Chapter - I

INTRODUCTION
1. INTRODUCTION

The pioneering and invaluable work of Waksman and Woodruff (1942) at Rutgers state university in U.S.A., not only brought to light the ability of actinomycetes to produce antibiotics, the magic cure to many dreadful diseases, but also stimulated the intensive research in this area. Thousand and one actinomycetes were isolated and screened in research laboratories of American pharmaceutical companies and in Krassilnikov's laboratory in Moscow (1959). Actinomycetes are recognised as an important group of microflora and stand out as a unique group of prokaryotic organisms in two respects; the diversity of their morphology and their metabolic products. No wonder actinomycetes became a focus of attention of many microbiologists.

Our first knowledge of actinomycetes dates back to 1875 when Ferdinand Cohn named the organisms found in the tear duct of the human eye as Streptothrix foersteri. Harz (1877) described the organism Actinomyces bovis found in the pus of cattle suffering from the disease now called "Actinomycosis" or Lumpy jaw of cattle. Harz used the term "Actinomyces" to describe the radial arrangement of the branching, mold-like thread of the organism when growing in infected tissues. (Gr. Actino = radial emanations, e.g. Sunlight, Mykes = fungus; hence "ray fungus"). Soon after, other species of actinomycetes were found in soil (Globig, 1888), manure (Tsiklinsky, 1899), grain (Brocq-Rousseau, 1904), compost and hay (Lacey, 1973).

Actinomycetes play an important role in the degradation of organic matter. They produce the highest chemical diversity, which include secondary metabolites of novel structure. About 61% of all the bioactive microbial metabolites were isolated from actinomycetes, especially from streptomycetes and also from some rare actinomycetes (Moncheva et al., 2002).

1.1 Characteristic of actinomycetes

1.1.1 They are prokaryotic bacteria with elongated cells or filaments (0.5 to 2.0 μm dia) usually showing some degree of true branching. They are considered as bacteria
with the ability to form branching hyphae at some stage of their development. They are intermediate in characters between bacteria and fungi.

1.1.2 They are capable of producing a variety of spores which facilitate their rapid dispersal in aquatic habitats (Actinoplanes - zoospores), in air and in soil (conidia). The endospores produced by few actinomycetes ensure viability over many decades.

1.1.3 They are able to attack and metabolize a wide variety of substrates including naturally occurring and synthetic compounds that are usually resistant to microbial decomposition.

1.1.4 They produce a wide variety of secondary metabolites which include antibacterial antibiotics which gives them main advantage in certain microsites.

1.1.5 Their ability to form a mycelium allowing radial growth facilitates colonization on organic debris away from the initial growth centre.

1.1.6 The actinomycetes are nutritionally versatile, being able to grow on nutrient media with rich substrates and also on those containing a minimum or even with an apparent lack of nutrients. For example, a common isolation medium consisting of tap water (Lechevalier 1964) will allow the development of taxa capable of growing on richly supplemented media such as blood agar.

1.2 Isolation of actinomycetes

Most ecological studies of actinomycetes have been carried out with the dilution plate technique. However, their detection by this method does not give an idea about their existence in the natural habitat whether in a spore or hyphae form and it thus becomes difficult to assess the role played by them in it. Moreover, there are inherent limitations of dilution plate technique. The actinomycetal population in soils usually lies between bacteria and fungi. Thus bacteria predominate in number during the isolation. Although the fungal colonies are numerically small their large radial spread interferes in the estimation of actinomycetes.
1.3 Identification of actinomycetes

The actinomycetes have always been a strong group of organisms to the bacterial taxonomists. Taxonomy and nomenclature of actinomycetes has been in a state of chaos until recently because of the lack of:

a. adequate description of the species,
b. uniformity in criteria,
c. standard methods for their characterization.

The number of investigators studying this group of microbes have always been small and these organisms have been neglected by bacteriologists, physiologists and biochemists. Many aspects of their nature, physiology and their role in various natural processes are little understood. This is due to certain factors, among which the prominent is the confusion regarding their morphology, life cycle, systematic position and difficulty in their cultivation. Their biochemical activities are also little understood. Indeed the exact composition and boundaries of the order actinomycetes remain open to question and modification forms the continued application of new taxonomic methods.

