CHAPTER FOUR

TAXONOMY OF ACIDOPHILIC ACTINOMYCETES
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4.1 Introduction:

Identification is the practical side of taxonomy. It is an essential component in a clinical set-up and is important to biotechnologists, ecologists and too many others. On the basis of a small number of characters, phenotypic, genotypic or a combination of both, an attempt is made to put a name to an isolate by assigning it to an already established group. Phenotypic characterization is easy to do and some basic tests, morphology and general type of metabolism may be a good starting point. A new isolate may be placed into a broad category on the basis of such initial tests. Additional tests are performed to further narrow the range of possibilities. At this stage one has some idea about the category or group the isolate belongs to and is likely to be in a position to use diagnostic tables or keys and perform specific tests for establishing the identity (Chakrabarti et al., 1998).

The term 'taxonomy' has been used with somewhat different definitions by microbiologists. According to Cowan, (1955) it is a mixture of three components, i.e. classification, nomenclature and identification. (1) The classification includes orderly arrangement of units. (2) The nomenclature involves naming and labeling of units. (3) Identification of the unknown with a unit defined and named by 2 constitute third component. In bacterial taxonomy the system of classification in Bergey's Manual of Determinative Bacteriology has been widely employed. The bacterial isolates are identified by testing the cultural appearance, cell morphology, biochemical characteristics, physiological characteristics, cell wall sugar and amino acids, cellular fatty acid composition and ecological characteristics and by referring to the system of classification (Shejul, 1998).

In any microbiological study, identification and classification of an isolate is of most importance for studying fundamental science, development of biotechnological processes, treatment of diseases, if an isolates is a pathogen, manipulation of environmental conditions, if an isolate is a producer of industrially important products.

Characterisation of a micro-organism first involves obtaining a pure culture. This normally necessitates lengthy periods of incubation. Next, a wide variety of morphological, serological, nutritional and biochemical tests are usually performed. Many of these tests have to be incubated, read and interpreted. Conventional diagnostic microbiology is therefore inherently slow because of the need to grow the organism and subsequently to carry out diagnostic tests. The time taken to characterise a microorganism is thus dependent on its growth rate (members of the slow growing mycobacterial group may take three to six weeks to grow). Thus, in just about every area of microbiology the more rapid, but still accurate, characterisation of microorganisms is a desirable objective. In medicine, shortening the time taken to identify a
pathogenic bacterium, yeast or fungus will accelerate targeted prescription and would lead to improvements in epidemiological studies. In industry, speedy characterisation will allow for better quality control procedures on both raw materials and finished products, and allow accurate microbial screening for isolates producing novel pharmacophores, thus saving time and money. In pure science the ability to characterise large numbers of microorganisms quickly will be beneficial, for example, in ecological studies involving bacteria from aquatic or soil habitats (Goodacre, 2004).

Several characters of an isolate are used in identification and classification of actinomycetes. Classification of actinomycetes dates back to Cohn, (1875), who first described actinomycetes. Prior to 1943, all the species of actinomycetes were included in a single genus which was frequently designated by different names, two common names amongst these were *Streptothrix* and *Actinomyces*. Since Rossi-Doria's, (1891) description of actinomycetes, several workers used different criteria for classification of actinomycetes. Krainsky, (1914), Waksman and Curtis, (1916) and Waksman, (1919) emphasized the use of synthetic substrates in addition to organic media in identification. Drechsler and Waksman, (1919) stressed the use of micromorphology of actinomycetes in classification. Gause *et al.*, (1957) emphasized the use of colour of the substrate mycelium and aerial mycelium as well as morphology of sporulating hyphae, in classification of actinomycetes. Krassilnikov, (1949) insisted on the importance of shape and morphology of spores in actinomycetes identification. Baldacci *et al.*, (1953) suggested the use of micromorphological criteria viz. segmentation and branching of vegetative generic classification (Shejul, 1998).

Bacterial systematics has undergone revolutionary change in the last 25 years. The application of new and reliable biochemical, chemical, genetical, numerical and molecular techniques have been responsible for rapidly changing view on how bacteria ought to be classified and identified.

Most of the new taxonomic techniques, such as DNA-DNA association, 16s' rRNA cataloguing (Fox *et al.*, 1992), G+C content determination, are used in identification and classification of actinomycetes (Stackebrand and Woese, 1981). Cell wall types based on cell wall and whole cell sugar analysis are used to classify actinomycetes. (Lechevalier and Lechevalier, 1979).

Chemical systematics is a rapidly expanding discipline in which information from chemical analysis of whole organisms or cell fractions is used for identification and classification, and for tracing evolutionary trends. A wide array of chemical methods is now commonly used to determine DNA base composition (Owen and Pitcher, 1985). Whole cell
sugar, cell wall amino acid and lipid composition of bacteria, is being increasingly used as chemical markers (Schleifeld and Schaal, 1980). Simple qualitative analysis of actinomycetes classified into eight large groups or wall chemotypes based on the limited distribution of a small number of wall components (Becker et al., 1965).

Actinomycetes are usually rich in lipids and the structural variations that exist amongst the various lipid classes are being used for taxonomic purposes (Minnikin and Goodfellow, 1980) especially of coryneform and nocardioform actinomycetes. Lipid analysis is being used to a limited extent in the classification of sporoactinomycetes (Lechevalier et al., 1981).

Numerical taxonomy has been the most effective modern method used to establish relationships between actinomycetes at the subgeneric level (Jones et al., 1983). Numerical taxonomy involves the construction of a large database for many strains that are grouped into clusters on the basis of shared similarities. Numerical phenetic methods have been applied relatively sparingly in the classification of sporoactinomycetes as many investigators have continued to rely on a small classification of these organisms (Kutzner et al., 1978).

4.1.1 Characters used in the Identification of Acidophilic Actinomycetes:

Poor systematics and a dearth of suitable selective isolative methods accounted for many of the problems experienced by those searching for new and rare actinomycetes. Actinomycetes are a very large group of bacteria comprising several genera, having numerous characters. The distinguishing characters of them are used to identify them from other bacteria. Until recently, actinomycetes were derived from a small number of subjectively weighted morphological and behavioral properties. Strains were assigned to taxa based on colony, micromorphology, staining properties, the presence and absence of spore, colour of the spores, spores chains morphology, spores surface, grow on various media, production of diffusible pigments, the ability to produce acid from sugars, and the capacity to grow on different carbon sources, cell wall analysis, whole cell sugar analysis, molecular techniques such as DNA-DNA association and 16 's' rRNA cataloguing (Goodfellow and O'Donnell, 1989).

Taxometric methods have also revealed that strictly acidophilic actinomycetes, which grow between pH 3.5 and 6.5 but not at pH 7.0, with an optimal pH 4.5, and neutrotolerant acidophilic actinomycetes which grow from pH 3.5 to around neutral pH with an optimal range between 5 and 5.5, form a diverse groups (Seong et al., 1995). These organisms are readily recovered on different acidified media. There enzymes adapted to function at lower pH than those of neutrophilic actinomycetes (Williams and Robinson, 1981).
1. Cultural Characters:

i) Colony:

Cultural and colonial features may provide presumptive evidence for the taxonomic position of an unknown isolate. Colonies can be characters on the basis of some morphological feature and colour, but very few taxa can be recognized with any degree of certainty on selective media. The morphological appearances and colour of most of the colonies depend upon the growth media (Nolan and Cross, 1998).

ii) Formation of Pigment:

The characters of large numbers of colonies by a preliminary grouping basis on pigmentation characteristics. Growth media composition is important basis for pigment formation. The formation of deep brown to black pigments on organic media containing proteins and protein derivatives, notably the amino acid tyrosine, is an important characteristic (Williams and Vickers, 1988).

2. Morphological Characters:

Among the morphological exotic forms, actinomycetes occupy a special position, exhibiting a structure which ranges from relatively 'simple' rods and cocci to a complex mycelium organization similar to that of some eukaryotes (Locci and Schaal, 1980).

i) Substrate Mycelium:

When an actinomycetes propagate, such as a spore, is allowed to grow on a solid substrate, it usually gives rise to hypha, which branch at intervals and spread radically. The resulting mycelium, consisting of hypha that either penetrate the substrate or grow along its surface and colour represents of the main characters of actinomycetes. The majority of spores-forming actinomycetes including the genus Streptomyces produce a nonfragmenting, branched substrate mycelium. In Thermoactinomyces, Micromonospora, Micropolyspora, and Microellobosporia, the substrate mycelium occurs regularly (Cross and Al-Diwany, 1981). The mycelium growth habit has been related to their ability to break down insoluble organic materials by extracellular enzymes. Rhodococci is produced pseudomycelium; the filament extends and subsequently fragments into rods. In Nocardia, a true mycelium is formed, in the early stages of growth and subsequently undergoes fragmentation into smaller units. Further morphological development is represented by the appearance of elementary branching, a phenomenon that has been reported in mycelial bacteria. Extreme care should be taken, however, in distinguishing between 'true' and 'false' branching (Locci and Schaal, 1980).
ii) Aerial Mycelium:

After a certain amount of growth of the substrate mycelium, vertically developing filaments may be formed. In some cases, such as aerial hyphae arise as side ramifications of single, isolated and scarcely branched substrate mycelia. Claims to the existence of special structures controlling aerial filament formation appear doubtful. With further development, a network of aerial hyphae may cover the colony surface, giving it atypical 'hairy' or 'powdery' appearance. The presence, absence, colour and appearances of the aerial mycelium is important criteria in the identification of actinomycetes. The production of an aerial mycelium is influenced by a number of factors including the composition of the growth medium, the incubation temperature and the presence of specific stimulating compounds (Locci and Schofield, 1989).

In the genus *Sporichthya*, growth consists only of aerial filaments adhering by holdfasts to the surface of the growth medium (Williams and Cross, 1971). Single, mostly unbranched, vertically hyphae frequently coalescing into 'synnemata' can often be observed in a mycelial *Mycobacterium* and *Actinomyces* species (Locci and Schofield, 1989). In some *Nocardia*, aerial growth may be quite abundant, although substrate hyphae organization is rather elementary. If sporulation takes place, the entire filament is involved in the process, with no differentiation into sporophore and spore chain (Locci, 1976). *Streptomyces* form a mycelial 'mat' on which sporulating structures are differentiated. Hyphal arrangement in *Streptovorticillium* species confers a loses constancy to the aerial mat, which is 'cottony' in appearance, contrasting with the 'powdery' texture of most streptomycetes. In *Actinoplanes* and *Streptosporangium* aerial mycelium development usually ceases with the onset of sporulation. Lytic processes also take place in ageing colonies and may play a role in motile or non-motile spore liberation of some species (Locci and Schaal, 1980).

iii) Spores:

Presence, or absence of the spores, spore chain morphology, absence of sheath on the spore chains, spore morphology, spores enclosed in the sac, motility of spores, number of spores in chain, colour of the spores on the specific nutrients medium and hygroscopicity of the spores, are the specific spore characteristics useful in identification of actinomycetes. Actinomycetes spores have been categorized as being either endogenous or of 'hyphal origin' (Williams et al., 1971). Pattern of sporulation, such as spiral, e.g. *Streptomyces*, straight e.g. *Streptovorticillium*, and *Actinopolyspora*, rectiflexibles, e.g. *Actinomadura*, retinaculaparti, e.g. *Streptomyces*. The spore number on mycelium, single, e.g. *Thermoactinomyces*, *Thermomonospora*, *Micromonospora* and *Saccharomonospora*, or in pairs, e.g. *Microbispora*, or in short chains,
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e.g. Microtetraspora, and Catellatospora. The spore may be borne within sporangia, which are produced on well developed aerial hyphae or on the substrate mycelium, with non-motile spore. e.g. Streptosporangium, with motile spores, e.g. Actinoplanes (Locci and Schofield, 1989).

Spore shape and morphology also play an important role in characteristics of actinomycetes. Spore shape may be oblong, oval, spherical and rod shaped. Kiss et al., (1945), first used electron microscope for studying spore morphology. Spores have different morphologies such as hairy spores, spores with smooth surfaces and warty surfaces (Shejul, 1998).

iv) Special Structures:

Some actinomycetes form unusual structures, spherical structures on their aerial hyphae or embedded in an amorphous matrix, e.g. Kibdelosporangium, or sclerotia are globose structures formed by some of the streptomycetes. These sclerotia contain not spores but cells filled with lipids. They germinate as a whole, as do the pseudosporangia of Kibdelosporangia (Holt et al., 1994).

3. Physical Characters:

i) Growth at Different pH:

As most actinomycetes are neutrophiles, the pH of the media is usually between 6.7 and 7.5. However, it is necessary to reduce the medium pH 4.5-5.0 to identify acidophilic, and acidotolerant actinomycetes groups (Khan and Williams, 1975). Acidoduric actinomycetes have ability to grow at pH below 7.0 but the optimum pH 7.0 (Williams and Wellington, 1982a). Micromonospora has ability to grow below 6 and optimum pH 6 (Holt et al., 1994).

ii) Growth at Different Temperature (°C):

Actinomycetes usually grow at 25°C to 30°C. Thermophiles grow at 45°C to 55°C and psychrophiles at 4°C to 10°C, thought little attention has been paid to the latter group. The mesophiles genera grow, such as Streptomyces and Micromonospora for 7 to 14 days, and common thermophiles, such as Thermoactinomyces, which require only 1 to 3 days incubation (Williams and Wellington, 1982b).

iii) Growth at Different NaCl (%):

Marine microbial population in general is still limited for discovery. The growth of halophilic, Actinopolyspora halphila, from contaminated laboratory media and a halophilic Streptomyces sp. from a salt farm (Kayamura and Takada, 1970), suggested that actinomycetes may grow in any salt-rich media. Mackay, (1977), claimed that the grow of Streptomyces on starch casein agar was improved by addition of 4.6% NaCl. Li, et al., (2003), isolate novel moderately halophilic actinomycetes, the optimum growth temperature was at 35-37°C and
growth occurred optimally in 10% NaCl, which was identified as *Saccharomonospora homophile.*

3. **Biochemical Characters:**
   i) **Utilization of Carbon Sources:**
   The ability of different species of actinomycetes to utilise sources of carbon and energy such as carbohydrates, alcohols, salts of organic acids, fats and amino acids compounds can be considered of diagnostic value. None of the actinomycetes produces gas. Utilisation of carbon compounds is tested by observation of the growth, production of acid and production of gum (Williams et al., 1983).

   ii) **Utilization of Nitrogen Sources:**
   Utilisation of amino acids, urea, and creatinine are important in species characterisation. Gordon and Miham, (1959), suggested that the ability of utilizing casein, tyrosine, or xanthine can be considered of some significance in characterizing species (Goodfellow and O'Donnell, 1993).

   iii) **Other Properties:**
   The reduction of nitrate to nitrite, production of H2S, and acid fast test, has been universally used among the criteria for species differentiation (Shejul, 1998).

