# The Bioprospecting Potentials of Microorganisms for the Synthesis of Bioactive Molecules

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By
Bi Bi Zainab Mazhari,
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The authors dedicate this book to the countless scientists who work under the most dangerous and difficult of field conditions to bring compassionate assistance to those in need.

*Microorganisms are role models.....*
*Learn from their experiences!!*
*Never give up in life .....*
*You will find a way to adapt yourself against*
*any problem you encounter!!*
*—Dr. Bi Bi Zainab Mazhari*

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# FOREWORD

This book is an introduction to isolation, identification, characterization of actinomycetes, Synthesis of extracellular Tyrosinase and Nanoparticles by Actinomycetes. Hopefully, it will act as a motivation to both research scientists and those engaged even in focused applied work. We will briefly explore some major divisions within microorganisms, and hopefully, highlight some interesting facts and differences within each group.

I am honored by Dr. Zainab Mazhari for having offered me the privilege of introducing the reader of this book. I imagine that her choice is driven by the intention to provide a broader framework rather than one of science information alone.

This book provides absorbing insights into Isolation, Identification of Actinomycetes, and also the synthesis of bioactive molecules by actinomycetes. This book is a comprehensive work on the sound knowledge of Dr. Zainab's experience in studying Actinomycetes. She is undoubtedly one of the leading researchers in the field of Microbiology. I am assured this book will be recognized as a reference guide for anyone with a keen interest in the microbiology of actinomycetes, enzymes, and nanoparticles.

My hearty congratulations to Dr. Zainab Mazhari and her team for this authoritative book. I wish them all the very best.

**Er. Mohammed Asad Sheikh**

# PREFACE

Microorganisms constitute a huge and almost unexplained reservoir of resources likely to provide innovative applications useful to man. The fact that bacteria have survived ever since the origin of life on the earth reveals that this group of organisms is a storehouse for changes that occurred over millions of years of evolution in response to changing environmental conditions. They are the most abundant and highly diverse group among all organisms living today. Relevant available literature reveals that actinomycetes are poorly studied and are the neglected group of organisms among all bacteria. In the recent past, actinomycetes have been exploited for the production of important industrial enzymes, although actinomycetes are well established for the production of antibiotics. Increased interest in the actinomycetes is due to the need for delineating specific potential actinomycetes and exploring them as a source of new desired bioactive molecules. There have been several changes in the criteria used for the isolation and screening of actinomycetes in the past several years. It is known that actinomycetes with few exceptions are prevalent in almost all geographical conditions. More importance shall be given to the unique ecological niche. Generally, novel isolates of microorganisms have been isolated from harsh or unique ecological habitats and niches for the synthesis of bioactive molecules.

I hope the book will be useful and exciting to read and instigate more enthusiasm for the subject.

**Dr. Bi Bi Zainab Mazhari**

# ACKNOWLEDGMENTS

A few people have been contributory in allowing this book to be completed. Foremost I would like to pen my sincere thankfulness to Er. Mohammed Asad for taking the time to go through the manuscript. I also extend my gratitude to the Co-author of this book Prof. Dayanand Agsar and Prof. Mohammed Saiem Aldhar for their constant support and valuable suggestions. Their suggestions and detailed critical analysis greatly improved the final product. I would also like to acknowledge my Husband Mohammed Asad Sheikh for his endless care and encouragement in this endeavor. I do not have enough words to express my gratitude to my parents, my family members for their love, care, and support in this endeavor. I am proud to express my deep gratitude to my Kids for their affection. I also sincerely thank Cambridge Scholars Publishing, UK for the excellent editorial assistance and for taking a keen interest in producing this book.

**Dr. Bi Bi Zainab Mazhari**

# CHAPTER 1

# INTRODUCTION: ISOLATION AND IDENTIFICATION OF ACTINOMYCETES

Microorganisms constitute a huge and almost unexplained reservoir of resources likely to provide innovative applications useful to man. Organisms have been evolving for nearly 4 billion years and are capable of exploiting a vast range of energy sources and thriving in almost every habitat. It has been estimated that only between 1 and 5% of all microorganisms on earth have been studied. A large proportion of thesis of unknown species is thought to reside in the soil estimates of the possible number of existing species of different groups are staggering, 1.5 million species of fungi, 3,00,000 species of bacteria, 4,00,000 species of nematodes, and 40,000 species of protozoa (Colin Campbell, 2002).

