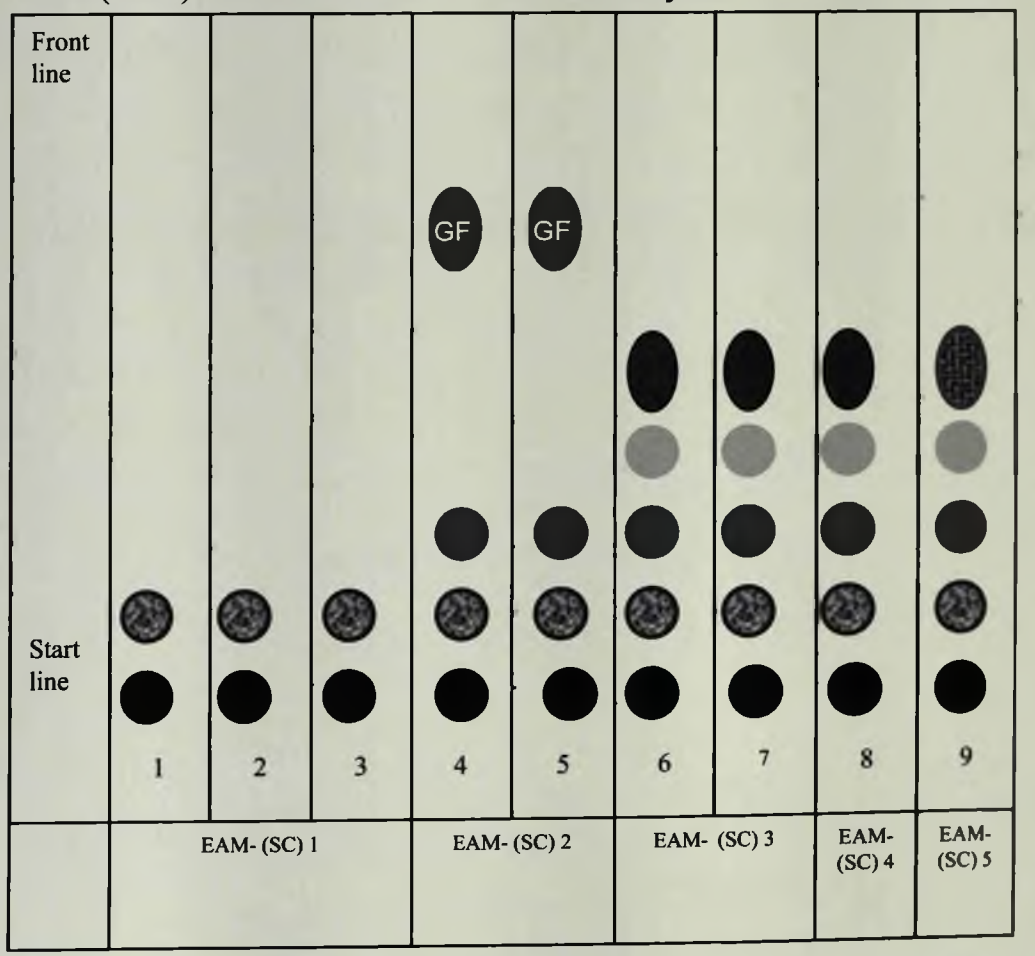


Figure 4.3cA representative diagram of chromatogram of EAM (S)-1-9 fraction run in Butanol: Acetic acid: Water (BAW) and visualized in UV and with ninhydrin. All spots are ninhydrin active spots with GF representing Green fluorescence under UV

column (60-120 mesh) using a gradient of Methanol:Chloroform solvent system to get seven fractions namely EAM (SG)1 - EAM (SG) 7 **(Figure 4.3d&e)**. Out of these, ninhydrin positive and bioactive fraction EAM (SG) 5 was subjected to MS/MS analysis. All the above fractions were individually chromatographed on silica gel aluminium sheets (Merck; 20 x 20 cm^2; Butanol : Acetic acid : Water 4 : 1 : 5, v/v) and the spots were visualized under UV at 254 and 365 nm and by chemical reagents such as sulphuric acid and ninhydrin. The fractions showing positive ninhydrin test were selected for further analysis. Detailed representation of steps used in purifying the compound is given in the flowchart below:

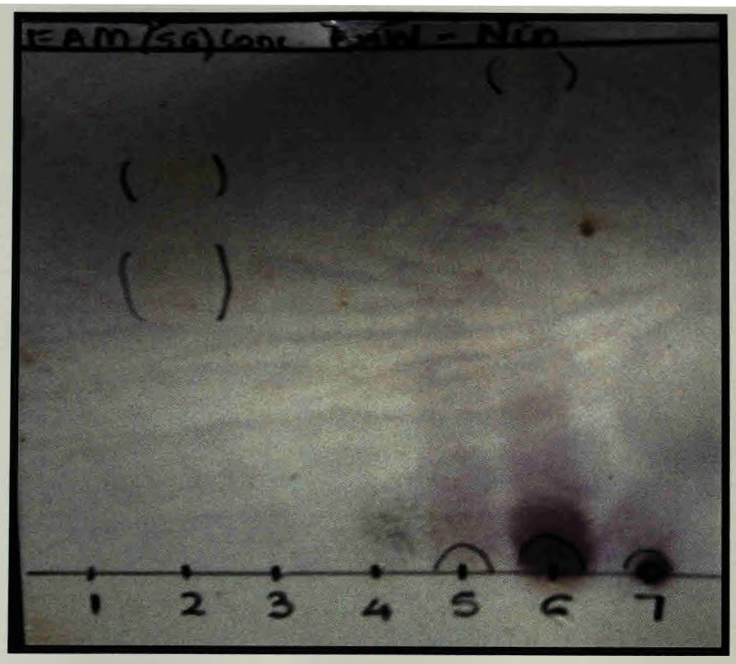


Figure 4.3d Chromatogram of the EAM (SG)-1-7 fraction run in Butanol: Acetic acid: Water (BAW) and visualized in UV and with ninhydrin. All spots are ninhydrin active spots with GF representing green fluorescence under UV and the brown spot (BS) was visible with the naked eye

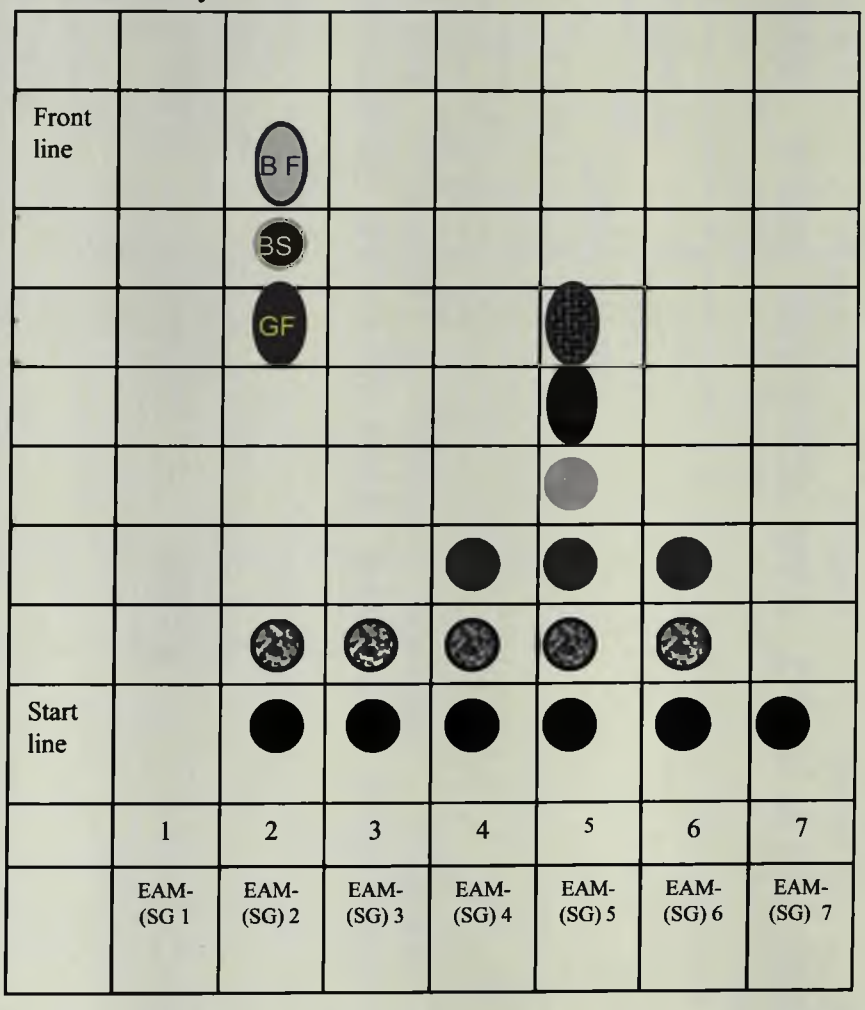
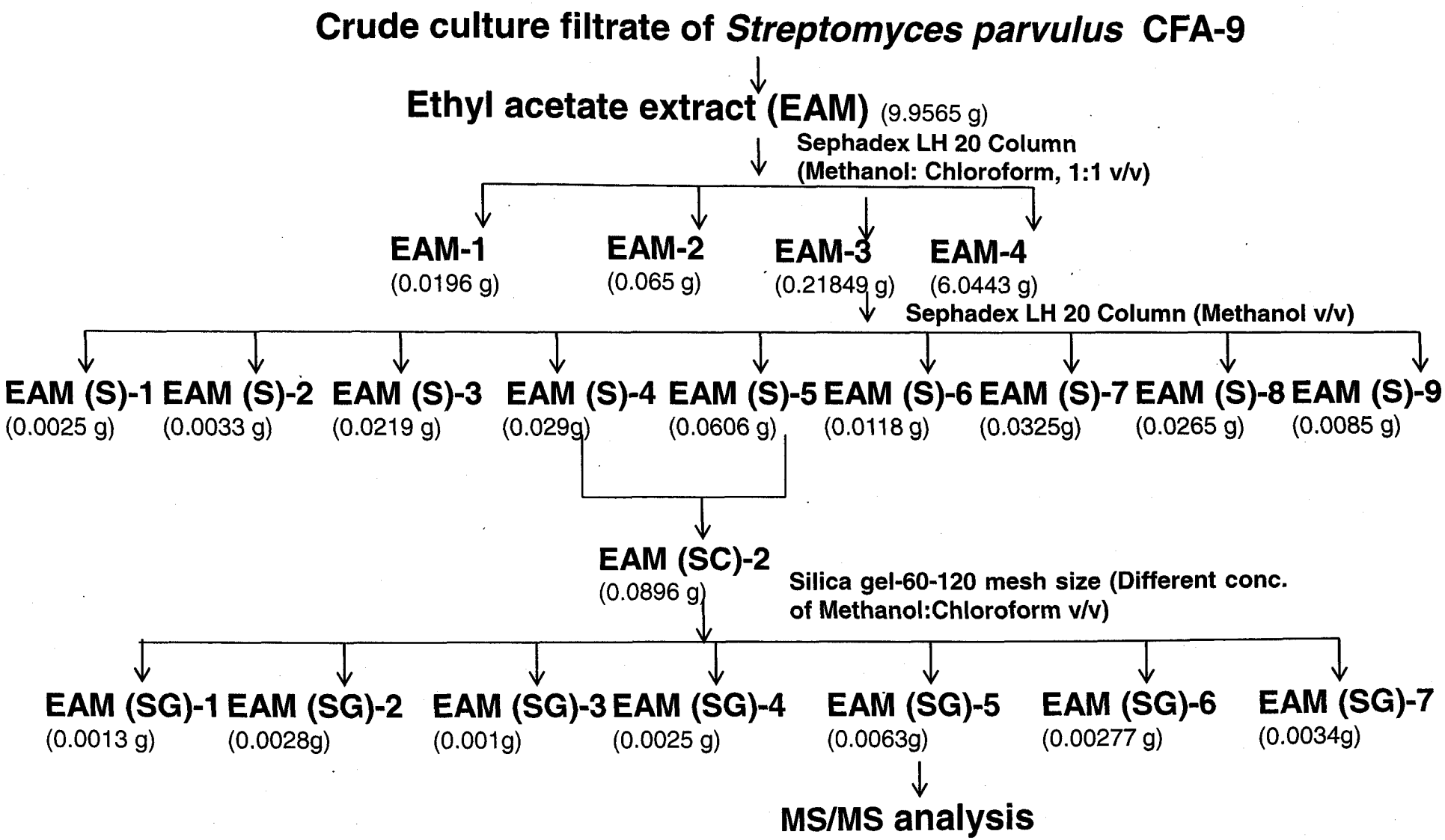


Figure 4.3e A representative diagram of chromatogram of the EAM (SG)-1-7 fraction run in Butanol: Acetic acid: Water (BAW) and visualized in UV and with ninhydrin. All spots are ninhydrin active spots with GF representing green fluorescence under UV and the brown spot (BS) was visible with the naked eye



A representative flowchart showing all the purification steps

4.2.12 Chemical characterization of partially purified antibiotic

This part of the work was carried out at Division of chemical Oceanography, National Institute of Oceanography, Goa. The partially purified fraction EAM (SG) 5 was in trace amounts as a result further NMR analysis could not be recorded for final confirmation and determination of stereochemistry of the compound. However, the compound was subjected to ESI-MS/MS (Electrospray Ionization Mass Spectrometry) analysis and its structure was elucidated. The mass spectrophotometer used was a QTOF-XL MS/MS Applied Biosystem instrument equipped with the MDS Sciex Analyst software (Concord, Ontario, Canada). The sample was dissolved in 1:1 MeOH: 0.1 % TFA and was directly infused at a constant flow rate of 10 µl/min into the ion spray source using an integrated syringe pump. The instrument was operated in positive ionization mode with the following settings: nebulizer gas (N 2) 28 (arbitrary units); curtain gas (N 2) 18 (arbitrary units); ion spray voltage 5700 V; declustering potential (DP) 120 V; focusing potential (FP) 365 V; declustering potential (DP2) 14 V and collision gas (CAD) 3 (arbitrary units). Full-scan data acquisition was performed, scanning from m/z 100 to m/z 2000 in the profile mode and using a cycle time of 1 s. A collision energy (CE) of 40 V and mass analysis was carried out using the TOF analyzer of the instrument.

Using Streptom dp (Lucas *et al.*, 2013) the predicted molecular formular of the compound was determined.

4.3 RESULTS

4.3.1 Preliminary screening of pigmented strains for antimicrobial activity

Out of 13 pigmented cultures tested for antimicrobial activity, four cultures showed positive activity against six test organisms as shown in **Table 4.4** and **Figure 4.4a**. *Streptomyces parvulus* CFA-9 **(Figure 4.4b)** showed positive activity against three test organisms with zone of inhibition given in parenthesis; *E. aerogens* (27 mm), *S. aureus* (26 mm) and *S. citreus* (25 mm). *Streptomyces* sp. MAS-11 showed positive activity against two test organisms *E. aerogens* (4 mm) and *S. aureus* (5mm). Unidentified actinomycetes MMA-2, showed positive activity against three test organisms *Candida albicans* (20 mm), *Aspergillus niger* (10 mm) and *B. subtilis* (11 mm). Unidentified actinomycetes TMK-34, showed positive activity against *Candida albicans* (44 mm).

Table 4.4 Antimicrobial activity of the pigmented actinomycetes strains

Actinomycetes strains	Test organisms [Zone of inhibition (mm)]						
	Enterobacter aerogens	*Escherichia coli*	*Bacillus subtilis*	*Staphylococcus aureus*	*Staphylococcus citreus*	*Candida albicans*	*Aspergillus niger*
Streptomyces sp. KFA-1	-	-	-	-	-	-	-
Streptomyces parvulus CFA-9	+ (27)	-	-	+ (26)	+ (25)	-	-
Unidentified actinomycetes TMK-34	-	-	-	-	-	+ (44)	-
Micromonospora sp. BSK-3	-	-	-	-	-	-	-
Micromonospora sp. BSK-15	-	-	-	-	-	-	-
Streptomyces sp. MAS-11	+ (4)	-	-	+ (5)	-	-	-
Streptomyces sp. MAS-12	-	-	-	-	-	-	-
Streptomyces sp. MAS-27	-	-	-	-	-	-	-
Streptomyces sp. MAS-31	-	-	-	-	-	-	-
Streptomyces sp. MAS-33	-	-	-	-	-	-	-
Streptomyces sp. MAS-35	-	-	-	-	-	-	-
Actinomadura sp. FCP-4	-	-	-	-	-	-	-
Unidentified actinomycetes MMA-2	-	-	+ (11)	-	-	+ (20)	+ (11)

Legend: (+) inhibition, (-) no inhibition

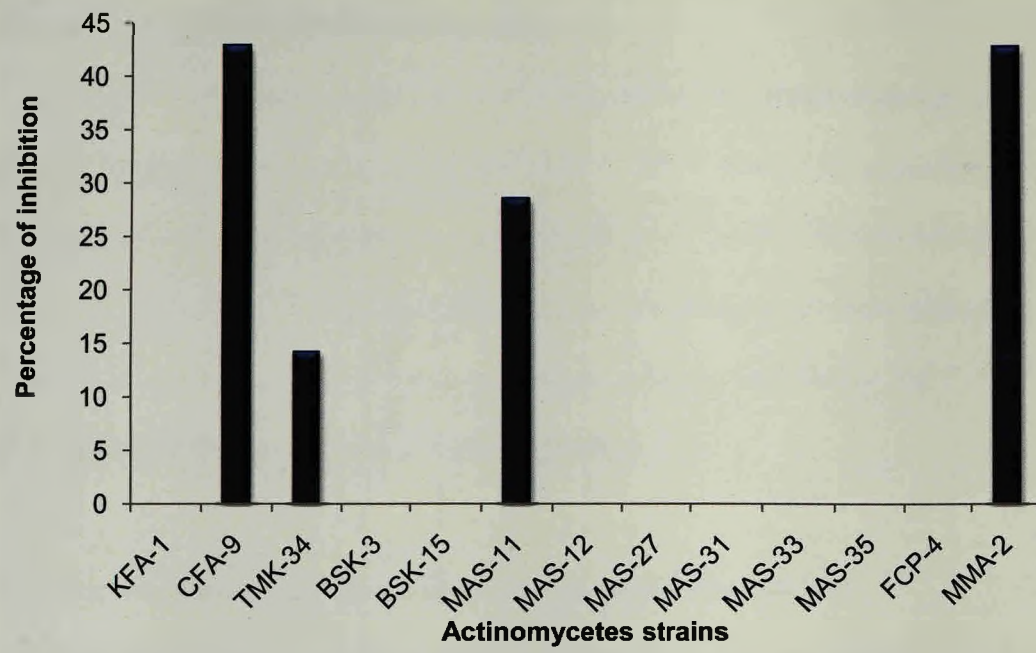


Figure 4.4a Average percentage inhibition of selected strains against test organisms on primary screening

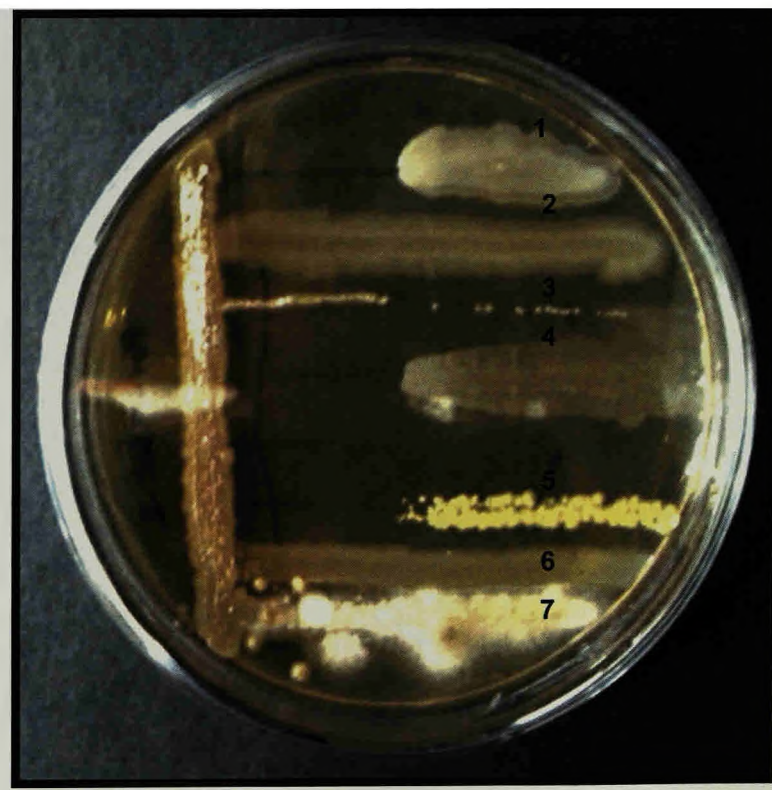


Figure 4.4b *Streptomyces* strain CFA-9 showing positive activity against *E. aerogens* (27 mm), *S. aureus* (26 mm) and *S. citreus* (25 mm) where 1, *Enterobacter aerogens*; 2, *E. coli*; 3, *Bacillus subtilis*; 4, *S. aureus*; 5, *S. citreus*; 6, *Candida albicans*; 7, *Aspergillus niger*

4.3.2 Selected strains for antibiotic screening

Based on the spore morphology and mycelium, the isolated strains were classified into various genera (Cross & Goodfellow, 1973). Out of 30 actinomycetes isolates, 16 isolates were identified as genus *Streptomyces* (spore chain with coiling, spiral and looped), 3 as *Actinomadura* (spore chains are straight and open hooked), 4 as *Micromonospora* (clusters of single conidia on substrate mycelium) and 7 were unidentified actinomycetes strains as shown in **Table 4.2**.

4.3.2.1 *Antimicrobial activity by modified cross-streak method (MCSM)*

Out of 30 cultures screened for antimicrobial activity, 28 cultures showed positive activity i.e 93 % of actinomycetes isolates associated with soil showed the ability to produce antimicrobial compounds against eight test pathogens. Forty percent of the strains inhibited *S. typhi*, 37 % strains inhibited *E. aerogens*, 77 % strains inhibited *S. aureus*, 57 % strains inhibited *B. subtilis* whereas 47 % strains inhibited *S. flexneri* and *S. marcescens* respectively and 60 % of the strains inhibited *Proteus* sp. and *C. albicans* respectively.

4.3.2.2 *Quantitative antibiotic activity score for better interpretation of results*

Streptomyces sp. MMA-5 showed high PASDAAS against *S. marcescens* (87), *Salmonella typhi* (80), *S. aureus* (80), *B. subtilis* (80), and *C. albicans* (80) while *Streptomyces* sp. MAS-11 showed high PASDAAS against *S. marcescens* (87) and *S. flexneri* (80) as shown in **Table 4.5**. *Streptomyces* sp. SIA-2 and *Streptomyces* sp. MAS-30 were found to be active against *B. subtilis* (80) and *C. albicans* (80) respectively **(Table 4.5)**. Unidentified actinomycetes KFA-1Gb was found active against *S. flexneri* (80) and *C. albicans* (80). *Streptomyces* sp. KFA-5 was found

active against *S. aureus* (80). *Streptomyces* sp. strain KFA-1 was found to be active against *S. flexneri* (80) where as *Micromonospora* sp. KFA-9 and *Micromonospora* sp. MMA-8 was found to be active against *S. flexneri* (80) as shown in **Table 4.5**. *Streptomyces* sp. MMA-5 showed the highest PMSAES (59) value **(Figure 4.5a)**. Broad antibiotic spectrum activity against eight test pathogens was exhibited by *Streptomyces* sp. MMA-2 and *Micromonospora* sp. MMA-8 **(Table 4.5)**. The highest POSES was exhibited against *S. aureus* followed by *Proteus* sp. and *C. albicans* **(Figure 4.5b)**.

Table 4.5 PASDASS and POIES for each actinomycetes strain

Actinomycetes strain	TPI	TP2	TP3	TP4	TP5	TP6	TP7	TP8	POIES
Streptomyces sp. KFA-5	20	0	47	0	0	**80**	73	73	63
Unidentified actinomycetes KFA-8	0	0	60	0	0	0	0	0	13
Micromonospora sp. KFA-9	0	**80**	60	0	67	53	60	0	63
Micromonospora sp. KFA-3	13	73	73	0	20	67	0	0	63
Streptomyces sp. KFA-6	0	0	0	0	0	0	0	0	0
Unidentified actinomycetes KFA-1Gb	0	**80**	0	27	20	60	0	**80**	63
Streptomyces sp. KFA-1	0	**80**	67	0	0	67	20	53	63
Streptomyces sp. CFA-1	0	0	0	0	0	60	0	0	13
Unidentified actinomycetes CFA-2	0	53	73	73	60	60	40	0	75
Unidentified actinomycetes CFA-3	0	27	47	0	0	0	53	53	50
Streptomyces sp. CFA-4	0	73	0	60	0	73	0	0	38
Actinomadura sp. TMK-33	0	60	0	0	0	53	67	27	50
Streptomyces sp. MAS-2	0	0	40	20	27	60	60	67	75
Actinomadura sp. MAS-4	27	0	0	20	0	0	0	73	38
Streptomyces sp. MAS-11	0	**80**	0	0	**87**	20	0	0	38
Streptomyces sp. MAS-14	0	0	0	0	0	0	0	0	0
Streptomyces sp. MAS-28	7	0	0	0	0	0	0	13	25
Streptomyces sp. MAS-30	20	0	40	60	67	67	**80**	**80**	88
Streptomyces sp. MMA-18	60	67	53	40	40	27	47	53	**100**
Micromonospora sp. MMA-4	20	53	40	0	20	20	40	13	88
Micromonospora sp. MMA-8	73	**80**	40	53	20	67	60	60	**100**
Actinomadura sp. MMA-9	0	40	73	0	0	53	40	73	63
Streptomyces sp. MMA-2	0	0	0	27	0	40	53	53	50
Streptomyces sp. MMA-5	**80**	0	67	0	**87**	**80**	**80**	**80**	75
Streptomyces rubrolavendulae PM-7	0	7	67	0	40	73	0	0	50
Unidentified actinomycetes BOA-10	0	0	0	0	0	20	0	0	13
Streptomyces sp. SIA-2	0	0	27	53	0	67	**80**	**80**	63
Streptomyces sp. SIA-2G	40	0	40	0	60	67	47	0	63
Unidentified actinomycetes SIA-2Gb	7	0	20	0	40	0	0	53	50
Unidentified actinomycetes SIA-12	20	0	0	27	0	73	73	73	63

Legend: TP1, *Salmonella typhi*; TP2, *Shigella flexneri*; TP3, *Proteus* sp.; TP4, *Enterobacter aerogens*; TP5, *Serratia marcescens*; TP6, *Staphylococcus citreus*; TP7, *Bacillus subtilis* and TP8, *Candida albicans*

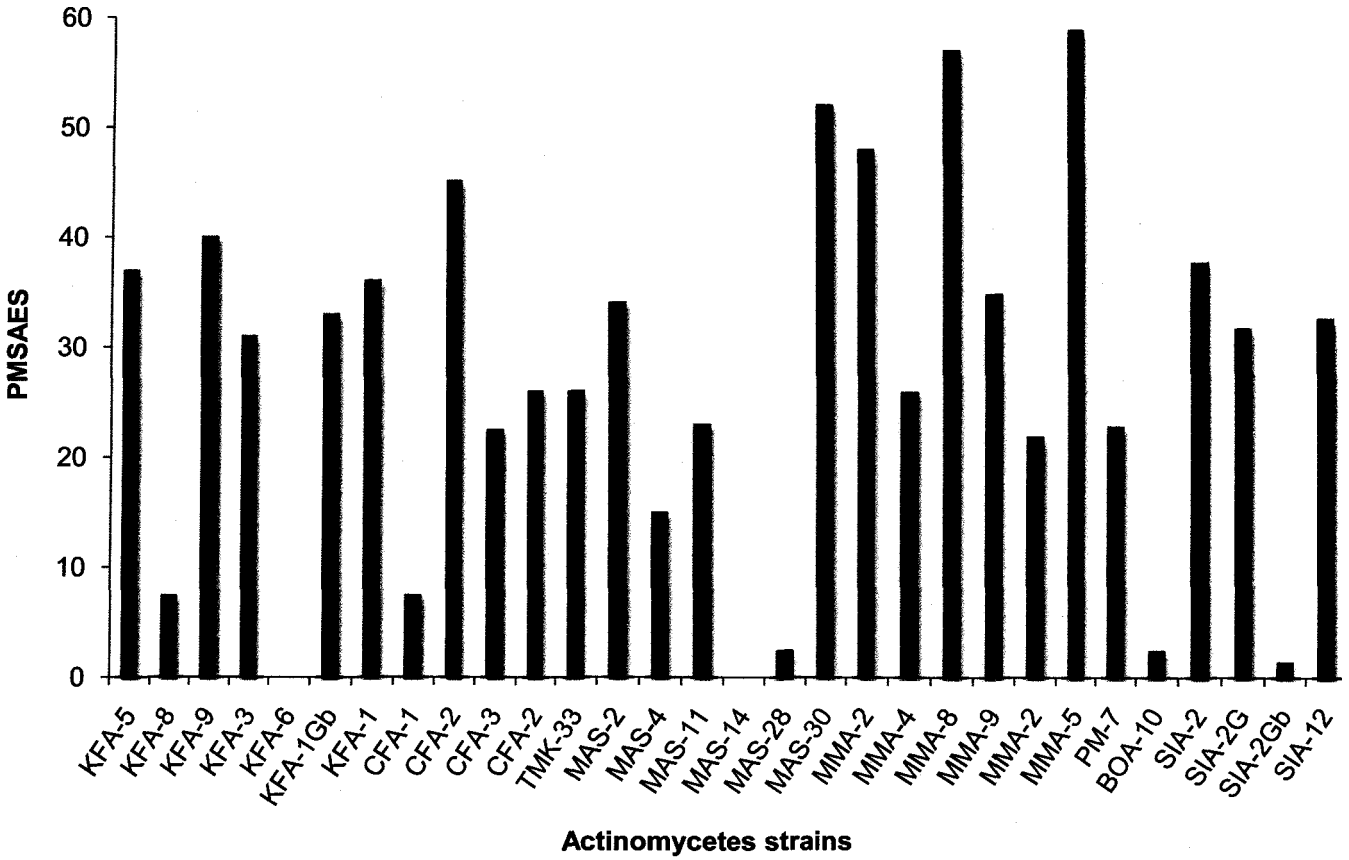


Figure 4.5a Percent multispecific antibiosis efficiency score exhibited by the actinomycetes strains (PMSAES)

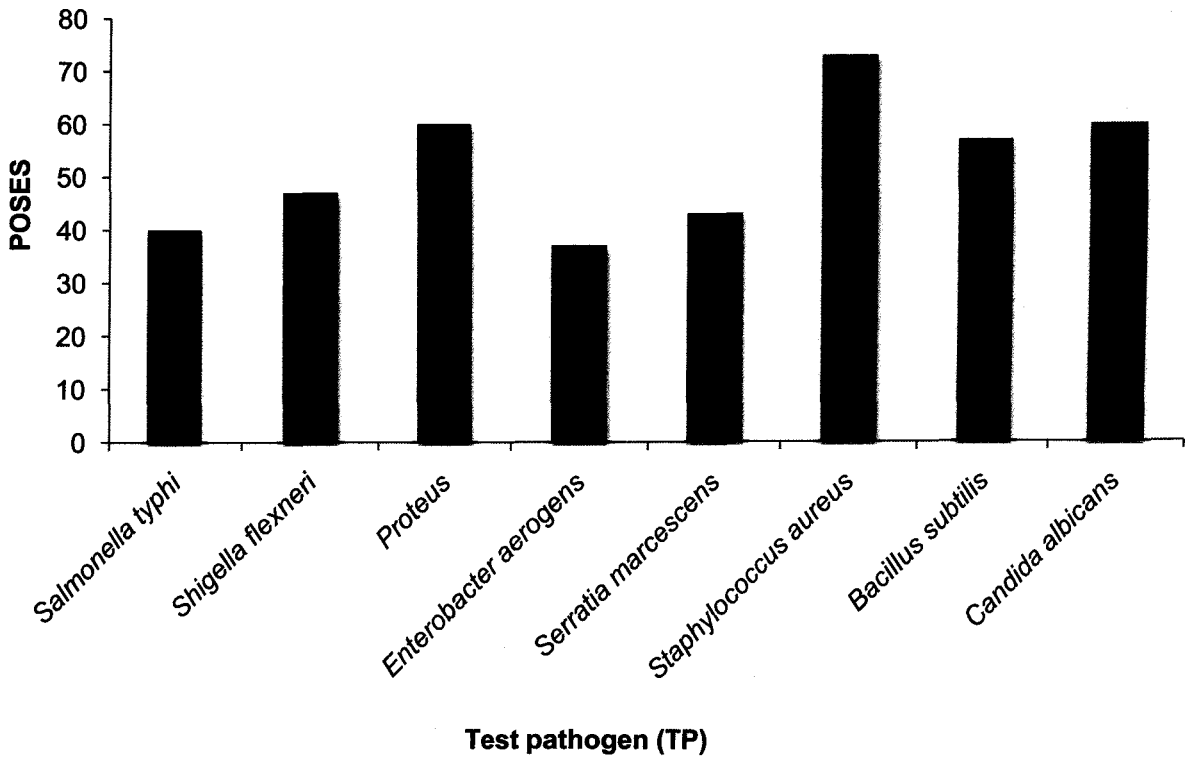


Figure 4.5b Percentage Overall Screening Efficiency Score (POSES) exhibited by the actinomycetes strains

4.3.3 Detection of Calcium Oxalate Crystals

Calcium Oxalate Crystals (COC's) were detected on MHA positive plates for antibiosis. The cumulative counts of the crystals are tabulated in **Table 4.6.**

Table 4.6 The cumulative count of Calcium Oxalate Crystals (COC's)

Actinomycetes strains	**COC's**								
	AS	**TP1**	**TP2**	**TP3**	**TP4**	**TP5**	**TP6**	**TP7**	**TP8**
Streptomyces sp. KFA-5	42	0	0	2	0	100	21	70	2
Unidentified actinomycetes KFA-8	1	3	0	0	0	4	1	0	1
Micromonospora sp. KFA-9	120	27	4	36	6	12	5	8	6
Micromonospora sp. KFA-3	0	0	1	0	0	1	2	4	1
Streptomyces sp. KFA-6	0	0	0	0	0	6	0	0	0
Unidentified actinomycetes KFA-1Gb	5	7	7	2	4	8	3	7	9
Streptomyces sp. KFA-1	3	1	2	1	3	2	1	1	11
Streptomyces sp. CFA-1	4	0	0	0	3	5	1	1	0
Unidentified actinomycetes CFA-2	120	0	3	4	3	15	4	14	27
Unidentified actinomycetes CFA-3	2	0	1	2	1	10	11	3	4
Streptomyces sp. CFA-4	0	1	1	2	7	1	1	0	0
Actinomadura sp. TMK-33	3	0	4	5	1	7	12	7	2
Streptomyces sp. MAS-2	3	2	2	3	5	4	7	4	3
Actinomadura sp. MAS-4	8	1	0	0	1	9	2	11	19
Streptomyces sp. MAS-11	2	0	0	1	7	4	7	5	2
Streptomyces sp. MAS-14	8	0	64	0	5	200	25	14	2
Streptomyces sp. MAS-28	40	1	0	6	4	17	2	5	5
Streptomyces sp. MAS-30	9	1	9	5	11	5	6	8	3
Streptomyces sp. MMA-18	25	1	0	3	8	11	16	10	5
Micromonospora sp. MMA-4	13	3	1	1	1	0	2	4	1
Micromonospora sp. MMA-8	19	7	0	0	0	2	8	7	8
Actinomadura sp. MMA-9	21	4	2	2	0	7	12	4	5
Streptomyces sp. MMA-2	26	7	1	1	1	23	1	0	9
Streptomyces sp. MMA-5	26	7	6	4	5	0	3	9	9
Streptomyces rubrolavendulae PM-7	40	6	10	7	8	4	8	10	6
Unidentified actinomycetes	37	15	0	2	0	6	2	8	5

BOA-10									
Streptomyces sp. SIA-2	80	10	0	1	20	17	5	4	100
Streptomyces sp. SIA-2G	12	15	0	1	5	2	3	2	1
Unidentified actinomycetes SIA-2Gb	2	5	5	4	8	6	6	2	8
Unidentified actinomycetes SIA-12	1	4	0	0	0	2	4	0	2

Legend: TP1, *Salmonella typhi*; TP2, *Shigella flexneri*; TP3, *Proteus*; TP4, *Enterobacter aerogens*; TP5, *Serratia marcescens*; TP6, *Staphylococcus aureus*; TP7, *Bacillus subtilis*; TP8, *Candida albicans*; AS, Actinomycetes strain

4.3.3.1 *Confirming the presence of COC's*

The test plates **(Plate 4.1a)** showed presence of COC's of different shapes surrounding all the wells. Around 100 % crystals were seen surrounding the well containing TP-7. Other TP also radiated flux of crystals mostly florets. Flux of crystals was also radiated from the central well containing actinomycetes culture. These were seen partially submerged and were less in number in comparison to flux radiated from TP-7.

Control plate 1 **(Plate 4.1b)** - showed flux of fine microcrystalline which were rhomboidal, small square like COC's radiating around the central well filled with *Streptomyces* sp. MAS-11 culture. The crystals were partially submerged. No crystals were formed surrounding the 8 wells filled with 0.01M $CaCl_2.2H_2O$ and corresponding 8 wells filled with sterile distilled water. On the basis of this result it could be inferred that the *Streptomyces* sp. MAS-11 culture has the potential of producing COC's termed as Calcium Oxalate Actinoliths (COAL's) on MHA medium and this does not get triggered in the presence of the test pathogens. No significant lines of precipitation were observed between actinomycetes culture and the surrounding wells filled with sterile 0.01M $CaCl_2.2H_2O$

Control plate 2 (**Plate 4.1c**) - showed no crystal formation surrounding the central well but crystals were formed surrounding the test pathogens. The crystals were mostly druzes. The well containing TP-7 showed presence of different types of crystals shapes other than florets such as sharp edged, pointed and rectangular. Crystals were also seen formed inside the well. On the basis of this result it could be said that test pathogens on their own have the potential of producing various calcium oxalate crystals. These crystals could be coined as 'Bacterioliths' and they are very different in appearance when compared to COAL's.

Control plate 3 (**Plate 4.1d**) - showed flux of crystals radiating from the central well and the wells containing the test pathogens in the similar manner as in control 1 and control 2. On basis of this result it could be said that actinomycete strain as well as the test pathogens have on their own the ability of producing various COC's whose production gets triggered on MHA medium without the presence of 0.01M $CaCl_2.2H_2O$. The crystals were mostly druzes of different shapes and sizes (**Plate 4.2a-y**). Plain rhomboidal (**Plate 4.3ab-pq**) crystals were also observed.

