Practical Guidebook to Actinomycete Biology and Technology Applications

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Autonomous Institute of the Department of Science and Technology (DST), Government of India
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Actinomycetes - the ubiquitous and widely distributed group of microbes have sustained the interest of academics, industrial microbiologists and biotechnologists in their search for bioactive metabolites. They are prokaryotes distinguished by possessing morphological features which parallel those observed in the eukaryotic fungi and the very term Actinomycete means "ray fungi" which was applied to denote the mycelial nature of the growing colonies. The discovery of streptomycin as a useful therapeutic antitubercular drug from *Streptomyces griseus* by Selman Abraham Waksman who was awarded the Nobel Prize for this discovery sparked worldwide interest and enthusiasm in the study of actinomycetes. Recent years have seen an explosion in our knowledge and understanding of these organisms from the point of basic biology, molecular biology and technological applications as a potent source of bioactive metabolites of value to mankind. These developments necessitate compilation and upgrading of information related to their biology and biotechnology in a readily accessible form for the benefit of students and research workers interested in undertaking studies on this fascinating group. The literature on actinomycetes is vast and widely scattered in microbiology and biotechnology journals, and recent studies have led to several conceptual changes in the understanding and classification of these organisms.

The initiative to write this book stems from a desire to fulfil this need, albeit in a preliminary fashion with ample scope for future improvements. Special emphasis has been laid on their ecology and distribution, biodiversity and taxonomic trends, techniques in selective isolation and "in vitro" conservation for biotechnology applications. It is hoped that the information compiled will be useful in a practical manner to those involved in studies on actinomycetes. References to original articles and review papers have been given wherever necessary, which will enable more in-depth information to be obtained on specific topics.

**M.C. Srinivasan**

**S.K. Singh**
We are happy to release this book on the occasion of the Platinum Jubilee (1946-2021) of Maharashtra Association for the Cultivation of Science and its Agharkar Research Institute.

In the spirit of Prof. Agharkar's legacy, we have made this book freely accessible for the benefit of researchers and students in Microbiology and Biotechnology to help them formulate innovative research projects involving species of Actinomycetes.

We are thankful to Dr. Prashant Dhakephalkar, Director, Agharkar Research Institute for his keen interest and support in releasing this book.

M.C. Srinivasan
S.K. Singh
It is a privilege and honour to dedicate our humble effort in compiling information on Actinomycetes for the benefit of students in the memory of two great personalities in the field, Professor Selman A. Waksman and Dr. M.J. Thirumalachar.

Prof. Waksman is an acclaimed authority on the genus *Streptomyces* and won the Nobel Prize for his discovery of the life-saving antitubercular drug Streptomycin from a species of *Streptomyces*. Dr. Thirumalachar, a reputed mycologist and microbiologist discovered a new genus of actinomycete *Chainia* from Indian soils and also discovered the therapeutically useful antifungal drug 'Hamycin' and 'Aureofungin' for the control of fungal diseases of plants, both from cultures of actinomycetes isolated by him.

The photograph is a historic one taken during Prof. Waksman's visit to Hindustan Antibiotics Ltd., Pune in which they are engaged in a serious discussion on the taxonomy of *Chainia*, a culture of which Prof. Waksman is holding in his hand.

A. Colony of *Chainia* growing on MGYP agar slant
B. Photograph of sclerotial development on MGYP agar medium
MANDAYAM CHAKRAVARTHI SRINIVASAN (age 86 years) is a senior Biologist with over sixty years of research experience in Mycology and Plant Pathology, Microbiology and Enzyme Technology. His areas of specialization include Microbial Biodiversity, taxonomy and exploring novel microbial strains isolated from natural sources for bioactive metabolites and industrially useful enzyme applications. He is an elected Fellow of the prestigious Indian National Science Academy (FNA) and has served the World Federation of Culture Collections (WFCC) as a nominated panel member for several years. He has been a member of the Project Advisory Committee of the Department of Science and Technology (DST) and Department of Biotechnology (DBT) of the Government of India. As a member of the International Editorial Board of World Journal of Microbiology and Biotechnology, he has reviewed manuscripts for publication for many years.

SANJAY KUMAR SINGH is associated with MACS' Agharkar Research Institute (ARI) with research experience of over 25 years in the field of mycology, plant pathology, in vitro culture conservation and applications of fungi. He has been instrumental in establishing a state-of-the-art National Facility for Culture Collection of Fungi at ARI. NFCCI is a recognized affiliate
member of World Federation for Culture Collections (WFCC). NFCCI houses over five thousand indigenous fungal cultures which are conserved and distributed to researchers. Dr. Singh has visited Montana State University, USA and undergone training with Prof. Gary Strobel on biology of endophytes and their applications. He is an elected Fellow of Maharashtra Academy of Sciences (Life Sciences), Indian Phytopathological Society (IPS), and Society of Applied Biotechnology (SAB). He has been an active member of Mycological Society of India (MSI). He has delivered several invited lectures in conferences/seminars and guided students for M.Sc. and Ph.D. degrees. He has more than 100 research papers in journals of repute. Dr. Singh has organized several national level workshops in which practical training has been imparted to young researchers in studying fungi in pure cultures, their systematics and conserving them for biotechnological applications.
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1.1 Introduction

The classical definition of actinomycetes has been that they are filamentous gram positive bacteria that exhibit true branching. They have been recognised for over a hundred years primarily on their morphological criteria. The first published description of an actinomycete was by Ferdinand Cohn in 1875 who observed a filamentous organism in a concretion from a human lachrymal duct. The organism was named as *Streptothrix foersteri*. Harz in 1877 observed the organism causing bovine lumpy Jaw, which he designated, *Actinomyces bovis*. The widespread saprophytic occurrence of the actinomycetes in soil was largely recognised through the outstanding contributions of Beijernick, Krainsky, Cohn and Waksman during the early years of the 20th century.

1.2 Biodiversity, Classification and Taxonomy

The term Biodiversity has gained attention after the U.N. Earth Summit held at Rio-de-Janiero in 1992, when the need for conservation of natural diversity of living organisms on the Planet Earth was resolved as something vital for the overall well-being of mankind. From a biological point of view, biodiversity may be defined as the totality of genes, species and ecosystems of a region. The United Nations Convention on Biological Diversity recognizes biodiversity as the variability among living organisms from all sources including 'inter alia' terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part. This includes diversity within species, between species and ecosystems. The year 2010 was declared by U.N. as International Year of Biodiversity and emphasised the value of biodiversity for life on earth.
While conservation of endangered species of plants and animals has been the principal aim of biodiversity programs, microbial biodiversity which has so much relevance to the ecosystem for the recycling of organic matter, has received scant attention. Unlike plants and animals which can be enumerated to identify endangered species, microbial biodiversity enumeration and characterization involves painstaking cultural studies.

The fact that the diversity of microbial life forms is of a dimension, far greater and far more varied than that of higher forms of life is now well accepted by all biologists and biotechnologists. Diversity at the taxonomical, ecological and physiological levels of microorganisms has tremendous relevance to the identification of novel metabolites from different groups of microorganisms. Let us consider the aforesaid parameters to evaluate actinomycete biodiversity. Since their first discovery by Ferdinand Cohn, morphology has played the most crucial role in the recognition and cataloguing of actinomycetes, especially those with filamentous mycelial vegetative growth.

Parallel to the morphological basis of classification of fungi, the actinomycete diversity was readily recognised on the morphological basis. Several genera were designated solely on morphological features of distinction such as sclerotia formation in the vegetative mycelium (Chainia), formation of septa in several planes (Dermatophilus, Geodermatophilus), formation of spore chains on aerial outgrowths from the vegetative mycelium (Streptomyces), formation of sporangia with motile spores (Actinoplanes) and formation of synnema like- structures (Actinosynnema).

The classical approaches to taxonomy and differentiation of diverse actinomycetes relied upon morphological, physiological and biochemical characters. In the species differentiation of the most widely studied genus Streptomyces, the classical methods have proved quite useful and formed the basis of species descriptions in the Bergey's Manual of Determinative Bacteriology (1974). These features include the colour of the mature sporulating mycelium, pigmentation of the reverse side of the colony, formation of melanoid and other soluble pigments, which diffuse around the growing colony, spore chain morphology and spore surface ornamentation (smooth, spiny, hairy or warty) and assimilation of carbohydrates, like arabinose, rhamnose, raffinose, xylose, inositol and mannitol.
Advances in our knowledge at the biochemical and molecular level led to the belief that the actinomycete biodiversity cannot be adequately appreciated only on the basis of morphological differences. Cell wall composition, presence of menaquinones and 16s ribosomal RNA sequence data have gained great importance in the identification and recognition of actinomycete biodiversity. Chemotaxonomy is the study of chemical variation in organisms, which can be meaningfully applied to evaluate differences useful for taxonomic purposes. **C.S. Cummins and H. Harris (J. Gen. Microbiol. 15: IX, 1956)** established that actinomycetes have a cell wall composition similar to that of gram positive bacteria. The chemical composition of the cell wall could furnish practical leads towards the differentiations of various types of actinomycetes. The presence of 2, 6 Diaminopimelic acid (DAP) isomers is one of the most important traits characteristic of gram positive bacteria and actinomycetes. DAP as a key amino acid is widely distributed in the cell wall peptidoglycan and occurs in the form of either of two isomers viz. LL form and meso-form.

**M.P. Lechevalier and H.A. Lechevalier (Int. J. Syst. Bact. 20: 435-43, 1970)** evaluated chemical composition as a criterion in the classification of aerobic actinomycetes. Based on cell wall composition, most oxidative (aerobic) actinomycetes can be separated into four groups. Type I cell wall exemplified by the genus *Streptomyces* has L-DAP and glycine in its cell wall. Genus *Micromonospora* has meso-DAP and glycine in its peptidoglycan (Type II cell wall). Type III cell wall present in the genus *Actinomadura* has meso-DAP and a characteristic sugar madurose (3-O-methyl D-galactose) in its cell wall. Type IV cell wall has meso-DAP along with arabinose and galactose, for example in *Nocardia*. Some genera with type IV cell walls possess in addition, a β-branched β- hydroxylated fatty acid termed mycolic acid. These lipids have different molecular weights,- the largest are found in *Mycobacterium* and the smallest in some members of *Corynebacterium* while intermediate molecular weight lipids are present in species of *Nocardia* (**M.P. Lechevalier et al. Can. J. Microbiol. 19: 965-72, 1973**).

Phospholipids have also been considered in actinomycete taxonomy and five different groups of nitrogenous phospholipids have been recognised among actinomycetes (**M.P. Lechevalier et al. Biochemical Ecology and Systematics 5: 249-260, 1977**).
As a result of application of chemotaxonomic data to classification of actinomycetes, several drastic amendments to classical concepts of distinguishing genera have been proposed. Genera established on the basis of distinctive morphological features have been de-recognised on the basis of chemotaxonomy. For instance in the family Streptomycetaceae, as many as six genera with distinct morphological features such as *Chainia, Streptovercillium, and Kitasatoa* have been merged with *Streptomyces* in recent literature based on cell wall composition similarities.

A new trend of actinomycete classification was proposed by E. Stackebrandt et al. (*Int. J. Syst. Bact. 47: 479-491, 1997*) who named *Actinobacteria* as a new class “proposed for the actinomycete line of descent as defined by analysis of small sub-unit (16s ribosomal RNA) and genes coding for this molecule (rDNA). In the classification proposed, phylogenetically neighbouring taxa are clustered into families, suborders, orders, subclasses and a class irrespective of their phenotypic characteristics on which delineation of the taxa had been based in the past. Rather than being based on a listing of a wide array of chemotaxonomic, morphological and physiological properties, the delineation is based solely on 16s rDNA/rRNA sequence-based phylogenetic clustering and the presence of taxon-specific 16s rDNA/RNA signature nucleotides”

To the relatively less specialized microbiologists, the present status of actinomycete taxonomy and classification would certainly appear puzzling and somewhat confusing. The nomenclatural changes made are relevant also for industrial biotechnologists apart from academic actinomycete taxonomists. For example, Rifamycin the anti-tubercular antibiotic was originally described from an actinomycete designated *Streptomyces mediterranei*. Based on subsequent chemotaxonomic and molecular studies, the strain was successively designated *Nocardia mediterranei* and *Amycolatopsis mediterranei*, which is the presently accepted name. This brings home the message regarding the importance of understanding the basic principles of actinomycete taxonomy even by those not actively involved in hard core taxonomic studies of actinomycetes.

Early studies were involved with controversy concerning the nature of actinomycetes, whether they were filamentous bacteria or minute fungi.
Even the name *Actinomycete* derived from Greek language meant 'Ray fungus'. Electron microscopic studies as well as understanding the basic biology of the actinomycetes at the biochemical and molecular level have resolved the controversy successfully to place the actinomycetes firmly as members of the bacterial kingdom with little or no phylogenetic relationship to the eukaryotic fungal groups.

The properties in common with the bacteria include hyphal diameter of only 1µ, prokaryotic nature of the genome, cell walls with mucopeptides, sensitivity to phages and sensitivity to antibacterial antibiotics. Their resemblance with fungi lies only in the formation of hyphae with true branching and formation of 'spores' as agents of dissemination. Endospores akin to those present in spore forming bacteria are not known among actinomycetes.

Application of modern techniques such as nucleic acid pairing and RNA oligonucleotide sequencing brought out several unique features and it was realized that definition of actinomycetes only on a morphological basis was not correct and tenable. Coryneform bacteria, *Mycobacterium*, *Arthrobacter*, *Cellulomonas*, and *Microbacterium* with little or no mycelial formation were shown to be phylogenetically related to the Actinomycetes. The possession of a high guanine (G) and cytosine (C) content in their DNA (>55 mol %) characterized all these forms placed under actinomycetes. On the other hand, mycelia-forming *Thermoactinomyces* forming spores was excluded from the actinomycetes and classified with the endospore forming Bacillaceae due to their low G+C content and the spores having similarity to the endospores of *Bacillus*.

As defined by Goodfellow and Williams (*Ann. Rev. Microbiol. 87: 189-216, 1983*) the actinomycetes “are gram positive bacteria with a high guanine (G) plus cytosine (C) content in their DNA (>55 mol %) which are phylogenetically related from the evidence of 16S ribosomal (r) cataloguing and DNA: rRNA pairing studies”. Evidently “possession of branched hyphae should not automatically place a bacterium within the order Actinomycetales, nor should the inability of an organism to form branching filaments necessarily exclude it from this taxon.

The actinomycetes have been separated into groups on the basis of
physiology (fermentative vs. oxidative metabolism), morphology (type and stability of mycelium, types, number and disposition of spores, formation of sclerotia, sporangia or synnemata, formation of flagellated elements, physical qualities (heat resistance, and chemistry (cell wall and whole cell composition, types of lipids, isoprenoid quinones).

The classification system proposed by E. Stackebrandt et al. (Intern. J. Syst. Bact. 47: 479-91, 1997) involves clustering phylogenetically neighbouring taxa at the genus level into families, suborders, and subclasses together under class Actinobacteria irrespective of those phenotypic characteristics on which the delineation of the taxa had been based earlier.

The delineation was based solely on the 16S rDNA/RNA signature nucleotides. Chemotaxonomic, morphological and physiological properties which were given weightage in earlier systems of classification were not taken into consideration in the present taxonomic scheme.

1.3 Ecology and Distribution

Actinomycetes are widely distributed in both terrestrial and aquatic ecosystems, the majority of them being saprobic while some of them are important plant and animal pathogens. Actinomycetes are also associated with select plants as nitrogen-fixing symbionts or as endophytes colonizing different tissues. Actinomycetes present in the rhizosphere region of plants have also been projected to play a role in biocontrol against root disease pathogens of plants.

1.4 Actinomycetes in the Terrestrial Ecosystem

1.4.1 Soil

Soil is a major source of actinomycete biodiversity and in most soils an estimated population of over one million per gram has been envisaged. Saprophytic actinomycetes are important primary colonizers of soil organic matter, the bulk of which occur as insoluble biopolymers. Ability of actinomycetes to degrade these polymers through enzymes allows them to colonize and establish themselves on residue such as lignocellulose from plants and chitin from animal sources.
Following colonization, heavy sporulation in forms like *Streptomyces* or mycelial fragmentation in other forms generate propagules for widespread distribution in the ecosystem. Soil inhabiting members of the Actinoplanaceae produce motile spores within desiccation resistant sporangia which are dispersed by moisture droplets or rain drops. While the actinomycetes are largely neutrophilic (growing best at or around neutral pH), true acidophilic forms growing best at pH 4.0-5.5 as well as alkalophilic forms having optimum growth above a pH of 8.5-9.0 have also been identified. A new genus *Streptoacidiphilus* has been established for an obligately acidophilic actinomycete from acidic soils (S.B. Kim et al. *Antonie van Leeuwenhoek* 83: 107-116, 2003).

Soil is a complex habitat and in analysing the actinomycete population various important factors need consideration among which the vegetation cover, soil type, depth of sample, physical nature, organic content, moisture and pH are the most important to take into account.

Actinomycete populations show a wide range of adaptability and their distribution in salt marshes, compost heaps and self-heating bagasse piles bear testimony to their wide ranging capability to survive and grow under diverse ecological environments.

Species of *Streptomyces* survive for prolonged periods in soil as resting arthrospores which under favourable conditions colonize fragments of plant debris or dead fungal hyphae, grow and eventually sporulate. Non-mycelial actinomycetes such as *Arthrobacter* possibly survive long periods as resting coccii.

Biochemical studies on acidophilic and acidoduric actinomycetes growing best at pH 3.5-6.5 have widespread distribution in soils and producing amylases or chitineses with pH optima lower than the corresponding enzymes of neutrophilic actinomycetes. Most soil actinomycetes are strict aerobes. Species of *Oerskovia* are capable of anaerobic growth. The saprophytic *Actinomyces humiferus* is microaerophilic.

Actinomycetes, and species of *Streptomyces* in particular have been shown to be capable of producing several enzymes which degrade naturally occurring polymers such as hemicellulose, cellulose, pectin, keratin and chitin.
Addition of hydrocarbons or petroleum to soil had a stimulatory effect on populations of select actinomycetes like *Rhodococcus* and *Arthrobacter*.

Degradation of man-made soil pollutants such as pesticides by actinomycetes has also received considerable attention. For example a *Nocardiopsis* strain isolated from chlordane treated soil degraded the insecticide during “in vitro” studies (R.W. Beeman and F. Matsumura. *J. Agr. Food Chem.* 29: 84-89, 1981).

### 1.4.2 Plant Litter

Relatively few studies on litter actinomycetes are available and no systematic surveys of their pattern of succession as well as degradation of litter particles have been explored. However the widespread occurrence of cellulolytic enzymes among the actinomycetes points to their potential significant role in the recycling of plant biomass in the natural environment.


### 1.4.3 Compost and Related Materials

Initial degradation of organic substrates during composting takes place by the activities of diverse microorganisms, including mesophilic actinomycetes. This process is often accompanied by self-heating under the compact moist conditions and the ecosystem now favours the selective enrichment of thermophilic bacteria and actinomycetes.

*Thermoactinomyces*, presently excluded from actinomycetes and classified under Bacillaceae along with members of different actinomycete genera such as *Thermomonospora*, *Saccharomonospora*, *Micropolyspora*, *Pseudonocardia* and thermotolerant *Streptomyces* constitute the representative forms encountered in composts.

*Rhodococcus coprophilus* is a mesophilic species associated with animal dung and enumeration of its population in aquatic habitats is regarded as a
biological indicator to the extent of faecal pollution arising from farm animal wastes.

In composts, predominance of actinomycetes is well established and can even be directly observed as a surface bloom termed as “fire fang”. *Thermomonospora* species occur in large numbers during manures undergoing composting for the purpose of mushroom cultivation. Activated sewage sludge shows several non-filamentous actinomycetes such as *Corynebacterium, Microbacterium, Arthrobacter* and *Rhodococcus*. The predominant form observed in the foam of activated sludge is *Nocardia amarae* which causes demulsification of the sludge.

Thermophilic actinomycetes like *Thermomonospora curvata* produce powerful extracellular enzymes of the cellulase complex and play an active role in municipal waste decomposition.

Decomposition of moist fodder and grains (improperly dried) during storage is a serious loss and actinomycetes play a major role. Both mesophilic and thermophilic forms have been studied for their successive colonization during degradation of hay. The moisture content closely regulates the temperature attained and acts as a selection pressure for the mesophilic or thermophilic forms to predominate. The pattern of succession involves mesophilic forms like *Streptomyces albus* yielding to more thermophilic and thermotolerant forms such as *Micropolyspora faeni* and *Saccharomonospora viridis*. Stored barley grains with a moisture content of 35-40% attained temperatures of 50-60°C which encouraged growth of *Micropolyspora faeni* and related species.

Baled sugarcane bagasse is yet another material with approximately 50% moisture content and 3-6% sugar which provides favourable conditions for self heating up to 50°C and development of a wide range of actinomycetes.

The health hazard to humans from inhaled spores developing on the deteriorated materials is of serious consequence. Actinomycete spores are involved as the causal agents of allergic syndromes variously referred to as Farmers' hay lung, bagassosis and mushroom worker's lung. *Micropolyspora faeni* and *Thermoactinomyces vulgaris* are dominant forms associated with the farmer's hay lung.
1.5 Actinomycetes in the Aquatic Ecosystem

1.5.1 Freshwater Actinomycetes

Aquatic habitats, both freshwater and marine, show populations of actinomycetes and have been widely studied. Many of the forms encountered such as *Rhodococcus coprophilus* and *Thermoactinomyces vulgaris* are deemed to have terrestrial origins and are present as wash-ins from neighbouring terrestrial habitats. Many terrestrial forms washed into aquatic habitats many get deposited in mud and sediments but while surviving in the aquatic habitat may not show active biological growth and metabolism. It is necessary to focus on genera and species which are indigenous to the aquatic habitat and well adapted to grow and exhibit biological activity.

*Actinoplanes* and related actinomycetes are found in rivers and lakes where they are commonly found in association with and colonizing decomposing aquatic vegetation. The spore bearing vesicles or sporangia are capable of withstanding considerable desiccation and when hydrated release motile zoospores which have a tuft of flagella. These zoospores colonize organic substances available in the aquatic environment.

*Micromonospora* species are commonly isolated from lake muds and river sediments and are also believed to be an integral part of the aquatic actinomycete microflora. *Micromonospora* species have even been considered to play a role in the recycling of cellulose, chitin and other polymers in the aquatic ecosystem. It is difficult to identify any actinomycete which is specifically adapted only to living in the freshwater ecosystem. Many actinomycete spores can remain viable in aquatic habitats for varying periods and become biologically active to varying degrees under favourable conditions. Actinomycetes have also been identified as contaminants of potable water where they cause a strong earthy odour due to formation of compounds such as geosmin and methyl iso-borneol.

1.5.2 Marine Actinomycetes

Actinomycetes over the early years have been reported sporadically from marine habitats and the general feeling among the microbiologists was that those isolated from marine mud were forms not indigenous to the sea.
However, C.E. Zobell and his associates as early as the 1940's had noted the occurrence of *Streptomyces*, *Nocardia* and *Micromonospora* growing on dead marine algae. Zobell et al. (*Bull. Amer. Assn. Petroleum Geologists* 27: 1175-93, 1943) reported that the majority of hydrocarbon oxidising microbes isolated from marine enrichment cultures were species of *Nocardia*, *Streptomyces*, and *Micromonospora*. Y.M. Freitas and J.V. Bhat (*J. Univ. Bombay* 23: 53-59, 1954) isolated species of *Nocardia* and *Streptomyces* from deteriorating fish nets in Bombay, which grew well on media containing up to 8% sodium chloride and showed significant cellulolytic activity.

Recent years have witnessed tremendous enthusiasm in exploring actinomycetes from marine habitats in the quest for novel bioactive molecules with pharmacological applications. T.J. Mincer et al. (*Appl. Env. Microbiol.* 68: 5005-11, 2002) reported isolation of major taxa of obligate marine actinomycetes from ocean sediments which was designated MAR-1. More than 200 isolations possessing MAR-1 morphologies were examined and they displayed an obligate requirement of sea water for growth.

Phylogenetic characterization based on almost complete gene sequencing including 16s Ribosomal RNA yielded a monophyletic clade within the family of Micromonosporaceae and suggested a novelty at the genus level. This report was the first evidence for widespread populations of obligate marine actinomycetes, which also showed remarkable biological activities in culture.

L.A. Maldonado et al. (*Int. J. Syst. Envol. Microbiol.* 53: 1759-66, 2005) reported on the taxonomy of MAR-1 and designated a new genus *Salinispora* with *S. arenicola* as type species. It is a point of interest that a species of *Salinispora*, *S. tropica* produced Salinosporamide-A which has been identified as a very potent anticancer drug and has been “fast tracked into clinical trials” (D.A. Hopwood. *Nature Chemical Biology* 3(b): 457-58, 2007). H.P. Fiedler et al. (*Antonie van Leeuwenhoek* 87: 37-42, 2005) isolated over 600 actinomycetes from various sites in Pacific and Atlantic oceans and found many of them to be producers of novel bioactive metabolites. A new genus of actinomycetes, *Verrucosispora* isolated from peat bog in Germany has been shown to produce novel inhibitors of p-aminobenzoic acid pathway


*Actinopolyspora: Gochnauer et al.* (Can. J. Microbiol. 21: 1300-11, 1975) isolated an actinomycete as a contaminant of a culture medium containing 25% sodium chloride. The organism could use a variety of carbon sources and ammonium salts for growth. A minimum sodium chloride concentration of 10-12% was essential for supporting growth in solid or liquid media. *Actinopolyspora halophila* was described as the type species.