Microbial diversity is the key to human survival and economic well-being. It provides a huge reservoir of resources to advance biotechnology for human welfare. New technologies, particularly, nucleic acid analysis, computer science, analytical chemistry, habitat sampling, and characterization placed the study of microbial diversity on the cutting edge of Science. In the past few years, due to advances in molecular methods and techniques, our knowledge of microbial diversity has increased dramatically (Manish Kapoor and Rakesh Kumar, 2004). Our first knowledge of actinomycetes dates back to 1875 when Ferdinand Cohn named the organism found in the tear duct of the human eye as Streptothrix forester. Harz (1877) described the organisms actinomyces bovis found in the pus of cattle suffering from the disease now called “Actinomycosis’ or ‘Lympy Jaw’ of cattle. The Harz used the term “actinomyces” to describe the radial arrangement of the branching, mold-like thread of the organisms when growing in infected tissues (Gr. Actino-radial emanation, e.g., sunlight, mykes = fungus, hence ray fungus). Soon after other species of actinomycetes were found in soil (Globig, 1888), manure (Tsiklinsky, 1899), grain (Brocqu-Rousseau, 1904), compost, and hay (Lacey, 1973).

Selman (1939) narrated isolation, basic identification, and classification of actinomycetes concerning bacteria and fungi. Actinomycetes were identified and classified as bacteria, like fungi, and also as a special group, either derived from one of the above or giving rise to both. With the accumulated knowledge concerning the morphology of the actinomycetes, it is being recognized more and more that they are an independent group of organisms, which is closely related to the bacteria through some of the constituent forms, but which has adopted a fungus-like form of growth. Actinomycetes are characterized by the formation of normally branching threads or rods, frequently giving rise to a typical mycelium that is unicellular, especially during the early stages of growth. The mycelium is either vegetative and growing in the substrate, or aerial, where a special mycelium is produced above the vegetative growth. Actinomycetes reproduce through special sporulating bodies or from parts of the vegetative mycelium.

As everywhere in biology, the most complicated, but at the same time most important aspect of the study of microorganisms is the isolation and basic identification. It is important to understand various criteria for the isolation and identification of actinomycetes (Krasilnikov, 1959). He has reported that cultural characteristics of actinomycetes vary on media to a greater extent with diverse sources of mineral contents. Minerals containing phosphorus, potassium, sodium, calcium, magnesium, and other salts were reported to influence the pigmentation pattern of actinomycetes. Further, he suggested that even minute quantities of mineral elements, especially metals such as iron, copper, zinc, and some others have great significance in pigmentation. Morphological features of hyphae, such as thickness, curving, branching, length, aerial mycelium, and substrate mycelium are unstable and vary easily depending on cultural conditions (Krasilnikov, 1959). Various means have been employed to use and evaluate color as a criterion for the identification of actinomycetes. As early as 1914, Krainsky attempted the use of a standard color code (Klincksieck and Valette, 1908) in evaluating the isolates of actinomycetes and he prepared colored illustrations of cultures. Kransilnikov (1959) also reported that incubation of temperature will have a greater influence, not only on the growth rate but also on the pigmentation pattern of actinomycetes, which can be an important feature to characterize actinomycetes. Waksman and Curtis (1916) and Waksman (1919) used Ridgway's (1912) color standards and color nomenclature in characterizing the isolates of actinomycetes.

A principal criterion of long-standing for the identification of actinomycetes has been, the color of the mycelium, branching nature of the mycelium, and color of the diffusible pigments in recent years, the surge in studying all phases of the actinomycetes especially the Streptomycetes has been tremendous. Whole the criterion has been re-evaluated and closer attention has been given to the identification of new isolates with more objective characteristic features. Such identification would serve as a base for better isolation and accurate identification of actinomycetes (Pridham, 1965). Color discrimination and the significance of color in distinguishing the isolates of actinomycetes constitute problems of considerable magnitude.

Rapid identification of isolates, at least to the genus level, can provide valuable information for later stages. Identification of isolates to the species level will often give the isolation biochemist a clue as to whether or not a metabolite is a novel. The use of selective isolation pressures to generate isolates from nature generally will increase the probability that particular actinomycete genera will be recovered (Labeda, 1986). Morphology, as previously mentioned, has always been an important characteristic used to identify actinomycete strains, and it was the only character used in many early descriptions, particularly of *Streptomyces* sp. in the first few editions of Bergey's Manual (Labeda, 1986).

Shirling and Gottlieb (1976) suggested several media for the International Streptomyces Project (ISP) to distinguish and characterize actinomycetes at different levels. Further, Shirling and Gottlieb (1968a; 1968b; 1969 and 1976) used a combination of mycelia characters, sporulation feature, production of melanin, and diffusible pigments in combination with the utilization of various sugars for the identification of actinomycetes in general and Streptomyces in particular under International Streptomyces Project.

