CHAPTER TWO

ECOLOGY OF ACIDOPHILIC ACTINOMYCETES
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2.1 Introduction:

Assessing microbial biosynthetic diversity has historically involved the isolation and cultivation of chemically prolific taxa and, of those now recognized as such, bacteria within the order Actinomycetales are without rival. The value of actinomycetes to society in terms of providing useful drugs, especially antibiotics and anticancer agents, and to the pharmaceutical industry for providing revenue generating discovery platform, is indisputable.

Actinomycetes have been recognized for over hundred years primarily on morphological criteria. They are usually considered to be bacteria with ability to form branching hyphae at some stage of their development. This attribute can be difficult to detect and is not always sufficient to distinguish actinomycetes with a transient mycelium from some other gram-positive bacteria (Goodfellow and Williams, 1983).

Actinomycete metabolites or derivatives thereof accounted for approximately two thirds of the naturally occurring antibiotics discovered as of 2003, making them one of the single most important sources of prescription drugs (Kanavade, 2003). These bacteria, which are best known from soils, have been the focus of aggressive research efforts since the discovery of actinomycin in 1940 from *Actinomyces antibioticus* by Selman Waksman at Rutgers University. Additional actinomycete products such as the clinically important antibiotics streptomycin and novobiocin helped fuel the post-war antibiotic revolution and firmly cemented these chemically prolific bacteria in the centre stage of natural products drug discovery research. Exhaustive studies of soil actinomycetes over the last 60 years have led to many significant discoveries, but the effectiveness with which chemically rich yet common soil taxa, such as *Streptomyces*, have been studied has created a situation where new classes of compounds are increasingly difficult to discover (Toivola *et al.* 2002). This decline can, in large part, be attributed to the repeated isolation of common species and the subsequent rediscovery of unacceptably large numbers of previously described metabolites. The process of evaluating the chemical novelty of microbial metabolites, also called ‘dereplication’, is time consuming and, if too many known substances have to be processed, takes resources away from the more important tasks of structure elucidation, molecular derivatization, and preclinical development. In an effort to improve the ratio of new to known compounds discovered, there has been a push within the pharmaceutical industry to work with novel or rare actinomycete taxa. This strategy has proved successful, leading to important discoveries such as the extraordinarily potent calicheamicin family of enediyne antitumor antibiotics from *Micromonospora echinospora* and the clinically useful aminoglycoside antibiotic, gentamicin from *Micromonospora purpurea*. These studies, however, have largely been restricted to actinomycetes from traditional habitats, such as neutral soils, and
it remains possible that unique actinomycete populations remain undetected in unexplored environments, such as the acidic soils (Jensen et al., 2003).

Microbial ecology can be defined as the study of the behavior and activities of microorganisms in their natural environments (Brock, 1993). Microbial ecology had its beginning in the work of Koch and Pasteur. It was Pasteur who first discussed the role of the microbes in natural habitats. Koch, the medical bacteriologist, proposed postulates that provided the basis for the study of the microbes in natural habitats. Sergei Winogradsky, first provided evidence that microorganisms were responsible for a specific transformation in nature. Goals of microbial ecology (Marshal, 1992) are:

- To define population dynamics in the microbial community,
- To define the physicochemical characteristics of the microenvironment, and
- To understand metabolic processes carried out by microorganisms in their natural habitat.

2.1.1 Microbial Community:

Progress in microbial population ecology has been very slow as a result of our inability to isolate and describe even a minor population of any microbial community. Detailed community analysis can provide data on population changes over space and time that should allow the construction of accurate models to predict population responses to environmental perturbation, description of biogeochemical cycling, biodegradation, bioremediation and description of new organisms with unique physiological properties in various environments.

The biological activity in soil is largely concentrated in the top soil, the depth of which may vary from a few to 30 cm. In top soil, the biological components occupy a tiny fraction (<0.5%) of the total soil volume and make up less than 10% of the total organic matter in soil. These biological components consist mainly of soil organisms, especially microorganisms. Despite their small volume in soil, microorganisms are key players in the cycling of nitrogen, sulphur, phosphorus, and the decomposition of organic residues. Thereby, they affect nutrient and carbon cycling on a global scale (Pankhurst et al., 1997). That is, the energy input into the soil ecosystems is derived from the microbial decomposition of dead plant and animal organic matter. The organic residues are in this way, converted to biomass or mineralized to CO₂, H₂O, mineral nitrogen, phosphorus and other nutrients (Bloem et al., 1997). Mineral nutrients immobilised in microbial biomass are subsequently released when microbes are grazed by microbivores such as protozoa and nematodes (Bloem et al., 1997). Microorganisms are further associated with the transformation and degradation of waste materials and synthetic organic compounds (Torstenson et al., 1998).
In addition, microorganisms also affect the physical properties of soil. Production of extra-cellular polysaccharides and other cellular debris by microorganisms help in maintaining soil structure, as these materials function as cementing agents that stabilise soil aggregates. Thereby, they also affect water holding capacity, infiltration rate, crusting, erodibility, and susceptibility to compaction (Elliott et al., 1996).

Microorganisms possess the ability to give an integrated measure of soil health, an aspect that cannot be obtained with physical/chemical measures and/or analyses of diversity of higher organisms. Microorganisms respond quickly to changes, hence they rapidly adapt to environmental conditions. The microorganisms that are best adapted will be the ones that flourish. This adaptation potentially allows microbial analyses to be discriminating in soil health assessment, and changes in microbial populations and activities may therefore function as an excellent indicator of change in soil health (Kennedy and Papendick, 1995).