They cannot be satisfactorily distinguished from coryneform genera such as \textit{Arthrobacter} and \textit{Corynebacterium} which also have a tendency to produce branched elements. There is therefore some doubt as to wherever the actinomycetes form a natural group or merely a convenient artificial taxon. The little evidence available suggests that actinomycetes form a reasonably tight group which can be usually distinguished from other procaryotic orders. However the evidence from DNA homology experiments suggest that actinomycete DNA is rich in guanine and cytosine and all but a few thermophilic strains have a base composition within the range 62-78.5\% (De ley; 1970). The theoretical predictions of homology and numerical taxonomy all show that the actinomycetes are a related group of nacters. However within the order, there is considerable morphological and physiological diversity.
1.4 Biochemical activities of actinomycetes

Actinomycetes are able to utilize simple compounds like organic acids, sugars, nitrates and aminoacids. They are also able to hydrolyze complex molecules like cellulose, starch, chitin, proteins, lipids and aliphatic hydrocarbons. Chitin hydrolysis is especially characteristic of the actinomycetes. The ability to assimilate atmospheric nitrogen or to carry out denitrification is absent.

Thus actinomycetes together with other saprophytic microorganisms play a significant role in the soil in the breakdown of complex organic compounds to simple forms of carbon, nitrogen, sulphur, phosphorus and other trace elements which then become easily available for absorption by plants.

1.5 Antibiotics of actinomycetes

A number of actinomycetes have antibiotic producing potential. Some important actinomycetes and the type of antibiotics produced by them are-

- Nocardicins - *Nocardia uniform*
- Gentamycin - *Micromonospora purpurea*
- Rifamycin - *Nocardia mediterranei*
- Azaserine - *Actinoplanes deccanensis*
- Carminomycin - *Actiomadura carminata*
- Azureomycin - *Pseudonocardia azurea*
- Midiomycin - *Streptoverticillium rimofaciens*
- Clavulanic acid - *Streptomycyes clavuligerus*
- Olivanic acid - *Streptomycyes olivaceus*

1.6 Aquatic actinomycetes

Actinomycetes are widely distributed in aquatic habitats. They are an integral part of the microflora of fresh-water and have been isolated from rivers, lakes and water...
Many research workers have studied the ecology of aquatic actinomycetes. (Cross, 1981; Kutzner, 1981; Goodfellow and Williams, 1983; Goodfellow and Simpson, 1987).


The most frequent genera of actinomycetes in freshwater habitats include Streptomyces, Micromonospora, Nocardia, Actinoplanes, Streptosporangium (Williams and Cross, 1971; Cross, 1981; Williams et al., 1983). The lakes and reservoirs exhibited a higher number of actinomycetes compared to streams and rivers and have a different specific composition. The mesophilic actinomycetes are common in lakes and rivers whereas thermophilic actinomycetes are less (Willoughby, 1976; Niemi et al., 1982).

The lake and river margins, plant communities at the margins of lakes and rivers particularly where water level fluctuates, will provide ideal substrate and conditions for growth and sporulation of actinomycetes (Silvery and Roach, 1975). Similarly, littoral mid periodically submerged and exposed will also provide wet aerobic conditions for growth and sporulation of actinomycetes. Shallow lakes with high summer temperature and a supply of degradable animal and plant remains would provide ideal conditions for actinomycete growth.

The Actinoplanetes spore vesicles withstand prolonged dessication and release motile spores when rehydrated. The Actinoplanetes are amphibious in nature. They colonize substrates in presence of water; survive under dessication in the form of sporangium and on rehydration release spores (Makkar and Cross, 1982). Micromonospora occur infrequently in soils, but in relatively high numbers in aquatic habitats such as lake-mud and river sediments. The occurrence of Micromonospora in lake systems has been confirmed.
Micromonosporae are frequently present in water samples from streams and rivers.

Nutrient availability is a major factor governing the number and types of Streptomyces in fresh water habitats. Streptomyces grow on the chitinous exoskeletons. The primary reservoir of Nocardia is the soil. Nocardia species also inhabit fresh water habitats (Williams et al., 1983).