Biosystematics studies of members of the cultivable Streptomyces community in soil are also hampered by problems associated with representative sampling. Soil aggregates need to be thoroughly dispersed as many microorganisms, notably those showing mycelial growth, may be bound to aggregate soil particles (Hattori and Hattori, 1976; Ramsay, 1984; Nishiyama *et al.,* 1992). A procedure used to promote the dissociation of microorganisms from particulate matter includes the use of buffered dilutions (Niepold *et al.,* 1979), chelating agent (MacDonald, 1986), elutriation (Hopkins *et al.,* 1991b), and mild sonication (Ramsay, 1984), techniques which address the problem of quantitative and

representative sampling to varying degrees. The dispersion and differential centrifugation (DDC) technique, a multi-step extraction procedure introduced by Hopkins *et al.* (1991b), combines several physicochemical treatments and has proved to be effective for representative sampling of bacteria, including actinomycetes, from diverse soils (Hopkins *et al.,* 1991a; MacNaughton and O' Donnell, 1994).

Actinomycetes have been isolated and identified from many natural environmental samples. Several studies have been focused on the isolation and identification of actinomycetes from geographically varied areas (Goodfellow *et al.,* 2010b). Few reports are also available regarding the exploration of actinomycetes from harsh environmental samples. In recent years, large numbers of researchers across the world have been involved in the isolation and identification of actinomycetes from diverse habitats or geographical conditions. The important studies regarding the identification and characterization of actinomycetes isolated from various natural sources are as follows.

**Table 1. Identification and characterization of actinomycetes from various sources**

| Sl. No. | Natural sources | References |
|---|---|---|
| 1. | Agricultural field soil | Dastager *et al.,* 2007, 2007a and 2008a; Shivaveerakumar *et al.,* 2013; Jeffery, 2010 |
| 2. | Desert soil | Mayilraj *et al.,* 2005 and 2006a and b |
| 3. | Fresh water | Grant *et al.,* 1999 ; Ress *et al.,* 2004 ; Joshi *et al.,* 2007 ; Surakasi *et al.,* 2007 |
| 4. | Forest soil | Vijaybhaskar and Dayanand,2010 |
| 5. | Hot springs | Li – Hua Xu *et al.,* 1998 |
| 6. | Limestone quarries | Nimaichand *et al.,* 2012 ; Quadri and Agsar, 2013; Mazhari *et al.,* 2014 |
| 7. | Marine water | Cross, 1981 ; Moran *et al.,* 1993 ; Sivakumar *et al.,* 2007 ; Manivasagam *et al.,* 2010 ; Gulve *et al.,* 2011 |
| 8. | Mining areas | Shirley *et al.,* 2008 ; Dhanjal *et al.,* 2010 and 2011 |

**Streptomyces**

Among actinomycetes producing bioactive compounds, the commonest genera that are reported are *Streptomyces* spp. (73%) and the rest are rare genera (27%). Streptomyces are known to produce varied novel antibiotics and bioactive compounds. *Streptomyces* spp. is a prominent genus of the Streptomycetaceae family which is nowadays deeply being studied because of their capacity to produce various important bioactive compounds including novel ones such as nanoparticles, auto regulators, etc.

Their metabolic diversity is due to their extremely large genome which has hundreds of transcription factors that control gene expression, allowing them to respond to specific needs. Hence, due to this property nowadays these organisms are widely tested for their ability to produce nanoparticles. And as per expectations, these organisms have turned out to be good producers of nanoparticles with more uniformity in their size, shape, etc. as compared to fungi and other bacteria.

## 1.1. Isolation of actinomycetes

The standard serial dilution plate culture method (El-Nakeeb and Lechevalier, 1963) was used to isolate the actinomycetes from different pretreated soil samples. Adequate serial dilutions from the soil samples. 0.1 ml of the inoculum from respective dilutions were inoculated on starch casein agar (Kuster and Williams, 1964), and incubate plates were at 35 $^0$C for one week. The initial pH of the medium was adjusted to 7.0. Growth of colonies of actinomycetes on the medium was observed at every 24 h.

The distribution of actinomycetes has been recorded in a terrestrial environment especially those in extreme conditions like frozen soil and the hot deserts of India (Dey and Chaphalkar, 1998), Africa (Marilize and Paul, 2005), and the Americas (Porter *et al*., 1960). Since 1978, about 4,200 soil samples have been collected from different areas of vegetational and climatic types throughout the province of Yunnan. Some genera like *Thermoactnomycetes* and *Saccharomonospora* were strictly thermophilic. Thermophilic actinomycetes grow well on animal manure. *Thermomonospora* sp. particularly grows during the second indoor phase of preparation of manure for mushroom cultivation (Waksman, 1959). The petroleum or heavy environments have been studied by few scientists (Mansour, 2003), and still of interest to explore the different genera of actinomycetes that can survive and be adapted to such habitats. In aquatic habitats,