In more recent years *Actinopolyspora* cultures have been isolated from extremely saline soil in Iraq. J.S. Ruan* et al.* (Int. J. Syst. Bact. 44: 759-63, 1994) designated *Actinopolyspora iraqiensis* growing on high salt content media and *A. mortivallis* was isolated from a soil sample collected in Death Valley, California, USA (M. Yoshida* et al.* Int. J. Syst. Bact. 41: 15-20, 1991). C.R. Kokare* et al.* (Current Science 86: 593-97, 2004) reported culturing a strain of *Actinoployspora* AH-1 from marine sediment collected at Alibagh in Maharashtra, India. The organism grew well at 30-36°C on culture media with 15% sodium chloride.

### 1.5.3 Mangrove Actinomycetes

The mangrove ecosystem worldwide is a largely unexplored source for actinomycetes with biotechnology potential. K. Hatano (IFO Research Commun. 18: 26-31, 1997) investigated actinomycete populations in rhizosphere soils of mangrove forests in Japan and found that their populations ranged from 10² to 10⁴ colony forming units per gram of soil. *Streptomyces* and *Micromonospora* isolates were obtained frequently while *Nocardia, Rhodococcus, Actinomadura* and some additional rare forms were also encountered.
In a study of mangrove actinomycetes from China, **Ku Hong et al.** (*Mar. Drug* 7: 24-44, 2009) reported studies on 2000 actinomycetes classified under 13 genera. *Micromonospora* and *Streptomyces* were sources of interesting bioactive metabolites including antitumor, antidiabetic and anti-neurodegenerative disease.

Isolation of novel actinomycetes from Bangladesh mangrove rhizosphere mud has been reported by **Ismel Ara** and co-workers. A new species of *Nonomuraea* (*N. maheshkhaliensis*) has been identified on the basis of morphological, physiological and chemotaxonomic studies supplemented with 16S rRNA gene sequence comparisons and DNA-DNA hybridization (*I. Ara et al. J. Gen. Appl. Microbiol.* 53: 159-166, 2007). It would not be surprising to observe in future years greater research inputs with a consequent better understanding of the actinomycetes present in the marine and mangrove ecosystems. Novel molecules with bio-activities useful to mankind could possibly be identified from their isolates.

### 1.6 Plant Associated Actinomycetes

Three categories of actinomycete association with different plants can be recognised:

- Nitrogen-fixing symbionts referable to the genus *Frankia*.
- Causing pathogenesis and disease symptoms.
- Non-symptomatic association as endophytes in plant tissues.

#### 1.6.1 Nitrogen-fixing Symbionts

Symbiotic associations are formed in more than 170 species of woody dicotyledonous plants by actinomycete referred to genus *Frankia* often as root nodules. The host plants are taxonomically unrelated. Pure culture isolation from hosts such as *Alnus, Comptonia, Elaegnus* and *Myrica* was achieved and all the strains were slow-growing while not having any unusual or complex nutritional requirements. Species differentiation based on host plant was not found to be satisfactory as “cross-infectivity” could be established in inoculations with isolates originating from taxonomically unrelated hosts.

Plant infection occurred through the root hairs in all cases followed by
subsequent developmental stages leading to nodule formation. Nitrogen fixation process information has been derived from studies on nodule homogenates or cell-free nodule extracts. It has been stated that the rates of nitrogen fixation in nodulated plants are comparative with those observed in legumes with root nodule bacterial association.

1.6.2 Phytopathogens

Species of *Streptomyces* causing scab disease of potato and sugar beet is widespread and is designated *Streptomyces scabies*. Infection causes disfiguration of potato tubers which may appear as either superficial skin roughening or forming discrete superficial or deep lesions. Species of *Corynebacterium* cause diseases on a variety of plants. Example includes bean wilt by *C. flaccumfaciens*, wilt and stunting of alfalfa (*Medicago sativa*) by *C. inisidiosum* and *C. michiganense* causing canker of tomato and some other solanaceous hosts. A. species of *Rhodococcus*, *R. fasciens* has been reported to cause fasciation in sweet pea and leaf galls on several hosts.

1.6.3 Non-symptomatic Endophytic Associates

Endophytic microbes including fungi, bacteria and actinomycetes are ubiquitous in most plant species growing in the natural environment. Fungal endophytes have received considerable attention and many aspects of benefit to plants such as improvement of plant growth or reducing disease symptoms caused by plant pathogens has been demonstrated. More recently the ability of fungal endophytes to acquire the capability and synthesise plant metabolites of value has gained attention. For example an endophytic fungal associate of the pacific yew plant *Taxus brevifolia* could produce the valuable anticancer drug ’Taxol’ and this endophytic fungus has been designated as a new taxon, *Taxomyces andreanae* (A. Steirle et al. *Science* 260: 214-216, 1993).

J. Hallmann et al. (*Can. J. Microbiol.* 41: 895-914, 1997) defined bacterial endophytes as those which do not visibly harm the plant host and which can be isolated from the surface disinfected plant tissue or extracted from inside the plant. Their definition encompasses internal colonizers with apparent neutral behaviour or with symbiotic relationship. Several reports in literature describe the diversity of actinomycetes isolated from a variety of hosts plants as well as some of their potential metabolites of value.

M. Rosenblueth and E. Martinez-Romero (Plant Microbe Interact. 19: 827-837, 2006) reviewed bacterial endophytes and their interaction with hosts. They have reported the isolation of *Streptomyces*, *Nocardia*, *Arthrobacter*, *Corynebacterium* and rare genera, like *Kocuria* as plant endophytes. It is generally observed that all vascular plants harbour several endophytic actinomycetes and their numbers are especially more in roots. A multiplicity of forms can be isolated from a single plant tissue by careful planning of isolation protocols and the biodiversity of actinomycetes residing within plant tissues could hold tremendous potential for academic interests as well as industrial research. Reports of metabolites from endophytic actinomycetes exhibiting antagonistic activity against phytopathogenic fungi include Fistupyrone from an endophytic *Streptomyces* inhibiting infection of Chinese cabbage by *Alternaria brassicicola* (Y. Igarashi et al. J. Antibiotics 53: 1117-1122, 2000) and an endophytic *Streptomyces* from *Aucuba japonica* producing novel novobiocin analogues (T. Sasaki et al. J. Antibiotics 54: 441-447, 2001).

A novel peptide antibiotic complex designated as coronamycin was isolated from an endophytic *Streptomyces* occurring in the stems of *Monstera* sp. and the antibiotic exhibited fungicidal activity towards species of *Pythium* (D. Ezra et al. Microbiology 150: 785-793, 2004). A strain of *Streptomyces hygroscopicus* isolated from the fern *Pteridium aquilinum* elaborated Pteridium acid A and B having plant growth promoting activity which has auxin, like-properties (Y. Igarashi et al. J. Antibiotics 55: 764-767, 2002).

Ten actinomycete strains isolated from herbaceous and woody tissues of *Taxus* plants were reported capable of producing the taxanes/anticancer drug Taxol (M. Caruso et al. Ann. Microbiol. 50: 3-13, 2000). A thermostable glucoamylase from an endophytic *Streptosporangium* in maize leaves has been reported by T.L.M. Samford et al. (Bioresource Technol. 83: 105-109, 2002). The promising potential of diverse endophytic actinomycetes as resource
pools for a variety of useful metabolites is evident from the examples cited. It is obvious that more intensive exploration of endophytic actinomycetes is warranted for discovering novel metabolites of value and use in diverse applications.

### 1.7 Actinomycetes Associated with Humans and Animal Hosts

Actinomycetes incite human and animal infections which manifest as actinomycetomas caused by *Actinomadura madurae*, *Nocardia asteroides*, *N. brasiliensis*, etc. Actinomycosis including lumpy jaw formation in humans and animals by the anaerobic *Actinomyces israelii* and *A. bovis* are the earliest reports of actinomycetes with pathogenic potential. Tuberculosis and leprosy caused by *Mycobacterium tuberculosis* and *M. leprae*, diphtheria caused by *Corynebacterium diphtheriae* are among the most dreadful diseases faced by mankind. The hazardous effect of actinomycete spore inhalations leading to farmens' lung syndrome has been already discussed.

Reports of opportunistic actinomycete infections in immuno-compromised patients have been on the increase in recent years. Strains of *Gordona* referable to *G. terrae* have been implicated in primary pulmonary disease as well as wound infections in immuno-compromised patients. *Gordona* is closely allied to *Rhodococcus* and *Mycobacterium* possessing mycolic acids.

Actinomycetes are known to cause water pollutions and cause problems in sewage treatment plants such as 'bulking' and formation of foams and scums. In a study of actinomycete diversity associated with foaming in activated sludge plants, **M. Goodfellow et al.** (*J. Ind. Microbiol. 17: 268-280, 1996*) isolated number of mycolic acid containing actinomycetes, many of which had chemical properties consistent with their classification in the genus *Gordona*. Actinomycetes also play an active role in the biodeterioration of natural materials and are responsible for biodeterioration and spoilage of a variety of materials including wood pulp, wool, rubber, plastics, straw, grains and plant fibers.
Chapter-2

Selective Isolation Techniques for Pure Culture Studies on Diverse Actinomycetes

2.1 Introduction

Diverse genera and species of actinomycetes are widely distributed in soil, plant litter and other natural substrates. The top 4-inch layers of soils have a high level of microbial activity and are best suited for actinomycete exploration. Under natural environmental conditions, the actinomycete populations have been estimated to be ten orders of magnitude less than the bacterial populations and hence they are easily overrun on conventional media rich in organic nutrients, if used for the actinomycete exploration studies. Among actinomycetes too, the heavy sporing *Streptomyces* are the most abundant and easily overtake the other oligosporous and slower growing forms.

It is essential to have knowledge on the relative survival potential of diverse genera and species and their storage conditions in the laboratory. Also, knowledge of the habitats and ecosystems rich in specific genera and species should be highly useful. For example, high population counts of *Micromonospora* are encountered in soil samples collected near the edge of ponds or the lake bottom core samples. Survival of actinomycete biodiversity during the storage of soil samples is influenced by and varies with the type of soil in organic content, pH and temperature of storage. Soil sample storage at -20°C in polythene bags showed better survival compared with survival at 4°C. Drying of soil samples followed by storage at room temperatures for long periods yielded satisfactory counts of both *Streptomyces* and *Micromonospora*. In planning experiments to explore actinomycete biodiversity in natural substrates, it is essential to adopt techniques which facilitate overcoming the competition from the more abundant and rapidly
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growing forms and creating such conditions favourable for the slower growing forms to establish colony growth on the agar media in the isolation plates.

The actinomycetes have been a most versatile group of microorganisms from the point of the variety and diversity of secondary metabolites produced, several of which have proved to be of immense value as therapeutic agents for combating diseases. When conventional isolation techniques are applied, most of the isolates recovered belong to the genus *Streptomyces*, which are predominant in the soil. *Streptomyces* species have long been recognized as prolific producers of bioactive compounds. They have provided more than 50% of the naturally occurring antibiotics discovered to date and continue to be a major source of new bioactive metabolites. However, the rate of discovery of new molecules/metabolites in the quest for new drugs and pharmaceutical compounds has considerably declined because of repeated re-isolation of species of *Streptomyces* already investigated widely. In the context of modern strategies for natural product screening, adoption of improved methodologies for isolating the uncommon and less investigated or "rare" actinomycetes is required to avoid re-isolating strains that produce known bioactive metabolites and to improve the quality of the natural products screened (*A. Lazzarinit et al. Antonie van Leuwenhoek 78: 399-405, 2001; Y. Takahashi and S. Omura. J. Gen. Appl. Microbiol. 49: 141-154, 2003*).

The role of rare actinomycetes as potential resource pools for novel bioactive molecules is amply evident from the finding that during 1975-1980, 255 of the novel actinomycete antibiotics originated from non-Streptomycete rare actinomycetes. (*L.J. Nisbeth. J. Chem. Technol. Biotech. 32: 251-270, 1982*). Aerial spores of most actinomycete genera show considerable resistance to wet and dry heat and this property has been taken advantage of in isolation experiments to minimize serious competition from bacterial species. Several actinomycetes have shown multiple resistance to a range of chemical additives and antibiotics and by a judicious choice of these in the isolation media, innovative techniques have been developed to achieve selective isolation of the so called "rare" actinomycetes. Unconventional nutrient media such as humic acid vitamin agar (*M. Hayakawa and H. Nonomura. J. Ferm. Technol. 65: 609-616, 1987*) and hair hydrolysate vitamin agar (*S.H. Cho et al. Korean J. Appl. Microbiol. Biotechnol. 22: 561-563, 1994; C.N. Seang et al.*).
have been successfully employed and these media while supporting growth and sporulation of the slow growing actinomycetes are less favourable for bacterial growth. Competition from fungi can be effectively controlled by addition of Nystatin (50 µg/ml) and Nalidixic acid (20 µg/ml).

An overview of some of these techniques and media formulations reported in the literature for exploring the actinomycete biodiversity in natural substrates and habitats is presented. Reference to the original publications is also included, wherever possible to enable the readers to get more details, if required.

2.2 Dry Heat Pre-treatment with/without Chemicals

Heating air dried soil samples at 100-120°C reduced the numbers of filamentous bacteria and *Streptomyces* and favoured the isolation of *Micromonospora* and *Streptosporangium* colonies. (H. Nonomura and Y. Ohara. *J. Ferm. Technol.* 47: 463-469, 1969). Chi Nam Seong et al. (*J. Microbiol.* 39: 17-23, 2001) observed that all kinds of microorganisms including rare actinomycetes decreased when soil was heated at 100°C for one hour. However, heating a soil suspension at 70°C for 15 minutes inhibited fungal and bacterial colonies facilitating the recovery of rare actinomycete colonies in high numbers.

Selective isolation of *Microtetraspora glauca* was achieved by dry heating soil at 110 °C for 1 hr. and subjecting a water suspension of the soil to chemical treatment with 0.05% Benzethenium chloride. The isolation medium designated LSV-55 agar contained a commercial lignin (Dealkaline) at one gm per liter as major carbon source. The medium supplemented with Kanamycin, Norfloxacin and Nalidixic acid supported adequate growth and sporulation of *M. glauca* while inhibiting non filamentous bacteria and fast growing common *Streptomyces* colonies (M. Hayakawa et al. *J. Appl. Bact.* 80: 375-386, 1996).

For selective isolation of *Streptosporangium* and *Dactylosporangium* colonies, air dried soil samples were heated at 120°C for 1 hr and a water suspension of the heated samples was treated with 0.01% Benzethenium chloride, diluted

Phenol treatment of soil suspensions lowered the numbers of fungi and other bacteria while actinomycetes are less affected. Treatment of soil suspension with 1.5% phenol at 30°C for 30 min prior to inoculation on humic acid vitamin agar plates enabled selective isolation of *Streptomyces violaceus-niger* phenotype cluster in high percentage while suppressing other ubiquitous streptomycetes. *S. violaceus-niger* strains are characterized by high antimicrobial and anti-tumor activities (M. Hayakawa et al. J. Appl. Microbiol. 96: 973-981, 2004).

Dry heat treatment of soil at 120°C for one hour and treatment with a solution of 1.5% phenol and 0.03% Chlorhexidine gluconate resulted in a 90% selective isolation of *Microbispora* species on humic acid vitamin agar supplemented with nalidixic acid (M. Hayakawa et al. J. Ferm. Bioeng. 72, 320-326, 1991). Similarly, dry heating at 110°C for one hour followed by 1.0% phenol treatment and plating on humic acid vitamin agar supplemented with Kanamycin, Josamycin, Lysozyme and Nalidixic acid was selective for *Actinomadura viridis* (M. Hayakawa et al. J. Ferm. Bioeng. 79, 287-289, 1995).

For isolation of *Streptosporangium* air dried soil was suspended in water and subjected to treatment with 1.0% chloramine T (a chlorine releasing biocide), diluted and cultured on Humic acid-vitamin agar. Chloramine T treatment reduced non-filamentous bacteria and undesirable streptomycetes. By this procedure apart from *Streptosporangium* other rare actinomycetes like *Herbidospora, Microbispora* and *Microtetraspora* were isolated (M. Hayakawa et al. J. Ferm. Bioeng. 89: 599-602, 1997).

Pretreatment of soil by incubation with calcium carbonate has been found to favour isolation of actinomycetes. El Nakaceb and H.A. Lechevalier (Appl. Microbiol.11: 75-77,1963) mixed 1 gm air dried soil with 1 gm CaCO₃ in a mortar followed by incubation for ten days at 28°C in a close inverted Petri-dish in which a high relative humidity was maintained by water saturated discs of filter paper. An arginine-glycerol salts medium was found to be suitable for the isolation of actinomycetes. M. Otogero et al. (J. Appl. Microbiol. 91: 118-
developed an integrated method for the enrichment and selective isolation of the motile-spore forming genus *Actinokineospora*. The method consists of initially incubating the source material with calcium carbonate under humid conditions followed by air drying. A second stage of operation involves re-hydration by immersing the amended substrate in phosphate buffer soil extract solution to permit release of zoospores. This is followed by centrifugation to settle the non-motile microbial associates while enriching the supernatant with the motile spores of *Actinokineospora*. Plating out the supernatant on humic acid vitamin agar supplemented with Fradimycin, Kanamycin, Nalidixic acid and Trimethoprim consistently enabled the selective isolation of *Actinokineospora* colonies.

A sucrose density gradient centrifugation technique for selective enrichment of *Nocardia* strains in the 20% sucrose layer followed by plating out on humic acid vitamin agar supplemented with Nalidixic acid and chlortetracycline has been described by H. Yamamura et al. (*J. Appl. Microbiol. 95: 677-685, 2003*). Methods to isolate specific forms based on the understanding of their basic physiology and nutritional capabilities have also been described in literature. A mineral salts sodium propionate (0.02%) agar designated M-3 medium was formulated for selective isolation of *Rhodococcus*. Filter sterilized Cycloheximide (50 pg/ml) and Thiamine-HCl (4 ug/ml) were added to the medium after autoclaving (*J. Rowbotham and T. Cross. J. Gen. Microbiol. 100: 231-240, 1977*).

The relative resistance of *Streptoverticillium* strains to oxytetracycline at concentrations inhibitory to several other actinomycetes including *Streptomyces*, *Nocardia* and *Micromonospora* was utilized to formulate a selective isolation protocol for isolating *Streptoverticillium* strains from soil (*L.J. Hanka et al. FEMS Microbiol. Lett. 30: 365-368, 1985*). Isolation of *Thermomonospora chromogena* from mushroom compost was successfully achieved on media containing Kanamycin at 25 µg/ml and incubation at 50°C (*A.J. MacCarthy and T. Cross. J. Appl. Bact. 51: 299-301, 1981*).

### 2.3 Baiting/Enrichment Technique

One of the earliest published record for baiting for actinomycete isolation is that of R.E. Gordan and W.A. Hagan (*J. Infec. Dis. 59: 200-206, 1936*) who
employed glass rods coated with paraffin wax to dip in a soil suspension prepared in a carbon free broth and incubating. The authors demonstrated the presence of *Nocardia asteroides*, *N. brasiliensis* and *N. caviae* in soils of USA and India. The affinity of *Nocardia* to colonize and grow on paraffin was made use of by P.V. Kurup and J.A. Schmitt (*Mycologia* 63: 175-177, 1971) to develop a technique in which a soil suspension in water was allowed to stand for 30 minutes at 30°C and 1 ml of supernatant was inoculated into a carbon-free broth containing (g/l) NaNO₃ 2, KH₂PO₄ 0.003, MnCl₂ 0.002, pH 7.2. Paraffin coated rods sterilized by overnight steeping in alcohol were dipped in the diluted soil suspension in the carbon-free broth and incubated at 37°C for 15 days. Colonies of *Nocardia* developed after 7-10 days as brown, yellow or pink turfs which were picked up and further purified on glucose nutrient agar plates. The paraffin baiting technique has been successfully adapted to isolate human pathogenic *Nocardia asteroides* strains from clinical specimens (S.K. Mishra and H.S. Randhawa. *Appl. Microbiol.* 18: 686-687, 1969).

Members of the genus *Actinoplanes* are widely distributed in soil around the world. These organisms were first isolated by J.N. Couch through the application of baiting procedure that he had employed for the isolation of aquatic chytrid fungi. Soil placed in a petri dish was covered with sufficient charcoal treated water and diverse sterilized baits like pollen, grass blades or hair are floated. After incubation the baits colonized by the actinomycete are picked up and those bearing sporangia are cultured on nutrient media. *Actinoplanetes* routinely colonize plant material in aquatic habitats and such material when incubated in moist chambers develop sporangia of the actinomycetes. Dehydrating by air drying the sample before suspending in water and incubating for one hour releases the zoospores which can then be plated out on agar media to isolate pure cultures without serious problems of bacterial contamination (N.S. Makkar and T. Cross. *J. Appl. Bact.* 22: 209-218, 1982).

Use of autoclaved human hair as a bait for the isolation of a new keratinophilic member of the Actinoplanaceae designated Pilimelia has been published (W.D. Kane. *J. Elisha Mitchell Sci. Soc.* 82: 220-230, 1966). A new keratinolytic nocardioform actinomycete *Amycolatopsis keratiniphila* has been isolated from Kuwait marsh soil using defatted sterilized animal wool as bait
(A.A. Mussallum et al. *Int. J. Syst. Evol. Microbiol.* 53: 871-874, 2003). Chemotactic attraction of Actinoplanete zoospores to chloride ions has been the basis of an enrichment technique developed for their selective isolation (N.J. Palleroni. *Arch. Microbiol.* 123: 53-55, 1980). After flooding soil samples with water, it is incubated for one hour followed by insertion of a capillary tube filled with phosphate buffer containing potassium chloride. The orifice of the capillary is placed a cm below the surface of the water and left for an hour during which time the zoospores migrate towards the KCl attractant and get enriched in the capillary tube. The capillary is carefully washed externally with jet of sterile water and its contents blown out for plating on suitable agar medium. R-collidine as a chemotactant was successfully employed for the selective isolation of *Actinoplanes* and *Dactylosporangium* strains (M. Hayakawa et al. *J. Ferment. Bioeng. 72: 426-432, 1991). Another chemotactant for actinomycete zoospores is Vanillin which has been used to enrich the planospores of *Catenuloplanes* (M. Hayakawa et al. *Actinomycetologica 9: 152-163, 1995) and a new genus *Virgosporangium* (T. Tamura et al. *Int. J. Syst. Evol. Microbiol. 51:1809-1816, 2001).

D.L. Arora (J. Gen. Microbiol. 132: 1657-1663, 1986) observed chemotaxis of *Actinoplanes missouriensis* planospores to fungal conidia, chlamydospores and sclerotia, presumably due to the exudates produced by these structures. Baiting with *Pinus* pollen grains followed by desiccation at 30°C for 2 hours and then carrying out a water immersion, released zoospores of *Actinoplanes* which could be easily isolated on HV-agar supplemented with 10 mg/ml of Nalidixic acid. The desiccation step was helpful in almost completely eliminating serious bacterial contamination on the isolation plates (M. Hayakawa et al. *J. Ferm. Bioeng. 72: 433-438, 1991). Suspending soil samples at 40°C for 30 min in a solution containing 6% yeast extract and 0.05% sodium dodecyl sulphate (SDS) caused activation of dormant actinomycete spores leading to increased numbers of actinomycete colony forming units (H. Nonomura and M. Hayakawa in "Biology of Actinomycetes" 88, p. 288-293).

An enrichment procedure to selectively isolate *Sporichthya* strains from soil consisted of flooding soil with a solution containing 0.1% skimmed milk in 10 mM morpholinepropane sulphonic acid (MOPS) at pH 8.0, incubating at 27 °C for 60 min and centrifuging at 1000 X g for 10 min followed by plating

2.4 Screening Unconventional Sources and Habitats for Actinomycetes Diversity

K. Iwai et al. (Actinomycetologica 23: 8-15, 2009) isolated several non-filamentous actinomycetes from spider material belonging to Micrococcaceae after pre-treatment of the spider web with 70% ethanol. A short coccoid or rod shaped non-motile actinomycete isolated from lichen thallus was identified as a strain belonging to Nocardioides (Bing-Li et al. Actinomycetologica 21: 22-26, 2007).

2.4.1 Marine Actinomycetes

Recent years have witnessed explosive interest in the screening of marine habitats including deep sea sediments for novel actinomycetes and their bioactive metabolites. Several active schools of research such as for example Prof. W. Fenical’s group at the Scripps Institute of Oceanography, USA have carried out intensive research and successfully identified actinomycetes that are different from other terrestrial forms based on 16 S rRNA gene sequences, some of which like the new genus Salinispora produce novel and therapeutically useful anticancer drugs like Salinosporamide A. (T.J. Mincer et al. Appl. Environ. Microbiol. 68: 5005-5011, 2002; R.H. Feling et al. Angew. Chem. Int. Ed. 42: 355-357, 2003; L.A. Maidonado et al. Int. J. Syst. Evol. Microbiol. 55:1759-1766, 2005).