Microorganisms also respond quickly to environmental stress compared to higher organisms, as they have intimate relations with their surroundings due to their high surface to volume ratio. In some instances, changes in microbial populations or activity can precede detectable changes in soil physical and chemical properties, thereby providing an early sign of soil improvement or an early warning of soil degradation (Pankhurst et al., 1993). An example is the turnover rate of the microbial biomass. This is much faster, e.g. 1-5 years, than the turnover of total soil organic matter (Carter et al., 1999). Observations in the Dutch soil monitoring programme have shown that most microbial indicators indeed have discriminating power relative to different soil treatments (Schouten et al., 2000). This has also been shown for microbial biomass and basal respiration at a regional scale in the USA (Brejda et al., 2000).

The bioavailability of chemicals, e.g. heavy metals or pesticides, is also an important issue of soil health because of its connection with microbial activities. The impact of such chemicals on soil health is dependent on microbial activities. For example, the concentration of heavy metals in soil will not change over small time periods, but their bioavailability may. It has thus been shown that the bioavailability of poly-aromatic hydrocarbons was lower in autumn compared to early spring due to a higher microbial activity after the growing season. Therefore, the total content of chemicals in soil is not a reliable indicator of its bioavailability and thereby, soil health. Instead, bioavailability has to be measured in relation to bioassays and specific microbial processes. In context of this, microbial responses also integrate the effect of chemical mixtures, information not obtained by studying the chemical mixtures themselves (Logan, 2000).
2.1.2 Ecology of Actinomycetes:

Soil is not only important as a means of supporting crop production. It is an essential natural resource which should be maintained for future generations. A measure of soil quality is important for determining whether soil is being degraded. Microorganisms may constitute less than 0.5% of the total soil mass, but they are essential for nutrient cycling and exert a major influence on soil fertility. It is not clear whether the genetic diversity of soil microbes is an indicator of soil quality, or if the diversity of microbial functions is the most important factor. These are not necessarily the same. The toxic effects of heavy metals on soil microorganisms are well reported (Havlin et al., 1999). However, a few studies have examined the more subtle effects of heavy metal pollution on the genetic diversity of particular groups of microorganisms. Studies that have been conducted tend to concentrate on the loss of microbial function in soil, with little consideration of the effect of bacterial biodiversity. A number of conventional methods were used to compare populations in the soils with different levels of contamination, while functional diversity was tested using BIOLOG™ (NCIMB Ltd.). They had applied a molecular technique, ERIC DNA fingerprinting, which allowed them to study biodiversity at a molecular level, with the resolution required to see subtle changes in community structure (O'Flaherty et al., 1999).

Actinomycetes are found in a wide range of different soil types in diverse geographical locations and play an important role in the degradation of polysaccharides, cellulose and chitin. Chitinases are produced in abundance by bacteria and majority of the actinomycetes have chitinolytic activity. The chitinolytic activity in the soil indicates that streptomycete chitinases are highly conserved and distributed within the streptomycetes. A soil that was known to have high chitin content and a bacterial community that was almost entirely dominated by actinomycetes (Miyashita et al., 1997).

Successful ecosystem management depends on an understanding of the mechanisms controlling the functioning and stability of these ecosystems. It is recognised that soil microorganisms mediate important ecosystem processes such as nutrient cycling. However, the identity and functional significance of these organisms is poor due to the lack of methodologies to study microbial diversity. Samples were taken over one-year period to determine the degree to which temporal variation and management influence microbial community variability. It is apparent that soil microbial communities are strongly influenced by vegetation type and site. It is also clear that broad scale measures of microbial biomass show little temporal variation as...
compared to measures of community structure, which vary significantly with time (Grayston et al., 1999).

Soil is dominated by a solid phase consisting of particles of different size surrounded by water and gases, the amount and composition of which fluctuate markedly in time and space. Water is normally discontinuous, except when the soil is water saturated. The pore space without water is filled with air and other gases and volatiles (Stotzky, 1997). There is continual interchange of molecules and ions between solid, liquid and gaseous phases which are mediated by physical, chemical and biological processes (Doran et al., 1994). These processes represent a unique balance between physical, chemical and biological components. Maintaining this balance is of great importance to soil health (Doran et al., 1994).

2.1.3 Acidic Soils:

About 25 to 30% of the soils in the world are classified as acidic and represent some of the world's most important food-producing regions. In the United States most of the acidic soils occur in the east and northwest regions, where annual precipitation usually exceeds 24 hrs. (Havlin et al., 1999).

Soil organic matter or humus, contains reactive carboxylic and phenolic groups that behave as weak acids releasing H⁺. The soil organic matter content varies with the environment, vegetation and soil; thus, its contribution to soil acidity varies accordingly. In peat and many soils and in mineral soils containing large amounts of organic materials, organic acids contribute significantly to soil acidity. The sources of soil acidity are organic materials, humus, clay, oxide minerals, aluminium, iron polymers, soluble salts and carbon dioxide. The acidic, neutral or basic salts in the soil solution originate from mineral weathering, organic materials decomposition or addition as fertilizers and manures. Soil behaves like a weak acid that will buffer the pH. In acidic soil adsorbed Al⁺³ will maintain equilibrium with Al⁺³ in the soil and the acidity of the soil buffered by aluminium hydrolysis reactions.

Increasing soil acidity in ecosystem is caused by use of commercial fertilizers, especially NH₄ sources that produce H⁺ during nitrification; plant removal of cations in exchange for H⁺; leaching of cations being replaced first by H⁺ and subsequently by Al⁺³; and decomposition of organic residue. Natural acidification of soils is enhanced with increasing rainfall since rain has a pH of 5.7 or less, depending on pollutants such as SO₂, NO₂, and others. Soil acidity dose not develop in a year or two, but the extent and rate of pH vary among soils. A problem might
develop in five years on a sandy soil or ten years on a silt loam soil but may take 15 years or more on clay loam (Havlin et al., 1999).