taxonomically diverse actinomycetes exhibit unique physiological and structural characteristics. Actinomycetes are found to occur in aquatic environments; freshwater and marine habitats (Fenical and Jensen, 2006; Singh *et al*., 2006; Pathomaree *et al*., 2006). Micromonospora is the dominant actinomycetes isolated from several samples from streams, rivers, lake mud, river sediments, beach sands, sponge, and marine sediments (Rifaat, 2003; Jensen *et al*., 2005a, b; Eccleston *et al*., 2008). Besides *Micromonospora*, other actinomycetes genera were found from aquatic habitats such as *Amycolatopsis, Marinophilus, Rhodococcus, Salinispora, Streptomyces,* and *Williams* (Mincer *et al*., 2005; Kim *et al*., 2006; Kwon *et al*., 2006; Pathomaree *et al*., 2006). Actinobacterial diversity in water and sediment samples from the marine environment of Tamil Nadu and various genera viz., *Streptomyces, Actinopolyspora, Actinomadura, Nocardiopsis, Micromonospora,* and *Actinomyces* have been reported by Manivasagam *et al.* (2010). Actinomycetes have been isolated from rocks and stone by several workers (Webley *et al.,* 1963; Agarossi *et al.,* 1985). Actinomycetes were the most common microorganisms isolated from grey surface rocks in Sicily (Urzi and Realini, 1998).

## 1.2. Identification of actinomycetes

Basic identification of actinomycetes was approved based on mainly colony characters and microscopic features, which are explained in brief as follows**.**

### 1.2.1. Colony characters

The typical colony characters of actinomycetes were observed. Aerial mycelium, substrate mycelium, and colony pigmentation were recorded after the adequate growth on starch casein agar as per the standard methods described in *Bergey's Manual of Systematic Bacteriology* (Goodfellow and O'Donnell, 1989). A pigmentation study of the colonies was carried out according to Flaig and Kutzer (1960).

### 1.2.2. Microscopic features

The colonies grown on starch casein agar were examined for Gram's staining (Gram, 1884) and mycelial branching (Shirling and Gottlieb, 1966) under a simple light microscope. Typical colonies of actinomycetes were re-inoculated on starch casein agar in which a coverslip was placed on an inclined position at $45^0$ angles and incubated for three days. After the growth of culture, the coverslip was removed and observed for the

nature of the substrate (Shinobu, 1958) and aerial mycelia (Kawato and Shinobu, 1959). The arrangement of spore mass was also recorded (Schaeffer, 1969 and Mandelstam, 1976)

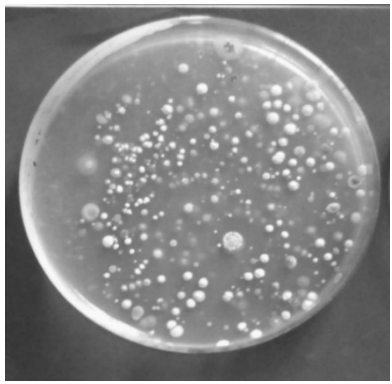

Figure 1: Colonies of actinomycetes starch casein agar

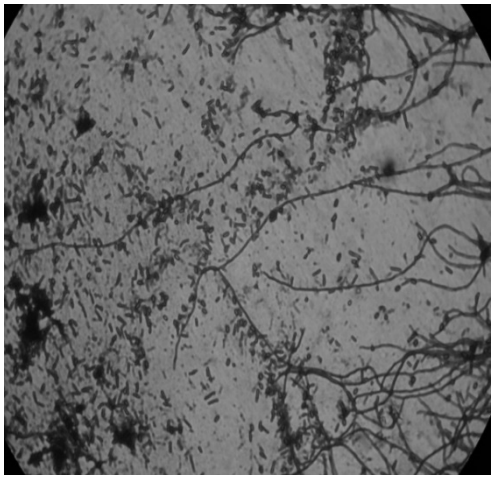

Figure 2: microscopic fields showing Gram's property and mycelial branching of actinomycetes

# CHAPTER 2

# BIOCHEMICAL AND PHYSIOLOGICAL CHARACTERIZATION OF ACTINOMYCETES

Important biochemical and physiological properties mainly including the ability of the isolates to assimilate certain carbon sources (Benedict *et al.,* 1955) and nitrogen sources (Burkholder *et al.,* 1954) have been preferred to characterize the actinomycetes. However, Waksman (1957) considered both physiological and cultural attributes to characterize actinomycetes.