A unique selective enrichment technique for the isolation of new taxa of marine actinomycetes that are able to produce bioactive metabolites has been described by N.A. Magarvey et al. (Appl. Environ. Microbiol. 70: 7520-7529, 2004). 50 mg of wet sediment was inoculated on synthetic salts agar medium...
prepared with artificial sea water and supplemented with 25 µg/ml Cycloheximide. Whatman no. 1 sterile filter paper discs pre-cut to fit the agar surface were placed on the agar and the sediment material was evenly dispersed on the surface of the cellulose discs. The plates were incubated in a humid chamber for 30-90 days. Selected colonies were streaked on ISP2 medium prepared with artificial sea water containing 25 µg/ml Cycloheximide and 25 µg/ml Nalidixic acid. Based on their extensive studies on pure culture isolations, the existence of unique indigenous marine actinomycete taxa has been firmly established and several rare and even new genera identified such as Dietzia, Salinospora, Marinophilus, Solwarospora, Salinibacterium, Aeromicrobium marium, Williamsia maris and Verucossispora. Brief notes on some of these novel taxa is given in Appendix-A.

2.4.2 Endophytic Actinomycetes

Details of the diversity of endophytic actinomycetes along with relevant references have already been discussed under the section on Ecology and Distribution (Chapter 1). Some of the techniques applied for the isolation of endophytic actinomycetes in pure culture are presented in brief here. P. Sardi et al. (Appl. Environ. Microbiol. 58: 2691-2693, 1992) surface sterilized washed roots, 1-5 cm in diameter, by exposure to propylene dioxide vapours for one hour followed by inoculating 1 cm segments aseptically on 2.5% water agar or starch casein medium (E. Küster and S.T. Williams. Nature 203: 928-929, 1964) supplemented with 50 ppm Nystatin and 50 ppm Cycloheximide to suppress fungal growth. Incubation at 25°C up to 21 days and purification of the actinomycete colonies growing from the roots was carried out based on differences in morphological characteristics. T.J.M. De Aruto et al. (Brazilian Archives of Biology and Technology 43: 47-53, 2000) studied actinomycetes endophytic in stems and roots of maize (Zea mays). Washed plant tissues, were briefly treated with 70% alcohol followed by disinfection with sodium hypochlorite (3-5% available chlorine) for three minutes and then the samples were extensively rinsed with sterile water. Roots and leaves were divided into 1 cm fragments and aseptically transferred to water agar or Küster-Williams agar supplemented with 50 µg /ml each of nystatin and cycloheximide.

Presently interest in the study of endophytic actinomycetes is on the increase.
from the point of their utilization as plant growth regulators, plant disease control and as sources for novel bioactive metabolites. **S. Compant et al.** *(Appl. Environ. Microbiol. 72: 4951-4959, 2005)* reviewed the use of plant growth promoting bacteria for bio control of plant diseases. **M. Caruso et al.** *(Ann. Microbiol. 50: 3-13, 2000)* isolated several actinomycetes from woody and herbaceous tissues of *Taxus* and several of them belonging to *Actinomadura*, *Kitasatospora* and *Micromonospora* exhibited the potential to produce taxanes including the anticancer drug taxol.

### 2.4.3 Extremophiles

Life forms which grow well and carry out their metabolic processes effectively under environmental conditions which are hostile or unfavourable for most of the living organisms have been termed extremophiles. Thermophiles (high temperature tolerant) alkalophiles (tolerant to high alkaline pH) halophiles (salt tolerant), barophiles (pressure tolerant) are examples of extremophiles which are widely encountered in the microbial kingdom. Thermophilic actinomycetes are widely distributed in soil and particularly in association with decomposing plant residues such as mushroom compost, bagasse piles etc. in which heat generation during the natural process of decomposition provides ecological niches favouring the development of thermophiles. *Thermoactinomyces* (presently classified as a member of the Bacillaceae and excluded from the actinomycetes), *Thermomonospora*, *Saccharomonospora*, and thermophilic members of important genera such as *Streptomyces* and *Micromonospora* are among the well investigated thermophilic forms. Incubation at high temperatures (50-65°C) and ensuring that the media do not dry out by providing adequate humid conditions during incubation of the isolation plates are important factors to consider in their exploitation from the habitats mentioned earlier. [Use of antibiotics for selective isolation has bee published. For example addition of 5 µg/ml Rifampicin to Tryptone soya casein hydrolysate agar plates incubated at 55°C facilitated isolation from hay and straw, large number of colonies of *Thermomonospora chromogena* as well as *Saccharomonospora viridis* (**Amaiye et al. J. Appl. Bact. 51: 289-297, 1980**).

Use of colloidal silica instead of agar as solidifying agent was found to be suitable for isolating thermophilic actinomycetes at 60°C. It provided the
surface which inhibited the spread of bacteria and permitted sporulation of actinomycetes (J.E. Uridil and P.A. Tetrault. J. Bact. 78: 243-246, 1960). Halophilic actinomycetes have received comparatively less attention and Actinopolyspora halophila isolated by M.B. Gochenauer et al. (Can J. Microbiol. 21: 1500-1511, 1975) as a contaminant on an agar plate containing 25% NaCl remains example of an extremely halophilic actinomycete. Actinopolyspora iraquiensis was isolated from extremely saline soil samples of Iraq and required 10-15 (-25%) sodium chloride for growth (J.S. Ruan et al. Int. J. Syst. Bact. 44: 759-763, 1994). Recent years have witnessed widespread interest in the study of alkalophilic bacteria, actinomycetes and fungi especially in relation to their potential as sources of enzymes and biocatalysts tolerant to harsh alkaline environments.

Alkalophilic actinomycetes can be isolated from soils using agar media in which the pH has been adjusted with 10% sodium carbonate or bicarbonate to pH 10.0 or 11.0. Media containing meat extract or peptone appear to be effective in the isolation of alkalophilic actinomycetes (Y. Mikami et al. J. Gen. Microbiol. 128: 1709-1712, 1982). Most importantly the Na$_2$CO$_3$ or NaHCO$_3$ must be aseptically added to pre-sterilized medium to provide an effective concentration of 1% and a pH above 9.5. For pH above 9.5, sodium hydroxide is preferably used. Addition of alkali to the medium prior to autoclaving should be totally avoided. Incubation at 27°C for 7 or more days yields actinomycete colonies. After primary isolation, the growth response is evaluated on media variously adjusted from pH 6.0 to 11.5 by incubation at 27°C for 7-15 days. Isolates unable to grow at pH 6.0 and able to grow at pH 11.5 or above should be considered truly alkalophilic actinomycetes. Many actinomycetes having broad range of pH tolerance (e.g. from pH 7.5 to 9.5, may be considered to be alkalotolerant while those exhibiting better growth at pH 10.0 and above compared to 7.5 can be considered alkalophilic.

2.5 Relevance of Actinomycete Biodiversity Screening to the Indian Context

India with its diversity of tropical climatic conditions is a recognized biodiversity "Hot Spot". Looking at the current global trends of exploring for novel microbial strains for biotechnology exploitation, it appears worthwhile to undertake serious screening programs, adapting diverse selective techniques to culture, identify and conserve "in vitro" in pure cultures,
diverse actinomycete taxa including rare genera and possibly even genera new to science. This calls for a greater emphasis to be laid to our academic programs with the required expertise, aptitude and technical skills with reference to the actinomycetes. In a globally competitive environment discovery of novel bioactive metabolites from actinomycetes holding the potential for newer beneficial applications is undoubtedly an area of prime importance for future developments in Indian biotechnological research and development. Commitment to strengthen research efforts in this fruitful area would be most beneficial for the academic as well as the application-oriented results from the studies on Indian actinomycetes.
3.1 Morphological Diversity

Morphological diversity of actinomycetes ranges from "diptheroid bacilli to filamentous forms with intricate modes of sporulation" (H.A. Lechevalier and M.P. Lechevalier. In "Prokaryotes". Ed. M.P. Starr et al. 1981). As generally recognized they are characterized by the formation of branching filaments which results in a somewhat fungal appearance. The vegetative growth is similar to bacteria in its diameter and very much smaller than any of the fungal hyphae. In general, the actinomycetes are gram positive and based on molecular data they are presently classified as closely related to the coryneform bacteria and mycobacteria under the order Actinomycetales. Besides, the genera forming filamentous growth, Corynebacterium, Mycobacterium and Arthrobacter are also included. These forms have morphology akin to bacteria and the mycelial morphology exhibited by strains could be rudimentary, evanescent or even totally absent.

The controversies of the earlier years regarding the true nature of actinomycetes, whether they should be grouped with fungi or bacteria - has been resolved following the scientific advances in electron microscopy, cytology and molecular biology. They are now unquestionably recognized as prokaryotes and deemed as having affinities with the bacteria. As discussed earlier under Taxonomy and Classification, the term Actinobacteria has been introduced and regarded as a class distinct from Eubacteria under the kingdom of Prokaryotes.

3.2 An Overview of Some of the Salient Morphological Features of Representative Genera

3.2.1 Nocardia species are gram positive, aerobic and acid fast, forming a
primary mycelium which fragments into coccoid and bacillary elements. An aerial mycelium that may be differentiated into arthrospores may also be observed. *Rhodococcus* colonies appear mucoid with a smooth or rough contour and are usually brightly pigmented, being pink, red or orange in colour. The primary mycelium fragments early and the growth appears pleomorphic. Both *Nocardia* and *Rhodococcus* possess mycolic acids and have a chemotype IV cell wall composition.

The family Streptomycetaceae is perhaps the most important and widely studied with several genera of industrial importance included in them. They all have LL-diaminopimelic acid in their peptidoglycan. *Streptomyces, Streptoverticillium, Chainia* and *Kitasatospora* are among the industrially useful genera, being the source of valuable secondary metabolites and industrial enzymes. Members of the family are characterized by non-fragmenting substrate mycelium and the formation of aerial mycelium and characteristic chains of arthrospores.

3.2.2 *Streptomyces* is most widely distributed with a large number of species described and explored for their potential as antibiotic producers. The genus *Streptoverticillium* is characterized by a verticillate arrangement of sporogenous hyphae. It is closely similar to *Streptomyces* and in some situations problems have been encountered in assigning the identity of strains to either *Streptomyces* or *Streptoverticillium*. The genus *Chainia* described from India by M.J. Thirumalachar (*Nature* 176: 934-935, 1955) is characterized by formation of spherical sclerotic granules or aggregate masses as a feature of differentiation of the vegetative mycelium. Formation of aerial mycelium and spore chains parallel the situation observed in *Streptomyces*. Studies on the fine structure of the sclerotia have indicated that these structures originate by extensive branching and cell division followed by rounding up to form morphologically distinct structures with intracellular accumulation of fat globules (M.P. Lechevalier et al. *Int. J. Syst. Bact.* 23: 2157-170, 1973; P.L. Ganju and M.R.S. Iyengar. *J. Gen. Microbiol.* 82: 25-48, 1974; G.P. Sharplees and S.T. Williams. *Microbiol* 15: 27-47, 1976). While the cell wall peptidoglycan of growing mycelium had LL-diaminopimelic acid, the presence of an unusual chemical constituent 2,3 diaminopropionic acid was reported in colonies having sclerotial differentiation (M.P. Lechevalier
et al. 1973 see above ref.). Sclerotial formation was observed on all media supporting good vegetative growth of the culture. On prolonged subcultures on sugar-rich artificial media, a tendency towards decrease or even loss of sclerotial development was observed, possibly due to factors such as dual phenomena (M.J. Thirumalachar and R.S. Sukapure. Hindustan Antibiotics Bulletin 6: 157-166, 1964).

Considerable discussion on the taxonomic status of sclerotia formation for differentiating Chainia from Streptomyces has been recorded in literature. Similarity of cell wall composition and sporulation pattern to Streptomyces has led to a school of thought of not recognizing sclerotia formation as valid criterion to distinguish from Streptomyces. Despite this trend, new species assigned to the genus Chainia continue to be published in literature, such as Chainia kunmingiensis described from China (Ji Sheng Ruan et al. Int. J. Syst. Evol. Microbiol. 35: 164-168, 1985).

3.2.3 Kitasatoa was described as a new a genus from Japanese soil sample by A. Matsume et al. (J. Antibiotics 21: 616-623, 1968). It exhibits morphological features of Actinoplanaceae and Streptomycetaceae. Formation of club shaped sporangia enclosing zoospores, carrying a single polar flagellum, both on the substrate and aerial mycelium together with the aerial mycelium producing long chains of arthrospores similar to Streptomyces characterise the genus. Classified originally under the Actinoplanaceae, the genus is now placed under the Streptomycetaceae based on the arthropore formation along with similarity of biochemical characters.

Members of the Actinoplanaceae comprise of forms with gram positive, non acid fast non-fragmenting mycelia, bearing sporangia with motile or non-motile spores. Under favourable conditions, the spores germinate forming a germ tube which branches to colonize the substratum. From the substrate mycelium branches arise which grow upward and develop the sporangia bearing sporangiospores. The sporangia are globose and multisporated in Actinoplanes and Streptosporangium or finger shaped to pyriform with one to four spores in genera like Planomonospora and Planobispora.

The sporangial wall arises from a thin sheath of material secreted outside the hyphal wall. The sporogenous hyphae may branch to form parallel rows
within the sporangium and eventually differentiate oval or rod-shaped motile spores in *Actinoplanes* and *Pilimelia* respectively. The spores developing inside the sporangia are non-motile in *Streptosporangium*.

In the animal pathogen *Dermatophilus* and the related saprophytic soil inhabitant *Geodermatophilus*, the branching filaments of the vegetative mycelium divide by longitudinal and transverse septa resulting in the formation of coccoid elements which may be motile.

A number of actinomycetes having great importance and industrial significance are characterized by single spores and have been widely studied. These include both mesophilic and thermophilic forms.

### 3.2.4 *Micromonospora*

*Micromonospora* is a source of several bioactive metabolites and the members of the genus are mesophilic. Widely distributed in soils, especially abundant in pond or lake mud samples, the genus is characterized by single spores borne on the substrate mycelium. Aerial mycelium is lacking and hence the colonies appear flat on the agar media. Growth temperatures range from 30-37°C.

### 3.2.5 *Thermomonospora* and *Saccharomonospora*

*Thermomonospora* and *Saccharomonospora* are thermophilic genera widely distributed in compost and self heated plant substances like fodder and grains. *Thermomonospora* strains possess type III cell wall and single spores which are heat sensitive (killed at 70°C) are formed on the aerial as well as substrate mycelium. Isolates may vary widely in their temperature optima for growth and a sharp demarcation between thermophilic (40-45°C growing up to 50°C) and mesophilic (35-40°C with no growth at 50°C) is proving to be difficult.

### 3.2.6 *Thermoactinomyces*

*Thermoactinomyces*, a thermophilic genus also producing single spores is now excluded from Actinomycetes and classified under Bacillaceae in view of its heat-stable endospores and low G+C content (48.7-52.2%).

### 3.2.7 *Saccharomonospora*

*Saccharomonospora* is slow growing at 30°C, but grows optimally at 45°C forming colonies with a blue green to grey-green aerial mycelium. A dark green soluble pigment may also be formed on media such as tryptone soy agar. Heat sensitive (killed at 70°C) spores are produced on the aerial mycelium and appear sessile or borne on lateral sporophores.
3.2.8 *Actinomadura* is characterised by branched substrate mycelium and formation of short chains of arthrospores on the aerial mycelium. The cell wall contains meso-diaminopimelic acid and whole cell hydrolysates show the presence of madurose (3-O-methyl-D-galactose). Some of the isolates like *A. dassonvillei* not possessing madurose and showing other differences have been placed by some authors under a separate genus *Nocardiopsis*.

3.2.9 *Microbispora* was described by H. Nonomura and Y. Ohara (*J. Ferm. Technol. 35: 307-311, 1957*) exhibiting the formation of paired spores on the aerial mycelium, cell wall with meso-diaminopimelic acid and presence of madurose in whole cell hydrolysates. In the same year, an isolate with identical features was described under the name *Waksmania* by M.P. Lechevalier and H.P. Lechevalier (*J. Gen Microbiol. 17: 104-111, 1957*). Based on the priority of publication date, *Microbispora* is recognized as the valid name and *Waksmania*, its synonym.

3.2.10 *Microtetraspora* described by J.E. Thiemann et al. (*J. Gen. Microbiol. 50: 295-304, 1968*) as a new genus having short sparsely branched aerial mycelium bearing chains of four spores. The cell wall composition was reported to be composed of meso-diaminopimelic acid along with traces of LL-diaminopimelic acid, glycine and lysine. The presence of chemotype III cell wall along with madurose was reported in species of *Microtetraspora* described by H. Nonomura and Y. Ohara (*J. Ferm. Technol. 49: 1-7 & 887-894, 1971*) who also described selective techniques for their isolation from soil.

3.2.11 *Micropolyspora* described by H.A. Lechevalier et al. (*J. Gen. Microbiol. 26: 11-18, 1961*) forms chains of spores on the substrate as well as aerial mycelium. The cell wall is of type III with mesodiaminopimelic acid along with the sugars arabinose and galactose.

3.2.12 *Saccharopolyspora* isolated from sugarcane bagasse shows fragmentary substrate mycelium bearing moniliform spore chains on the aerial mycelium. The genus has chemotype IV cell wall composition (J. Lacey and M. Goodfellow. *J. Gen. Microbiol. 88: 75-85, 1975*).

3.2.13 *Actinopolyspora* an extremely halophilic actinomycete growing optimally on media containing 15-20% sodium chloride was described by M.B. Gochenauer et al. (*Can. J. Microbiol. 21: 1500-1511, 1975*).
Fragmentation of substrate mycelium and an aerial growth bearing chains of spores and wall chemotype IV characterize the genus.

The range of morphologic and chemotaxonomic diversity exhibited by actinomycetes would be evident from the representative examples cited above. In more recent years there have been several new genera described especially from the marine habitats, and the primary basis for generic differentiation has been based on molecular biology criteria, particularly the 16 S rDNA gene sequencing data.

3.3 Physiology and Nutritional Regulation of Vegetative Growth and Reproduction

Saprophytic and soil inhabiting actinomycetes would grow on ordinary laboratory media without requirement for specific growth factors or supplements. Pathogenic forms for animal and humans like anaerobic *Actinomyces* will grow slowly on complex media with special nutrient supplements.

*Mycobacterium tuberculosis* is slow growing in culture with a generation time of 15 hours under optimal conditions. The leprosy inducing *Mycobacterium leprae* has not been grown and maintained successfully "in vitro".

Even the saprophytic actinomycetes widely distributed in soil and plant debris are much slower in growth rates compared to most eubacterial strains. A division cycle of 2-3 hours for actinomycetes compared to 20 minutes for *E. coli* will give an idea of their relative growth rates. The plant associated nitrogen fixing actinomycete *Frankia* is again very slow growing and successful "in vitro" culture and conservation often pose serious problems.

Germination of an actinomycete propagule such as a spore results in the formation of hyphae which branch at intervals and spread rapidly on the substrate establishing a colony that is generally leathery, tough and easily recognizable on isolation plates. In the natural environment, because of their ability to penetrate the substrate as well as production of extracellular hydrolytic enzymes to break down diverse organic substances, the actinomycetes are capable of colonizing on a variety of natural substrates. The spores are often capable of withstanding prolonged desiccation and some of them even survive dry heat at 100-120°C for 30-60 min (Chapter 2).
On solid media most actinomycetes form a mycelium (substrate or primary) that grow on and into the agar. In addition there may be a mycelium (aerial or secondary) growing away from the medium. In some cases, the primary mycelium is short lived and soon breaks up into bacillary or coccoid elements that may be flagellated. In some species the mycelium may be so transient as to escape notice or be non-existent. Distinguishing morphological features helpful in differentiating diverse actinomycetes include type and stability of mycelium types, numbers and disposition of spores, formation of sclerotia, sporangia or synnemata and formation of flagellated elements.

*Streptomyces* species forming copious numbers of arthrospores survive well in dry soils but are relatively sensitive to water-logged conditions and its consequent exposure to increased levels of carbon dioxide.

While most species prefer neutral to alkaline pH for growth and survival, isolates from acidic forest soils adapted to grow in acidic media such as *Streptomyces acidophilis* have been identified. A separate genus *Streptoacidophilis* has been created to accommodate such isolates (*S.B. Kim et al. Anotnie van Leeuwenhoek 33: 107-116, 2003*). *Streptomyces* cultures generally are nutritionally non-fastidious and can be readily grown on media containing a carbon source like starch, glucose or glycerol, an inorganic nitrogen source such as nitrates or ammonium salts and mineral salts. However use of complex media containing protein hydrolysates e.g. tomato paste oatmeal agar are generally preferred for long term in vitro conservation as well as mass propagation for industrial biotechnology. Careful microscopic examination of *Streptomyces* colonies to decipher the relative proportion of aerial mycelium and spore chains is essential. The composition of the growth medium should be selected to enhance the proportion of spore chains in comparison with sterile aerial hyphae.

Japanese investigators like *Masayasu Nomura* and *Y. Hasegawa* have formulated media with low concentrations of glucose (0.3 g/l) and arginine (0.3 g/l) along with mineral salts and B-vitamin mixture as the preformed medium for isolating rare genera of actinomycetes.

Humic acid in low concentrations supplemented with vitamins was shown to be very favourable for ready recognition of the relatively rare genera of actinomycetes on the isolation plates. Humic acid is poorly metabolised by the bacterial microflora whereas the actinomycetes generally have the ability...
to grow on utilizing humic acid as a nutrient substrate. Use of humic acid based media for actinomycetes isolation has been discussed in the chapter on selective isolation techniques (Chapter 2).

From the foregoing discussion, a perspective outlook on the basic requirements for recognizing, culturing and identifying diverse actinomycetes would have been generated. An overview presentation on the physiology and nutritional aspects of actinomycetes has been published (M.C. Srinivasan et al. World J. Microbiol. Biotechnol. 7:171-183, 1991).

### 3.4 Signal Molecules Regulating Differentiation and Secondary Metabolism

Streptomyces species have been the most extensively studied and have merited considerable attention as the source of diverse antibiotics. The vegetative mycelial growth (substrate mycelium) is non-fragmenting and continuous. The colonies are readily recognized by the extensive formation of aerial mycelium and spore chains. The morphological developmental features and their correlation with elaboration of bioactive secondary metabolites has been the subject of extensive investigation. Studies by D.A. Hopwood and co-workers using the model strain Streptomyces coelicolor A3(2) has led to several salient findings (D. A. Hopwood. Proc. Royal Soc. London B 215: 121-138, 1988).

Isolation of gene clusters linked to antibiotic biosynthesis and sporulation has facilitated a better understanding of the regulatory events in morphological and physiological differentiation. Antibiotics are metabolites resulting from the co-ordinated action of several genes and the complex interplay of these genes with regard to determination of the structure of the antibiotic molecule(s). Protection to the producing organism from the effects of the antibiotic molecule(s) is a fascinating topic which will be discussed in some detail in Chapter 5 on secondary metabolism and bioactive secondary metabolites.

The organisms seem to have an in-built mechanism to commit a number of genes for the purpose of metabolite production and regulation and this has been found to coincide with the transition between active vegetative growth and development of sporulation (D.A. Hopwood. in "Biology of Actinomycetes", Ed. Y. Okami et al. 1988, 3-10).
Streptomyces strains often produce more than one antibiotic, each requiring its own set of structural, regulatory and resistance genes. Production of aerial mycelium and spores also necessitates the co-ordinated ability of several genes. It is a challenging proposition to fully understand the molecular events involved in the structural organization and metabolite formation in Streptomyces strains. Secondary metabolite formation can be regarded as a physiological differentiation occurring in the idiophase at the end of the main period of rapid growth and assimilative metabolism (Y. Ohnishi. Actinomycetologica 17: 23-27, 2003). In the streptomycin producing S. griseus, a low molecular weight chemical signaling molecule termed A-factor (2-isocapryloyl-3-R- hydroxymethyl-\(\beta\)-butyrolactone) has been identified which triggers both morphological and physiological differentiation. The A-Factor was discovered as a diffusible extracellular molecule that stimulated sporulation in a 'bald' mutant of S. griseus (A.S. Khokhlov et al. Doki. Akad. Nauk. USSR, 177). It was essential for both aerial mycelium formation and streptomycin biosynthesis in this mutant. D. Hara and T. Beppu (J. Antibiotics 35: 349-358, 1982) confirmed the observations of Khokhlov et al. with a stereochemically synthesized A-Factor at concentrations as low as 10^{-9} M.


Other signal molecules that positively control the differentiation process are metabolites such as Pamamycin 607 isolated from Streptomyces alboniger (M. Natsume. Actinomycetologica 13: 11-19, 1999) and Goadsporin produced by Streptomyces sp. TP A0584 (M. Onaka et al. J. Antibiotics 54:1036-1044, 2001).