As compared to terrestrial and fresh water habitats actinomycetes are less in marine habitats (Weyland, 1969; Cross, 1981; Goodfellow and Williams, 1983). Actinomycetes genera such as Actinoplanes, Geodermatophilus, Microbispora, Micromonospora, Micropolyspora, Nocardia, Rhodococcus, Streptomyces, Streptosporangium, Streptoverticillium and Thermoactinomyces have been isolated from marine habitats (Grein and Meyers, 1958; Weyland, 1969; Goodfellow and Williams, 1983; Eliaiah and Reddy, 1987). Streptomyces predominate in shallow areas of Pacific and Atlantic oceans, whereas micromonosporae and nocardioforms dominate deep-sea sediments (Williams et al., 1983).

1.7 Benificial and deterimental role of actinomycetes

Actinomycetes play both detrimental and beneficial roles in nature. Among their negative attributes is their opportunistic pathogenic nature in disease of animals, humans and forestry and plants. The disease 'lumpy jaw' is characterized by draining abscesses and progressive destruction of the underlying bony structures in cattle. The agent in this case is Actinomyces bovis. Nocardiae produce a number of human diseases. These include pulmonary, neural, and/or systemic nocardiosis; actinomycotic mycetomas, which are tumor like growths of the organisms within the tissues; and localized cutaneous or subcutaneous infections. Nocardia asteroides, Nocardia brasiliensis and Nocardia otitidiscaviarum cause these infections. Nocardia africana sp. now a new pathogen isolated from patients with pulmonary infections (Hamid et al., 2001).
Streptomyces scabies causes blemishing disease of potato known as potato scab. Streptomyces is found to infect variety of other root crops such as sugar beet. Streptomyces ipomoeae causes 'soil rot of pox' of sweet potato.

Streptomyces and Thermomonospora species are being implicated in aflatoxin production. The spores of many actinomycete species easily become airborne following mechanical disturbance, others may be liberated in aerosols from humidifiers and other equipments to pollute the air, and as a result, diseases such as farmer’s lung, and hypersensitivity pneumonitis can occur. They also pollute water with their cells, causing bulking and scumming in sewage treatment plants and tainting of drinking water with their metabolites. The production of earthy tastes and odors in reservoirs and water supplies has often been attributed to Streptomyces (Wood et al., 1983, Zaitlin et al., 2003). Geosmin (trans-1, 10-dimethyl-trans-9decalol) and Certain strains of the species belonging to the genera Actinoplanes, Amorphosphorangium, Micromonospora and Spirillospora have been shown to parasitise the spores of phytophthora megasperma var. sojae (Sneh et al., 1977).

1.8 Chitin

Chitin, a linear β-(1,4)-linked N-acetylglucosamine (GlcNAc) polysaccharide (Cabib et al., 1986) and (Gooday et al., 1990), is a main structure component of fungal cell wall and the exoskeletons of invertebrates, such as insects and crustaceans (Flach et al., 1992 and Jeuniaux et al., 1966). It is one of the most abundant, naturally occurring polysaccharides and has attracted tremendous attention in the fields of agriculture, pharmacology and biotechnology. (Muzzarelli et al., 1985, 1987). This linear polymer can be hydrolysed by bases, acids or enzymes, such as lysozyme, some glucanases and chitinases.

Chitinases (EC 3.2.1.14), essential enzyme catalysing the conversion of chitin to its monomeric or oligomeric components, have been found in a wide range of organisms, including bacteria (Cody, 1990), plants (Shinshi et al., 1987), Fungi (Bartnicki et al., 1968) and crustaceans (Koga et al., 1987). Plants produce chitinases as a defence against fungal pathogens (Mauch et al., 1984). Because chitin is not found...
in vertebrates, it has been suggested that inhibition of chitinases may be used for the treatment of fungal infections and human parasitosis. In addition to the potential applications of chitinase itself, the chito-oligosaccharides (GlcNAc) have been found to function as anti-bacterial agents, elicitors, lysozyme inducers and immuno-enhancers. Due to these biological interests, the preparation of chito-oligosaccharides is becoming one of the new targets in the carbohydrate industry.

1.9 Occurrence of Chitin

Chitin occurs widely in nature as a structure polymer in lower animals, fungi and algae. In lower animals it is found principally in the phyla Annelida, Arthropoda (crustacean insects, etc) and mollusca (snails, squids etc.) and to a lesser extent in coelenterata (marine organisms such as hydrids and jellyfish) and Nematoda (unsegmented worms) (Deshpande, 1986).

Amongst microorganisms the occurrence of chitin is confined to fungi and green algae. All fungi belonging to Phycomycetes, Ascomycetes, Basidiomycetes, fungi imperfectii, with possible exception of some Oomycetes and Monoblepharidales, have chitinous cell wall. In nature chitin is not found alone, and usually forms a part of very complex system, such as chitin-protein complex, calcium carbonate in addition to protein, and organic substances (Hackmen, 1954).

1.10 Structure of Chitin

X-ray diffraction studies of chitin structure indicates three different types of crystallographic patterns among chitins, alpha, beta and gamma chitins. These forms differ with respect to arrangements of GlcNAc chains, binding to water molecules with the chain, unit cell structure and dimensions, compactness of the chains, crystallinity of the molecule intramolecular and intermolecular electrostatic interaction. Alpha chitin is a compact structure as compare to beta and gamma chitins. Beta and gamma chitins are more open structure and therefore, easily attacked by chemical agents such as acid and alkali. These chitins are more easily hydrolysed by chitinases (Carlstrom, 1962; Ramchandran and Ramkrishnan, 1962).
1.11 Chitinolytic enzyme system

The enzymatic hydrolysis of chitin to its monomers, dimers and oligomers is performed by a chitinolytic enzyme system consisting of three hydrolases (Flach et al., 1992; Deshpande 1986).

1. Endochitinase (E.C.3.2.1.14)
   
   This enzyme cleaves glycosidic linkages randomly within the chitin polymer into short oligomers of N-acetyl glucosamine (NAG), generally dimers or trimers (Muzarrelli; 1993).

2. Exochitinase (E.C.3.2.1.14)
   
   This enzyme also called as exo-N,N'-diacetyl chitobiohydrolase or chitobiosidase. It catalyzes the progressive release of diacetethylchitobiose in a step-wise fashion from a non-reducing end of (GlcNAc)ₙ.

3. 1,4-3-N acetyl-glucosaminidase (E.C.3.2.1.30)
   
   This enzyme splits chitin polymer chain and chitobiose into GlcNAc monomers in an exotype fashion (Haran et al., 1996).

In addition to the major pathway of chitin degradation by chitinases there are other enzymes involving initial deacylation of chitin to chitosan and further breakdown of chitosan.

i. Chitin deacetylase:

   Chitin polymer is deacetylated to chitosan by chitin deacetylase e.g. in *Mucor rouxii* (Araki and Ito, 1974).

ii. Chitosanase:

   This enzyme depolymerises chitosan e.g. in *Rhizopus rhizopodiformis*, *Myxobacter* sp. and *Bacillus* R-4 (Tominaga, 1975).

1.12 Occurrence of chitinases in microorganisms

Chitinases have widespread distribution in microorganisms, plants and animals. Among bacteria, chitinases are produced by *Serratia*, *Chromobacter*, *Klebsiella*, *Pseudomonas*, *Clostridia*, *Vibrio*, *Arthrobacter*, *Beneckea*, *Aeromonas* and *Erwinia*. Among actinomycetes, *Streptomyces*, *Micromonospora* and *Actinoplanes* produce...
chitinases, (Iverson et al., 1984; Clarke and Tracey, 1956).

Among fungi Trichoderma, Penicillium, Verticilium, Neurospora, Mucor Beauveria, Lycoperdon, Aspergillus, Myrothecium, Conidiobolus and Agaricus produce chitinases (Ulhoa and Peberdy, 1992; Deshpande, 1986).

1.13 Transformation of Xenobiotics

Transformation of xenobiotics is defined as the structural modification of components foreign to an organism’s metabolism which occur in its chemical environment. The most characteristic reactions in transformation of Xenobiotics are oxidative, reductive, hydrolytic, dehydraion and condensation.

The ability of actinomycetes to perform a variety of microbial conversions of organic compounds is an important factor in the complicated processes of biodegradation of pollutants in soil and water and in the biodegradation of pesticides, oil spills, chemical waste decomposition and the like.