Krasilnikov's (1959) observations and experimental data showed that biochemical manifestations appear to be the essential species criteria in actinomycetes, as appears in several other bacteria, yeast, and fungi. Many of the regular biochemical tests have little or no use in the recognition and subdivision of species of actinomycetes. Such properties as the ability to liquefy gelatin, hydrolyze starch, peptonize milk, invert sucrose, reduce nitrates, etc., are possessed by almost all actinomycetes. Only quantitative differences are observed some cultures liquefy gelatin faster than others in some forms the ability to hydrolyze starch is expressed strongly, in others weakly some cultures grow on cellulose well, others poorly, etc. These differences often disappear when optimal growth conditions are established. Cultures with low activity may start to show certain physiological activities more distinctly and faster. Gottlieb (1961) reported the reduction of catalase and nitrate, production of $H_2S$, hydrolysis of starch, casein, and gelatin as important biochemical characters to characterize the isolate of actinomycetes. One characteristic alone, whatsoever, is inadequate for the recognition and especially for the identification of a species. Only the sum of properties can characterize and differentiate a species. The more detailed and comprehensive the study of an organism, the sharper are the species boundaries and hence the more exact their identification. The characteristics used to deal with species are largely dependent upon the genus being studied. In addition to the morphological characteristics, a wide range of physiological characteristics (including utilization of carbohydrates, nitrogen sources, and degradation

or hydrolysis of numerous substrates) have been suggested by Labeda (1986).

Benedict *et al.* (1995) and Zahnner and Ettlinger (1957) reported that almost all actinomycetes can liquefy gelatin, hydrolyze starch, peptonize milk and reduce nitrates. They emphasize that more significance should be given to the assimilation of certain sources of carbon and nitrogen to identify and characterize actinomycetes at the level of genus and species. Effect of pH, temperature, and sodium chloride on the growth of selected isolates of actinomycetes was reported (Aman, 2001) as major physiological properties to characterize the actinomycetes.

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utilization of carbohydrates, nitrogen sources, and degradation or hydrolysis of numerous substrates) have been suggested (Labeda 1986).

**Molecular characterization**

The species concept in actinomycetes genera are still poorly understood (Labeda, 1986) and an evaluation of strains within these genera by classical, numerical, and molecular taxonomic techniques was necessary to clarify the situation.

The biology of most actinomycete genera has been covered by Balows *et al.* (1992) in the Prokaryotes some of them have been reclassified or dissected since then. Many additional genera were added recently to the actinobacteria proper, increasing the phylogenetic and epigenetic diversity of the class, even though the medical, ecological, and biotechnological importance of these new taxa has not yet been evaluated thoroughly (Stackebrandt and Goodfellow, 1991; Stackebrandt and Goebel, 1994). During the past 25 years, comparative analysis of sequences of homologous and genetically stable semantides has demonstrated that several classification systems based on morphology and physiology do not reflect the natural relationships among actinomycetes and related organisms (Stackebrandt and Goodfellow, 1991 Stackebrandt and Goebel, 1994). It is their tempo and mode of evolution that makes actinomycetes and their relatives difficult to classify outside the context of phylogeny though the majority of taxa evolved rather late in earth's history, individual characteristics (traditionally used in early classification) are highly diverse and only rarely reflect phylogenetic relationships.

Since the mid-1980s, the use of small subunit ribosomal ribonucleic acid (SSU rRNA) based technique has facilitated a culture-independent approach of investigating microorganisms as they occur in nature (Olsen *et al.,* 1986; Ward *et al.,* 1992 and Amman *et al.,* 1995). The comparison of these molecular "signature" sequences transformed microbial taxonomy from a pure identification system to an evolutionarily based framework (Gray *et al.,* 1984; Woese, 1987; Olsen *et al.,* 1994 and Norman, 1997). The most popular probe-target by for is the SSU rRNA molecule or its larger counterpart. These are present in high copy numbers ($10^4$ to $10^5$ molecules per bacterial cell), thus greatly improving the sensitivity of the hybridization within these genes are regions that are highly variable and differ significantly between species where as other areas are more conserved and suitable for identification at the generic level (Amann and

Ludwig, 2000). This technique is the forerunner of the hybridization micro and macro arrays that have now been adopted for bacterial identification.

DNA hybridization has played a pivotal role in bacterial systematics and provided one of the first molecular methods for identification. If the organism has a particular and specific trait, hybridization reactions are valuable for identifying members of the taxon from the environment, food, or clinical samples (Stackebrandt and Goodfellow, 1991). It offers identification possibilities indeed chromosomal DNA hybridization forms the basis of the generally accepted species definition in bio systematics, that members of the same species should hybridize more than 70% with minimal mismatch as displayed by the reduction in melting temperature (Wayne *et al.,* 1987). DNA-DNA reassociation is a method (Cho and Tiedje, 2001) measuring the DNA relatedness of two organisms and has proved to be suitable for the investigation of relationships between closely related taxa, such as species (Stackebrandt and Goebel, 1994). Strains belonging to the same species will generally have greater than 70% DNA-DNA relatedness. The method has been used in numerous studies dealing with Streptomycetes. In some cases, it has shown a good correlation with the identification based on morphology and physiology, but in other cases, there was no apparent correlation (Healy and Lambert, 1991). While DNA-DNA reassociation has shown to be useful in the identification of *Streptomyces* sp. because of the instability of the genome, it should not be used alone, but in connection with other tests (Anderson and Wellington, 2001).