Stimulation of aerial mycelium formation and antibiotic biosynthesis in various Streptomyces strains at 1-20 ng/ml of indoleacetic acid (IAA) has been

### 3.5 Giant linear Plasmids

*Streptomyces* species have been found to carry a 8-9 Mb linear chromosome. In addition, they have giant linear plasmids which are widely distributed and involved in antibiotic production, degradation of aromatic compounds and other functions. In his extensive studies on *Streptomyces coelicolor* and its plasmid SCP-1, Hopwood and his co-workers demonstrated that the plasmid SCP-1 carried the biosynthetic genes for the antibiotic methylenomycin (R. Kirby et al. Nature 284: 265-267, 1973; R. Kirby and D.A. Hopwood. J. Gen Microbiol. 98: 239-252, 1977).

Characterization of the plasmid SCP-1 was facilitated by the application of pulse field gel electrophoresis (PFGE) to *Streptomyces* DNA which revealed that SCP-1 was a giant linear plasmid of about 350 Kb (H. Kinashi et al. Nature 328: 454-456, 1987). A linear plasmid PSLA2 was isolated from a strain of *Streptomyces roche* which produced two structurally unrelated polyketide antibiotics, lankacidin and lankamycin (T. Hayakawa et al. J. Gen. Appl. Microbiol. 25: 255-260, 1979). Several additional examples of the presence of giant linear plasmids and their role in antibiotic production in industrially important strains such as *S. rimosus* and *S. clavuligenus* has been critically reviewed (H. Kinashi. J. Antibiotics 54: 19-25, 2011) which may be consulted for more details on this topic.
4.1 Introduction

The actinomycetes represent an important component of the soil microflora and are widely distributed. Prior to 1940, interest in the actinomycetes mainly centered around the morphological identification of diverse forms and assigning names to the different isolates. The majority of these studies were related to the widely occurring and abundant forms referable to the genus *Streptomyces*. Beyond the 1950's, following the discovery of the therapeutically useful anti-tubercular drug streptomycin from *Streptomyces griseus*, commercial interest from Pharma companies focused on isolating a variety of actinomycetes and exploring them for their potential as sources of novel bioactive compounds and drugs useful to man. Several success stories have been scripted out of these efforts, notably between 1950 and 1980, and the acceptance of actinomycetes as the most prolific producers of bioactive secondary metabolites became unquestionable.

This development necessitated that the cultures producing these valuable products have to be properly conserved ensuring that the ability to produce the product of interest is not lost over a period of time. This requires application of conservation methods which would guarantee morphological and genetic stability in the strains revived by sub-culture after prolonged in-vitro conservation. As the number of new molecules of actinomycete origin with commercial interest increased, patenting of the process of manufacture as well as the product became mandatory for the protection of intellectual property rights. The strain was required to be deposited in one of the recognized international culture collections under the Budapest treaty and a complete morphological description of the strain was required to be mentioned in the patent application.
Problems faced in the correct technical description especially of the genus *Streptomyces* as well as the tendency to describe a strain producing a novel molecule as a new species without regard to careful comparison with previously described species became serious. An International *Streptomyces* Project (ISP) organised by a group of competent expert microbiologists carried out in-depth studies on specific media under defined conditions to redescribe all authentic cultures of *Streptomyces* species. The different ISP media recommended were also made commercially available in dehydrated form by Difco for ready use.

It is well recognised that microorganisms, when propagated in the laboratory by periodic serial transfer, tend to undergo changes in certain of their biochemical properties, in their reproductive capacity and in some of their phenotypic characteristics.

Actinomycetes, and in particular members of the genus *Streptomyces* are subject to certain detrimental changes during lengthy laboratory cultivation on artificial media. They have a tendency to show high degree of genetic instability which may manifest within a colony as sectors with altered pigmentation, extent of sporulation and in physiological terms may also affect important traits like antibiotic production. Upon repeated restreaking variant strains can easily be isolated, but the extent of the retention of the variation in a stable form is often unpredictable. Genetic instability often affects only specific genes in a strain and sometimes occurs at high frequencies spontaneously. It may also be stimulated by treatments such as UV-irradiation, which means genetic instability could be a serious problem in the case of high yielding strains for antibiotic production after different kinds of mutagenic treatment.

The presence of giant linear plasmids in several antibiotic producing strains has been demonstrated and in some cases like methylenomycin production, the biosynthetic genes have been shown to be present on a giant linear plasmid. To ensure sustained production of such metabolites, the loss of giant plasmids during frequent serial subcultures should be avoided, and this makes it obvious that long-term conservation strategies for actinomycetes culture is a must for any technology development based on these cultures.

The filamentous nature of *Streptomyces* and other forms is similar in their
morphology to fungi and as in the case of many fungi, heterokaryosis is an additional feature contributing to genetic instability. For example F. Reusser et al. (Appl. Microbiology 9: 342-346, 1961) observed novobiocin production by Streptomyces niveus decreased drastically as the culture was transferred regularly under both sporulating and non-sporulating conditions. They speculated that culture instability of S. niveus was due to heterokaryosis.

### 4.2 Methods of Conservation

Lyophilization, liquid nitrogen storage and preservation in sterile soil are practised universally for actinomycetes culture conservation. Several important factors which influence the survival and physiological features of different strains include the medium of cultivation, culture age including whether it was taken for preservation in the lag, log or post logarithmic phase of growth, method of dehydration prior to lyophilization, storage in the liquid of gas phase for liquid nitrogen conservation, type of soil used for sterile soil conservation etc. Revival and restoration of conserved cultures and their physiological behaviour may be influenced considerably by the composition of culture medium and incubation temperature.


### 4.3 Soil Preservation

Sandy loam soil or farmyard soil rich in organic matter and free from fertilizers or pesticides is heated for six hours in shallow tray at 150°C. The soil is sieved through 20 or 30-mesh sieve, distributed in test tubes and plugged with gauze covered non-absorbant cotton. The tubes are autoclaved for 60 minutes on two successive days.

Random check is carried out for sterility from the autoclaved tubes by transfer to broth tubes and it is ensured that no bacterial growth occurs, indicating successful sterilization of the soil tubes. In a slight modification to the above procedure (M.C. Srinivasan, unpublished) the soil in the tubes was
moistened by adding minimum amounts of distilled water and after autoclaving heated in a hot air oven at 140-160°C for 2 hours or until the soil particles appear homogenous and free-flowing. The culture is added as a spore suspension and dispersed by shaking the soil tubes and air dried at room temperature. Sporulating culture discs from thinly poured agar plates may also be added to the soil tubes and shaken to disperse the inoculum. The spore masses get released and mix with the soil as the thin agar medium undergoes desiccation. The soil culture tubes are stored at 5-10°C. Viability and freedom from contamination can be periodically checked by transferring small quantities of the soil culture to agar plates or broth tubes.

4.4 Lyophilization

This method is most widely used for long term storage of microbial cultures. Frozen cultures are subjected to vacuum and under reduced pressure, the liquid is removed and the dried cells can be conserved for indefinite periods. The cells are required to be protected from damage by intracellular ice crystal formation during the freezing portion of the lyophilization process. This is achieved by adding a cryoprotective agent such as sterile 15% skim milk solution in which the spores can be suspended. Spore suspensions so prepared are distributed as small aliquots in sterile borosilicate glass vials plugged with sterile cotton wool. The ampoules are frozen in a dry-ice solvent bath. They are then transferred to the freeze dryer and vacuum pump started. When vacuum is below 100 μm Hg, the shell temperature of the freeze dryer can be raised to 10°C. The lyophilizer is run overnight to dry the cell suspension. The ampoules are then attached to a vacuum manifold and sealed under vacuum using oxygen gas torch. The sealed ampoules are checked for leaks and kept stored at 4-5°C.

To revive, the ampoules are broken open in a sterile chambers and the pellet aseptically dropped into an appropriate broth of growth medium and incubated.

4.5 Cryopreservation

Cryopreservation refers to preservation of microbial cultures by freezing using cryoprotectant(s). About 10% glycerol or dimethyl sulfoxide is used as
a satisfactory cryoprotectant. Well sporulated culture is shaken with 10% glycerol and aliquots placed in sterile silicate glass vials having teflon –lined screw caps. Alternatively aliquots of a 72 hours old shake cultures mixed with equal volumes of 20% sterile glycerol may also be used for cryopreservation. Vials are frozen in the gas vapour phase of the Liquid Nitrogen freezer or in a -80°C mechanical freezer.

Poor survival rates due to cell damage are recorded when the frozen storage temperature is -20°C as in home refrigerators. For revival, rapid thawing at 37°C and immediate use give best results. An important factor is freedom from contamination of cultures prepared for frozen storage, which must be rigorously ascertained.

Suitability of frozen storage also requires to be monitored for individual strains from stability of morphological, physiological and biochemical traits following cryopreservation and revival.

**H.D. Tresner et al. (Applied Microbiology 8: 339-341, 1960)** preserved over 400 *Streptomyces* cultures grown on malt extract yeast extract glucose slants “in a deep freeze unit at -22°C and were allowed to freeze at their own rate”. After 2-3 years storage, morphological stability and stability of physiological features were reported as unchanged and maintained.

**M.A. Zippel and M. Neigenfind (J. Gen. Appl. Microbiol. 34: 7-14, 1988)** made a comparative evaluation of lyophilization, storage in frozen glycerol medium (-20°C) and in liquid nitrogen (-196°C). “Storage of *Streptomyces* in frozen glycerol is recommended as quick method for frequent studies in the laboratory. Storage in liquid nitrogen is recommended as a long term preservation method”.

Among other techniques used mention may be made of cryopreservation of agar plugs of cultures in the gas phase of liquid nitrogen. From 10-14 day cultures growing on agar plates, plugs are cut and transferred to sterile polypropylene sterile straw bits which are transferred to sterile screw-cap vials. Agar acts as a cryoprotectant and the results of revival of such frozen cultures have been satisfactory.

*Streptomyces* cultures which are heavily sporulating and having xylanolytic activity have been successfully grown on autoclaved grass leaf bits placed on
2.5% plain agar plates adopting the technique developed by M.C. Srinivasan et al. (Trans. Br. Mycol. Soc. 86: 31-35, 1971) for sporulation of seed-borne fungi. The colonization of the grass leaf and copious sporulation on the leaf surface facilitates easy observation for morphological features of the sporulation under the stereo-binocular microscope. The grass leaf sporulated cultures can be stored in the refrigerator or even transferred to sterile water in screw capped tubes. Good survival and conservation of xylanase productivity was observed in stock cultures so conserved (M.C. Srinivasan-unpublished data).

4.6 Conclusions

From the discussion presented the need for long-term conservation of actinomycetes cultures with minimum change in vital characteristics is obvious. Individual strains vary in their response to specific methods of conservation and a careful evaluation of the most suitable techniques may need to be carried out for various strains. Also more than one method of preservation is always advisable to ensure sustained and long-term conservation of actinomycetes.
Chapter-5

Secondary Metabolism and Bioactive Secondary Metabolites: An Overview

5.1 Introduction

The discovery of antibiotic effects of penicillin triggered a surge of interest in metabolites produced by microorganisms. Besides primary metabolites which participate in the basic life processes, microorganisms produce many complex metabolites which apparently take no part in their basic life processes. These substances termed secondary metabolites possess a remarkable diversity in their chemical structures and several studies on their biosynthesis involving multiple enzymatic steps have been published. Understanding the regulatory processes related to the biosynthesis of secondary metabolites is an important criterion in optimizing their production in high yields leading to commercial scale manufacturing processes. This presents an opportunity as well as challenge to geneticists to engineer microorganisms with overproduction capacity for the secondary metabolites of value. Industrial strains with several fold biosynthetic capabilities for secondary metabolites compared to the wild strain have been selected in the laboratory and employed in commercial manufacture. It is noteworthy that these hyperproductive strains differ from the wild type in their "enzyme equipment and in the relative activities of individual enzyme systems (V. Behal. Trends Biochem. Sci. 11: 88-91, 1986).

The synthesis of enzymes of secondary metabolism is normally repressed and must be de-repressed for their expression. One of the factors leading to de-repression is the exhaustion of rapidly used sources of carbon, nitrogen and phosphorous (for example, glucose, ammonium salts, inorganic phosphate). Secondary metabolites are produced in higher quantities after the rapid growth phase and it is observed that it is during this transition from
active growth phase to stationary phase that maximum synthesis of the enzymes of secondary metabolism takes place. For example, it has been observed that enzymes of the tetracycline synthetase complex are synthesized only for a short time following the depletion of inorganic phosphate in the medium and until the concentration levels of the product formed may exert an inhibitory effect on the enzymes. Likewise production of polypeptide antibiotics also follow a similar pattern in which the biosynthetic enzyme activity peaks up during the stationary phase. Addition of amino acids which are part of the polypeptide antibiotic to the growth medium enhanced the levels of antibiotic production.

Definition of secondary metabolism and secondary metabolites has been a topic of interesting discussion. In a review entitled “What is in a name? Microbial secondary metabolism” J.W. Bennett and R. Bentley (Adv. Appl. Microbiol. 34: 1-28, 1989) suggest defining them as special or specific metabolites and have characterised them as "a metabolic intermediate of product found as a differentiation product in restricted taxonomic groups, not essential to growth and life of the producing organism and biosynthesized from one or more "general" (primary) metabolites by a wider variety of pathways than are available in general (primary) metabolism." Secondary metabolites are often materials of low molecular weight and are frequently accumulated in large quantities after the cessation of growth. Examples of secondary metabolites include alkaloids, polyketides, terpenes, non-ribosomal oligopeptides etc. many of which exhibit pharmacological activity as drugs (e.g. antibiotics, toxins etc.) The exact role of secondary metabolites in the life of the producing organism is not fully understood. However, they have been regarded as providing certain selective advantages which are of an ecological nature.

Actinomycetes as a group have been the most prolific source of diverse secondary metabolites with interesting and useful biological activities. Most notable are the various antibiotics discovered, many of which have found pharmacological and therapeutic applications. Actinomycetes make 74% of all described antibiotics (S. Miyadoh. Actinomycetologica 7: 100-106, 1993) and the genus Streptomyces has been the source of 78% of all the known secondary metabolites of actinomycete origin. The diverse and unusual chemical structures of the secondary metabolites elaborated include β-lactam rings,
cyclic peptides containing "unnatural" and non-protein amino acids, unusual/rare sugars, nucleotides, polyenes and large macrolide rings.

In a review on "Emerging concepts of secondary metabolism in actinomycetes" A.L. Demain and A. Fang (Actinomycetologica 9: 98-117, 1995) have discussed the complex signals and regulatory events associated with the onset of secondary metabolism. Exhaustion of a nutrient and/or growth rate decrease generates signals effecting 'a cascade of regulatory events resulting in chemical differentiation (secondary metabolism) and morphological differentiation (morphogenesis).’ The signal is often an auto-regulatory factor in the form of low molecular weight butyrolactone inducers which has been observed to bind to a regulatory protein which prevents secondary metabolism and morphogenesis during rapid growth and under conditions of sufficient nutrient availability. The growth phase is termed the "trophophase" and the phase of secondary metabolite production is referred to as the "idiophase". The secondary metabolites are also given the name "idiolites'. Depending on the composition of the medium and other fermentation parameters, the trophophase and idiophase may either be distinctly separated or show considerable overlap.

5.2 Fermentation Parameters Influencing Secondary Metabolite Production

Rapidly metabolized sugars such as glucose, while supporting biomass build up are unfavourable for the formation of many secondary metabolites. Complex polysaccharides such as starch, oligosaccharides like lactose and oils such as soya bean oil when incorporated in media yield secondary metabolites which are favoured by the slow release of the essential nutrients from these substrates. Likewise readily utilized ammonium salts are unfavourable while complex, nitrogen-rich natural substrates like soybean meal favour secondary metabolite formation. Ammonium ions have been shown to repress certain key enzymes like valine dehydrogenase involved in the production of macrolide antibiotics such as tylosin and streptomycin. The role of phosphates as deterrents for secondary metabolite formation appears to be due to inhibition/repression of phosphatases, which are required in the biosynthesis of intermediates of aminoglycoside antibiotics, which are sensitive to phosphate concentration. Enzymatic hydrolysis of
dihydrostreptomycin-6-phosphate is inhibited, for example, by inorganic phosphate. Levels below 10 \( \text{nM} \) of phosphate, while being suboptimal for growth are favourable for secondary metabolite formation. Satoshi Omura and his collaborators in Japan investigated the effect of ammonium and phosphorous trapping agents in antibiotic fermentations. Production of many antibiotics was enhanced by addition of magnesium phosphate (ammonium ion trapping) and magnesium carbonates or allophone (aluminosilicate) (phosphate trapping agents). Several new bioactive molecules such as nanomycins, globopeptin, thiotetromycin and Jietacin were identified from these studies (S. Omura. *The search for bioactive compounds from microorganisms*. Springer 32, 1992).

Endogenous metabolites, notably A-factor induce both morphological and chemical differentiation in *S. griseus*, characterized by aerial mycelium and spore chains as well as induction of streptomycin synthetases leading to streptomycin formation. As mentioned earlier the A-factor butyrolactones exert their effect through receptor proteins which are repressors of chemical and morphological differentiation.

### 5.3 Antibiotics: The Largest Group of Known Secondary Metabolites

Among the known bioactive secondary metabolites, antibiotics constitute the largest group. Waksman defined antibiotics as "chemical substances produced by microorganisms and possessing the ability to kill or to inhibit the growth of bacteria and other microorganisms." The impact of this definition is obvious from the world-wide focus on screening microorganisms for their potential to produce chemical moieties exerting inhibitory effect on particularly pathogenic microorganisms, while being low in toxicity towards humans and animals. Primarily the search for antibacterial, antifungal and to a lesser extent, anticancer molecules has led to identification of thousands of natural bioactive molecules of which only a small fraction has found application in therapeutic medicine.

The diversity of chemical structures of antibiotics encompasses virtually every group and class of organic compounds known. Also these compounds exhibit their bioactivity towards microbial cellular processes ranging from DNA replication, translation and transcription to biosynthetic processes.
such as fatty acid synthesis or cell wall peptidoglycan and membrane synthesis and functioning. (Y. Aharonowitz and G. Cohen. *Scientific American* 245: 141-152, 1981). While there is gross similarity in cellular processes in all the living systems, characteristic differences exist which allow the antibiotics to discriminate the metabolic activity of one organism from another. This phenomenon of selective toxicity exhibited by the antibiotics enabled their application as antibacterial, antifungal, antitumor, antiviral or antiparasitic drugs. The key element however is their lack of toxicity or adverse effects on the hosts while being antagonistic to the microbial pathogens. The range and diversity of antibiotics produced by actinomycetes include β-lactams, aminocyclitols, tetracyclines, macrolides, polyether and peptide antibiotics.

### 5.4 Protection Mechanisms in Producer Organisms to "Prevent Suicide"

When a survey is made of various molecules with antibiotic activity, which are synthesized by microbes, we can identify two major groups:

1. Molecules which do not have inhibitory effects on the organism producer strain
2. The molecules which can exert an inhibitory effect on the organism producing the antibiotic.

As an example of the first category may be mentioned penicillin biosynthesis by *Penicillium chrysogenum* and as is well known, penicillin does not have antifungal activity. The actinomycetes which produce diverse antibacterial antibiotics are examples of the second type and this necessitates an understanding and appreciation of the subtle mechanisms by which these producer organisms avoid getting inhibited by their own antibacterial product(s) or as has been more emphatically stated "how do they prevent suicide?" (A.L. Demain. *Ann. N.Y. Acad. Sci.* 235: 601-612, 1974; E. Cundliffe. *Ann. Rev. Microbiol.* 43: 207-233, 1989).

As discussed in detail by Cundliffe (1989), antibiotic producing organisms have "a range of diverse options" which include replacement of the target site at which a given drug normally acts, inactivation or sequestration of intracellular drug molecules, or crection of permeability barriers possibly coupled with an efficient efflux mechanism. Systematic investigations have
brought to light different biochemical mechanisms operating to enable producer organisms to acquire resistance to their own autotoxic antibiotics. For example, elaboration of either a chromosomal or plasmid encoded enzyme which modifies the antibiotic molecule leading to its inactivation is one such mechanism. Thus aminocyclitol phosphotransferase found in streptomycetes producing aminocyclitol antibiotics plays a role in the resistance of the producer strains. In Erythromycin and Thioestrepton producing *Streptomyces* sp., the resistance mechanism is through alteration of the target ribosome by ribosomal methylases. Novobiocin resistance in the producer organism, *Streptomyces sphaeroides* is determined by a gyrB gene whose product, the DNA gyraseB protein, is resistant to the drug. These are just a few examples to illustrate the diverse biochemical mechanisms relevant to making the producer strains insensitive to their own secondary metabolites with autotoxic antibiotic activity.

**5.5 Therapeutically useful Antibiotics Produced by Actinomycetes**

Actinomycetes and particularly members of the genus *Streptomyces* are the largest antibiotic producers from the microbial world. Out of the 12,000 antibiotics known in 1985, 53% were produced by streptomycetes and an additional 11% by other actinomycete genera. The diversity of antibiotic molecules originating from actinomycetes include several life-saving drugs which have revolutionized medical treatment of microbial infections. It has been estimated that the doubling of our life span in the 20th century was mainly due to the use of plant and microbial secondary metabolites and undoubtedly actinomycetes have contributed substantially towards this success in combating pathogenic infections and disease. Recent years have witnessed the natural products of actinomycetes as well as their semi-synthesis derivatives becoming increasingly significant to the drug industry. For example, the macrolide antibiotic Erythromycin A synthesized by *Saccharopolyspora erythraea* and its synthetic derivatives had an annual sale of over $2.6 billion in 2000. Likewise the glycopeptide antibiotic vancomycin produced by *Amycolatopsis orientalis* had a sale value of $4.24 million in the same year. The tetracyclines produced by *Streptomyces aureofaciens* and anticancer drugs like Doxorubicin produced by *Streptomyces peucetius* also recorded substantial sales and increasing market demand.
A glimpse into the specific modes of action of these antibiotics in inhibiting bacterial infections is highly interesting. The aminoglycoside antibiotics like streptomycin, gentamycin, kanamycin and neomycin bind to bacterial 30 S ribosomes and also cause a misreading of the m-RNA whereby the bacteria are unable to synthesize proteins vital for their growth. Others like the carbapenam and glycopeptide antibiotics (e.g. Teicoplanin, vancomycin) as well as monobactams (e.g. Aztreonam) inhibit cell wall and bacterial peptidoglycan synthesis. Thienamycin, a carbapenem antibiotic developed by Merck from *Streptomyces cattleya* is a very potent broad spectrum and medically important antibiotic. More potent beta-lactamase resistant derivatives like Imipenem have been developed and marketed. Inhibition of protein synthesis by binding to the 50S subunit of bacterial RNA is characteristic of Lincosamide antibiotics like Clindamycin and Lincomycin as well as Erythromycin. Lipopeptides like Daptomycin bind to the membrane causing rapid depolymerization to impair normal cell wall synthesis. Also DNA, RNA and protein synthesis can be adversely affected. The tetracyclines exemplified by oxytetracycline, tetracycline, doxycycline, minocycline etc. inhibit the binding of aminoacyl t-RNA to the m-RNA ribosome complex and they do so by binding to the 30 S ribosomal subunit in the m-RNA translation complex. The antitubercular drugs streptomycin and rifampicin act by binding to the b-subunit of RNA polymerase to inhibit transcription. Chloramphenicol inhibits bacterial protein synthesis by binding to the 50S subunit of the ribosome.

### 5.6 Antibiotics in Plant Disease Control

Plant diseases, in particular those caused by fungi result in serious crop losses and in extreme situations as in the case of potato blight in Ireland due to *Phytophthora infestans*, a famine situation arose. Conventionally plant disease control by fungicidal chemicals has been practiced. In recent years, serious infections due to phytopathogenic bacteria have added a significant dimension. For example, the high-yielding varieties of rice are severely affected by bacterial blight due to *Xanthomonas campestris* pv. *oryzae*. Against the bacterial infections there have been no effective means of chemical control. With the advent of the antibiotic era and increased awareness of the hazards of environmental pollution by chemicals, efforts to use antibiotics
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for plant disease control gained momentum. While initial efforts were
directed towards evaluating antibiotics not suited to human therapy as agents
to control plant disease, more recent efforts have focussed on finding effective
antibiotic molecules exclusively for controlling devastating crop diseases.

Bacterial diseases were effectively combated by the use of antibacterial
antibiotic formulations derived from those used in human therapy. For
example, a combination of streptomycin and Chlortetracycline
("streptocycline") as well as a streptomycin-oxytetracycline preparation
("Agrimycin-100") were reported to significantly control the bacterial blight
disease of rice under Indian conditions.

Examples of antibiotic compounds discovered and effectively used to
combat major plant diseases include Blasticidin-S and Kasugamycin
developed in Japan to control the blast diseases of rice (*Pyricularia grisea*) and
Aureofungin developed at the Hindustan Antibiotics Ltd., India. The topic
of antibiotics in the control of plant pathogens has been comprehensively
Blasticidin was discovered as an antifungal agent produced by *Streptomyces
griseochromogenes* and Besticidin-S is a benzyl amino benzene sulphonate
derivative) Blasticidin which has been used effectively on rice crop at 10-40 g
of the antibiotic per hectare.