4. **Sensitivity Characters to:**
   i) **Antibiotics:**
   The ability of actinomycetes to grow in the presence of antibiotics is used for their characterisation (Williams et al., 1989). Actinomycetes characterise by their resistance to wide variety of antibiotics, mainly aminoglycosides showed individual patterns of resistance to certain ranges of these antibiotics. Analysis of the mechanisms of the antibiotics resistance revealed that the resistance patterns of the strains tested were unique, being exclusively dependent on self-resistance determinants such as inactivating enzymes and ribosomal resistance. This result opened up a promising way of predicting the type of antibiotics that each actinomycetes strain can be expected to produce as far as aminoglycoside antibiotics are concerned. The same result can be expected in strains that produce other families of antibiotics. Similar antibiotics resistance patterns have been reported in macrolide antibiotic producers (Fujisawa and Weisblum, 1981).

   Characterization of actinomycete strains in terms of their resistance to certain ranges of antibiotics makes it possible to avoid the repeated screening of isolation with the production of unknown antibiotics and also to predict or select the ability of isolates to produce new antibiotics. Thus multiple antibiotic resistance patterns can be regarded as useful marker
phenotypes for predicting the types of antibiotics actinomycete produce (Okami and Hotta, 1988).

ii) Detergents:

The ability of actinomycetes to grow in the presence of different detergents, such as anionic, nonionic and cationic are used for their characterisation.

iii) Heavy Metals:

Recently, resistance to different metals is used as a characteristic for identification of bacteria. Mineral rock weathering and anthropogenic sources provide two of the main types of metal inputs to soils. According to Ross, (1994) the anthropogenic sources of metal contamination can be divided to five main groups: (1) metalliferous mining and smelting (arsenic, cadmium, lead and mercury); (2) industry (arsenic, cadmium, chromium, cobalt, copper, mercury, nickel and zinc); (3) atmospheric deposition (arsenic, cadmium, chromium, copper, lead, mercury and uranium); (4) agriculture (arsenic, cadmium, copper, lead, selenium, uranium and zinc); and (5) waste disposal (arsenic, cadmium, chromium, copper, lead, mercury and zinc). In Finland, most cases of soil metal contamination have been caused by waste treatment plants, sawmills, wood impregnation plants, shooting ranges, garages and scrap yards. In 2001, a total of 20,000 metal contaminated sites were identified. Because 38% of this metal contaminated sites are located in groundwater areas or close to settled areas, metal contaminated soil sites are of great concern (Haavisto, 2002).

Metals play an integral role in the life processes of microorganisms. Some metals, such as calcium, cobalt, chromium, copper, iron, potassium, magnesium, manganese, sodium, nickel and zinc, are essential, serve as micronutrients and are used for redox-processes; to stabilize molecules through electrostatic interactions; as components of various enzymes; and for regulation of osmotic pressure (Bruins et al., 2000). Many other metals have no biological role (e.g. silver, aluminum, cadmium, gold, lead and mercury), and are nonessential (Bruins et al., 2000) and potentially toxic to microorganisms. Toxicity of nonessential metals occurs through the displacement of essential metals from their native binding sites or through ligand interactions (Nies and Silver, 1995). For example, Hg\textsuperscript{2+}, Cd\textsuperscript{2+} and Ag\textsuperscript{+} tend to bind to SH groups, and thus inhibit the activity of sensitive enzymes (Nies, 1999). In addition, at high levels, both essential and nonessential metals can damage cell membranes; alter enzyme specificity; disrupt cellular functions; and damage the structure of DNA (Bruins et al., 2000). To have a physiological or toxic effect, most metal ions have to enter the microbial cell. Many divalent metal cations (e.g. Mn\textsuperscript{2+}, Fe\textsuperscript{2+}, Co\textsuperscript{2+}, Ni\textsuperscript{2+}, Cu\textsuperscript{2+} and Zn\textsuperscript{2+}) are structurally very similar. Also, the structure of
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Oxyanions such as chromate resembles that of sulfate, and the same is true for arsenate and phosphate. Thus, to be able to differentiate between structurally very similar metal ions, the microbial uptake systems have to be tightly regulated. Usually, microorganisms have solved this problem by using two types of uptake systems for metal ions. One is fast, unspecific, and driven by the chemiosmotic gradient across the cytoplasmic membrane of bacteria. Since this mechanism is used by a variety of substrates, it is constitutively expressed (Nies, 1999). The second type of uptake system has high substrate specificity, is slower, often uses ATP hydrolysis as the energy source and is only produced by the cell in times of need, starvation or a special metabolic situation (Nies and Silver, 1995).

The oxyanions of arsenic enter bacterial cells via transporters for other compounds. In bacteria, \( \text{As}^{3+} \) is taken up by phosphate transport systems, such as the ATP-coupled Pst pump. One route of entry for \( \text{As}^{3+} \) is via the GlpF polyol transporters (Rensing et al., 1999). The bacterial detoxification of arsenic is often based on inducible ion efflux systems that reduce the intracellular concentration of arsenic by active export (Ji and Silver, 1995). Since anion export from bacterial cells is driven by the chemiosmotic gradient, simple \( \text{As}^{3+} \) efflux systems are composed of just one efflux protein. \( \text{As}^{3+} \) cannot, however, be transported with this system. The solution to the problem of \( \text{As}^{3+} \) efflux is the enzyme arsenate reductase, which catalyzes the reduction of \( \text{As}^{3+} \) to \( \text{As}^{5+} \), the substrate of the efflux system (Rensing et al., 1999). Thus, this enzyme extends the spectrum of resistance to include both \( \text{As}^{3+} \) and \( \text{As}^{5+} \). The lead resistance may also be based on metal ion efflux. For example, zinc and cadmium specific pumps are able to export lead from bacterial cells (Rensing et al., 1999). In addition, lead resistance can be due to precipitation of lead phosphate within the cells of resistant bacteria (Nies, 1999).

iv) Actinophage:

The phage typing of actinomycetes is considered one of the characteristics in classification but there are certain pitfalls to the system especially, *Streptomyces* sp. Actinophages vary greatly in their host range and most of them are polyvalent (Williams et al., 1989).

5. Chemotaxonomic Characters:

Chemical taxonomy is the study of the chemical variation in living organisms and the use of selected chemical characters in classification and identification (Goodfellow and Minnikin, 1985). However, useful to restrict the definition to refer to the distribution of specific chemical components such as lipids, cell wall amino acids, sugars and proteins among microbial taxa.
Chemical information can be used at all taxonomic levels and it is likely that chemical properties will become an important part of the minimal descriptions of many genera and species (O'Donnell, 1986). Lechevalier and Lechevalier, (1965), pointed out that the actinomycetes can be separated into broad groups on the basis of morphological and chemical criteria. This approach is still the simplest and best for generic identification (Holt et al., 1994).

1) Whole Cell Sugars:

The presence of xylose and arabinose in whole cell hydrolysate will usually indicate a type II cell wall type, and the organism belonging to Actinoplanes and other genera, e.g. Catellatospora, Micromonospora and Glycomyces. The presence of madurose will indicate a cell wall of type III of the Thermoactinomyces, Actinomadura variety and some strains of Frankia and to the genus Dermatophilus (Goodfellow and Cross, 1984).

The presence of rhamnose without other diagnostic sugars indicates a possible Saccharothrix. Organisms containing fucose may belong to the genus Frankia or to Actinoplanes. Arabinose and galactose present in the whole cell hydrolysates, cell wall type IV will point to many organisms, such as Nocardia, Kibdelosporangium, Amycolata, Amycolatopsis, Saccharomonospora, Saccharopolyspora, and Actinopolyspora (Holt, et al., 1994). The absence of any of the above-mentioned sugars in the whole cell hydrolysates is an indication of cell walls of type I and the organisms belonging to Streptomyces (Table 4.1) (Locci and Schofield, 1989).

Table 4.1: Guide to the chemical properties of the genera of actinomycetes*

<table>
<thead>
<tr>
<th>Cell Wall Type</th>
<th>DAP Acid Isomer</th>
<th>Glycine in Interpeptidoglycan Bridge</th>
<th>Diagnostic Sugars</th>
<th>Genera</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>L,L</td>
<td>Yes</td>
<td>No</td>
<td>Streptomyces sp.</td>
</tr>
<tr>
<td>II</td>
<td>meso</td>
<td>Yes</td>
<td>Xylose and arabinose</td>
<td>Micromonospora, Actinoplanes</td>
</tr>
<tr>
<td>III</td>
<td>meso</td>
<td>No</td>
<td>madurose</td>
<td>Thermoactinomyces, Actinomadura, Frankia</td>
</tr>
<tr>
<td>IV</td>
<td>meso</td>
<td>No</td>
<td>Arabinose and galactose</td>
<td>Saccharomonospora</td>
</tr>
</tbody>
</table>

ii) Cell Wall Amino Acids:

Peptidoglycans contain various significant criteria for actinomycete taxonomy. Determination of diaminopimelic acid isomers by thin-layer chromatography is one of the most essential techniques for identification of actinomycetes. Amino acid composition of the hydrolyzate of peptidoglycan is helpful information to speculate the peptidoglycan structure. A thin-layer chromatography procedure which separates D- and L-isomers of amino acids was applied to the peptidoglycan analysis (Fig. 4.1) (Suzuki, 1997).

![Cell wall composition of Gram + bacteria such as actinomycetes](image)


iii) Fatty Acid Analysis:

Fatty acid composition of a microorganism is an important taxonomic character (Goodfellow and Minnikin, 1985) but, despite the high diagnostic value of fatty acid profiles, they are still interpreted qualitatively for the presence or absence of particular compositions (O'Donnell, 1985). It has limited the analysis of fatty acid profiles to definitions of taxa at generic and suprageneric levels. It has been demonstrated that fatty acid profiles can be analyzed quantitatively providing useful taxonomic information at species and in some cases subspecies (Saddler et al., 1991).

Actinomycetes are usually rich in lipids and the structural variations that exist amongst various lipids classes are being used for taxonomic purposes. Some of the organisms from actinomycetes and related to actinomycetes with type IV cell wall produce α-branched, β-hydroxylated fatty acids called mycolic acids. These lipids fall into three broad groups on the...
basis of molecular weight: the largest are typical of the genus *Mycobacterium*, the smallest are produced by some members of the genus *Corynebacterium* and those of intermediate molecular weight are found in species of *Nocardiia* (Lechevalier, 1977). Phospholipids patterns have recently been shown to cast considerable light on the interrelationships of various aerobic actinomycetes genera. There are five groups of phospholipids are recognizable on the basis of their content of nitrogenous phospholipids (Lechevalier et al., 1981).

6. Genotaxonomic Characters:

i) DNA-DNA Hybridisation:

This approach also revealed evidence of subspecific grouping within the taxa, the DNA pairing method is particularly useful in resolving important taxonomic dilemmas at the species level, but thus could not be substantiated because of the small number of strains analysed. Relatively few DNA pairing studies have been carried out on actinomycetes and the importance of them has been offset by the use of unrepresentative reference strains (Shejul, 1998). This method which minimized the risks outlined above was applied to chromosomal DNA extracted and cleaved with restriction enzyme. The DNA fragments were separated on a polyacrylamide gel and resultant banding pattern digitized using a scanning densitometer. The multivariate profile was then analysed using SIMCA pattern recognition (O’Donnell, 1986).

ii) 16’s’ rRNA Sequencing:

Base sequences of 16’s’ rRNA cistrons are more highly conserved than most of the genes in the bacterial genome (Moore and McCarthy, 1967). Comparison of such sequences helps in detection of supergeneric and evolutionary relationship amongst actinomycetes. In this method purified RNA is digested by T1 ribonuclease, the oligonucleotides are separated by two-dimensional electrophoresis and are sequenced by a combination of endonuclease digestion procedures. The oligonucleotide catalogues of any two strains are compared one with another and oligonucleotides of six residues or larger, common to these are scored to produce a ‘SAB value’ characteristic of that pair of organisms. The function SAB is equivalent to twice the total number of residues in sequences common to a pair of catalogues, divided by the total number of residues in all of the sequences in the two catalogues. SAB values are analysed using standard clustering algorithms and data presented as dendrograms or as evolutionary trees (Stackebrandt and Woese, 1981).

ii) 5’s’ rRNA Sequencing:

5’s’ ribosomal RNA sequencing has been used to establish evolutionary relationships between diverse prokaryotes including *Micrococcus* and *Staphylococcus* sp. (Dekio et al., 1984),
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Mycoplasma sp. (Rogers et al., 1985), Thiothrix and Thiobacillus (Lane et al., 1985), the Vibrionaceae (MacDonell and Colwell, 1985), coryneform actinomycetes (Park et al., 1987) and acidophilic actinomycetes (Lonsdale, 1985) were compared with marker neutrophilic streptomycetes using data derived from 5′rRNA sequencing studies.

The phylogenetic data generated from the 5′rRNA sequence shows that the acidophilic, neutrotolerant and neutrophilic actinomycetes form a distinct evolutionary group that can readily be distinguished from the marker 'coryneform' actinomycetes and representatives of the genera Bacillus, Lactobacillus and Staphylococcus. It is evident, therefore, that acidophilic and neutrotolerant actinomycetes with a combination of chemical, morphological and physiological properties corresponding to those characteristic of neutrophilic Streptomyces should be assigned to the genus Streptomyces (Park et al., 1991).

4.1.2 Analysis Methods used for Identification grouping of Acidophilic Actinomycetes:

Numerical phenetic and chemical data have revolutionized the classification of several actinomycetes genera, notably Actinomadura (Williams and Wellington, 1981), Actinomyces (Schofield and Schaal, 1982), Corynebacterium (Goodfellow and Minnikin, 1985), Frankia (An et al., 1983), Nocardia (Goodfellow and Minnikin, 1985) and Streptomyces (Williams et al., 1983). Microbial ecologists are not primarily concerned with classification but do require workable diagnostic tests to identify isolates from natural habitats. A combination of morphological, chemical, and spore characters can be used to identify actinomycetes to the genus level (Goodfellow et al., 2001), but problems abound, especially at the species level. Probability matrices and identification programs are available for the recognition of slowly growing actinomycetes and Streptomyces (Williams et al., 1983).

1. Numerical Taxonomy:

Conventional numerical taxonomy has been one of the most effective methods used to establish relationships between actinomycetes at both sub-generic and generic levels (Goodfellow and O'Donnell, 1993). Classification derived using numerical taxonomic methods have high information contents and are polythetic that is they based on a complete set of recorded characters rather than on the presence or absence of single characters. The introduction of numerical taxonomy has led to improvements in the classification of many actinomycete taxa. Numerical classification also provides an ideal basis for the construction of identification matrices (Sneath and Sokal, 1973). Such matrices ideally contain the minimum of characters, selected from the data base, necessary to discriminate between the relevant taxa (Seong et al., 1995). To date, only a few probabilistic identification matrices have been designed for the identification of actinomycetes. One of the examples of workable, polythetic identification
system is that devised by Williams et al., (1983), for the identification of *Streptomyces* isolates. Using this identification matrix, over 81% of the unknown isolates were identified to cluster groups. Although it was theoretically and practically sound, minor clusters and single-membered clusters were excluded in the matrix. Therefore, separate matrices were constructed for major and minor cluster, and a diagnostic table for single-membered clusters was also produced by Langham et al., (1989). The data in those matrices was derived from previous numerical classification of *Streptomyces* species (Williams et al., 1983). A more comprehensive numerical survey of *Streptomyces* species was carried out by Kämpfer and Kroppenstedt, (1991), and the results of this numerical study were used to construct a single probability matrix for members of all major and minor clusters.