Restriction analysis and DNA/RNA hybridization methods are suitable for distinguishing some *Streptomyces* sp. The RNA/DNA sequencing analysis is a method that registers a phylogenetic relationship. The determination of the full 16S rRNA sequencing of *S. lividans* and *S. coelicolor* has given a possibility for the 2 species to be classified as *S. violaceoruber* (Anderson and Wellington, 2001). The registered partial sequencing of 16S rRNA belonging to genera Streptomycetes and Streptoverticillium together with the data of the numerical phenotypic analysis and chemotaxonomic properties gave a possibility to include genus Stretoverticillium in genus Streptomycetes.

The profound analysis showed the advantages of the molecular, biological methods for actinomycetes taxonomy and indicated that none of these methods applied independently could solve the existing problems in the taxonomy of its largest genus Streptomycetes (Krassimira *et al.,* 1995). A rapid method for identifying filamentous actinomycetes genera was

developed based on 16S rRNA gene restriction patterns. The pattern was generated by using specific restriction endonucleases to perform in silico digestion on the 16S rRNA sequence of all validly published filamentous actinomycete species (Cook and Meyers, 2003).

**Biochemical and physiological characterization of actinomycetes**

The selected efficient isolates of actinomycetes were subjected to their biochemical and physiological characterization and explained in brief as follows.

## 2.1. Biochemical properties

Test isolates of actinomycetes were examined for major biochemical properties such as gelatin liquefaction, $H_2S$ production, reduction of nitrate and hydrogen peroxide (Gottleib, 1960), and tyrosine utilization (Lerch and Ettlinger, 1972), as per the standard protocols.

## 2.2. Physiological properties

The growth of test isolates of actinomycetes at various physiological conditions such as pH (7.0 to 10.0), temperature (30 to 50 $^0$C), and salt concentration (1 to 5%) was assessed. The utilization of sugars (Arabinose, Glucose, Fructose, Galactose, Lactose, Maltose, Mannitol, Sucrose, Xylose) and amino acids (Cysteine, Arginine, Histidine, Tyrosine, Proline, Methionine, Asparagine, Tryptophan, Valine) sources on the growth of test isolate was also observed (Benedict *et al.,* 1955 and Gottleib, 1960).

**Pigmentation profile**

Various means have been employed to use and evaluate color as a criterion for the identification of actinomycetes. As early as 1914, Krainsky attempted the use of a standard color code (Klincksieck and Valette, 1908) in evaluating the isolates of actinomycetes and he prepared colored illustrations of cultures. Kransilnikov (1959) also reported that incubation of temperature will have a greater influence, not only on the growth rate but also on the pigmentation pattern of actinomycetes, which can be an important feature to characterize actinomycetes. Waksman and Curtis (1916) and Waksman (1919) used Ridgway's (1912) color standards and color nomenclature in characterizing the isolates of actinomycetes.

A principal criterion of long-standing for the identification of actinomycetes has been, the color of the mycelium, branching nature of the mycelium, and color of the diffusible pigments in recent years, the surge in studying all phases of the actinomycetes especially the Streptomycetes has been tremendous. Whole the criterion has been re-evaluated and closer attention has been given to the identification of new isolates with more objective characteristic features. Such identification would serve as a base for better isolation and accurate identification of actinomycetes (Pridham, 1965). Color discrimination and the significance of color in distinguishing the isolates of actinomycetes constitute problems of considerable magnitude.

**Electron microscopic sporulation pattern**

Colonial growth on agar plates can be scanned microscopically under high-dry magnification for the presence of sporulation. Scanning electron microscopy can provide far more detailed information concerning the sporulation micromorphology of actinomycetes, particularly those whose spore structures are associated with the vegetative mycelium (e.g., *Micromonospora*). Scanning electron microscopy provides information not only on spore surface characteristics but also on sporophore and spore arrangement and the presence of a 'sheath' or sporangium surrounding spores (Labeda, 1986).

Understanding of systematics of microorganisms has undergone spectacular changes in recent years because of the advantages of current development in molecular biology and computer technology. This has improved the understanding of the relationship between microorganisms and the underlying genetic mechanism on which they are based. A variety of techniques are being employed routinely for microbial classification. However, it is of utmost importance to understand at which level these methods carry and reveal essential information. The kind of information that each technique retrieves is directly related to its resolving power and the correct use of this information is essential to ensure the systematic position of a tax on (Rassello and Aman, 2001).