PLATE 4.1

a-Test plate showing the central well, filled with inoculum of actinomycetes strain, surrounded by 8 wells of 0.01M $CaCl_2.2H_2O$ and corresponding 8 wells filled with inoculum of test pathogens

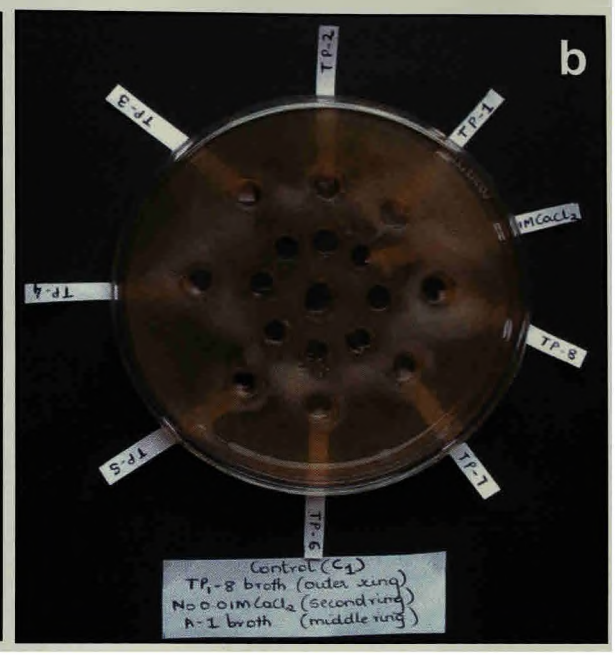
b-Control plate 1 showing the central well, filled with inoculum of actinomycetes strain , surrounded by 8 wells of 0.01M $CaCl_2.2H_2O$ and corresponding 8 wells filled with sterile distilled water

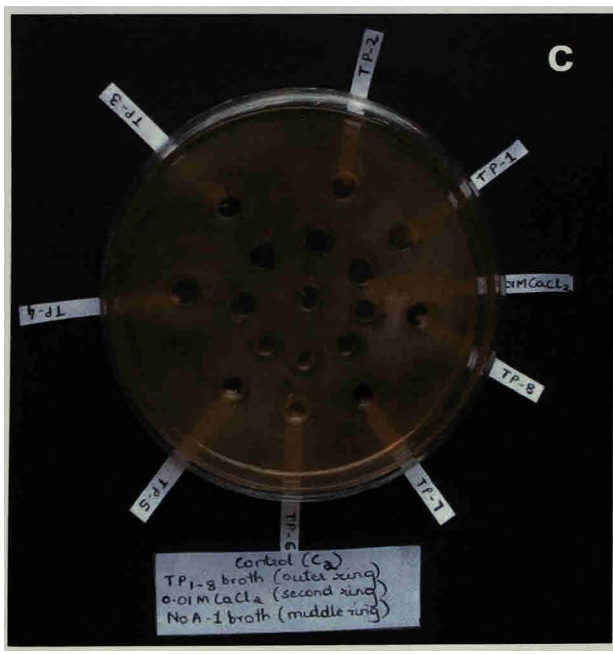
c-Control plate 2 showing the central well, with sterile distilled water, surrounded by 8 wells of 0.01M $CaCl_2.2H_2O$ and corresponding 8 wells filled with inoculum of test pathogens

d-Control plate 3 showing the central well, filled with inoculum of actinomycetes strain, surrounded by 8 wells of sterile distilled water and corresponding 8 wells filled inoculum of test pathogens

PLATE 4.1

a
TP-2
TP-1
A-1
TP-8
1M CaCl2
TP-7
TP-6
TP-5
TP-4
TP-3
Set-2
TP1-8 broth (outer ring)
0.01M CaCl2 (second ring)
A-1 broth (middle ring)


b
TP-2
TP-1
1M CaCl2
TP-8
TP-7
TP-6
TP-5
TP-4
TP-3
Control (C1)
TP1-8 broth (outer ring)
No 0.01M CaCl2 (second ring)
A-1 broth (middle ring)


c
TP-2
TP-1
1M CaCl2
TP-8
TP-7
TP-6
TP-5
TP-4
TP-3
Control (C2)
TP1-8 broth (outer ring)
0.01M CaCl2 (second ring)
No A-1 broth (middle ring)

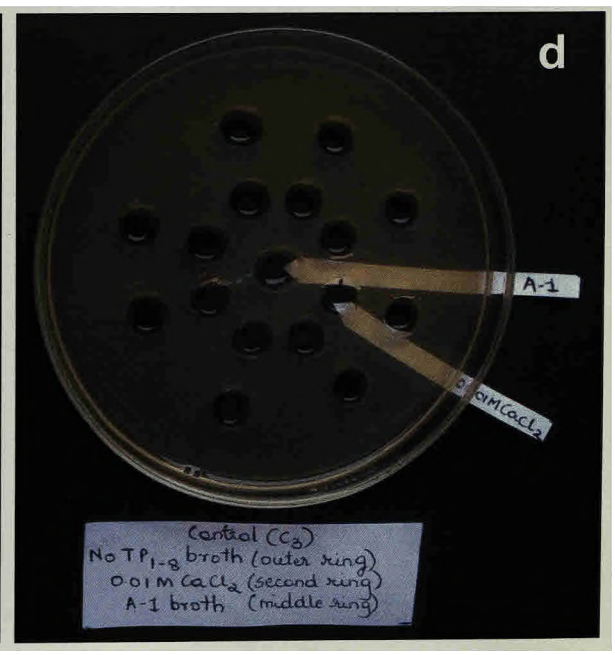

d
A-1
0.1M CaCl2
Control (C3)
No TP1-8 broth (outer ring)
0.01M CaCl2 (second ring)
A-1 broth (middle ring)

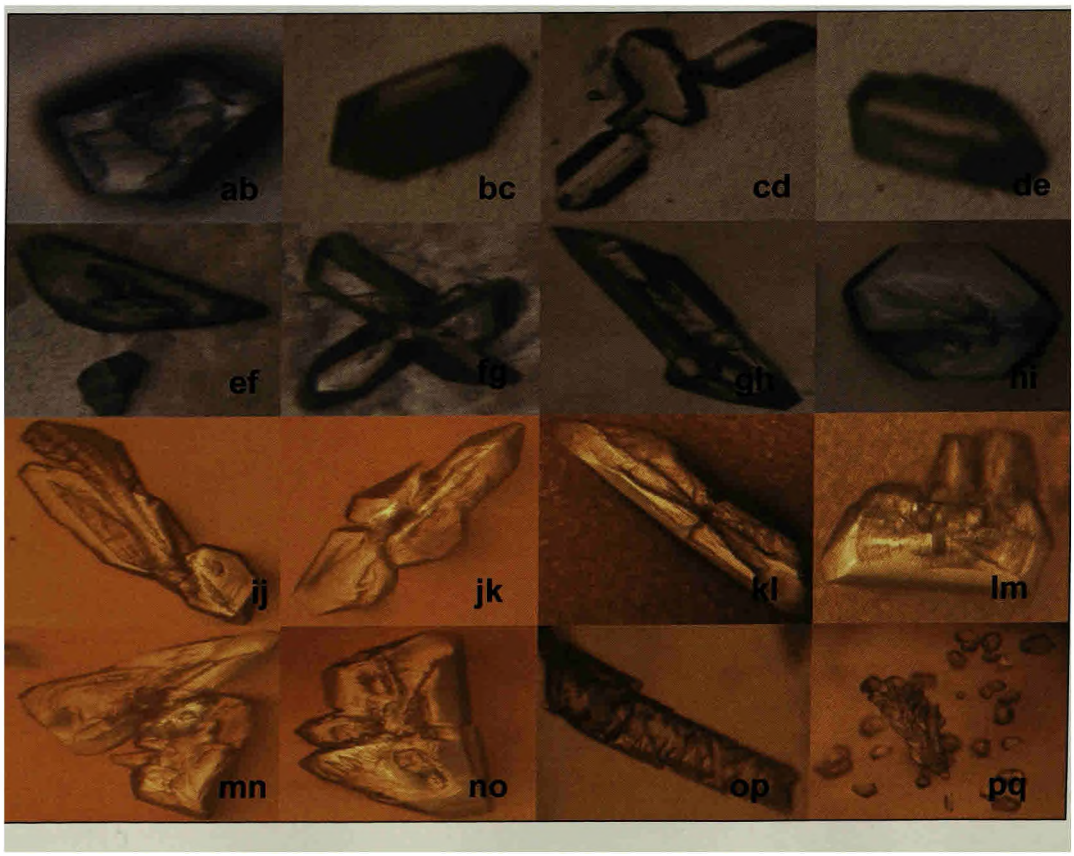
PLATE 4.2

a-y Calcium oxalate crystals in the shape of druzes. Magnification at 40 X

ab-pq Rhomboidal shaped calcium oxalate crystals

PLATE 4.2

a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r
s
t
u
v
w
x
y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4.3.4. Selection of a hyper producer antibiotic strain

Among all the strains tested for potential bioactivity; *Streptomyces* sp. CFA-9, with broad spectrum antimicrobial activity of 66.7 % against human pathogens namely Gram negative bacteria *S. flexneri*, *E. aerogens* and Gram positive bacteria *B. subtilis*, *S. typhi* and *S. citreus* was selected. Highest zone of inhibition (31 mm) was observed against *S. citreus*.

4.3.4.1 *Taxonomic and molecular identification*

The strain CFA-9 exhibited grey aerial mycelium, with spiral spore chains and produced a bright yellow pigment. Sporophores monopodially branched, with open, regular spirals (**Figure 4.6a-c**).

The strain was identified as *Streptomyces parvulus* (GenBank Accession Number: KC904376) based on nucleotide homology and phylogenetic analysis as shown in **Figure 4.6.**

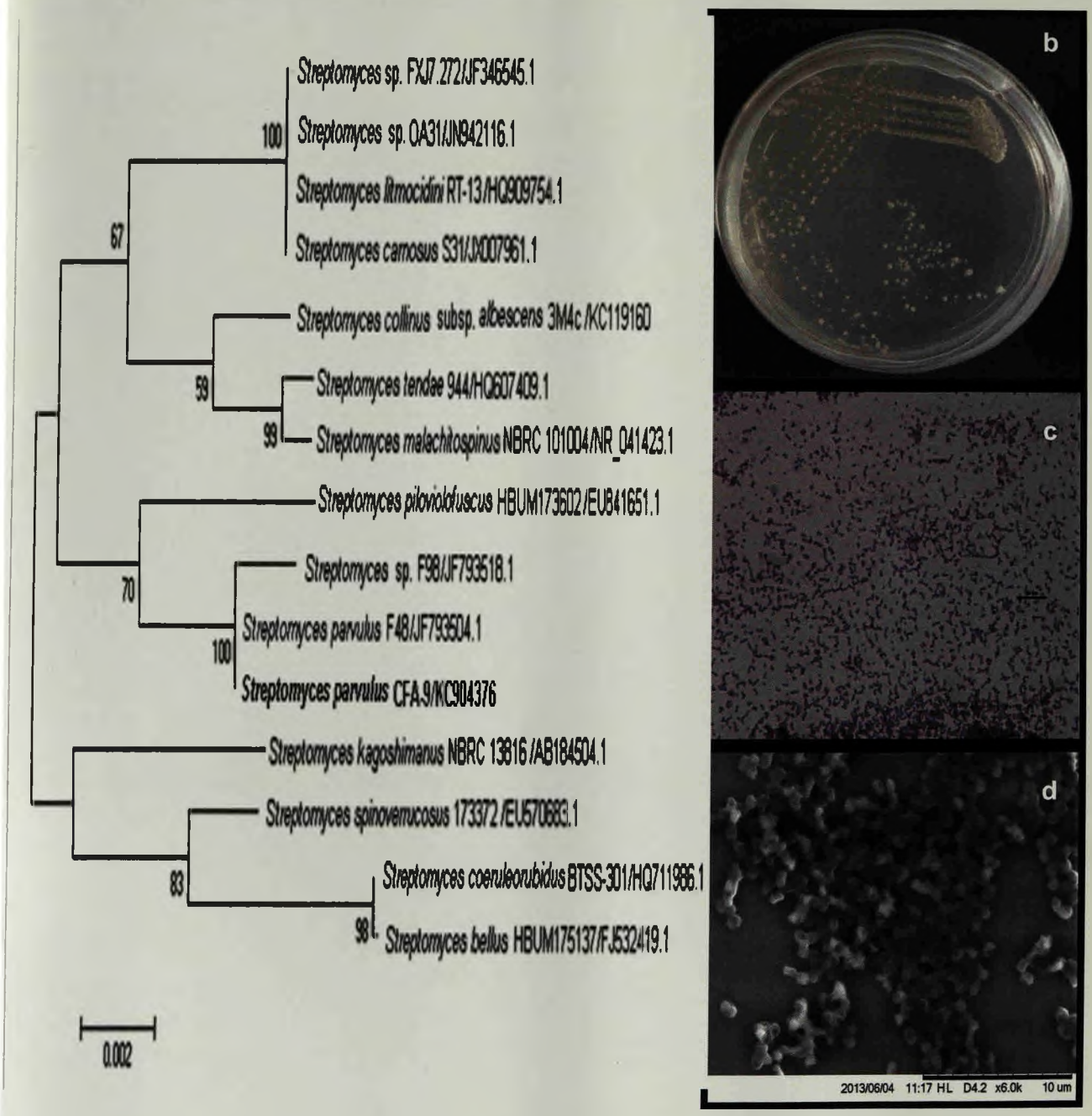


Figure 4.6a Maximum-likelihood tree based on 16S rRNA gene sequence showing the relations between antibiotic producer strain *Streptomyces parvulus* strain CFA-9 and type species of the genus *Streptomyces* within the order actinomycetales under actinobacteria. The numbers at the nodes indicate the levels of bootstrap support based on maximum-likelihood analyses of 1000 resampled data sets (only values >50% are shown). The scale bar indicates 0.002 substitutions per nucleotide positions, **b)** *Streptomyces parvulus* CFA-9 culture streaked on AVA medium, **c)** *Streptomyces parvulus* CFA-9 culture stained in crystal violet as seen under compound light microscope (Olympus BX41, Tokyo, Japan), **d)** SEM photographic image of *Streptomyces parvulus* CFA-9

4.3.5 Secondary screening

4.3.5.1 *Bioassay*

Streptomyces parvulus CFA-9 was found promising as it showed maximum zone of inhibition (22.5 mm, 19.5 mm, 19 mm) against *Enterobacter aerogens* **(Figure 4.7a&b)**, *Bacillus subtilis* **(Figure 4.8a&b)** and *S. typhi* **(Figure 4.9a&b)** on the 12th day respectively. Zone of inhibition of 31 mm and 17.5 mm was exhibited against *S. citreus* **(Figure 4.10a&b)** and *Shigella flexneri* **(Figure 4.11a&b)** on the 14th and 16th day respectively. The control (Kuster's broth devoid of culture) showed no activity.

4.3.5.2 *Spectroscopy measurments*

The UV-Spectral scan (190-700 nm) of the CFCF on the 4th , 12th , 16th and 20th day of incubation showed two peaks at 331 nm and 290 nm appearing consistently with an increase in absorbance (1.533 (331 λ) and 2.113 (293 λ))on the 18th day. The spectral data of day 14th showed absorbance maxima of 1.849 at λ 289.5 indicating the presence of peptide moiety/bonds. There was a gradual increase in absorbance at 280 nm from the 7th to the 14th day followed by gradual decline.

FTIR spectral analysis of the 14th day CFCF showed absorbance at 3428.55 cm^{-1} indicating an amino and hydroxyl group of amino acids, absorbance at 2916.7, 1056.4, 1113 and 1380.7 cm^{-1} indicated presence of carbon and hydrogen groups, aliphatic carbon and nitrogen groups and methyl group respectively.

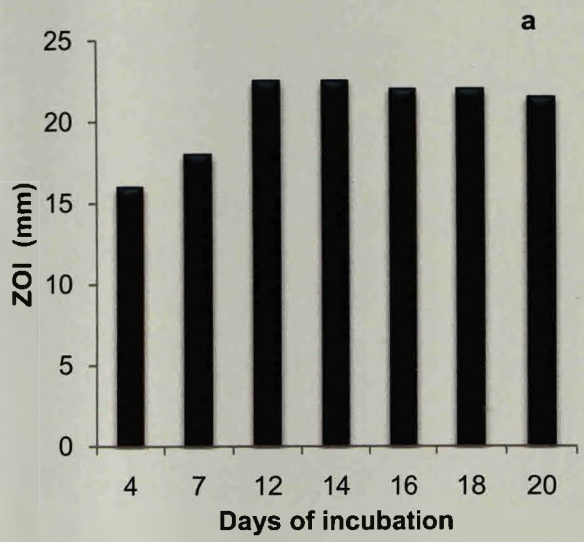
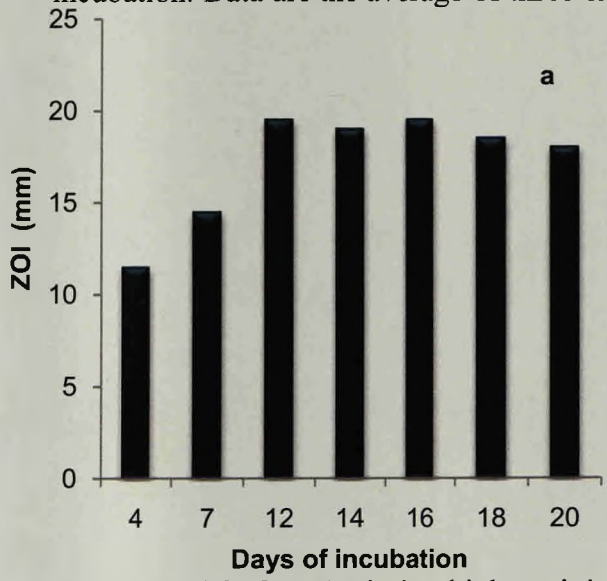


Figure 4.7a&b Antimicrobial activity against *E. aerogens* on varying days of incubation. Data are the average of three experiments



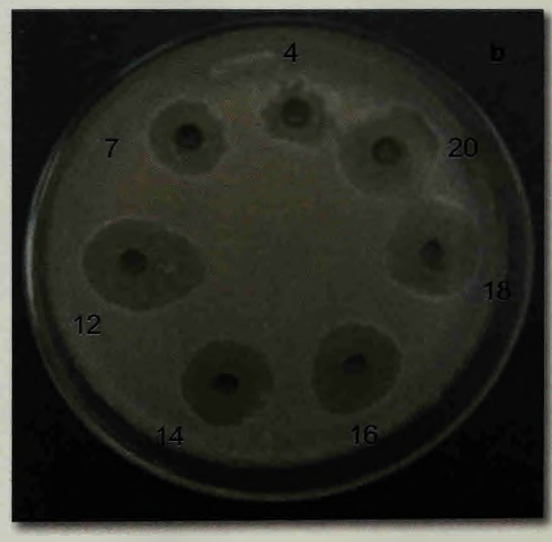
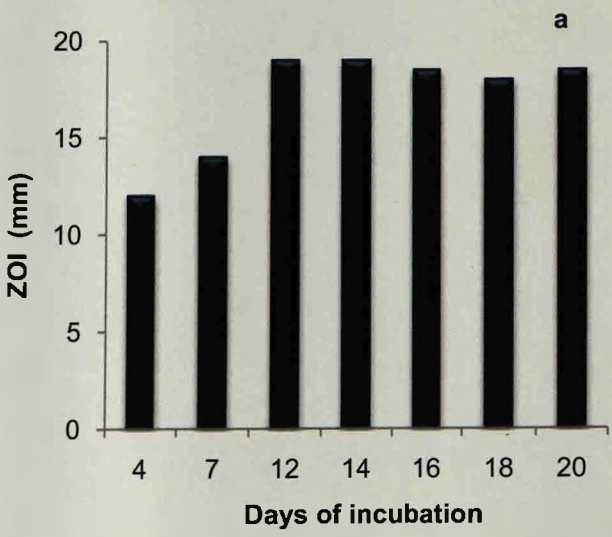


Figure 4.8a&b Antimicrobial activity against *B. subtilis* on varying days of incubation. Data are the average of three experiments



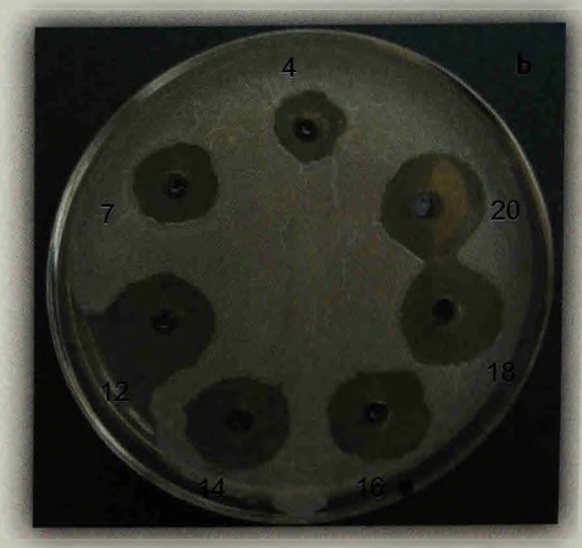
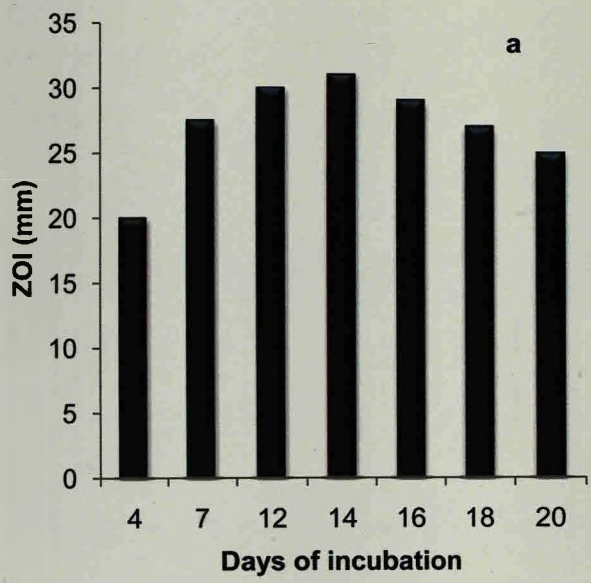


Figure 4.9a&b Antimicrobial activity against *S. typhi* on varying days of incubation. Data are the average of three experiments



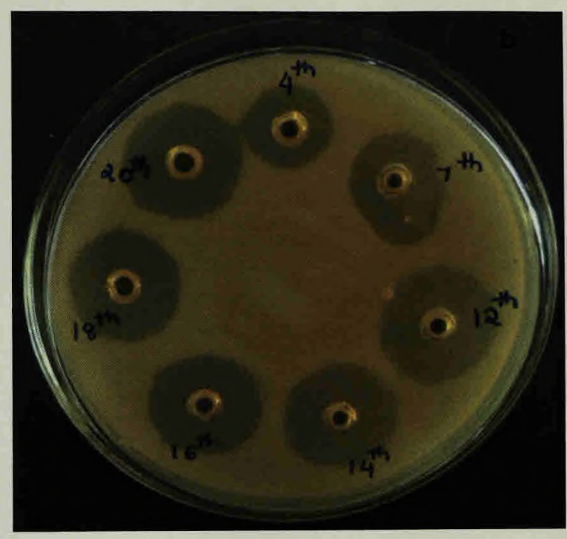
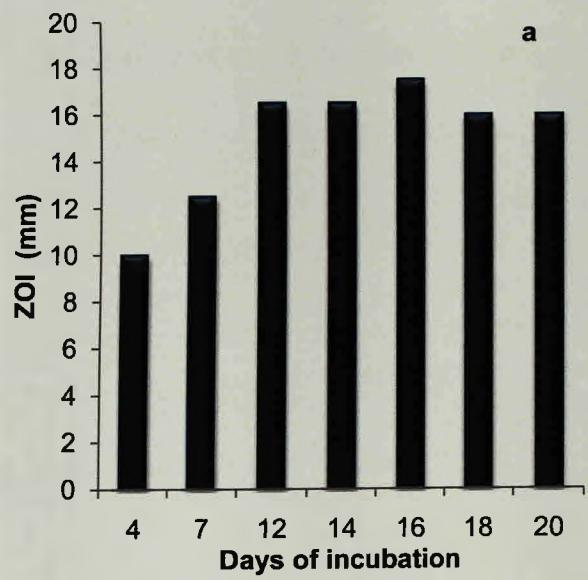


Figure 4.10a&b Antimicrobial activity against *S. citreus* on varying days of incubation. Data are the average of three experiments



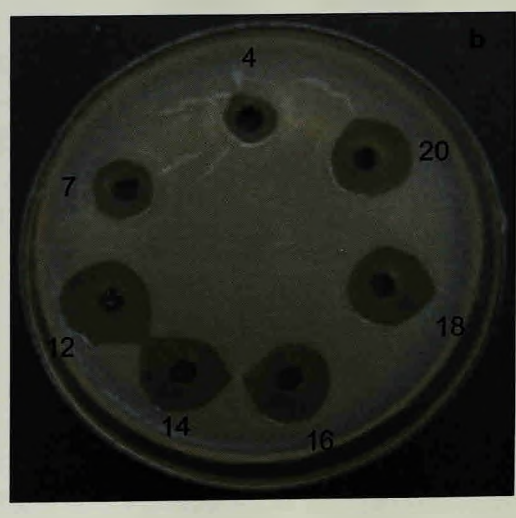


Figure 4.11a&b Antimicrobial activity against *Shigella flexneri* on varying days of incubation . Data are the average of three experiments

4.3.6 Impact of different process parameters on antibiotic production

4.3.6.1 *Physiological parameters*

Incubation period

The maximum antibiotic production resulting in a mean ZOI of 35 mm was recorded over a period of 14 days. The activity was observed from the 4th day of incubation and reached a maximum on the 14th day showing significant variation ($p \leq 0.05$). Thereafter, with the increase in incubation period, the antibiotic production decreased **(Figure 4.12)**.

Temperature

As seen in the **Figure 4.13**, incremental temperature rise led to an increase in the antibiotic production till it reached the optimum, further increase in temperature was accompanied by a decrease in the antibiotic production. Maximum yield of bioactive metabolites was observed when the strain was cultured at optimum temperature of 28 °C with a mean ZOI of 37 mm which was significant ($p \leq 0.05$). 25°C and 32 °C also showed an appreciable mean ZOI of 29 and 32 mm respectively. The lowest mean ZOI of 24 mm was observed at 37 °C .

pH

Maximum antibiotic activity occurred at pH 7.0, exhibiting mean ZOI of 37 mm which was significant ($p \leq 0.05$). No antibiotic production was observed at pH 3, 4 and 11. Increasing the pH value led to an increase in the antibiotic production up to certain threshold limit and further increase in values resulted in decrease in the antibiotic production **(Figure 4.14).**

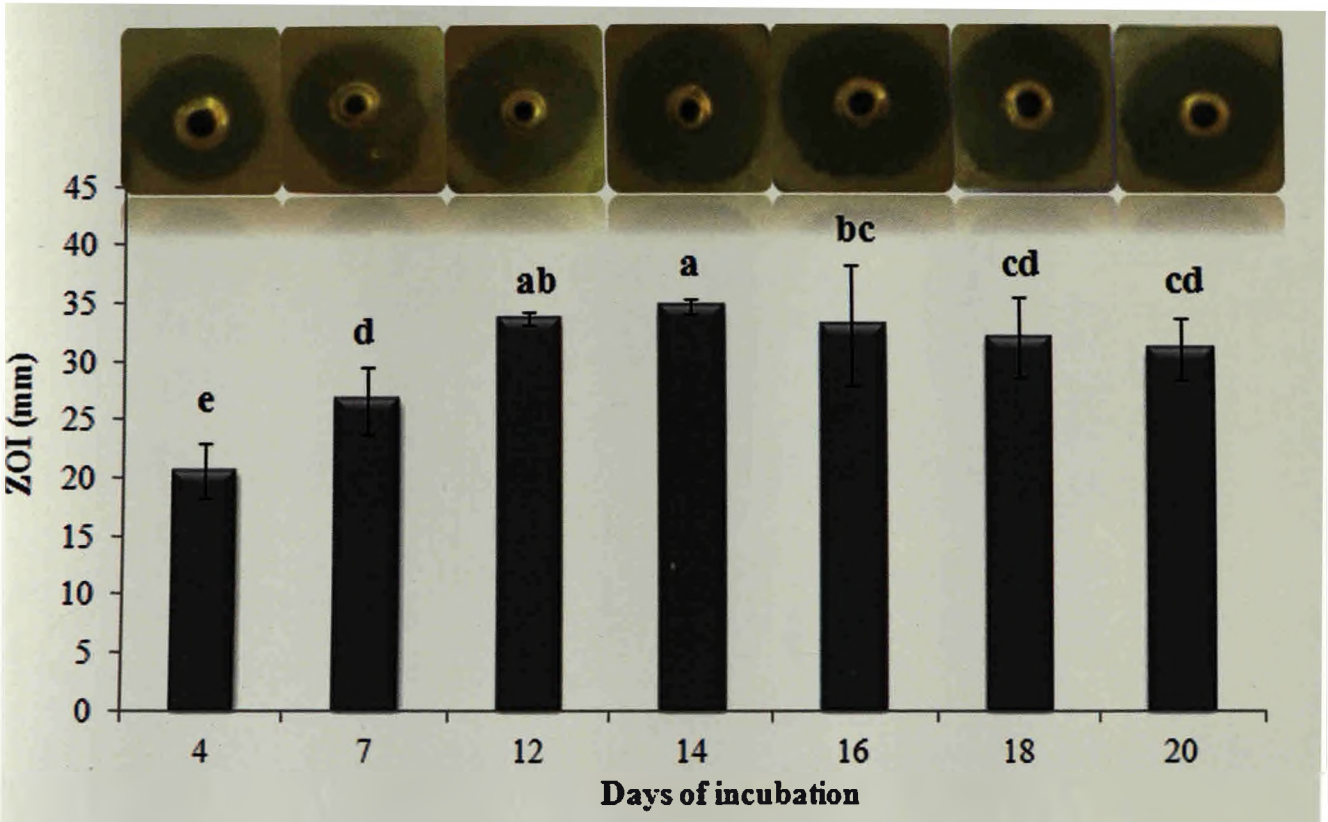


Figure 4.12 Effect of incubation period on antibiotic production. Bars indicate standard deviation of the mean and the superscripts, significant differences ($p \leq 0.05$)

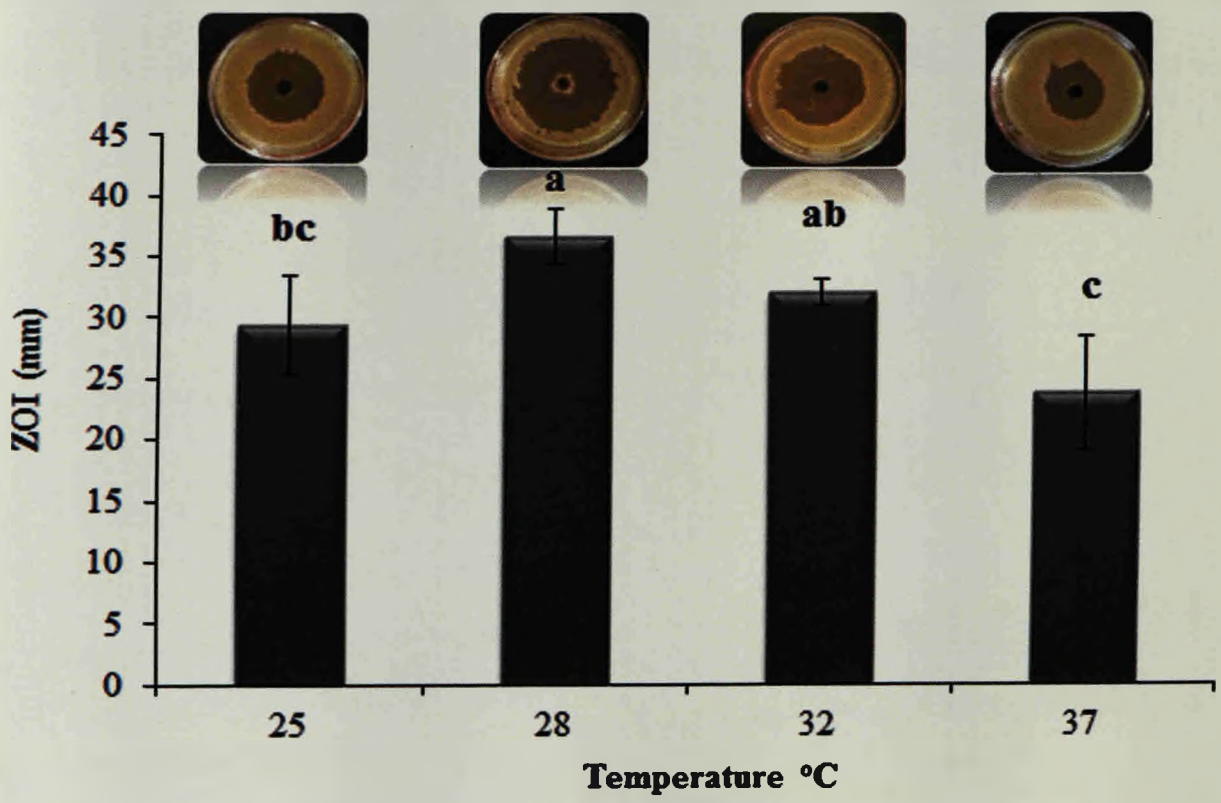


Figure 4.13 Effect of temperature on antibiotic production. Bars indicate standard deviation of the mean and the superscripts, significant differences ($p \leq 0.05$)

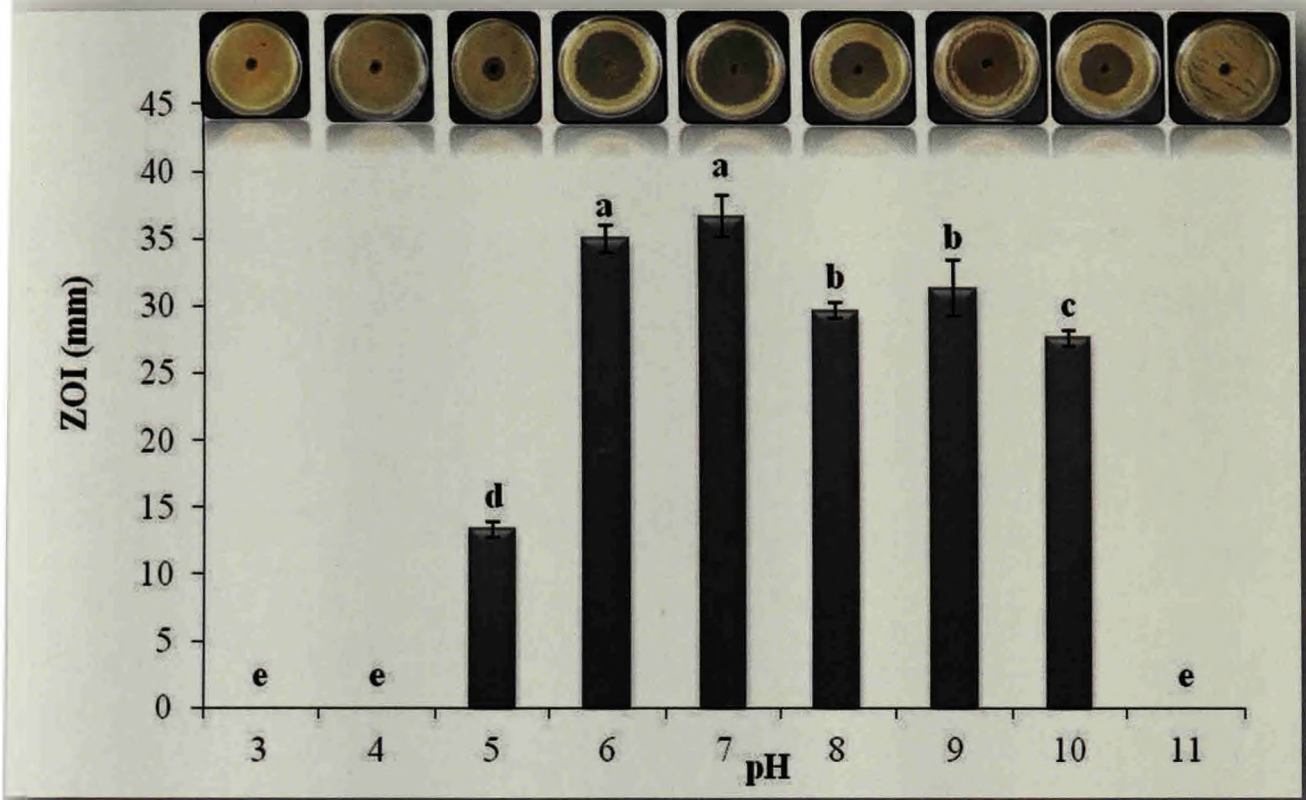


Figure 4.14 Effect of pH on antibiotic production. Bars indicate standard deviation of the mean and the superscripts, significant differences ($p \leq 0.05$)

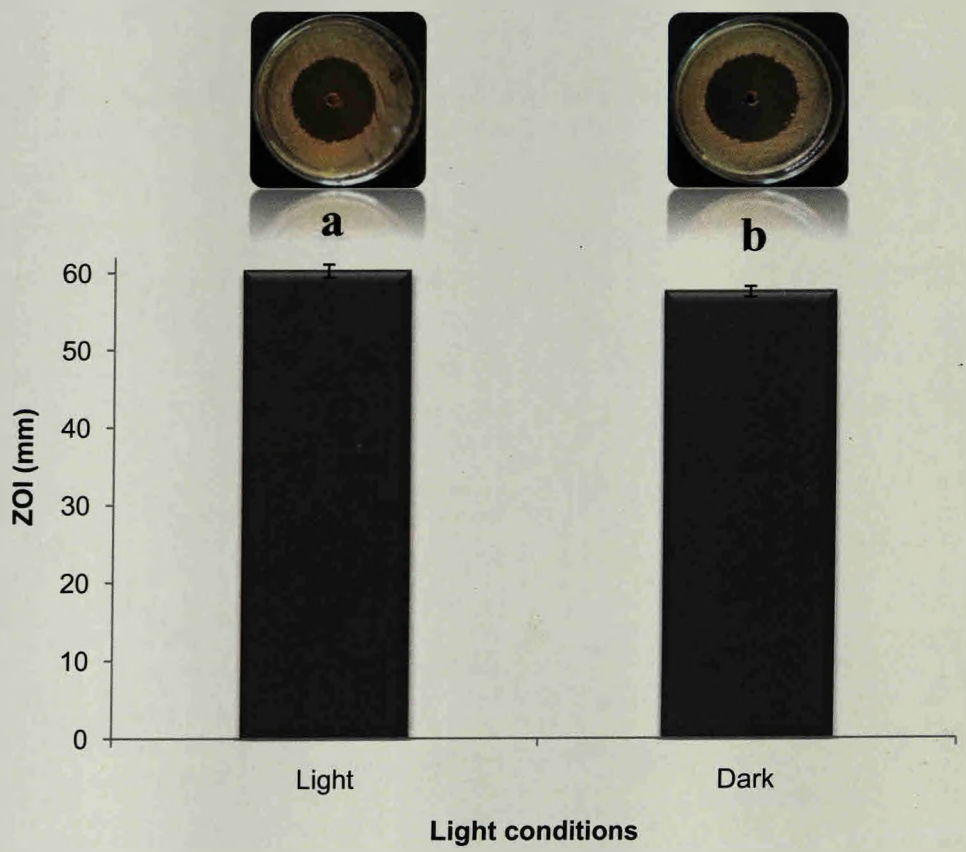


Figure 4.15 Effect of light on antibiotic production. Bars indicate standard deviation of the mean and the superscripts, significant differences ($p \leq 0.05$)

Effect of light on antibiotic production

The present findings showed no significant difference in the effect of antibiotic production when exposed to light or dark conditions as shown in **Figure 4.15.**

Effect of autoclaving on antimicrobial activity

It was observed that heat treatment did not affect antibiosis but only decreased the size of the zone of inhibition, which was significant ($p \leq 0.05$) as shown in **Figure 4.16.**

4.3.6.2 *Nutritional parameters*

NaCl concentration

Maximum antibiotic production in terms of mean ZOI of 37 mm, was obtained without NaCl and was significant ($p \leq 0.05$). A significant difference was found over control (20,000 ppm concentration) exhibiting a mean ZOI of 33 mm. However activity was observed at all concentration of NaCl **(Figure 4.17).**

Organic Carbon and Nitrogen concentration (%)

The onset and intensity of secondary metabolism is dependent on various nutritional factors like carbon and nitrogen sources.

Maximum antibiotic activity was observed with 0.7 % (v/v) glycerol concentration showing a mean ZOI of 35 mm and was significant ($p \leq 0.05$). Data indicated that increasing concentration of glycerol from 0.5-0.7 % (v/v) led to an increase in the antibiotic production, thereafter further increase in values from 0.8-1.2 % (v/v) resulted in its decrease **(Figure 4.18).**

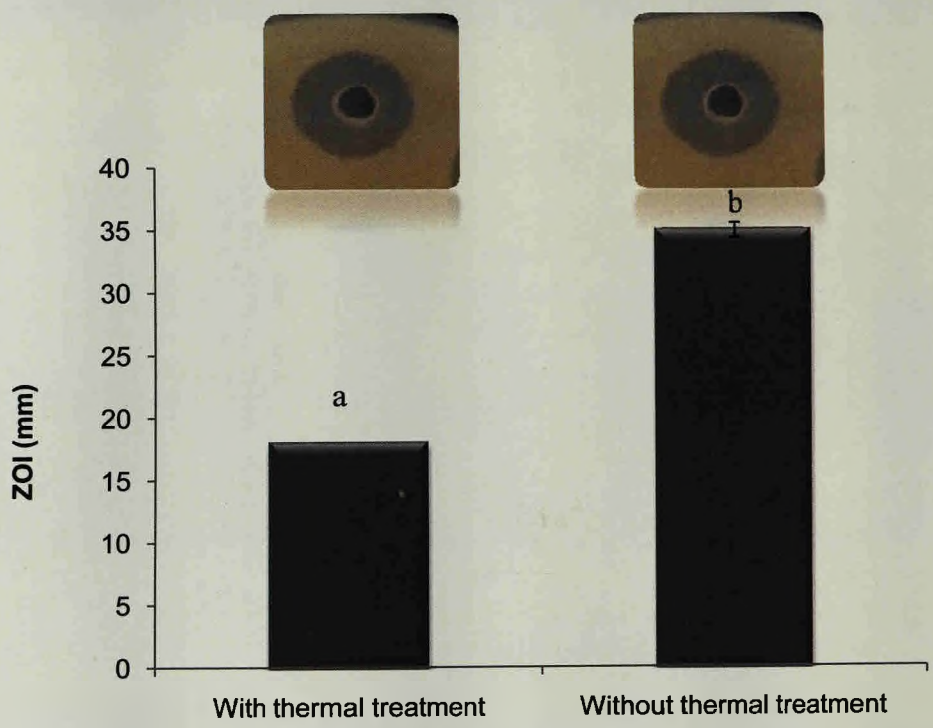


Figure 4.16 Effect of autoclaving on antimicrobial activity. Bars indicate standard deviation of the mean and the superscripts, significant differences ($p \leq 0.05$)

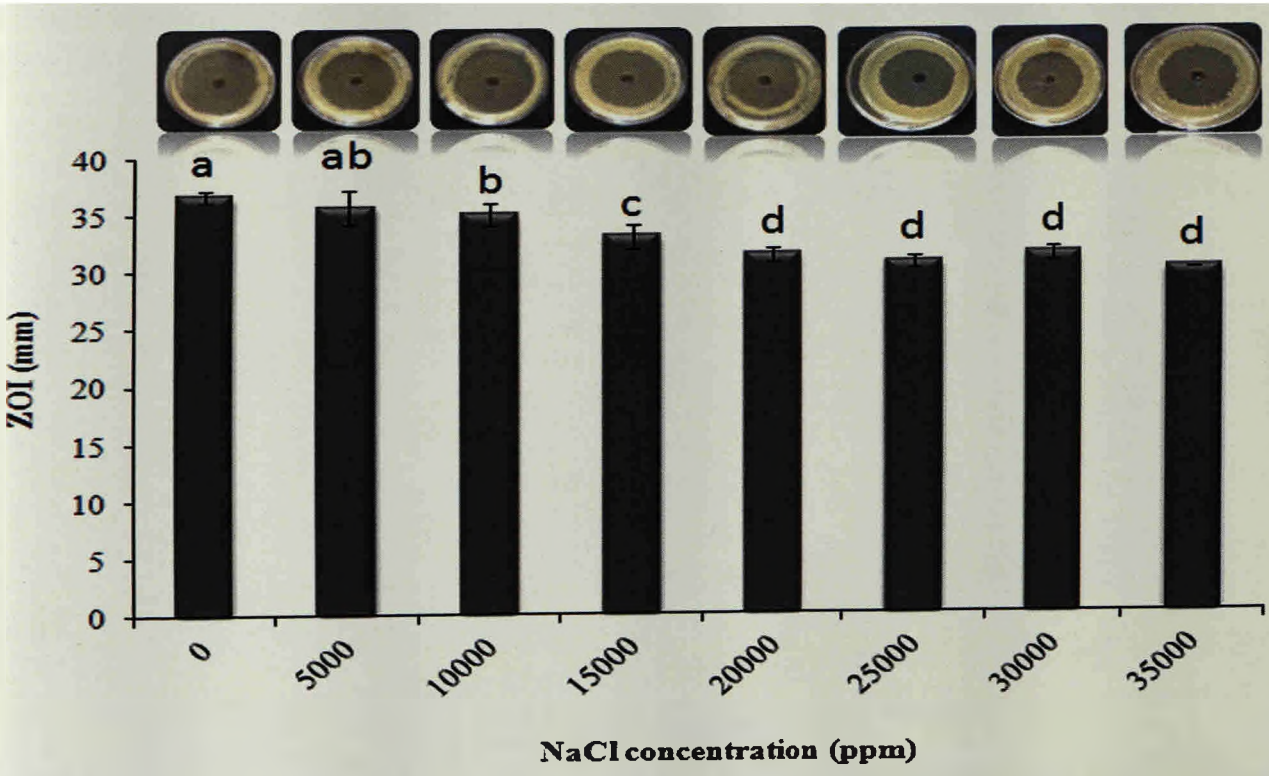


Figure 4.17 Effect of NaCl (ppm) on antibiotic production. Bars indicate standard deviation of the mean and the superscripts, significant differences ($p \leq 0.05$)

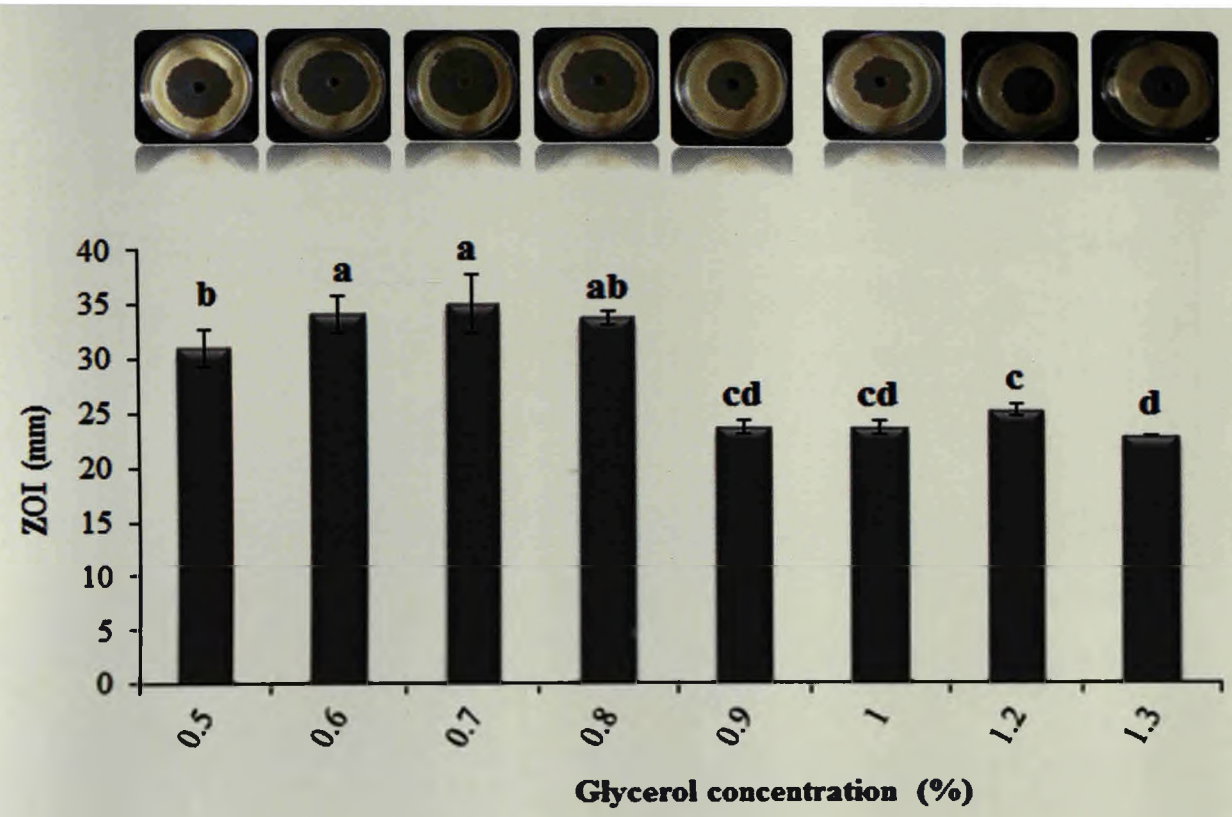


Figure 4.18 Effect of different concentrations of glycerol (%) as organic carbon source on antibiotic production. Bars indicate standard deviation of the mean and the superscripts, significant differences ($p \leq 0.05$)

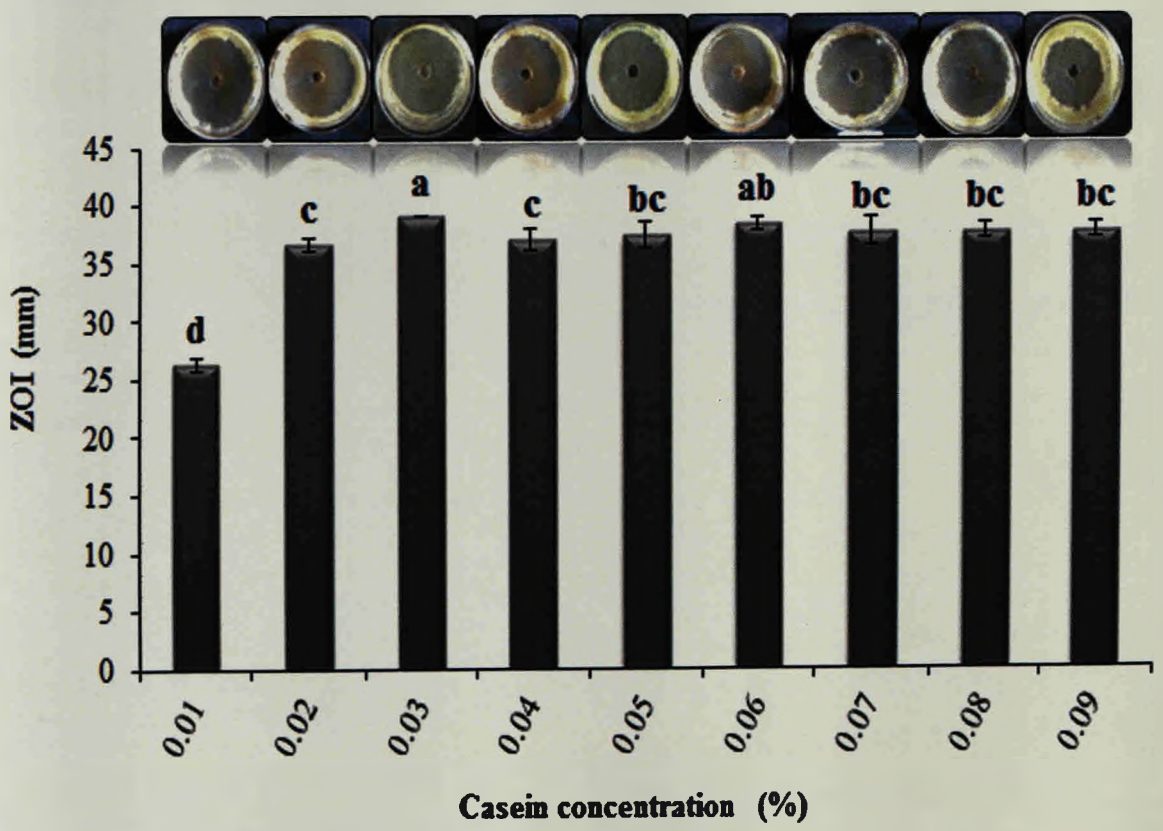


Figure 4.19 Effect of different concentrations of casein (%) as organic nitrogen source on antibiotic production. Bars indicate standard deviation of the mean and the superscripts, significant differences ($p \leq 0.05$)

Maximum antibiotic activity was observed with 0.03 % (w/v) casein concentration showing a mean ZOI of 39 mm and was significant ($p \leq 0.05$). However, other concentration of casein also favored the production of antibiotic compounds **(Figure 4.19)**.

Phosphate concentration (%)

As seen in **Figure 4.20,** 0.25 % (w/v) concentration of phosphate resulted in maximum yield of antibiotic exhibiting a mean ZOI of 35 mm and was significant ($p \leq 0.05$). The results also indicated that other concentrations of phosphate also exhibited an appreciable ZOI.

Inorganic Nitrogen and Carbon concentration (%)

Maximum antibiotic activity was observed using 0.1 % (w/v) KNO_3 concentration exhibiting a mean ZOI of 36 mm which was much higher as compared to that of control (0.2 % w/v) with a mean ZOI of 29 mm and was significant ($p \leq 0.05$) **(Figure 4.21).**

Maximum antibiotic activity was observed with 0.0015% and 0.002% $CaCO_3$ concentration with a mean ZOI of 37 mm in both cases and was significant ($p \leq 0.05$) **(Figure 4.22).**

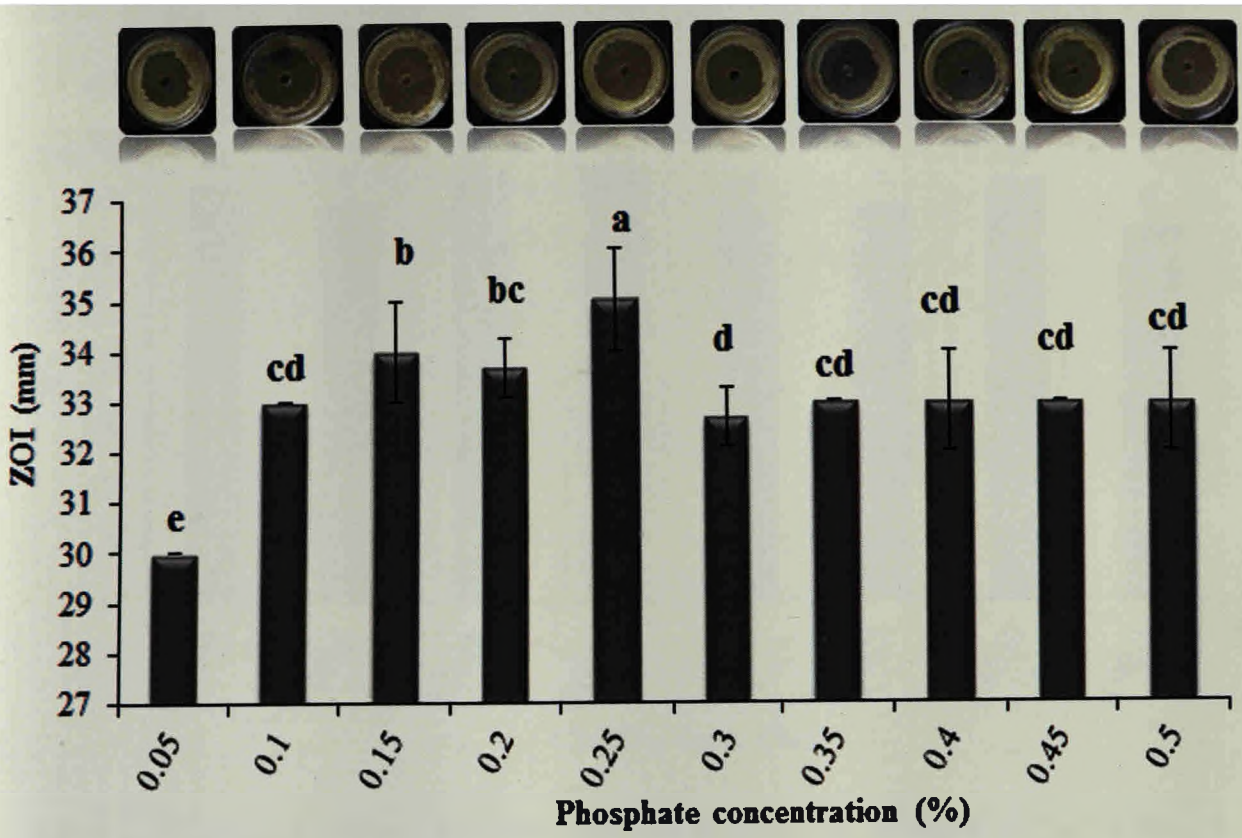


Figure 4.20 Effect of phosphate (%) on antibiotic production. Bars indicate standard deviation of the mean and the superscripts, significant differences ($p \leq 0.05$)

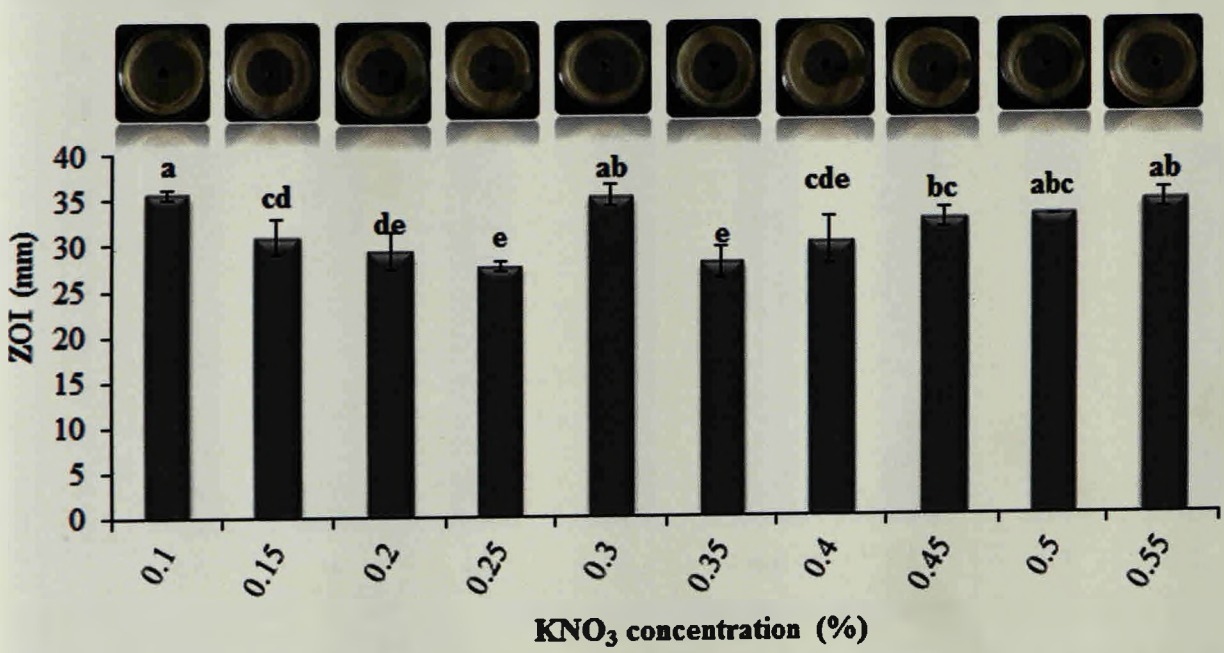


Figure 4.21 Effect of different concentrations of KNO_3 (%) as inorganic nitrogen source on antibiotic production. Bars indicate standard deviation of the mean and the superscripts, significant differences ($p \leq 0.05$)

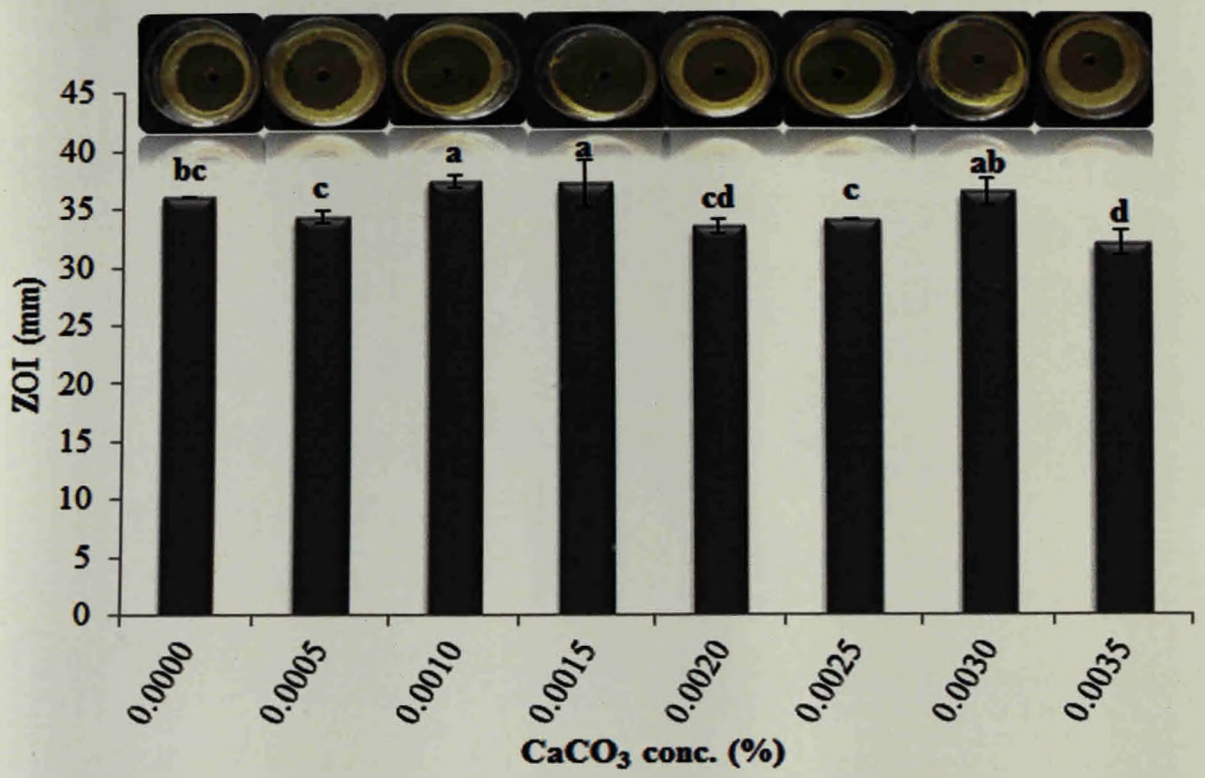


Figure 4.22 Effect of different concentrations of $CaCO_3$ (%) as inorganic nitrogen source on antibiotic production. Bars indicate standard deviation of the mean and the superscripts, significant differences ($p \leq 0.05$)

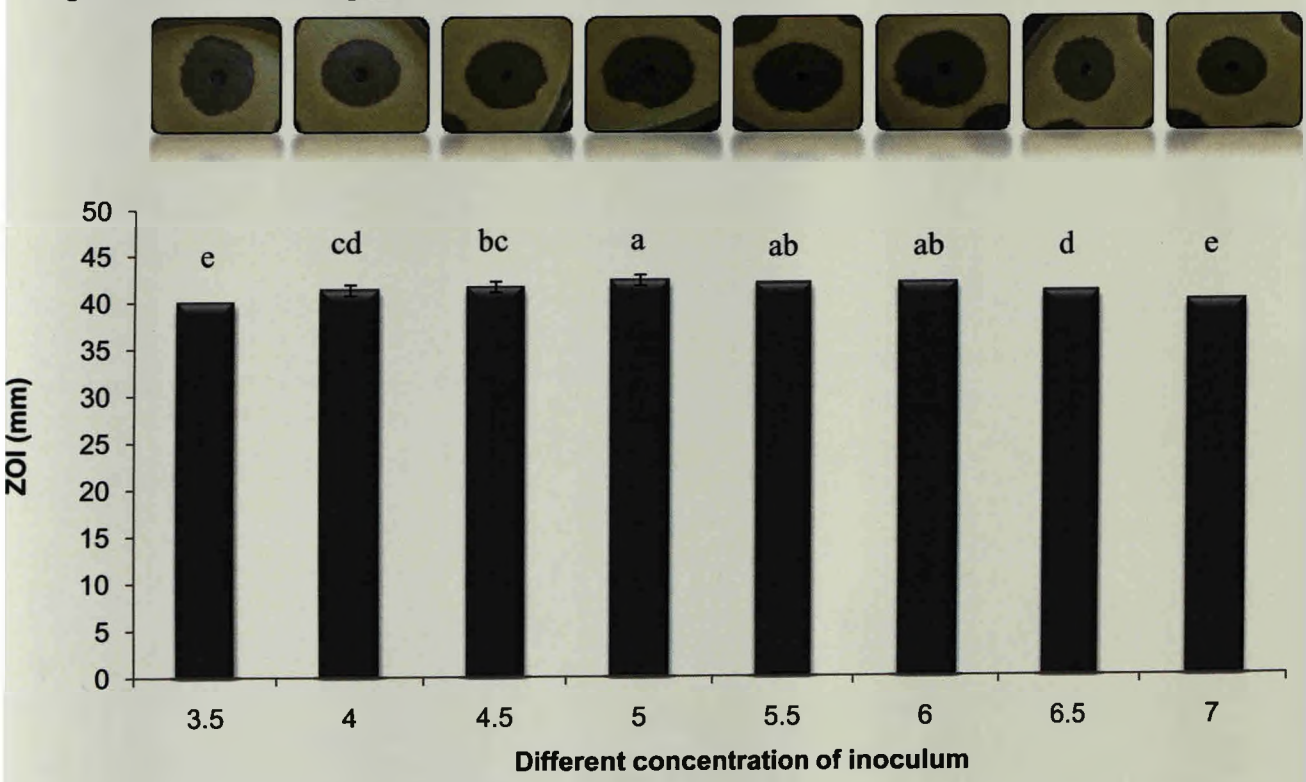


Figure 4.23 Effect of culture inoculum (%) on antibiotic production. Bars indicate standard deviation of the mean and the superscripts, significant differences ($p \leq 0.05$)

Effect of culture inoculum (%)

Maximum antibiotic activity was observed with 5 % as the optimal inoculum concentration which was significant ($p \leq 0.05$) as shown in **Figure 4.23.**

Comparing the antibiotic production efficacy by differential sampling of its aerial (AM) and substrate mycelium (SM)

SM showed 14 % higher efficacy of producing antibiotic/s than AM which was significant ($p \leq 0.05$) as shown in **Figure 4.24**

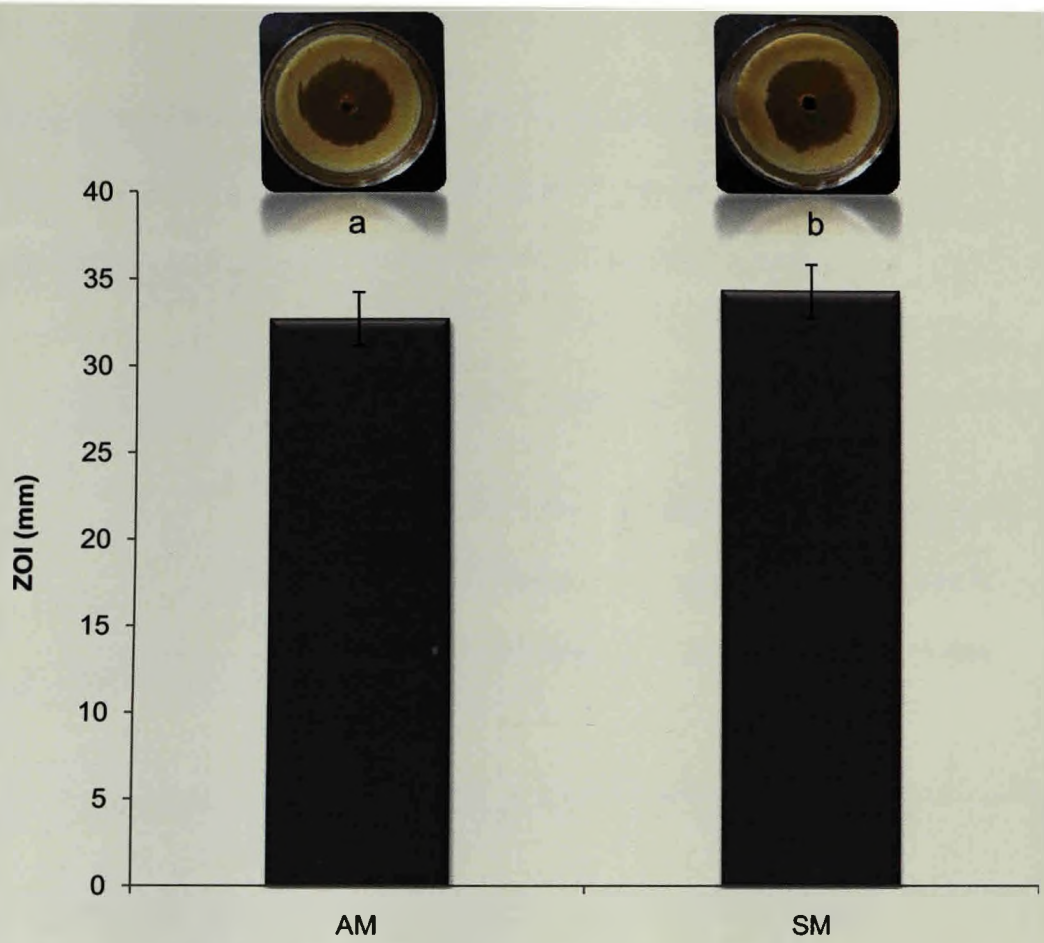


Figure 4.24 Efficacy of aerial (AM) and substrate mycelium (SM) in antibiotic production. Bars indicate standard deviation of the mean and the superscripts, significant differences ($p \leq 0.05$)

4.3.7 Testing the relative efficacy of CFCF with standard antibiotics

The CFCF showed higher efficacy to almost 35 out of 36 antibiotics used against *Staphylococcus* infections as shown in **Table 4.7** and **Plate 4.3a&b**.

Table 4.7 Comparative ZOI of standard antibiotics to CFCF of *Streptomyces parvulus* CFA-9

List of standard antibiotics	**Mean ZOI (mm) against *S. citreus* (experimental data)**	**ZOI of standard antibiotics against *S. aureus* ATCC 25923 (mm) (Hi Media catalogue)**
Amikacin	29	20-26
Amoxyclav	0	28-36
Ampicillin	0	27-35
Augmentin	0	NR
Azithromycin	34	NR
Ceflodoxime	0	NR
Ceftriaxone	30	NR
Cephalothin	28	29-37
Chloramphenicol	0	19-26
Ciprofloxacin	27	22-30
Clarithromycin	36	26-32

Clindamycin	25	24-30
Colistin	30	0
Co-Trimoxazole	31	24-32
Erythromycin	24	22-30
Gatifloxacin	41	27-33
Gentamicin	26	0
Imipenem	18	0
Kanamycin	27	19-26
Levofloxacin	42	25-30
Linezolid	40	25-32
Methicillin	13	17-22
Moxifloxacin	41	28-35
Nalidixic acid	25	0
Norfloxacin	20	17-28
Novobiocin	40	22-31
Ofloxacin	34.5	24-28
Oxacillin	0	18-24
Penicillin	10	26-37

Sparfloxacin	34	27-33
Streptomycin	25	14-22
Teicoplanin	36	15-21
Tetracycline	30	24-30
Ticarcillin	0	29-37
Tobramycin	19	19-29
Vancomycin	32	17-21
Unknown CFCF	**42**	**NA**

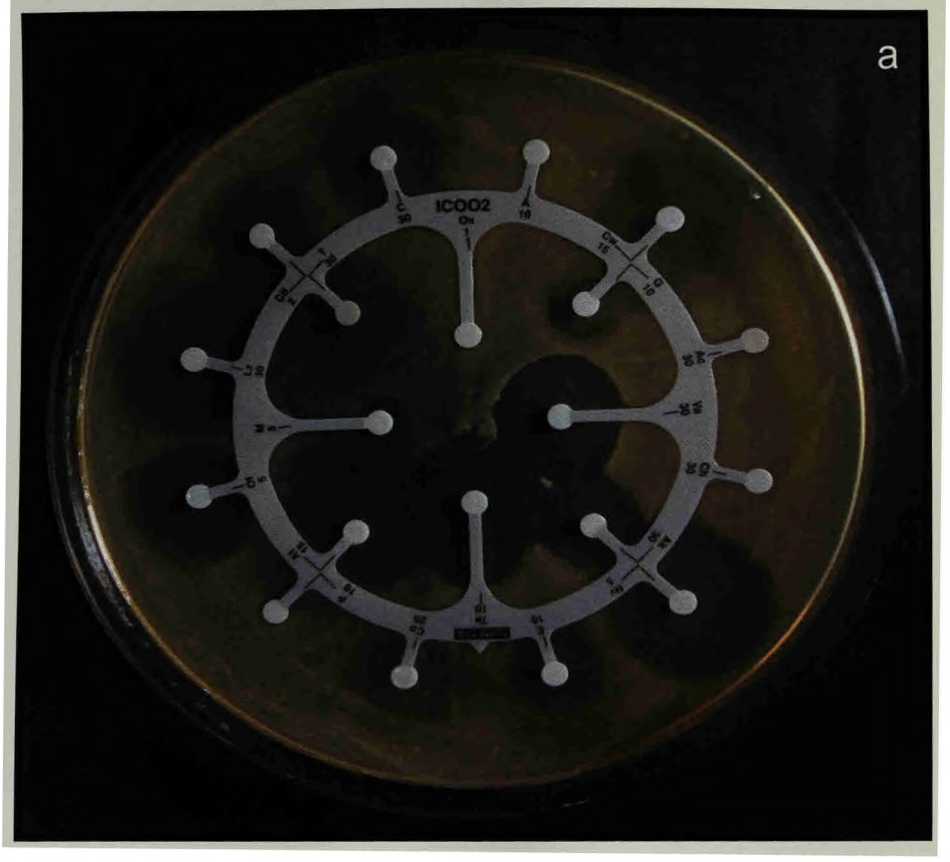
Legend: *NR, not reported; NA, not applicable

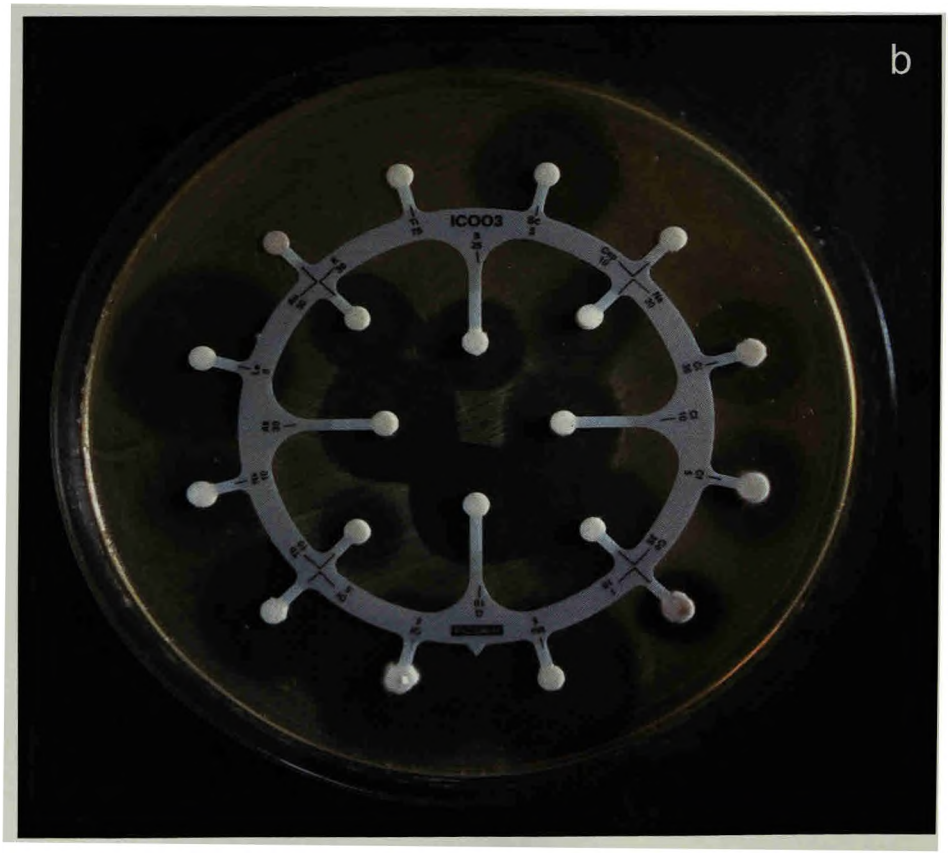
PLATE 4.3

a- Anti *S. citreus* activity using standard ICOO2 antibiotic discs (Hi Media, Mumbai, India)

b- Anti *S. citreus* activity using standard ICOO3 antibiotic discs (Hi Media, Mumbai, India)

PLATE 4.3





4.3.8 Extraction of antibiotic compounds from CFCF using various solvents

Methanolic extract, showed maximum antibiosis against all test pathogens thus, validating its capability as an ideal solvent for extraction of compounds as shown in **Figure 4. 25 – 4.29.**

4.3.8.1 *Spectroscopic measurements (UV-Visible and FTIR spectral analysis)*

The UV-Visible spectral data is shown in **Table 4.8**. The control readings obtained were matched with the test readings and the ones matching were then deleted as they could probably be the interfering substances present in the broth medium. The spectral data of methanol extracted CFCF showed absorbance maxima at 405 nm.

FTIR spectrum of the methanolic extract **(Figure 4.30)** exhibited absorption at ~3300 cm^{-1} and ~1051.20 cm^{-1} which indicated an amine group; at ~2939 cm^{-1} and ~2877.79 cm^{-1} which indicated carboxylic acids group; at ~1708.93 cm^{-1} which indicated aldehydes, ketones, carboxylic acids and esters group and absorption at ~1571.99 cm^{-1} which indicated nitro group.

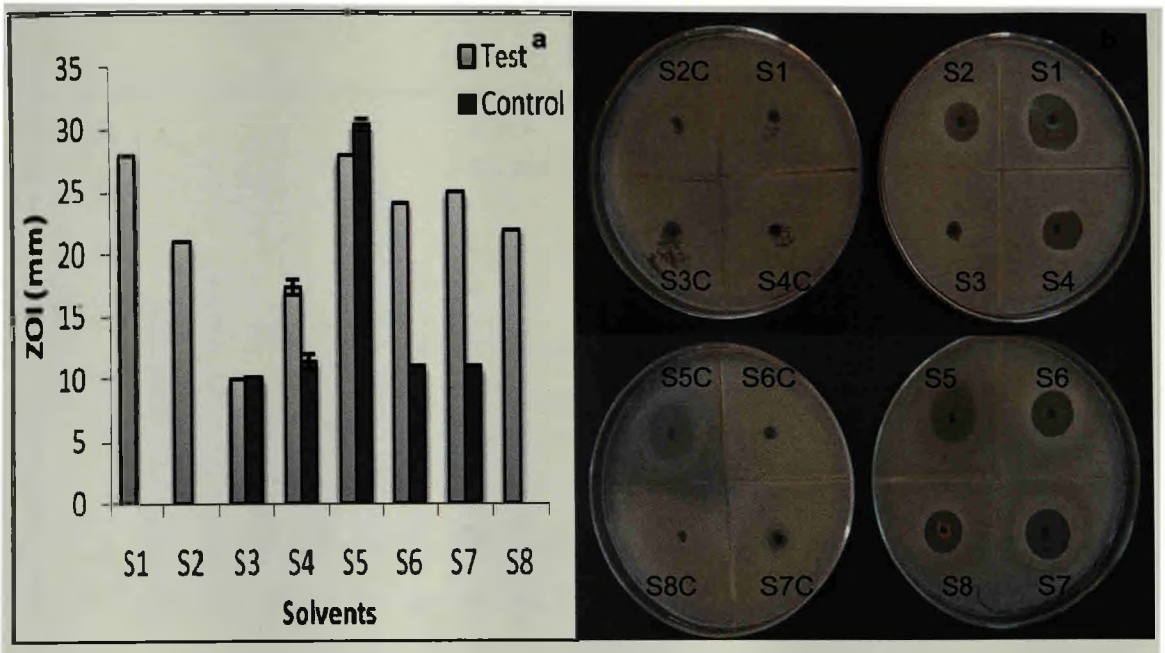


Figure 4.25a&b Antimicrobial activity against *E. aerogens*

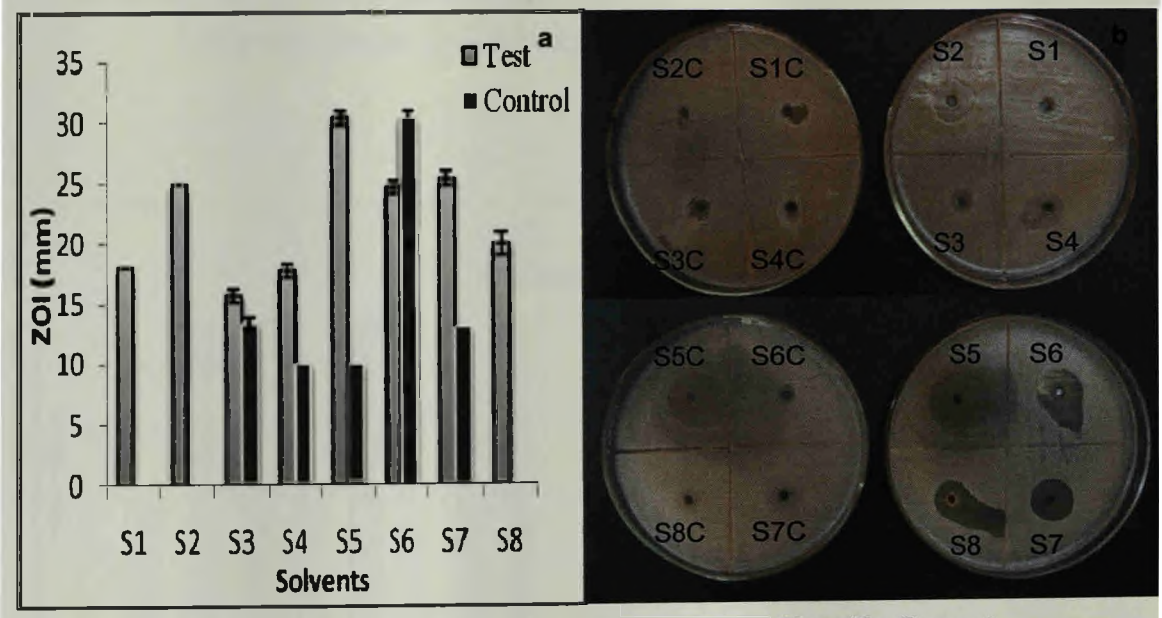


Figure 4.26a&b Antimicrobial activity against *Shigella flexneri*

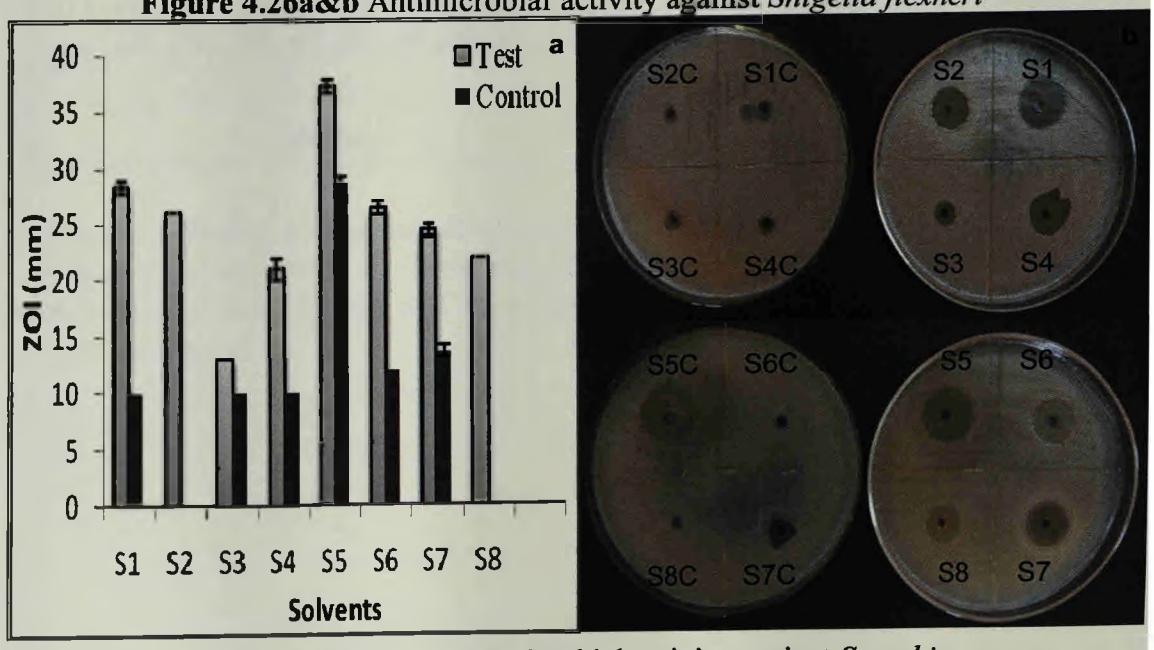


Figure 4.27a&b Antimicrobial activity against *S. typhi*

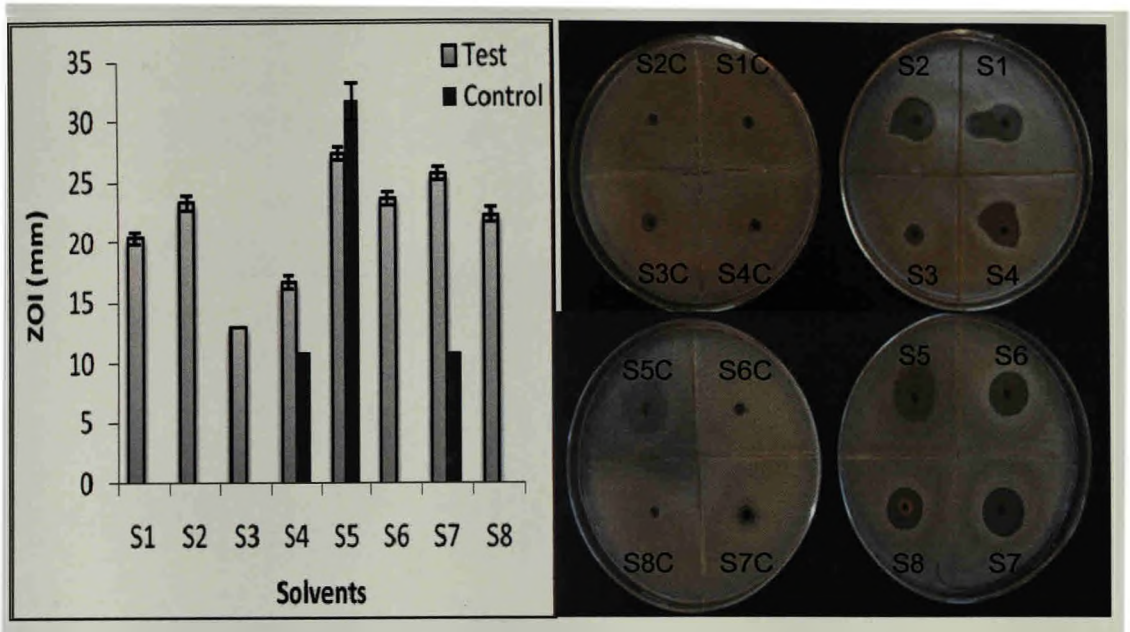


Figure 4.28a&b Antimicrobial activity against *B. subtilis*

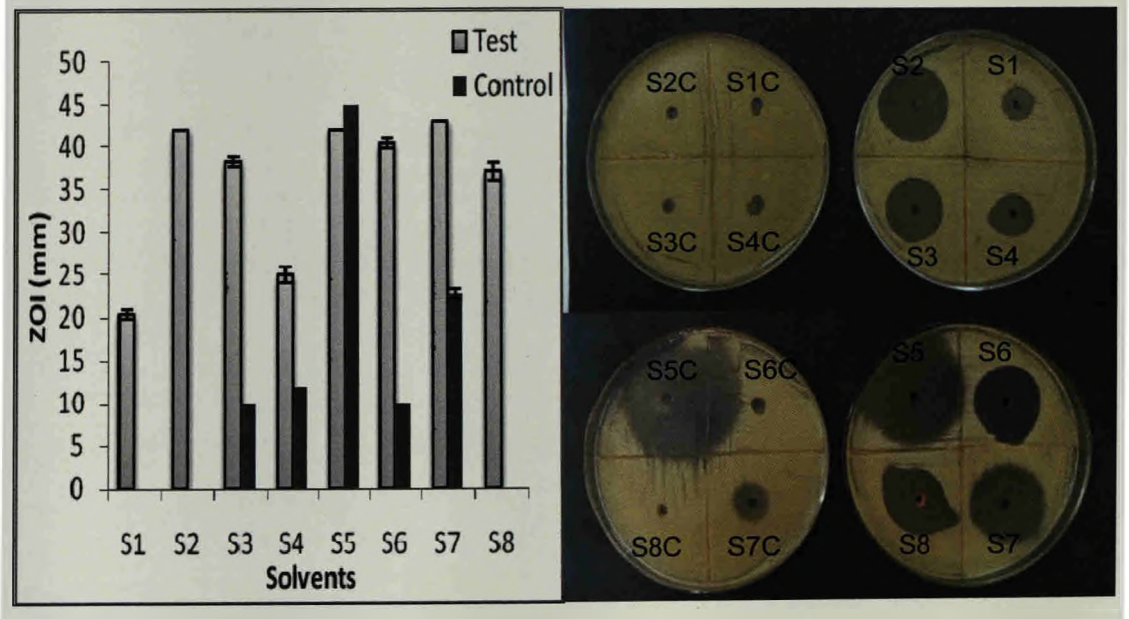


Figure 4.29a&b Antimicrobial activity against *S. citreus* using various solvents where S1, Chloroform; S2, Methanol; S3, Butanol; S4, Propanol; S5, Aniline; S6, Acetone; S7, Ethyl acetate and S8, n-hexane and S1C-S8C represent the control (pure solvents)

Table 4.8 Absorbance profile of solvent extracts in UV-Visible

	Polar				Dipolar		Non polar	
Wavelenght (λ)	Chloroform	Methanol	Butanol	Propanol	Acetone	Ethyl acetate	n-hexane	Aniline
209.5								
217.5								
238								
240.5								
241								
252.5								
261.5								
261.5								
295								
323								
335.5								
340								
345								
405.5								
407								
407.5								
425.5								
469								
550.5								
551								
601.5								
662								
705								
708.5								
731.5								
733								
739								
749.5								
766.5								
771								
785								

Keys for colour in cells	
0-0.5	
0.6-1	
1.1-1.5	
1.51-2	
2.1-2.5	
2.6-3	
3.1-3.5	
3.6-4	
4.1-4.5	

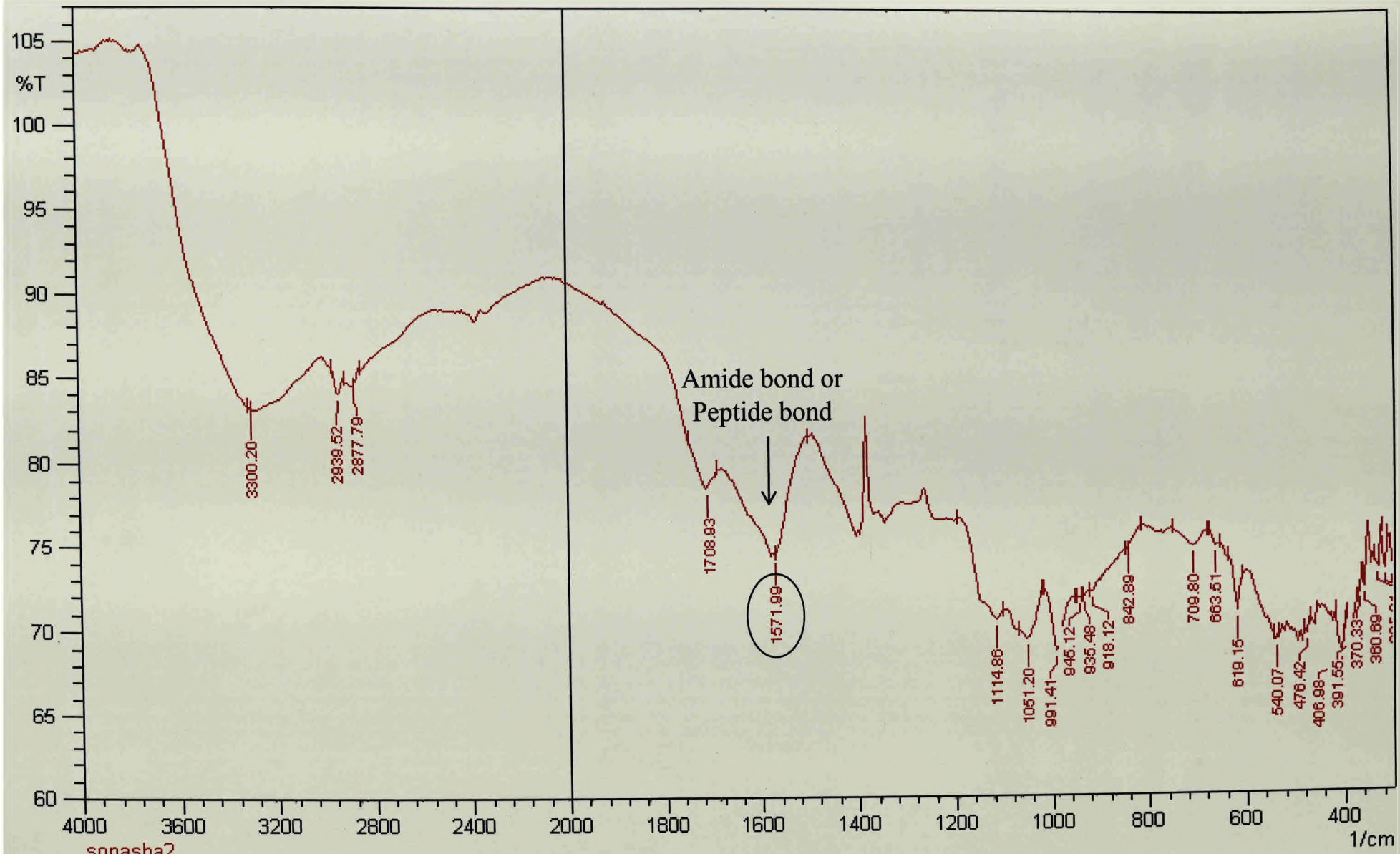


Figure 4.30 FTIR spectrum of the methanolic extract of CFCF of *Streptomyces parvulus* CFA-9

4.3.9 Testing of the crude methanolic extract for number of components by TLC

Chloroform: methanol (5:1 v/v) was selected as the best solvent system. Use of smaller TLC plates restricted the number of spots to five and longer TLC plates produced nine distinct spots. The developed coloured spots were easily detected with the naked eye and are tabulated in **Table 4.9** and **Plate 4.4a**.

Table 4.9 Rf values of spots in chloroform: methanol (5:1 v/v) solvent system

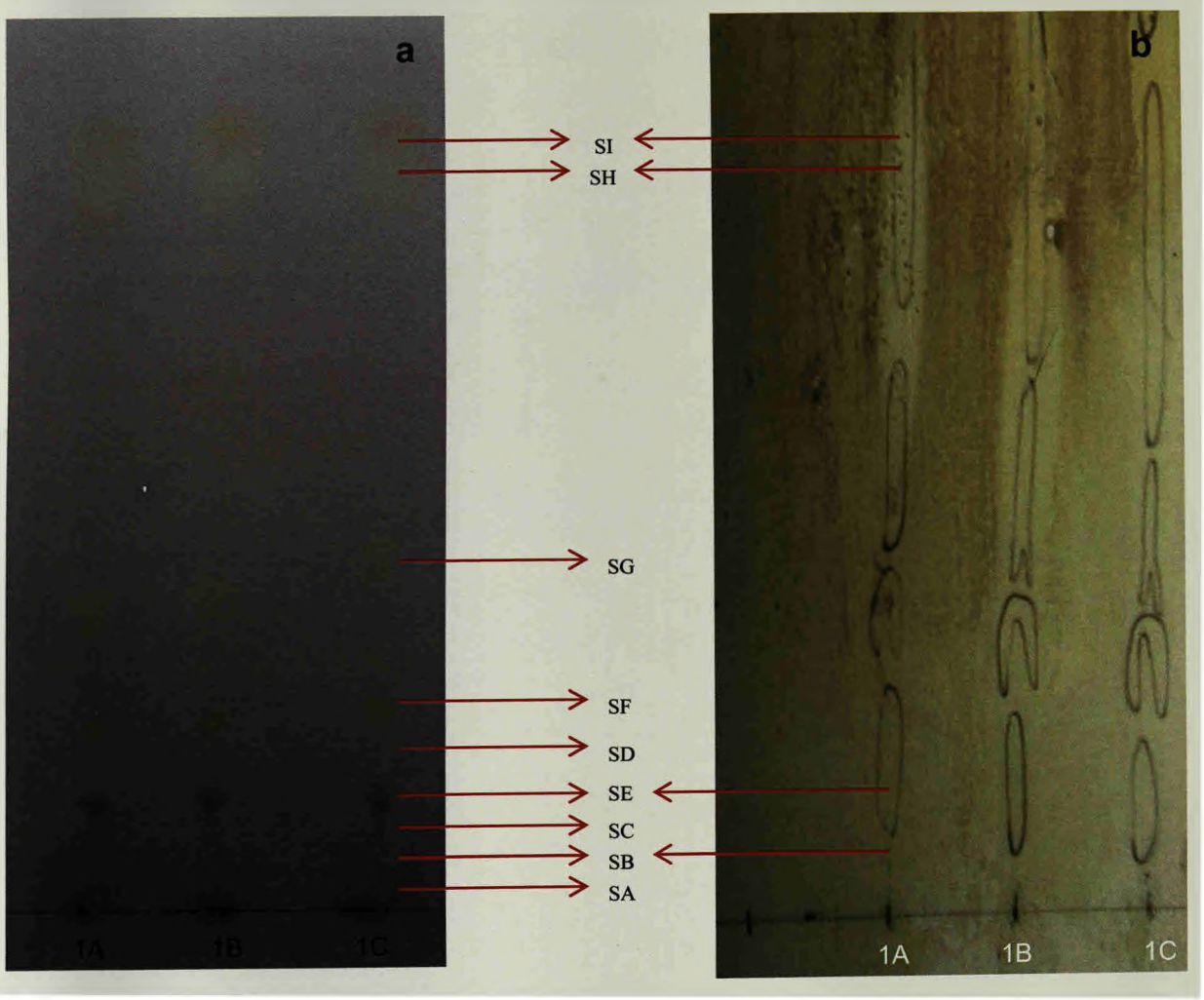
Spot designation	Mean R_f value (%)	Colour
S_A	7.4	Brown
S_B	12.4	Light brown
S_C	14.2	Faint brown
S_D	17.3	Red
S_E	22.2	Faint yellow
S_F	28.4	Orange
S_G	42	Yellow
S_H	86.4	Lemon yellow
S_I	88	Bright lemon yellow

PLATE 4.4

a- TLC plate with the marked spots (S_A - S_I)

b- Bioautographic -TLC plate showing zone of clearance

PLATE 4.4



4.3.10 Bioautography and chemical identity of antibiotics

Bioautography studies revealed that out of nine spots (SA-SI), four spots (SB, SE, SH and SI) showed clear visible inhibition against the test pathogen as shown in **Table 4.10, Plate 4.4b.**

Table 4.10 Bioautography- spots showing visible inhibition

Spot designation	Visible inhibition*
S_A	-
S_B	+
S_C	-
S_D	-
S_E	+
S_F	-
S_G	-
S_H	+
S_I	+

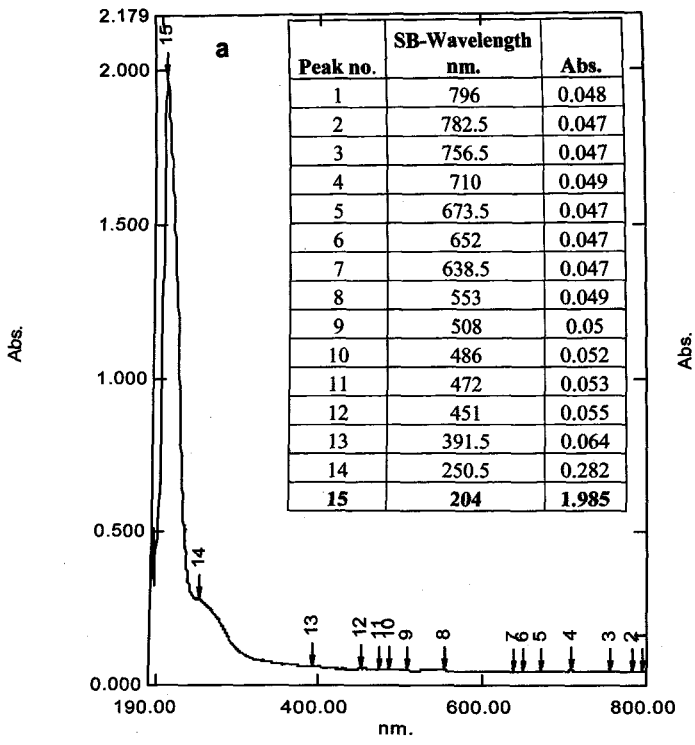
Legend: *(+), present; (-), absent

UV-Visible spectral data of all the four positive TLC fractions **(Figure 4.31 a-d)** showed most characteristic peak with absorption maxima (λ max) at 202 nm. Absorbance peaks were also observed at 220 nm. Minor absorbance peaks were observed between 255 - 257 nm.

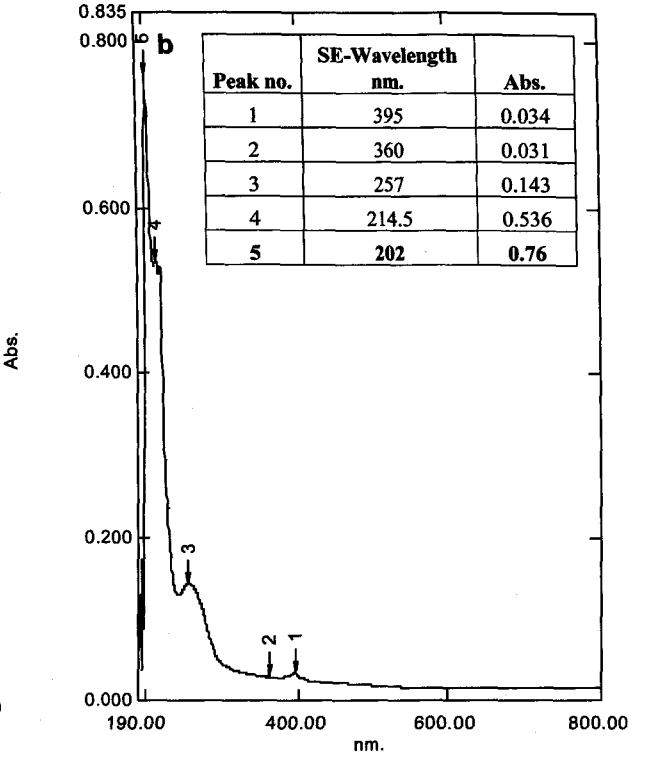
4.3.11 Chemical characterization of partially purified compound

ESI-MS/MS (Electrospray Ionization Mass Spectrometry) spectrum of EAM-5 showed peak at m/z 587 $[M+H]^+$ **(Figure 4.32)**. The literature report suggests that the molecular mass m/z 586 is close to Streptolydigin molecule (Horna *et al.*, 2011). The structure was elucidated based on MS/MS fragmentation wherein the precursor ion at m/z 587 $[M+H]^+$ was selected and subjected to fragmentation at collision energy 30-40 v. The product ion spectrum showed peaks at m/z 569, 497, 407, 365, 310, 279, 197, 169, 137 and 112 **(Figure 4.33)**. The possible fragmentation pattern confirmed that the modification takes place at the tetramic acid lateral side chain with demethylation of Streptolydigin **(Figure 4.34a&b).**

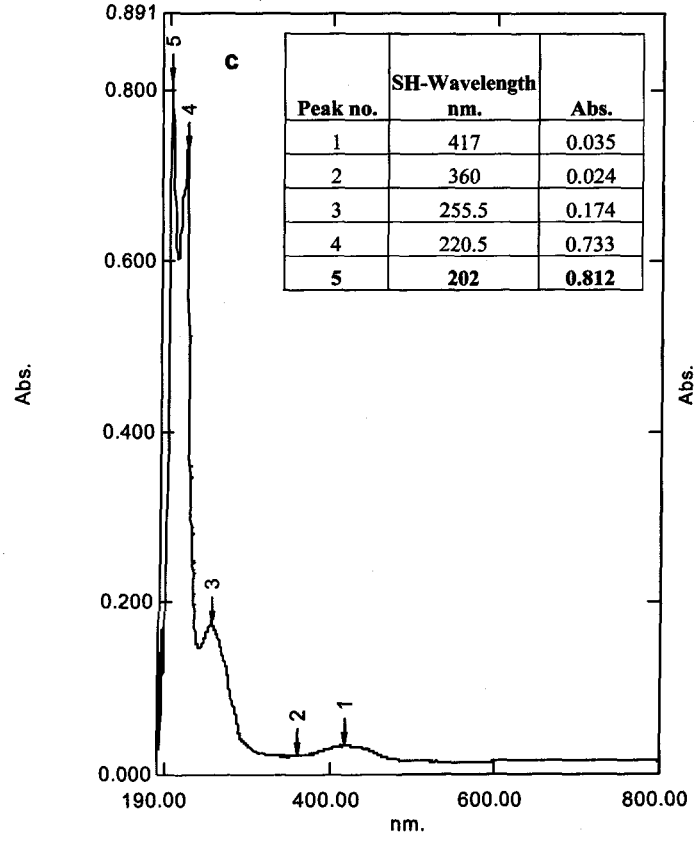
Results of predicted elemental analysis showed the presence of C, H, O and N in 63.36 %, 7.37 %, 4.77 %, 24.50 % using the Strepdom database (Lucas *et al.*, 2013). The predicted molecular formula of compound EAM (SG) 5 as $C_{31}H_{43}N_2O_9$ was determined by the interpretation of data from elemental analysis.



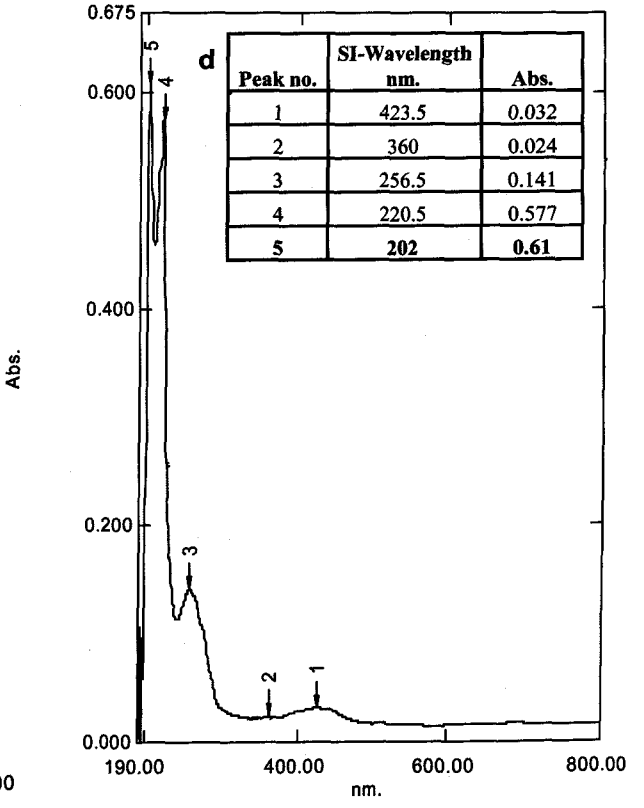
Peak no.	SB-Wavelength nm.	Abs.
1	796	0.048
2	782.5	0.047
3	756.5	0.047
4	710	0.049
5	673.5	0.047
6	652	0.047
7	638.5	0.047
8	553	0.049
9	508	0.05
10	486	0.052
11	472	0.053
12	451	0.055
13	391.5	0.064
14	250.5	0.282
15	**204**	**1.985**



Peak no.	SE-Wavelength nm.	Abs.
1	395	0.034
2	360	0.031
3	257	0.143
4	214.5	0.536
5	**202**	**0.76**



Peak no.	SH-Wavelength nm.	Abs.
1	417	0.035
2	360	0.024
3	255.5	0.174
4	220.5	0.733
5	**202**	**0.812**



Peak no.	SI-Wavelength nm.	Abs.
1	423.5	0.032
2	360	0.024
3	256.5	0.141
4	220.5	0.577
5	**202**	**0.61**

Figure 4.31UV-Visible spectral data of four bioactive spots [**a)** SB, **b)** SE, **c)** SH and **d)** SI]

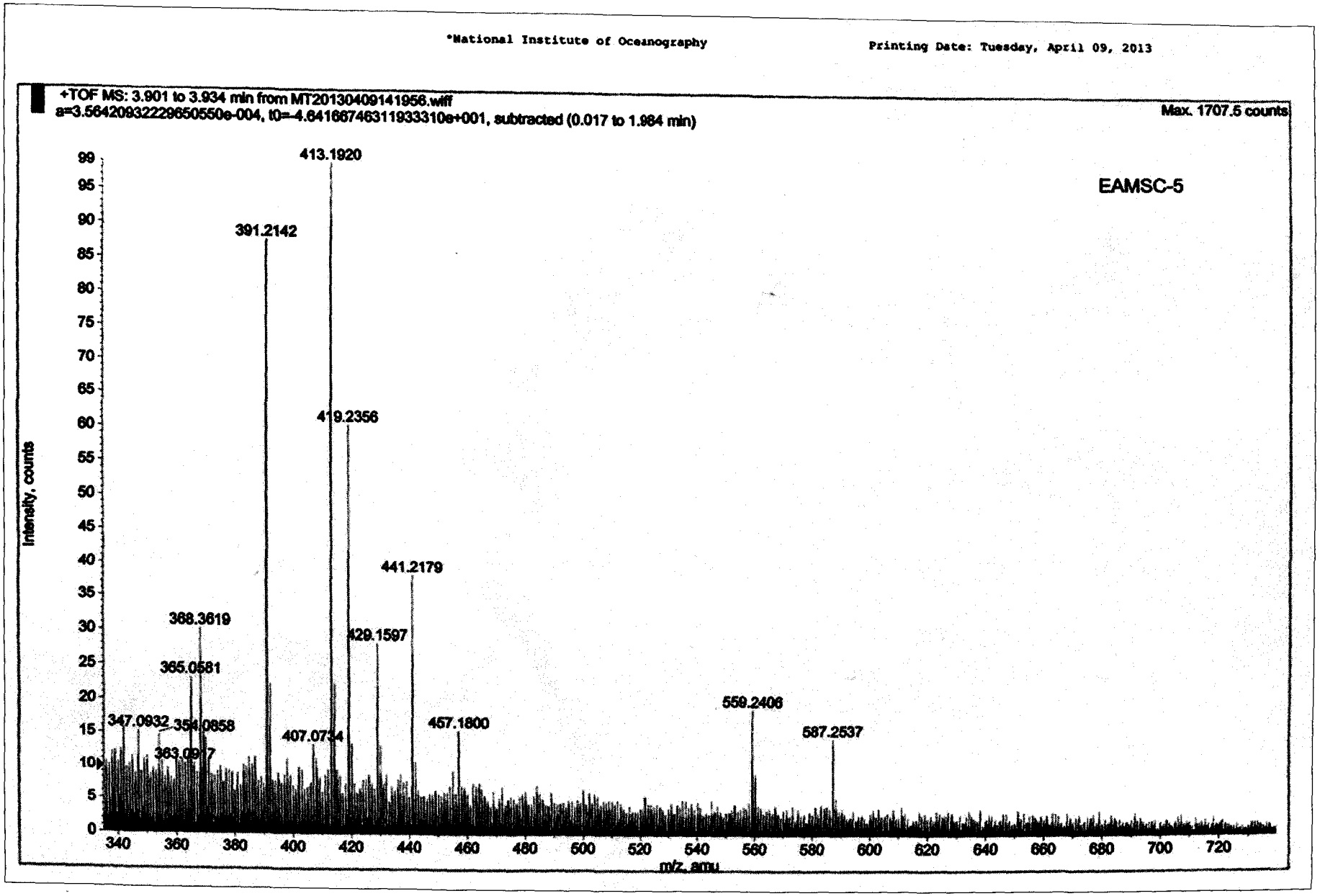


Figure 4.32 ESI-MS (Electrospray Ionization Mass Spectrometry) spectrum of EAM-5 showing a peak at m/z 587 $[M+H]^+$

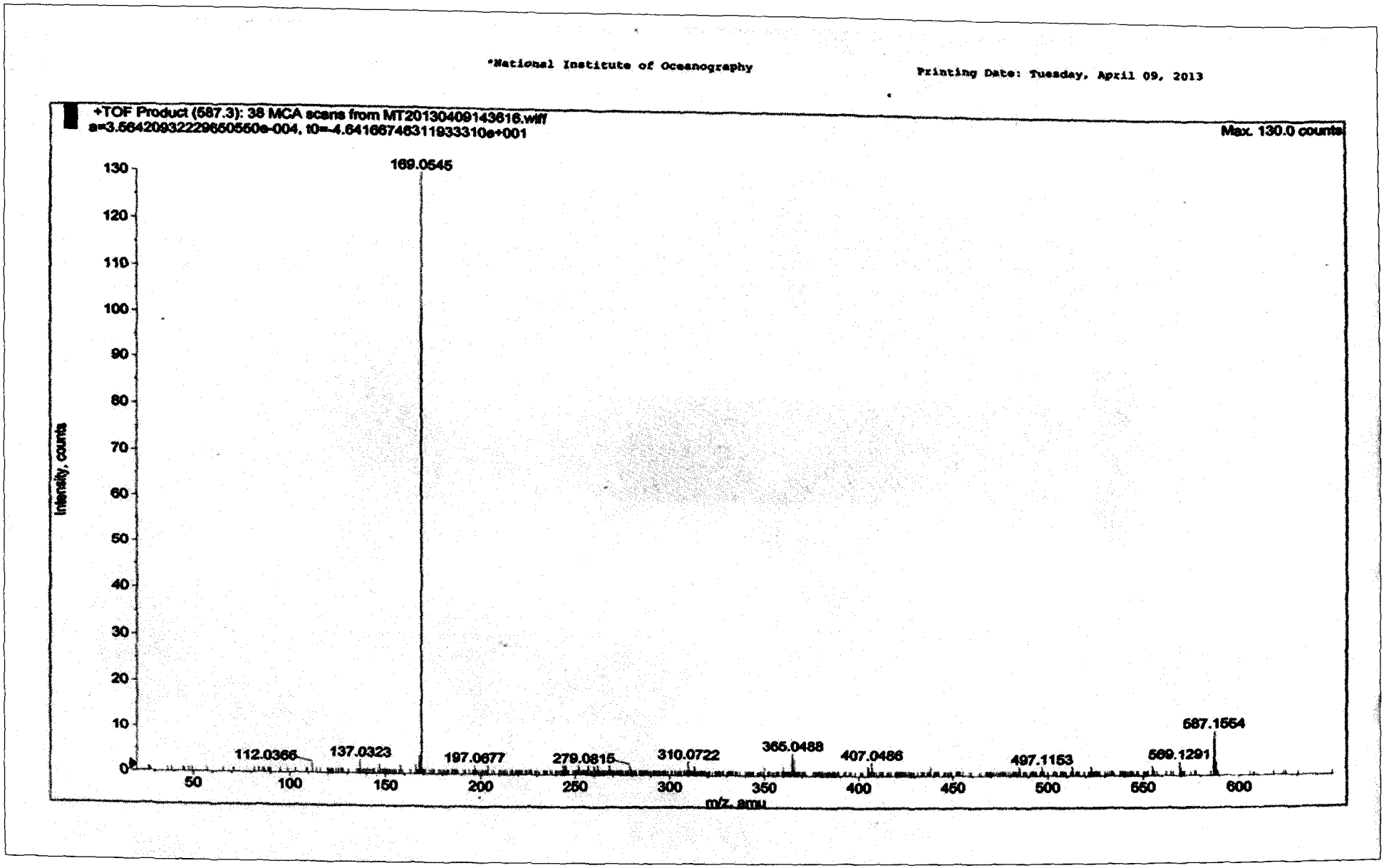


Figure 4.33 MS/MS fragmentation of precursor ion at m/z 587 $[M+H]^+$ at collision energy 30- 40 v.

m/z 137

m/z 365

m/z 407

m/z 569 $[M-H_2O]^+$

m/z 112

m/z 587 $[M+H]^+$

m/z 515

$-H_2O$

m/z 497

m/z 197

m/z 310

m/z 169

Figure 4.34a The proposed fragmentation pattern and possible structures of its productions

Molecular Formula	$C_{31}H_{43}N_2O_9$
Molecular Weight	587.68
Composition:	
Carbon	63.36 %
Hydrogen	7.37 %
Nitrogen	4.77 %
Oxygen	24.50 %

Figure 4.34b The elucidated structure of Streptolydigin compound

4.4 DISCUSSION

4.4.1 Preliminary screening of pigmented strains for antimicrobial activity

Preliminary screening efforts for determination of antimicrobial activity of the pigmented actinomycetes strains have clearly demonstrated the ability of these strains to produce antimicrobial compounds. Actinomycetes are known producers of natural products, which find extensive applications in clinical fields as antibacterial, antifungal, antiparasitic and anticancer agents (Butler, 2008). In the present study, 13 pigmented strains isolated from different unexplored soil habitats were screened for potential antagonistic activity of which 31 % showed positive activity. Baltz, (2007) estimated that a fraction of the earth's soil diversity still needs to be explored and one approach to accelerate antibiotic discovery from actinomycetes is by isolating strains from untapped random and exotic soils. The geographic variation in Indian soil type and their contents, throws the possibility of variable distribution of antibiotic producing actinomycetes (Velho-Pereira & Kamat, 2013).

According to Waksman, (1961) the ability to produce pigments is the characteristic property of actinomycetes specifically Genus *Streptomyces* wherein it plays a defensive role against foreign cells. In this study, the antibiotic producing pigmented strains mainly belonged to genus *Streptomyc*es. Soil Streptomycetes are producers of diverse antibiotics and other biologically active compounds. They are known for continued production of commercially important antibiotics like daptomycin, erythromycin, fosfomycin, lincomycin, neomycin, streptomycin and tetracycline (Mahajan & Balachandran, 2012). Being filamentous they are ubiquitously distributed in nature (Antony-Babu *et al.*, 2008) thus, making them a highly explored genus for generations of microbiologists (Hopwood, 2007).

4.4.2 Antimicrobial activity by modified cross-streak method (MCSM)

Antimicrobial activities of 30 actinomycetes cultures were determined by modified cross-streak method (MCSM) (Velho-Pereira & Kamat, 2011). Among the genera selected in the present study, *Streptomyces* was the most predominant as compared to others. The dominance of *Streptomyces* among the actinomycetes especially in soils has been reported by many workers (Vijayakumar *et al.*, 2007; Ceylan *et al.*, 2008). Preliminary tests for antimicrobial activity of isolated strains obtained from different locations in Goa, clearly demonstrated that 53 % of actinomycetes strains belonging to *Streptomyces*, 13 % belonging to *Micromonospora* and 10 % belonging to *Actinomadura* genera have the ability of producing antimicrobial compounds. Results demonstrated the fertility of the sampled soils in possessing such microbes which are valued for their unparalleled ability to produce bioactive molecules of biotechnological and pharmaceutical importance.

There are many techniques for detecting antimicrobial activity; most of them are based on methods involving diffusion through solid or semi-solid culture media to inhibit the growth of sensitive microorganisms (Lertcanawanichakul *et al.*, 2008). The cross-streaking is a easy and relatively rapid method for screening cultures in search for new antibiotics and thus establishes a spectrum of inhibiting properties of any bacterium, mold, or actinomycetes which will grow discretely on an agar plate (Williston *et al.*, 1947). Studies have revealed that the 'cross streak method' resulted in higher inhibition zones on indicator bacteria than those obtained by agar well diffusion method (Lertcanawanichakul *et al.*, 2008) but the major drawback of the 'cross streak method' was difficulty in obtaining quantitative data, since the margins of the zone of inhibition were usually very fuzzy and indistinct (Williston *et al.*, 1947).

Thus in the present study, the cross streak method was modified as the streak non uniformity caused a problem with respect to quantification of results (Williston *et al.*, 1947). Besides mutual antibiosis due to use of multiple cultures in the test panel could not be ruled out. The distance between the test streak and the actinomycetes strain was kept constant to reduce the effect of probable mutual antibiosis, as multiple cultures were used in the test panel. Since the test pathogen colonies were delimited within a defined streaking zone, antibiosis would indicate its impact with respect to that zone and thus the antibiosis reaction could be easily quantified.

Inorder to provide enough time for the active organism to produce the antibiotic substance, which will diffuse into the agar medium (Waksman, 1962), the plates were initially inoculated with actinomycetes cultures by a single streak and incubated at 28 °C for 7 days. The plates containing active organisms were kept for 14-28 days to determine whether the test pathogens made any further growth towards the master streak, or they remained stationary, or whether lysis of the test pathogens occurred (Fernando, 1947).

The modified cross streak technique revealed, 93 % of actinomycetes strains with the ability to produce antimicrobial compounds against eight multidrug resistance test pathogens. The multidrug resistance is presently an urgent focus of research and new bioactive compounds are necessary to combat these multidrug resistant pathogens (Selvin *et al.*, 2009; Selvameenal *et al.*, 2009).

4.4.3 Detection of Calcium Oxalate Crystals

In the present study, oxalic acid fluxes diffusing from actinomycetes colony margin and interacting with the growing colonies of test pathogens was observed on Mueller Hinton Agar (MHA) medium. It was thus hypothesized that the excess oxalic

acid, probably bind with the soluble calcium in the medium to precipitate calcium oxalate. Besides, a probable correlation between synthesis and release of oxalic acids and the action of unknown soluble bioactive substances could not be ruled out. As for better antibiosis perhaps oxalic acid production could be necessary and vice versa.

In order to prove or disprove the above hypothesis, an experiment was carried out. On the basis of the experiment, it could be said that actinomycetes strains as well as the test pathogens individually have the ability of producing extracellular calcium oxalate crystals (COC's) on MHA medium and its production was not influenced in the presence of each other. This finding corroborates with that of Gombert *et al.*, (2011), which states that oxalate formation is detrimental to increasing antibiotic yields. The phenomenon also clearly indicates that it is the calcium in the MHA medium which probably triggers oxalate production and its role may be purely to immobilize the heavy cation flux i.e calcium (Li *et al.*, 2003). According to Hi media technical data (NCCLS, 2000), calcium and magnesium ion concentrations are adjusted in the medium to provide the amounts recommended by CLSI to give correct MIC values. The recommended concentration is 20-25 mg Ca per liter or 0.000625 moles (M) or 6.25×10^{-4}. To offset this, a concentration of 2.2 times that is 0.001375 M or 1.375×10^{-3}M oxalate ions are required to produce calcium oxalate as reaction product.

Postulated reaction= $[Ca^{++}] + [C_2O_4^-] = CaC_2O_4^- \rightarrow$ Nucleation>> Crystallization >> simple forms>> Accretion>>elongation >> complex forms>> Druzes.

Another plausible explanation for detecting COC's on specific MHA medium could be the presence of ingredient casein acid hydrolysate in the medium (NCCLS, 2000) which provides casaminoacids and small peptides acting as nucleating templates for crystallogenesis (Sayan *et al.*, 2009).

The COC's produced by actinomycetes was termed as Calcium Oxalate Actinoliths (COAL) and could be defined as typical forms of a biomineral-calcium oxalate in a solid crystalline phase produced in extracellular environment. Its nomenclature is similar to uroliths (kidney stone), otoliths (in fish ear), phytoliths (plants), mycoliths (fungi) etc. In the present study, 47 % of strains producing COAL belonged to Genus *Streptomyces*, which is consistent with the earlier findings that *Streptomyces* produce a wide range of organic and inorganic acids (Abdel-Haliem *et al.*, 2013).

Calcium oxalate crystals have been reported in plants (Frey-Wyssling, 1981), filamentous fungi (Gombert *et al.*, 2011) and bacteria (Chen *et al.*, 2007). A recent study by Chutipongtanate *et al.*, (2013), has shown pathogenic bacteria such as *E. coli*, *K. pneumoniae*, *S. aureus*, and *S. pneumoniae* promoting COC's crystal growth and aggregation in vitro. In plants, COC's usually occur as the monohydrate (whewellite) or as the dihydrate weddellite (Frey-Wyssling, 1981). A number of crystal habits have been reported for both hydration states: raphids, prisms, styloids, druses and crystal sand (Franceschi & Horner, 1980; Monjer & Baran, 2002). Other less common shapes could be considered as variations of the mentioned forms. Till date there is not a single report on detection of calcium oxalate crystals or COAL's on MHA medium during antibiosis.

4.4.4 *Streptomyces parvulus* strain as a potential antibiotic producer

Streptomyces parvulus CFA-9 was selected as a potential antibiotic hyper producer strain for further analysis. The strain was isolated from a forest soil in Canacona, Goa and as mentioned in **Chapter II,** the soil sample is from a lateritic profile on the basement rock known as Peninsular gneiss which is more than 3 billion

years old. Previous studies in India, have shown that *S. parvulus* has been isolated from Pitchavaram mangrove forests (Sivakumar, 2001; Usha, 2011) and Gaderu mangroves composed of mixed vegetation of *Avicennia* and *Excoecaria* (Raghavendrudu & Kondalarao, 2007). There is a single report on *S. parvulus* of terrestrial origin being isolated form gardens of CMS College of Science and Commerce, Coimbatore (Rasheed *et al.*, 2013).

S. parvulus has been reported to produce antibiotics like Actinocin, Actinomycin, Borrelidin, Hydroxyectoine and Manumycin (StreptomeDB, www.pharmaceuticalbioinformatics.de/streptomedb) (Foster & Katz, 1981; Souza *et al.*, 2001). *Streptomyces* species are a proven source of medicinally useful compounds with diverse structures (Baltz, 2008). The genus stands apart from the most bacterial genomes by possessing a detectable gene cluster that encode enzymes for production of secondary metabolites like polyketides, lactams, nonribosomal peptides and terpenes (Omura *et al.*, 2001; Bentley *et al.*, 2002). Recent genome sequencing data has indicated the potential of this genus to produce many more bioactive compounds, many of which are yet to be elucidated (Hwang *et al.*, 2013). It is been estimated that only a fraction of the antibiotics produced by *Streptomyces* strains have been discovered (Watve *et al.*, 2001) thereby, highlighting the substantial effort needed to screen more of these strains for potential bioactive compounds.

4.4.5 Secondary screening

The preliminary broad-spectrum inhibitory action exhibited by *Streptomyces parvulus* CFA-9 was verified during secondary screening (Agar well diffusion assay). The average inhibition zone size observed in preliminary screening was slightly different for the secondary screening stage, but the spectrum of activity remained

unchanged. Agar well diffusion assay showed better and clearer ZOI. The motion in shaken cultures condition, helped keep the organism well dispersed in the liquid medium, permitting good contact between the cell surfaces and the nutrients, thus resulting in maximal growth and better formation of antibiotics (Waksman, 1961). According to Kollef, (2008) use of broad-spectrum antibiotics lowers the risks of death, morbid complications, increased duration of hospital stay and emergence of resistance.

Since assay methods combined with spectroscopic readings result in not only qualitative but also quantitative data, which further help resolve the fate of various components in a CFCF (Porter, 1963). In the present study, the spectroscopic analysis of CFCF revealed it to be a mixture of unidentified small molecular weight aromatic secondary metabolites, peptides or glycopeptides. All of which may be accountable for composite antibiotic action. Antibiotics produced by a particular organism are reported to be rarely formed alone and are often mixtures of complexes of related or totally unrelated antibiotics (Kumar *et al.*, 2009; Oskay, 2011). Besides, the same culture may have the capacity of forming, in the same medium and at the same time, several antibiotics or various chemical modifications of the same antibiotic (Waksman, 1961).

The FTIR spectrum of CFCF offered valuable information about the probable structure of the antibiotic compound by revealing the information on various functional groups present. Analysis of the spectrum showed typical absorption bands corresponding to N-H stretching of proteins and peptide bonds (Maquelin *et al.*, 2002) with concrete evidence that the substance contained is peptide in nature. Additional absorption valleys indicating aliphatic chains may be related to other secondary metabolites present in the crude CFCF.

4.4.6 Impact of different process parameters on antibiotic production

As mentioned earlier, a broad-spectrum antibiotic producing strain *Streptomyces parvulus* CFA-9 exhibiting maximum activity against human pathogenic bacteria *S. citreus* was subjected to various optimization parameters.

Studies dealing with optimizing antibiotic compounds produced by *Streptomyces parvulus* are scarce with the exception of Actinomycin (Foster & Katz, 1981; Souza *et al.*, 2001). However, studies by Genilloud *et al.*, (2011) have shown that inspite of taxonomic relatedness of these strains, the conditions for antibiotic production were strain dependent.

Staphylococcus citreus needs a special mention on account of its pathogenicity. Pure line strains of pathogenic *Staphylococcus aureus* due to spontaneous split gave rise to *S. citreus*, a virulent strain (Pinner & Voldrich, 1932). It causes skin infections, pneumonia, meningitis, endocarditis, toxic shock syndrome, septicemia and arthritis (Dilsen *et al.*, 1961; Chambers, 2001). Novel antibiotics against *S. citreus* has not been reported, although concerted efforts to harness drugs against multi drug resistant strains of *S. aureus* such as MRSA is underway (Demain & Sanchez, 2009).

The ability of actinomycetes cultures to produce antibiotics is not fixed and arises from intracellular intermediates through defined biochemical pathways. It can either be greatly increased or completely lost depending on the conditions in which they are grown (Kavitha & Vijayalakshmi, 2009). The production of most antibiotics is regulated by complex biosynthetic pathways encoded by physically clustered genes (Sevcikova & Kormanec, 2004).

Earlier studies on optimization of various process parameters has led to increase in yields of actinorhodin by *Streptomyces coelicolor* by almost 32 % (Elibol,

2004); natamycin by *Streptomyces natalensis* (Farid *et al.*, 2000); meilingmycin production by *Streptomyces nanchangensis* by 4-fold (Zhuang *et al.*, 2006); penicillin G acylase by *Bacillus* spp. by 2-fold (Rajendhran *et al.*, 2002); and bacteriocin production by *Lactococcus lactis*, also by 2-fold (Li *et al.*, 2002).

4.4.6.1 *Physiological parameters*

Incubation period

Incubation period had a profound effect on antibiotic production with maximum ZOI exhibited after 14 days. Further increase in incubation period led to decrease in the antibiotic production which is in accordance to the previous studies reporting that antibiotic production usually occurs in late exponential and stationary phase (El-Nasser *et al.*, 2010; Singh & Rai, 2012).

Temperature

Maximum antibiotic production at 28 °C was in agreement to the previous reports indicating that optimal temperature for antibiotic production is usually in the range of 26 °C to 35 °C exhibited by several *Streptomyces* species (Elleuch *et al.*, 2010; Mangamuri *et al.*, 2012; Singh & Rai, 2012; Gunda & Charya, 2013; El-Nasser *et al.*, 2010 Elliah *et al.*, 2004; Rizk *et al.*, 2007; Mustafa, 2009; Ghosh and Prasad, 2010; Atta *et al.*, 2011; Vijayakumar *et al.*, 2012).

pH

Antibiotic production in the present study was affected by change in pH of growth medium which is a significant factor affecting nutrient solubility and uptake, enzyme activity, cell membrane morphology by product formation and oxidative

reduction reactions (Bajaj *et al.*, 2009; Vijayabharathi *et al.*, 2012). This study found pH 7 as the optimum for antibiotic production and decrease or increase in these values led to complete loss of antibiotic production. Thus, the data is in accordance to the previous reports illustrating pH 7 as optimal for enhancing antibiotic production, exhibited by most *Streptomyces* sp. strains (Elleuch *et al.*, 2010; Singh & Rai, 2012; Gunda & Charya, 2013).

Besides, *S. coelicolor* (Bystrykh *et al.*, 1996), *S. hygroscopicus* D1.5 (Battacharyya *et al.*, 2009), *S. torulosus* KH-4 (Atta *et al.*, 2010), *S. viridodiastaticus* (El-Nasser *et al.*, 2010), *Streptomyces cheonanensis* (Mangamuri *et al.*, 2012) also showed maximum activity at pH 7. This phenomenon could be attributed to the adaptation of the strain to neutral/alkaline soils from which it has been isolated (Rezuanul *et al.*, 2009).

Effect of light on antibiotic production

According to Waksman, (1961), actinomycetes do not need light for their activities and sudden exposure to light causes its protoplasm to contract and darkness causes the reverse to take place. Besides due to the rise in temperature, partial degradation of the cell contents also occurs. Our results have shown that there is no much difference to the antibiotic production when exposed to either of the conditions.

Effect of autoclaving on antimicrobial activity

Combined temperature and pressure treatment by autoclaving had a considerable effect on the stability and activity of antibiotic compound. However antibiosis with reduce ZOI was still observed indicating either incomplete or partial denaturation of peptide antibiotic or existence of a non peptide secondary metabolite

not destroyed by the treatment. The findings corroborates with those of Singh *et al.*, (2013) and Uddin *et al.*, (2010) who have reported a similar result with respect to *Streptomyces* sp. strain SS12 and *Streptomyces fulvoviridens* respectively. Heat stable active component production by *Streptomyces* has also been reported by Yucel & Yamac, (2010).

4.4.6.2 *Nutritional parameters*

NaCl concentration

Sodium chloride had no significant effect on the antibiotic production and were consistent with previous report with respect to neomycin production (Kavitha & Vijayalakshmi, 2009).

Antibiotic production has been reported to be affected by salt concentration due to the osmotic pressure effect to the medium (Pelczer *et al.*, 1993). The present finding contradict the above mentioned statement but is consistent with the results reported by Usha, (2011) that change of NaCl concentration did not result in significant variation in the antibiotic production.

Organic Carbon and Nitrogen concentration (%)

Antibiotic synthesis is highly dependent on utilization of the preferred carbon sources. The results of this study revealed that maximum antibiotic activity was observed with 0.7 % (v/v) glycerol concentration. Glycerol as the better carbon source for enhancing antibiotic production by *Streptomyces* has been reported in the previous studies (Selvin *et al.*, 2009; Elleuch *et al.*, 2010; Singh & Rai, 2012; da Silva *et al.*, 2012)

According to Shikura *et al.*, (2002) when glycerol is used as the precursor, it forms a β-ketoacyl-CoA, a process similar to polyketide biosynthesis where a dihydroxyacetone-type-C_3 unit is derived from glycerol to create a β-keto ester leading to a γ-butyrolactone autoregulators which is regarded as *Streptomyces* hormones that trigger the onset of secondary metabolism in general and that of antibiotic production in particular.

Other than the carbon, assimilation of nitrogen source is also crucial for antibiotic production and is regulated by complex mechanisms of glutamate synthetases (Selvin *et al.*, 2009; Kavitha & Vijayalakshmi, 2009; Vijayabharathi *et al.*, 2012; da Silva *et al.*, 2012; Rodríguez-García *et al.*, 2009; Saha *et al.*, 2010). Our study revealed 0.03 % casein concentration as the optimal for maximum antibiotic production.

Phosphate concentration (%)

Phosphate is also a major factor in antibiotic biosynthesis and expression of phosphate-regulated genes in *Streptomyces* species, is modulated by the two-component system PhoR-PhoP (Martin & Demain, 1980; Rodríguez-García *et al.*, 2009), Dipotassium hydrogen phosphate (K_2HPO_4) is being reported as the most favorable salt for its production. Our results showed 0.25 % (w/v) phosphate as optimal for antibiotic production. This data corroborates with the findings of Harold, (1996); Kishimoto *et al.*, (1996), Kavitha & Vijayalakshmi, (2009); El-Nasser *et al.*, (2010); Mangamuri *et al.* (2012).

Inorganic Nitrogen and Carbon concentration (%)

Among the inorganic carbon and nitrogen sources, maximum antibiotic production was obtained with 0.0015 % and 0.002 % (w/v) concentration of $CaCO_3$ and 0.1 % (w/v) concentration of KNO_3. $CaCO_3$ being used as a source of Ca^{+2} enhances antibiotic production and also aids in maintaining the pH of the medium (Hamedi *et al.*, 2004; Vastrad & Neelagund, 2011). Potassium nitrate as a superior to other inorganic nitrogen sources has been reported El-Nasser *et al.*, (2010) and present finding confirms the same.

Effect of culture inoculum (%) at varying days of incubation

Optimizing the cell density in industrial fermentation is being considered an important strategy to improve the productivity of final product, shorten its fermentation time and also reduce the volume of fermenters (Cheng *et al.*, 2013). Dobson *et al.*, (2008) demonstrated that the inoculum size not only had an impact on the morphological profiles of *Streptomyces hygroscopicus* var. *geldanus*, but also causes the variations in the production of secondary metabolites.

In the present study, optimal inoculum concentration of 5 % by *Streptomyces parvulus* CFA-09 against *S. citreus* was observed. Antibiotic production decreased with increase in inoculums level thus showing a direct effect on the antibiotic production. This phenomenon could be attributed to depletion of nutrients including the carbon source or directed for the formation of biomass (Usha, 2011). Previous studies by Himabindu & Jetty, (2006) reported 6 % inoculum concentration as optimum for the production of antibiotic Gentamicin. According to Sen & Swaminathan, (2004) the inoculum size plays a crucial role in cell growth and

production yield, which has an impact on the lag phase duration, the biomass yield of the strain, its specific growth rate and the final product quality.

Comparing the SCA production efficacy by differential sampling of its aerial (AM) and substrate mycelium (SM)

Two thirds of all antibiotics are synthesized by Genus *Streptomyces* which makes it an important bacterium for the pharmaceutical industry. The traditional life cycle of *Streptomyces* describes it as a mycelium or branching type hyphae which consists of network of multinucleoid hyphae known as the 'substrate mycelium' which after days of growth produces 'aerial hyphae' that eventually develop chains of spores (Manteca & Snachez, 2010). Antibiotic production switches from substrate to aerial mycelium at the time of development.

The present study, helped in understanding the specific cellular localization of antibiotic production with respect to AM and SM. The results revealed ZOI to be 14 % higher in SM as compared to AM. According to Rigali *et al.*, 2008 the onset of antibiotic production and development has been fairly studied but the detailed understanding of the control mechanisms that tie chemical differentiation is yet to be fully explored.

4.4.7 Testing the relative efficacy of CFCF with standard antibiotics

The study was carried out mainly to compare the relative efficacy of CFCF with standard antibiotics. It showed anti*Staphylococcus* activity was negative with respect to commercial broad spectrum antibiotics namely, Chloramphenicol, Amoxycalv, Ampicillin, Oxacillin, Ticarcillin, Cefodoxime. The results of the present

study were encouraging, with the CFCF showing high efficacy to 35 commercially available antibiotics, indicating presence of a mixture of compounds.

4.4.8 Extraction of antibiotic compounds from CFCF using various solvents

It has been reported that organic solvents always provides a higher efficiency in extracting compounds for antimicrobial activities compared to water based methods (Lima-Filho *et al.*, 2002). Besides, separation of the antibiotics from accompanying impurities is done by taking advantage of differences in solubility between compounds. Previous studies have confirmed extraction method to have a definite effect on the isolation of bioactive principles (Selvin *et al.*, 2009). In this study, substances in the broth were targeted and extracted with both polar and non-polar solvents. The successful extraction of the antibacterial agent with polar solvents indicated that it was a polar organic molecule. The data corroborates with the findings of other workers that the majority of antibiotic compounds are polar (Lancini & Lorenzetti, 1993).

In the present study, all the solvent extracts were subjected to spectroscopic analysis. As mentioned earlier, antibiotic spectra are considered important and useful. The data revealed absorption peaks at various wavelengths for all the solvent extracts. In case of methanolic extract, absorption maxima were observed at 405 nm. An earlier finding by Atta *et al.*, (2009) has shown lipopeptides exhibiting absorption maxima of 350-359 nm. FTIR studies showed characteristic absorption valleys at 1571.99 cm^{-1} indicating presence of a peptide bond. But, the presence of other functional groups indicated that the compound needs further purification.

4.4.9 TLC, Bioautography and chemical identity of antibiotics

Thin layer chromatography (TLC) is the most commonly used preparative technique for separation of chemical compounds and when combined with biological detection method it is known as TLC Bioautography (Marston, 2011).

Bioautography (BAG) is a simple, time saving, benchtop assay technique useful for rapid detection of bioactive compounds such as antibiotics, using TLC plates overlaid with desirable microbial test pathogen. Besides, the technique has an edge over others, as it requires very small amount of the sample and is a target-directed isolation. Among the BAG assays, agar overlay assay provides well defined zones of inhibition and is also not prone to contamination (Marston, 2011). Previous studies has shown the use of this technique for yeasts such as *C. albicans* and bacteria such as *B. subtilis* (Rahalison *et al.*, 1991), *E. coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Saxena *et al.*, 1995).

The present study demonstrated successful application of BAG for detection of antibiotics from soil *Streptomyces*. *Staphylococcus citreus* was used as test pathogen and the technique revealed four distinct TLC spots with differential zone of inhibition.

Thus, the lead provided by BAG could aid in rapid screening of tropical actinomycetes for potential antibiotics as its saves time and cost involved in the battery of bioassay guided extract purification processes (Choma & Grzelak, 2011). Besides, the technique being effective and inexpensive, could be used for rapid screening of a large number of samples not only microbial extracts but also extended to plants and animals extracts (Suleiman *et al.*, 2010; Marston, 2011).

According to Choma & Grzelak, (2011) the bioassay when combined with spectroscopic methods, provides information on both bioactivity and the structure of

the analytes. The present study showed a characteristic peak with absorption maxima (λ_{max}) at 202 nm being observed for all the four positive TLC fractions. It also gave an indication of the presence of a probable peptide amido chromophore moiety and phenoxazone ring (Tai *et al.*, 1976; Silverstein & Webster, 1998). Besides, absorbance at 190-220 nm also showed presence of secondary structural elements like α-helix, β-sheets and random coils (Rosenheck & Doty, 1961).

4.4.10 Chemical characterization of the partially purified antibiotic

The structure of the partially purified compound was elucidated using ESI-MS/MS. Tandem mass spectrometry has become a powerful technique for sequencing peptides and small proteins. Besides due to its sensitivity, it does not require a highly purified material, making detection of minor peptides possible (Tilvi & Naik, 2007). In the present study, the use of the ESI-MS/MS technique to characterize a hybrid polyketide-nonribosomal peptide compound Streptolydigin has been reported. According to Gomez *et al.*, 2011, majority of antibiotic and antitumor drugs belong to the polyketide or non-ribosomal families of natural products.

Streptolydigin production by *Streptomyces lydicus* has been reported by many workers (Schobert & Schlenk, 2008; Liangzhi *et al.*, 2007) but till date there are no reports on production of Streptolydigin by *Streptomyces parvulus.* Although, earlier studies have shown *Streptomyces parvulus* producing notable antibacterial compounds such as Actinocin, Actinomycin, Borrelidin, Hydroxyectoine and Manumycin (StreptomeDB, www.pharmaceuticalbioinformatics.de/streptomedb)

Efforts to investigate the biological activities of Streptolydigin are still in progress with antimicrobial (Temiakoc *et al.*, 2005), anticancer and antitumor activities (Di Ciocci *et al.*, 1980) been reported. The antimicrobial activity is

attributed to the inhibitory action to the bacterial RNA polymerase β- subunit (RNAP) (Horna *et al.*, 2011) and its proposed pathway has been described (Gomez *et al.*, 2011).

SUMMARY

Efforts undertaken to exploit the chemical diversity of these microbial sources, relies on proper understanding of their biological diversity. This study was formulated with the fact that, actinomycetes are widely distributed in the natural soil environment and synthesize numerous natural products of paramount importance. The study provides an overview of the integrated approaches to exploit the diversity and biosynthetic potential of actinomycetes.

The state of Goa as a goldmine of biodiversity has been amply justified by the richness of microbial diversity through these studies. Besides, use of innovative strategies for recovering actinomycetes diversity maximized the chances of exploiting its chemical diversity from vast microbial collections.

In this study, a novel baiting technique was adopted for diversity assessment of soil actinomycetes (Velho-Pereira & Kamat, 2012). The technique showed evidence of actinomycetes dominating the slides at different time intervals. It also showed better improvement over previously described baiting techniques as direct visualization of the growing actinomycetes colonies was accompanied by their subsequent isolation. This in turn, permitted real time still image and video documentation of the growing actinomycetes colonies. Out of the two approaches namely '*Insitu*' and '*Exsitu*' initially adopted for this study, '*Exsitu*' being a much easier and practical approach as compared to '*Insitu*' was applied to all other sampling sites for studying its diversity. Two hundred and fifty five actinomycetes isolates, belonging to six known taxa of actinomycetes were isolated from 23 unexplored soil habitats of Goa. The frequency of the genus *Streptomyces* was 53 %, followed by *Micromonospora* (25 %) and *Actinomadura* (10 %). The taxa *Streptosporangium* (3.9 %), *Saccharomonospora* (2.4 %) and *Nocardia* (1.2 %) showed low percentage of frequency. Highest generic richness was observed in the

sampling site BT, followed by VSN and BTM-1. Thus, the use of this novel baiting technique facilitated specific capturing of actinomycetes diversity and would be useful for rapid isolation efforts in tropical countries rich in biodiversity.

Since actinomycetes are a proven source of industrial enzymes (Prakash *et al.*, 2013). The next objective of the present study was to screen for potential enzymes. Previous enrichments techniques have shown the success of obtaining specific enzyme producers using different baits. Based on this previous knowledge and the success of novel slide baiting technique, a modified technique, was used to bait specific enzyme producers. The strains were screened for xylanase, pectinase, amylase, ligninase and protease (gelatin and casein as substrates) activity. Enzyme producing actinomycetes morphotypic diversity in all sites were high with respect to xylan utilizers (24 %) followed by pectin (19 %), casein (18 %) Poly-R-478 (16 %), gelatin (15 %) and starch (9 %) utilizers. High EPA diversity was observed at PM (21 %) site, followed by MMA-2 (16 %), BOA (16 %), KFA (15 %) and CFA (14 %). Further confirmatory tests of the enzyme activity, revealved 47 % cultures with positive amylase activity. *Streptomyces* sp. CFA-5 exhibiting highest zone of clearance of 4.9 cm was selected for further studies. The strain showed white aerial mycelia, with spiral spore chains. The sporophores were monopodially branched, with open, regular spirals.

Studies on impact parameters for enhancing amylase production revealed 1 % starch and yeast extract as the best carbon and nitrogen sources at pH 6.4 at 60 °C on the 9th day of incubation. The enzyme produced was calcium independent with manganese chloride as an activator showing a comparably better effect on amylase production.

The partially purified enzyme showed a specific activity of 24.22 units/mg protein with a yield of 40.1 % on 23.8 fold purification. The molecular mass of partially purified enzyme was ~41 KDa using diastase as the standard enzyme marker. The partially purified enzyme showed similar pH (6.4), temperature (60 °C) optima and stability as that of the crude enzyme.

Since there is an urgent need for new antibiotics against current antibiotic resistant and opportunistic pathogens, 43 selected actinomycetes strains were screened for antimicrobial activity against various human pathogens of which 32 strains showed positive activity.

For the purpose of better quantification of results and also in ruling out probable mutual antibiosis the cross streak method was modified. Data was computed for the quantification of: Percent area specific differential antibiotic activity score (PASDAAS), Percent multispecific antibiosis efficiency score (PMSAES), Percent overall inhibition efficiency score (POIES), Percent overall screening efficiency score (POSES). Forty percent of the strains inhibited *S. typhi*, 37 % strains inhibited *E. aerogens*, 77 % strains inhibited *S. aureus*, 57 % strains inhibited *B. subtilis* whereas 47 % strains inhibited *S. flexneri* and *S. marcescens* respectively and 60 % of the strains inhibited *Proteus* sp. and *C. albicans* respectively.

During the screening efforts, oxalic acid fluxes diffusing from actinomycetes colony margin and interacting with the growing colonies of test pathogens was observed on Mueller Hinton Agar (MHA) medium. The study further led to the understanding that actinomycetes strains as well as the test pathogens individually have the ability of producing extracellular calcium oxalate crystals (COC's) on MHA medium. The study ruled out the possibility that its production was not influenced in the presence of each other but it is due to the calcium in the MHA medium which

probably triggered its production to immobilize the heavy cation flux. The COC's produced by actinomycetes was termed as Calcium Oxalate Actinoliths (COAL) and was defined as typical forms of a biomineral-calcium oxalate in a solid crystalline phase produced in extracellular environment.

Further confirmation of the antimicrobial activity revealed an *Streptomyces parvulus* CFA-9, with broad spectrum antimicrobial activity of 66.7 % against human pathogens namely Gram negative bacteria *S. flexneri*, *E. aerogens* and Gram positive bacteria *B. subtilis*, *S. typhi* and *S. citreus* was selected. Highest zone of inhibition (31 mm) was observed against *S. citreus*. The strain exhibited grey aerial mycelium, with spiral spore chains and produced a bright yellow pigment. Sporophores were monopodially branched, with open, regular spirals.

Nutritional and physiological parameters produced by *S. parvulus* under shaken flask conditions were determined. Optimization of these parameters led to 35.5 % increase in zone of inhibition. Highest antibiotic production was obtained at 250 rpm for 14 days with optimum temperature of 28 °C and pH 7 with 5 % (v/v) concentration of the preculture. Kuster's modified medium containing glycerol, 0.7 % (v/v); casein, 0.03 % (w/v); phosphate, 0.25 % (w/v); KNO_3, 0.1 % (w/v) and $CaCO_3$, 0.0015 % (w/v) concentration was found ideal and statistically significant ($p \leq 0.05$). The present findings also showed no significant difference in the effect of antibiotic production when exposed to light or dark conditions. It was observed that heat treatment did not affect antibiosis but only reduced the size of the zone of inhibition. Study on effect of submerged (SM) and aerial mycelium (AM) revealed SM showing 14 % higher efficacy of producing antibiotic/s than AM and the data was statistically significant ($p \leq 0.05$).

The crude antibiotic when tested for its relative efficacy with standard antibiotics showed higher efficacy to almost 35 out of 36 antibiotics used against *Staphylococcus* spp. infection. The study to extract compounds using various solvents revealed methanol as an ideal solvent for extraction of compounds. FTIR studies of the methanolic extract showed characteristic absorption valleys at 1571.99 cm^{-1} indicating presence of a peptide bond. But, the presence of other functional groups indicated that the compound needed further purification.

Inorder to isolate antibiotic compounds from the crude methanolic extract by TLC, Chloroform: methanol (5:1 v/v) was selected as the best solvent system. Thin layer chromatography (TLC) was then combined with biological detection method - TLC Bioautography (Marston, 2011). Bioautography studies revealed that out of nine spots (SA-SI) detected in TLC, four spots (SB, SE, SH and SI) showed clear visible inhibition against the test pathogen.

Mass cultivation of the culture for large scale production of the antibacterial compound was carried out. Purification of the crude antibiotic compound using column chromatography (Sephadex LH-20) involving appropriate separation technique was done. The structure of the partially purified compound was elucidated using ESI-MS/MS which showed peak at m/z 587 $[M+H]^+$. The literature report suggested that the molecular mass m/z 586 was close to Streptolydigin molecule (Horna *et al.*, 2011). The possible fragmentation pattern confirmed that the modification takes place at the tetramic acid lateral side chain with demethylation of Streptolydigin.

Results of predicted elemental analysis showed the presence of C, H, O and N in 63.36 %, 7.37 %, 4.77 %, 24.50 % using the Strepdom database (Lucas *et al.*, 2013). The predicted molecular formula of compound EAM (SG) 5 was elucidated as

$C_{31}H_{43}N_2O_9$ and was determined by interpretating the data from elemental analysis. The present finding highlights the importance for further investigation towards the goal of obtaining compounds from this untapped habitat. Thus suggesting that the microbial diversiy of the state of Goa has to be further explored in order to get benefit out of the precious biowealth.

Outcome of research

- Fills the gap in knowledge as there is sporadic work on actinomycetes diversity reported from soils of Goa.
- Formulation of a novel modified medium (AVA) and its antibacterial/antifungal cocktail.
- Development of simple technique for rapid and high frequency isolation of soil actinomycetes.
- Modified baiting slide technique to specifically capture, detect and isolate enzyme producers.
- Identified a industrially important *Streptomyces* strain which produced an thermostable and calcium independent enzyme, a desirable feature for industrial applications.
- Identified another industrially important *Streptomyces* strain with broad spectrum activity against various antibiotic resistant clinical test pathogens.
- Modified the cross streak antimicrobial screening technique for better quantification of the antibiotic principle (Velho-Pereira & Kamat, 2011).
- Reports for the first time a hybrid polyketide peptide antibiotic from soil dwelling *Streptomyces parvulus.*

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APPENDIX

Media

Kuster's broth

Composition

Glycerol	0.8 % v/v
Casein	0.03 % w/v
NaCl	20,000 ppm w/v
KNO_3	0.2 % w/v
K_2HPO_4	0.2 % w/v
$MgSO_4.7H_2O$	0.005 % w/v
$CaCO_3$	0.001 % w/v
pH	7±0.1

Suspend the ingredients in 100 ml of distilled water. Heat to boiling to dissolve the medium completely. Adjust the pH. Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 minutes. Mix well before pouring.

Mueller Hinton Agar

Composition	**Gms/Litre**
Beef infusion form	300.00
Casein acid hydrolysate	17.5
Starch	1.5
Agar	17
pH	7.3±0.1

Suspend 38 grams in 1000 ml of distilled water. Heat to boiling for dissolving the medium components completely. Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 minutes. Mix well before pouring.

Nutrient Agar

Nutrient Agar	37 g
Distilled water	1000 ml

Reagents

Iodine potassium iodide solution

Iodine Crystals	0.6 % (w/v)
Potassium iodide	6 % (w/v)
Distilled water	100 ml

Congo red solution

Congo red	0.5 % (w/v)
Ethanol	5 % (w/v)
Distilled water	100 ml

Crystal violet

Crystal violet	2 g
Ethyl alcohol	20 ml 95%
Ammonium citrate monohydrate	0.8 g
Deionized water	80 ml

Dissolve 2 g crystal violet in 20 ml of 95% ethyl alcohol. Dissolve 0.8 g ammonium oxalate monohydrate in 80 ml deionized water. Mix the crystal violet and ammonium

oxalate monohydrate solutions to make the crystal violet stain. Filter the stain if necessary.

DNS reagent

Potassium sodium tartarate (Rochelle salt)	15g
DNSA	0.5 g in 10 ml of 2N NaOH
Distilled water	50 ml

Dissolve by stirring (Remi, India) at room temperature (RT) DNSA, then added Rochelle salt. Mix well. The reagent to be stored at RT.

Folin-Lowry

Reagent A: 2% Na_2CO_3 in 0.1 N NaOH

Reagent B: 0.5 % $CuSO_4$ in 1% Rochelle salt solution

Reagent C (alkaline copper solution): 50 mL of Reagent A + 1 mL of Reagent B

Folins-phenol reagent: Commercial Folins-phenol reagent was used after dilution in a 1:1 ratio with ddH2O.

Ammonium sulphate saturation chart

Initial concentration of ammonium sulfate	Percentage saturation at 0°																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
	Solid ammonium sulfate (grams) to be added to 1 liter of solution																
0	106	134	164	194	226	258	291	326	361	398	436	476	516	559	603	650	697
5	79	108	137	166	197	229	262	296	331	368	405	444	484	526	570	615	662
10	53	81	109	139	169	200	233	266	301	337	374	412	452	493	536	581	627
15	26	54	82	111	141	172	204	237	271	306	343	381	420	460	503	547	592
20	0	27	55	83	113	143	175	207	241	276	312	349	387	427	469	512	557
25		0	27	56	84	115	146	179	211	245	280	317	355	395	436	478	522
30			0	28	56	86	117	148	181	214	249	285	323	362	402	445	488
35				0	28	57	87	118	151	184	218	254	291	329	369	410	453
40					0	29	58	89	120	153	187	222	258	296	335	376	418
45						0	29	59	90	123	156	190	226	263	302	342	383
50							0	30	60	92	125	159	194	230	268	308	348
55								0	30	61	93	127	161	197	235	273	313
60									0	31	62	95	129	164	201	239	279
65										0	31	63	97	132	168	205	244
70											0	32	65	99	134	171	209
75												0	32	66	101	137	174
80													0	33	67	103	139
85														0	34	68	105
90															0	34	70
95																0	35
100																	0

SDS-PAGE

Composition of separating and stacking gel

Stock	Separating gel (10%)	Stacking gel (4%)
Acrylamide solution (30%) Bis acrylamide (0.8%)	3.3 ml	1.5 ml
Tris-HCl (1.875M, pH 8.8)	2.5 ml	---
Tris-HCl (0.6M, pH 6.8)	---	2.5 ml
Water	4 ml	6 ml
SDS (10%)	0.1 ml	0.1 ml
APS (5%)	50 µl	50 µl
TEMED	25 µl	20 µl

Tank buffer

Tris-HCl	0.05M
Glycine	0.192M
SDS	0.1%

Staining solution

1 % Coomassie brilliant blue in methanol, glacial acetic acid and distilled water

Destaining solution

Methanol	50 %
Glacial acetic acid	10 %
Distilled water	

Acetate buffer

Stock solutions

A: 0.2M solution of acetic acid (11.55 ml in 1000 ml)

B: 0.2M solution of sodium acetate (16.4g of $C_2H_3O_2Na$ or 27.2g of $C_2H_3O_2Na.3H2O$ in 1000ml)

x ml. of A + Y ml. of B, diluted to a total of 100ml.

x	y	pH
46.3	3.7	3.6
30.5	19.5	4.4
10.5	39.5	5.2

Phosphate buffer

Stock solutions

A: 0.2M solution of monobasic sodium phosphate (27.8 g in 1000 ml)

B: 0.2M solution of dibasic sodium phosphate (53.65 g of $Na_2HPO_4.7H2O$ in 1000 ml)

x ml. of A + Y ml. of B, diluted to a total of 200 ml.

x	y	pH
92.0	8.0	5.8
73.5	26.5	6.4
51.0	49.0	6.8
39.0	61.0	7.0
19.0	81.0	7.4
8.5	91.5	7.8

Tris (hydroxymethly) aminomethane (Tris) Buffer

Stock solutions

A: 0.2M solution of Tris (hydroxymethly) aminomethane (24.2 g in 1000 ml)

B: 0.2M HCl

50 ml. of A + Y ml. of B, diluted to a total of 200 ml.

x	pH
5.0	9.0
12.2	8.6

PUBLICATIONS

Research publications

Velho-Pereira S. & Kamat N. M. Optimization of anti *Staphylococcus* antibiotic produced by tropical soil dwelling *Streptomyces parvulus*. Indian J. Exp. Biol. (*communicated*).

Velho-Pereira S. & Kamat N. M. Actinobacteriological research in India. Indian J. Exp. Biol. 2013; 51: 573-597. **(Appendix I)**

Velho-Pereira S. & Kamat N. M. A novel baiting technique for diversity assessment of soil actinobacteria in a laboratory microcosm. Int. J. Biotech & Biosci. 2012; 2(2): 217-220. **(Appendix II)**

Velho-Pereira S. & Kamat N. M. Screening of actinobacteria for antimicrobial activities by a modified cross streak method. Ind. J. Pharm. Sci. 2011; 73(2): 125-254. **(Appendix III)**

Research presentations:

Poster

Velho-Pereira S. & Kamat N. M. **"Exploring hidden dimension of soil fungal biodiversity: A simple technique to detect soil fungi resistant to antifungal compounds"** at the National Seminar on 'Fungal Biodiversity and Bioprospecting in the Age of Global Warming' and 36th Annual meeting of the Mycological Society of India organized by UGC-SAP, Department of Botany, Goa University, Goa during 29-30th Oct, 2009 at Goa University, Goa.

Oral

Velho-Pereira S. & Kamat N. M. **"Application and potential of bioautography for rapid screening of tropical actinobacteria for antibiotic activity"** at the National

Conference on Plant Sciences organized by Department of Botany, Parvatibhai Chowgule College of Arts and Science, Margao, Goa on 7-8th February, 2013. **Awarded second prize for best presentation.**

Velho-Pereira S. & Kamat N. M. Presented a research paper entitled **"How Natural Catastrophes and Human Interference could affect soil microbial diversity of Goa: Ecological and Economic implications and an action plan for sustainable soil microbial resources management"** at the State level Seminar on 'Environment: Issues, Concerns and Alternatives' organized by Research cell at Government College of Arts and Science, Marcela, Goa on 8th January, 2010.

Workshops participated:

'**National Workshop on Immunoinformatics**' organized by Bioinformatics centre, University of Pune, Pune on 5th - 10th April, 2011. **Awarded for best performance in hands-on session.**

'**Biodiversity Awareness**' workshop organized by Department of Botany, Goa University, Goa on 3rd July 2010 and assisted Dr. Nandkumar M. Kamat, the coordinator of the workshop in editing a DVD on different aspects of biodiversity.

Indian Journal of Experimental Biology
Vol. 51, August 2013, pp. 573-596

Review Article

Actinobacteriological research in India

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Actinobacteria are important sources of compounds for drug discovery and have attracted considerable pharmaceutical, chemical, agricultural and industrial interests. Actinobacteriological research is still in its infancy in India. Early work on actinobacteria started in the 20th century and mostly focused on studying the diversity, identification and screening for antibiotics, enzymes and enzyme inhibitors. Exploration of diverse habitats for the isolation of actinobacteria, have yielded till date 23 novel species. Screening of actinobacteria for antagonistic activity, has led to the discovery of four novel antibiotics. Research on enzymes mostly covered lipases, amylases, proteases, endoglucanases, α-galactosidases, pectin lyases, xylanases, L-asparaginases, L-glutaminase and cellulases. Research on exploiting actinobacteria for other purposes such as production of enzyme inhibitors, single cell protein, bioemulsifier and biosurfactants is still in the experimental stage. This review compiles the work done in last few years, with an emphasis on actinobacterial diversity and bioprospecting for pharmaceutically important compounds like antibiotics, enzymes and other important applications. The chemical creativity and biotechnological potential of Indian actinobacterial strains are yet to be fully explored. A national strategy is required consistent with the opportunities provided by CBD-Nagoya protocol.

Keywords: Actinobacteria, Antibiotics, Biodiversity, Bioprospecting, Enzymes, India

Being ubiquitous in nature, actinobacteria are sources of bioactive compounds for pharmaceutical purposes. According to Baltz[1] only a fraction of the world's biodiversity has been explored with less than one part of the Earth's soil surface screened for potential actinobacteria. The terrestrial soil has been widely exploited for isolation of actinobacteria wherein they perform significant biogeochemical roles contributing to the turnover of complex biopolymers[2]. However, despite scarce studies on diversity of actinobacteria from marine environment, they are proved to be potential producers of novel bioactive compounds[3]. Actinobacteria hold a prominent position as targets in screening programmes due to their diversity and account for the production of most of the discovered bioactive secondary metabolites, primarily antibiotics[2,4], immunosuppressive agents, enzymes[5] and enzyme inhibitors[6]. With the rise in infectious diseases and ~25-30,000 clinically described human diseases, accounting for many deaths, novel antibiotics are in demand as the prevalent antibiotics are slowly loosing their existing potencies[7]. Owing to the globally growing challenge of antibiotic resistant microbial strains, antibiotics drug discovery programmes have been undertaken and novel approaches are being followed[8]. Such approaches include, searching for the less or unexploited ecosystems for isolation of less studied rare actinobacteria expected to yield novel metabolites[9] or by using modern strategies like proteomic signatures with high throughput *in vitro* assays[8], whole cell screening methods, sequencing of the actinobacterial genomes and combinatorial biosynthesis[1,7]. This review focuses on biodiversity and bioprospecting of actinobacteria from India, aimed at pharmaceutically important antibiotics, enzymes and other applications.

In view of a rapid decline in the rate of discovery of new genera and metabolites observed in the beginning of the 20th century and the period from 2011-2012, being the most productive, in terms of actinobacterial diversity and biosprospecting (Fig. 1), actinobacteriological research is still in its infancy in India. However, there are still active groups making useful contributions. Top five institutions/laboratories, on the

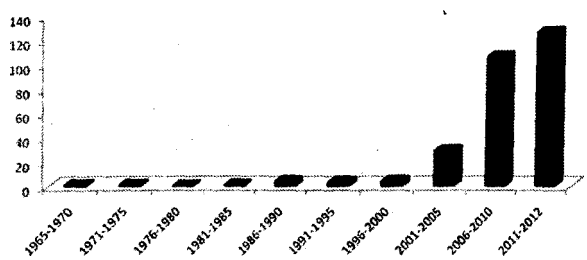

Fig. 1—Indian publications on actinobacterial diversity and bioprospecting since 1965.

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basis of interity of research efforts as evidenced by publications are indicated in Table 1.

Biodiversity and taxonomy

Baltz[1] opined that approaches to explore random and exotic soil, could accelerate the production of bioactive substances from actinobacteria. In view of this, in India diverse habitats and locations (Fig. 2) have been screened for studying actinobacterial diversity. Soil habitats (96%) have been largely surveyed as compared to other resources like endophytic plants (3%) and animal guts (1%). On the other hand, the percentage of actinobacterial isolations from marine resources (85%) is much higher than freshwater resources (15%).

Table 1—Top five institutions engaged in actinobacteriological research

Universities	Research laboratories
VIT University	CSIR-National Chemical Laboratory, Pune
Bharathidasan University	Research Centre, Hoechst India Limited, Mulund
Annamalai University	CSIR-National Institute for Interdisciplinary Science and Technology, Trivandrum
Andhra University	National Bureau of Agricultural important Microorganisms, Mau
Periyar University	CSIR-Central Drug Research Institute, Lucknow

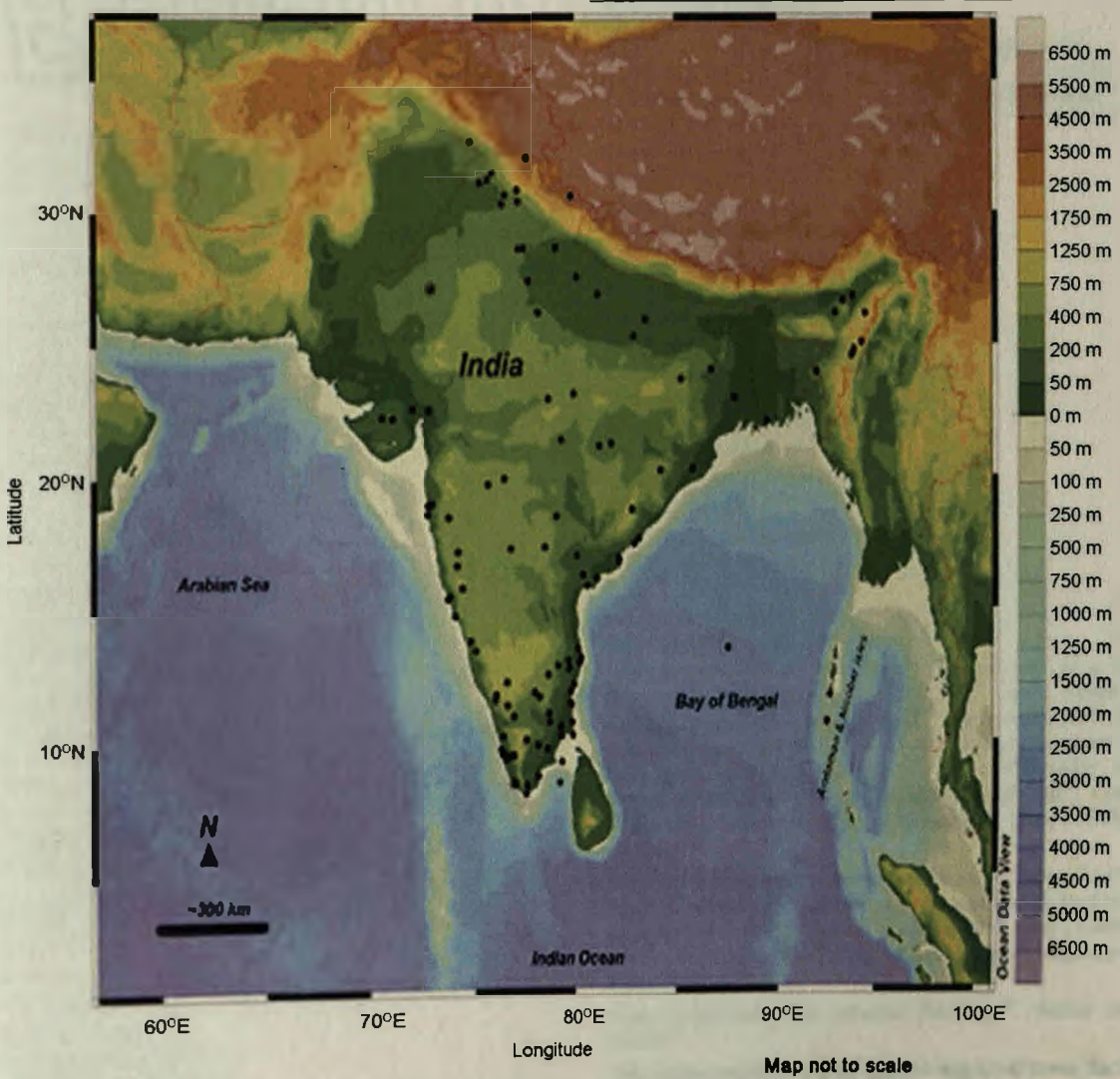

Fig. 2—Distribution of habitats sampled in India for actinobacteria.

Research on actinobacterial diversity from underexplored ecological niches of terrestrial ecosystems from India, has led to the isolation of 26 actinobacterial genera, with genus *Streptomyces* being the most dominant, followed by *Micromonopora, Actinomadura, Rhodococcus, Microbispora, Nocardia* and others (Table 2).

Table 2—Actinobacteria from terrestrial ecosystems

Species[ref]

Soil inhabitants

Genus *Streptomyces* [4, 10-43]

S. aurantiacus[•], *S. citricolor*[•], *S. clavuligerus*[•], *S. gulbargensis* sp. nov.[•], *S. kavamyceticus*[•], *S. platensis*[•], *S. spectabilis*[•], *S. albidoflavus*[♦], *S. erumpens** *S. griseoruber*[■], *S. hyderabadensis* sp. nov.[▲], *S. hygroscopicus* subsp. *ossamyceticus*,[◊] *S. manipurensis* sp. nov.[■], *S. sannanensis*[▶], *S. sannanensis*,[♦] *S. tendae*[○], *S. viridis*[○], *Streptomyces* sp.[•,■,▲,■,▶,♦,◊,■,♂,♀]

Genus *Micromonospora*[23, 40, 43, 44]

Micromonospora sp.[▲,▶,♦]

Genus *Actinomadura*[12, 41-43]

A. roseale[•], *Actinomadura* sp.[■,♂,θ]

Genus *Rhodococcus*[45-48]

R. kroppenstedtii sp. nov.[Ψ], *R. canchipurensis* sp. nov.[■], *R. imtechensis*[Ω], *Rhodococcus* sp.[■]

Genus *Microbispora*[23, 49]

M. indica sp. nov.[♦], *M. karnatakensis* sp. nov.[♦], *Microbispora sp.*[▶]

Genus *Nocardia*[21, 40, 42]

Nocardia sp.[▲,♂]

Genus *Kitasatospora*[23, 50]

Kitasatospora sampliensis sp. nov.[♦], *Kitasatospora* sp[▶]

Genus *Kocuria*[51, 52]

Kocuria himachalensis sp. nov.[Ψ], *Kocuria* sp.[▶]

Genus *Streptosporangium*[41, 42]

Streptosporangium sp.[■,♂]

Genus *Thermoactinomyces*[42, 53]

T. thalophilus[▶] *Thermoactinomyces* sp.[♂]

Genus *Actinoplanes*[23]

Actinoplanes sp.[▶]

Genus *Actinoalloteichus*[54]

A. spitiensis sp. nov.[Ψ]

Genus *Agrococcus*[55, 56]

A. carbonis sp. nov.[ς], *A. lahaulensis* sp. nov.[Ψ]

Genus *Arthrobacter*[57]

Arthrobacter sp.[§]

Genus *Brevibacterium*[48]

Brevibacterium caseí[■]

Genus *Microbacterium*[58]

M. immunditiarum sp. nov.[♦]

Genus *Micrococcus*[57]

M. lactis sp. nov.[§]

Genus *Norcardiopsis*[59]

N. prasina[■]

Genus *Planomonospora*[23]

Planomonospora sp.[▶]

Genus *Planococcus*[60]

P. stackebrandtii sp. nov.[Ψ]

Genus *Saccharomonospora*[61]

S. saliphila sp. nov.[◊]

Table 2—Actinobacteria from terrestrial ecosystems

Genus *Saccharopolyspora*[42]

Saccharopolyspora sp.[♂]

Genus *Streptoverticillium*[23]

Streptoverticillium sp.[▶]

Genus *Thermomonospora*[62]

Thermomonospora sp.[Þ]

Genus *Yaniella*[63]

Y. fodinae sp. nov.[ς]

Plant endophytes

Genus *Streptomyces*[64, 65, 66]

S. aureus[ю,ψ], *S. hydroscopicus*[ω,ψ], *S. greseofuscus*[ю,ψ], *S. albosporus*[ю,ψ], *S. cinereus*[ω,ю,ψ], *S. flavus*[ψ], *S. cyaneus*[ψ], *S. globisporus*[ю,ψ], *S. viridis*[ψ], *S. glaucus*[ω], *S. lavendulae*[ю,ω], *S. griseorubroviolaceus*[ω], *Streptomyces* sp.[•,ω,ю,ψ,ε,◄,☼,ϰ,ĉ,d]

Genus *Nocardia*[64, 66]

Nocardia sp.[ω,ю,ⓜ,ǂ,й,≈]

Genus *Actinomadura*[66]

Actinomadura sp.[F,Θ,η]

Genus *Microbispora*[64, 66]

Microbispora sp.[ε,ω,ю,ψ]

Genus *Nocardiopsis*[66]

Nocardiopsis sp.[б]

Genus *Sacchromonospora*[64]

Sacchromonospora sp.[ψ]

Genus *Saccharopolyspora*[66]

Saccharopolyspora sp.[ч]

Genus *Streptosporangium*[64]

Streptosporangium sp.[ω,ю,ψ]

Genus *Streptoverticillium*[64]

Streptoverticillium sp.[ω,ψ]

Gut

Genus *Streptomyces*[67, 68]

S. tritolerans sp.nov.[й], *S. noursei*[ъ]

Soil inhabitants: Black soils[•], Surface soil of a landfill[•], Brick-kiln soil*,Rhizospheric soil[■], Red soil[▲], Desert soil,[■] Limestone quarry[◊], Alkaline soil[▶], Agricultural soil[♦], Lateritic soil[○], Bitumen (heavy crude oil) soil[■], Solitary wasp and swallow bird mud nest[♂],Tree Hollow[♂],Forest soil[♦],Termite mounds[ψ], Indian Himalayas[Ψ], Pesticide-contaminated soil[Ω], Coal mine soil[ς],Dairy industry effluent treatment plant[§], Self-heating compost[Þ]

Plant endophytes: *Azadirachta indica* stem[ω], leaves[ю] and roots[ψ]; *Vigna mungo* nodule surface[•]; *Coleus* leaf[≈], root[ψ]; *Barleria* root[◄]; *Coelogyne ovalis* root[☼], leaf[ϰ], seed[ĉ], petiole[η], stem[ǂ]; *Carthranthus* leaf[d]; *Plumbago* leaf[ⓜ], stem[ч]; *Citrullus* root[й]; *Asparagus* root[F]; *Aloevera* stem[Θ], leaf[б]

Gut: Earthworm gut (*Eisenia foetida*)[й], Indian silkworm breeds[ъ]

The novel actinobacterial species reported from India have been underlined

Isolation strategies like novel baiting bag[69], baiting slide[41] and enrichment culture[70] techniques to isolate uncommon and rare genera have also been reported. Besides soil habitats, nature's other reservoirs rich in therapeutic compounds are endophytic plants and animals. Oceans cover 70% of the earth's surface and support richest ecosystems of the earth in terms of microbial diversity. Indian efforts to isolate marine actinobacteria have yielded 30 actinobacterial genera, with genus *Streptomyces* being the major component of the total actinobacterial population followed by genus *Micromonospora, Actinopolyspora, Saccharopolyspora, Actinomadura* and others. Both freshwater and marine habitats are considered dynamic in nature, however, there are sporadic reports on actinobacteria from sponges, bivalves, corals and guts of marine organisms (Table 3).

Table 3—Actinobacteria from Aquatic ecosystems

Species[ref]

Fresh water ecosystem

Genus *Streptomyces*[71-76]
S. tanashiensis[Φ], *S. sindenensis*[Φ], *Streptomyces sp.*[ώ]

Genus *Georgenia*[77]
G. satyanarayanai sp. nov.[ж]

Genus *Kocuria*[78]
K. assamensis sp. nov.[ч]

Marine ecosystem

Genus *Streptomyces*[79-108]
S. afghaniensis[δ], *S. albus*[δ], *S. albus* gangavarams[δ], *S. carpaticus*[δ], *S. cheonanensis*[Ђ], *S. griseoloalbus*[Ђ], *S. marinensis*[δ], *S. peucetius*[d], *S. rochei*[±], *S. sundarbansensis* sp. nov.[Ђ], *S. cyaneus*[δ], *Streptomyces* sp.[δ,Ђ]

Genus *Micromonospora*[80, 91, 101, 103, 106, 108, 109]
M. echinospora[δ], *Micromonospora* sp.[δ,Ђ]

Genus *Actinopolyspora*[90, 91, 103, 106, 110, 111]
Actinopolyspora sp.[δ,Ђ,d]

Genus *Saccharopolyspora*[80, 91, 103, 106, 112, 113]
S. hirsute[δ], *S. salina*[δ], *Saccharopolyspora* sp.[δ,Ђ]

Genus *Actinomadura*[80, 91, 103, 106, 109]
A. citrea[δ], *Actinomadura* sp.[δ,Ђ]

Genus *Actinoplanes*[91, 106, 109]
Actinoplanes sp.[δ]

Genus *Microbispora*[91, 106, 109]
Microbispora sp.[δ,Ђ]

Genus *Nocardiopsis*[99, 103, 106]
Nocardiopsis sp.[δ,Ђ]

Genus *Actinomyces*[89, 106]
Actinomyces sp.[δ,d]

Genus *Kitasatospora*[106, 108]
Kitasatospora sp.[Ђ]

Genus *Nocardia*[106, 108]
Nocardia sp.[Ђ]

Genus *Pseudonocardia*[108, 114]
P. endophytica[Ђ], *Pseudonocardia* sp.[Ђ]

Genus *Streptoverticillium*[80, 106]
S. album[δ], *Streptoverticillium* sp.[Ђ]

Genus *Actinobispora*[80]
A. yunnanensis[δ]

Genus *Actinodassonvillei*[108]
Actinodassonvillei sp.[Ђ]

Genus *Actinosynnema*[108]
Actinosynnema sp.[Ђ]

Table 3—Actinobacteria from Aquatic ecosystems

Genus *Agromyces*[115]
Agromyces indicus sp. nov.[Ђ]

Genus *Amycolatopsis*[116]
Amycolatopsis alba var. nov[δ]

Genus *Gordona*[108]
Gordona sp.[Ђ]

Genus *Intrasporangium*[108]
Intrasporangium sp.[Ђ]

Genus *Microtetrospora*[80]
M. fastidiosa[δ]

Genus *Nocardiodes*[109]
Nocardiodes sp.[Ђ]

Genus *Rhodococcus*[108]
Rhodococcus sp.[Ђ]

Genus *Saccharomonospora*[80]
S. viridis[δ]

Genus *Streptoalloteichus*[106]
Streptoalloteichus sp.[Ђ]

Genus *Streptosporangium*[106]
Streptosporangium sp.[Ђ]

Genus *Thermomonospora*[80]
T. mesophila[δ]

Marine organisms

Genus *Streptomyces*[117-125]
S. noursei[□] *S. canus*[≠] *S. rimosus*[•] *Streptomyces* sp.[□,ч,Ξ,□] ◄

Marine Wastes/Polluted areas

Genus *Rhodococcus*[126]
Rhodococcus sp.[σ]

Genus *Streptomyces*[127]
Streptomyces sp.[θ]

Fresh water ecosystem: lake[Φ], spring[ώ], soda lake[ж], river[ч]

Marine ecosystem: Sandy shores[δ], Mangroves[Ђ], Estuarine[d], Surface waters[±]

Marine organisms: Sponge , Finfish-*Mugil cephalus*[≠], Gut contents of *Chanos chanos*[•], *Chaetodon collare* (Red tail butterfly) and *Archamia fucata* (Orange-lined cardinal)[□], Fish red snapper[Ξ], *Villorita cyprinoids*[□], Bivalves Meretrix casts (Gmelin) ◄

Marine Wastes/Polluted areas: Oil polluted coastal region[σ], Heap of marine wastes[θ]

The novel actinobacterial species reported from India have been underlined

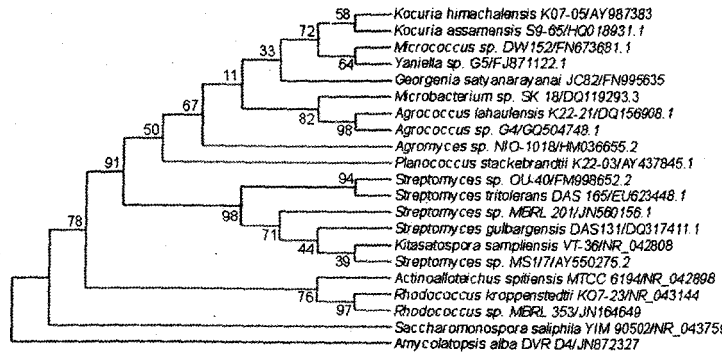

Fig. 3—Phylogram indicating the placement and relatedness of 21 novel Indian actinobacterial strains within the order Actinomycetales under Actinobacteria. Numbers given at the branch nodes indicate (%) bootstrap values. Bar 0.01 substitutions per 100 nucleotide positions.

Exploration of such unique habitats has led to the discovery of 23 novel taxa (Tables 2 and 3). Among these novel strains, five belong to genus *Steptomyces;* two to genus *Agrococcus, Rhodococcus, Microbispora, Kocuria* each and strains belonging to genus *Actinoalloteichus, Planococcus, Kitasatospora, Micrococcus, Georgenia, Saccharomonospora, Microbacterium, Yaniella, Amycolatopsis* and *Agromyces*.

Phylogenetic relationship of novel Indian actinobacterial taxa—It is interesting to study the phylogenetic relationship of these taxa, so using MEGA5 software and 16S rDNA sequences available at NCBI (http://www.ncbi.nlm.nih.gov/genbank/), phylogenetic relationship of 21 Indian actinobacterial species was determined by neighbor joining method (Fig. 3).

Bioprospecting for pharmaceutically important compounds

Antibotics—Actinobacteria are noteworthy antibiotic producers and have yielded ~3,000 known antibiotics. Genus *Streptomyces* produces 75% of all these[128]. Baltz[129] estimated that only 1-3% of Streptomycete antibiotics are so far discovered. Thus there is an urgent need of producing novel antibiotics keeping in view the ever increasing rise of resistant pathogens.

Strategies employed elsewhere in the world to obtain novel antibiotics from unexplored habitats have been used in India. Screening efforts made to obtain antibiotics against human pathogens is much higher than those against plant and animal pathogens.

Since Indian research on antibiotic discovery, has focused mostly on preliminary screening and optimization of the various culture conditions, for antibacterial and antifungal compounds, further studies to reveal the identity of the bioactive molecules is on decline. A few reports however hold much promise for future. These include a report from Gorajana *et al.*[130], reporting a cytotoxic compound 1, hydroxyl-1-norresistomycin from marine actinobacteria *Streptomyces chibaensis*. Resistomycin an anticancer compound, also showing antimicrobial activities produced by *S. aurantiacus* was reported by Vijayabharathi *et al.*[10]. Parthasarathi *et al.*[131], reported a broad spectrum antimicrobial compound, 7, demethoxyrapamycin produced by *Streptomyces hydroscopicus*. Besides, four novel antibiotics include namely Swalpamycin, Butalactin, Alisamycin and 1(10-aminodecyl) Pyridinium salt (Table 4).

Process optimization—Reports on optimization of physiological and biochemical parameters to increase the yield of the metabolites revealed that, different concentrations of glucose[133-136], glycerol[137,138], starch[139], arabinose and sucrose[140] as carbon sources and nitrogen sources such as yeast extract[141], peptone[137,140], soyabean[71,133,139], sodium nitrate and potassium nitrate[138], ammonium dihydrogen phosphate[136], liver extract[137] as optimal biochemical parameters and alkaline *p*H ranging from 7-8[11,59,71,99,134-137,139-141] and temperature ranging from 25-35 °C [11,59,69,99,135-141,180] are optimal physiological parameters.

Antimicrobials vis á vis humans pathogens—Dawn of antibiotic era witnessed a decline of infectious diseases however, the new threat or multidrug resistant strains has created an urgency to promote novel antibiotic discoveries centered at antinobacteria[1,8].

Indian research, on harnessing antibiotics from actinobacterial resources has accelerated. However, the work is so far limited to mostly preliminary screening. Large number of actinobacterial strains

Table 4—Novel metabolites from Indian actinobacterial strains

Strain[ref]	Novel metabolite	Antibiotic group	Reported activity
Streptomyces sp. Y-84,30967[35]	Swalpamycin	Macrolide	Against Gram-positive bacteria including erythromycin-resistant strains
Streptomyces sp. HIL Y-86,36923[37]	Butalactin	Butanolide	Against Gram-positive and Gram-negative bacteria
Streptomyces sp. HIL Y-88,31582[89]	Alisamycin	Manumycin	Against Gram-positive bacteria and fungi and weak antitumour activity
Amycolatopsis alba var. nov. [132]	1(10-aminodecyl) Pyridinium salt	Pyridinium compound	Potent cytotoxic and antibacterial activities

have been screened against various human test pathogens, including the multidrug resistant strains (Tables 5 and 6).

Several researches have reported genus *Streptomyces* as the profilic producer of antimicrobials compounds, followed by *Micromonospora*, *Nocardia* and others (Fig. 4).

Indian patent claims on antibiotics—Patents have been obtained on varied aspects of antibiotic research (Table 7).

Antimicrobials vis á vis plants pathogens—The need to screen actinobacteria against fungal and bacterial plant pathogens is mainly because these pathogens play a threatening role in food security, economic prosperity and natural environments. Indian efforts to study antimicrobials against plant pathogens is gaining considerable importance, although the work is limited to mostly preliminary screening (Table 8).

Antimicrobials vis á vis marine organisms—Marine fauna are also susceptible to a large number of bacterial and fungal diseases which in turn are a posing threat to humans and animals who consume them. Indian efforts to screen Actinobacteria " against these harmful test pathogens are given in Table 9.

Other bioactive compounds

Anticancer—Since, cancer is recognized as one of the most dreaded diseases, there is a need for developing drugs to combat it. The most frequent cancers in men are of the lung, lip, oral cavity and in women these include those of the cervix, uteri, breast and ovary[221]. Anticancer drug research in India based on lead molecules from actinobacteria is highlighted in Table 10.

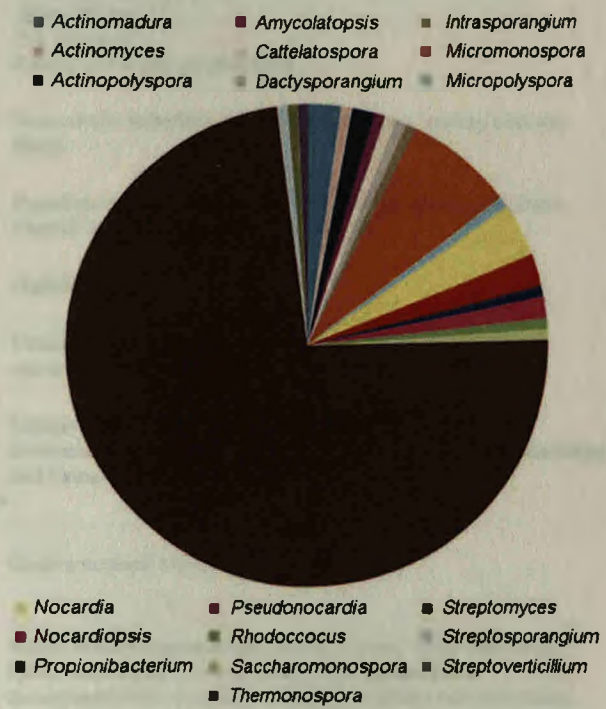

Fig. 4—Relative importance of actinobacteria in antibiotic screening programme based on published work (1965-2012).

Antioxidants—Thenmozhi *et al.*[224] evaluated the antioxidant activity of intracellular and extracellular metabolites of *Streptomyces* sp. VITTK3.