2. Taxonomical Computer Software:

Lapage et al., (1973), established first computer programming for identification of bacteria, which they used morphological, physiological, and chemical characteristics. Now there are many software program which can be used for identification of bacteria in general and actinomycetes genera in particular, e.g. CHARSEP a computer programme for determining the ability of individual characters in a frequency matrix to separate taxa, e.g. DIACHAR a computer program which lists the most diagnostic characters for a taxon in a frequency matrix (Williams et al., 1983), MOSTTYP a computer program which calculates the hypothetical median organism of a taxon from an identification matrix and then attempts to identify it (Langham et al., 1989), and many other programs. Bryant, (1993), developed a software programme for *Streptomyces, Actinoplanes, Rhodococcus, Streptoverticillium* and Corynoform identification by probability identification and called Probability Identification of Bacteria (PIB).

3. Other Methods:

Pyrolysis is the thermal degradation of complex material in an inert atmosphere or a vacuum. It causes molecules to cleave at their weakest points to produce smaller, volatile fragments called pyrolysate (Irwin, 1982). Curie-point pyrolysis is a particularly reproducible and straightforward version of the technique, in which the sample, dried onto an appropriate metal is rapidly heated to the Curie-point of the metal. A mass spectrometer can then be used to separate the components of the pyrolysate on the basis of their mass to charge ratio (m/z) to produce a pyrolysis mass spectrum (Meuzelaar et al., 1982), which can then be used as a "chemical profile" or fingerprint of the complex material analysed. The combined technique is then known as pyrolysis mass spectrometry (PyMS) (Goodacre, 2004).
4.2 Materials and Methods:

4.2.1 Cultural Characterization:

i) Colony:
Morphology and colour of the colony was determined by inoculating the isolates on different media and incubated at 28°C for 21 days (Shirling and Gottlieb, 1972).

ii) Formation of Pigment:
Diffusible pigmentation was determined on glycerol asparagine agar. Plates were inoculated with actinomyces and incubated at 28°C for 21 days and observed for pigmentation. Melanin pigment formation was determined on tyrosine agar. Isolates were inoculated at 28°C for 21 days and observed for production of melanin pigment (Williams and Wellington, 1982a).

4.2.2 Morphological Characterization:
To study growth and morphological characters of the acidophilic actinomycetes isolates were grown on following media: (1) Starch Casein Agar (SCA) (Nawani, 2002), (2) Glycerol Asparagine Agar (GAA) (Kanavade, 2003), (3) Nutrient Agar (NA) (Goodfelow and O'Donnel, 1989), (4) Yeast extract-Malt extract Agar (YMA), (5) Czapeck's Dox-Thom Agar (CDTA) (Collins et al., 1995) and (6) Sabouraud's Dextrose Agar (SDA) of pH 4.5. The inoculated plates were incubated at 28°C for 7-14 days and observed for colony characters, sporulation and pigmentation. The isolates were subjected to slide culture by:

i) A thin agar block, cut from poured plate, is placed on sterile microscope slide and inoculated, and a sterile coverslip is applied. After incubation in moist chamber, view the slide culture directly on the phase contrast microscope stage (Nikon co., Japan), when it should be possible to see the aerial, substrate mycelium and spores morphology within the agar (Holt et al., 1994).

ii) Inclined coverslips for observing actinomycete morphology. Inoculate the agar plates with coverslips inserted at an angle. After incubation, withdraw the cover slips and mount them, upper surface down, in the slide culture and directly put it on the phase contrast microscope stage (Nikon co., Japan), observe the aerial, substrate, spores morphology (Holt et al., 1994).

4.2.3 Physiological Characterization:

i) Growth at Different Temperature:
Acidophilic actinomycete isolates were inoculated on starch casein agar at 20, 28, 30.37, 45 and 60°C. Growth was assessed after 14 days.

ii) Growth in Presence of NaCl:
Sodium chloride ranging from concentration 0 to 10% in starch casein broth. Each concentration tube was incubated with loopful of spore suspension, and incubated at 28°C. Presence or absence of growth was noted after 14 days.
4.2.4 Biochemical Characterization:

i) Utilization of Carbon Sources:

The ability of acidophilic actinomycetes isolates to utilize different carbon sources was tested by incorporating 20 different carbon sources into carbon utilisation agar (1% w/w). Isolates were spot inoculated on agar medium and incubated at 28°C. Results, acid and gum production were recorded and after 7, 14, and 21 days by comparing growth with positive control unsupplemented basal medium containing glucose. Carbon compounds included in the medium under study were glucose, mannose, sucrose, galactose, maltose, inositol, lactose, mannitol, cellobiose, xylose, arabinose, raffinose, rhamanose, fructose, fucose, ribose, dextran, citric acid, lactic acid and glutamic acid (Williams et al., 1983).

ii) Utilization of Nitrogen Sources:

The ability of acidophilic actinomycete isolates to use nitrogen compounds was tested by incorporating compounds into a basal medium for nitrogen utilisation (1% w/w). Isolates were spot inoculated on agar medium and incubated at 28°C. Results were determined after 14 days by comparing the growth on each medium with that on the unsupplemented basal medium and on a positive control containing asparagine. The nitrogen sources included in the medium under study were glycine, asparagine, alanine, phenylalanine, histidine, praline, tyrosine, serine, cysteine and valine (Langham et al., 1989).

iii) Nitrate Reduction:

Nitrate reduction ability of acidophilic actinomycete isolates was determined in nutrient broth supplemented with KNO₃ (0.2% w/v). Spore suspension was inoculated in the broth and incubated at 28°C. After 14 days of incubation nitrate reduction was tested by addition of 0.2 ml each of Griess-Ilosvay reagents I and II (Collins et al., 1995).

iv) Acid Fast Staining:

Actinomycetes which are not readily decolorized with acid-alcohol after staining with hot carbol fuchsin are said to be acid fast. These actinomycetes contain considerable quantities of wax like lipoidal material which combines tenaciously with this red dye. This stain is used primarily in the identification of Nocardia. After decolorization methylene blue is added to the smear to counterstain any material that is not acid fast (Colwell and Hill, 1992).

1. Cover smear with carbol fuchsin, heat gently by spirit lamp until the steam rises and repeat 3 to 4 times in the course of 5 minutes. Added additional stains if stains dry up. After the slide has cooled, decolorize with acid-alcohol for 15 to 20 seconds. Stop decolorization of acid-alcohol by rinsing briefly with water.
2. Counterstain with methylene blue for 30 seconds. Rinse briefly with water to remove excess methylene blue. Blot dry with filter paper, examine directly under oil immersion.

4.2.5 Sensitivity of Acidophilic Actinomycetes to:

4.2.5.1 Antibiotics:

All the isolates were tested for antibiotic sensitivity to grow in the presence of 14 antibiotics such as: Gentamycin (50 μg), Nalidixic acid (30 μg), Polymyxin-B (100 units), Rifampicin (30 μg), Kanamycin (30 μg), Novobiocin (30 μg), Tetracycline (30 μg), Penicillin-G (10 units), Oxytetracycline (30 μg), Amphotericin-B (100 units), Clotrimazole (10 μg), Fluconazole (10 μg), Nitrofurazone (100 μg) and Nystatin (100 units) (Combined Microbial Sensitivity Disc, Himedia co. Ltd. India). Sterile antibiotic discs (6 mm diameter) were tested using method recommended by Collins et al., (1995).

4.2.5.2 Detergents:

Sensitivity of acidophilic actinomycete isolates to six detergents of different concentration. Minimal inhibitory concentration (MIC) of the detergents was determined by modified E-test and the MIC value ranged from 0.01 μg/ml to 100 μg/ml and for Nonionic detergents the MIC value ranged from 0.01 μl/ml to 100 μl/ml. Detergents used in the study are:

i) Anionic: SDS (Sodium dodosil sulphate), SLS (Sodium laryl sulphate).

ii) Nonionic: Tween-80 (Polyoxyethyleneserbitan monolaurate), Tween-20 (Polyoxyethyleneserbitan monooleate), Triton X 100.

iii) Cationic: Cetrimide.

4.2.5.3 Heavy Metals:

Sensitivity of acidophilic actinomycete isolates to eight heavy metals salts on different concentration. Minimal inhibitory concentration (MIC) of the heavy metals was determined by modified E-test and the MIC value ranged from 1 μg/ml to 1000 μg/ml. Heavy metals used in the study are: Cu\(^{2+}\), Zn\(^{2+}\), Ag\(^{+}\), Hg\(^{2+}\), Mo\(^{6+}\), As\(^{3+}\), Pb\(^{2+}\), and Cd\(^{2+}\) (Turpeinen, 2002).

4.2.6 Chemotaxonomy:

Cell wall amino acids and whole cell sugars of acidophilic actinomycete isolates were analysed as per the following protocol (Staneck and Roberts, 1974).

i) Protocol for Biomass Production:

1. Isolates were streaked on starch casein agar to get well isolated colonies. Plates were incubated at 28°C for 7 days. Well isolates colony was inoculated into 50 ml. brain heart infusion broth in 250 ml. Erlenmeyer flask and incubated on rotary incubator shaker, at 200 rpm at 28°C for 7 days.
Taxonomy of Acid...

2. Cell mass was collected by centrifugation at 4000 rpm. for 10 minutes. Cell mass was washed thrice with distilled water and finally washed with 95% ethanol. Cell mass was dried at 45°C for two days.

ii) Protocol for Cell Wall Amino Acid:

The amino acid analysis was carried out only for actinomycetes isolates:

1. 3 mg. dry cell mass was placed in 5 ml. Pyrex test tube, 1.5 ml. 6N HCl was added to it. The test tube was sealed and kept at 100°C for 18 hours. After 18 hours, test tube was opened and the content was filtered through Whatman filter paper No.1. The cell extract was dried at 50°C. 300 ul. distilled water was added to the dried cell extract and again dried at 50°C. This procedure was repeated once again.

2. The dried cell extract was collected in 300 ul. distilled water and stored in microfuse tube. This was used for amino acid analysis. 5 ul. cell extract was applied to the baseline of Whatman filter paper No.1. 5 ul. of DL-DAP (Sigma) and glycine mixture (1 μg/ul.), and Streptomyces purpureus MTCC 5022 contain LL-DAP were applied to the line on the same paper, as the stander amino acids.

3. Ascending chromatography was performed using, methanol: distilled water: 6N HCl: pyridine (80: 26: 4: 10 v/v), for approximately 4 hours. Chromatography paper was air dried and developed with 0.2% Ninhydrin in acetone and kept at 100°C for 3 minutes.

iii) Protocol for Whole Cell Sugar Analysis:

1. 25 mg. dry cell mass was placed in an ampoule with 1.5 ml. of 1N H2SO4 and sealed. Ampoule was heated in the boiling water bath for 2 hours. After cooling cell hydrolysate was transferred to 15 ml conical centrifuge tube saturated Barium hydroxide was added dropwise to it until the pH was between 5.2 and 5.5. The tube was centrifuged at 5000 rpm. for 15 minutes. Supernatant was collected in 50 ml. beaker and evaporated under a stream of air.

2. Residue was dissolved in 300 ul. Distilled water and collected in microfuse tube. It was centrifuged at 5000 rpm. for 15 minutes to remove any insoluble material, if left. 5 ul. of the hydrolysate and 5 ul. of each of two standard sugar solutions were applied at the baseline of the Whatman filter paper No.1.

3. Ascending chromatography was performed in the solvent system, n-Butanol: Distilled water: Pyridine: Toluene (10: 6: 6: 1 v/v), approximately for 4 hours. Sugar spots were developed by spraying acid aniline phthalate and by keeping at 100°C for 4 minutes.

4. Hexoses appear as yellowish, brown spots and Pentoses appear as maroon colored spots. Hexoses such as glucose, mannose, rhamanose and galactose. Pentoses such as riboses, arabinose and xylose.
4.2.7 Identification of Acidophilic Actinomycetes by PIB Computer Software:

Based on microscopic, cultural and cell wall characteristics the acidophilic actinomycete isolates were identified to genus level. Biochemical and other characters were used to identify to genus species level. Members of Streptomyces, Actinoplanes, Rhodococcus, Streptovericillium genus were identified by using PIB software (Bryant, 1993). Probabilistic identification matrices were Streptomyces species major cluster (Williams et al., 1989), Streptomyces species minor cluster (Langham et al., 1989), Streptomyces species minitest single cluster (Kämpfer and Kroppenstedt, 1991), Streptovericillium species (Williams et al., 1985), Actinoplanes species (Long, 1994) and Rhodococcus species (Kämpfer and Seiler, 1993), Isolates belonging other genera were identified manually (Williams et al., 1989; Grund and Kroppenstedt, 1990; Evtushenko et al., 2000; Kämpfer et al., 1993; Holt, et al., 1994; Seong et al., 1995; Sarkonen et al., 2001). The identification routine used in PIB is based on Willcox's implementation of Bayes theorem for use with bacteria (Willcox et al., 1973).

4.2.8 Identification of Acidophilic Actinomycetes by Numerical Classification Matrices:

The primary aim of numerical taxonomy is to provide classification that are precise, reproducible and have large information content. The application of numerical techniques to the construction of biological classification led to the realization that the circumscription of taxa follows certain established principles and dose not just happen. The logical and sequential steps involved, though not always consciously separated in practice, can be applied to all branches of systematics. These steps, in the case of actinomycetes (Garrity et al., 2003), area:

1. The actinomycetes (n) to be classified are collected and examined for a large number of test (t)-morphological, nutritional, physiological and biochemical tests. A data matrix (n x t) is then prepared. 2. Using results from the data matrix, the strains are classified on the basis of similarities or differences. 3. Strains which resemble one another closely are grouped together using clustering algorithms. 4. Numerically defined groups are examined and any characters which distinguish them are extracted from the data matrix and weighted for identification.

Numerical taxonomists recognize these logically successive steps. One numerical taxonomic technique is called 'Adansonian' after Michel Adanson who was the first to announce the principles on which it is based. There are five in number (Holt et al., 1994):

1. The idea taxonomy is that in which the taxa contain the most information and which is based upon as many characters as possible. 2. Every character is giving equal weight in generating natural taxa. 3. The degree of relatedness between any two operational taxonomic units (OTUs) is an expression of the similarity of the many characters for which they are compared. 4. Distinct
groups can be constructed because diverse character correlations exist between the taxa. 5.
Taxonomy is a strictly empirical science.

i) Operational Procedure:
1. Collection of Data:
   In Adansonian taxonomy, the actinomycetes should be studied for at least 50 characters.
   Little confidence can be placed in groups based on fewer characters. Positive characters should
   score 1 or +, negative ones 0 or -.

2. Measurement of Resemblance:
   Each culture will be compared with every other culture and the degree of resemblance
   found using the Matching Coefficient ($S_{sm}$):
   \[
   \% S_{sm} = \frac{\text{Number of shared character}}{\text{Total number of characters}} \times 100
   \]
   \[
   \times a + d \times 100
   \]
   \[
   \text{Total number of characters} \quad 1 \quad n \quad 1
   \]
   Where $a$ is the number of shared positive characters between two strains, $d$ the number of
   negative characters shared and $n$, the total number of characters. Initially, the results of
   numerical taxonomic analysis are presented as unsorted similarity matrices and unsorted shaded
   diagrams.

ii) Cluster Analysis:
   The strains from the unsorted similarity matrix now need to be arranged into groups.
   The strains are arranged into groups. This is called Cluster Analysis (CA) and many clustering
   logarithms are available. One such a logarithm is the Single Linkage Cluster Analysis Technique
   (SLCA). With this technique the unsorted similarity matrix is scanned visually for similarity values of
   99% and this process is repeated using levels reduced consecutively by 1%. Each strain is extracted
   from the matrix at its highest similarity level to any other strain. If actinomycetes are already a member of
   an established group, the newly selected actinomycete joins that group. If neither of the two
   actinomycetes belonging to an established group they form the nucleus of a new cluster.
   Established clusters join at the highest similarity existing between any two constituent members.
   This process will complete when all the clusters have joined to form one aggregate group.
   Cluster analysis is then completed. The results of cluster analysis are usually expressed in the
   form of a Dendrogram. Alternatively or additionally, the results may be expressed in a Sorted
   Shaded Diagram (SSD). The latter is constructed by placing the actinomycetes in the same order
   (Williams et al., 1989).
4.3 Results:

In all, 147 acidophilic actinomycete isolates were identified as belonging to different genera as shown in Fig. 4.2. Out of them, 85 were strictly acidophilic and 62 neutrotolerant acidophilic. Out of 147 isolates, 108 belonging to the genus *Streptomyces*, and 39 belonging to other genera. Out of them, 7 isolates were belonging to *Streptovercillium*, 7 isolates identified as *Nocardia*, 6 isolates were belonging to *Actinomadura*. 6 isolates were identified as *Actinoplanes*, other 6 isolates were belonging to *Thermomonospora*, 4 isolates were belonging to *Thermoactinomyces*, and 3 isolates were identified as other genera (Fig. 4.2).

4.3.1 Cultural, Morphological, Physiological and Biochemical Characterization:

All acidophilic actinomycete isolates results are recorded in appendix I and II. Cultural morphology and pigment results of some acidophilic actinomycetes appear in Plate 4.4 a,b,c, and d for pigments. The cultural morphology results of some acidophilic actinomycetes are presented on Plate 4.4 e, f, g, and h. Utilization of different sugars and acid production of some acidophilic actinomycetes are presented in Plate 4.5.

i) Streptomyces:

There were 76% of the isolates which belonged to these genera out of them 62% strictly acidophilic and 38% belonged to neutrotolerant acidophilic. Substrate mycelium was nonfragmenting and branched. Aerial mycelium formed short or long spore chains. Spore chains were straight, hooked or spirals. On starch casein agar, glycerol aspargine agar, nutrient agar, the growth was good and fast. They produced a wide variety of diffusion and melanin pigments. The isolates were growing within the psychrophilic and thermophilic. Cell wall amino acids were L-DAP and glycine (cell wall type I). No whole cell sugar was found (Table 4.2). All *Streptomyces* isolates identified to 27 species as:

*Streptomyces flaveolus*:

Isolates 4/1, 4/14, 4/16/ 5/22, 11/2, 12/16, 15/2, 16/5, 17/1, 18/2, 18/12, 19/1, 19/6, 20/12, and 21/13 were identified as *Streptomyces flaveolus*. The spore chains were rectiflexibles and spirales. The spore mass was gray. Diffusible and melanin pigments were produced. They can grow at 45 °C, utilized meso-inositol and D-fructose as a carbon source (Plate 4.1c and d).

*Streptomyces prasinosporus*:

There are 13 isolates 1/1, 1/8, 3/3, 9/13, 9/18, 10/4, 13/1, 13/4, 20/19, 22/13, 28/8, 29/5, and 30/3 were identified as *Streptomyces prasinosporus*. The spore chains were spirales. The spore mass was gray and the reverse was yellow to brown. Most of the isolates did not produced diffusible and melanin pigments. They utilized histidine and mannitol (Plate 4.1g and h).
**Streptomyces glaucescens:**

Isolate numbers 3/4, 8/13, 10/19, 11/19, 11/22, 12/19, 12/20, 13/5, 13/6, 15/7, and 20/9 were identified as *Streptomyces glaucescens*. The spore chains were spirales. The spore mass was gray. Diffusible and melanin pigments were produced. They can grow at 45 °C and some of them tolerated 7% NaCl. They utilize lactose and most of strains utilized meso-inositol as a carbon source (Plate 4.1b).

**Streptomyces antibioticus:**

There were 11 isolates which were identified as *Streptomyces antibioticus*; the isolates are 6/3, 10/2, 10/21, 11/3, 11/17, 12/6, 13/12, 21/1, 26/9, 28/6, and 30/2. The spore chains were rectiflexibles and spirales. The spore mass was gray. None of the isolates produced diffusible and melanin pigments. Most of the isolates tolerated 7% NaCl, not grown at 45 °C and utilized meso-inositol as a carbon source. Antimicrobial activity against *Candida albicans* was observed (Plate 4.1e and f).

**Streptomyces nogalater:**

Isolates 1/5, 4/11, 5/8, 5/15, 5/20, 5/24, 18/18 and 31/2 were identified as *Streptomyces nogalater*. Most of the isolates spore chains were rectiflexibles and spirales. The spore mass was gray and the reverse was yellow to brown. Diffusible pigment was produced but no melanin pigment. They utilized D-lactose and meso-inositol as a carbon source (Plate 4.1a).

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![Graph](image)

**Fig. 4.2:** Distribution of acidophilic actinomycete genera to different acidophilic groups

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Table 4.2: List of acidophilic *Streptomyces* isolates identified up to species level.

<table>
<thead>
<tr>
<th>Cluster No.</th>
<th><em>Streptomyces</em> sp.</th>
<th>Cluster</th>
<th>Total isolates</th>
<th>Strictly acidophilic isolates</th>
<th>Neutrotolerant acidophilic isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Streptomyces antibioticus</em></td>
<td>Major</td>
<td>11</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>2.</td>
<td><em>Streptomyces cyaneus</em></td>
<td>Major</td>
<td>7</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>3.</td>
<td><em>Streptomyces diastatis</em></td>
<td>Major</td>
<td>6</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>4.</td>
<td><em>Streptomyces rochei</em></td>
<td>Major</td>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>5.</td>
<td><em>Streptomyces exfoliatus</em></td>
<td>Major</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>7.</td>
<td><em>Streptomyces violaceoniger</em></td>
<td>Major</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>8.</td>
<td><em>Streptomyces atrodiavicus</em></td>
<td>Major</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>9.</td>
<td><em>Streptomyces albicus</em></td>
<td>Major</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>10.</td>
<td><em>Streptomyces lydicus</em></td>
<td>Major</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>11.</td>
<td><em>Streptomyces albidoiavicus</em></td>
<td>Major</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>12.</td>
<td><em>Streptomyces chromogenus</em></td>
<td>Major</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>13.</td>
<td><em>Streptomyces flavoavicus</em></td>
<td>Minor</td>
<td>15</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>14.</td>
<td><em>Streptomyces prasinosporus</em></td>
<td>Minor</td>
<td>13</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>15.</td>
<td><em>Streptomyces glycocenum</em></td>
<td>Minor</td>
<td>11</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>16.</td>
<td><em>Streptomyces nogalater</em></td>
<td>Minor</td>
<td>8</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>17.</td>
<td><em>Streptomyces chattanoogenesis</em></td>
<td>Minor</td>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>18.</td>
<td><em>Streptomyces xanthochromogenus</em></td>
<td>Minor</td>
<td>4</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>19.</td>
<td><em>Streptomyces pautum</em></td>
<td>Minor</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>20.</td>
<td><em>Streptomyces cuma</em></td>
<td>Minor</td>
<td>2</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>21.</td>
<td><em>Streptomyces longispironavibus</em></td>
<td>Minor</td>
<td>2</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>22.</td>
<td><em>Streptomyces gruminifaciens</em></td>
<td>Minor</td>
<td>2</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>23.</td>
<td><em>Streptomyces thermovulgaris</em></td>
<td>Minor</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>24.</td>
<td><em>Streptomyces grisonavibus</em></td>
<td>Minor</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>25.</td>
<td><em>Streptomyces viridochromogenes</em></td>
<td>Minor</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td>67</td>
<td>44</td>
<td>23</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>38 Major, 67 Minor and 3 Single cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>26.</td>
<td><em>Streptomyces longissimus</em> Single 2 1 1</td>
</tr>
<tr>
<td>27.</td>
<td><em>Streptomyces ulboavibus</em> Single 1 1 -</td>
</tr>
<tr>
<td></td>
<td>3 2 1</td>
</tr>
</tbody>
</table>

*Streptomyces cyaneus*:

Isolates numbers 5/1, 7/3, 7/9, 9/28, 15/1, 16/2, and 17/3 were identified as *Streptomyces cyaneus*. The spore chains were spirales. The spore mass was gray and the reverse was yellow to brown. Diffusible and melanin pigments were produced. Antimicrobial activity against *Aspergillus niger* was observed.
Streptomyces diastaticus:

There are 6 isolates 8/20, 11/14, 12/7, 28/7, 29/1 and 31/1 were identified as *Streptomyces diastaticus*. The spore chains were spirales. The spore mass was white to gray. The isolates 8/20 and 28/7 produced diffusible and melanin pigments. All isolates utilized L-histidine and sucrose. Antimicrobial activity against *Aspergillus niger* was observed. Isolate 11/14 tolerated 7% NaCl.

Streptomyces rochei:

Isolates 5/16, 6/4, 24/2, and 31/4 were identified as *Streptomyces rochei*. The spore chains were spirales or rectiflexibles. The spore mass was gray. Diffusible and melanin pigments were not produced. Isolate 6/4 was lipolytic. Most of the isolates grow at 45 °C and isolate 24/2 tolerated 7% NaCl.

Streptomyces chattanoogensis:

There were 4 isolates 4/12, 7/27, 8/16 and 13/13 which were identified as *Streptomyces chattanoogensis*. The spore chains were spirales or rectiflexibles. The spore mass was gray. Isolates 8/16 and 13/13 were produced diffusible and melanin pigment. Isolates 8/16 grown at 45 °C. All isolates were utilized phenylalanine and histidine as a nitrogen source. They utilized lactose and xylose as a carbon source.

Streptomyces xanthochromogenes:

Isolate numbers 8/2, 10/11, 21/14 and 25/1 were identified as *Streptomyces xanthochromogenes*. The spore chains were spirales or rectiflexibles. The spore mass was gray. Diffusible and melanin pigments were not produced. They utilized phenylalanine and histidine as a nitrogen source. Isolate 8/2 tolerated 7% NaCl.

Streptomyces pactum:

There were 3 isolates 5/23, 7/23 and 26/8 that were identified as *Streptomyces pactum*. The spore chains were spirales. The spore mass was gray. The reverse was yellow to brown. They produced diffusible and melanin pigments. They utilized phenylalanine and histidine as a nitrogen source. They utilized sucrose as a carbon source. Isolates 5/23 and 7/23 grow at 45 °C and 7% NaCl.

Streptomyces canus:

Isolate numbers 7/28 and 23/4 were isolated as *Streptomyces canus*. The spore chains were spirales and rectiflexibles. The spore mass was gray. Diffusible and melanin pigments were not produced. They can grow at 45 °C and isolate 23/4 tolerated 7% NaCl. They utilized histidine, arabinose, sucrose, xylose, fructose and raffinose.
Plate 4.1: Photograph of Streptomyces sp.:

a) Streptomyces nogalater.
b) Streptomyces glaucescens.
c) Streptomyces flaveolus.
d) Mycelium of Streptomyces flaveolus.
e) Streptomyces antibioticus.
f) Substrate mycelium of Streptomyces antibioticus.
g) Streptomyces prasinosporus.
h) Substrate mycelium of Streptomyces prasinosporus.
Streptomyces longisporoflavus:
There were 2 isolates 13/2 and 26/1 that were identified as Streptomyces longisporoflavus. The spore chains were spirales or rectiflexibles. The spore mass was gray. Diffusible and melanin pigments were not produced. They utilized pectin, xylan, arabinose, fructose, raffinose and lactose as a carbon source. They utilized phenylalanine as a nitrogen source. Isolate 13/2 tolerated 7% NaCl.

Streptomyces graminofaciens:
Isolates 7/21 and 16/6 were identified as Streptomyces graminofaciens. The spore chains were spirales and rectiflexibles. The spore mass was gray and the reverse was yellow to brown. The diffusion pigments were produced. They utilized phenylalanine, histidine as a nitrogen source. They utilized sucrose, xylose, inositol, mannitol and raffinose as a carbon source. Isolate 7/21 tolerated 7% NaCl.

Streptomyces exfoliatus:
There were 2 isolates 5/2 and 5/7, which were identified as Streptomyces exfoliatus. The spore chains were rectiflexibles. The spore mass was gray and the reverse was yellow to brown. Diffusion and melanin pigments were produced. Antimicrobial activity against Micrococcus luteus and Aspergillus niger were observed. They are lipolytic and grown at 45 °C. They utilized valine, phenylalanine and histidine as a nitrogen source. They utilized inositol and raffinose as a carbon source.

Streptomyces halstedii:
Isolate numbers 4/13 and 26/2 were identified as Streptomyces halstedii. The spore chains were spirales and rectiflexibles. The spore mass was gray. Diffusible and melanin pigments were not produced. Antimicrobial activity against Aspergillus niger was observed. They are lipolytic, isolates 4/13 can grow at 45 °C and isolate 26/2 tolerated 7% NaCl. They utilized histidine as a nitrogen source. They utilized sucrose as a carbon source.

Streptomyces longissimus:
There were 2 isolates 11/9 and 14/10, which were identified as Streptomyces longissimus. The spore chains were spirales. The spore mass was gray and the reverse was orange. Diffusible pigment was produced. They utilized alanine, glycine and valine as a nitrogen source. They utilized arabinose, fucose, lactose, sucrose, inositol, mannitol, lactate and glutamate as a carbon source.

Streptomyces alboflavus:
Isolate 21/5 was identified as Streptomyces alboflavus. The spore chains were straight. The spore mass was white. Diffusible and melanin pigments were not produced. Isolate was
Taxonomy of Acid ...

utilized alanine, glycine, and valine as a nitrogen source and utilized arabinose, fucose, raffinose, inositol, mannitol and glutamate as a carbon source.

*Streptomyces violaceoniger*:

Isolate number 9/12 was *Streptomyces violaceoniger*. The spore chains were spirales. The spore chains were gray. Diffusible and melanin pigments were not produced. Antimicrobial activity against *Candida albicans* was observed. Isolate was lipolytic and grow at 45 °C. Isolate was utilized cysteine, phenylalanine, histidine and valine as a nitrogen source and utilized inositol and raffinose, as a carbon source.

*Streptomyces atroolivaceus*:

Isolate number 29/10 was identified as *Streptomyces atroolivaceus*. The spore chains were rectiflexibles. The spore mass was gray to white. Diffusible and melanin pigments were not produced. Antimicrobial activity against *Aspergillus niger* and *Candida albicans* were observed. The isolate was lipolytic. Isolate was utilized phenylalanine and cysteine as a nitrogen source, and utilized sucrose and raffinose as a carbon source.

*Streptomyces albus*:

Isolate 12/23 was identified as *Streptomyces albus*. The spore chains were spirales. The spore mass was gray. Diffusible and melanin pigments were not produced. Isolate was utilized alanine, glycine and valine as a nitrogen source and utilized lactose, sucrose, mannitol, lactate and glutamate as a carbon source.

*Streptomyces lydicus*:

The isolate, 29/2 was identified as *Streptomyces lydicus*. The spore chains were spirales. The spore mass was gray and reverse yellow to brown. Diffusible and melanin pigments were not produced. Isolate was utilized alanine, glycine and valine as a nitrogen source and utilized fucose, raffinose, sucrose, inositol, mannitol and glutamate as a carbon source.

*Streptomyces albidosflavus*:

Isolate 27/4 was identified as *Streptomyces albidosflavus*. The spore chains were straight. The spore mass was white. Diffusible and melanin pigments were not produced. Antimicrobial activity against *Aspergillus niger* and *Candida albicans* were observed. Isolate was tolerated 7% NaCl, utilized cysteine, phenylalanine and histidine as a nitrogen source. They utilized raffinose and dextran as a carbon source.

*Streptomyces chromogenus*:

Isolate 22/6 was identified as *Streptomyces chromogenus*. The spore chains were spirales and rectiflexibles. The spore mass was gray and the reverse was yellow to brown. Diffusible and melanin pigments were produced. Antimicrobial activity against *Candida albicans* was...
observed. Isolate was utilized cysteine, phenylalanine and valine as a nitrogen source. Isolate was utilized inositol, mannitol, raffinose and dextran as a carbon source.

*Streptomyces thermovulgaris*:

Isolate number 9/14 was identified as *Streptomyces thermovulgaris*. The spore chains were straight. The spore mass was white and reverse was orange. Diffusible pigment was produced. Isolate was utilized glycine and alanine as a nitrogen source. Isolate was utilized lactose, sucrose, inositol, mannitol and lactate as a carbon source.

*Streptomyces griseoluteus*:

Isolate 10/15 was identified as *Streptomyces griseoluteus*. The spore chains were spirales and rectiflexibles. The spore mass was gray. Diffusible and melanin pigments were not produced. Isolate was utilized xylan. Isolate was utilized phenylalanine and histidine as a nitrogen source. Isolate was utilized xylose, inositol, mannitol and raffinose as a carbon source.

*Streptomyces viridochromogenes*:

The isolate, 22/3 was identified as *Streptomyces viridochromogenes*. The spore chains were spirales and rectiflexibles. The spore mass was gray. Diffusible and melanin pigments were produced. Isolate was grown at 45 °C and tolerate 7% Na Cl. Isolate was utilized xylan. Isolate was utilized histidine as a nitrogen source. Isolate was utilized pectin, arabinose, sucrose, xylose, inositol, mannitol and raffinose as a carbon source.

ii) *Streptoverticillium*:

There were 18% of isolates belonging to *Streptoverticillium* genus, out of them 42% strictly acidophilic and 58% neutrotolerant acidophilic, substrate mycelium was nonfragmenting and branched. Aerial mycelium forms long spore chains. Most of the spore chains were straight and some of them hook. On starch casein agar, glycerol aspargine agar, nutrient agar, the growth was good and fast. They produced a wide variety of diffusion and melanin pigments. The isolates were growing mesophilic. Cell wall amino acids were L-DAP and glycine (cell wall type I). No whole cell sugar was found (Table 4.3). All the isolates identified to species as: *Streptoverticillium olivoreticulum*:

There were 4 isolates 8/1, 20/15, 22/1 and 25/2, that were identified as *Streptoverticillium olivoreticulum*. The spore chains were straight and rectiflexibles. The spore mass was white to gray. Diffusible and melanin pigments were not produced. Isolate utilized histidine as a nitrogen source. Isolate utilized sucrose, mannitol and lactose as a carbon source (Plate 4.2 g and h).
Strepotoverticillium netropsis:

The isolate, 24/9 was identified as *Strepotoverticillium netropsis*. The spore chains were straight. The spore mass was white to yellow. Diffusible pigment was produced and melanin pigment was not produced. Isolate utilized proline and tyrosine as a nitrogen source. Isolate utilized mannitol, raffinose, inositol and fructose as a carbon source. Isolate was grown at 12 °C, 5% NaCl and degradation of Tween 20. Antimicrobial activity against *Aspergillus niger* was observed.

*Strepotoverticillium griseocarneu*:

Isolate number 9/38 was identified as *Strepotoverticillium griseocarneu*. The spore chains were straight. The spore mass was yellow. Diffusible pigment was produced and melanin pigment was not produced. Isolate was utilized proline and tyrosine as a nitrogen source. Isolate was utilized mannitol, raffinose, inositol, fructose and citrate as a carbon source. Isolate was grown at 12 °C and 5% NaCl. Antimicrobial activity against *Aspergillus niger* and *Candida albicans* were observed.

*Streptoverticillium abikoense*:

The isolate, 27/6 was identified as *Streptoverticillium abikoense*. The spore chains were straight. The spore mass was yellow. Diffusible and melanin pigments were produced. Isolate was utilized proline and tyrosine as a nitrogen source. Isolate was utilized mannitol, galactose, inositol, fructose and citrate as a carbon source. Isolate was tolerated 5% NaCl. Antimicrobial activity against *Aspergillus niger* and *Candida albicans* were observed.

iii) Nocardioform:

There were 18% of isolates belonging to Nocardioform group, out of them 42% strictly acidophilic and 58% neutrotolerant acidophilic. Substrate mycelium was branched and fragmented into bacillary elements. Most of genera don't have aerial mycelium and the other genera have being sparse and invisible aerial mycelium. *Nocardia* genus was acid fast bacteria. Cell wall amino acids were meso-DAP (cell wall type IV). Whole cell sugars were arabinose and galactose. All the isolates identified to species as:

*Nocardia brasiliensis*:

There were 2 isolates 4/10 and 9/39, that were identified as *Nocardia brasiliensis*. Substrate mycelium was colorless and aerial mycelium form short spore chains. The spore mass was orange to red. Diffusible and melanin pigments were produced. The isolates tolerated 7% NaCl. The isolates were utilized chitin, xylan, pectin, tyrosine and cellulose. The isolates utilized inositol, fructose and rhamnose as a carbon source.
Taxonomy of Acid....

*Nocardia otitidiscaviarum:*

There were 2 isolates 18/3 and 23/5, that were identified as *Nocardia otitidiscaviarum*. Substrate mycelium was colorless and aerial mycelium form short spore chains. The spore mass was yellow to orange. Diffusible pigment was produced. The isolates tolerated 5% NaCl. The isolates utilized chitin, xylan, pectin and cellulose. The isolates utilized inositol, fructose and mannitol as a carbon source.

*Saccharomonospora caesia:*

The Isolate number 9/4 was identified as *Saccharomonospora caesia*. Substrate mycelium was white and aerial mycelium form single spore. The spore mass was green. Diffusible pigment was produced. The spore was heat sensitive. The isolates utilized chitin, xylan, pectin and cellulose. The isolates utilized mannose, inositol, arabinose, galactose, fructose and mannitol as a carbon source.

*Saccharopolyspora erythraea:*

The isolate. 26 10 was identified as *Saccharopolyspora erythraea*. Substrate mycelium was branched and aerial mycelium form long and straight spore chains. The spore mass was gray. Diffusible and melanin pigments were not produced. The isolate growing at 37 °C. The isolates utilized chitin, pectin and cellulose. The isolate was utilized sucrose, galactose, inositol, xylose, arabinose, fructose, rhamanose and mannitol as a carbon source.

*Saccharopolyspora hirsuta:*

The isolate number 29/4 was identified as *Saccharopolyspora hirsuta*. Substrate mycelium was white and aerial mycelium form long, spirals and straight chains. The spore mass was green. Diffusible pigment was produced. The isolate was growing at 45 °C. The isolates utilized chitin, xylan, pectin and cellulose. The isolates utilized galactose, inositol, xylose, fructose, rhamanose and as a carbon source.

iv) *Actinoplanes:*

There are 15% of isolates belonging to *Actinoplanes* genus, out of them 83% strictly acidophilic and 16% neutrotolerant acidophilic. Substrate mycelium was fine and branched. Aerial mycelium was scanty in all isolates. On starch casein agar, glycerol asparagine agar, nutrient agar, the growth was good. The substrate mycelium was white to orange. The isolates did not grow at 45 °C and sensitive to gentamicin and rifampicin. Cell wall amino acids were meso-DAP and glycine (cell wall type II). Whole cell sugars were xylose and arabinose. The isolates identified to species as:
### Table 4.3: List of acidophilic Non-Streptomyces isolates identified up to species level.

<table>
<thead>
<tr>
<th>Sr.</th>
<th>Non-Streptomyces</th>
<th>Total isolates</th>
<th>Strictly acidophilic isolates</th>
<th>Neutrotolerant acidophilic isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Streptoveriicillium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Streptoveriicillium olivorectum</em></td>
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<td>2</td>
<td></td>
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<tr>
<td></td>
<td><em>Streptoveriicillium metrogis</em></td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Streptoveriicillium griessкорнеu</em></td>
<td>1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Streptoveriicillium abokuense</em></td>
<td>1</td>
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<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>7</strong></td>
<td><strong>3</strong></td>
<td><strong>4</strong></td>
</tr>
<tr>
<td>2.</td>
<td>Nocardiia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Nocardiia brasiliensis</em></td>
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<tr>
<td></td>
<td><em>Nocardiia unisccavirum</em></td>
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<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><em>Saccharomonospora caesia</em></td>
<td>1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Saccharomonospora erythrae</em></td>
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<td></td>
</tr>
<tr>
<td></td>
<td><em>Saccharomonospora hissata</em></td>
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<td>1</td>
<td>-</td>
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<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>7</strong></td>
<td><strong>3</strong></td>
<td><strong>4</strong></td>
</tr>
<tr>
<td>3.</td>
<td>Actinoplanes</td>
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<tr>
<td></td>
<td><em>Actinoplanes humidas</em></td>
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<td>1</td>
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</tr>
<tr>
<td></td>
<td><em>Actinoplanes palleronii</em></td>
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<td></td>
<td><em>Cattellaiospora citrea</em></td>
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<tr>
<td></td>
<td><em>Micromonospora purpureochromogenes</em></td>
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<td></td>
<td><em>Micromonospora inostotile</em></td>
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<tr>
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<td><em>Micromonospora halophylica</em></td>
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<tr>
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<td><strong>5</strong></td>
<td><strong>1</strong></td>
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<tr>
<td>4.</td>
<td>Actinomadura</td>
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<td><em>Actinomadura fibrosa</em></td>
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<td><em>Microtenispora flexuosa</em></td>
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</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
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<td><strong>3</strong></td>
<td><strong>3</strong></td>
</tr>
<tr>
<td>5.</td>
<td>Thermomonospora</td>
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<td><em>Thermomonospora curvata</em></td>
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<td><em>Thermomonospora mesophila</em></td>
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<td>-</td>
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</tr>
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<td></td>
<td><em>Nocardiosis allbrubidus</em></td>
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<td>-</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>6</strong></td>
<td><strong>2</strong></td>
<td><strong>4</strong></td>
</tr>
<tr>
<td>6.</td>
<td>Thermuscinomycetes</td>
<td></td>
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</tr>
<tr>
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<td><em>Thermuscinomycetes putidus</em></td>
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<td>-</td>
</tr>
<tr>
<td></td>
<td><em>Thermuscinomycetes sacchari</em></td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><em>Thermuscinomycetes thalpophilus</em></td>
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<td>-</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><em>Thermuscinomycetes dichotomicus</em></td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>4</strong></td>
<td><strong>2</strong></td>
<td><strong>2</strong></td>
</tr>
<tr>
<td>7.</td>
<td>Others</td>
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<td><em>Glycomyces rungersensis</em></td>
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<td></td>
<td><em>Kitasatospora setae</em></td>
<td>1</td>
<td>-</td>
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</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>3</strong></td>
<td><strong>-</strong></td>
<td><strong>3</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>39</strong></td>
<td><strong>18</strong></td>
<td><strong>21</strong></td>
</tr>
</tbody>
</table>
Plate 4.2: Photograph of non-Streptomyces spp. I:

a) Glycomyces rutgersensis.

b) Actinomadura verrucospora.

c) Kitasatospora setae.

d) Actinomadura kijianiate.

f) Nocardopsis albrubidus.

e) Actinomadura fibrosa.

g) Streptoverticillium olivoreticulum.

h) Substrate mycelium of S. olivoreticulum.
Actinoplanes humidus:

The isolate, 5/17 was identified as Actinoplanes humidus. Diffusible pigment was produced. The isolate was utilized chitin, pectin and tyrosine. The isolate utilized arabinose, inositol, lactose, mannitol and raffinose as a carbon source.

Actinoplanes palleronii:

The isolate number 21/1 was identified as Actinoplanes palleronii. Diffusible and melanin pigment was not produced. The isolate utilized chitin, pectin and tyrosine. The isolate utilized arabinose and inositol as a carbon source.

Catellatospora citrea:

The isolate number 4/9 was identified as Catellatospora citrea. Only substrate mycelium was present. Straight and spiraled short spore chains were observed arising on the colony surface. The spore mass was orange. Diffusible pigment was produced. The isolate utilized chitin, xylan, pectin and cellulose. The isolate utilized mannose, sucrose, maltose, xylose and inositol as a carbon source.

Micromonospora purpureochromogenes:

The isolate, 7/24 was identified as Micromonospora purpureochromogenes. Only substrate mycelium was present. Spores were single and sessile observed arising on the substrate mycelium. The spore mass was white. Diffusible and melanin pigments were not produced. The isolate utilized chitin, xylan, pectin and lipids. The isolate utilized mannose, arabinose and raffinose as a carbon source.

Micromonospora inositole:

The isolate number 4/17 was identified as Micromonospora inositole. Only substrate mycelium was present. Spores were single and sessile observed arising on the substrate mycelium. The spore mass was white. Diffusible and melanin pigments were not produced. The isolate was growing at 37 °C and utilized chitin, xylan, pectin and lipids. The isolate utilized mannose, arabinose and raffinose as a carbon source.

Micromonospora halophytica:

The isolate, 12/3 was identified as Micromonospora halophytica. Only substrate mycelium was present. Spores were single and sessile observed arising on the substrate mycelium. The spore mass was white. Diffusible and melanin pigments were not produced. The isolate utilized chitin, xylan, pectin and lipids. The isolate utilized mannose, sucrose, fructose and raffinose as a carbon source.
Plate 4.3: Photograph of non-Streptomyces spp II:

a) *Actinoplanes humidus.*

c) *Saccharopolyspora hirsuta.*

e) *Catellatospora citrea.*

g) *Thermoactinomyces thalophilus.*

b) Substrate mycelium of *A. humidus.*

d) Substrate mycelium of *S. hirsuta.*

f) *Microtetraspora flexuosa.*

h) *Nocardia brasiliensis.*
v) Actinomadura:

There are 15% of isolates belonging to Actinomadura genus, out of them 50% strictly acidophilic and 50% neutrotolerant acidophilic. Substrate mycelium was nonfragmenting and branched. Aerial mycelium forms short or long spore chains. Spore chains were straight, hooked or spirals. On starch casein agar, glycerol asparagine agar, nutrient agar, the growth was good. The substrate mycelium was orange, yellow, pink or violet. The isolates were growing at 45 °C. Cell wall amino acids were meso-DAP (cell wall type III). Whole cell sugar was madurose. The isolates identified to species as:

Actinomadura kijianiate:

There were 2 isolates 7/1 and 14/9, that were identified as Actinomadura kijianiate. The spore chains were hooked and spore mass was pink for 7/1 and orange for 14/9. The isolate was utilized chitin, xylan, pectin and keratin. The isolate utilized mannose, sucrose, inositol and raffinose as a carbon source.

Actinomadura verrucosorsora:

There were 2 isolates 9/47 and 22/12, that were identified as Actinomadura verrucosorsora. The spore chains were hooked and spirals. The spore mass was violet for 9/47 and colorless for 22/12. The isolate was utilized chitin, xylan, cellulous and keratin. The isolate utilized mannose, sucrose, arabinose and fructose as a carbon source.

Actinomadura fibrosa:

The isolate number 17/2 was identified as Actinomadura fibrosa. The spore chains were straight and spore mass was colorless. The isolate was utilized chitin, xylan, pectin and cellulose. The isolate utilized mannose, sucrose, inositol, arabinose, fructose and raffinose as a carbon source.

Microtetraspora flexuosa:

The isolate, 28/2 was identified as Microtetraspora flexuosa. The spore chains were straight and spore mass was yellow. There are four spores on the spore chain. The isolate utilized chitin, pectin and keratin. The isolate utilized sucrose, inositol, fructose and raffinose as a carbon source.

vi) Thermomonospora:

There were 15% of isolates belonging to Thermomonospora genus, out of them 33% strictly acidophilic and 67% neutrotolerant acidophilic. Substrate mycelium was nonfragmenting and branched. Aerial mycelium forms short single spore. On starch casein agar, glycerol asparagine agar, nutrient agar, the growth was good. Most of the isolates were growing around 45
Taxonomy of Acidophilic Actinomycetes

°C. Cell wall amino acids were meso-DAP (cell wall type III). No diagnostic sugar was found. The isolates identified to species as:

*Thermomonospora chromogena*:
The isolates number 4/7, 16/1 and 29/9 were identified as *Thermomonospora chromogena*. The spore mass was gray to brown. They utilized tyrosine and galactose. They were resistant to kanamycin.

*Thermomonospora curvata*:
The isolate, 11/4 was identified as *Thermomonospora curvata*. The spore mass was gray and spore reverse was yellow. The isolate utilized chitin, xylan and cellulose. The isolate utilized mannose, ribose, sucrose, inositol and fructose as a carbon source. The isolate tolerated 6% NaCl.

*Thermomonospora mesophila*:
The isolate number 11/21 was identified as *Thermomonospora mesophila*. The spore mass was green and spore reverse was brown. The isolate utilized chitin, xylan and keratin. The isolate utilized mannose, raffinose and fructose as a carbon source. The isolate tolerated 7% NaCl.

*Nocardiopsis albrubidus*:
The isolate, 11/1 was identified as *Nocardiopsis albrubidus*. The substrate mycelium and aerial mycelium were fragmented into short rod like and the spore chains were straight. The spore mass was white to colorless and reverse was brown. The isolate utilized chitin, xylan keratin and lipids. The isolate utilized mannose, inositol, xylose, raffinose and fructose as a carbon source. The isolate was growing at 37 °C.

Thermoactinomyces:
There were 10% of isolates belonging to *Thermoactinomyces* genus, out of them 50% strictly acidophilic and 50% neutrotolerant acidophilic. Substrate mycelium was nonfragmenting and branched. On substrate and aerial mycelium form a single spore on branched and unbranched sporophores. On starch casein agar, glycerol aspargine agar, nutrient agar, the growth was good and fast growing. All the isolates were growing at 60 °C and heat resistance. Cell wall amino acids were meso-DAP (cell wall type III). No diagnostic sugar was found. The isolates identified to species as:

*Thermoactinomyces putidus*:
The isolate, 9/25 was identified as *Thermoactinomyces putidus*. Aerial mycelium was white and spore mass was yellow. Diffusible and melanin pigments were produced. The isolate
Taxonomy of Acid....

was utilized chitin, xylan keratin and lipids. The isolate utilized galactose, inositol, xylose, sucrose, arabinose, raffinose and fructose as a carbon source.

*Thermoactinomyces sacchari:*

The isolates number 14/4 was identified as *Thermoactinomyces sacchari*. Aerial mycelium was white and spore mass was yellow. Diffusible and melanin pigments were produced. The isolate utilized chitin, xylan, keratin and lipids. The isolate utilized galactose, inositol, xylose, sucrose, arabinose, raffinose and fructose as a carbon source.

*Thermoactinomyces thalpophilus:*

The isolates number 18/17 was identified as *Thermoactinomyces thalpophilus*. Aerial mycelium was white and spore mass was red. Diffusible and melanin pigments were produced. The isolate utilized chitin, xylan, pectin and lipids. The isolate utilized mannose, inositol, xylose, sucrose, arabinose, raffinose and fructose as a carbon source.

*Thermoactinomyces dichotomius:*

The isolates number 22/10 was identified as *Thermoactinomyces dichotomius*. Aerial mycelium was white and spore mass was yellow. Diffusible and melanin pigments were not produced. The isolate utilized chitin, xylan and keratin. The isolate utilized mannose, inositol, lactose, sucrose, arabinose, raffinose and fructose as a carbon source.

viii) Other genera:

There were 7% of isolates belonging to other genera, all of them neutrotolerant acidophilic. Substrate mycelium was nonfragmenting and branched. Aerial mycelium formed a long and short spore chains on branched and unbranched sporophores. On starch casein agar, glycerol aspargine agar, nutrient agar, the growth was good. Cell wall amino acids and whole cell sugars type were different from species to another in these genera. The isolates identified to species as:

*Glycomyces rutgersensis:*

There are 2 isolates 7/16 and 25/3 were identified as *Glycomyces rutgersensis*. Aerial mycelium was white and spore mass was white. Aerial mycelium formed short spore chains. Diffusible and melanin pigments were produced. Cell wall amino acids were meso-DAP and glycine (cell wall type II). Whole cell sugars were xylose and arabinose. The isolates utilized chitin, xylan, keratin and cellulose. The isolates utilized galactose, xylose, raffinose and fructose as a carbon source. The isolates tolerated 6% NaCl.

*Kitasatosporia setae:*

The isolates number 28/13 was identified as *Kitasatosporia setae*. Aerial mycelium was white and spore mass was yellow. Diffusible and melanin pigments were produced. Aerial
mycelium formed long spore chains. Diffusible and melanin pigments were produced. Cell wall amino acids were meso-DAP and L-DAP. Whole cell sugar was galactose. The isolate utilized chitin, lipids and cellulose. The isolate utilized mannose, galactose, inositol, arabinose and fructose as a carbon source.

4.3.2 Sensitivity of Acidophilic Actinomycetes to:

4.3.2.1 Antibiotics:

There are 2% of the isolates resistant to gentamycin all of them belonged Micromonospora strictly acidophilic. There are 71% of the isolates resistant to nalidixic acid most of them belonged strictly acidophilic (62%) and most of them Sterptomyces spp. For polymyxin-B there are 49% of the isolates resistant to this antibiotic and distributed equal between the two groups of acidophilic actinomycetes. There are 68% of the isolates 58% of them belonging strictly acidophilic actinomycetes resistant to rifampicin. Only 7% of the isolates belonged to Actinomadura genera 60% of them strictly acidophilic actinomycetes resistant to kanamycin. Resistant to novobiocin isolates belonged to Micromonospora and Thermoactinomyces genus and they are 11% of them 70% belonging to strictly acidophilic actinomycetes. For tetracycline, only 7% of the isolates resistant to this antibiotic, most of them belonged strictly acidophilic. There are 22% of the isolates resistant to Penicillin-G half of them belonged to neutrotolerant acidophilic. For oxytetracycline there are 11% of the isolates out of them 50% belonged strictly acidophilic. Amphotericin-B is polyene antibiotic most of the isolates resistant to this antibiotic (88%). Out of them 61% strictly acidophilic actinomycetes. For clotrimazole antibiotic there are 20% of the isolates resistant, most of them belonged neutrotolerant acidophilic actinomycetes (62%). There are 94% of the isolates resistant to fluconazole, most of them strictly acidophilic. For nitrofurazone antibiotic there are 65% of the total isolates resistant. Out of them 56% belonged strictly acidophilic actinomycetes. For nystatin all the isolates resistant to this antibiotic, most of them 57% belonged to strictly acidophilic actinomycetes (Fig. 4.6).

4.3.2.2 Detergents:

Three are three groups of detergent anionic, nonionic and cationic. Cationic detergents have less resistant from acidophilic actinomycetes. For anionic, acidophilic actinomycetes more resistant. For SDS 63% of total isolates, out of them only 37% neutrotolerant acidophilic were resistant to this detergent. There are 37% of the total isolates resistant to SLS and most of them belonged strictly acidophilic actinomycetes (64%). Nonionic detergents are more resistant of acidophilic actinomycetes, for Tween 80 all the isolates resistance more than the half resistant belonged to strictly acidophilic. Three are 93% of the total isolates resistant to Tween 20, out of
them 58% belonged to strictly acidophilic actinomycetes. Last Nonionic detergent, are Triton x 100 which there are 89% of the isolates resistant to this detergent. Out of them 56% belonged to strictly acidophilic. Cationic detergent, were more effective than other detergents. Only 49% of the total isolates resistant to cetrimide, from them 60% belonged to strictly acidophilic actinomycetes. Strictly acidophilic actinomycete isolates have ability to resist detergent more than neutrotolerant acidophilic actinomycetes (Fig. 4.7. Plate 4.4i).

![Graph showing resistance to antibiotics](image)

**Fig 4.6: Resistance to antibiotics**

The results obtained from Minimal Inhibitory Concentration (MIC) of detergents to acidophilic actinomycetes indicate that cationic detergent (Cetrimide) has showed more effect against acidophilic actinomycetes. Strictly acidophilic more resistant to cetrimide than neutrotolerant acidophilic actinomycetes. While, the nonionic detergents. Tween 80 showed less sensitivity than other nonionic detergents Tween 20 and Triton X 100. The minimal inhibitory concentration of anionic detergents was nearly same (Table 4.4).
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**Table 4.4: Minimal Inhibitory Concentration (MIC) ranges of detergents to acidophilic actinomycetes.**

<table>
<thead>
<tr>
<th>Detergents</th>
<th>Total acidophilic actinomycetes</th>
<th>Strictly acidophilic actinomycetes</th>
<th>Neutrotolerant acidophilic actinomycetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Nonionic: (µg/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i. Tween 80</td>
<td>$10^2 - R$</td>
<td>$10 - R$</td>
<td>$10^2 - R$</td>
</tr>
<tr>
<td>ii. Tween 20</td>
<td>$10^2 - R$</td>
<td>$10 - R$</td>
<td>$10 - 10^2$</td>
</tr>
<tr>
<td>iii. Triton X 100</td>
<td>$10^2 - R$</td>
<td>$10^2 - R$</td>
<td>$10 - 10^2$</td>
</tr>
<tr>
<td>2. Anionic: (µg/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i. SDS</td>
<td>$10^2 - 10^3$</td>
<td>$10^2 - 10^3$</td>
<td>$10^2 - 10^3$</td>
</tr>
<tr>
<td>ii. SLS</td>
<td>$10^2 - 10^4$</td>
<td>$10^2 - 10^4$</td>
<td>$10^2 - 10^4$</td>
</tr>
<tr>
<td>3. Cationic: (µg/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i. Cetrimide</td>
<td>$7.7 \times 10 - 7.7 \times 10^3$</td>
<td>$7.7 \times 10 - 7.7 \times 10^3$</td>
<td>$7.7 \times 10^2 - 7.7 \times 10^3$</td>
</tr>
</tbody>
</table>

Determined of MIC by E-test on SCA media, at 37°C and pH 4.5.

![Fig 4.8: Resistance to Detergents](image)
4.3.2.3 Heavy Metals:

The acidophilic actinomycetes have different reactions with the 8 heavy metals used in this test. For copper, there are 69% of the isolates resistant to it, most of them (68%) belonged strictly acidophilic actinomycetes. The isolates were less resistant to zinc, 57% of the total isolates resistant to it. Out of them 69% strictly acidophilic and 31% neutrotolerant acidophilic actinomycetes. For silver, similar reaction 59% of the isolates resistant to it, neutrotolerant acidophilic actinomycetes were slightly more resistant (55%) than another group. Mercuric have very less action, only 29% of the total isolates resistant to it. Neutrotolerant acidophilic actinomycetes have more resistant (59%). There are 76% of the isolates were resistant to molybdate. Out of them 55% strictly acidophilic actinomycetes. Similar results were found for arsenate, 74% of the total isolates were resistant to this metal. Neutrotolerant have less resistant isolates 35% from them. Most of the isolates resistant to arsenate belonged to strictly acidophilic actinomycetes. Half number of isolates was found resistant to lead, slightly strictly acidophilic actinomycete isolates were more resistance to this metal (55%). There are 74% of the isolates resistant to cadmium salt. Out of them, 62% belonged to strictly acidophilic actinomycetes. Neutrotolerant acidophilic less resistant from other group in 6 metals out of 8 at 200 ug/ml (Fig. 4.8, Plate 4.4f).
Response of acidophilic actinomycete isolates to eight metal ions indicates that most of the acidophilic actinomycetes are resistant to As$^{5+}$ and Mo$^{6+}$ ions which distributed to group I. Acidophilic actinomycetes are resistant to this group were resistant at high concentration. Group II contain Zn$^{2+}$, Cu$^{2+}$ and Cd$^{2+}$ which have less resistant for acidophilic actinomycetes. Acidophilic actinomycetes are sensitive to group III which contain Ag$^{+}$, Hg$^{2+}$ and Pb$^{2+}$. All acidophilic actinomycetes groups have same similar action against heavy metals which were used in this experiment (Table 4.5).

<table>
<thead>
<tr>
<th>Heavy metal ions</th>
<th>MIC (µg/ml)</th>
<th>Total acidophilic actinomycetes</th>
<th>Strictly acidophilic actinomycetes</th>
<th>Neutrotolerant acidophilic actinomycetes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1. Group I:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i. As$^{5+}$</td>
<td>1.5×10$^1$ - R</td>
<td>1.5×10$^2$ - R</td>
<td>1.5×10$^3$ - R</td>
<td></td>
</tr>
<tr>
<td>ii. Mo$^{6+}$</td>
<td>2.5×10$^2$ - R</td>
<td>2.5×10$^3$ - R</td>
<td>2.5×10$^4$ - R</td>
<td></td>
</tr>
<tr>
<td><strong>2. Group II:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i. Zn$^{2+}$</td>
<td>3×10$^1$ - 3×10$^3$</td>
<td>3×10$^2$ - 3×10$^3$</td>
<td>3×10$^3$ - 3×10$^3$</td>
<td></td>
</tr>
<tr>
<td>ii. Cu$^{2+}$</td>
<td>2.5×10$^2$ - 2.5×10$^3$</td>
<td>2.5×10$^3$ - 2.5×10$^3$</td>
<td>2.5×10$^4$ - 2.5×10$^4$</td>
<td></td>
</tr>
<tr>
<td>ii. Cd$^{2+}$</td>
<td>7.7×10$^2$ - 7.7×10$^3$</td>
<td>7.7×10$^3$ - 7.7×10$^3$</td>
<td>7.7×10$^4$ - 7.7×10$^4$</td>
<td></td>
</tr>
<tr>
<td><strong>3. Group III:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i. Ag$^{+}$</td>
<td>3×10$^1$ - 3×10$^3$</td>
<td>3×10$^2$ - 3×10$^3$</td>
<td>3×10$^3$ - 3×10$^3$</td>
<td></td>
</tr>
<tr>
<td>ii. Hg$^{2+}$</td>
<td>3×10$^2$ - 3×10$^3$</td>
<td>3×10$^3$ - 3×10$^3$</td>
<td>3×10$^4$ - 3×10$^4$</td>
<td></td>
</tr>
<tr>
<td>iii. Pb$^{2+}$</td>
<td>5.5×10$^2$ - 5.5×10$^3$</td>
<td>5.5×10$^3$ - 5.5×10$^3$</td>
<td>5.5×10$^4$ - 5.5×10$^4$</td>
<td></td>
</tr>
</tbody>
</table>

Determined of MIC by E-test on SCA media, at 37°C and pH 4.5.

### 4.3.3 Identification of Acidophilic Actinomycetes by PIB Computer Software:

This software programme which is used for identification of acidophilic actinomycetes depends upon the morphological, physical, antimicrobial activity, and chemical characterization. Three are 53 characters which used for identification of Actinoplanes genera. All the 6 Actinoplanes' isolates identified up to species level by this software all of them belonging to strictly acidophilic actinomycetes group and the high percent for Catellatospora citrea AK49 and Actinoplanes palleronii AK212 (98%). For Streptoverticillium genera there 41 characters and there 7 isolates belonged to these genera, 3 of them belonging to strictly acidophilic actinomycetes. The maximum percentage found for Streptoverticillium olivoreticulum AK81.
4.3.4 Identification of Acidophilic Actinomycetes by Numerical Classification Matrix:

This matrix was further evaluated by applying it to the identification of 147 unknown acidophilic actinomycete isolates. The characters in the matrix were determined for the isolates, using the methods of Seong et al., (1993). The test date was assessed against this matrix using the PIB programme to determine identification score. The matrix consisted of 215 characters derived from numerical classification data. The characters covered a wide range of attributes, including morphology, pigmentation, antibiotics, antibiotic resistance, growth tolerances and utilization of carbon and nitrogen sources. The positive characters calculated and form unsorted similarity matrix and sorted shaded diagram. The results constructed in the sorted shaded diagram placing them by organism in the same order in first dendogram for Streptomyces and Streptoverticillium species. These two genera confirmed to join in 82% similarity then Streptomyces divided into three major groups at 84% similarity. For major cluster group start to distributed to different species at 86% similarity. The maximum similarity percentage was found in this group with S. diastaticus which consist 6 strains (94%). But for minor cluster, single cluster and Streptoverticillium species started at 95% similarity. The maximum similarity was found at 92% for S. canus of minor cluster, for S. longisporoflavus of single cluster and Str. olivoreticulum of Streptoverticillium species (Fig. 4.8). Second dendogram for acidophilic actinomycetes other than Streptomyces and Streptoverticillium genera. At 73% similarity the isolates divided into 6 genera. Actinomadura genera start divided to different species at 80% similarity. For Thermomonospora species were divided at 77% similarity. Thermoactinomyces were started distribute to different species at 79%. Actinoplanes and other genera start too
Taxonomy of Acid....

divided to different species at 80% similarity. For Nocardia species start too divided at 81%
similarity. Maximum similarity was found with Glycomyces rutgersensis strains 91% (Fig. 4.9).
Table 4.6: List of acidophilic actinomycete isolates identified up to species level by PIB programme.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Identification isolate species</th>
<th>%*</th>
<th>Group</th>
<th>Isolate</th>
<th>Identification isolate species</th>
<th>%</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/1</td>
<td>Streptomyces prasinosporus</td>
<td>98</td>
<td>Minor</td>
<td>8/13</td>
<td>Streptomyces glaucescens</td>
<td>98</td>
<td>Minor</td>
</tr>
<tr>
<td>1/5</td>
<td>Streptomyces nogalater</td>
<td>96</td>
<td>Minor</td>
<td>8/16</td>
<td>Streptomyces chattanoogensis</td>
<td>84</td>
<td>Minor</td>
</tr>
<tr>
<td>1/8</td>
<td>Streptomyces prasinosporus</td>
<td>87</td>
<td>Minor</td>
<td>8/20</td>
<td>Streptomyces diastaticus</td>
<td>98</td>
<td>Major</td>
</tr>
<tr>
<td>3/3</td>
<td>Streptomyces prasinosporus</td>
<td>96</td>
<td>Minor</td>
<td>9/4</td>
<td>Saccharomonospora caviae</td>
<td>-</td>
<td>Noca.</td>
</tr>
<tr>
<td>3/4</td>
<td>Streptomyces glaucescens</td>
<td>97</td>
<td>Minor</td>
<td>9/12</td>
<td>Streptomyces violaceoniger</td>
<td>96</td>
<td>Major</td>
</tr>
<tr>
<td>4/1</td>
<td>Streptomyces flavoelus</td>
<td>95</td>
<td>Minor</td>
<td>9/13</td>
<td>Streptomyces prasinosporus</td>
<td>98</td>
<td>Minor</td>
</tr>
<tr>
<td>4/7</td>
<td>Thermomonospora chromogena</td>
<td>-</td>
<td>Therm.</td>
<td>9/14</td>
<td>Streptomyces thermovulgaris</td>
<td>91</td>
<td>Major</td>
</tr>
<tr>
<td>4/9</td>
<td>Catellatospora citrea</td>
<td>98</td>
<td>Actinp.</td>
<td>9/18</td>
<td>Streptomyces prasinosporus</td>
<td>98</td>
<td>Minor</td>
</tr>
<tr>
<td>4/10</td>
<td>Nocardia brasiliensis</td>
<td>-</td>
<td>-</td>
<td>9/25</td>
<td>Thermactinomyces putidus</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4/11</td>
<td>Streptomyces nogalater</td>
<td>98</td>
<td>Minor</td>
<td>9/28</td>
<td>Streptomyces cyanus</td>
<td>95</td>
<td>Major</td>
</tr>
<tr>
<td>4/12</td>
<td>Streptomyces chattanoogensis</td>
<td>98</td>
<td>Minor</td>
<td>9/38</td>
<td>Streptosporiellum griseocarnen</td>
<td>87</td>
<td>Streve.</td>
</tr>
<tr>
<td>4/13</td>
<td>Streptomyces halstedii</td>
<td>74</td>
<td>Major</td>
<td>9/39</td>
<td>Nocardia brasiliensis</td>
<td>-</td>
<td>Noca.</td>
</tr>
<tr>
<td>4/16</td>
<td>Streptomyces flavoelus</td>
<td>63</td>
<td>Minor</td>
<td>10/2</td>
<td>Streptomyces antibioticus</td>
<td>64</td>
<td>Major</td>
</tr>
<tr>
<td>4/17</td>
<td>Micromonospora inoizotae</td>
<td>-</td>
<td>Actinp.</td>
<td>10/4</td>
<td>Streptomyces prasinosporus</td>
<td>93</td>
<td>Minor</td>
</tr>
<tr>
<td>5/1</td>
<td>Streptomyces cyanus</td>
<td>97</td>
<td>Major</td>
<td>10/11</td>
<td>Streptomyces xanthochromogenes</td>
<td>66</td>
<td>Minor</td>
</tr>
<tr>
<td>5/2</td>
<td>Streptomyces exfoliatus</td>
<td>96</td>
<td>Major</td>
<td>10/15</td>
<td>Streptomyces griseolatus</td>
<td>98</td>
<td>Minor</td>
</tr>
<tr>
<td>5/7</td>
<td>Streptomyces exfoliatus</td>
<td>80</td>
<td>Major</td>
<td>10/16</td>
<td>Streptomyces graminificaciens</td>
<td>96</td>
<td>Minor</td>
</tr>
<tr>
<td>5/8</td>
<td>Streptomyces nogalater</td>
<td>95</td>
<td>Minor</td>
<td>10/19</td>
<td>Streptomyces glaucescens</td>
<td>98</td>
<td>Minor</td>
</tr>
<tr>
<td>5/15</td>
<td>Streptomyces xanthochromogenes</td>
<td>85</td>
<td>Minor</td>
<td>10/21</td>
<td>Streptomyces antibioticus</td>
<td>98</td>
<td>Major</td>
</tr>
<tr>
<td>5/16</td>
<td>Streptomyces rochei</td>
<td>63</td>
<td>Major</td>
<td>11/4</td>
<td>Nocardiosis albusdatus</td>
<td>-</td>
<td>Therm.</td>
</tr>
<tr>
<td>5/17</td>
<td>Actinoplanes humida</td>
<td>96</td>
<td>Actinp.</td>
<td>11/2</td>
<td>Streptomyces flavoelus</td>
<td>87</td>
<td>Minor</td>
</tr>
<tr>
<td>5/20</td>
<td>Streptomyces nogalater</td>
<td>98</td>
<td>Minor</td>
<td>11/3</td>
<td>Streptomyces antibioticus</td>
<td>98</td>
<td>Major</td>
</tr>
<tr>
<td>5/23</td>
<td>Streptomyces pactum</td>
<td>94</td>
<td>Minor</td>
<td>11/9</td>
<td>Streptomyces longissimus</td>
<td>85</td>
<td>Single</td>
</tr>
<tr>
<td>5/24</td>
<td>Streptomyces nogalater</td>
<td>98</td>
<td>Minor</td>
<td>11/14</td>
<td>Streptomyces diastaticus</td>
<td>98</td>
<td>Major</td>
</tr>
<tr>
<td>6/3</td>
<td>Streptomyces antibioticus</td>
<td>98</td>
<td>Major</td>
<td>11/17</td>
<td>Streptomyces antibioticus</td>
<td>98</td>
<td>Major</td>
</tr>
<tr>
<td>6/4</td>
<td>Streptomyces rochei</td>
<td>88</td>
<td>Major</td>
<td>11/19</td>
<td>Streptomyces glaucescens</td>
<td>96</td>
<td>Minor</td>
</tr>
<tr>
<td>7/1</td>
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## Taxonomy of acid...

### Continue Table 4.6:

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<th>Group</th>
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<td><em>Streptomyces prasinosporus</em></td>
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<td>29/1</td>
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<td>Streve.</td>
<td>31/4</td>
<td><em>Streptomyces rochei</em></td>
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</table>

* Percentage of the Probability Matrix by PIB software.
Taxonomy of Acidophiles

Fig 4.8: Dendrogram showing the relationships between acidophilic Streptomyces and Streptoverticillium species recovered by Ssm and PIB programme analysis

<table>
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<th>Similarity (%)</th>
<th>No. of Strains</th>
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<tr>
<td>100</td>
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</table>

Streptomyces sp.

Major 86%

1. Sp. thermovulgaris
2. Sp. vaginalis
3. Sp. griseus
4. Sp. griseus
5. Sp. thermovulgaris
6. Sp. vaginalis
7. Sp. griseus
8. Sp. griseus
9. Sp. griseus
10. Sp. griseus
11. Sp. griseus
12. Sp. griseus
13. Sp. griseus
14. Sp. griseus
15. Sp. griseus
16. Sp. griseus
17. Sp. griseus
18. Sp. griseus
19. Sp. griseus
20. Sp. griseus

Minor 85%

Single 85%

1. Sp. thermovulgaris
2. Sp. vaginalis
3. Sp. griseus
4. Sp. griseus

156
Fig. 4.9: Dendrogram showing the relationships between other acidophilic actinomycete species recovered by Ssm and PIB programme analysis.
Plate 4.4: Pigments, Colony and MIC:
  a, b, e and d) Pigment production.
e, f, g and h) Colony morphology.
i) E-test for MIC of detergents for acidophilic actinomycetes.
j) E-test for MIC of heavy metals for acidophilic actinomycetes.
Plate 4.5: Utilization of different sugars and acid production.

a) Glucose agar.
b) Sucrose agar.
c) Mannose agar.
d) Fructose agar.
e) Galactose agar.
f) Maltose agar.
4.4 Discussion:

The actinomycetes have been traditionally considered to be prokaryotic bacteria with elongated cells or filaments that usually showed some degree of true branching. Although the morphology of these organisms ranges from simple to complex, most strains of most species can be assigned to one of two broad morphological groups, nocardioform- and sporo-actinomycetes (Goodfellow and Cross, 1984).

Actinomycetes have for many years been grouped together solely on morphological grounds even though they have never been satisfactorily distinguished from coryneform bacteria on this basis (Goodfellow and Minnikin, 1985).


Actinomycetes are distinguished from other groups in the second category by formation of branched filaments bearing arthrospores. Actinomycetes group is further divided into seven groups on the basis of morphological features, diagnostic cell wall amino acids and whole cell sugars according to Bergey's Manual of Determinative Bacteriology. Those seven groups included group 22 was contain Nocardioform actinomycetes, group 23 was contain genera with multilocular, group 24 was contain actinoplanetes, group 25 was contain Streptomyces and related genera, group 26 was contain maduromycetes, group 27 was contain thermoactinomycetes, and group 29 was contain other genera (Holt et al., 1994).