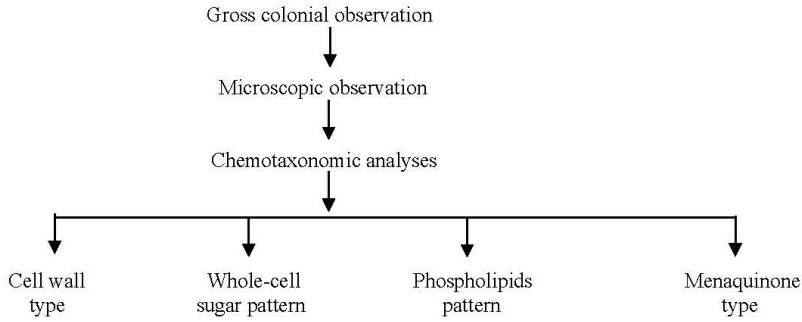
Actinomycetes are analyzed at various levels to gain information suitable for constructing databases and effecting identification. The highest level is the genome and its direct expression as RNA. Sequence analysis of various genes provides a stable classification and accurate identification, which has become the cornerstone of modern phylogenetic taxonomy. The regions of 16S rRNA genes are highly variable and differ significantly

between species, whereas other areas are more conserved and suitable for identification at the generic level (Amann and Ludwig, 2000). This technique is the forerunner of molecular analysis and has now been followed for bacterial identification.

**Molecular characterization of actinomycetes**

Although, morphology is not adequate in itself to differentiate between many genera. That is why molecular methods and chemical methods are being employed for the classification of not only actinomycetes but also for all the prokaryotes and eukaryotes. Actinomycetes can be analyzed at various levels to gain information suitable for constructing databases and effecting identification. The highest level is the genome and its direct expression as RNA. The sequence analysis of the genes coding for the ribosomal subunits (16S, 23S, and 5S rRNA), in particular, the 16S rRNA gene has become an important tool in bacterial identification, since it provides information about the phylogenetic placement of species (Brenner *et al*., 2001). However, the 16S rRNA sequence information was not sufficient for species identification. DNA hybridization offers identification possibilities, indeed chromosomal DNA hybridization forms the basis of the generally accepted species definition in biosystematics, that members of the same species should hybridize more than 70% with minimal mismatch as displayed by the reduction in melting temperature (Wayne *et al*., 1987). Amplified 16S rDNA of soil actinomycetes was restricted with selected endonucleases and electrophoresed on agarose gels. The restriction fragment patterns of the unknown isolates were easily compared to the established patterns (Andrew *et al*., 2003). The application of the bioinformatics-based approach is very promising in microbial ecology, particularly for the understanding of organisms such as Actinobacteria in marine environments (Bull and Stach, 2007). Some investigators propose ELISA (Krassmira *et al.*, 1995), electrophoretic mobility of total protein extracts and computer programmed analysis of radiolabeled protein binding patterns as new approaches in the biosystematics of Streptomycetes. In addition to these many more advanced molecular biological approaches are there for species identification of *Streptomyces* sp. but, none of these methods applied independently could solve the existing problems in the taxonomy of the genus *Streptomyces* sp. (Krassmira *et al*., 1995).

A general outline for the identification of actinomycete isolates to the genus level prepared by Labeda (1986) is as follows.


Gross colonial observation
Microscopic observation
Chemotaxonomic analyses
Cell wall type
Whole-cell sugar pattern
Phospholipids pattern
Menaquinone type

# CHAPTER 3

# SYNTHESIS OF TYROSINASE BY ACTINOMYCETES

Actinomycetes are known to produce several enzymes, degrading complex organic matter in soil or sediments. In India, ample reports are published on actinobacterial enzymes. Diverse environmental conditions affect the populations in a specific microbial niche and regulate the production of various extracellular enzymes. Gulve *et al.* (2011) has reported various enzymes such as proteases, gelatinases, amylases, lecithinases, cellulases, and ureases from the actinomycetes strains isolated from the coastal sediments of Konkan Coast of Maharashtra.

The commercial and research applications of their enzymes are listed in Table 2.

The discovery of different actinobacterial enzymes is a significant contribution in the field of biotechnology viz., thermostable cellulases (2001), proteases, alkali tolerant xylanases (2008), and inulinases are of important industrial application and L-asparaginases have clinical applications as anti-leukemic compounds (2011).

Raja *et al.* (2010) have reported marine actinobacteria producing amylase inhibitors against both prokaryotic and eukaryotic amylases isolated from mangrove rhizosphere of *Rhizophora mucornata* in Vellar estuary, East coast, India.