For many years the optimum soil pH for agriculture production was considered to be between 6.5 to 7.0; however, lime sufficient to reduce pH to about 5.6 or 5.7 and reduce exchangeable Al** to less than 10% (Havlin et al., 1999).

2.1.4 Non-Acidic Soils:

Actinomycetes constitute a significant component of the microbial population in natural soils and counts over 1 million per gram are commonly obtained. The soil is also the most prolific source of isolates, which includes many microbes found to produce antibiotics and other useful metabolites. It is, therefore, the most intensively studied habitat, but despite this there are still many gaps in our knowledge of the rules played by actinomycetes in soil process. Many studies have been primarily concerned with the enumeration and identification of isolates.

The growth of actinomycetes in natural soil has been studied by various microbial techniques. It appears that actinomycetes exist for extended periods as resting arthrospores that germinate in the occasional presence of exogenous nutrients particular by organic substrate, such as root fragments and dead fungal hyphae are rapidly colonized by mycelium, which soon produce spores above the substrate; several different strains may grow together in a restricted area. Most soil actinomycetes also behave as neutrophilic in culture, growing between pH 5.0 – 9.0 with an optimum close to neutrality.

2.1.5 Types of Actinomycetes in Acidic Habitats:

i) Streptomycetes:

Streptomycetes are gram-positive, filamentous soil bacteria that undergo morphological differentiation during their life cycle. They normally occur as spores, but in the presence of sufficient moisture and nutrients, the spores can germinate and form vegetative mycelium. In response to environmental signals, such as a shortage of nutrients or water, the process of differentiation is set in motion and spores resistant to desiccation and starvation are formed again. At the same time, the production of pigments, antibiotics and other secondary metabolites is initiated (Williams et al., 1989).

Streptomycetes are common in soil, but also found in composts, fodder and aquatic habitats. Due to their characteristic life cycle, they are good survivors under the fluctuating growth conditions predominating in nature (Kutzner, 1986). Streptomycetes have also been isolated from indoor environments, from air, building material and dust samples (Hyvärinen et
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They are considered as indicators of moist conditions in buildings that are favorable for microbial growth (Samson et al., 1994).

Streptomycetes are known to be producers of many secondary metabolites, which have different biological activities, such as antibacterial, antifungal, antiparasitic, antitumor and immunosuppressive actions (Demain, 1999). They are not particularly pathogenic, although some species can also cause infections (Mishra et al., 1980), but they have been shown to be potent inducers of inflammatory responses in vitro and in vivo (Jussila et al., 2002).

Culture methods are currently used for the exposure assessment of microbes in indoor environments. However, it has been estimated that only 0.001-15 % of the environmental microbial population can be cultured (Amann et al., 1995); for indoor environments a value of 1% cultivability has been reported (Toivola et al., 2002).

The number of species in the genus Streptomyces is increasing continually. In 1997, 464 validly described species and 45 subspecies were reported (Hain et al., 1997), in September 2002 there were over 650 species listed in the German Collection of Microorganisms and Cell Cultures (GCMCC). Thus, the genus is the largest of the order Actinomycetales within the class Actinobacteria (Stackebrandt et al., 1997). The genera Streptoverticillium and Kitasatospora have been included in the genus Streptomyces (Wellington et al., 1992), although the taxonomic position of Kitasatospora is unclear (Zhang et al., 1997).

ii) Actinoplanes:

Actinoplanes currently include those hyphae-forming, gram-positive, non-acid-fast members of the order Actinomycetales in which the spores (motile or non-motile depending on the genus) are produced within spore vesicles or sporangia. About 15 genera have, at one time or another, been reduced to the following genera: Actinoplanes, Ampullariella, Dactylosporangium and Pilimelia. This classification has been based almost entirely on the morphology of the spore vesicle or sporangium (Stackebrandt and Woese, 1981).

The studies of peptidoglycan composition and DNA-DNA reassociations have shown that this classification is artificial and that at least two major DNA homology clusters with distinct peptidoglycans can be recognized (Stackebrandt and Woese, 1981). The genera in DNA homology cluster I, which we will now call the Actinoplanetes, have a wall chemotype II. The peptidoglycan contains meso- or 3-hydroxy diaminopimelic acid and glycine; the latter amino acid would appear to replace L-alanine in the peptide to muramic acid which is glycolated (Kawamoto et al., 1981). Whole-organism hydrolysates contain xylose and arabinose with variable amounts of other sugars. The same composition has been found in the genus Micromonospora which forms single spores not enclosed within a vesicle or sporangium on the
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substrate mycelium and *Catellatospora* which forms vegetative hyphae are branched but not fragmented, no true aerial mycelium is produced, short chains of nonmotile spores arise singly or in tufts from the vegetative hyphae on the surface of agar media. The genera which grow in acidic habitats include *Micromonospora* and *Catellatospora* (Kawamoto et al., 1981).

iii) **Nocardioform Actinomycetes:**

Nocardioform actinomycetes are aerobic, gram-positive, catalase-positive actinomycetes that show considerable morphological diversity. This aggregate group includes organisms classified in 18 genera viz., *Gordona*, *Nocardia*, *Rhodococcus*, *Tsukamurella*, *Actinobiospora*, *Actinokinospora*, *Actinopolyspora*, *Amycolata*, *Amycolatopsis*, *Kibdelosporangium*, *Pseudoamycolata*, *Pseudonocardia*, *Saccharomonospora*, *Saccharopolyspora*, *Nocardoides*, *Terrabacter*, *Jonesia* and *Oerskovia*. Cell wall peptidoglycan is based upon meso-diaminopimelic acid, major cell wall sugars are arabinose and galactose. The genera found in acidic soils are *Nocardia*, *Saccharomonospora* and *Saccharopolyspora* (Goodfellow and Cross, 1984).