In the second edition of Bergey's Manual of Systematic Bacteriology, which classified bacteria into five volumes. Volume 1 contained the Archaea, the deeply branching and phototrophic Bacteria. Volume 2 contained the Proteobacteria. Volume 3 was containing the low G + C Gram-positive Bacteria. Volume 4 was containing the high G + C Gram-positive Bacteria which included order Actinomycetales. Volume 5 was containing the Planctomycetes, Spirochetes, Fibrobacteres, Bacteroidetes and Fusobacteria. In this edition Actinomycetales order located under subclass V Actinobacteridae. This order contains ten suborder, 38 family and 121 genera (Garrity et al., 2003).

There are some differences between Bergey's Manual of Determinative Bacteriology by Holt et al., (1994) and Bergey's Manual of Systematic Bacteriology by Garrity et al., (2003) for classification of actinomycetes. Bergey's Manual of Determinative Bacteriology by Holt et al., (1994) classified actinomycetales order to seven groups but in Bergey's Manual of Systematic Bacteriology by Garrity et al., (2003) classified to ten groups or suborders including group I Actinomycetia (such as Actinomyces genus), group II Micrococcineae (such as Micrococcus,
Taxonomy of Acidophilic Actinomycetes

Acidophilic actinomycete isolates selected from different sites and acidophilic groups were identified to genus and species level by Bergey's Manual of Determinative Bacteriology by Holt et al., (1994). Isolates identified belonged to eight different genera. Isolates belonged to Streptomyces were predominant in all sites and two acidophilus groups. Isolates belonged to Streptomyces, Streptoverticillium, Nocardia, Actinoplanes, Actinomadura, Thermomonospora, Thermoactinomyces, Saccharopolyspora, Saccharomonospora, Catellatospora, Micromonospora, Microtetraspora, Nocardiopsis, Glycomyces and Kitasatosporia.

Isolates belonging to the genus Streptomyces were identified on the basis of cell wall amino acids, whole cell sugars, morphological, physiological and cultural characters, spore chain patterns, shape and ornamentation of spore surface and many other characters. They were distinguished from other related genera on the basis of morphology, spore chain patterns, cell wall amino acid and whole cell sugars. The closely related genera to Streptomyces are Streptoverticillium and Kitasatosporia. Arrangement of spore chains on the aerial mycelium distinguishes Streptomyces from Streptoverticillium. In Streptoverticillium spore chains are arranged in verticals. Kitasatosporia do not form spiral and hook like spore chains.

There are twenty seven different species of Streptomyces which were found in both acidophilic groups and all sites. All isolates belonged to major, minor and single clusters. All isolates belonging to Streptomyces genus were identified to the species level by software programme and probability matrix. The high score of similarity by software reach 98% indicates about the identical species. Predominant Streptomyces species were Streptomyces flaveolus,
**Streptomyces prasinosporus, Streptomyces glaucescens, Streptomyces antibioticus, Streptomyces nogalater and Streptomyces cyaneus.**

Some isolates belonging to *Streptomyces* species showed deviations from standard descriptions in Bergey's Manual of Determinative Bacteriology Holt *et al.*, (1994) and Bergey's Manual of Systematic Bacteriology Williams *et al.*, (1989). Deviations from standard descriptions were found especially in the production of diffusible and melanin pigment, salt tolerance, growth at different temperatures and biochemical characters. Many isolates did not produce diffusible and melanin pigment. Some showed deviations in growth at 45°C. Deviations were found in utilisation of histidine, phenylalanine and meso-inositol. Isolates which showed variations from standard description belonged to *Streptomyces diastaticus, Streptomyces rochei, Streptomyces xanthochromogenes and Streptomyces albidoflavus.*

*Streptoverticillium* and *Streptomyces* species to be closely related, both genus have a wall chemotype I. Predominant *Streptoverticillium* species were *Streptoverticillium olivoreticulum* in the both acidophilic *Streptoverticillium* and *Streptoverticillium metropsis* showed deviations from standard description in Bergey's Manual of Determinative Bacteriology Holt *et al.*, (1994). Classifying species into series based on colour of the aerial, substrate mycelium, nonfragmenting substrate mycelium and a wall developed aerial mycelium which bears short chains of spores. In Bergey's Manual of Systematic Bacteriology by Garrity *et al.*, (2003) were classified *Streptoverticillium, Streptomyces* and *Kitasatospora* in group VII suborder Streptomycineae.

The genus *Nocardia*, a member of Nocardioform group form rudimentary to extensively branched vegetative and aerial mycelium that fragments into coccoid to rod shaped elements. Spore chains are produced on the aerial mycelium. This is the distinguishing character of this genus from other related genera. The predominant *Nocardia* species were *Nocardia brasiliensis* and *Nocardia otitidiscaviarum*. Bergey's Manual of Systematic Bacteriology by Garrity *et al.*, (2003) were classified *Nocardia* with *Rhodococcus* in group II under Micrococcineae suborder.

The other *Nocardia* genus is *Saccharomonospora* which have nonfragmenting, nonmotile sporactinomycetes typically forming a branched substrate mycelium. Single sensitive spores are densely packed along the aerial hyphae on unbranched sporophores of variable length. There is one species which belonged to this genus *Saccharomonospora cavia.*

*Saccharopolyspora* genus is belonging to *Nocardia* by Holt *et al.*, (1994). *Saccharopolyspora* and *Saccharomonospora* genera in Bergey's Manual of Systematic Bacteriology by Garrity *et al.*, (2003) were classified under suborder Pseudonocardineae group.
V1. *Saccharopolyspora* genus is nonacid fast actinomycetes, substrate mycelium fragmented into rod shaped elements. This genus presented by two species *Saccharopolyspora erythraea* and *Saccharopolyspora hirsuta*.

The genus *Actinoplanes* is classified under the group Actinoplanetes in Bergey's Manual of Determinative Bacteriology Holt *et al.*, (1994). Genera in this group are distinguished from each other on the basis of shape of the sporangium, number, shape and arrangement of spores in the sporangium, motility and flagellation of the spores and other such characters. Two isolates were identified belonging to *Actinoplanes*. Identification of isolates to the species level was done on the basis of morphology of sporangium, presence or absence of aerial mycelium and assimilation of carbon sources. Species belonging to this genus were identified as *Actinoplanes humidus* and *Actinoplanes pulleronii* both species have nonmotile spores. They are belonging to strictly acidophilic actinomycetes.

The genus *Catellatospora* is distinguished from other related genera of *Actinoplanes* by its unique feature that it not produce sporangium. Spore chains are produced on the vegetative mycelium which is seen on the surface of the medium. Member of this genus is classified to the species level by differences in cell wall components, resistance to novobiocin, growth requirements and utilisation of sugars. One isolate was identified as *Catellatospora citrea* from strictly acidophilic actinomycetes.

The genus *Micromonospora* is classified under Actinoplanetes group in Bergey's Manual of Determinative Bacteriology Holt *et al.*, (1994). It is characterized by its morphology, single spores on the substrate mycelium, cell wall amino acid and whole cell sugars. *Micromonospora* could be distinguished from genera which do not form aerial mycelium by morphology, number of spores and spores in sporangia. It could by distinguished from the genera which form single spores as these form aerial hypha and their cell wall chemotypes and whole cell sugar pattern is different. Three isolates were identified to species belonging to *Micromonospora* such as *Micromonospora purpureochromogenes*, *Micromonospora inositole* and *Micromonospora halophytica*. The first two belonging to strictly acidophilic actinomycetes. Bergey's Manual of Systematic Bacteriology by Garrity *et al.*, (2003) were classified the *Actinoplanes*, *Catellatospora* and *Micromonospora* under suborder IV Micromonosporineae.

Five isolates were identified to species belonging to *Actinomadura* such as *Actinomadura kijianiate*, *Actinomadura verrucospora* and *Actinomadura fibrosa*. The first two species were predominant. This genus distinguished from other genera by cell wall amino acids, whole cell sugars, spore pattern and chains. Most species of this genus belonging to strictly acidophilic actinomycetes.
The genus *Microtetraspora* is classified under *Actinomadura* group in Bergey's Manual of Determinative Bacteriology *Holt et al.* (1994). There is only one species as *Microtetraspora flexiosa* which belonging to neutrotolerant acidophilic actinomycetes. This genus distinguished from other by aerial mycelium, spores number and cell wall chemotypes. *Microtetraspora* and *Actinomadura* genera classified by Bergey's Manual of Systematic Bacteriology by *Garrity et al.*, (2003) under group VIII Streptosporangineae suborder.

The genus *Thermomonospora* is classified in separate genera in Bergey's Manual of Determinative Bacteriology *Holt et al.*, (1994). An artificial grouping of mesophilic and thermophilic actinomycetes with a cell wall containing meso-DAP and no other characteristic sugars or amino acids (wall chemotype III). Predominant *Thermomonospora* species is *Thermomonospora chromogena*. Other species were *Therm. curvata* and *Therm. mesophila*.

Genus *Nocardiopsis* is non-acid fast, non-motile, sporoactinomycetes which form a branched substrate mycelium that fragments into rod elements. It is classified by *Holt et al.*, (1994) with *Thermomonospora* genus. *Thermomonospora* and *Nocardiopsis* were classified by Bergey's Manual of Systematic Bacteriology by *Garrity et al.*, (2003) under suborder or group VIII Streptosporangineae.

The genus *Thermoactinomyces* is single spores which borne on both substrate and aerial hyphae with extensive branch forming compact colonies. This genus is classified in separate genera according to Bergey's Manual of Determinative Bacteriology *Holt et al.*, (1994).

Predominant of other genera is *Glycomyces* genus with long chain spores on aerial mycelium. It is distinguish from other genera by cell wall chemotype. Recently classified under separate group or suborder X Glycomycineae by Bergey's Manual of Systematic Bacteriology by *Garrity et al.*, (2003).There are two species of this genus identified as *Glycomyces rugersensis*.

Second genus of other genera is *Kitasatospora* with club shaped sporangia on substrate and aerial hypha contain long chain spores and identified by Bergey's Manual of Determinative Bacteriology *Holt et al.*, (1994). In recent classification this genus is classified under Streptomycineae group by Bergey's Manual of Systematic Bacteriology by *Garrity et al.*, (2003). Specie of this genus is *Kitasatospora setae*.

In order to be able to exploit the potential of bioremediation and phytoremediation, basic environmental phenomena like the effects of acidophilic actinomycetes on metals have to be understood. This thesis gives insight how these phenomena could be exploited when developing...
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bioremediation methods for metal, especially Cu\(^{2+}\), Zn\(^{2+}\), Ag\(^{+}\), Hg\(^{2+}\), Mo\(^{6+}\), As\(^{3+}\), Pb\(^{2+}\) and Cd\(^{2+}\) contaminated soils.

Unlike organic pollutants that can be mineralized to harmless products such as Cu\(^{2+}\), Zn\(^{2+}\), As\(^{3+}\), Pb\(^{2+}\) and Cd\(^{2+}\) cannot be biodegraded, but persist indefinitely, complicating the remediation of contaminated soils. Therefore, the main strategy is to reduce the bioavailability, mobility and toxicity of the metal. Biological methods for remediation of Cu\(^{2+}\), Zn\(^{2+}\), Ag\(^{+}\), Hg\(^{2+}\), Mo\(^{6+}\), As\(^{3+}\), Pb\(^{2+}\) and Cd\(^{2+}\) contaminated soils include detoxification, bioleaching and phytoremediation. In arsenic-contaminated soils, microbial methylation of inorganic arsenic to water soluble methylated arsenic forms, monomethyl arsonic acid (MMAA) and dimethyl arsinic acid (DMAA), may function as a detoxification method. However, in soils biomethylation of arsenic was of minor importance. Also, the possibility that MMAA and DMAA may be transformed to highly toxic volatile arsines by biomethylation has to be taken into account in the bioremediation of arsenic-contaminated soils.

The bioavailability of heavy metals was low at all sites when compared to the acid-soluble metals concentration. This can explain why biomethylation was not a common microbial transformation process in the soils. Even though the results of this study revealed that biomethylation activity in metals-contaminated soils was low, metals were bioleached in laboratory microcosms. Therefore, as a result of enhanced microbial activity due to addition of nutrient rich or organic soil, the mobilization of metals from soil may be increased and result in pollution of groundwater and downstream lakes. Thus, bioleaching is not a suitable in situ bioremediation method. More likely, bioleaching could be a potential ex-situ bioremediation method in which the contaminated soils are treated in closed tanks or reactor vessels.

Taxometric methods have also revealed that acidophilic actinomycetes, which grow between pH 3.5 and 6.5 but not at pH 7.0, form a diverse group. These organisms, which are numerous and wide-spread in natural and man-made acidic habitats, are readily recovered on acidified isolation media (Khan and Williams, 1975).

More recently, probability matrix has been assembled for the identification of unknown acidophilic actinomycetes (Seong et al., 1995; Seung et al., 2004). This matrix is based upon 21 characters drawn from the 150 unit characters used in the original classification (Lonsdale, 1985).

Our study was based upon 13 characters drawn from the 215 unit characters used. The results were used to group the isolates with help of software PIB to classify the isolates to species and all isolates grouped in unsorted similarity matrix and unsorted similarity matrix to
construct a dendrogram for all isolates. In Bergey's Manual of Systematic Bacteriology by Garrity et al., (2003) which classified acidophilic actinomycetes into two group. The first group is under suborder X Frankinea, Family Acidothermaeae, which consist of one genus Acidothermus. This genus is contain the actinomycetes grow in acidic and high Temperature. Second group is under order Acidimicrobiales, suborder Acidimicroineae, and family Acidimicrobiaeae which contain one genus Acidimicrobium. This genus has similar characters as actinomycetes genera. Seung, et al., (2003), classified acidophilic streptomycetes into separate genus Streptacidophilus which can be distinguished from neutrotolerant streptomycetes.

Numerical classification result in objective groupings based on a large number of phenetic characters. The large data content of numerical classification is important and can be applied to a variety of purposes. However, a large number of characters sometimes leads to restriction in practical usefulness. To be practicable, the identification matrices are to contain the minimum number of characters needed to discrimination between the clusters (Nolan and Cross, 1998). Such probabilistic matrices have been designed for Streptomyces and related genera (Kämpfer et al., 1991). The matrices were shown to be practically sound, and are widely used to identify unknown isolates other than Streptomyces (Yun and Lee, 1994).

The results of this and earlier studies (Lonsdale. 1985; Park et al., 1991; Seong, 1992; 1993; Seung et al., 2003) indicate that strictly and neutrotolerant acidophilic actinomycetes form a heterogeneous group actinomycetes with difference in colours, morphology, antimicrobial, enzymes activity, resistance to antibiotics, detergents and heavy metals of isolates reveals that there is a high degree of diversity among acidophilic actinomycte groups in soils.
4.5 References:


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