Kasugamycin from *Streptomyces kasugaensis* was also developed in Japan as a
specific and effective antibiotic to control the rice blast disease. Applied as a
20 mg/ml spray, it was effective as a protective as well as curative agent. The
antibiotic molecule comprises three moieties viz. D-inositol, kasugamine
and an imino acetic acid side chain. Aureofungin is a polyene antibiotic
(heptaene) from *Streptoverticillium cinnamomeum var. terricola*, discovered by
**M.J. Thirumalachar** in India. The antibiotic was successfully used in seed
treatment to control seed-borne infections of rice due to *Helminthosporium
oryzae* and of wheat by the internally seed-borne loose smut (*Ustilago tritici*)
disease. Soaking seeds in antibiotic solution (20 µg/ml) prior to planting
effectively prevented transmission of the seed-borne infections to the
growing seedling. The antibiotic was also inhibitory to the rice blast pathogen
at less than 0.1 µg/ml and when sprayed, the antibiotic was readily absorbed
and translocated all over the plant, as a result of which even new shoots
growing after the spraying were effectively protected against infection. Additional examples of actinomycete metabolites found effective against serious phytopathogens include Phthoramycin inhibitory to *Phytophthora* (*S. Omura et al. J. Antibiotics 41:1910-1912, 1988*) and Rustmicin active against wheat rust (*Puccinia graminis*) produced by *Micromonospora chalacea* (*H. Nakayama et al. Agr. Biol. Chem. 51: 853-59, 1987*).

Despite their positive attributes, antibiotic treatments for large scale combating of plant diseases, has not been practiced. Instability under field conditions, loss of potency during storage due to limited shelf-life and high cost of production have been some of the contributory factors for this limited application of antibiotics for plant disease control.

### 5.7 Biocontrol

Biocontrol with the aid of antagonistic microbes is a developing frontier area, especially for root disease fungi. Treatment of seeds or planting material such as rhizomes with the antagonist microbe has been reported to be effective in containing the extent of loss from fungal root infection of young seedlings.

Control of damping off and root rot of tomato and capsicum by *Pythium aphanidermatum* by coating seeds with a heavy spore suspension of an antagonistic strain of *Streptomyces griseus* strain 38, has been reported (*M.J. Thirumalachar et al. Hindustan Antibiotics Bulletin 12: 135-141, 1970*).

A commercial preparation from Finland (Verdera OY) based on *Streptomyces griseoviridis* and designated "Mycostop" has been reported effective for the control of diseases of cucumber, tomato, pepper, and greenhouse ornamentals. Effective results were obtained with root rots, wilts or damping off caused by fungal species belonging to *Fusarium, Alternaria, Phytophthora* and *Pythium*. The streptomycete outcompetes the pathogen by colonizing the plant roots and effectively inhibits by producing enzymes and metabolites. Plant growth promoting metabolites are also produced by the streptomycete ensuring vigorous growth and higher yields.

### 5.8 Other Bioactive Metabolites

Apart from the clinically useful antibiotics already discussed, actinomycetes
also produce novel blood sugar lowering agents like acarbose and potent immunosuppressive compounds such as Tacrolimus (FK-506). Enzyme inhibitors with potent therapeutic applications have also been identified from actinomycetes. Actinomycete metabolites useful in agriculture and collectively termed "Agroactive metabolites" (Y. Tanaka and S. Omura. Ann. Rev. Microbiol. 47: 57-87, 1993) have found widespread application. These include insecticidal, antiprotozoal, antihelminthic and herbicidal compounds.

5.8.1 **Acarbose**, an α-glucosidase inhibitor produced on a commercial scale employing strains of *Actinoplanes*. It has been in use since 1990 and is marketed in Europe under the name "Glucobay". This α-glucosidase inhibition slows down digestion of carbohydrates thereby lengthening the time it takes to release glucose into the blood stream. It has found application in Type I diabetes therapy and enables patients to exercise better control on their blood sugar levels.

5.8.2 **Tacrolimus (FK-506)** discovered at the Fujisawa Pharmaceutical Laboratories, Japan as a metabolite of *Streptomyces tsukubaensis*. Tacrolimus (FK-506) is a potent immunosuppressant. It slows "in-vitro" T-cell proliferation and also disrupts Calcineurine -mediated signal transduction in T lymphocytes. It is a macrolide compound and its immunosuppression potency is rated as 10-100 times greater than cyclosporine A. It is widely used in controlling immuno-rejection during organ transplants.

5.9 **Enzyme Inhibitors**

Pioneering studies of **Prof. Hamao Umezawa** in Japan led to the discovery of several low molecular weight enzyme inhibitors from streptomycetes with potential medical applications. Most significant among these are a variety of inhibitors of diverse proteolytic activities. Leupeptin inhibitory to cathepsin B, papain, plasmin and trypsin is widely distributed among members of the genus *Streptomyces* (H. Umezawa. "Enzyme inhibitors of Microbial Origin", University of Tokyo Press, Tokyo, 1972). Inhibitors of aminopeptidase A and B discovered from *Streptomyces* showed enhanced immune response in mice and these include bestatin from *S. olivoreticuli*, Forphenicins from *S. fulvoviridis* var *acarbodicus*, Amastatin, Ebelactone and others. The review by H.

5.10 Insecticidal and Acaricidal Metabolites

Avermectin and Milbamycin are among the most important metabolites having this biological activity. The Avermectins are a family of fused 16-membered macrolides produced by *Streptomyces avermitilis* and jointly developed by Merck Sharp and Dome laboratories and Kitasato Institute in Japan. Avermectin inhibits signal transmission at the gamma amino butyric acid (GABA) level as a neuroactive substance. It is a potent metabolite with insecticidal, nematocidal and acaricidal activities. It is active against mites, arthropods and aphids and finds application in combating them for crop protection. It is used as an acaricide against worm infestations in cattle. A derivative of this compound named Ivermectin has currently been used to combat onchocerciasis or African River Blindness disease in humans caused by a microfilaria, *Onchocerca volvulus*. Satoshi Omura and W.C. Campbell won the 2015 Nobel prize in Physiology or Medicine for their discovery of Avermectin and its therapeutically useful derivative Ivermectin effective against river blindness and lymphatic filariasis.

Milbamycins are structurally closely related to the Avermectins. It was discovered by Y. Takaguchi et al. (*J. Antibiotics* 33: 1120-1127, 1980) and has been commercially available for acaricidal and veterinary applications. Other examples of metabolites with insecticidal potential are setamycin from *Kitasatosporia* sp. (K. Otoguro et al. *J. Antibiotics* 41, 250-252, 1988), Jietacin and Allosamidins. Nikkomycins produced by *Streptomyces tendae* are nucleosidal compounds with insecticidal as well as fungicidal activities by virtue of their interference with chitin synthesis. The compound was projected as unique one for applications in pest as well as fungal disease control on plants. Its efficacy, however, under field conditions was rather poor and has failed to qualify for large scale field applications.
5.11 Herbicidal Activity

Herbicides are an important component of agricultural practice aimed at controlling weed populations during crop cultivation. A variety of synthetic chemical compounds are being widely used as herbicides. A search for actinomycete metabolites exhibiting herbicidal potential has yielded promising results. Bialophos, a product of *Streptomyces hygroscopicus* fermentation has been the first microbial product to be used in agriculture as a herbicide. Its broad spectrum activity as well as high fermentation yields are contributory to its viability for large scale applications (H. Takebe et al. *J. Ferm. Bioeng.* 67: 226-232, 1989). The compound is an inhibitor of glutamine synthetase. Other metabolites with herbicidal activity from actinomycetes include Hydantocidin, also from a strain of *S. hygroscopicus*, which has herbicidal activity towards monocot and dicot annuals comparable in efficacy to Bialophos as well as the widely used synthetic herbicide, Glyphosate (M. Nakajima et al. *J. Antibiotics* 44: 293-300, 1991). Herboxidine, a herbicidal metabolite from *Streptomyces chromofuscus* has been reported to successfully control weeds such as rape and wild buckwheat while cultivated crops like wheat, rice and soyabean exhibited little or no sensitivity (M. Miller-Wideman et al. *J. Antibiotics* 45: 914-921, 1992).

5.12 Conclusion

The diversity of secondary metabolites from actinomycetes is immense and as more new and useful molecules are discovered either from new taxa or through innovative approaches in their cultivation as well as novel analytical techniques, it appears certain that there is still a lot more to be discovered and the actinomycetes would continue to remain in the forefront as a virtual goldmine for useful secondary metabolites for a long time to come. What has been presented in this chapter is just a glimpse into the vast potential that has been explored and exploited over the years. If the information presented in this chapter will have a stimulatory and positive effect on ardent students of microbiology and biotechnology to focus on actinomycetes and their metabolites with a spirit of discovery and innovation, the objective of writing this chapter may be deemed as fulfilled.
6.1 Introduction

The intrinsic ability of natural populations of microorganisms to degrade and recycle organic residues has tremendous ecological significance. Harnessing the biodegradative and biotransformation abilities of microorganisms for making the environment free of toxic and harmful residues is called bioremediation and is an interesting and challenging aspect of microbial biotechnology. Microbial biotransformation also has enormous potential for industrial production of fine and commodity chemicals as the processes based on biocatalysis, can be carried out under mild conditions and thus much more environmentally acceptable.

Actinomycetes constitute a major group of microbes in the natural environment and play a very active role in both organic matter recycling and biodegradation. Actinomycetes are known to degrade or transform a wide variety of natural as well as man-made materials, especially those which are toxic or recalcitrant. In this chapter, we intend to present a brief overview of the broad spectrum of the biodegradation and biotransformation activities and capabilities of actinomycetes. In the end, a few examples of industrial biotransformation processes based on actinomycete enzymes are stated. Details of molecular mechanisms of biodegradation are beyond the scope of this review and hence will not be discussed.

6.2 Biodegradation of Natural Organic Substances

Plant residues and herbivorous excretion wastes constitute the major organic material subjected to degradation and recycling in the natural environment. The main constituent of these wastes is lignocellulose consisting of cellulose,
hemicellulose and lignin. Starch is another organic substrate available in nature for degradation, although to a lesser extent. Chitin from insect residues and keratin from hair, feathers and similar material are also subjected to microbial degradation. Actinomycetes possess very strong ability to metabolize these natural substrates and detailed studies on various aspects of their biodegradation have been published.

### 6.2.1 Lignocellulose Degradation

Lignocellulose is a highly complex, insoluble, polymeric substance consisting of cellulose (40-45%), hemicellulose (20-30%) and lignin (15-25%). It is the major component of plant biomass. In natural environment, complete degradation of lignocellulose occurs due to concerted activities of a range of hydrolytic and oxidative enzymes which are secreted by several microorganisms that coexist in a particular ecosystem. Specific contribution of actinomycetes in lignocellulose degradation is not clear so far. However, their ubiquitous and almost obligatory presence in lignocellulosic wastes and diversity of their species in such environments indicates their potential role in degradation of lignocellulose.

**A.J. McCarthy** and **S.T. Williams** *(Gene 115: 189-192, 1992)* discussed various strategies that actinomycetes could have adapted to degrade and utilize insoluble, solid substrates like lignocellulose. These include a) mycelial nature of most of the actinomycetes which help them to invade and colonize the solid substrate, b) secretion of range of extracellular enzymes which aid in the degradation of the substrate to obtain nutrition, and ultimately, c) sporulation upon nutrient exhaustion, which helps them to sustain in the low nutrient environment. It is also proposed that the alkaline environment present in the lignocellulosic environments such as compost heaps favours the development of actinomycetes and reduces competition from fungal population. Indeed, actinomycetes have been shown to dominate in such alkaline, high temperature environments and diverse range of both thermophilic and mesophilic actinomycetes such as *Thermomonospora, Thermobifida, Saccharomonospora, Streptomyces, Pseudonocardia, Micromonospora, Microbispora* and *Rhodococcus* have been isolated from such substrates *(A.J. McCarthy. FEMS Microbiol. Lett. 46: 145-163, 1987; M.Hayakawa et al. Actinomycetologica 24: 58-62, 2010)*.
Actinomycetes have been shown to attack all the three components of lignocellulose, although cellulolytic activity of actinomycetes has received greater attention. Cellulose is a highly organized homopolymer consisting of D-glucose units linked by 1,4-β-glycosidic bonds. It is considered quite recalcitrant and is amenable to complete degradation by only those microorganisms which are able to produce multiple cellulolytic enzymes. The cellulolytic enzyme complex consists of; a) Endo-glucanases (1,4-β-glucan hydrolases, Cx, carboxymethylcellulase) which act randomly, disrupting the crystalline structure of cellulose and exposing individual cellulose chains, b) exo-glucanases (C1 or 1,4-β-cellobiohydrolases) which cleave exposed cellulose chains to generate tetra or disaccharides units such as cellobiose, and c) cellobiases or β-glucosidases which hydrolyze cellobiose to individual glucose units. Cellulose degradation is the result of combined activities of all these enzymes.

Actinomycetes secrete extracellular, inducible cellulolytic enzyme complex which can degrade cellulose in variety of forms. Cellulolytic actinomycetes have been isolated from both thermophilic and mesophilic environments. They prefer neutral to slightly alkaline pH for optimum activity. *Thermomonospora curvata*, a thermophile isolated from municipal waste compost, has been shown to degrade ~75% of the cellulose in the form of ground cotton within 10 days at 55°C, pH 8.0 (F.J. Stutzenberger. *Appl. Env. Microbiol.* 24: 77-82, 1972). *Microbispora* strains also possess around 51-82% cellulolytic activity. Mesophilic *Cellulomonas* strains were shown to degrade cotton wool (W.Y. Choi et al. *Australian J. Biological Sciences* 31: 553-564, 1978) and also a printed newspaper (P. Rapp et al. *Biotechnol. Bioeng.* 26: 1167-1175, 1984).

S.M. Betrabet et al. (*Textile Research Journal* 38: 1189-1197, 1968) reported degradation of cotton and fabric indigenous to the Bombay region in India, by highly cellulolytic *Streptomyces* strains. Cellulolytic strains of *Streptomyces, Kibdelosporangium, Nocardoides* and *Micromonospora* were reported to degrade rice straw pieces to the extent of 50-61% (H.M. Abdulla and S.A. El-Shatoury. *Waste Management* 27: 850-853, 2007). Cellulolytic *Micromonospora* may have an important role in lignocellulose degradation in soil and fresh water sediments. They have also been isolated from decaying wood and implicated in the biodeterioration of timber foundation piles.
Some of the cellulolytic *Micromonospora* strains have been isolated from fresh water lakes using cellulose baits (*A.B. deMenezes et al. Appl. En. Microbiol. 74: 7080-7084, 2008*).

Isolation of cellulolytic *Streptomyces* and *Micromonospora* strains from the niche environment such as hindguts of termites indicates their specific role and possible symbiotic association to degrade lignocellulose in the guts (*M.B. Pasti and M.L. Belli. FEMS Microbiol. Lett. 26: 107-112, 1985*).

Many actinomycetes co-express hemicellulolytic activity along with cellulases. Hemicellulose is structurally much more complex than cellulose. It is a nonlinear heteropolymer with beta-1, 4 glycosidic bonds and contains xylan and mannan as major sugar polymers. Other sugars such as galactose, arabinose, glucuronic acid, rhamnose and fucose are present in variable amounts. The complexity of hemicellulose structure requires a high degree of coordination between the enzymes involved in hemicellulose degradation. Co-production of xylanases and other xylanolytic enzymes with cellulases is demonstrated during the degradation of bagasse by *Streptomyces* and *Micromonospora* strains (*W.H. van Zyl. Biotechnol. Bioeng.27:1367-1373, 1985*).

Molecular studies have revealed that hemicellulose degrading systems of actinomycetes are quite complex and non-uniform, comprising range of activities, some of which are expressed in multiple forms. For example, *Streptomyces coelicolor* A3 (2) possess 11 different hemicellulase genes coding for (in some cases multiple forms of) xylanases, xylosidases, arabinofuranosidases and arabinases whereas *Cellulomonas fimi* possesses 5 hemicellulase genes coding for xylanases, mannanases and mannosidases (*D. Shallom and Y. Shoham. Curr. Opin. Microbiol. 6: 219-228, 2003*).

The complex nature and presence of variety of hemicellulolytic enzymes has also been experimentally demonstrated by several workers. *W. Zimmerman et al. (FEMS Microbiol. Lett. 55: 181-185, 1988)* showed presence of xylanase, acetyl xylan esterase and arabinofuranosidase activities in the strains of *Streptomyces, Actinomadura* and *Saccharomonospora viridis* when grown on ball-milled barley straw. In *Streptomyces flavogriseus*, simultaneous production of xylose isomerase in addition to cellulases and xylanases is described (*M. Ishaque and D. Kluepfel. Biotechnol. Lett. 3: 48-486, 1981*). *Streptomyces*
olivochromogenes and Streptomyces flavogriseus growing on wheat bran were shown to produce significant levels of acetyl xylan esterases and ferulic acid esterases (K.G. Johnson et al. Ens. Microb. Technol. 10: 403-409, 1988; K. Pontanen et al. J. Biotechnol. 6: 49-60, 1987).

Actinomycetes tend to degrade xylan polymer backbone outside the cells, however, breakdown of oligosaccharides might occur by cell associated or intracellular enzyme. A.S. Ball and A. J. McCarthy (J. Gen. Microbiol. 134: 2139-2147, 1988) described saccharification of wheat straw by the strains of Streptomyces, Thermobifida fusca and Microbispora bispora which co-produced-xylosidase, acetyl xylan esterase and arabinofuranosidase along with b-glucosidase. b-xylosidase activity in most of these strains was found to be largely cell associated whereas other xylan polymer degrading enzymes were extracellular. Membrane bound xylosidase has also been described in Streptomyces species (K. Nakanishi et al. J. Ferm. Technol. 65: 1-6, 1987; N. Belfaqih and M.J. Penninckx. Enz. Microb. Technol. 27:114-121, 2000). The intracellular or cell bound degradation of oligosaccharides could be significant in preventing the non-xylan degrading competitors from getting benefited by the extracellular degradation products (D. Shallow and Y. Shokam. Cur. Opin. Microbiol. 6: 219-228, 2003).

The most challenging component for lignocellulose degraders is lignin. Lignin is a complex, three dimensional, phenolic polymer with variety of bonding arrangements. Rate of lignin degradation by actinomycetes is poor compared to that of fungi (A.J. McCarthy and P. Broda. J. Gen. Microbiol. 130: 2905-2913, 1984). Actinomycetes can attack grasses which are low in lignin content much more easily than lignin-rich wood. However, in neutral to alkaline environment, lignin solubilizing actinomycetes may get an edge over lignolytic fungi. Streptomyces viridosporus has been reported to solubilize lignin from corn lignocellulose at pH 8.4-8.8 (A.L. Pometto III and D.L. Crawford. Appl. Env. Microbiol. 52: 246-250,1986). M. Hernández et al. demonstrated modification of alkali lignin produced from wheat straw and paper mill effluent by Streptomyces strains (Biodegradation 12: 219-223, 2001; J. Chromatography 919: 389-394, 2001).

B. Godden et al. (J. Gen. Microbiol. 138: 2441-2448, 1992) proposed that lignin degradation may not contribute significantly to the carbon and energy
metabolism of actinomycetes, but could be useful in destabilizing lignocellulose to increase the accessibility of hemicellulose and cellulose. Primary product from lignocellulose degradation by actinomycetes is demonstrated to be a soluble, high molecular mass, lignocarbohydrate complex (D.L. Crawford et al. Appl. Env. Microbiol. 45: 898-904, 1983) and was termed as acid precipitable polymeric lignin (APPL). Extraction and analysis of low molecular weight compounds from APPL indicated the similarity of APPL with humic acids (C. Trigo and A.S. Ball. Microbiology 140: 3145-3152, 1994). Hence it was proposed that lignin degradation by actinomycetes might play important role in humification processes in natural soils and composts.


### 6.2.2 Degradation of Starch

Starch can be easily and rapidly degraded compared to lignocellulose and serves as an excellent carbon source for actinomycetes. Starch consists of
amylose (10-30%), a linear glucose polymer with α, 1-4 linkages and amyllopectin (70-90%), a highly branched glucose polymer with α, 1-6 linkage at the branching point. Raw starch is insoluble in water and most of the microorganisms degrade starch only when it is liquefied or solubilized by heating it in water.

Starch degrading enzymes are generally extracellular, and include α-amylases, isoamylases, glucoamylases, maltogenic β-amylases and α-glucosidases, α-amylases are endoenzymes which randomly cleave α,1-4 linkages in amylose and amyllopectin generating, maltose, maltotriose and dextrins. Isoamylases hydrolyze α, 1-6 linkages at the branch points in amyllopectin. Glucoamylases and maltogenic β-amylases are exoenzymes which cleave amylose and amyllopectins from the non-reducing end generating β-glucose and maltose, respectively. Glucosidases yield α-glucose from maltose and maltotriose.


Some of the actinomycetes are capable of attacking and hydrolyzing raw starch granules without the need of pre-liquefaction. This could be highly beneficial especially in an industrial set up, as the liquefaction of starch is an energy intensive process. Raw starch degrading actinomycetes mainly belong

### 6.2.3 Rubber Degradation

Actinomycetes seem to dominate rubber degradation activity amongst microorganisms and are shown to decompose natural as well as, to some extent, vulcanized rubber. They are widely distributed in nature and have been isolated from variety of sources such as soil of rubber tree plantation, waste water ponds of rubber producing companies (D. Jendrossek et al. FEMS Microbiol. Lett. 150: 179-188, 1997), fouling water inside deteriorated car tyres (A. Linos et al. Int. J. Syst. Bacteriol. 49: 1785-1791, 1999) and also vulcanized rubber gaskets (J.J. Rook. Appl. Microbiol. 3: 302-309, 1955).

Rubber mainly consists of poly-isoprene units which occur in two different double bond configuration viz. cis (poly (cis-1,4 isoprene) as in Natural rubber (NR) or trans (poly (trans-1,4 isoprene)) as in Gutta percha (GP). Natural rubber degrading microorganisms are divided into two groups based on their growth characteristics and mechanism of rubber degradation. Both the groups predominantly contain various actinomycete genera. Actinomycetes belonging to the first group are mycelial in nature and metabolize the polyisoprene by extracellular enzymes. This group includes species of *Streptomyces, Actinomadura, Actinoplanes, Micromonospora, Thermomonospora* and *Dactylosporangium* (K. Rose and A. Steinbüchel. Appl. En. Microbiol. 71: 2803-2812, 2005). Most representatives of this group show relatively weak and very slow growth on natural or synthetic rubber. Members of the second group which require direct contact with rubber show relatively strong growth on rubber and belong to *Corynebacterium-Nocardia-Mycobacterium* group. Some novel strains belonging to this group are *Gordonia polyisoprenivorans* VH2 and Y2K and *G. westfalica* strain Kb1 (M. Arenskötter et al. FEMS Microbiol. Lett.
During colonization these organisms show strong adhesion and formation of biofilm over the surface of rubber material.

Actinomycetes have also been shown to degrade vulcanized rubber material. Two *Streptomyces* strains were isolated from vulcanized gaskets of cement water tubes, which were the cause of 1.5 mm diameter holes in the material after 12 months of incubation (J.J. Rook. *Appl. Microbiol.* 3: 302-309, 1955). Based on these studies, Leefgang test bath, in which rubber test material is examined in a steady aquatic stream to test its stability against microbial degradation, was developed (K.W.H. Leefgang. *J. Am. Water Works Assoc.* 53: 1523-1535, 1963). Poly (trans-1,4 isoprene) or Gutta percha (GP), generally considered to be biologically recalcitrant, has been reported to be degraded by *Nocardi*a strains (S. Warneke et al. *Microbiology* 153: 347-356, 2007). *Nocardia nova* Lib and SE126, and *Nocardia takedensis* WE30 were shown to mineralize 42-54% of the supplied GP to CO₂ after 80 days of incubation.

Mechanism of enzymatic rubber degradation is so far mostly unknown. K. Rose and A. Steinbüchel (Appl. En. Microbiol. 71: 2803-2812, 2005) have proposed hypothetical rubber degradation pathway based on the studies on various microorganisms including *Streptomyces*, *Nocardia* and *Gordonia*. They suggested involvement of enzymes such as latex clearing protein (Icp), oxidoreductase (OxiAB) and rubber oxygenase (RoxA) in rubber degradation and proposed that the first step in this pathway could be an oxidative cleavage of the double bond in poly (cis-1,4 isoprene) backbone.

### 6.2.4 Degradation of Petroleum Hydrocarbons

Petroleum hydrocarbons are fast emerging as leading pollutants of the environment because of the wide scale of production, use, transport and disposal of crude oil and petroleum products at global level. Petroleum hydrocarbons mainly include alkanes, cycloalkanes and polycyclic aromatic hydrocarbons such as hexane, benzene, toluene, xylene, naphthalene and fluorene, pyridine, thiophene etc.