iv) **Maduromycetes:**

The term Maduromycetes has been introduced for a collection of sporoactinomycetes that have a wall chemotype III and contains the sugar madurose. Cell wall peptidoglycan is based upon meso-diaminopimelic acid, gram-positive, non-acid-fast. The group currently contains seven genera viz., *Actinomadura*, *Microbiospora*, *Microtetraspora*, *Planobiospora*, *Planomonospora*, *Spirillospora* and *Streptosporangium*, all of which can be readily distinguished from *Dermatophilus* and *Frankia*, which also contain madurose, on morphological ground. The genera present in acidic soils are *Actinomadura*, *Microbiospora* and *Microtetraspora* (Goodfellow, 1994).

v) **Thermomonospora:**

These aerobic spore-forming actinomycetes produce a branched vegetative mycelium bearing aerial hyphae. The cell wall contains meso-diaminopimelic acid but not characteristic sugars or other amino acids. This group contains four genera viz., *Actinosynnema*, *Nocardiopsis*, *Streptotilateichus* and *Thermomonospora*. The genera *Thermomonospora* and *Nocardiopsis* grow in acidic soils (McCarthy, 1994).

vi) **Thermoactinomycetes:**

Well developed substrate and aerial mycelium is typical of actinomycetes. Single spores are borne on both aerial and vegetative hyphae. The spores are true bacterial endospores and are heat resistant. The wall structure is gram-positive; it contains meso-diaminopimelic acid but not characteristic sugars or other amino acids. They are aerobic and saprophytic chemoorganotrophs.
utilize a range of sugars as carbon and energy sources and are able to degrade various polymeric substrates. This group contains one genus which can grow at acidic pH e.g. *Thermoactinomyces* (Goodfellow and Cross, 1984).

**vii) Other Genera:**

This group comprises three genera whose combination of morphological and chemotaxonomic characterstic do not permit simple assignment to an established group of actinomycete genera. They do, however, have two properties in common: the production of long chains of spores on the aerial mycelium and the absence of mycolic acids in the cell envelope. Cells are aerobic and chemoorganotrophic. Cell wall peptidoglycan contains meso-diaminopimelic acid. This group contains three genera viz., *Glycomyces* whose whole cell sugars are xylose and arabinose, and can grow at acidic pH. Other two genera are *Kitasatosporia* whose cell wall contains L- and meso-diaminopimelic acid and galactose as whole cell sugar, and *Saccharothrix* whose whole cell sugars are galactose and rhamnose (Holt et al., 1994).

### 2.1.6 Extreme Sites:

There are two extreme acidic regions, and 21 soil samples were collected from seven different sites of these regions:

**i) Mahabaleshwar:**

Mahabaleshwar is a hilly region in Satara district of Maharashtra state, India. On the basis of Agro Climatic Zones in Maharashtra, it comes under Western Ghat Zone. Soil in this area is light lateritic, reddish brown, distinctly acidic in nature. Most of the area is under forest and the average rainfall recorded is about 4000-6000 mm at various places. Soil samples were collected from three different sites of Mahabaleshwar i.e. *Wilson point*, which has vast barren rocks with juncos litter of soil, *Bombay point* which is covered with dense forest, black soil and *Lingmala point* where Lingmala waterfall is one of the famous waterfalls in Mahabaleshwar which is coming from Venna lake and Cascades down in steep drop has juncos litter of soil and the pH of the soils from these locations is 5.4, 5.5 and 5.7 respectively.

**ii) Sana'a:**

Sana'a is the capital city of Yemen, situated in the inland, 100 km from the coast of Red Sea, on a plateau at an elevation of more than 2000 meters above sea level. It was selected as the spot of acidic soil however, it dose not identify with any podsol. The pH of the soil was found to be 5.5 and thus samples were taken from this region for the isolation of acidophilic actinomycetes. The site of sampling remarks cultivable garden soil, although it is very unusual that the soil has an acidic pH despite any specific vegetation. However, one cannot exclude the possibility of it coming under a specific geographic belt. There are four sites in Sana'a viz.,
Madbah Garden, Science College Garden Sana'a University, Main Building Garden Sana'a University and Al-Saiaji Garden, from which soil samples were collected.

### 2.1.7 Non-Extreme Sites:

There are seven non-extreme regions from which the soil samples were collected:

- **Pune:**
  
  Pune, one of the very few places in India which has great and wonderful geographical location. Located at an altitude of 2,000 ft above the sea level and partially hidden by the mighty ranges of the Sahyadri Mountain. The soil samples were collected from three sites, two of which were acidic viz., Main Building Garden of Pune University and Botanical Garden, Aundh.

- **Aurangabad:**
  
  This city is located 356 km. north east of Mumbai, India, the climate of which is very hot in the summer. It is sugarcane growing agriculture area. The sampling sites included Ajanta was 100 km. from Aurangabad, Ellora which is 30 km. from Aurangabad, and last site was Aurangabad Garden.

- **Ahmednagar:**
  
  Ahmednagar is small city located 100 km. north east of Pune, India. It is agriculture city and the site of sampling is located in the centre of the city from where three agriculture soil samples were collected from Ahmednagar Garden.

- **Alibag:**
  
  Alibag, a coastal city located 175 km. south of Mumbai, India, and situated in the east coastal side of India on the Arabian Sea. The nine soil samples were collected from three sites in this location included two beach sand and one beach mud samples.