Antiacaricidal/antilarvicidal/antifeedant—Deepika *et al.*[225] studied the acaricidal and larvicidal property of marine actinobacterial compound (2S, 5R, 6R)-2-hydroxy-3, 5, 6-trimethyloctan-4-one isolated from *Streptomyces* sp. VITDDK3. The compound showed maximum efficacy against the larvae of *Rhipicephalus microplus*, *Anopheles subpictus* and *Culex quinquefasciatus*. Vijayakumar *et al.*[226]

Table 5—Human bacterial pathogens used to screen actinobacterial lead molecules

Human bacterial pathogens used in screening[ref]	Symptoms/Diseases
Genus *Acinetobacter*[42, 66, 142]	
A. baumanii, Acinetobacter sp.	Severe pneumonia and Urinary Tract Infection
Genus *Aeromonas*[118, 137, 143, 144]	
*A. formicans, A. hydrophila, A. veronii**	Gastroenteritis; Wound infections and diarrhoea*
Genus *Alcaligenes*[35]	
A. faecalis	Urinary Tract Infection
Genus *Arthrobacter*[107]	
A. protophormiae	Skin infection
Genus *Bacillus*[2, 4, 10, 12, 15, 20, 22, 31, 32, 35, 38, 39, 40, 42-44, 66, 68, 69, 72-74, 79, 81, 86, 93, 96, 98,105, 107, 109, 110, 112, 120, 125, 130, 131, 135, 136, 138, 140-142, 145-178]	
Bacillus sp., B. amyloliquefaciens, B. cereus, B. coagulans, B. megaterium, B. pumilis, B. saccharolyticum, B. sphaericus, B. subtilis[∞] *B. thuringiensis*[#]	Foodborne illness; Skin infection[∞], Eye infection[∞]; Emetic syndrome and diarrhoea[#]
Genus *Citrobacter*[35, 37]	
C. freundii	Nosocomial infections of the respiratory tract, urinary tract and blood
Genus *Clostridium*[120, 179]	
Clostridium sp., C. botulinium	Pseudomembranous colitis, food poisoining, tetanus, infections, Flaccid muscular paralysis
Genus *Corynebacterium*[154]	
C. diptheriae	Diphtheria
Genus *Enterobacter*[35, 43, 105, 138]	
E. cloacae[♂], *E. aerogens*♠	Urinary and respiratory tract infections[♂]; Gut infections, opportunistic infections♠
Genus *Enterococci*[10, 145, 153, 159, 164, 177, 180, 181]	
Enterococci sp., *E. faecalis*♦	Urinary Tract Infections, bacteremia, bacterial endocarditis, diverticulitis and meningitis; Gut infection, Meningitis, endocarditis and Urinary Tract Infections♦
Genus *Escherichia*[2,10-12, 22 23, 28, 31, 35, 37-40, 42, 44, 64, 68, 69, 72-74, 79, 81, 89, 93, 94, 98, 108, 109, 110, 112, 116, 120, 130, 131, 136, 138, 139-142, 144-161, 163-165, 167, 169-175, 179-186]	
E. coli	Gastrointestinal infection
Genus *Klebsiella*[2,10,12, 22, 31, 32, 39, 40, 69, 86, 93, 96, 98,105, 107, 109, 112, 120, 125, 135, 136, 138-142, 145-147, 151, 153, 161, 166, 167, 169, 170, 172, 175, 179, 180, 183]	
Klebsiella sp., K. aerogenes[Ξ], *K.oxytoca*[Φ] *K. planticola*[γ], *K. pneumonia*[¤]	Nosocomial pneumonia septicaemia, Urinary Tract Infections[Ξ]; Colitis and sepsis[Φ]; Severe pancreatitis[γ]; Pneumonia thrombophlebitis, Urinary and upper respiratory tract infections, cholecystitis, diarrhea, wound infection, osteomyelitis, meningitis, bacteremia and septicemia[¤]
Genus *Lactobacillus*[98, 107, 139, 159]	
L. acidophilus■, *L.casei*[§], *L. lactis*[€] *L. plantarum*[δ], *L. vulgaris*[ψ]	Vaginal infections, mild gastrointestinal discomfort or gas■; Opportunistic infections[§, ψ, €]; Mild gastrointestinal discomfort or gas[δ]
Genus *Leuconostoc*[98]	
L. mesenteroides	Bacteremias meningitis, breast abscess, abdominal abscess, peritonitis
Genus *Listeria*[98]	
L. monocytogenes	Listeriosis
Genus *Micrococcus*[35, 37, 68, 69, 72, 74, 93, 98, 120, 144, 145, 146, 157, 185]	
Micrococcus sp., M. flavus▲, *M. luteus*[♂]	Skin infections, recurrent bacteremia, septic shock, septic arthritis, endocarditis, meningitis, and cavitating pneumonia▲; Intracranial abscesses, pneumonia, septic arthritis, endocarditis and meningitis[♂]

Contd.

Table 5—Human bacterial pathogens used to screen actinobacterial lead molecules (Contd.)

Human bacterial pathogens used in screening[ref]	Symptoms/Diseases
Genus *Mycobacterium*[15, 42, 69, 98, 107, 186, 188]	
M. phlei, M. smegmatis, M. tuberculosis[fi]	Tuberculosis[fi]
Genus *Pseudomonas*[10, 12, 22, 31, 32, 35, 37, 38, 40, 42, 44, 66, 68, 69, 72-74, 79, 86, 93,105, 109, 110, 116, 120, 123, 130, 138, 140-149, 151-160, 163-164, 167, 170, 173, 174, 177, 179, 180, 182, 185, 186]	
Pseudomonas sp., P. aeruginosa, P. fluorescence[≈], *P.luteola*[◊] *P. putida*[‡], *P. staetrolens*[Θ]; *P. solanacearum*[Θ]	Nosocomial infections, Gut Lung infections, Urinary tract infections; Infections in compromised immune systems[≈]; Peritonitis, cellulitis, and bacteremia[◊]; Bacteraemia, sepsis in neonatal, neutropenic and cancer patients, Urinary tract infections[‡]; Nosocomial infections[Θ]
Genus *Proteus*[10, 35, 37, 39, 40, 43, 44, 69, 81, 86, 93, 98, 107, 109, 130, 136, 138, 139, 142, 145, 154, 158, 159, 163, 172, 175, 179, 180]	
Proteus sp., P. mirabilis, P.vulgaris	Infections, septicemia, pneumonias - mostly in hospitalized patients, Urinary tract infections, wound infections
Genus *Rhodococcus*[98, 185]	
R. rhodochrous	Infections in immunocompromised hosts
Genus *Salmonella*[10,12, 22, 39, 43, 66, 69, 86, 105, 108, 109, 120, 131, 132, 138, 139, 160, 167, 171-173, 180]	
Salmonella sp., S. bovis, S. enteritidis, S. mgulani, S. paratyphi[Ψ], *S. senftenberg, S. typhi*[Ψ], *S. typhimurium*[Ψ], *S. weltsverden*	Gut infection, salmonellosis; Typhoid[Ψ]
Genus *Sarcina*[138]	
Sarcina lutea	Skin infection
Genus *Serratia*[39, 28, 35, 43, 98, 107, 149, 150, 154]	
Serratia liquefaciens, S. marcescens[d]	Nosocomial infections, Gut infection[d]
Genus *Shigella*[10, 22, 43, 98, 138, 159, 167]	
Shigella sp., *Shigella flexneri*	Dysentery, Gastrointestinal infection
Genus *Staphylococcus*[4, 10, 20, 22, 23, 28, 31, 32, 35, 37-40, 42-44, 66, 67, 72-74, 79, 81, 86, 89, 93, 96, 98, 105, 109, 110, 112, 120, 125, 130, 131, 136, 138-149, 152-161, 163, 164, 166, 169-181, 183-185, 189-191]	
Staphylococcus sp., *S. aureus*[≠], *S. epidermidis,S. haemolyticus* (MDR)[‖]	Food poisoning, Skin infection[≠], Nosocomial infections, endocarditis, septicemia, peritonitis, urinary tract, wound, bone, joint infection[‖]
Genus *Streptococcus*[31, 35, 37, 89, 98, 125, 135, 142, 145, 148, 159, 161, 170, 192]	
Streptococcus sp., *S. faecalis, S. mutans*[■], *S. oralis, S. pneumoniae*[♦], *S. pyogenes*[£], *S. viridians*[∂]	Streptococcal pharyngitis, meningitis, bacterial pneumonia, endocarditis, erysipelas, necrotizing fasciitis, Dental infections/decay[■], Pneumococcal infections[♦], Pharyngitis, skin infection, Erysipelas, cellulitis streptococcal pharyngitis Acute glomerulonephritis, inflammation of the renal glomerulus[£], Mouth or gingival infections[∂]
Genus *Streptomyces*[116, 183]	
Streptomyces sp., *S. griseus*	Mycetoma
Genus *Vibrio*[12, 22, 98, 108, 139,142, 143, 185, 193]	
Vibrio sp., *V. alginolyticus*[‡], *V. cholerae*[•], *V. parahaemolyticus*[∞], *V. vulnificus*[€]	Foodborne infection, Otitis and wound infection[‡], Cholera[•], Gastrointestinal illness[∞], Cholera, cellulitis or septicemia[€]
Genus *Yersinia*[98]	
Y. enterocolitica	Yersiniosis, mild self-limiting entero-colitis or terminal ileitis

reported marine actinobacteria *Streptomyces* sp. and *Streptosporangium* sp. having notable larvicidal activity. Antifeedant activity was reported against *Helicoverpa armigera* and *Spodoptera litura*[195].

Enzymological research-enzymes and enzyme inhibitors—Enzymes of actinobacterial origin have triggered scientific interest due to their wide range of applicability in textile, beverage, food, feed and other industries. The Indian efforts of screening useful enzymes have been encouraging (Fig. 5). Genus *Streptomyces* has been largely studied, followed by few reports on genus *Nocardiopsis, Micromonospora* and *Thermoactinomyces*. Thus, this data lays an emphasis on the need to screen other genus of actinobacterial origin like *Acinetobacter, Actinobispora, Kocuria, Microbispora, Microtetrospora* and *Thermomonospora* for obtaining industrially important enzymes.

Table 6—Human fungal pathogens used to screen actinobacterial lead molecules

Human fungal pathogens used in screening[ref]	Symptoms/Disease
Genus *Alternaria*[22, 39, 69, 81, 154]	
Alternaria sp., *A. alternata, A. awamori*	Allergies like hay fever or asthma, opportunistic infections in immunocompromised patients, Lung disease
Genus *Aspergillus*[21, 38, 96, 99, 110, 112, 140, 141, 145, 153, 154, 156, 157, 159, 161, 162, 182, 186, 194, 195]	
Aspergillus flavus, A. fumigatus[Ω], *A. niger*[∞], *A. terreus*[▲]	Aspergillosis of the lungs, corneal, otomycotic, and nasoorbital infections; Invasive fungal infection chronic pulmonary infections or allergic disease in immunosuppressed individuals, Skin infection[Ω], Serious lung disease, aspergillosis[∞], Opportunistic infection in immunocompromised patients[▲]
Genus *Botrytis*[22, 89, 93, 195]	
Botrytis cinerea	Pneumonitis
Genus *Candida*[12, 21-23, 35, 37-40, 43, 44, 64, 69, 72-74, 81, 86, 89, 93, 96, 98, 105,107, 109, 110, 112, 124, 131, 135, 141, 142, 144-146, 148, 150, 151, 153, 154, 156, 159, 161, 163, 171, 172, 175, 185, 194-199]	
Candida albicans, C. glabrata, C. krusei[F], *C. lipolytica*[Ю], *C. neoformans, C. tropicalis*	Opportunistic oral and genital infections in humans, candidiasis, Candidemia; Nosocomial infections in immunocompromised and hematological malignancies patients[F]; Refractory oral candidiasis[Ю]
Genus *Cryptococcus*[23, 69, 110, 148, 171, 172]	
Cryptococcus sp., *C. neoformans*[Δ], *C. terreus*	Cryptococcosis, meningitis, in immunocompromised patients; Meningitis and meningo-encephalitis in HIV/AIDS patients[Δ]
Genus *Epidermophyton*[94, 154, 195, 200]	
E. floccosum	Tinea pedis, tinea cruris, tinea corporis, onychomycosis
Genus *Fusarium*[12, 22, 23, 44, 69, 110, 151, 154, 155, 159, 163, 201]	
Fusarium sp., *F. moniliforme*[‡], *F. oxysporum*[♦], *F. solani*[♂]	Opportunistic infections, onychomycosis, keratomycosis or mycotic keratitis; Skin infection[‡]; Opportunistic infections in immunocompromised patients[♦]; Neutropenia, aggressive fusarial infections[♂]
Genus *Microsporum*[64, 94, 135, 194, 198, 200, 202]	
Microsporum sp., *M. canis, M. gypseum, M. nanum*	Tinea capitis, tinea corpus, ringworm, and other dermatophytoses
Genus *Penicillium*[12, 39, 69, 110, 152, 154, 159, 162, 201]	
Penicillium sp., *P. chrysogenum*[☼], *P. citrinum*[¥], *P. ochrochloron*	Penicilliosis, Keratitis, endophtalmitis, otomycosis, necrotizing esophagitis, pneumonia, endocarditis, peritonitis, UTI; Opportunistic infections in immunocompromised patients[☼]; Balkan nephropathy and yellow rice fever[¥]
Genus *Scopulariopsis*[195]	
Scopulariopsis sp.	Infections in immunocompromised patients
Genus *Trichoderma*[110]	
Trichoderma sp.	Opportunistic infections in immunocompromised patients
Genus *Trichophyton*[64, 69, 94, 194, 195, 200]	
Trichophyton sp., *T. mentagrophytes*[⊥], *T. rubrum*[υ], *T. simii*[§]	Malabar itch, athlete's foot, ringworm, jock itch, infections of the nail, beard, skin and scalp; Fungal nail infections, tinea corporis, tinea cruris and tinea capiti[⊥]; Athlete's foot, jock itch and ringworm[υ]; Tinea corporis, tinea cruris and tinea capiti[§]

Table 7—Antibiotic Patents from India

Description/claims	Patent number	Claimants
Actinomycete strain SKF-CWI-785 producing novel glycopeptide antibiotics of the CWI-785 complex	US Pat 4742045	Verma *et al.* [203]
Streptomyces species producing a novel macrolide antibiotic Swalpamycin	US Pat 4988677	Franco *et al.* [204]
Actinomycete strain Y-86,21022 producing a novel glycopeptide antibiotic Balhimycin	US Pat 5571701	Nadkarni *et al.*[205]
Novel strain, *Streptomyces* sp. BICC 7522 producing macrolides	US Pat 7704725	Kulkarni *et al.*[206]
Novel strain of *Streptomyces* sp., CIMAP A1 producing anti-microbial activity against phytopathogenic fungi	US Pat 6558940	Alam *et al.*[207]
Streptomyces species (PM0626271/MTCC 5447) producing antibiotic compounds	US Pat 2012/0156295	Mishra *et al.*[208]
Actinoalloteichus spitiensis producing bipyridine compound bioactive molecule- Caerulomycin A, derivatives and analogs thereof as effective immunosuppressive agents	US Pat 8114895	Singla *et al.*[209]
Actinomycete strain Y-86,36910 producing a novel glycopeptide antibiotic Decaplanin	EP0356894	Franco *et al.*[210]
Actinomycete strain Y-88,31582 producing a novel antibiotic Alisamycin	EP0436935	Franco *et al.*[211]
Actinomycete strain MTCC 5597 producing antibacterial and antifungal compound, (I) 5-(4-bromobutyl)-N-(but-3-enyl) dodec-11-enamide or a derivative thereof	WO/2012/104793	Kumar *et al.*[212]

The studies on enzymes have prioritized lipases, amylases, proteases, endoglucanases, α-galactosidases, pectin lyases, xylanases, L-asparaginases, L-glutaminases and cellulases (Table 11).

Enzyme inhibitors—Enzyme inhibitors are gaining importance due to wide applications in chemotherapeutic drugs, metabolic control, pesticides, herbicides and natural poisons. Indian research in this area is still in infancy, with more interest in amylases, α-glucosidases and proteases inhibitors. Raja *et al.*[271] studied amylase inhibitors producing actinobacterial strains SSR-10 and SSR-2 using *Bacillus subtilis* and *Aspergillus niger* as test organisms. Ganesan *et al.*[272] tested marine actinobacterial strains for their ability to produce yeast and rat α-glucosidase inhibitors and Pandhare *et al.*[6] reported actinobacterial strains producing alkaline protease inhibitors.

Research on other useful products and processes—Besides the normal areas of interest reviewed earlier, Indian actinobacterial research has attempted to move in novel directions for assessment of actinobacterial processes such as bioemulsification, biodegradation, biosorption; biosurfactants, osmolytes, nanoparticles production, mineral biosynthesis which are still in the experimental stage (Table 12).

Other applications include genomic studies by Ramachander and Rawal[285] who reported first putative PHA synthase gene from a *Streptomyces* sp. with serine as the active nucleophile in the conserved lipase box, Bajpai *et al.*[286] have worked on the genome sequence of the bacteriophage (phage) PIS136 isolated from a strain of *Saccharomonospora*, Vikram *et al.*[47] investigated the 8.231-Mb genome sequence of *Rhodococcus imtechensis*. Besides studies on co-production of caffeic acid and p-hydroxybenzoic acid by *Streptomyces caeruleus*[287], production of poly-e-lysine by *Streptomyces noursei*[288] and biotransformation of the anti-inflammatory compound meloxicam by *Streptomyces griseus* NCIM 2622 and *S. griseus* NCIM 2623[289] was also reported.

Problems and challenges related to study of actinobacteria in India

Early work on actinobacteria in India started in the 20th century, made slow and steady progress despite lack of success in discovery of new genera and metabolites. According to Berdy[7], the major cause of such declining trends in microbial metabolite

Table 8—Plant pathogens used to screen biocontrol agents from actinobacteria

Phytopathogens type[ref]	Symptoms/Disease
Bacterial pathogens	
Genus *Bacillus*[67]	
B. cereus	Damping off
Genus *Rhizobium*[65, 149]	
Rhizobium sp., *R. japonicum*	Diseases in legumes
Genus *Xanthomonas*[22, 67, 68, 154, 159, 168]	
Xanthomonas sp., *X. capsicii, X. campestris*[κ]	Bacterial leaf spot; Citrus canker, leaf spot[κ]
Fungal pathogens	
Genus *Alternaria*[67, 73, 151, 167, 199, 213]	
Alternaria sp., *A. alternata, A.brassicicola*[ж]	Leaf spot; Brassica dark leaf spot on most Brassica species[ж]
Genus *Aspergillus*[67, 167, 213]	
Aspergillus sp., *A. flavus*[ч], *A. niger*[¤]	Disease on many grain crops, especially maize; Storage problems in stored grains[ч]; Black mold on certain fruits and vegetables such as grapes, onions, and peanuts[¤]
Genus *Bipolaris*[72]	
B. oryzae	Brown spot disease in rice
Genus *Collectotrichum*[67, 189, 214]	
C. falcatum; C.capsici[Ŧ]	Red rot disease of sugarcane; Leaf blight on *Chlorophytum borivilianum*, basil, chickpea and pepper, dieback in pigeonpea and anthracnose in poinsettia[Ŧ]
Genus *Curvularia*[72]	
C. oryzae	Pecky rice (kernel spotting)
Genus *Fusarium*[67, 72, 167, 189, 213]	
Fusarium sp., *F. moniliforme*[♠], *F. oxysporium*[ȣ], *F. solani*[Θ], *F. udum*[Ψ]	Damping off, Percky rice (kernel spotting), Bud rot[♠]; Fusarium crown root, Fusarium wilt[ȣ]; Fusarium crown rot, bud rot[Θ]; Fusarium wilt in pigeonpeas[Ψ]
Genus *Helminthosporium*[191, 214]	
H. oryzae	Stem rot in rice
Genus *Macrophomina*[67, 215, 216]	
M. phaseolina	Charcoal rot on many plant species
Genus *Penicillium*[169]	
Penicillium sp.	Postharvest decay of stored apples
Genus *Phytophthora*[167]	
Phytophthora sp.	Damping off
Genus *Pyricularia*[72, 89, 195, 215]	
Pyricularia oryzae	Rice Blast (leaf, neck, nodal and collar)
Genus *Pythium*[167]	
Pythium sp.	Pythium root rot, Damping off
Genus *Rhizoctonia*[12, 67, 69, 73, 93, 189, 215, 214, 215]	
R. oryzae, R. solani[ε]	Aggregate sheath spot; Rhizoctonia root and crown rot, Web blight, Damping off[ε]
Genus *Rhizopus*[213]	
R. stolonifer	Rhizopus blight
Genus *Trichoderma*[151, 167, 199]	
Trichoderma sp., *T. viride*	Green mould rot of onion, green mold in button mushrooms
Genus *Verticillium*[22]	
V. alboatrum	Yellowing and tiger-striping effect on hop leaves

Table 9—Animal pathogens used for preliminary screening of bioactive molecules

Bacterial pathogens in animals[ref]	Symptoms/Disease
Genus *Aeromonas*[90, 121, 136, 179, 217, 218]	
A. hydrophlia, A. sobria, Aeromonas sp.	Aeromonosis, ulcers, tail rot, fin rot, and hemorrhagic septicemia in fish
Genus *Bacillus*[90, 179, 218]	
Bacillus sp., *B. subtilis*	Intestinal infections in fish
Genus *Escherichia*[218]	
E.coli	Intestinal infections in fish
Genus *Flavobacterium*[90]	
Flavobacterium sp.	Mycobacteriosis infections of invertebrates
Genus *Micrococcus*[90]	
Micrococcus sp.	Intestinal and skin infections in fish
Genus *Serratia*[121, 179]	
Serratia sp., *S. marcescens*	White pox disease in corals
Genus *Vibrio*[90, 121-124, 179, 219, 220]	
Vibrio sp., *V. alginolyticus*[±], *V. parahaemolyticus*[Ø], *V. anguillarum*[Ħ], *V. cholera*[σ], *V. harveyi*[ϕ]	Infections in fish and shellfish; Disease in pufferfish[±]; Disease in squids, mackerels, tunas, sardines, crabs, shrimps, and bivalves, such as oysters and clams[Ø]; Vibriosis in salmonid fish, or red pest of eels[Ħ]; Infections fish, eels[σ]; Luminous vibriosis in penaeid prawns[ϕ]

Table 10—Promising actinobacterial strains used in anticancer assays

Species[ref]	Human cell lines (IC_{50}/ LC_{50} values)				
	A549 (lung)	HeLa (cervical)	HEP G2 (liver)	MCF-7(breast)	U87MG (brain)
Streptomyces sp.[178]	+ (NA)	NT	NT	NT	NT
Streptomyces sp.[222]	NT	+ (IC_{50} 21.50μg/ml)	NT	NT	NT
Streptomyces sp.[109]	NT	NT	NT	+ (NA)	NT
Streptomyces aurantiacus[10]	NT	+ (LC_{50} 0.013 μg/ml)	+ (LC_{50} 0.010 μg/ml)	NT	NT
Streptomyces avidinni[223]	NT	NT	+ (IC_{50} 64.5 μg)	NT	NT
Saccharopolyspora salina[112]	NT	+ (IC_{50} 26.2 μg/ml)	NT	NT	NT
Amycolatopsis alba var. nov.[132]	NT	+ (NA)	NT	+ (NA)	+ (NA)
Unidentified actinobacteria[147]	NT	+ (IC_{50} 4.9 μg/ml)	NT	NT	NT

+, positive activity; NT, not tested; NA, data not available; LC, lethal concentration; I_{C50}, Half maximal inhibitory concentration
*Has also reported activity against VERO cell line (Ic_{50} 250 μg/ml)

research is human responsibility, scientific failure mainly due to limitations of use of modernised techniques like high-throughput screening and combinatorial synthesis and problematic economic-regulatory environment. Such limitations may be prime cause of declining productive actinobacterial research in India. Many institutions working in India might not be well equipped with all the facilities required to expedite the research. Besides other research limitations like extremely slow doubling time of the actinobacteria in comparison to other microorganisms, making the isolation programmes to take several months is a major issue. Also, despite high frequency sampling and isolation efforts, one cannot ensure isolation of novel genera which could lead to a novel antibiotic lead molecule. But all these problems could be reduced through a national strategy to boost actinobacterial research aiming at using more advanced natural isolation and screening strategies. Besides providing new avenues, facilities and funds is a must in all leading research institutions so as to upgrade the standards of research which will lead to positive outcomes.

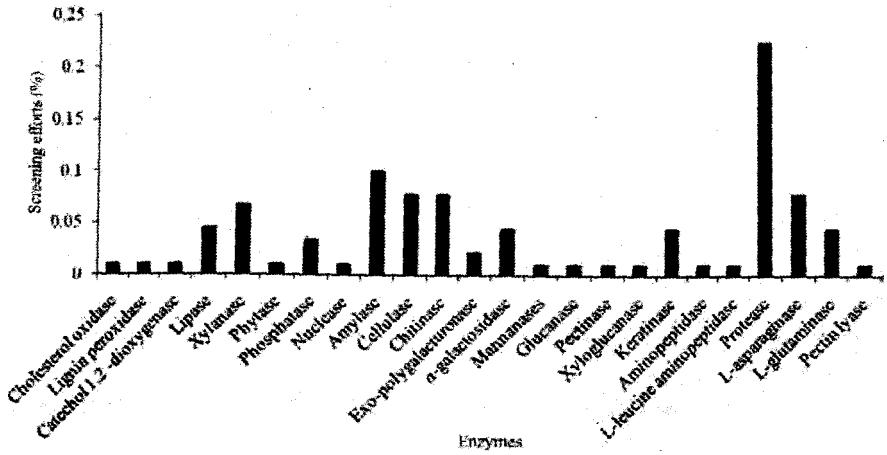

Fig. 5—Preliminary enzyme screening activity reported in India [Cholesterol oxidase[227], lignin peroxidase[228], catechol 1,2-dioxygenase[229], lipase [29,185, 230,231], xylanase[28,52,53,75,232,233], phytase[234], phosphatase[80,235,236], nuclease[237], amylase[14,113,185,230, 237-241], cellulase[75,118,185,201,242,243,244], chitinase[29,127,155,186,223,245,246], exo-polygalacturonase[247,248], α-galactosidase[84,249-251], mannanases[252], glucanase[29], pectinase[75], xyloglucanase[253], keratinase[254-257], aminopeptidase[258], l-leucine aminopeptidase[259], protease[4,13,20,24,34,59,76,82,167,185,214,238-240,260-265], l-asparaginase[95,117, 235, 265-268], l-glutaminase[102,119,269,270], pectin lyase[247]].

Table 11—Indian research on enzymes from actinobacteria

Enzyme	Sources strain[ref]
Cholesterol oxidase	*Streptomyces lavendulae*[227]
Lignin peroxidase	*Streptomyces psammoticus*[228]
Catechol 1,2-dioxygenase	*Rhodococcus* sp.[229]
Lipase	*Nocardia dassonvillei*[185], *Streptomyces griseus*[230]
Xylanase	*Kocuria* sp.[52], *Thermoactinomyces thalophilus*[53], *Steptomyces* sp.[75], *Steptomyces rameus*[232]
Phytase	*Nocardia* sp.[234]
Phosphatase	*Actinobispora yunnanensis*[80], *Microtetraspora fastidiosa*[80], *Micromonospora echinospora*[80], *Saccharopolyspora hirsute*[80], *Saccharopolyspora viridis*[80], *Thermoactinomyces mesophila*[80], *Streptomyces albus*[80], *Streptomyces cyaneus*[80], *Acinetobacter* sp.[235], *Streptomyces* sp.[235], *Nocardiopsis* sp.[235]
Nuclease	*Streptomyces thermonitrificans*[237]
Amylase	*Streptomyces erumpens* [14], *Saccharopolyspora* sp.[113] *Streptomyces* sp.[240]
Cellulase	*Streptomyces actuosus*[118], *Streptomyces* sp.[75, 240], *Nocardiopsis dassonvillei*[185],*Streptomyces noboritoensis*[243], *Microbispora* sp.[244]
Chitinase	*Streptomyces canus*, *Streptomyces pseudogriseolus*[245], *Streptomyces* sp.[245], *Micromonospora brevicatiana*[246]
Exo-polygalacturonase	*Streptomyces erumpens*[247], *Streptomyces lydicus*[248]
α -galactosidase	*Streptomyces griseoloalbus*[84, 250]
Mannanases	*Streptomyces* sp.[252]
Pectinase	*Streptomyces* sp.[75]
Xyloglucanase	*Thermomonospora* sp.[253]
Keratinase	*Streptomyces thermoviolaceus*[254], *Streptomyces sclerotialus*[255]
Aminopeptidase	*Streptomyces gedanensis*[258]
L-leucine aminopeptidase	*Streptomyces mobaraensis*[259], *Streptomyces gedanensis*[259], *Streptomyces platensis*[259]
Protease	*Streptomyces clavuligerus*[13], *Nocardiopsis prasina*[59], *Streptomyces carpaticus*[82, 240], *Streptomyces moderatus*[161], *Streptomyces megasporus*[261], *Streptomyces gulbargensis*[262], *Nocardiopsis alba*[263], *Streptomyces roseiscleroticus*[264]
L-asparaginase	*Streptomyces canus*[91, 268], *Streptomyces noursei*[117], *Streptomyces griseoloalbus*[235], *Streptomyces ceolicolor*[265], *Streptomyces* sp.[265], *Streptomyces aureofasciculus*[268], *Streptomyces chattanoogenesis*[268], *Streptomyces hawaiiensis*[268], *Streptomyces orientalis*[268], *Streptomyces olivoviridis*[268]
L-glutaminase	*Streptomyces rimosus*[119], *Streptomyces* sp.[270]
Pectin lyase	*Streptomyces lydicus*[247]

Table 12—Other useful actinobacterial products and processes

Other uses	Strains
Biodegradation	*Streptomyces rochei*[26♦], *S. krainskii*[273♦], *Rhodococcus* sp.[170 Ψ]; *Rhodococcus* sp.[101]*, *Nocardia* sp.[101]*, *Gordonia* sp.[101]*, *Dietzia* sp.[101]*, *Brevibacterium casei*[48¤], *Rhodococcus* sp.[48¤], *Slackia exigua*[274Ю], *Corynebacteium liquefaciens*[275Ш]
Biosurfactants	*Streptomyces* spp.[276, 277], *S. gedanensis*[278], *Nocardiopsis alba*[279]
Biosorption of heavy metals	*Streptomyces* sp.[100ß]
Osmolytes	*Actinopolyspora* sp.[111]; *Streptomyces* sp.[280], *Nocardiopsis* sp.[280]
Biominerals	*Thermomonospora* sp.[281]
Pigments	*Streptomyces* sp.[360]; *Streptomyces* spp.[282◊]
Nanoparticles	*Thermomonospora* sp.[62•], *Rhodococcus* sp.[283•], *Streptomyces* sp.[284▫]

Biodegradation- 3-4 ring PAH compounds degraders (anthracene, fluorene, phenanthrene and pyrene)♦, textile dye reactive blue-59 degraders♦, aliphatic and aromatic crude oils degraders[Ψ], hydrocarbon degraders*, p-nitrophenol degraders[¤], agar degraders[Ю], chlorobenzoates degraders[Ш]; **Biominerals**-produced extra and intracellular formation of $CaCO_3$; **Pigments**- melanin[◊]; carotenoids mainly phytoene[◊]; **Nanoparticles**- synthesis of gold nanoparticles•; synthesis of metal oxide nanoparticles▫

Future prospective on actinobacteriological research

A vast area is opened by Nagoya protocol[290,291] for biodiversity and bioprospecting of microbial resources. India can reap rich dividends through a national strategy and action plan to explore and exploit actinobacterial resources. Considering the diverse physiological and climatic conditions, various regions in mainland India and the islands offer huge scope for sampling and isolation of many interesting and novel actinobacterial strains. Besides modern metagenomic tools can also be employed for mapping the unculturable actinobacterial diversity. Promising strains of culturable actinobacteria can be subjected to national bioprospecting efforts in which the corporate biotech and pharma R&D sector could play a major role. Biotechnological applications of culturable actinobacteria especially in bioremediation and nanobiotechnological processes and products are another promising area of research.

There is further scope to generate nationally important and useful intellectual property from industrially useful strains considering the positive trends emerging out of almost a century of actinobacterial research. An attempt has been made to suggest an outline of such plan to benefit from Nagoya protocol (Fig. 6). Since India has already ratified this protocol, it would be advantageous to make an early start. If this strategy or action plan is launched systematically and successfully, it could make India a global leading player in identification of useful strains, novel products and processes from actinobacterial diversity, both, from pure academic and practical technological angles. The future scope for advancement of actinobacterial research in India appears bright.

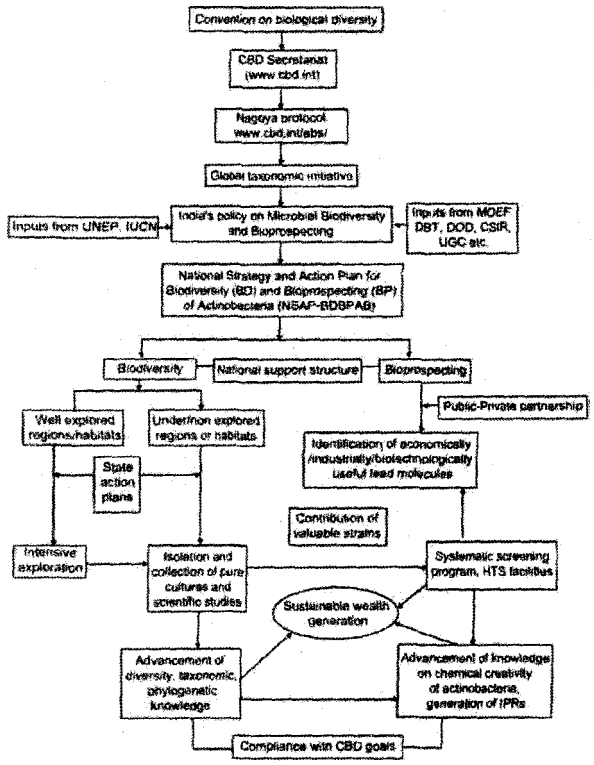

Fig. 6—Schematic representation of basic elements of a national strategy and action plan for boosting actinobacteriological research in India.

Conclusions

Actinobacteria have proven the potential as chemically creative species which could be sustainably utilized for human welfare. In India, actinobacterial research has not accelerated despite the country having interesting and fertile habitats waiting to be exhaustively explored. The Indian research so far has progressed in a few areas and needs more thrust through a national strategy and action plan for biodiversity and biosprospecting of actinobacteria. Such strategy would advance knowledge, build indigenous capacities, throw up new techniques, spawn novel technologies and continue to sustain wealth generation and national development. The present century offers promising challenges and opportunities for Indian actinobacteriological research.

Acknowledgement

Authors would like to acknowledge UGC-SAP Phase II at the Department of Botany for providing necessary facilities. The first author received the support of UGC-JRF Maulana Azad National Fellowship.

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Original Paper
Velho-Pereira & Kamat

INTERNATIONAL BOOK HOUSE P. LTD.

Int. J.Biotech Biosci, ISSN 2231-0304
Vol 2 (3) : 217-220 (2012)

A NOVEL BAITING TECHNIQUE FOR DIVERSITY ASSESSMENT OF SOIL ACTINOBACTERIA IN A LABORATORY MICROCOSM

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Abstract
A novel slide technique which employed Arginine Vitamin Agar was used for isolation of diverse actinobacteria from tropical soil habitat in a laboratory microcosm. About ~60% of the total actinobacteria were recovered comprising mainly Streptomyces, Streptosporangium and Actinomadura as the common genera. The technique demonstrates the potential for 'ex situ' visualization and rapid diversity assessment of soil actinobacteria, especially under demanding tropical conditions.

Keywords: *Actinobacteria, Arginine Vitamin Agar, Microcosm, Tropical diversity, Isolation*

INTRODUCTION

Actinobacteria are producers of biologically useful substances, such as antibiotics, vitamins and enzymes, (Basilio et al 2003; Takahashi and Omura 2003; Berdy 2005) thereby necessitating development of novel methods to access new representatives of this group (Gavrish et al 2008). Laborious and time consuming techniques such as dilution plating are commonly being used for isolating soil actinobacteria (Waksman and Martin 1939). Novel technologies like high throughput screening, whole genome sequencing, make targeted isolation possible but being expensive these are beyond the reach of large culture collections in Third World Countries. Difficulties encountered using older techniques for specific isolation, have led to development of baiting techniques which uses addition of natural substrates to soil as baits to study the population and succession of microbes in different habitats (Warcup 1965; Tribe 1957; Warnes and Randles 1980). This note describes a technique with slides specifically coated with an actinobacterial isolation medium combining direct visualization with isolation techniques. Instead of glass slides, previous modifications had employed, nylon mesh (Warcup 1965), pieces of glass, or cover slips (Warcup 1965; Linford 1942; Takahashi and Omura 2003), or strips of cellophane attached to cover slips (Tribe 1957, 1961) and hair (Griffin 1960) which were buried in soil to study the succession of microbes by direct examination. These techniques however do not permit isolation of morphological dissimilar colonies on plate. Gavrish et al (2008) developed semi-permeable membrane trap for specifically capturing and cultivating actinobacteria *in situ,* but it employs relatively expensive polycarbonate membrane filters and a diffusion chamber. Lewis et al (2010) on the other hand, introduced isolation chips (ichips) which are more advanced than diffusion chambers for invitro isolation of actinobacteria but in the same year, Nichols et al (2010) showed the limitations of the technique in isolating the same. Thus, emphasizing the need for an effective equipment for specific actinobacterial isolation (Hame°-Kocaba° and Uzel 2012). We describe a simple, inexpensive, 'ex situ' slide based technique with defined coating of AVA medium to capture actinobacterial diversity under 'Laboratory Soil Microcosm' (LSM) setup. AVA is fairly clear medium, yields a large number of actively growing actinobacterial colonies (Nonomura and Ohara 1969), supports good sporulation and permits microscopic detection of the individual colonies

including spores/cells diversity with relative ease and had emerged in our routine isolation work as a suitable candidate for slide coating medium (Velho-Pereira and Kamat 2011).

For testing the technique, humus rich soils from the lateritic plateau Taleigao, Goa University campus, Goa, India were collected at a depth of 10-20 cm in clean polythene bags from 5 x 5 m quadrat below the canopy of two indigenous keystone plant species namely *Ficus benghalensis* L., a sacred tree of 400 years age as per local knowledge and *Bombax ceiba* L. Samples were transported to the laboratory and stored at 22°C before use. The LSM was prepared in triplicates in brand new Garden PVC pots (length x width x depth; 13.5 x 13.5 x 10.5 cm, 1.2 kg capacity) which were filled with the moistened soil samples. Equidistant (1cm) vertical slits of upto 5-8 cm depth were made radially with a surface sterilized knife blade to create seven identical 'Slide Insertion Slots' (SIS) to enable periodic removal of colonized slides for observations. AVA was prepared and used as 'Slide Coating Medium' (SCM) (Velho-Pereira and Kamat 2011).