Mycobacterium, Nocardia and Rhodococcus are the predominant genera which are encountered in the soil contaminated with crude oil and are known for their ability to degrade wide range of petroleum hydrocarbons.

Mycobacteria are dominant amongst the microflora in polycyclic aromatic hydrocarbon (PAH) contaminated environment and seem to be specialised in PAH degradation. Different species of mycobacteria such as \( M. \) austroafricanum, \( M. \) frederiksbergens and \( M. \) pyrenivorans have been reported to display high mineralization activity of PAHs like phenanthrene and pyrene (M. Uyttebrock et al. Environ. Microbiol. 8: 836-847, 2000). S.J Kim et al. (Appl. Env. Microbiol. 72: 1045-1054, 2006) described cloning and expression of novel dioxygenase from \( Mycobacterium \) vanbaalenii PYR-1 which was able to metabolize wide variety of high molecular weight PAHs including biphenyls, naphthalene, anthracene, fluoranthene, pyrene,1-nitropyrene, phenanthrene, benzo(a) pyrene, benza(a) anthracene and 7, 12-dimethylbenza(a)anthracene.

Several strains of \( Rhodococcus \) have been shown to degrade and mineralize alkanes from diesel and crude oil (L.G. Whyte et al. Appl. Env. Microbiol. 64: 2578-2584, 1998; S.L. Sharma and A. Pant. Biodegradation 11: 289-294, 2000; D. Takei et al. Biotechnol. Lett. 30: 1447-1452, 2008). \( Rhodococcus \) strains TMP2 and T12 were shown to be capable of degrading highly resistant branched alkanes including 2,6,10,14, tetramethylpentadecane (pristane) (N. Kunihiro et al. J. Biotechnol. 115: 129-136, 2005). Naphthalene degrading \( Rhodococcus \) opacus strain M213 was isolated from fuel contaminated soil from Idaho, USA (I.Uz et al. FEMS Microbiol. Lett. 185: 231-238, 2000). U. Walter el al. (Appl. Microbiol. Biotechnol. 34: 671-676, 1991) reported \( Rhodococcus \) strain UW1 which was able to degrade variety of PAHs such as pyrene, phenanthrene, anthracene, fluoranthene and chrysene.

Hydrocarbon degrading actinomycetes have been isolated from extreme environments indicating their potential of bioremediation under such environments. Different strains of psychrophilic rhodococci which were able to degrade alkanes as well as PAHs at low temperatures have been isolated from extremely cold regions such as Arctic (A.K. Bej et al. Polar Biol. 23: 100-105, 2000), Antarctic (L.G. Whyte et al. FEMS Microbiol. Ecol. 41: 141-150, 2002; A.D. Luz et al. Can. J. Microbiol. 50: 323-333, 2004) and Alpine (R.
Margesin et al. *Appl. Env. Microbiol.* 69: 3085-3092, 2003) soils. Thermophilic strain of *Nocardia otitidiscaviarum* TSH1 which could efficiently degrade phenol, alkanes and some PAHs at 50°C has been suggested to be useful in bioremediation of oil contaminated desert soil (M. Zeinali et al. *J. Basic Microbiol.* 47: 534-539, 2007). R. AL-Mueini et al. (Environmental Chemistry 4: 5-7, 2007) described an extremely halophilic *Actinopolyspora* strain isolated from saline and arid surroundings of an oil field from Oman which was able to efficiently degrade alkanes and fluorene.

To facilitate crude oil or hydrocarbon uptake many crude oil utilizing microorganisms produce cell wall associated or extra-cellular surface active agents or biosurfactants. Several strains of alkane degrading rhodococci have been shown to produce biosurfactants (J. Philp et al. *Appl. Microbiol. Biotechnol.* 59: 318-324, 2002; F. Peng et al. *J. Appl. Microbiol.* 102: 1603-1611, 2007) which are mainly glycolipid in nature. P. Rapp and L.H.E. Gabriel-Jürgens (*Microbiology* 149: 2879-2890, 2003) demonstrated degradation of alkanes as well as highly chlorinated benzenes with simultaneous production of biosurfactant by *Rhodococcus* sp. strain Ms11.

Actinomycetes are active in metabolism of organic sulfur and nitrogen present in fuels such as coal and crude oil. Organic sulfur mainly in the form of dibenzothiophene (DBT) and other thiophene derivatives produces sulfur dioxides upon combustion that can cause acid rains and are considered as one of the worst air pollutants and hence needs to be removed. *Rhodococcus* (J.J. Kilbane II and K. Jackowski. *Biotechnol. Bioeng.* 40: 1107-1114, 1992; B. Yu et al. *Appl. Env. Microbiol.* 72: 54-58, 2006) and *Mycobacterium* (F.L. Li et al. *FEMS Microbiol. Lett.* 223: 301-307, 2003) strains have been shown to selectively remove sulfur from coal, diesel oil and crude oil converting dibenzothiophene to 2-hydroxybiphenyl (2-HBP). The biodesulfurization of gasoline using *Rhodococcus erythropolis* strains, has also been described (B. Yu et al. *FEMS Microbiol. Lett.* 258: 284-289, 2006). This indicates potential of actinomycetes in biorefining of crude oil and other petroleum products.

6.2.5 Degradation of Xenotoxic Compounds

Pesticides, herbicides and other toxic compounds generated through activities of various industries are major source of soil and groundwater contamination. Actinomycetes belonging to the suborder Corynebacterineae such as *Rhodococcus*, *Nocardia* and *Mycobacterium*, have considerable potential for biotransformation of these compounds and can contribute significantly in bioremediation efforts. Most notable genus is *Rhodococcus*, members of which exhibit remarkable metabolic versatility and degrade almost all types of organo-pollutants such as short and long chain hydrocarbons, halogenated hydrocarbons, aromatics, heteroaromatics and polycyclic aromatics (M.J. Larkin et al. *Adv. Appl. Microbiol*. 59: 1-29, 2006).

6.3 Pesticides

Organochlorine pesticides and herbicides are a large class of chlorinated hydrocarbon chemicals which include DDT (1,1,1-trichloro-2,2-bis [4-chlorophenyl]ethane), lindane (g-hexachlorocyclohexane), endosulfan, aldrin, dieldrin and the herbicides dalapon and 2,4,5-T. They are known for their long term persistence and accumulation in fatty tissues of animals. Many of them are considered to be neurotoxic and their use is banned. However, some are still being used, especially in developing countries, mainly for economic reasons. Several actinomycetes namely *Streptomyces*, *Rhodococcus*, *Arthrobacter*, *Nocardioides*, *Micromonospora* and *Brevibacterium* have shown potential to degrade or detoxify organochlorine pesticides.

G-hexachlorocyclohexane (lindane), one of the most commonly used pesticides, is listed as a priority pollutant by US EPA (Environmental Protection Agency) because of its toxicity. M.K. Speedie et al. (*The Actinomycetes* 20: 315-335, 1987) indicated potential use of *Streptomyces* strain PS1/5 to dechlorinate and thereby detoxify it. C.S. Benimeli et al. (*International Biodeterioration and Biodegradation* 61: 233-239, 2008) studied lindane bioremediation capabilities of aquatic *Streptomyces* M-7 by artificially contaminating the soil with various concentrations of lindane. The maize plants seeded in this soil showed increased vigor in presence of *Streptomyces* M-7 (2 g/kg soil) which was capable of removing ~68% lindane. Another heavily used, but severely toxic organochlorine pesticide is
endosulfan. **R. Martens** (*Appl. Env. Microbiol.* 31: 853-858, 1976) isolated several species of *Nocardia*, *Corynebacterium*, *Mycobacterium* and *Streptomyces* that are capable of degrading endosulfan, mainly producing endosulfate. Three different species of *Streptomyces* were shown to metabolize more than 30% of given endosulfan.

Organochlorine compounds are also used as herbicides. Species of *Arthrobacter* has been shown to use mono-, di- and tri-chloro derivatives of phenoxyacetate herbicides (4-chlorophenoxyacetate, 2-methyl-4-chlorophenoxyacetate, 2,4-dichlorophenoxyacetate, etc.) as sole source of carbon and energy (**M.A. Loos et al. J. Agric. Food. Chem.* 15: 858-860, 1967). Mixture of herbicides 2, 4 D and 2,4,5 T makes defoliating agent, Agent Orange, which was extensively used in Vietnam war as a chemical warfare. 2,4 D is easily biodegradable but 2,4,5 T is known to be relatively recalcitrant. *Nocardioides simplex* 3E has shown the capability of completely mineralizing 2,4 D as well as 2,4,5 T (**L.A. Golovleva et al. Biodegradation* 1: 263-271, 1990).

PCP (pentachlorophenol) is another organochlorine herbicide which is also used as a disinfectant for wood treatment and can be released in the environment from wood treating and manufacturing sites. This toxic and probably carcinogenic compound is comparatively less recalcitrant and easily biodegradable. *Mycobacterium chlorophenolicus* PCP-1 is shown to use it as a sole carbon and energy source (**J.H.A. Apajalahti and M.S. Salkinoja-Salonen. Microb. Ecol.* 10: 359-367, 1984). Other actinomycetes which can degrade PCP are *Streptomyces* and *Rhodococcus* (**O. Zaborina et al. J. Env. Sci. Health (B),* 32: 55-70, 1997; **M.M. Häggblov et al. Appl. Env. Microbiol.* 54: 3043-3052, 1988).

S-triazines are heterocyclic nitrogen derivatives and are used as herbicides. Atrazine which belongs to this class is heavily used throughout the world, especially in corn growing areas. A number of *Rhodococcus* strains have been reported to metabolize atrazine and also other triazines such as propazine, simazine, cyanazine (**R. Bekki et al. Appl. Env. Microbiol.* 59: 1955-1959, 1993). **M.C. Giardina et al. (Agric. Biol. Chem.* 46: 1439-1445, 1982) isolated a strain of *Nocardia* that utilizes atrazine as sole carbon and nitrogen source.
Organophosphates and carbamates find wide application as herbicides, insecticides and fungicides in agriculture. Carbamates are also used as a household insecticide at the restaurants, kitchens, home and gardens. Both organophosphate and carbamates are much less persistent in the environment than organochlorines and are, known for their rapid degradability. Also unlike, organochlorines they are not fat soluble and do not accumulate in the food chain. But since they are water soluble, contamination of surface and ground water by these compounds is of concern. Organophosphates are known to cause reproductive toxicity in mammals, if they accumulate beyond the toxic level. *Rhodococcus* and *Arthrobacter* strains have been demonstrated to metabolize Organophosphate and carbamates. J. Nagy et al. (*Hungarian Patent 202058, 1987*) described the use of *Rhodococcus erythropolis* NI86/21 as a protector or biosafener of maize against soil applied thiocarbamates. Glyphosate, the most widely used organophosphate herbicide, is degraded by *Arthrobacter atrocyaneus* (R. Pipke and N. Amrhein. *Appl. Env. Microbiol. 54: 1293-1296, 1988*). D.R. Shelton et al. (*Biodegradation 7: 129-136, 1996*) demonstrated rapid degradation of several other herbicides such as alachlor, linuron, tebuthiuron and bromacil by *Streptomyces* species PS1/5.

Actinomycetes follow variety of metabolic pathways for degradation of pesticides having diverse chemical structures. Information regarding the molecular mechanisms of pesticide degradation is scattered and limited. However, a review by A. De Schrijver and R. De Mot (*Crit. Rev. Microbiol. 25: 85-119, 1999*) which elaborates on the metabolic pathways and the catabolic genes involved can be a good starting point for those who are interested.

### 6.4 Other Xenotoxic Compounds

Actinomycetes have been shown to be involved in degradation or transformation of many other xenotoxic compounds and some examples are given below.

Dibutyltin (DBT) is widely used plastic stabilizer which is considered to be highly neurotoxic and immunotoxic. It has been detected in environment as well as in human tissues. *Streptomyces* strain was isolated which converted 90% DBT into a lesser toxic compound monobutyltin (MBT) within a day (P. Bernat and J. Dlugonski. *J. Hazardous Materials 17: 660-664, 2009*).
RDX (Hexahydro-1,3,5-trinitro-1,3,5-triazine) is one of the most powerful and widely used military explosive which has contaminated the soil and groundwater at army ammunition and other military sites through manufacturing and testing. RDX is toxic compound which affects central nervous system of animals and is potential human carcinogen. *Rhodococcus* strains were shown to aerobically degrade RDX ([N.V. Coleman et al. Soil Biol. Biochem. 30: 1159-1167, 1998; D. Fournier et al. Appl. Env. Microbiol. 68: 166-172, 2002; H.M.B. Seth-Smith et al. Appl. Env. Microbiol. 68: 4764-4771, 2002]) and mineralize ~160-250 mM RDX within 20 hrs. Scientists at US Army Engineer Research and Development Centre at Mississippi isolated two relatively newly described actinomycete genera, namely, *Williamisia* sp. KTR4 and *Gordania* sp. KTR9 capable of using RDX as sole source of carbon and nitrogen ([K.T. Thompson et al. Appl. Env. Microbiol. 71: 8265-8272, 2005]).

The fuel oxygenates methyl tert-butyl ether (MTBE), ethyl tert-butyl ether (ETBE) and tert-amyl methyl ether (TAME) are added to fuel to reduce environmental pollution caused by toxic emissions during burning of the fuel. Although not very toxic, these oxygenates are odorous, recalcitrant due to the presence of ether bond, and are emerging as a major pollutant in ground water supply. *Rhodococcus ruber* IFP 2001 and *Rhodococcus zopfü* 2002 were shown to degrade ETBE ([S. Chauvaux et al. J. Bacteriol. 183: 6551-6557, 2001; A. Francois et al. Appl. Env. Microbiol. 68: 2754-2762, 2002]). ([ ]) demonstrated ability of newly isolated *Mycobacterium austroafricanum* IFP2012 to degrade MTBE and TAME.

2-mercaptobenzothiazole (MBT) is used in rubber industry as a vulcanization accelerator and is known to be toxic, odorous, recalcitrant pollutant. This compound can be biotransformed and partially mineralized by a strain of *Rhodococcus rhodochrous* ([N. Haroune et al. Appl. En. Microbiol. 70: 6315-6319, 2004]).

Actinomycetes also seem to play major role in degradation of some of the not so easily biodegradable plastics. [H. Pranamuda et al. (Appl. En. Microbiol. 63: 1637-1640, 1997)] reported polylactide (PLA) degradation using a strain of *Amycolatopsis* isolated from soil, which was able to reduce 100 mg film of PLA by 60% within 14 days in liquid culture at 30°C. [Y. Tokiwa and I. Tsuchiurashi (European Patent, 1227158 (Al), 2002)] demonstrated the ability
of several different strains of actinomycetes (Saccharothrix, Actinopolyspora, Saccahromonospora, and Actinokineospora, Kibdelosporangium) to degrade polylactide resin at the rate ranging from 10-50% at 30°C in 4 weeks. Two strains of Thermomonospora fusca K7a-3 and K13g isolated from compost exhibited degradation of co-polysterers of 1,4 butanediol, adipic acid and terephthalic acid (BTA) at high rates (I. Kleeberg et al. Appl. Env. Microbiol. 64: 1731-1735, 1998). Another group of German scientists showed 99.9% degradation of the commercially available co-polyester Ecoflex® by a strain of Thermomonospora fusca within 22 days of incubation at 55°C (U. Witt et al. Chemosphere 44: 289-299, 2001).

6.5 Biosorption of Heavy Metals

Discharge of heavy metals from heavy metal processing industries causes adverse effects on the environment. Radioactive waste stored underground also is under constant threat of accidental escape and migration in sediments and ground water. Biosorption of heavy metals and radioelements using metabolically active microbial biomass is an environmentally friendly technology option to reduce the pollution in the aquatic bodies. Selective biosorption could also be helpful in recovery of metal ions or separation of the metal ions with similar chemical properties.

Actinomycetes show considerable heavy metal resistance and could be potential candidates for biosorption of heavy metals as well as radioactive elements. Y. Andres et al. (Appl. Microbiol. Biotechnol. 39: 413-417, 1993) reported adsorption of actinide (Th,U) and lanthanide (La, En, Yb) ions by Mycobacterium smegmatis. The strain showed selective uptake of thorium and was claimed to be useful for separation and concentration of natural radio elements present in high dilutions (Y. Andres et al. Appl. Microbiol. Biotechnol. 44: 271-276, 1995). Later on T. Tsuruta (J. Nucl. Radiochem. Sci. 6: 81-84, 2005) reported use of Streptomyces albus and Arthrobacter nicotianae, along with other bacteria, in separation of rare earth metals. Streptomyces albus selectively accumulated Lu, whereas Arthrobacter nicotiana accumulated Sm and Lu when screened for selectivity using mixture of 5 rare earth elements (Y, La, Sm, Er, Lu). Cadmium biosorption has been demonstrated by Streptomyces sp. F4 which was shown to accumulate cadmium at the level of 41.7 mg/g
Several actinomycete strains have been studied for their ability to accumulate uranium. Biosorption of lead and uranium by lyophilized cells of *Streptomyces longwoodensis* was studied and the cells were shown to have exceptionally high capacity for uranium (440mg/g dry wt) at pH 5.0 (N. Friis and P. Myers-Keith. *Biotechnol. Bioeng.* 28: 21-28, 1986). Z. Golab et al. (*Water Air Soil Pollut.* 60: 99-106, 1991) demonstrated the ability of both living and dead biomass of *Streptomyces* species to adsorb both lead and uranium at pH 5-6. M. Nedelkova et al. (*FEMS Microbiol. Ecol.* 59: 694-705, 2007) reported three *Microbacterium* strains isolated from the vicinity of the radioactive waste depository in Siberia, one of which could accumulate high amounts of uranium (240 mg/g dry biomass). A. Nakajima and T. Tsuruta (*I Nucl. Sci. Technol.* 3: 528-531, 2002) reported a strain of *Streptomyces levoris* capable of biosorption of uranium and thorium from aqueous systems. A strain of *Arthrobacter nicotianae* was reported to accumulate ions of uranium, thorium (T. Tsuruta. *J. Biosci. Bioeng.* 94: 23-28, 2002) and lithium (T. Tsuruta. *J. Biosci. Bioeng.* 100: 562-566, 2005) with high efficiency.

### 6.6 Biotransformation and Biocatalysis

Apart from bioremediation and biosorption, the incredible versatility of actinomycetes in transforming variety of substrates and compounds has been proven to be useful in the preparation of fine and commodity chemicals. Indeed, some of the actinomycete enzymes have shown enough potential to be used as industrial biocatalysts. The first successful manufacture of a commodity chemical involving biotransformation relates to the use of enzyme nitrile hydratase (NHase) from *Rhodococcus rhodochrous* J1 (H. Yamada and M. Kobayashi. *Biosci. Biotech. Biochem.* 60: 1391-1400, 1996). This enzyme converts acrylonitrile to acrylamide, an important commodity chemical used in coagulators, soil conditioners and stock additives for paper treatment and paper sizing, and for adhesives, paints and petroleum recovering agents. The process is developed on an industrial scale of several
thousand tons per annum by Mitsubishi Rayon (formerly Nitto Chemical Co. Ltd), Japan. Acrylamide is produced continuously in a series of fixed bed reactors using Rhodococcus cells immobilized in polyacrylamide gel. The process offers advantages over the conventional chemical process such as lacking requirement of heat or high pressure, elimination of use of heavy metals and easy recovery of the product. NHase from Rhodococcus rhodochrous J1 is termed as generation biocatalyst, the third as it is said to be better compared to the similar enzymes from other microbial systems in terms of heat stability and resistance to high concentrations of acrylonitrile and acrylamide (T. Nagasawa et al. Appl. Microbiol. Biotechnol. 40:189-195, 1993).

Another example of large scale industrial manufacture using nitrile hydratase from Rhodococcus is the conversion of nicotinonitrile to nicotinamide, an essential vitamin in animal and human nutrition. This process developed by Lonza utilizes cells of Rhodococcus rhodochrous J1 immobilized in polyacrylamide in a three step continuous biocatalytic reaction to produce several thousand tons of nicotinamide per year (N.M. Shaw et al. Adv. Synth. Catal. 345: 425-435, 2003). Nitrile hydratases and nitrilases from Rhodococcus and other actinomycetes have been shown to be useful in several other biocatalytic processes intermediates and for the production of many useful fine chemicals and drug have been reviewed by G. DeSantis and R. DeCosino. Biocatalysis for the Pharmaceutical Industry: Discovery, Development, and Manufacturing (Eds. J. Tao, G. Lin and A. Liese). John Wiley and Sons Asia (Pte) Ltd., 153-178, 2009.

Vanillin, which is used as a flavouring agent industry and as a precursor in food industry, as an aroma in cosmetic for the synthetic drugs in pharmaceutical industry, is produced using a biotransformation process involving Streptomyces setonii. A large scale process based on fed batch fermentation which converts ferulic acid into vanillin at the high titre of >15g/l (A. Muheim et al. US Patent 6: 235,507 BI, 2001) has been developed and commercialized by a Swiss Company, Givaudan Roure.

Actinomycete enzymes also have been useful in bio-transformations for the production of pharmaceuticals and their intermediates. A well known example is that of Pravastatin. Statins selectively inhibit hydroxymethyl
glutaryl-coenzyme A (HMG-CoA) reductase, the first enzyme in cholesterol biosynthesis, and are widely used to treat hypercholesterolemia. Pravastatin, a potent and tissue specific beta hydroxylated of mevastatin/compactin derivative can be produced by two step fermentation process, the first step being production of mevastatin followed by its biotransformation. *Streptomyces carpophilus* SANK 62585 was found to carry out this biotransformation efficiently. It could hydroxylate mevastatin in the C-6 position by cytochrome P-450 containing enzyme system with only small amount of byproducts. This led to successful development of pravastain production at industrial scale by Sankyo (M. Manzoni and M. Rollini. *Appl. Microbiol. Biotechnol.* 58: 555-564, 2002).

Specific acylases/deacetylases from actinomycetes have been shown to be useful in the synthesis of semi-synthetic derivatives of antifungal agents of Échinocandin class. Echinocandins are lipopeptides that possess cyclic peptide nucleus with fatty acid acyl side chain at the N-terminus. Micafungin or FK463 is a semi-synthetic derivative of naturally occurring sulfonated cyclic hexapeptide, an echinocandin-like antibiotic FR901379. It is developed by Fujisawa Pharmaceutical Co. Ltd., Japan and is marketed in Japan and United States as candin-class parenteral antifungal agent for life threatening mycoses (A. Fujie. *Pure Appl. Chem.*79: 603-614, 2007). Micafungin is prepared by enzymatic deacetylation of the hexapeptide nucleus to remove the fatty acid acyl side chain, followed by reacetylation with isoxazole containing benzoyl-like side chain. A specific cyclic lipopeptide acylase from 3 different strains of *Streptomyces amulatus* have been shown to be useful for the deacetylation of FR901379 (S. Ueda et al. *US Patent* 6537789, 2003). The acylase enzyme from *Actinoplanes utahensis* has broad substrate specificity and can deacylate echinocandins (L.-D. Boeck et al. *J. Antibiott.* 42: 382-388, 1989) as well as beta lactams such as penicillin (J. Torres-Bacete et al. *Appl. Env. Microbiol.* 73: 5378-5381, 2007) and later developed as a robust immobilized biocatalyst for their production (D. Hormigo et al. *Bioresource Technology* 101: 4261-4268, 2010).

Paclitaxel, an antitumor agent originally isolated from the bark of Yew (*Taxus brevifolia*) tree was developed commercially by Bristol-Meyers-Squibb (BMS) and is used to treat ovarian and metastatic breast cancer. The
hydrolytic enzymes from *Nocardioides* species has been shown to be useful in the semi-synthesis of paclitaxel. C-13 taxolase, from *Nocardia albus* SC 13911 was reported to remove C-13 side chain of the paclitaxel molecule resulting in formation of baccatin-III (R.L. Hanson et al. J. Biol. Chem. 269: 22145-22149, 1994). The deacetylace from *Nocardioides luteus* SC 13912 catalyzed the deacetylation of C-10 acetyl group from baccatin to produce 10-deacetyl baccatin (10-DAB), a key precursor in the semi-synthesis of paclitaxel (V.B. Nanduri et al. Biotechnol. Bioeng. 48: 547-550, 1995).

Amidase from *Mycobacterium aureum* ATCC 25795 has been reported to catalyze an enantioselective hydrolysis of racemic a-methyl phenylalanine amide and a-methyl-4-hydroxyl-phenylalanine amide to produce corresponding S-amino acids (R.N. Patel et al. J. Am. Oil Chem. Soc. 75: 1473-1482, 1998). These are intermediates for the synthesis of B-3 receptor agonists which are being developed for the treatment of gastrointestinal disorders, type II diabetes and obesity.

Actinomycetes have been reported to be involved in the biocatalytic formation of several other chiral pharmaceutical intermediates and the readers may refer to an excellent review by R.N. Patel (Food Technol. Biotechnol. 42: 305-325, 2004) for further information and examples.