- **Hudaydah:**
  
  Hudaydah is a city at the west coast of Red Sea in Yemen. Soil samples were obtained from Al-Shab and Al-Hudaydah Gardens near the coastal regions of Red Sea. Red Sea is also popularly known as Coral Sea rich in red coral reefs which leave a dull red tidal scum at the edge of water. Red Sea is remarkable by an explosive growth of blue - green algae, *Trichodesmium erythraeum* which makes the water orange-red. Four sites were selected; three of them have acidic soil.

- **Aden:**
  
  This is the important port of Yemen located at the south towards the Arabian Sea at Gulf of Aden. The bay of Aden has originated from crater of volcano and it is the only remains of the original. Out of four sites, two of them have acidic soil; Goldmore and Kormaksir Gardens which are located near the coastal regions of Arabian Sea.
vii) Makkah:

Makkah is a well-known holy city situated 80 km from the Red Sea coast of Saudi Arabia. Samples were obtained from Al-Abdia, 20 km from Makkah and very close to Arafat the place visited by millions of people and is surrounded by agriculture area and desert. Al-Azizia and Al-Setten Gardens are located in Makkah city which are agriculture soils.

viii) Jiddah:

Jiddah is the main port in Saudi Arabia situated at Red Sea. The region has oil refineries and the climate in this region is humid and hot. From the inside of the coast starts the desert that covers most of the region except mountains. Three soil samples were collected out of which, one was acidic (Plate 2.1, 2.2, and 2.3).
Plate 2.1:

a-c) Sampling regions.
Plate 2.2: a-d) India sampling sites.
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Plate 2.3:  
a-e) Saudi Arabia and Yemen sampling sites.
2.2. Materials and Methods:

2.2.1 Sampling:

Soil samples were collected from different sites. Soil samples were collected using handheld scoops (10 to 100 g capacity), spoons (100 to 500 g capacity), and shovels were used for sampling near surface soils. The top layer of soil was removed to the desired depth with a clean spade and then using a clean stainless steel scoop, plastic spoon or shovel, a thin layer of soil from the area which came in contact with the spade was removed and discarded. The samples were transferred to an appropriate, labeled sterile sample container with a sterile laboratory spatula or equivalent (Kanavade, 2003).

2.2.2 Estimation of pH of the Samples:

Soil samples were suspended in the distilled water in 1:1 ratio and mixed vigorously, and allowed to stand for 3-5 min and then the pH of the suspension was recorded using a digital pH meter (Lab. India, Thane). The system was calibrated with the standard buffers provided with the unit (Nawani, 2002).

2.2.3 Determination of Temperature at the Sampling Site:

The temperature of the samples was determined at the site using a digital thermometer (Orion Co., USA). The thermometer probe was retained in the sample until constant reading was attained. The temperature was recorded in degree Celsius (°C) (Shejul, 1998).

2.2.4 Preparation of Selective Media:

Starch Casein Agar (SCA) of pH 4.5 was used for isolation of acidophilic actinomycetes. To minimize changes in pH of the medium, SCA was buffered to desired pH using KH₂PO₄ buffer. Required volumes of 1 N HCl or 1 N NaOH were added to the medium after autoclaving to adjust the pH to desired value (Nawani, 2002).

2.2.5 Isolation of Acidophilic Actinomycetes from Different Sites:

Isolation of acidophilic actinomycetes was done by suspending 1 g of soil sample in 10 ml sterile water, which was vigorously shaken and allowed to settle for 5 min. The supernatant was serially diluted and plated on Starch Casein Agar (SCA) of pH 4.5, followed by incubation at 37 °C for 14 days. The isolates were enumerated and selected for further study. The pH of the buffered medium was checked during incubation (Kanavade, 2003).

2.2.6 Confirmation of Acidophilic Actinomycete Groups:

The selected isolates were grown on Starch Casein Agar (SCA) of pH 4.5, 7.0, and 10.0, to minimize changes in pH of the medium. SCA was buffered to desired pH using KH₂PO₄ buffer. Required volumes of sterile 1 N HCl or 1 N NaOH were added to the medium after
autoclaving to adjust the pH to desired value. The group of acidophilic actinomycetes depends upon the growth pH (4.5) (Seong, et al., 1993).

2.2.7 Growth of Acidophilic Actinomycetes on Different Media:

Growth characters of acidophilic actinomycete isolates were studied on following media of pH 4.5 viz., Starch Casein Agar (SCA), Glycerol Asparagine Agar (GAA), Nutrient Agar (NA), Yeast extract-Malt extract Agar (YMA), Starch Yeast extract Agar (SYA), Glycerol Yeast extract Agar (GYA), Czapeck's Dox-Thom Agar (C-Dox), and Sabouraud's Dextrose Agar (SDA) of pH 4.5. The inoculated plates were incubated at 28 °C for 14 days and observed for growth characters and pigmentation (Shejul, 1998).

2.2.8 Media used for the Maintenance of Acidophilic Actinomycetes:

Isolates of Acidophilic Actinomycetes (AA) were maintained on Starch Casein Agar (SCA) at 4 °C. They were also preserved at -20 °C in 20% glycerol in distilled water, where glycerol acted as a cryoprotectant (Kanavade, 2003).
2.3 Results:

2.3.1 Physicochemical Characteristics of the Samples:

pH:

There are two extremely acidic regions viz., Mahabaleshwar, India, and Sana'a, Yemen, which were selected for isolation of acidophilic actinomycetes. Nine soil samples from three sites in Mahabaleshwar were collected. The pH of all sites varied from 5.4 (Wilson Point) to 5.7 (Lingmala Point). The second region was Sana'a from which 51 isolates were selected. The pH of all Sana'a sites varied from 5.5 (Main Building Garden, Sana'a University) to 6.1 (Science College Garden) (Fig 2.1). In all 12 soil samples were collected from four sites in Sana'a region for isolation of acidophilic actinomycetes. The total isolates from seven extremely acidic sites were 194.