The first anti-cancer compound was discovered from obligate marine actinomycetes from the sediments of the Caribbean islands, named Salinosporamide A belonging to the genera Salinispora (2009). Suthindhiran *et al.* (2010) isolated actinomycetes from marine sediments of Puducherry coast of Bay of Bengal with cytotoxic activity on HeLa cells. Adinarayana *et al.* (2006) has reported marine actinomycete from sediments of the Bay of Bengal producing two potent cytotoxic compounds

**Table 2: List of enzymes from actinomycetes of Indian origin**

| Sl. No. | Enzyme | Species | Habitat | Location | Year |
|---|---|---|---|---|---|
| 1 | L-Asparaginase | *S. plicatus* | Alimentary canal of fish | Velilake, Kerala | 1997 |
| 2 | Protease | *S. megasporus* | Sediment | Lonarlake | 1998 |
| 3 | Keratinase | *S. thermoviolaceus* SD8 | Sediment | Maharashtra | 1999 |
| 4 | Xylanase | *Streptomyces* sp. | Decaying coconut fibre | Goa | 2000 |
| 5 | CM Cellulase | *Thermomonospora* sp. | Compost | Barabanki Dist., U.P. | 2001 |
| 6 | Inulinase | *Streptomyces* sp. *S. cyaneus, S. tendae* | Soil | Amritsar, Panjab | 2003 |
| 7 | Xylanases | Caelestis | Soil | Delhi | 2006 |
| 8 | L-Glutaminase | *S. rimosus* | Chanoschanos (Estuarine Fish) | Vellar Estuary | 2006 |
| 9 | L-Asparaginase | *Streptomyces* sp. | Marine sediment | Parangipettai coast | 2006 |
| 10 | α-galactosidase | Not identified | Mangrove sediment | West coast, India | 2006 |
| 11 | Cellulase | *S. actuosus* | Mugilcephalus, Estuarine Fin Fish | Vellar Estuary | 2007 |
| 12 | α-galactosidase | *S. greisoalbus* | Mangrove sediment | West coast, India | 2007 |
| 13 | Xylanase | *Kocuria* sp | Alkaline bauxite residue | Damanjodi | 2008 |
| 14 | Polygalacturonase | *S. lydicus* MTCC7505 | Estuarine sediment | West coast, India | 2008 |
| 15 | α-amylase | *S. gulbargensis* | Soil | Gulbarga, Karnataka | 2009 |
| 16 | Keratinase | *S. gulbargensis* | Soil | Gulbarga, Karnataka | 2009 |
| 17 | L-Asparaginase | *S. nouresi* MTCC10469 | Cally spongia diffusa | Kovalam coast, Kerala | 2011 |

identified as resistomycin and tetracenomycin D. They (2010) also reported an actinomycete strain with potent anti-cancer activity, identified as *Streptomyces albovinaceus*. The bioactive compound ($C_{62}H_{86}N_{12}O_{16}$) was active against both gastric adenocarcinoma as well as hepatic carcinoma. The 1HNMR spectrum of the compound showed a similarity to actinomycin.

Kharat *et al.* (2009) have reported a *Streptomyces* sp. from Lonar Lake with significant anti-cancer activity against Human Lung carcinoma cells exhibiting a cytotoxic effect on a monolayer of cells within 48 hours.

Ravikumar *et al.* (2012) have used the metabolite extracts from the actinomycetes isolated from the mangrove sediments of the Manakkudi mangrove ecosystem, Kanyakumari, Tamil Nadu for the cytotoxic assays against the breast cancer cell lines viz., MCF-7 and MDA-MB-231.

Dharmaraj *et al.* (2009) isolated a *Streptomyces* sp. from the marine sponge *Mycale mytilorum* tissue producing carotenoids.

Naik *et al.* (2001) have shown the presence of an extracellular alkaloid "Pimprinine" in the culture filtrate of a *Streptomyces* sp. The compound showed anti-convulsant activity, analgesia in mice models and also inhibited tremoring-induced tremors which contribute to its pharmacological importance.

Kokare *et al.* (2007) have studied the production of emulsifiers from actinomycetes of Alibag, Janjira, and Goan coastal marine sediments.

Deepika *et al.* (2010) have studied the production of biosurfactants from actinomycetes isolated from Ennore saltpan, Tamil Nadu. The isolates were first screened for lipase on Tributyrin agar plates with an ability to collapse the mineral oil drop in a 96 well plate.

Marine actinobacterium *Brevibacterium aureum* MSA13 isolated from the sponge *Dendrilla nigra* collected from the South West coast of India has been used for the production of biosurfactant by Kiran *et al.* (2010).

Lipopeptide biosurfactant has been reported from *Nocardiopsis alba* MSA10 isolated from marine sponge *Fasciospongia cavernosa* by Gandhimathi *et al.* (2009).

Actinomycetes isolated from different habitats of India have been successfully employed as the biological system for the production of nano-particles. Sastry *et al.* (2003) have reviewed the ability of fungi and actinomycetes to synthesize metal nano-particles intracellularly.

A novel alkalotolerant actinomycete, identified to be a *Rhodococcus* sp. isolated from the Fig tree (*Ficus carica*) was employed by Ahmad *et al.* (2003) for the synthesis of gold nano-particles from the aqueous solution of $HAuCl_4$.