Member of the genera Nocardia and Streptomyces have ability to perform highly selective chemical modification of complicated compounds of natural and synthetic origin. Nocardia strains have been found to degrade aromatic hydrocarbons, first by hydroxylation, followed by other reactions (Kieslich, 1976).

Actinomycetes indigenous in soil and water are probably the first line of attack on hydrocarbon molecules as these compounds are not broken down by the majority of microorganisms. In general actinomycetes have the ability to hydroxylate aliphatic chains of hydrocarbons in the terminal and subterminal positions and subsequently followed by shortening of the transformed chains (Golovelev et al., 1978).

Actinomycetes are able to degrade certain pesticides. The herbicide, dalapon, 2,2-dichloropropionic acid, was degraded by selected Nocardia strains isolated from soil (Hirtsch and Alexander, 1960). DDT (1,1,1-trichloro-2,2-di-4-chlorophenyl-ethane) is dechlorinated by Streptomyces aureofaciens, Streptomyces cinnamoneous and Nocardia erythorpolis (Chacko et al., 1966).

Actinomycetes are also involved in the transformation of steroids. Mixed culture of Arthrobacter simplex and Streptomyces roseochromogenes was used to insert a
double bond and hydroxy group in the desired position of the substrate 9-fluorohydrocortisone (Lee et al., 1969).

The application of actinomycetes in bioorganic chemistry to perform microbial transformation of organic compounds has become a field of great interest and of commercial value. The ability to effect subtle modifications of complicated organic compounds has been employed in the production of several interesting products, some of which have been subjected to further chemical synthesis to provide valuable pharmaceutical agents.

1.14 Biodegradation of textile dyes

Azo dyes represent the largest class of dyes used in textile processing and other industries connected with producing colored materials like different plastics. They are extremely versatile colorants and constitute about 50% of dyes produced (Manu and Chaudhari). The history of industrial production of azo dyes is more than 100 years and on the time being it is produced in the amount of more than 7,00,000 tons per year. For example now in textile industry for each 25 tons of fibers are usually used 2 tons of azo dyes. Nowadays, there are about 5000 commercial dye products. The main characteristics of azo dyes is N=N connection between aromatic rings. These N=N bonds give azo dyes the ability to absorb electromagnetic spectrum in visible region due to extended conjugated pi electron system. Because of that ability, azo dyes can have different colors and can be used in industry as dyes.

Taking into account that fixation rate of azo dyes in dyeing process can be as low as 50% to about 10-15% (Donlon et al., 1997; Tan and Field, 2000), as much as 30% of the initial azo dye applied remains unfixed and ends up in the effluents (Manu and Chaudhari, 2003). Dyes are synthetic in nature and generally alien to the natural biotic environment and hence persist in nature. The relationship is mentioned between azo dye chemical structure and its biological characteristics. All azo dyes containing a nitro group are mutagenic and toxic. On contrary, sulfonated azo dyes show decreased or no mutagenic effect compared to unsulfonated azo dyes due to their electric charge and low lipophilicity, which prevent them from uptake and metabolic activation (Tan and Field, 2000).

There exist different methods of physical and chemical azo dye degradation such as photochemical degradation with hydrogen peroxide, electrochemical destruction, and
adsorption and so on. But effective physical and chemical treatment usually needs specific conditions and material (high pressure, temperature, radiation and so on) that make the process expensive, high energy consuming and not ecofriendly (Rittmann, 2002). On contrary biological treatment of wastewater, including azo dye effluents can be performed in soft conditions (Robinson et al., 2001). In general azo dyes are considered as stable compounds difficult for biodegradation. Degradation of azo dyes can be performed by two ways, first is direct oxidation of azo dye molecule that has serious obstacles in biological process. Second way is a preliminary reduction of N=N bond which result in splitting of azo dye molecule to another products, usually aromatic amines and following oxidation of products. This process is more preferable from thermodynamic point of view (Rajaguru et al., 2000) and usually used by activated sludge in biological azo dye wastewater treatment.