The pH of other 24 sites varied from 6.2 (Al-Hudaydah Beach Garden) to 8.2 (Mula River, Pune), from 24 non-acidic sites were 411 (Fig 2.2). The maximum 51 acidophilic actinomycete isolates were from Sana'a region Main Building Garden, Sana'a University site no.9. From India 35 acidophilic actinomycetes were isolated from Wilson Point site no. 5 from Mahabaleshwar region.

Temperature:

The temperature of the sites varied from 18.7°C in Lingmala site, Mahabaleshwar region to 43.4°C in Al-Setten Garden site, Makkah region. Maximum isolates from Main Building Garden, Sana'a University site no. 9 were 51, where temperature was 20.1°C and from India maximum isolates were from Wilson Point site no.5 from Mahabaleshwar region were 35, where temperature was 19.4°C, and Ellora site from Aurangabad region were 35 acidophilic actinomycetes, where temperature was 39.8°C.

The extremely acidic sites had temperature between 18.7°C Lingmala site, Mahabaleshwar region and Science College Garden, Sana'a University site 21.2°C Sana'a region. The non-acidic sites had temperature between 25.0°C at Main Building Garden, Pune University site, Pune region and 43.4°C Al-Setten Garden site, Makkah region.

Soil Types:

Most of the soil samples were collected from agriculture fields. In extremely acidic sites, the soil types were Juncus litter from Mahabaleshwar region and agriculture soil from Mahabaleshwar and Sana'a regions. The maximum acidophilic actinomycete isolates from agriculture soil were 51 at site no. 9 from Sana'a region and from juncus litter 35 at site no.5 from Mahabaleshwar region and pH of this site was 5.4 (Fig. 2.3).
Fig 2.1: Frequency of occurrence of acidophilic actinomycetes at extreme acidic sites and their pH and temperature.

Fig 2.2: Frequency of occurrence of acidophilic actinomycetes at non-acidic sites and their pH and temperature.
In non-acidic sites, the soil samples were mostly from agriculture fields from 14 sites out of 24 and the remaining were beach mud, beach sand, sand, and sand dune from 10 sites. Maximum isolates from non-acidic sites in agriculture fields in Ellora, Aurangabad were 35 from site no. 29 and minimum eight isolates were from site no. 13 from beach mud on Hudaydah (Fig. 2.4).

Soil Colour:

Acidic sites soil color varied from red to brown and maximum isolates were from site no. 9 from Sana'a region where agriculture soils are brown. Other Sana'a region sites have same color and same soil type. Mahabaleshwar region soil color is between red and brown and maximum isolates were from site no. 5, Wilson Point, where soil type is juncus litter. Red soil is acidic but most of the brown soils are acidic and some of them are neutral (Fig. 2.5).

At non-acidic sites, the soil color varies from black to yellow. Maximum isolates were from site no. 29 where soil is brown and minimum isolates from site no. 13 where soil is black. All yellow and black soils are neutral and most the dark and light brown soils were neutral to alkaline. Desert and beach sand are poor in acidophilic actinomycetes. pH and colour of these soils is above 7 and yellow to black respectively (Fig. 2.6).

Country:

In all 605 acidophilic actinomycetes were isolated from three countries, 241 were from India, of which 8% were from Pune region soils the pH of which was between 6.6 and 8.2 and 11.5% of the isolates were from Mahabaleshwar region, the extremely acidic site the pH of which was between 5.4 and 5.7. Alibag region had 39 isolates of acidophilic actinomycetes (6.5%) the pH of which was between 7.2 and 7.7. Aurangabad region had 69 acidophilic actinomycete isolates (11%) the pH of which was between 6.9 and 7.3. The last region is Ahmednager from which 12 isolates (2%) were isolated; the pH of this region was 7.5.

From Yemen 257 acidophilic actinomycete isolates were obtained from three regions one of them is extremely acidic whose pH was between 5.5 and 6.1 and the number of isolates was 124 which equals to 20% of the total isolates and 48% of the total isolates from Yemen. At Al-Hudaydah region, 68 acidophilic actinomycetes were isolated which corresponds to 11% of the total isolates and 27% of the total isolates from Yemen. The pH of this region was between 6.2 and 7.4. At Aden region, 65 isolates were isolated which equals to 10.5% of the total isolates and 25% of the total isolates from Yemen.

In all 107 acidophilic actinomycetes from Saudi Arabia regions were isolated which equals to 18% of the total isolates. At Makkah region 67 isolates which equals 11% of the total were isolated, the pH of those region was between 6.7 and 7.2. Second region in Saudi Arabia is
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Fig 2.3: Soil-type wise frequency distribution of acidophilic actinomycetes at acidic sites and their pH and temperature.

Fig 2.4: Soil-type wise frequency distribution of acidophilic actinomycetes at non-acidic sites and their pH and temperature.
Fig 2.5: Soil colour type-wise frequency distribution of acidophilic actinomycetes at acidic sites and their pH and temperature.

Fig 2.6: Soil colour type-wise frequency distribution of acidophilic actinomycetes at non-acidic sites and their pH and temperature.
Jiddah from which 40 isolates were isolated (6.5%), the pH of this region was between 6.9 and 7.8 (Fig. 2.7).