Two treatments were used-in first the slides coated with AVA homogeneously mixed with antibacterials and antifungals and the second without. Standard plain microscope glass slides (HiMedia, Mumbai, India) 75 x 25 mm and 1.0 mm thick were used as inert coating surface. To permit routine direct stereomicroscopic observations in a marked pre-defined area, a quadrat of the size of a standard cover slip, app. 324 mm^2 (Corning Glass, USA, Type n No.1, 18 x 18 mm, thickness 0.16 mm) was chosen at the centre of the reverse side of the area to be coated and was marked carefully. The slides were then sterilized by autoclaving at 121°C for 20 min. All the operations were performed on a laminar air flow bench. The slides were transferred in an 11 cm diameter sterile petri plate (Himedia, Mumbai, India) for coating of the medium to the marked central area. Two sets of slides in triplicates one with the coating medium incorporated with antibacterial and antifungals and other without were prepared. Sterile homogenous molten AVA medium cooled to 40°C was carefully poured in a single thin stream from a 500 ml Erlenmeyer flask on each slide to get a uniform coating. The coated slides were then transferred back to the petri plate for solidifying and excess medium crossing the edges of the marked area was removed. The solidified coated slides were designated as per a preset scheme and used within 15-30 min. Preselected and labeled pair of coated test slides (one with and another without antibiotics) was then gently inserted in SIS in the LSM, taking care to bring the medium in the contact with the surrounding soil without disturbing coated surface. The LSM was then covered with aluminum foil and incubated under ambient conditions on a clean laboratory platform. A pair of slides from each of the three LSM was sequentially extricated from respective SIS in LSM on 4, 7, 12, 15, 22, 26 and 28 days. After removing loose extraneous soil particles, these were observed under Olympus BX41TF microscope (Tokyo, Japan) with digital photomicrographic attachment. Actinobacterial (AB) colonization was detected by presence of typical submerged, aerial mycelium and spore bearing chains. 'Non Actinobacterial' (NAB) colonization was detected by presence of typical growth or the colour, texture and overall appearance of non filamentous microcolonies. Fungal (F) colonization was detected by presence of typical filamentous microcolonies. The extent of microbial colonization was scored by noting the relative area under AB, NAB and F in each of the 200+ random optical fields and expressed as average percentage colonization by AB, NAB and F (results not shown). For sampling, AB colony forming units (CFUs) from colonized surface, jets of 2 ml of sterile distilled water were directed (for each pair of extricated slides) using a sterile accupipet model T1000 (Tarsons Products Pvt. Ltd., Kolkata, India). Washings were collected in a sterile glass beaker and an aliquot (0.1 ml) was then plated on AVA medium with antibiotics (as described previously) and incubated at 28-30°C for upto 30 days. Plates were observed daily under Olympus SZ2-ILST stereomicroscope (Tokyo, Japan) and morphologically dissimilar colonies were identified by recording growth, aerial spores-mass colour, texture, elevation; substrate mycelium colour, margin, pigment production and presence or absence of exudates (Cross and Goodfellow 1973). Isolates representing dissimilar morphotypes were then identified, selected and purified on AVA plates. Pure colonies were transferred to AVA slants. Isolated pure strains were designated and maintained at the ambient temperature of 26-34°C.

For recording micromorphological details, air dried smears were stained in 1% (w/v) crystal violet and the spore bearing hyphae were identified with entire spore chain along with other microstructures (Locci 1989). The technique demonstrated the usefulness of AVA as a coating medium for selective growth of actinobacteria. Selection of a marked quadrat allowed focused, prolonged, reproducible observations and quick visual estimates of colonization under 40 X objective. Placement of coated slides inside defined SIS within LSM was also an improvement over previous techniques. Rossi-Cholodny technique was limited by the inability of microbes to attach to the slide and the difficulty in further isolation, as the mycelia growing over the slides were usually sterile (Garrett 1951; Alexander and Jackson 1954) but in the present technique use of AVA with antibiotics helped to reduce competing microbes. Around 60-70 % colonization by actinobacteria was seen in all the slides extricated from the soil of *F. benghalensis* L., with soil from *B. ceiba* L. exhibiting the same percentage of colonization, indicating that the technique is not specific to rhizosphere soils and actinobacteria could be isolated from varied soil samples. This could be highly beneficial for rapid isolation of such forms. The actinobacterial diversity predominated on the 4th and 12th day of incubation with decrease in colonization after 26th day and sudden increase on 28th day. The decrease in colonization could be attributed to medium depletion caused by presumptive grazing of soil arthropods which could be avoided by preheating the soils prior to setting up the experiment. Distribution of actinobacterial colonies at different time intervals could be due to the appearance of late colonizers. Majority of actinobacterial colonies formed well developed microscopically distinguishable vegetative mycelia. Fructifications such as the formation of branching, stable substrate hyphae and aerial hyphae with long spore chains could be categorized as *rectiflexibiles, rectinaculiaperti* or *spirales* on basis of their characteristic morphology (Locci 1989).

Spiral spore chains with spores of Genus *Streptomyces* approx 1-1.5 ìm in length predominated the slides (Fig. 1). Around 30 isolates representing 24 dissimilar morphotypes and 39 isolates representing 15 dissimilar morphotypes were recovered from respective LSM of *F. benghalensis* and *B. ceiba*. Majority of these belonged to genus *Streptomyces*. Other actinobacteria isolated in small numbers included *Streptosporangium* and *Actinomadura*. The technique also permitted real time still image and video documentation of the growing actinobacterial colonies (www.youtube.com/watch?v=bJ8pDnXamPw). According to Durbin, the buried slide technique could also be used to obtain selective isolation of a particular organism rather than to study general microbial colonization by using many natural substrates as baits (Warcup 1965). Incorporating specific enzyme substrates in the baiting medium so as to isolate potential enzymatic actinobacterial producers has also been carried out using this technique (results not incorporated).Thus this novel baiting technique facilitated specific capturing of actinobacterial diversity and quick insight into community structure from mixed populations of microorganisms from different soil samples. In tropical countries it may be found useful for high frequency exploration and isolation of actinobacteria.

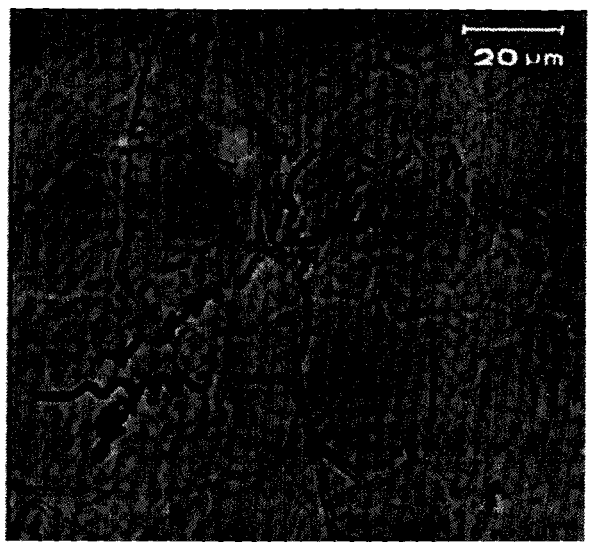

Fig. 1. Spiral spore chains with spores of *Streptomyces* sp. Approx 1-1.5 µm in length as seen under Phase Contrast Microscopy. Arrows indicates submerged mycelium.

ACKNOWLEDGEMENTS

Authors would like to acknowledge UGC-SAP for providing financial assistance to carry out the work. The first author duly acknowledges the support of UGC-JRF Maulana Azad National Fellowship.

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Accepted 5 March 2011
Revised 20 January 2011
Received 8 May 2010
Indian J. Pharm. Sci., 2011, 73 (1): 219-223

Antimicrobial Screening of Actinobacteria using a Modified Cross-Streak Method

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Velho-Pereira and Kamat: Antimicrobial Screening of Actinobacteria

Out of the 30 actinobacterial cultures screened for antimicrobial activity, 28 cultures were found to produce active products against various pathogenic microorganisms such as Gram-negative, Gram-positive bacteria and yeast, using a modified cross streak method. The modified method helped in easy quantification of results and also in ruling out probable mutual antibiosis. The actinobacterial strains that showed the ability to produce antimicrobial compounds belonged to *Streptomyces* (53%), *Micromonospora* (13%) and *Actinomadura* (10%) genera. *Streptomyces* sp. strain MMA-5 showed the highest multispecific antibiosis efficiency score value. Broad antibiotic spectrum activity was exhibited by *Streptomyces* sp. strain MMA-2 and *Micromonospora* sp. strain MMA-8. The multidrug resistant human pathogenic yeast strain *Candida albicans* was inhibited by 18 actinobacterial strains.

Key words: Actinobacteria, antibacterial and antifungal activity, modified cross streak method

As multidrug resistance is rising, there is a steep decline in antibiotic discovery and development[1]. Hence, there is a continuing and cyclical need of new antibiotics to combat antibiotic resistant strains of pathogenic bacteria[2]. The actinobacteria are a group of Gram-positive bacteria, majority of which are found in the soil playing a significant role in decomposition and humus formation. The discovery of new molecules from actinobacteria have marked an epoch in antibiotic research as most of its antibiotics are too complex to be synthesized by combinatorial chemistry[3,4]. Actinobacteria are the most prolific producer of antibiotics[5]. Out of more than eight thousand antimicrobial products described in antibiotic literature database (ABL database), 45.6% are produced by Streptomycetes and 16% by strains belonging to rare genera of actinobacteria[3], which forms an important component of the soil microflora[6]. The rate of discovery of new compounds from terrestrial actinobacteria has decreased, whereas the rate of reisolation of known compounds has increased[7].

Search of underexplored ecological niches have revealed new molecules which stresses the need to explore new groups of actinobacteria from such habitats as source of novel bioactive secondary

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metabolites supplemented by novel selected media and/or improvement of isolation methods[6-8]. Baltz has estimated that less than one part in 10^{12} of the earth's soil surface has been screened for actinobacteria[1]. Only 10% of natural products from screened strains and just 1% of molecules from the global consortium of microbial producers may have been discovered by researchers[2].

There are many techniques for detecting antimicrobial activity; most of them are based on methods involving diffusion through solid or semi-solid culture media to inhibit the growth of sensitive microorganisms[9]. The cross-streaking is a easy and relatively rapid method for screening cultures in search for new antibiotics and thus establish a spectrum of inhibiting properties of any bacterium, mold, or actinobacteria which will grow discretely on an agar plate[10]. Studies have revealed that the 'cross streak method' resulted in higher inhibition zones on indicator bacteria than those obtained by agar well diffusion method[9] but the major drawback of the 'cross streak method' was difficulty in obtaining quantitative data, since the margins of the zone of inhibition were usually very fuzzy and indistinct[10]. Hence, in the present study, we reported the isolation as well as the inhibitory effects of local actinobacterial isolates on various human pathogenic bacteria and yeast using a modified cross streak method.

For screening, the soil samples were collected from various locations in Goa (Table 1) at a depth of 10-20 cm in clean polythene bags. Several diverse habitats in different areas were selected for isolation of actinobacterial strains, which included forest, biogenic, market, orchard and sediment soils. Samples were transported to the laboratory and stored at 4° until use. The bacterial strains used were procured from the Department of Microbiology, Goa Medical College, Goa while the yeast strain was procured from Microbial Type Culture Collections, Chandigarh (MTCC). The bacteria strains were maintained on slants of Nutrient Agar (Himedia, India), whereas the yeast strain was maintained on slants of Potato Dextrose Agar (Himedia, India). Other chemicals used were of analytical grade and were purchased from Sigma, Germany.

A modified slide technique for isolation of soil actinobacteria in a natural tropical community was used, which employs slides coated with Arginine Vitamin Agar (AVA) medium to specifically capture *ex situ* actinobacterial diversity. The composition of AVA medium[11] is given in Table 2. The basal medium

TABLE 1: VARIOUS SITES SELECTED FOR SOIL SAMPLE COLLECTION

Sampling location	Latitude and longitude	Total no. of actinobacterial isolates	No. of actinobacterial isolates grouped in different taxa			
			Streptomyces	*Actinomadura*	*Micromonospora*	Unidentified Genera
Forest soils						
Forest Karmali, Tiswadi (KFA)	15°29'16"N 73°54'40"E	7	3	0	2	2
Forest Canacona (CFA)	14°59'45.76"N 74°03'02.17"E	4	2	0	0	2
Biogenic soils						
Termite mound, Karmali (TMK)	15°29'16"N 73°54'40"E	1	0	1	0	0
Hollow of *Terminalia* sp. tree, Arambol (MAS)	15°41'02.28"N 73°43'05.17"E	6	5	1	0	0
Market soils						
Mapusa market, Bardez (MMA)	15°35'16.73"N 73°48'32.66"E	6	3	1	2	0
Panjim market, Tiswadi (PM)	15°29'55.64"N 73°49'18.40"E	1	1	0	0	0
Orchard soils						
Orchard, Bicholim (BOA)	15°35'13.96"N 73°56'11.06"E	1	0	0	0	1
Sediment soil						
Siolim, Bardez (SIA)	15°37'39.35"N 73°48'38.79"E	4	2	0	0	2
Total		30	16	3	4	7

TABLE 2: AVA MEDIA COMPOSITION

Basal medium	
Glycerol	0.08 ml
Glucose	0.1 g
K_2HPO_4	0.03 g
L-arginine	0.03 g
NaOH	0.03 g
$MgSO_4.7H_2O$	0.02 g
Agar	1.8 g
Distilled water	100 ml
Stock trace element solution (Add 0.1 ml to the basal medium)	
$Fe_2(SO_4)_3$	50 mg
$CuSO_4.5H_2O$	50 mg
$MgSO_4.4H_2O$	50 mg
$ZnSO_4.7H_2O$	50 mg
Distilled water	50 ml
Stock vitamin solution (Add 0.1 ml to the basal medium)	
Thiamine	100 mg
Calcium panthothenate	25 mg
Meso-inositol	25 mg
Nicotinic acid	25 mg
P-aminobenzoic acid	25 mg
Pyridoxine	25 mg
Riboflavin	25 mg
Biotin	12.5 mg
Distilled water	50 ml
Antibacterials (Added to basal medium)	
Nalidixic acid	0.0176 mg/ml
Neomycin and polymyxin b sulphate	0.01 mg/ml
Antifungals (Added to basal medium)	
Nystatin	0.004 mg/ml
Actidione	0.00192 mg/ml

and the stock trace element solution were sterilized by autoclaving at 121° for 20 min and pH of the medium was adjusted to 6.8 by using 0.1N NaOH/HCl. The stock vitamin and antibiotic solution was membrane sterilized and added later to the presterilised basal medium. The soil samples were filled in clean polyvinylchloride pots and pair of test slides (with antibiotics) was then inserted so that the coated surface was in contact with the surrounding soil horizon. The sets of slides were sampled at predetermined time points on 4, 7, 12, 15, 22, 26 and 28 days without disturbing the colonized coated surface and processed immediately. The slides were then washed with 1 ml of sterile distilled water and 0.1 ml of the suspension was then plated on antibiotic based AVA medium (the same used for coating the slides) and incubated at room temperature of 28-30° till visible colonies were seen on the plates. Incubated plates were observed under Olympus SZ51 stereomicroscope (Tokyo, Japan) and the actinobacterial colonies were then transferred to AVA slants and incubated at the ambient temperature of 28° for 4-7 days.

The isolates were phenotypically grouped according to the traditional criteria of classification including growth, aerial spore-mass colour, texture, elevation, substrate mycelium colour, margin, pigment production and presence or absence of exudates. For micromorphological details air dried smears stained in 1% w/v crystal violet were used and morphology of the spore bearing hyphae with entire spore chain was studied along with other microstructures[11,12]. The criteria used to select the actinobacterial strains were based on *ex situ* slide observations. The strains, which were recovered from slides having less or no fungal bacterial colonization, were selected as it was assumed that these strains could be potential antibiotics procedures.

Determination of antimicrobial activities of 30 pure actinobacterial cultures was performed by modified cross-streak method (MCSM). Muller–Hinton agar (Himedia, India) plates[8] were prepared and inoculated with actinobacterial cultures by a single streak in the centre of the Petri dish and incubated at 28° for 7 days. This was done to provide enough time for the active organism to produce the antibiotic substance, which will diffuse into the agar medium[13]. The cross-streak method (CSM)[14] was modified by keeping the distance between the test pathogen streak fixed i.e. 1 cm; length of the test pathogen streak line, 3 cm; streak width, 0.5 cm; length of actinobacterial streak, 7 cm and width of the actinobacterial streak, 0.5 cm. The markings were drawn on a transparency sheet, which acted as a template and was attached below the plates to facilitate streaking and thereafter in quantifying the results.

The plates were seeded with test organisms by streaking perpendicular to the line of actinobacterial growth. The plates containing active organisms were kept for 14-28 days to determine whether the test pathogens made any further growth towards the master streak, or whether they remain stationary, or whether lysis of the test pathogens occurred[14]. Antagonism was observed based on the inhibitory interaction between the actinobacteria and test strains. The potential antagonistic producer was selected by screening the actinobacterial isolates against a group of pathogens which included human pathogenic Gram negative bacteria such as *Salmonella typhi,*

Shigella flexneri, Proteus sp., *Enterobacter aerogenes, Serratia marcescens,* Gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis* and dimorphic yeast such as *Candida albicans* (MTCC183).

Since the area of each test streak was fixed at 150 mm^2 it was possible to quantify regions of growth and measure the clear/inhibited areas with a graph paper template. The clear areas for different test species are indicators of antibiotic activity and are proportional to antibiotic concentration and diffusion. To express these results quantitatively and to prepare quantitative antibiotic activity score matrices, the following Eqns. were used, Percent area specific differential antibiotic activity score (PASDAAS)= (AWG/TSA)×100....(1), where, AWG is the area on the plate without growth for each test panel streak, TSA is the total streak area (150 mm^2) scored for each test panel streak.

Percent multispecific antibiosis efficiency score (PMSAES), gives the efficiency of tested actinobacteria strain to inhibit more than a single test pathogen. The ideal score would be 100. This is computed using the Eqn. PMSAES=($\Sigma PASDAASTP_{1-8}$/ TPS)×100.....(2), where, $\Sigma PASDAASTP_{1-8}$ is the percent area specific differential antibiotic activity score of test pathogens 1-8 and TPS is total possible score for all test pathogens (i.e. 100×8=800). Scoring total or percent overall inhibition efficiency score (POIES), since multiple microbial species were used in the test panel it was possible to calculate a total score for each actinobacteria using the following Eqn, POIES = (TNIS/TNTS)×100.....(3), where, TNIS is total number of inhibited species and TNTS is total number of test species. The ideal score for multispecific inhibition would be 100. Having calculated the total no of positive results of each test pathogen against 30 actinobacterial strains, it was possible calculate percent overall screening efficiency score (POSES). This is computed by the equation, POSES= (TPR/TAS)×100.....(4), where TPR is total no of positive results of each test pathogen and TAS is total no of actinobacterial strains.

The occurrence and distribution of different genera of actinobacteria isolated from different soil samples are presented in Table 1. Despite the addition of antibiotics in the slide coating medium (SCM) to inhibit growth of unwanted microbes and allow actinobacteria to dominate, bacterial and fungal spores were observed, which shows their partial resistance towards the incorporated antibiotics. These slides acted as reference to the slides in which less or no fungal and bacterial colonization was observed. The strains recovered from such slides were thus assumed to be potential producers of antibiotics (antifungal and antibacterial) and were selected for screening.

Based on the spore morphology and mycelium, the isolated strains were classified into various genera. Out of the 30 actinobacterial isolates, 16 isolates were identified as genus *Streptomyces* (spore chain with coiling, spiral and looped), 3 as *Actinomadura* (spore chains are straight and open hooked), 4 as *Micromonospora* (clusters of single conidia on substrate mycelium) and 7 were unidentified actinobacterial strains. Among the genera recorded in the present study, *Streptomyces* was the most predominant as compared to others. The dominance of *Streptomyces* among the actinobacteria especially in soils has also been reported by many workers[15,16]. Preliminary tests for antimicrobial activity of isolated strains obtained from different locations in Goa, clearly demonstrated that 53% of actinobacterial strains belonging to *Streptomyces* genera, 13% belonging to *Micromonospora* and 10% belonging to *Actinomadura* genera have the ability of producing antimicrobial compounds. Results demonstrate the fertility of the sampled soils in possessing such microbes, which are valued for their unparalleled ability to produce bioactive molecules of biotechnological and pharmaceutical importance. The cross streak method was modified as the streak non uniformity caused a problem with respect to quantifying the results[10]. Besides mutual antibiosis due to use of multiple cultures in the test panel could not be ruled out. The distance between the test steak and the actinobacterial strain was kept constant to reduce the effect of probable mutual antibiosis, as multiple cultures were used in the test panel. Since the test pathogen population was delimited within a defined streaking zone, antibiosis would indicate its impact with respect to that zone and thus the antibiosis reaction could be easily quantified.

Out of 30 cultures screened for antimicrobial activity, 28 cultures showed positive activity. Fifty percent of all antimicrobial producing strains had inhibited five Gram positive bacteria, thirty percent strains had inhibited two Gram negative bacteria and twenty

percent strains inhibited multidrug resistant human pathogenic strain of *Candida albicans*. The multidrug resistance is presently an urgent focus of research and new bioactive compounds are necessary to combat these multidrug resistance pathogens[8,17]. The results of the antimicrobial screening are represented in Table 3.

Streptomyces sp. strain MMA-5 showed high PASDAAS against *Serratia marcescens* (87), *Salmonella typhi* (80), *Staphylococcus aureus* (80), *Bacillus subtilis* (80), and *Candida albicans* (80). while *Streptomyces* sp. strain MAS-11 showed high PASDAAS against *Serratia marcescens* (87) and *Shigella flexneri* (80) whereas *Streptomyces* sp. strain SIA-2 and *Streptomyces* sp. strain MAS-30 were found to be active against *Bacillus subtilis* (80) and *Candida albicans* (80). Unidentified actinobacterial strain KFA-1Gb was found active against *Shigella flexneri* (80) and *Candida albicans* (80). *Streptomyces* sp. strain KFA-5 was found active against *Staphylococcus aureus* (80). *Streptomyces* sp. strain KFA-1 was found to be active against *Shigella flexneri* (80) where as *Micromonospora* sp. strain KFA-9 and *Micromonospora* sp. strain MMA-8 was found to be active against *Shigella flexneri* (80). *Streptomyces* sp. strain MMA-5 showed the highest PMSAES (59) value (fig. 1). Broad antibiotic spectrum activity against eight test pathogens was exhibited by *Streptomyces* sp. strain MMA-2 and *Micromonospora* sp. strain MMA-8 (Table 3). The highest POSES was exhibited against *Staphylococcus aureus* followed by *Proteus* sp. and *Candida albicans* (fig. 2).

From these results we concluded that, a total of 28 actinobacterial isolates associated with soil have shown the ability to produce antimicrobial compounds against 8 test pathogens, especially multiple antibiotic resistant Gram positive bacteria and yeast using 'modified cross streak method'. Further studies

TABLE 3: PASDAAS AND POIES FOR EACH ACTINOBACTERIAL STRAIN

Actinobacterial strain	TPI	TP2	TP3	TP4	TP5	TP6	TP7	TP8	POIES
Streptomyces sp. strain KFA-5	20	0	47	0	0	80	73	73	63
Unidentified actinobacteria strain KFA-8	0	0	60	0	0	0	0	0	13
Micromonospora sp.strain KFA-9	0	80	60	0	67	53	60	0	63
Micromonospora sp. strain KFA-3	13	73	73	0	20	67	0	0	63
Streptomyces sp. strain KFA-6	0	0	0	0	0	0	0	0	0
Unidentified actinobacteria strain KFA-1Gb	0	80	0	27	20	60	0	80	63
Streptomyces sp. strain KFA-1	0	80	67	0	0	67	20	53	63
Streptomyces sp. strain CFA-1	0	0	0	0	0	60	0	0	13
Unidentified actinobacteria strain CFA-2	0	53	73	73	60	60	40	0	75
Unidentified actinobacteria strain CFA-3	0	27	47	0	0	0	53	53	50
Streptomyces sp. strain CFA-2	0	73	0	60	0	73	0	0	38
Actinomadura sp. strain TMK-33	0	60	0	0	0	53	67	27	50
Streptomyces sp. strain MAS-2	0	0	40	20	27	60	60	67	75
Actinomadura sp. strain MAS-4	27	0	0	20	0	0	0	73	38
Streptomyces sp. strain MAS-11	0	80	0	0	87	20	0	0	38
Streptomyces sp. strain MAS-14	0	0	0	0	0	0	0	0	0
Streptomyces sp.strain MAS-28	7	0	0	0	0	0	0	13	25
Streptomyces sp. strain MAS-30	20	0	40	60	67	67	80	80	88
Streptomyces sp. strain MMA-2	60	67	53	40	40	27	47	53	100
Micromonospora sp. strain MMA-4	20	53	40	0	20	20	40	13	88
Micromonospora sp. strain MMA-8	73	80	40	53	20	67	60	60	100
Actinomadura sp. strain MMA-9	0	40	73	0	0	53	40	73	63
Streptomyces sp. strain MMA-2	0	0	0	27	0	40	53	53	50
Streptomyces sp. strain MMA-5	80	0	67	0	87	80	80	80	75
Streptomyces rubrolavendulae	0	7	67	0	40	73	0	0	50
Unidentified actinobacteria strain BOA-10	0	0	0	0	0	20	0	0	13
Streptomyces sp. strain SIA-2	0	0	27	53	0	67	80	80	63
Streptomyces sp. strain SIA-2G	40	0	40	0	60	67	47	0	63
Unidentified actinobacteria strain SIA-2Gb	7	0	20	0	40	0	0	53	50
Unidentified actinobacteria strain SIA-12	20	0	0	27	0	73	73	73	63

PASDAAS is percent area specific differential antibiotic activity score and POIES is percent overall inhibition effciency score. TP1: *Salmonella typhi*, TP2: *Shigella flexneri*, TP3: *Proteus*, TP4: *Enterobacter aerogens*, TP5: *Serratia marcescens*, TP6: *Staphylococcus aureus*, TP7: *Bacillus subtilis* and TP8: *Candida albicans*.

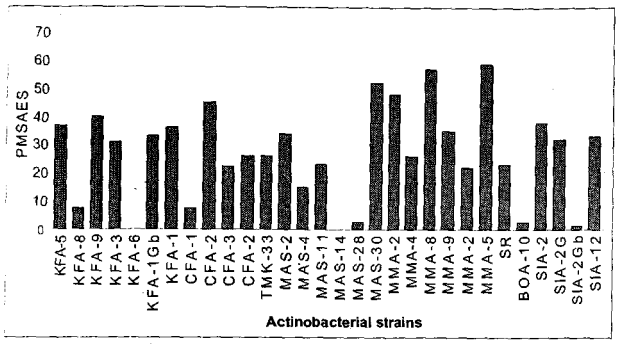

Fig. 1: Percent Multispecific Antibiosis Efficiency Score (PMSAES) exhibited by actinobacterial strains.

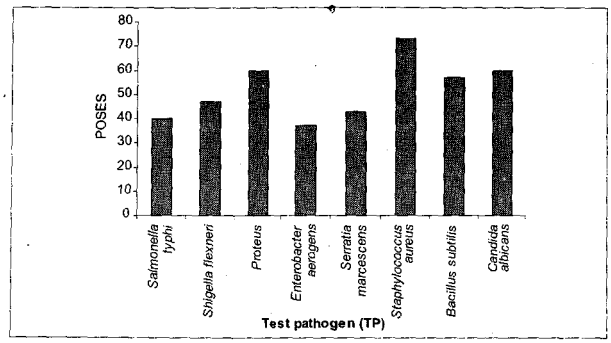

Fig. 2: Percent Overall Screening Efficiency Score (POSES).

on molecular taxonomical characterization of the potential organisms, purification, and characterization of antibacterial and anticandidial compounds are warranted.

ACKNOWLEDGEMENTS

The Authors would like to acknowledge UGC-SAP for providing financial assistance to carry out the above work.

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Accepted 8 March 2011
Revised 31 January 2011
Received 31 August 2010
Indian J. Pharm. Sci., 2011, 73 (1): 223-228

Diuretic Activity of Leaves of *Garcinia Cambogia* in Rats

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Annexure IV

Identification of a Microbial Culture using 16S rDNA based Molecular Technique (*Rajiv Gandhi Centre for Biotechnology, Kerala*)

Sample: CFA-5

I Protocols

DNA isolation using GenElute Plant Genomic DNA Miniprep Kit (Sigma)

The tissue/mycelium was transferred to a microcentrifuge tube and ground in 350µl of lysis solution A and 50µl of lysis solution B using a micro pestle. The mixture was incubated at 65°C for 10 minutes with occasional inversion. 130µl of precipitation solution was added to the mixture, mixed completely by inversion and the sample was placed on ice for 5 minutes. The sample was centrifuged at 14,000 rpm (Eppendorf Centrifuge 5804R) for 5 minute to pellet the cellular debris, proteins, and polysaccharides. The supernatant was transferred to the GenElute filtration column tube and centrifuged at 14,000 rpm for 1 minute. This removed any cellular debris not removed in the previous step. The filtration column was discarded and 700µl of binding solution was added directly to the flow through liquid and mixed thoroughly by inversion. 700µl of this mixture was added into GenElute nucleic acid binding column and centrifuged at 14,000rpm for 1 minute.

The flow through liquid was discarded and the collection tube was retained. The column was returned to the collection tube and the remaining sample was applied to the column. Centrifugation was repeated as above and the flow through liquid and the collection tube were discarded. The binding column was placed into a fresh 2ml collection tube. 500 µl ethanol-added wash solution was added to the binding column and centrifuged at 14,000rpm for 1 minute. The flow through liquid

was discarded and the collection tube was retained. The wash was repeated once more. The binding column was transferred to a new collection tube. 30 µl of elution solution (pre-warmed to 65°C) was added to the binding column and centrifuged at 14,000rpm for 1 minute. The stock DNA was properly labelled and stored at 4 °C.

Agarose Gel Electrophoresis for DNA Quality and Quantity check

The quality of the DNA isolated was checked using agarose gel electrophoresis. 1µl of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0) was added to 5µl of DNA. The samples were loaded to 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/ml ethidium bromide. Electrophoresis was performed with 0.5X TBE as electrophoresis buffer at 75 V until bromophenol dye front has migrated to the bottom of the gel. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad) (Figure 1).

DNA quantification was done by comparing the fluorescent intensity of the samples with a standard (100ng DNA). DNA was diluted to approximately 10ng/µl and stored at 4°C as working solution while the stock DNA (undiluted) was stored at -20°C.

PCR Analysis

PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X PCR buffer (100mM Tris HCl , pH-8.3; 500mM KCl), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 2.0mM $MgCl_2$, 20ng DNA, 1 unit of AmpliTaq Gold DNA polymerase enzyme, 0.15 mg/ml BSA and 3% DMSO, 0.5M Betaine, 5pM of forward and reverse primers.

Primers used

Target	Primer Name	Sequence (5' → 3')
16S rRNA	F243	GGATGAGCCCGCGGCCTA
	R513GC	CGGCCGCGGCTGCTGGCACGTA

The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

PCR amplification profile

COX1

95 °C - 5.00 min

95 °C - 0.30 min
60 °C - 0.30 min } 40 cycles

72 °C - 0.30 min

72 °C - 7.00 min

4 °C - ∞

Agarose Gel electrophoresis of PCR products

The PCR products were checked in 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 μg/ml ethidium bromide. 1 μl of 6X loading dye was mixed with 5 μl of PCR products and was loaded and electrophoresis was performed at 75V power supply with 0.5X TBE as electrophoresis buffer for about 1-2 hours, until the bromophenol blue front had migrated to almost the bottom of the gel. The molecular standard used was a 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad) (Figure 2).

ExoSAP-IT Treatment

ExoSAP-IT (GE Healthcare) consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for the removal of unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications.

Five micro litres of PCR product is mixed with 2 μl of ExoSAP-IT and incubated at 37°C for 30 minutes followed by enzyme inactivation at 80°C for 15 minutes.

Sequencing using BigDye Terminator v3.1

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems , USA) following manufactures protocol.

The PCR mix consisted of the following components:

PCR Product (ExoSAP treated)	-	10-20 ng
Primer	-	3.2 pM (either Forward or Reverse)
Sequencing Mix	-	0.28 µl
DMSO	-	0.30 µl
5x Reaction buffer	-	1.86 µl
Sterile distilled water	-	make up to 10µl

The sequencing PCR temperature profile consisted of a 1st cycle at 96°C for 2 minutes followed by 30 cycles at 96°C for 30 sec, 50°C for 40 sec and 60°C for 4 minutes.

Post Sequencing PCR Clean up

1. Master mix I of 10µl milli Q and 2 µl 125mM EDTA per reaction and master mix II of 2 µl of 3M sodium acetate pH 4.6 and 50 µl of ethanol were prepared.
2. 12µl of master mix I was added to each reaction containing 10µl of reaction contents and was properly mixed.
3. 52 µl of master mix II was added to each reaction.
4. Contents were mixed by inverting and incubated at room temperature for 30 minutes
5. Spun at 14,000 rpm for 30 minutes
6. Decanted the supernatant and added 100 µl of 70% ethanol
7. Spun at 14,000 rpm for 20 minutes.
8. Decanted the supernatant and repeated 70% ethanol wash
9. Decanted the supernatant and air dried the pellet.

The cleaned up air dried product was sequenced in ABI 3500 DNA Analyzer (Applied Biosystems).

Sequence Analysis

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1 (Drummond et al., 2010).

III Sequences

Sequences

>SR94-CFA-5-16S

TCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGG
CCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCT
ACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGC
AGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAG
CAGGGAAGAAGCGAGAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAAC

References

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- BigDye Terminator v3.1 Cycle sequencing Kit – User Manual, Applied Biosystems
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II Figures

Figure 1. DNA

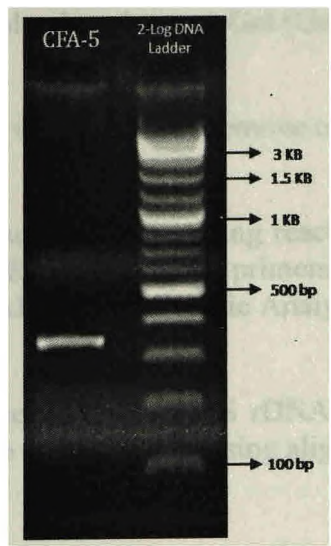


Figure 2. PCR product (16S)

Identification of a Microbial Culture using 16S rDNA based Molecular Technique (Xcelris Labs Ltd.)

SAMPLE I.D. F-9

Experimental Method

1. DNA was isolated from the culture provided by the scientist. Its quality was evaluated on 1.2% Agarose Gel, a single band of high-molecular weight DNA has been observed.

2. Fragment of 16S rDNA gene was amplified by PCR from the above isolated DNA. A single discrete PCR amplicon band of 1500 bp was observed when resolved on Agarose Gel (Gel Image-1).

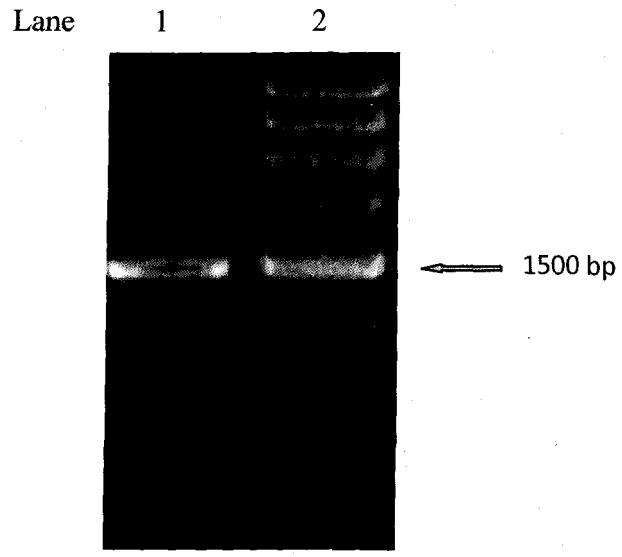
3. The PCR amplicon was purified to remove contaminants.

4. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.

5. Consensus sequence of **1347bp** 16S rDNA gene was generated from forward and reverse sequence data using aligner software.

6. The 16S rDNA gene sequence was used to carry out BLAST with the nrdatabase of NCBI genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 4.

Gel Image of 16SrDNA amplicon



Lane 1:16S rDNA amplicon band

Lane 2: DNA marker

Chromatogram data file detail:

F9_8F_S5361_051_G07.ab1: Data obtained with Forward primer

F-9_1492R_S5361_018_H04.ab1: Data obtained with Reverse primer

F9_8F_S5361_051_G07 (542bp)

ATGGATCCCCTTCGGTGGGGATTAGTGGCGAACGGGTGAGTAACACGTGG
GCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCG
GATACTGACCTTCACGGGCATCTGTGAGGGTCGAAAGCTCCGGCGGTGCA

GGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAACGGCTCACCAAG
GCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTG
AGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAA
TGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGG
GTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAG
AAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCG
CAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCA
CGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAG

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TCGGGTGTTACCGACTTTCGTGACGTGACGGGCGGTGTGTACAAGGCCCG
GGAACGTATTCACCGCAGCAATGCTGATCTGCGATTACTAGCGACTCCGA
CTTCATGGGGTCGAGTTGCAGACCCCAATCCGAACTGAGACCGGCTTTTTG
AGATTCGCTCCACCTTGCGGTATCGCAGCTCATTGTACCGGCCATTGTAGC
ACGTGTGCAGCCCAAGACATAAGGGGCATGATGACTTGACGTCGTCCCCA
CCTTCCTCCGAGTTGACCCCGGCGGTCTCCCGTGAGTCCCCAGCACCACAA
GGGCCTGCTGGCAACACGGGACAAGGGTTGCGCTCGTTGCGGGACTTAAC
CCAACATCTCACGACACGAGCTGACGACAGCCATGCACCACCTGTACACC
GACCACAAGGGGGCGCCCATCTCTGGACGTTTCCGGTGTATGTCAAGCCT
TGGTAAGGTTCTTCGCGTTGCGTCGAATTAAGCCACATGCTCCGCCGCTTG
TGCGGGCCCCCGTCAATTCCTTTGAGTTTTAGCCTTGCGGCCGTACTCCCC
AGGCGGGGCACTTAATGCGTTAGCTGCGGCACGGACAACGTGGAATGTTG
CCCACACCTAGTGCCCACCGTTTACGGCGTGGACTACCAGGGTATCTAAT
CCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTATCGGCCCAGAGA
TCCGCCTTCGCCACCGGTGTTCCTCCTGATATCTGCGCATTTCACCGCTAC
ACCAGGAATTCCGATCTCCCCTACCGAACTCTAGCCTGCCCGTATCGACTG
CAGACCCGGGGTTAAGCCCCGGGCTTTCACAACCGACGTGACAAGCCGCC
T

Consensus Sequence F-9 (1347 bp)

ATGGATCCCCTTCGGTGGGGATTAGTGGCGAACGGGTGAGTAACACGTGG
GCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACC
GGATACTGACCTTCACGGGCATCTGTGAGGGTCGAAAGCTCCGGCGGTGC
AGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAACGGCTCACCAA
GGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACT
GAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCAC
AATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTC
GGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGC
AGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG
GCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTT
GTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGAT
ACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGG

T-654

TGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCT
GGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATT
AGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCAA
CATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGG
GAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCAC
AAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCA
AGGCTTGACATACACCGGAAACGTCCAGAGATGGGCGCCCCCTTGTGGTC
GGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGG
GTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCC
TTGTGGTGCTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAG
GTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGT
GCTACAATGGCCGGTACAATGAGCTGCGATACCGCAAGGTGGAGCGAATC
TCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGA
AGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTT
CCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACACCC
GA