### 6.7 Conclusions

Incredible potential of actinomycetes to metabolize such extensive range of materials which helps them to survive and propagate in diverse and many times low nutrient habitats could be the basis of their ubiquitous existence in the biosphere and their ecological significance. This potential of actinomycetes has not been extensively exploited for bioremediation as well as industrial biotransformation technologies. With few exceptions, knowledge on molecular mechanisms of degradation of many compounds is also limited. The extent of metabolic diversity of actinomycetes from several unusual environments such as marine ecosystems is yet to be fully explored.

Aggressive approaches to explore and exploit microbial biodiversity together with advances in analytical and molecular techniques can lead to rapid and in-depth research on these aspects, which can be the basis of new and/or better bioremediation and biotransformation technologies of the future.
7.1 Introduction

The value of microorganisms in the production of enzymes is enhanced by their relatively high yields, cost effectiveness and amenability to genetic manipulation. Enzymes of microbial origin are widely used in food processing, detergent, textile, pharma industry and medical therapy. Enzymes of different microbial origin vary in their substrate specificity and optimal conditions for their bio-catalysis. Based on specific industrial requirements enzymes meeting the demands for substrate specificity, temperature and manufacturing processes are developed for large scale applications.

Actinomycetes, best known as the source of diverse secondary metabolites like the antibiotics have in recent years been the focus of attention for their potential as producers of industrial enzymes with novel properties. One of the enzymes with large industrial potential from the actinomycetes has been glucose (xylose) isomerase which has ability to convert D-glucose to D-fructose having greater sweetening power.

Exploitation of this enzyme in the food industry for the manufacturer of High Fructose Corn Syrup (HFCS) has been very successful in the western world to provide an effective alternative to beet or cane sugar for various food industry applications. This chapter has attempted to bring together information on several enzymes of actinomycete origin which are significant due to their established or potential industrial usefulness. Some information already discussed in Chapter VI under "Biodegradation" would be of direct relevance to the subject matter discussed in this chapter and it will be beneficial for the readers to study them in tandem.
7.2 Glucose (Xylose) Isomerase

The enzyme converting D-glucose to D-fructose is technically D-xylose ketoisomerase which is widely studied from bacteria and actinomycetes. In the majority of cases, the enzyme is cell bound and intracellular. A commercial process based on *Bacillus coagulans* by Novo Industries for HFCS manufacture was among the earliest technologies developed. Several species of *Streptomyces* as well as strains of *Actinoplanes, Microbispora, Micromonospora, Nocardia* and *Nocardiopsis* have been shown to be good producers of the enzyme. Commercial manufacturing processes have centred around *Streptomyces* species such as *S. albus, S. phaeochromogenes, S. olivochromogenes* as well as *Actinoplanes missouriensis*.


In most strains, glucose isomerase is produced intracellularly when cultivated on organic media containing protein hydrolysates, yeast extract and magnesium salts. An inducer such as xylose or xylan hydrolysates derived from xylan-rich natural substrates like corn cobs, straw or cereal brans stimulated enzyme production. Most glucose isomerases require a divalent cation such as Co++, Mg++ or Mn++ for catalytic reaction. The search and selection of strains/mutants producing glucose isomerase without obligate requirement of Co' ions has been carried out due to non-acceptability of toxic cobalt ions in food industry applications of the isomerase enzyme.

Another development significant from the industrial viewpoint has been the removal of the D-xylose inducer for glucose isomerase synthesis in *Streptomyces phaeochromogenes*. L-Lyxose, an anamorph of D-xylose does not support spore germination in wild strain, but growing mycelia on xylose
media can use L-Lyxose for growth. Selection for mutants which will germinate directly on lyxose will yield strains lacking the xylose inducer requirement for lyxose and concomitantly that for glucose isomerase. Constitutive mutants that are catabolic repression resistant may be screened for by the use of antimetabolites. Non-inducer requiring strains of Streplomyces phaeochromogenes are inhibited by 3-0-methyl glucose, a glucose analogue. If colonies are screened on Lyxose-3-0-methyl glucose plates, mutants showing good growth utilize the Lyxose and are catabolite repression -resistant for glucose (S.Sanchez and C.M. Quinto. *Appl. Microbiol.* 30: 750-754, 1975).

In the commercial process for the manufacture of HFCS, corn syrup is first produced by hydrolyzing gelatinized corn starch with α-amylase and amyloglucosidase enzymes (commonly from *Bacillus* and *Aspergillus* species, respectively). The glucose syrup is passed through a column loaded with the isomerase resulting in a syrup containing approximately 42% fructose. It is further purified to get 90% fructose required for special industrial applications. For more extensive details and information on the molecular and industrial aspects of glucose isomerase the review by S.H. Bhosale et al. (*Microbiol. Review* 60: 280-300, 1996) is recommended.

### 7.3 Amylases

Amylase production in actinomycetes is less investigated and applied to industry as compared to bacteria (e.g. *Bacillus*) and fungi (e.g. *Aspergillus*). Essentially the amylase from actinomycetes have been investigated for the production of maltose syrups or for the enzymatic preparation of trisaccharides like panose and isopanose from polysaccharides such as pullulan. A purified α-amylase preparation from *Thermoactinomyces vulgaris* (presently classified as a bacterium under the Bacillaceae) was used to hydrolyse pullulan and obtain panose at 70% yield (Y. Sakano et al. *Carbohydrate Research* 61: 175-179, 1978).

*Thermomonospora fusca* (presently re-designated as *Thermobifida fusca*) produced an extracellular amylase which generated maltose at 61% of the identified hydrolysis products of starch. Amylases which produce maltotriose as the end product of starch hydrolysis is characteristic of

α-amylases capable of catalyzing the production of high yields of maltose or specific malto-oligosaccharides on degradation of starch are of considerable commercial interest particularly in the food, pharmaceutical, biomedical and fine chemical industries. Y.B. Ammar et al. (*J. Biochem. Mol. Biol.* 35: 568-575, 2002) studied an α-amylase from a thermophilic *Streptomyces* isolated from Thailand, which produced maltose at 90% conversion levels from soluble starch. Its application in the baking industry has been described as facilitating increased bread volume together with longer maintenance of softness of the bread loaf.

Amylases active at high alkaline pH have been reported from alkalotolerant actinomycetes. A *Nocardiopsis* strain capable of growth between pH 5 and 12 produced alkaline amylase only at pH 10.5. Best yields were obtained in a 3% starch-yeast extract medium (M.A. El Maleigy et al. *Egyptian Journal of Microbiology* 31: 361-371, 1998). An isolate of *Nocardiopsis* originating from Antarctica soil was reported capable of producing: "cold adapted" amylase active at 35°C and at pH 8.0 (J.N. Zhang and R.Y. Zeng. *Marine Biotechnology* 10: 75-82, 2008).

### 7.4 Lignocellulose Degrading Enzymes

Actinomycetes are extensively isolated from natural habitats in which active decomposition of lignocellulose is taking place. They play an active role in the recycling of organic matter of plant origin (Chapter 6). Mesophilic as well as thermophilic forms isolated from habitats like mushroom compost have
been the subject of intensive study to evaluate their potential for the production of cellulolytic, xylanolytic and lignin-degrading enzymes.

7.5 Cellulases


7.6 Xylanase

Xylanases (hemicellulases) from actinomycetes have also received considerable attention, especially in recent years following the recognition of the potential of xylanase enzymes for bio-bleaching and bio-pulping operations in the paper and pulp industry as well as rayon grade pulp manufacture. Use of xylanase enzymes facilitates significant reduction in the levels of chlorine compounds used for bleaching. Particular attention has been given to xylanases free from associated cellulase activity and active at high alkaline pH. Several reports of extracellular xylanase production by mesophilic Streptomyces and thermophilic Thermomonospora strains are published in the literature. A.J. McCarthy et al. (Appl. Microbiol. Biotechnol. 21: 238-244, 1985) in their studies on extracellular xylanase activity of thermophilic actinomycetes reported that the enzyme from Saccharomonospora viridis Thermomonospora sp. was optimally active in the temperature range of 60-75°C and between pH 8.0-8.6. Xylanase production was induced by growth on xylan or xylan-rich natural materials such as cereal brans, and in all cases, xylanases are induced enzymes. Non metabolizable inducers like methyl-p-xyloside have also been reported to induce xylanase production (M. Marui et al. Agr. Biol. Chem. 49: 3399-3407, 1985).


An alkalophilic *Streptomyces* VP-5 secreting cellulase free xylanase active at pH 10.0 while growing optimally at high alkaline pH was reported by P. Vyas et al. (*Biotechnol. Lett.* 12: 225-228, 1990). From a thermotolerant *Streptomyces* T-7 isolated from vermicompost, a cellulase-free xylanase was characterized by S.S. Keskar et al. (*Biochem. Journal* 261: 49-55, 1989).

The impact of xylanase enzymes for the paper and pulp industry as well as for high quality dissolving pulp manufacture (for rayons) has been widely noticeable worldwide during the past few years. Intensive research efforts are on to identify more potent thermotolerant, cellulase-free and alkali stable xylanase enzymes to develop technologies which are commercially viable.

### 7.7 Lignin Degrading Enzymes

Several actinomycetes are believed to have the potential to act on lignin and the topic of lignin degradation by this group has been extensively investigated by D.L. Crawford and coworkers. *Streptomyces* strains decomposed grass lignocellulose more efficiently than hard and soft wood lignocellulose (S.P. Antai and D.L. Crawford. *Appl. Env. Microbiol.* 42: 378-380, 1981). Production of extracellular oxidative enzymes catalyzing specific oxidations lead to solubilization of water soluble polyphenolic polymeric lignin fragments. An enzyme system comparable or equivalent to the ligninase from the white rot fungi has so far not been established from actinomycetes. It is perhaps relevant to assume that during the process of recycling of plant residues, the lignin degrading ability of actinomycetes may have an added significance to overall process of hemicellulose and cellulose degradation capability which is widespread among its members.

### 7.8 Pectinase

Relatively few reports on the pectinolytic activity of actinomycetes exist in comparison with the vast literature available on the bacterial and fungal
pectinases. An inducible thermoalkalophilic polygalactouranase from *Thermomonospora fusca* has been reported ([F.J. Stutzenberger. *J. Bacteriol.* 169: 2774-2780, 1987](#)). [A. LadJama et al. (FEMS Microbiol. Lett. 79: 279-283, 1991)](#) have characterized the pectinolytic enzymes of a *Streptomyces* strain. It appears reasonable to assume that more extensive investigations are warranted on the pectinolytic enzyme systems from the actinomycetes.

### 7.9 Chitinase


### 7.10 Protease

Actinomycetes and in particular *Streptomyces* species have been the sources of a variety of novel proteases. While production of proteolytic enzymes for bulk industrial applications such as detergent or leather processing are mostly derived from species of *Bacillus* or *Aspergillus*, it is a matter of interest that unique proteases of actinomycete origin have also merited considerable attention for useful applications.

Pronase is a complex mixture of proteases and peptidases secreted by *Streptomyces griseus* and it is established that its components are essentially serine and metalloproteases ([W.M. Awad Jr. et al. *J. Biol. Chem.* 247: 4144-4154, 1972; Y. Narahashi. *Methods in Enzymology* 19: 651-664, 1970](#)). Pronase acts on a variety of proteins causing hydrolysis through the coordinated action of its different components and finds widespread application in analytical protein chemistry. Other proteases of actinomycete origin include


### 7.11 Lipase

Actinomycetes as sources of interesting lipolytic activity has been relatively less investigated. Reports of enzymes such as cholesterol esterase and acetyl hydrolase are available in the literature. A thermostable extracellular lipase from *Amycolatopsis mediterranei* has been investigated by D.S. Dheeman et al. (*J. Industrial Microbiol. Biotechnol.* 17: 1-17, 2010; *Bioresource Technol.* 102: 3373-3379, 2011). Enzyme production was studied in a linseed oil-fructose medium supplemented with phytone peptone and yeast extract. The enzyme was stable to pH 3-9 and up to 60°C. The lipase showed remarkable stability in the presence of a wide range of organic solvents at 25% (w/v) concentrations for 24 hours. The enzyme was reported to have potential in flavour ester synthesis especially flavour ester isoamyl acetate purified in flavour ester synthesis especially.

Lipase from *Streptomyces* displayed better activity than commercial lipase from *Candida rugosa* in the resolution of chiral secondary alcohols. Lipase from *Streptomyces halstedii* also showed very good activity in the synthesis of carbamates (E. Cardenas et al. *Biocatalysis and Biotransformation* 19: 325-329, 2001).
7.12 Clinical and Diagnostic Enzymes

Several enzymes of actinomycetes origin have found application in clinical chemistry as well as medical therapy. Their stability, activity at high temperature and unusual substrate specificity are attributes which make them more favoured in preference to similar enzymes from other sources.

Cholesterol oxidase from *Nocardia*, *Streptomyces* and *Rhodococcus* have been used both for estimation of cholesterol in blood serum and also to lower blood cholesterol in mammals. Cholesterol esterase has been studied from *Streptomyces lavendulae* and some other species. Phylogenetic analysis as well as database search have led to the conclusion that actinomycete cholesterol esterase are unique and constitute a novel family of these enzymes, distinct from the enzymes, originating from mammals, yeasts and bacteria (*Hongyu Klang et al. Biochem. Biophys. Acta 1774: 112-120, 2007*).

Choline oxidase from *Streptomyces nigrifaciens* has been employed in diagnostic tests to determine the levels of phospholipids in blood serum of patients with liver complaints such as obstructive jaundice.

Urate oxidase from *Streptomyces cyanogenus* has been used to treat patients suffering from hyperuremia and primary gout. Levels of uric acid and L-glutamate in blood serum can be determined using uric acid and L-glutamate oxidase, respectively. An extracellular L-amino acid oxidase isolated from *Streptomyces violaceus* oxidized L-glutamate, L-glutamine and L-histidine (*T. Kamei et al. Chem Pharm. Bull. 31: 1307-1314, 1983*). *Streptomyces X-1196* was reported to produce an extracellular enzyme highly specific to L-glutamate when cultivated in solid state fermentation on moist wheat bran (*M. Kusakabe et al. Agr. Biol. Chem. 47: 1323-1328, 1983*).

A hyaluronidase specifically degrading hyaluronate as an eliminase from *Streptomyces hyaluronicus* has been reported (*E. Shimada and G. Matsumura. J. Biochem. 88: 1015-1023, 1980*). Several additional examples can be given to provide evidence of the wide diversity of enzymes with application potential in diagnosis as well as therapy. For more details the following review on Actinomycete enzymes would be worth studying: *W. Puczynska* and *M. Mordarski* in "Actinomycetes in Biotechnology" (*Ed. M. Goodfellow et al. Academic Press, 219-283, 1988*). Inhibitors of Angiotensin Converting Enzyme
(ACE) are a group of drugs used primarily for treatment of hypertension and congestive heart failure. They inhibit the ACE which is a component of the blood pressure regulating renin-giotensin system. Several metabolites from actinomycetes have been identified with ACE inhibitor activity. These include Foroxymithine from *Streptomyces nitrosporeus* (H. Umezawa et al. *J. Antibiotics* 38: 1813-1815, 1985), Ancovenin (Y. Kido et al. *J. Antibiotics* 35: 1295-1299, 1983) and K-26, a water-soluble acidic peptide (M. Yamato et al. *J. Antibiotics* 39: 44-52, 1986) as a few representative examples. Other useful inhibitors from the actinomycetes include glycosidase inhibitors for weight loss, diabetes control (e.g. Acarbose discussed in Chapter 5), prolyl-4-hydroxylase inhibitors for treatment of fibrosis and phospholipase inhibitors for treatment of acute pancreatitis. A novel fibrinolytic enzyme, Actinokinase from a strain of *Streptomyces megasporous* was patented by R.R. Chitte and S. Dey from MACS-Agharkar Research Institute, Pune (Indian Patent No. 193435; US 6638503).

### 7.13 Conclusion

The versatility of actinomycetes as sources for novel enzymes would be evident from the examples cited. It appears reasonable to conclude that this group deserves even greater attention and the prospects of finding useful enzymes and related therapeutic molecules seem indeed very bright. So far, the research and development efforts on actinomycetes have mostly centred around antibiotic and serious efforts to explore them for enzymes of importance and commercial value are certainly warranted.
“If one had to answer the question, which group of microorganisms has been the most helpful to medicine and agriculture, the loud and clear answer would be the actinomycetes”. This statement was made by the renowned biotechnologist, Prof. A.L. Demain in his presentation "Actinomycetes: what have you done for us lately" at the "Biology of Actinomycetes" symposium in Japan in 1988. This evaluation of the potential of acitnomycetes is valid even today and over the last three decades increased global attention has significantly contributed to our knowledge and a better understanding of this group of microbes. The scientific and technological advances made can be broadly classified under the following three heads:

1. Understanding the biodiversity through improved selective techniques for isolation from diverse natural habitats and conserving them "in vitro" for technology exploration

2. Taxonomy and conceptual changes in classification primarily based on the recent advances in molecular techniques

3. Progress in the search for bioactive metabolites other than antimicrobial compounds as well as industrial enzymes.

The subject matter covered in Chapters 1-7 has dwelt upon in some detail on these aspects and in this 'perspectives' chapter we will aim at summing up the salient features and project some thoughts on the future trends in actinomycete research and development in the Indian context.

Biodiversity exploration through imaginative choice of natural habitats and substrates coupled with application of innovative selective isolation techniques have scripted several success stories in the identification of novel taxa. Recent years have witnessed greater application of molecular
techniques, in particular 16srRNA sequences, for recognition of biodiversity- 
a shift from the earlier practice of differentiation based essentially on 
morphological features. One significant development has been the focus on 
marine situations to study indigenous populations of novel marine 
actinomycetes. The successful results obtained have set at rest the long 
prevailing notion that actinomycete populations recovered from marine 
habitats were merely washed-in representatives originating from the terrestrial 
ecosystem. The discovery of *Salinispora* as an obligate marine actinomycete 
producing unique anticancer drug Salinosporamide A provides ample 
justification for intensifying the efforts further to look for novel forms and their 
unique metabolites. Fresh water habitats have also yielded zoosporic 
actinomycetes which were first recognized by the reputed mycologist Prof. 
**John N. Couch** when he designated the genus *Actinoplanes* in 1950. Several 
additional actinomycetes with motile propagules have been isolated from 
aquatic habitats, soil and plant litter, including specialized keratinophilic 
forms like *Pilimelia*. Members of the Actinoplanaceae have proved valuable as 
sources of novel antimicrobial compounds and industrial enzymes like 
glucose isomerase. Extremophilic actinomycetes studied include alkalophilic 
and halophilic forms. More widely studied have been the thermophilic forms, 
especially rich in self-heating composts, So far no hyperthermophilic 
actinomycetes have been identified and it is difficult to conclude whether such 
forms do not exist or they have escaped detection so far.

Existence of endophytic actinomycetes has attracted attention with 
presumed role in the plant’s life by way of synthesizing beneficial metabolites 
or possible antagonism to pathogens. Serious efforts are warranted to explore 
the endophytic actinomycetes of medicinal plants and use them to develop 
fermentation processes for the plant based drug molecules.

Revolutionary changes in the taxonomic concepts have taken place in the 
way we understand the origin and molecular phylogeny of actinomycetes. 
16srRNA sequences have provided actinomycetologists with a phylogenetic 
tree that permits us to study their evolutionary trends while providing a basis 
for classification. On the basis of-RNA sequence divergence calibration, it is 
accepted that the actinomycetes probably arose one billion years ago. Several 
phylogenetically homogenous but phenotypically heterogenous clades 
appear to have diverged within this group over a shorter evolutionary period
The phenotypic heterogeneity is correlated with the wide morphological diversity observed and it is remarkable that the actinomycetes are the members of the prokaryotes which exhibit the most profound morphological differentiation. The classification of Actinobacteria as a group distinct from Eubacteria proposed by E. Stackebrandt has gained widespread acceptance among taxonomists. The term Actinomycetes continues to be in use only out of greater familiarity and convenience to microbiologists, while fully appreciating that they have no phylogenetic relatedness to fungi. Morphological and chemotaxonomic features have their role in helping to differentiate the various forms from one another. The extent to which they should be given importance and validity for taxonomic classification has been a point of debate and serious discussion and there has been no unanimous opinion among the actinomycete taxonomists. From a practical point of view to explore and culture as many diverse forms as possible, morphology, chemotaxonomy and physiological traits will be of tremendous value. From this viewpoint, search for novel forms could essentially be based on these criteria and subsequent data based on molecular studies could be used to interpret the phylogenetic relationships of these novel isolates. It is necessary to realize that facilities as well as expertise for routine application of molecular techniques like 16s rRNA sequencing is not available in average microbiological laboratories, particularly in countries of the developing world. However, efforts focused on selective isolation of novel forms based on morphological and physiological characteristics can be more readily initiated in these laboratories and this can lead to the building up of a vast collection of valuable germplasm for future biotechnological exploration.

In the words of T. Cross and G. Alderson (Biology of Actinomycetes' 88: 216-220) "Future classification may be proposed largely on the basis of numerical and chemotaxonomic data with subsequent determination being undertaken with the aid of rapid methods and reference to data banks of properties, profiles and sequences. Nevertheless, we urge that the morphology of the products of these classifications, the genera and the species must continue to be described in detail."

The first thought which crosses the mind when actinomycetes are mentioned is the large number and structurally diverse drug molecules useful to combat
various infections, which are collectively termed as antibiotics. *Streptomyces* has been the largest antibiotic producing genus accounting for almost 70% of all the antibiotics known. Discovery of new antibiotics peaked in the 1970's, but during the subsequent decades there has been a steady decline in the intensity of screening and repeated re-isolation of previously known molecules. In an interesting mathematical modelling and evaluation study on the number of antibiotics produced by *Streptomyces*, it has been concluded that the total number of antimicrobial compounds that the genus is capable of producing is around 100,000 of which only a tiny fraction has been unearthed so far. Reduced intensity or lack of screening efforts rather than exhaustion of variety is responsible for the decline in the number of new antibiotics discovered (M.G. Watve et al. *Arch. Microbiol.* 176: 386-390, 2001). In a perspective evaluation, F. Palaez (*Biochem. Pharmacol.* 7: 981-990, 2006) has stated that lack of interest of industry in the field and strong competition from synthetic compound collections have led to an alarming scarcity" of new antibiotic classes. New approaches to improve efficiency of the process of new antibiotics discovery encompassing microbial diversity and cultural conditions to produce secondary metabolites are very critical and challenging factors.

Large scale use of antibiotics as pharmaceuticals over the last few decades has led to selection of several antibiotic resistant pathogenic bacteria. New antibiotics as therapeutic agents are needed to combat infections by these antibiotic resistant pathogens. M.A. Fischbach and C.T. Walsh (*Science* 325: 1089-1093, 2009) point out that most antibiotics have come from a small set of molecular scaffolding whose ‘functional lifetimes have been extended by generation of synthetic tailoring’. There is an urgent need to discover new scaffolding and the approaches for this would include mining unexplored niches for natural products, designing screens that avoid rediscovering old scaffolds and re-purposing libraries of synthetic molecules for use as antibiotics.

Advances in molecular techniques applied to bacterial systems have been applied also to actinomycetes. Gene cloning and heterogeneous gene expression have been widely studied. Species of *Streptomyces* such as *Streptomyces lividans* have been identified as ideal hosts for heterologous gene
expression. High levels of protein secretion and low proteolytic activity which characterize these strains make them well suited for expression of heterologous proteins. A review by R.H. Baltz (J. Ind. Microbiol. Biotechnol. 37: 759-772, 2010) has mentioned that advances made in whole genome sequencing of actinomycetes indicate that their large genomes encode multiple secondary metabolic pathways, most of which remain unexpressed ("cryptic"). Search for expression of possible cryptic pathways for novel biosynthetic pathways elucidated through bioinformatics, could be an approach to discovery of novel bioactive molecules.

Molecular genetics has spawned dramatic expansion of the biotechnology industry in the direction of products of single genes. However, when applied to antibiotics which are formed as a result of the concerted action of several genes, the technology is more complex and beset with many problems (K.F. Chater. BioTechnology 8:115-121, 1990). In attempts made to isolate the multiples of genes involved in the various enzymatic steps leading to the antibiotic synthesis and cloning them, success has been limited and achieved only in obtaining clones carrying either individual genes or incomplete gene sets and not the full gene complement.

Various techniques including molecular cloning involving transfer of genes between unrelated species have been studied for getting expression for novel molecules. Sequence based analysis of secondary metabolite biosynthesis has been a recent development with reference to non-ribosomal peptide synthetases as well as polyketide synthetases which are the two major lines of synthesis applied to most antibiotics (E.A. Gontong et al. Appl. Env. Microbiol. 76: 2487-2499, 2010).

It is beyond the scope of this write up to go into a detailed discussion of all the newer approaches related to discovery of novel biomolecules from actinomycetes. Suffice it to point out by the examples given, of the intense research activity that is in progress to harness the full potential of the actinomycetes for biotechnology.