In all 42% of the actinomycete isolates were from Yemen regions and Sana'a is the major region which constituted 20% of the total isolates. In India all regions constituted 40% of the total isolates and Mahabaleshwar region constituted 11.5% of the total isolates (Fig. 2.8).

### 2.3.2 Confirmation of Acidophilic Actinomycete Groups:

Starch casein agar (SCA) with varied pH was used to classify acidophilic actinomycete isolates into three groups i.e. acidoduric, neutrotolerant and strictly acidophilic actinomycetes based on optimum pH for their growth. Acidoduric actinomycete group which can grow at pH ranging from 4.5 to 7 and optimally at pH 7. This group constituted 279 acidophilic isolates which equals to 46% of the total and maximum isolates of this group were from site no. 20 at Makkah region and site no. 29 at Aurangabad region, the pH of both regions was near 7. All the sites contained acidoduric actinomycetes and minimum isolates of this group were at site no. 6 of Mahabaleshwar region (Fig. 2.9).

Neutrotolerant acidophilic actinomycete isolates constituted 36% of the total isolates. This group of actinomycetes can grow at pH ranging from 4.5 to 7, and optimally at pH 6.5. Maximum isolates of this group were at site no. 9 of Sana'a region, and minimum isolates from the site no. 24 of Jiddah region (Table 2.1).

Strictly acidophilic actinomycetes were isolated from 22 sites. This group of actinomycetes was present at the sites whose pH was below 7 and absent in the sites of pH above 7. This group can grow in the pH range between 3.5 and 6.5 and optimally at pH 4.5. This group constituted 18% of the total isolates which were mostly obtained from site no. 9 of Sana'a region and site no.5 of Mahabaleshwar region (Fig. 2.10).

Neutrotolerant and strictly acidophilic actinomycete groups were selected for further study. The total isolates of these two groups constituted 54% of the total isolates. The isolation of acidophilic actinomycetes was done on SCA of pH 4.5. Viable count of bacteria was enumerated on nutrient agar (NA) of pH 7.0. The results obtained using NA and SCA were compared. In the acidic soils the number of bacteria was $5 \times 10^5$ cfu g$^{-1}$ while actinomycetes were $1.3 \times 10^6$ cfu g$^{-1}$. The results indicated that 73% of the bacteria were other than actinomycetes.

### 2.3.3 Growth of Acidophilic Actinomycetes on Different Media:

Growth of acidophilic actinomycete isolates was studied on different media including ISP and other media i.e. starch casein agar (SCA), glycerol asparagine agar (GAA), yeast-extract malt extract agar (YMA), starch yeast extract agar (SYA), glycerol yeast extract agar (GYA),

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Fig 2.7: Country-wise distribution of acidophilic actinomycetes.

Fig 2.8: Site-wise distribution of acidophilic actinomycetes.

Fig 2.9: Different group of acidophilic actinomycetes and their frequency of occurrence.
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Fig 2.10: Site-wise distribution of acidophilic actinomycete groups.

Fig 2.11: Growth of acidophilic actinomycete groups on different media.
Ecology of Acid.

Sabouraud's dextrose agar (SDA), Czapek's dox-thom agar (C-Dox), and nutrient agar (NA). Interestingly, it was observed that same culture showed different spore mass colour and substrate mycelial colour on different media. Growth pattern was also different. The spore mass colour was significant on starch casein agar (SCA) and salt starch casein agar (0.5% NaCl), which had been selected as standard media, the spore colour on which was used in the identification of actinomycetes (Plate 2.4). On glycerol asparagine agar the colour of the substrate mycelium and diffusible pigment was prominent.

All acidophilic actinomycetes could grow very well on SCA, GAA, and YMA of pH 4.5. Most of the acidophilic actinomycetes could grow on NA, SYA, and GYA 65% of the isolates could grow on C-Dox agar and 82% could grow on SDA. All neutrotolerant and strictly acidophilic actinomycetes could grow on SCA, GAA, and YMA, but all neutrotolerant acidophilic actinomycetes could grow on GYA and NA, 64% could grow on C-Dox, 95% could grow on SDA, and 99% could grow on SYA. Strict acidophilic actinomycetes could grow very well on SYA, 98% could grow on GYA, 97% could grow on NA, and 66% could grow on C-Dox and SDA (Fig 2.11).
Table 2.1: Site-wise distribution of acidophilic actinomycetes.

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* 1 is no. of actinomycete isolates, 2 is no. of acidoduric isolates, 3 is no. of neutrotolerant isolates, 4 is no. of strictly acidophilic isolates.
Plate 2.4:

a-p) Acidophilic actinomycetes produced pigment on Starch Casein Agar (SCA).
2.4. Discussion:

There were no seasonal fluctuations in the pH of different sites. pH of Mahabaleshwar and Sana'a regions were higher than that of other regions. Low pH of those two regions was attributed to the high organic matter which decreases the pH. Same observation was made by Williams and Robinson, (1981) in acidic soil and litter. Both Sana'a and Mahabaleshwar regions have similar pH, temperature, soil types, color and height from the sea level around 2000 mts. and located on the mountain areas. Non-acidic sites were found less of organic matters, more salinity and have more pH from neutral to alkaline and soil types from coastal mud, desert sand to agriculture soils.

Temperature of acidic regions was not fluctuated with seasons. Temperature of Mahabaleshwar and Sana'a regions were lower than other regions. Forest trees and plants matters of acidic region attributed the amount of sunlight reaching the service and stable the temperature of the soil. For non-acidic region the fluctuation of the temperature was slightly more with seasons.