The azo group is substituted with benzene or naphthalene groups, which can contain many different substituents such as chloro (-Cl), methyl (-CH₃), nitro (-NO₂), amino (-NH₂), hydroxyl (-OH) and carboxyl (-COOH). A substituent often found in azo dyes is the sulfonic acid group (-SO₃H). The azo dyes containing this substituent are the so-called sulfonated azo dyes. Water-soluble azo dyes, like the sulfonated azo dyes, will enter the environment generally with wastewater discharges. Also these sulfonated azo dyes are widely used in different industries (Zollinger, 1987). Sulfonated and unsulfonated azo dyes have a negative aesthetic effect on the wastewater and some of these compounds and biodegradation products are also toxic, carcinogenic and mutagenic (Grover et al., 1996). Furthermore, some azo dyes can produce toxic degradation products such as substituted benzidines, like o-tolidine (Rosenkranz and Klopman, 1989; Rosenkranz and Klopman, 1990).

There exists clear evidence that sulfonated azo dyes show decreased or no mutagenic effect compared to unsulfonated azo dyes due to their electric charge and low lipophilicity, which prevents them from uptake and metabolic activation (Chung and Cemiglia, 1992; Jung et al., 1992; Levine, 1991; Rosenkranz and Klopman, 1990). Due to the above mentioned effects. It is clear that azo dyes should not enter the environment. An attractive method to prevent this is to apply microbial treatment method for their mineralization. Due to the fact that preliminary reduction is going better in an anaerobic condition and oxidation on contrary needs an aerobic condition, the reasonable scheme of azo dye water treatment is anaerobic-aerobic process (Kalyuzhnyi, 2000).
1.15 The aerobic biodegradation of (sulfonated) azo dyes

Although for a long time it was thought that azo dyes remained recalcitrant under aerobic conditions some specific aerobic bacterial cultures were found to be able to reduce the azo linkage via an enzymatic reaction. The azo reductases isolated from these organisms have a narrow substrate range (Kulla, 1981; Kulla et al., 1983; Zimmermann et al., 1982; 1984). The occurrence of aerobic conversions of sulfonated azo dyes were more recently reported by Heiss et al., (1992) and Shaul et al., (1991), and sometimes even a complete mineralization of a sulfonated azo dye was found under aerobic condition. A bacterial strain S5, derived from *Hydrogenophaga palleronii* S1, was able to reduce the azo dye 4-carboxy-4'-sulfobenzene and to mineralize the azo dye reduction products 4-aminobenzenesulfonic acid (4-ABS) and 4-aminobenzoic acid. The sulfonated azo dye was used as carbon energy source in this case (Blumel et al., 1998).

Also a bacterial strain M2, isolated from a biofilm reactor, was able to utilize Acid Orange 7 and 8 as sole carbon, nitrogen and energy source and the azo dye reduction product 4-ABS was also degraded (Coughlin et al., 1997). Furthermore, it was found that *Sphingomonas* sp. Strain ICX could use the sulfonated and unsulfonated azo dye, Acid Orange 7, Acid Orange 8, Acid Orange 10, Acid Red 4 and Acid Red 88 as sole carbon and nitrogen source. All these dyes contained a hydroxyl group next to the azo bond on a naphthalene ring. However, a complete mineralization of Acid Orange 7 was not obtained because the azo dye reduction product 4-ABS accumulated (Coughlin et al., 1999). In some studies, aerobic color removal of certain azo dyes was achieved, but all these strains required an additional energy and carbon source for their growth. Since the supply of this additional substrate could have easily led to the formation of anaerobic microniches, the occurrence of anaerobic azo dye reduction certainly cannot be excluded (Govindaswami et al., 1993; Horitsu et al., 1977; Hu, 1994; 1998; Wong and Yuen, 1996; Yatome et al., 1991; Zissi et al., 1997). Similarly, the degradation of azo dyes was also observed in aerobic biofilm reactors, but this also may have been a result of the presence of anaerobic microniches in the biofilm (Costerton et al., 1994; Harmer and Bishop, 1992; Jiang and Bishop, 1994). This certainly might prevail when additional substrate was supplied. In some cases the evidence for the occurrence of mineralization of the aromatic amines is poor (Harmer and Bishop, 1992; Ogawa and Yatome, 1990). The successful degradation of Acid Red 151 as sole carbon source was described using an aerobic sequenced biofilm reactor and mineralization experiments showed that 73% of the carbon was transformed into carbon dioxide (Quezada et al., 2000)