It is evident that with their importance and usefulness for biotechnological research and development firmly established, actinomycetes have triggered widespread global attention and studies on their biodiversity, morphology,
physiology and molecular biology have come into sharp focus. Search for novel taxa from diverse natural ecosystems have intensified in China, Japan and Thailand. What is the present status of actinomycete research and development in India? It is a matter of concern that sufficient effort and focus are not laid in our microbiology and biotechnology training programs on the actinomycetes and their relevance to biotechnology. We believe that it would be in our long term interest to effect this "course correction" and build up strong schools of research in actinomycete biology and biotechnology, both in academic institutions and industrial research laboratories. We need to aim at building a strong work force of microbiologists at the grass root level who would be well versed with the actinomycetes and would have the confidence to undertake and execute projects involving actinomycetes.

As stated in the Preface, information on actinomycetes is widely scattered in different scientific journals. There has been no comprehensive publication from which information on the biology and technological aspects of actinomycetes could be readily retrieved by interested researchers from academia as well as industry. Our attempt to compile such information may at best be considered as an appetizer for undertaking scientific studies on this group of microbes. We are certainly aware of our own limitations while presenting information from such a vast topic and hopefully the text could be improved further in later editions based on the feedback received from the community of microbiologists.

We dedicate this book to the ardent population of young microbiologists and biotechnologists and sincerely hope that the scientific information given in the chapters would stimulate their intellectual and innovative thinking to investigate the multifaceted dimensions of this fascinating group of actinomycetes. Our discussion can be concluded by quoting Prof. Takao Okazaki (Actinomycetologica 20: 15-22, 2006) as follows:

“As long as we continue search to find potentially versatile actinomycetes through new ideas and test treatments, I believe that the expectations of researchers will be successfully attained and even surpassed, throughout the future course of actinomycete research”.
Recommendation

*We wish to recommend to our readers the authoritative review articles/Award lectures, etc. pertaining to *Actinomycetes* published annually in *Actinomycetologica*, the official publication of the *Society for Actinomycetes, Japan (SAJ)*. Published twice in a year, it is fully accessible on internet, free to the readers. The latest issue *Vol.35 (No.2)* has been published in December 2021.*
**Chemotaxonomy Techniques**

**Determination of Whole Cell Amino Acid Composition**

1. Grow the biomass in MGYP medium in suitable shaking conditions.

2. When the culture is 4-5 days old treat it with 1% formaldehyde solution and incubate at room temperature for 24 hrs.

3. Harvest the cells by centrifugation and wash the biomass with distilled water followed by 95% ethanol.

4. Dry the biomass completely at 55°C.

5. Take the dried biomass (30 mg) and hydrolyze it with 6N HCl (10 ml) in a screw-capped vial at 110°C for 18 hrs.

6. Cool the hydrolysate to room temperature and filter the preparation. Collect the filtrate and dry using rotary evaporator.

7. Dissolve the dried contents in distilled water (0.3 ml).

8. Load the sample (1 ml) on cellulose coated thin layer chromatography sheet along with the standard (1 ml of 0.01M DL-Diaminopimelic acid).


10. Visualize by spraying the plate with 0.2% (w/v) Ninhydrin, prepared in acetone, and heating at 105°C.

Determine Rf values for the test and the standard. DAP appears as gray green spot fading to yellow.
Determination of Whole Cell Sugar Composition

1. Take dried biomass (100 mg) and add 2N H₂SO₄ (8 ml) in PTFE capped tubes.

2. Hydrolyze at 110°C for 2 hrs.

3. Cool the hydrolysate to room temperature and adjust the pH to 5.2-5.5 with barium hydroxide.

4. Centrifuge and collect the supernatant.

5. Dry the supernatant using rotary evaporator.

6. Dissolve the dried contents in 0.3 ml distilled water.

7. Apply the sample (1-2 ml) on TLC silica gel plate along with the standards.


Determine Rf values for the test and the standard. Hexoses such as glucose, galactose, mannose and rhamnose appear as yellow spots and pentoses such as xylose, arabinose and ribose appear as maroon spots.


Mycolic Acid Extraction and Analysis

1. Take dried biomass (100 mg), add methanol:toluene: H₂SO₄ (5:5:0.2, v/v) and keep for 12-16 hrs at 50°C.

2. Cool the preparation and extract with hexane (2 ml) by shaking.

3. Load the sample (1-2 ml) from the upper hexane layer on TLC plates (Merck Silica Gel H. 0.5 mm).

4. Develop the chromatograms in petroleum ether: diethylether (85:15 v/v).

5. Spray the plates by chromic acid solution (5 g H₂Cr₂O₇, in 5 ml water, made up to 100 ml with conc. H₂SO₄, then diluted 10 times with water) and heat them at 150°C.
Measure the Rf values. The components with Rf values between 0.1-0.5 usually correspond to methyl esters of mycolic acid.


**Extraction and Analysis of Menaquinones**

1. Take dried biomass (100 mg) and mix with 20 ml of Chloroform: methanol (2:1, v/v). Keep the suspension for 16-18 hrs.

2. Filter and evaporate the extract using rotary evaporator.

3. Dissolve the extract in small amount of hexane and load on TLC plate (Merck- Kiesel gel Hf254).


5. Detect the menaquinones by brief irradiation of short-wave UV light (254 nm).

6. Menaquinones appear as dark spots under UV illumination. Rf value of menaquinone is approximately 0.7 in this system.

Brief Description of Select Actinomycete Taxa

In this write up salient features of select actinomycete genera have been presented with focus on clearly recognizable morphological features which would be of help in recognizing their biodiversity. Recent years have witnessed erection of new genera based solely on 16S rDNA sequence analysis, signature nucleotides and DNA-DNA hybridization studies most of which have been published in International Journal of Systematic Bacteriology (presently named International Journal of Systematic and Evolutionary Microbiology) and these have not been exhaustively covered here. Readers are advised to refer to this Journal to get an idea of the newer trends in the identification of new actinomycete taxa.

For the sake of convenience, we have presented the descriptions under the following group headings.

**Group A:** Genera with motile spores from fresh water and soil habitats

**Group B:** Genera with non-motile spores, mesophilic or extremophilic, predominantly from terrestrial habitats

**Group C:** Novel genera recently described from the marine ecosystem and saline habitats

The genera under each group are listed in alphabetical order with no implication of phylogenetic relatedness. **Sketches depicting the salient features of sporulation in some of the genera has also been provided.**
Group A: Genera with Motile Spores

   Type species: *Actinokineospora riparia*

Mycelium colourless to brownish, whitish aerial mycelium bearing long chains of smooth walled conidia that can differentiate into motile zoospores in an aqueous environment. Zoospores are rod shaped with peritrichous flagella, germinating by multiple germ tubes. Sporangia not formed. Whole cell hydrolysates contain arabinose, galactose, glucose, mannose and rhamnose. Inhabitants of plant materials or soil, especially adjacent to ponds.

   Type species: *Actinoplanes philippinensis*

Mycelium non-fragmenting, fine. Colonies lacking aerial mycelium, brilliantly colored, pinkish orange. Spores produced in sporangia, spherical or sub-spherical borne on palisade layer of sporangiophores, spores arranged in coils in sporangia, discharged at maturity when flooded with water. Motile spores small, spherical to cylindrical with a polar flagellum. Whole cell hydrolysates contain meso DAP, glycine, D-xylose and L-arabinose, Inhabitants of fresh water, plant litter (aquatic and terrestrial soil). Source of several novel antibiotics (e.g. teicoplanin) and industrial enzymes (glucose isomerase).

   Type species: *Actinosynnema mirum*

Colonies with substrate mycelium growing on and penetrating agar medium, flat, from which dome like bodies of synnemata arise. Aerial hyphae arise
from these structures and bear chains of spores that can form flagella in the aqueous environment, zoospores with peritrichous flagella. Cell wall contains meso-DAP. Associated with surfaces of fresh plant tissue especially grass blades in aqueous ecosystem. Application of a differential centrifugation technique enable isolation of *Actinosynnema* strains from soil samples (M. Hayakawa et al. *Antonie van Leeuwenhoek* 78: 171-185, 2000).

   Type species: *Ampullariella regularis*

Branched, septate substrate mycelium, true aerial mycelium, sporangia produced above the surface of the substrate irregular, cylindrical, lobate, bottle shaped bearing numerous spores arranged in parallel chains. Spores rod shaped, motile. Substrate mycelium brightly colored. Meso-DAP and glycine in cell wall peptidoglycan, xylose and arabinose in whole cell hydrolysates. Inhabitants of soil, fresh water.


   Type species: *Catenuloplanes japonicus*

Colonies showing differentiation of aerial and substrate mycelium during growth cycle and produced motile spores arranged in chains in the aerial mycelium. Sporangia not observed. Cell walls contain L-lysine, serine, glycine, D-glutamic acid and D-alanine (wall type VI). No mycolic acid. Inhabitants of soil of Japan, India, USA.
Planopolyspora (P. crispa) with long curly and sometimes branching sporangia has been merged with the genus Catenuloplanes on the basis of chemotaxonomic and molecular data (T. Tamura et al. Int. J. Syst. Bact. 45: 858-860, 1995).

   Type species: Couchioplanes caeruleus
Colonies forming blue substrate mycelium and aerial mycelium with motile arthrospores. No sporangia observed. Lysine is present in cell wall. Actinoplanes caeruleus and A. azureus, described earlier, transferred under this genus on the basis of molecular data. Genus proposed on the basis of morphological, physiological and chemotaxonomic data as well as comparative 16s rRNA and DNA-DNA studies.

   Type species: Dactylosporangium aurentiacum
Substrate mycelium pale to deep orange, rose or wine colored to brown, sporangia finger shaped to claviform borne on short sporangiophores and containing a single row of 3-4 spores which are oblong, ovate ellipsoid or pyriform and motile with a tuft of polar flagella. Meso-DAP, glycine in cell wall xylose and arabinose in whole cell hydrolysates, mainly soil inhabitants.

   Type species: Pilimelia terevasa
Sporangia produced on the surface of substratum on sporangiophores,
spherical, ovoid pyriform, campanulate or cylindrical. Sporangia contain numerous spores in chains arranged in parallel or irregularly swirl like rows. Zoospores rod-shaped with lateral tufts of flagella. Colonies grow only on complex media, smooth compact, pasty or solid. Substrate mycelium pale brown yellow, golden orange or pale brown turning dark with age. Present in soil, decompose keratinous substrates (hair of mammals)

Type species: Planomonospora parontospor
Substrate mycelium growing profusely into the medium and forming a compact layer from which aerial mycelium arises, poorly branched, bearing sporangia developing a single motile sporangiospore. Soil inhabitants. Igarashi et al. (Can. J. Microbiol. 47: 253-263, 2001) developed a selective isolation procedure in which soil is flooded with 0.1% skimmed milk solution containing 5 mM N-acetyl 2-aminoethane sulfonic acid (pH 9.0), incubating at 32°C for 90 minutes, centrifuge and plate out supernatant on gellan gum medium.
Isolates of Planomonospora venezuelensis were obtained from several soil samples around the world including India.

Type species: Planobispora longispora
The organism is a typical mesophilic, aerobic actinomycete, producing a filamentous growth which is differentiated into a vegetative and aerial
mycelium. Sporangia are formed on the aerial mycelium contain a longitudinal pair of motile spores. Found in Ecuador, Egypt, French Guiana, India and Madagascar soils. S. Suzuki et al. (Can. J. Microbiol. 47: 979-86, 2001) described selective isolation of Planobispora by flooding the soil with an alkaline solution of skimmed milk containing antimicrobial agents or by dry heating at 90°C for 60 min and plating out on Humic acid vitamin-gellan gum medium at pH 9.0.

Type species: Planotetraspora mira


Type species: Spirillospora albida

Mycelium white to pale yellowish. Sporangia spherical to vermiform developing spores from one or more coils within. Spores weakly motile with one or more flagella, rod shaped to spiral. Soil inhabitants.
**Group B:** Genera with non-motile spores mesophilic or extremophilic, predominantly from terrestrial habitats

**Group B1: Monosporic Genera**

   Type species: *Micromonospora chalcea*

   Colonies lacking aerial mycelium forming yellow to orange colored growth bearing dark brown to black spores which are single borne in dense clusters imparting a waxy or mucoid consistency for the colony appearance. The cell walls are Type-I with meso-DAP, xylose and arabinose. A very important genus for biotechnology applications as a source of several antibiotics, secondary metabolites and enzymes. (e.g., Gentamicin produced by *M. purpurochromogenes*)

   Type species: *Saccharomonospora viridis*

   Aerobic, non-fragmenting substrate mycelium and a well-developed aerial mycelium bearing single aeurispores, either sessile or on short sporophores. No spores are formed on the substrate mycelium. Meso-DAP in cell walls madurose present in cell wall hydrolysates. Facultative thermophile, cell wall composition different than that of *Thermomonospora.*

Type species: *Thermomonospora curvata*

Aerobic with well-developed non-fragmenting mycelium of vegetative substrate and aerial hyphae. Single spores are formed at the tips of simple or branched short sporophores arising from the substrate as well as aerial mycelium. Species are common in soils and thermophilic forms occur widely in compost and self-heating plant biomass substrates. Spores are not heat resistant and are aleuriospores.

*Thermoactinomyces* with heat resistant endospores borne singly is classified under Bacillaceae by removing it from the actinomycetes group. *Thermomonospora fusca* and *T. alba* have been separated from the genus and classified under a new taxon *Thermobifida* on the basis of 16S rRNA sequence data as well as phylogenetic, chemotaxonomic and phenotypic evidence ([Z. Zhnag et al. Int. J. Syst. Bact. 48: 411-422, 1998](#))

**Group B2: Spores Borne in Chains**


Type species: *Actinomadura madurae*

Aerobic, non-acid-fast with branched substrate mycelium and sparse to well-developed aerial mycelium. Arthrosposores borne in short chains of 5-15 arising branches from aerial hyphae. Type III cell wall, several species from short lateral contain madurose.
   Type species: *Chainia antibiotica*

Non-fragmenting branched mycelium with the colonies showing development of numerous thick-walled sclerotic granules storing fatty materials, formation of aerial mycelium and sporophores often observed after prolonged cultivation. In the concomitant reduction in the extent of sclerotia laboratory on nutrient media with developed. Type I (LL-DAP) cell walls of vegetative mycelium.

The morphological basis on which the genus was established (sclerotial development) is presently debated and many authors from the western world have considered it synonymous with Streptomyces. A more detailed discussion on this genus described as new from India has been presented in the chapter on Morphology.

   Type species: *Microbispora rosea*

Spores borne in characteristic longitudinal pairs only on the aerial mycelium while no spores are formed on the substrate mycelium. Spores sessile or borne on short sporophores, often closely aggregated to present a catkin like appearance. Cell wall Type III. Facultatively anaerobic or thermophilic in some strains.

Type species: *Micropolyspora brevicatena*

Substrate and aerial mycelium formed. Short chains (1-20) of spores formed basipetally originating from both substrate and aerial mycelium. Cell wall type IV containing meso-DAP, arabinose and galactose.


Type species: *Microtetraspora glauca*

Short sparsely branched aerial mycelium is formed on which sporophores bearing chains of four spores are formed. Cell walls contain meso-DAP (with a trace of LL-DAP), lysine and glycine.


Type species: *Streptomyces albus*

Aerobic with extensively branched non-fragmenting substrate mycelium and aerial hyphae. Aerial mycelium bears long chains of spores observed in
diverse morphological appearance (straight, flexous, spiral etc.) Spores are arthrospores developed by regular septation of the hyphae and enclosed within a fibrous sheath Type I (LL-DAPA). Cell wall is characteristic. Most abundant in occurrence in soil and species of this genus most extensively investigated for bioactive metabolites. Source of several therapeutically useful antibiotics and industrial enzymes.

A. Species with straight sporophores
B. Species with flexuous sporophores


Non-fragmenting substrate mycelium bearing well developed aerial mycelium. Bearing short chains of arthrospores. Spore chains are borne in typical whorls or verticils which is a characteristic morpho-logical feature on which the genus was established. Type I (LL-DAP) cell wall as in *Streptomyces* and lacking characteristic sugars. The differentiation of the genus from *Streptomyces* on this morphological basis has been a debatable matter.
Group B3: Spores Developing Inside a Vesicle

   Type species: *Streptosporangium roseum*

Aerial mycelium bears spore vesicles often in clusters which develop non-motile aplanospores formed by the septation of a spiral unbranched hypha growing into the spore vesicle. Type I cell wall composition. Soil, dung and plant litter are rich sources for the isolates.

Group C: Novel Marine and Salt Tolerant Actinomycetes

   Type Species: *Actinopolyspora halophila*

Branching vegetative mycelium mostly non-fragmenting, aerial mycelium develops long chains of smooth walled spores, oval, cylindrical or coccoid to rod shaped. Moderately to extremely halophilic.

   Type species: *Aeromicrobium erythraeum*

*Aeromicrobium marinum* obligately salt-dependent, Gram positive bacterium, non-motile rods, isolated from surface waters of the German Wadden sea. Classified under Nocardioidaceae, LL-DAP in peptidoglycan, form ivory-colored colonies, characterized by a diffuse halo, optimal salinity for growth
is 53.5%. Utilize trehalose, cellobiose, unable to hydrolyse starch, casein, cellulose, chitin G+C content 70.6%.


First designated MAR-1. Classified under Micromonosporaceae. Formed a distinct taxon in the 16S rRNA. Micromonosporaceae gene tree. Type species: *Salinispora arenicola*

Comparative 16S-23S rRNA gene spacer region and DNA-DNA relatedness data was the basis for designating the new genus. A spore forming bacterium originating from a saline habitat. Aerobic, gram positive, non acid-fast forming extensively branched substrate hyphae carrying smooth surfaced spores singly or in clusters. Require sea water or a medium containing a sodium supplemented medium for growth. Colonies flat, slow, growing (3-6 weeks) orangish to black, lacking aerial mycelium. *S. tropica* is the source of Salinosporamide A, a highly cytotoxic proteosome inhibitor and a very promising compound in anticancer therapy.


Type species: *Streptomonospora salina*

Halophilic, forming a distinct branch in the 16s DNA phylogenetic tree adjacent to genera *Nocardiopsis* and *Thermobifida* (suborder Streptosporangiaceae). *Streptomonospora alba* isolated from soil in China on starch casein agar with a salt concentration of 20% (w/v) at pH 7.0. Substrate and aerial mycelium well developed, non-motile spores with wrinkled surface borne on the aerial mycelium. Meso-DAP, glucose and arabinose present in whole cell hydrolysate. Gram positive, aerobic, non-fragmenting substrate mycelium. Short chains of non-motile oval to rod shaped spores with wrinkled surface borne on aerial mycelium. Extensively branched
substrate mycelium bears single non-motile oval rounded spores borne on sporophores which may be dichotomously branched.


   Type species: *Verrucosispora gifhornensis*

Spores warty, classified under Micromonosporaceae. Morphological similarity to *Micromonospora*. Differs in the lack of arabinose in whole cell hydrolysate. Gram positive, aerobic, spore forming. Isolated from peat bog near Gifhorn, Germany. Designation of genus based on 16S rRNA family specific signature nucleotides produce Abyssomicins, novel antibacterial compounds inhibiting para aminobenzoic acid pathway.


   Type species: *Williamsia muralis*


   Type species: *Yania halotolerans*

Isolated from saline soil in China. Classified under Micrococcaceae. G+C content 53.5%. Optimal growth in media containing 10% potassium chloride. Coccoid, halotolerant actinobacteria forming yellow opaque circular lubricious small colonies, 2 mm in diameter after 7 days at 28°C.
Composition of media used for isolation, sporulation and vegetative growth are listed below. Sterilization is carried out at 121°C for 15-20 min by autoclaving. The vitamin and the antibiotic agents are separately sterilized by filtration and added to cooled (45-50°C) agar media.

The quantities are all per liter of distilled water.

### Media for Isolation

<table>
<thead>
<tr>
<th>Media Name</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1. Humic Acid-Vitamin Agar</strong></td>
<td></td>
</tr>
<tr>
<td>Humic acid (dissolved in 10 ml 0.2 N NaOH)</td>
<td>1.00 g</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>0.50 g</td>
</tr>
<tr>
<td>KCI</td>
<td>1.71 g</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>0.05 g</td>
</tr>
<tr>
<td>FeSO$_4$.7H$_2$O</td>
<td>0.01 g</td>
</tr>
<tr>
<td>CaCO$_3$</td>
<td>0.02 g</td>
</tr>
<tr>
<td>Vitamins stock solution</td>
<td>1.00 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>Agar</td>
<td>18.0 g</td>
</tr>
<tr>
<td>Adjust to pH 7.2</td>
<td></td>
</tr>
<tr>
<td>Cycloheximide (Added at 50 mg per liter of medium before pouring)</td>
<td></td>
</tr>
</tbody>
</table>
2. Vitamin Stock Solution

- Thiamine hydrochloride 10 mg
- Riboflavin 10 mg
- Niacin 10 mg
- Pyridoxine hydrochloride 10 mg
- Inositol 10 mg
- Calcium pantothenate 10 mg
- para-Aminobenzoic acid 10 mg
- Biotin 5 mg
- Distilled water 20 ml

3. Starch-Casein-Agar

- Starch 10.0 g
- Casein 0.3 g
- KNO$_3$ 2.0 g
- NaCl 2.00 g
- K$_2$HPO$_4$ 2.00 g
- MgSO$_4$·7H$_2$O 0.05 g
- CaCO$_3$ 0.02 g
- FeSO$_4$·H$_2$O 0.01 g
- Distilled water 1000 ml
- Agar 18.0 g

Adjust to pH 7.0 to 7.2.

4. Soil Extract Agar

First 150 g of garden soil is stirred in 600 ml of tap water, filtered immediately through Whatman No. 1 filter, and made up to 1 liter with tap water. The pH is adjusted to 7.2 and 18 g/L agar added. 50 mg/L Cycloheximide and Nystatin added after autoclaving.
5. Colloidal Chitin Agar (Composition of Minerals according to Hsu and Lockwood, 1975)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colloidal chitin (dry wt)</td>
<td>2.0 g</td>
</tr>
<tr>
<td>K$_2$HP0$_4$</td>
<td>0.7 g</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>0.3 g</td>
</tr>
<tr>
<td>FeSO$_4$.7H$_2$O</td>
<td>0.5 g</td>
</tr>
<tr>
<td>ZnSO$_4$.7H$_2$O</td>
<td>0.01 g</td>
</tr>
<tr>
<td>MnCl$_2$</td>
<td>0.001 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>Agar</td>
<td>20.0 g</td>
</tr>
<tr>
<td>Adjust to pH 7.0</td>
<td></td>
</tr>
</tbody>
</table>

6. Glycerol Asparagines Agar

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount (g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Asparagine</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Glycerol</td>
<td>10.0 ml</td>
</tr>
<tr>
<td>K$_2$HP0$_4$</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>Agar</td>
<td>20.0 g</td>
</tr>
<tr>
<td>Adjust pH to 7.0-7.4</td>
<td></td>
</tr>
</tbody>
</table>

**Preparation of Humic acid**

500 g of soil (forest soil) sample is suspended in 1 liter of 0.5% NaOH solution and left standing at room temperature for 24 hours, with occasional stirring. The precipitate of the suspension is removed by centrifugation (20 min at 7,000 rpm), and the supernatant is acidified to pH 1.0, with concentrated HCl. The resulting precipitate is centrifuged (20 min at 3,000 rpm), washed three times, by centrifugation with 150 ml of water and
suspended again in 150 ml of water. The suspension is frozen overnight at -20°C. After thawing, the granulated humic acid is filtered, washed and air dried.

**Preparation of Colloidal Chitin**

Crude chitin is washed alternately in 1 N NaOH and 1 N HCl for 24 hours period each, five times. Then, it is washed four times with 95% (9v/v) ethanol. 15 g of the purified white chitin is dissolved with 100 ml of conc. HCl and stirred in an ice bath for 20 min. The mixture is filtered through glass wool, and the solution is poured into distilled water to precipitate the chitin. The insoluble chitin on the glass wool is treated again with HCl, and the process is repeated until no more precipitate is obtained when the filtrate is added to the cold water. The colloidal chitin is allowed to settle overnight and the supernatant is decanted. The remaining suspension is neutralized to pH 7.0 with NaOH. The precipitated chitin is centrifuged, washed, and stored as a paste at 4°C.

**Preparation of Antibiotics**

To dissolve cycloheximide, warm water is recommended. Nystatin can be dissolved initially in dimethylsulfoxide and then diluted in 95% alcohol. To completely dissolve Nystatin, the pH of the aquatic solution can be adjusted using 1N NaOH to 11.0. Since Nystatin is unstable at high pH, the pH can be lowered to 7.0, immediately after complete dissolution.

**Media for Conservation**

<table>
<thead>
<tr>
<th>1. Yeast Extract Glucose Agar</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
</tbody>
</table>
### 2. Oatmeal-Tomato Paste Agar g/L

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tomato Paste</td>
<td>20.0 g</td>
</tr>
<tr>
<td>Precooked Oat meal</td>
<td>20.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>Agar</td>
<td>20.0 g</td>
</tr>
</tbody>
</table>

### 3. Yeast Malt Extract Agar g/L

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract</td>
<td>4.0 g</td>
</tr>
<tr>
<td>Malt extract</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>4.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>Agar</td>
<td>20.0 g</td>
</tr>
</tbody>
</table>

Adjust pH to 7.3