Soil types of non-acidic sites were slightly different from beach mud, beach sand, sand, sand dune, and agriculture soil, which contains less organic matters. For acidic sites the large number of acidophilic actinomycetes present in agriculture and juncus litter soils which decrease the pH of the soil. Maximum acidophilic actinomycetes isolated of acidic sites from agriculture soil compare to juncus soil because of the mineral and specific pesticide which added to this soil and made the soil flora suitable to plant and plant associated microorganisms like actinomycetes.

Colour of the soil affected by the soil composition and percentage of each mineral (Havlin et al., 1999). Most of the agriculture and forest soils colour which were collected from acidic and non-acidic sites were from yellow, red, brown to black. The pH of those soils was from acid to neutral. Red soil mostly present in forest and rich in organic matters. The population of actinomycetes was very high but the total population of the other microorganisms was very high, the fungi population is high too. Brown soil was agriculture soil and contains minerals like magnesium, sodium, and iron. Presence of the fertilizer and pesticide increase the population of acidophilic actinomycetes and decrease the fungi in this soil (Havlin et al., 1999). Most of the other soil types like yellow, light brown, and grey which contains more silicon, carbonate and form sand soil. Those soil present mostly in desert and costly regions. The population of acidophilic actinomycetes is very low because, the pH of this soil is neutral to alkaline and less in organic matters.

From the country-wise and site-wise results the acidophilic actinomycetes belong to Yemen sites in the first place mostly from Sana'a region. The region is representing the typical
acids from the number of the acidophilic actinomycetes population, geographical, pH, temperature, organic matters, type and colour of the soil. The diversity of the acidophilic actinomycetes present in this region gives this region specialty. Second country in the population of the total isolates is India. Mahabaleshwar is the region which give typical acidic soil and similar to Sana’a in Yemen. The similarity between two regions from the number of total isolates is the concentrate for soil which has same characters and properties. Saudi Arabia has very less population of acidophilic actinomycetes because the regions which we selected for sampling have very high temperature and the soil type is not suitable for acidophilic actinomycetes. Most of Saudi Arabia regions have same soil characters like Makkah and Jiddah. The desert and coastal sand soil type contain very less organic matters and minerals, which most regions in Saudi Arabia. The rain level in those regions is very low compare to other countries.

The isolation scheme used here, intended not only to study diverse sites and ecology but also to obtain actinomycetes with tolerance acidity and having unusual features. Isolation at acidic pH demonstrated the presence of three different groups of actinomycetes which tolerated acidic pH. In general, a higher predominance of actinomycetes are acidoduric actinomycetes, which was seen in most of the samples. This group of actinomycetes has a wide range of pH growth and belonging to neutrophilic actinomycetes. The second group is neutrotolerant actinomycetes which were classified by Seong et al. (1993) as acidophilic actinomycetes. This group has a wide range of pH growth more than the third group, strictly acidophilic actinomycetes. Those last two groups consider as acidophilic actinomycetes by Seong et al. (1995). Strictly acidophilic actinomycetes appear to be confined to acid soils only and neutrotolerant actinomycetes are numerous in acid soils close to neutrality. The optimum pH for hydrolysis of organic matters is broadly related not only to the optimum pH for growth but also to the pH of the soil from which the isolates come (Williams and Robinson, 1981), which was typical to our observation. Increases and decreases in the pH of the soil is generally not expected but may happen in some localized sites of decomposition which explains the occurrence of acidoduric in acid habitats.

Soil is very rich source of actinomycetes, where selection pressure leads to the enrichment of a particular group of actinomycetes and in competition these groups are expected to express certain phenotype, which confer upon them an advantage over other. The isolates capable of maximum organic matters hydrolysis at acidic pH can be important for recycling of those compounds in acidic soils (Livanainen et al., 1993).

Actinomycetes population in both the pH regions were significant and had correlation with seasonal variations, content of organic matter and other environmental factors. Acidophilic
actinomycetes population of both the pH regions were 15 fold higher than that of the other actinomycetes population (Nawani, 2002). Fungal population was very high in acidic regions which may be because of the pH of the soils. Acidic bacterial population of acidic regions was very high more than other type of bacteria (Lee and Hwang, 2002).

Acidophilic bacteria and actinomycete population in acidic soils were higher than that of the other soil samples because of the high organic matter and low pH content of this soil (Livanainen et al., 1993). During rainy season the bacterial and actinomycete count were higher. Cross and Attwell (1974), have reported that Streptomyces spores are being washed continually by rain into streams, rivers, lakes and sea.

Growth on different culture media showed interesting variations. Growth on starch casein agar formed bunch of spores along with spore chains, sporulation was also taking place in short time compared to other media and colonies were growing very well. Majority of the acidophilic actinomycetes isolates tolerated less salt concentration than the other groups of actinomycetes. Growth on glycerol asparagine agar has similar growth character to starch casein agar but the production of diffusible and melanin pigment was more in glycerol asparagine agar. Growth on sabouraud's agar and C-Dox agar were typical, colonies were gemlike and crystalline. Sporulation was also delayed on C-Dox medium. Sporulation was affected by number of factors such as. composition of the culture medium, temperature of incubation and presence of specific compound and growth factors stimulatory to its development (Kalakoutksi and Agre, 1976). For all the acidophilic actinomycetes groups the growth was similar on all tested media. Actually, the regulatory signals that commit certain portions of the mycelial biomass to sporulation are not well understood. It has been assumed that the sporulation is triggered by nutrient limitation (Ochi, 1987).

Diffusible and melanin were found to vary with culture medium to medium and with incubation condition. Production of diffusible and melanin pigment was rare in actinomycetes from all acidic sites. Even though, melanin is reported to be the protective mechanism from photoinactivation; it was produced by important isolates (Alexander, 1970).
2.5. References:


