BIOSYSTEMATICS
OF STREPTOMYCETES
3.1. BIOSYSTEMATICS

The mere existence of microbial life was recognized only about 300 years ago relatively recently in history with Leeuwenhoek’s invention of the microscope, even under the microscope, however, the simple rods and spheres, prevented their classification by morphology, the way that large organisms had always been related to one another. It was not until the late 19th century and the development of pure culture technique that microbial organisms could be studied as individual types and characterized to some extent, mainly by nutritional criteria. More ever, the morphological and nutritional criteria used to describe microbes failed to provide natural taxonomy, ordered according to evolutionary relationships (Bull et al., 1992).

Identification, the process of determining whether an unknown organism belongs to a previously defined group is seen by many microbiologists as the most important objective of systematics. This quite mistaken view overlooks the interdependence of classification, nomenclature and identification and stems from the fact that systematics is a pervasive discipline and often seen as ancillary to other areas of research. This is particularly true with respect to diagnostic microbiology where the role of the “taxonomist” is generally perceived to be the quick and reliable identification of pathogens. This view of systematics as an ancillary science has not only persisted but also has significantly influenced the
development of the subject with much of the research effort designed to produce rapid identification systems. However, with increased interest in the exploitation and conservation of microbial resources from natural habitats, the role of the taxonomist is changing such that rapid identification should no longer be considered the primary objective of taxonomic research (Bull et al., 1992).

Although the ultimate objective of classification should be to achieve a taxonomy that is independent of the individual types of data used, classification and identification remain markedly data dependent. As such classification is continually being reassessed it is hardly surprising that many of the major advances in microbial systematics in recent years have come as result of changes in the way data are collected and analyzed. In prokaryotic systematics, as in other areas of microbiology, there is a continuing trend towards quantification and automation with an increasing reliance placed on analytical instrumentation. The latter is particularly true for chemosystematics, which relies heavily on analytical chemical techniques such as gas chromatography, high-performance liquid chromatography, and mass and ultraviolet spectrometry (Goodfellow and O'Donnell, 1993).

### 3.1.2. CHEMO BIOSYSTEMATICS OF STREPTOMYCES

The earliest attempts for classification of the *Streptomycetes* have been made between 1914 and 1962, and are based on the results obtained from the investigations of limited number of morphological characteristics. Waksman (1961) defined the aerobic actinomycetes in genus *Streptomyces* in 1961. A general revision of the genus was made in 1966 with the International
Streptomyces Project, as a result of which in the approved list *Streptomyces* species remain just 459 of the initially described 1000 (Nonomura, 1974). This classification is based on relatively small number of morphological, cultural and physiological characteristics. The numerical taxonomy has been applied for *Streptomyces* since the 60s. Silvestri (1962) have studied 200 strains in 100 characteristics, grouped in 25 variation groups. The results of this analysis have shown that many of the characteristics used for classification of the *Streptomyces* species are strongly variable and hard for interpretation.

The sure and definite identification of microorganisms is difficult to achieve without restoring to the use of specialized techniques and expertise. In the actinomycetes, identification at the genus level and above can be achieved using a combination of morphological and chemical approaches (Joseph and Glenn, 1974). In recent years, the well established use of wall chemotypes and whole organisms sugar pattern has been supplemented by additional chemical information drawn from the analysis of fatty acids, including mycolic acids, menaquinones and polar lipids (Stackerbrandt and Goodfellow, 1991). In contrast, species identification especially in large genera like *Streptomyces*, remains a difficult and time consuming process.

Chemosystematics depends upon the chemical analysis of microbial cells and most chemotaxonomic procedures involve, to varying degrees, the extraction, fractionation, purification and resolution to target compounds. Although the introduction of new derivatization techniques and improved methodologies has led to significant advances in the extraction, fractionation and purification of cell
components, the major developments in chemosystematics have come as a result of improvements in the resolution of chemical constituents, with many of the procedures now automated and capable of giving reproducible. Electrophoresis analysis of whole cell protein has been an effective approach to classification and identification. Other cell components can be analyzed by a range of techniques applied to earlier whole cells or particular cell extracts.

The application of chemical methods influenced the development of the actinomyces systematics at the genus level and even at higher taxonomic levels. The separation of genus *Streptomyces* from other *Streptomyces* groups (on the same taxonomic level) is not a problem (Goodfellow and Cross, 1984). The *Streptomycetes* can be distinguished from all the rest actinomycetes in morphological and chemotaxonomical (amino acids, diaminopemilic acid, fatty acids, lipids, phospholipids, menaquinones, mycolic acids and sugars) characteristics (Goodfellow and O'Donnell, 1993).

At the same time the early studies by Cummins and Harris (1956) on the biochemistry of the cell wall of representatives of the actinomycetales and other bacteria were taken up by Lechevalier and co-workers (Becker *et al*., 1964,1965 and Yanaguchi, 1967). It was shown that *Streptomyces* contained LL-diaminopimelic acid (DAP) in its peptidoglycan, whereas DL-DAP were found in all other actinomycetes then known. Later several other Actinomycetes were described and as members of new genera, placed into the family *Streptomycetaceae* on the basis of biochemical markers. However, few other genera with LL- DAP exist among the actinomycetales. These belong either to
the family actinomycetaceae or are genera without family. It thus appears that Actinomycetes of family Streptomyceteaceae are now primarily recognized by the structure of their peptidoglycan, which is characterized not only by the LL-DAP (Lechevalier and Lechevalier, 1970; Lechevalier et al., 1971).

Various criteria have been used for taxonomic separation of groups of microorganisms. Cell wall composition determinations have demonstrated important differences not only by conventional bacteriological techniques. Such procedures of cell wall analyses, in particular, have clearly defined members of the genus Actinomyces. Becker et al. (1964) reported a method for rapid differentiation of Streptomyces, in which whole cells were hydrolyzed, and one dimensional descending paper chromatography was used to determine differences in diaminopimelic acids. Chemical criteria such as the isomer of the diaminopimelic acid (DAP) present in cell wall and the diagnostic sugars present in the whole cell hydrolysate have been used to separate the actinomycete genera into broad chemotaxonomic group.

The application of chemical methods influenced the development of the actinomycetes systematic at the genus level and even at higher taxonomic level. The Streptomyces can be distinguished from all the rest actinomycetes in morphological and chemotaxonomical (amino acid, diaminopimelic acid, fatty acids, lipids, phospholipids, menaquinones, mycolic acids, sugars) characteristics. The use of the above-mentioned chemotaxonomic methods leads to more profound and precise characterization of the genera. Among the sporoactinomycetes the presence of L-diaminopimelic acid in the cell wall is a
fact of diagnostic value for the *Streptomyces*. The registered differences in some chemotaxonomic characteristics are of limited importance for their species differentiation (Becker *et al.*, 1964).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of whole cell protein extracts (Morosoli *et al.*, 1997) has found major application in bacterial systematics and identification (Russell, 1988 and Jackman, 1985,1987).

### 3.1.3. MOLECULAR BIOSYSTEMATICS OF *STREPTOMYCETES*

Since the mid-1980s, the use of small subunit ribosomal ribonucleic acid (SSU rRNA) based technique has facilitated a culture independent approach of investigating microorganisms as they occur in nature (Olsen *et al.*, 1986; Ward *et al.*, 1992 and Amman *et al.*, 1995). The comparison of these molecular “signature” sequences transformed microbial taxonomy from a pure identification system to an evolutionarily based frame work (Gray *et al.*, 1984; Woese, 1987; Olsen *et al.*, 1994 and Norman, 1997).

DNA based molecular methods have been used for species differentiation and the identification of *Streptomyces*. DNA-DNA reassociation is a method (Cho and Tiedje, 2001) measuring the DNA relatedness of two organisms and has proved to be suitable for the investigation of relationships between closely related taxa, such as species (Stackebrandt and Goebel, 1994). Strains belonging to the same species will generally have greater than 70 % DNA-DNA relatedness. The method has been used in numerous studies dealing with *Streptomyces*. In some cases it has shown good correlation with the
identification based on morphology and physiology, but in other cases there was no apparent correlation (Healy and Lambert, 1991 and Kim et al., 1999). While DNA-DNA reassociation has shown to be useful in the identification of *Streptomyces* species, because of the instability of the genome, it should not be used alone, but in connection with other tests (Anderson and Wellington, 2001). The sequence analysis of the genes coding for the ribosomal subunits (16S, 23S, and 5S rRNA), in particular the 16S rRNA gene has become an important tool in bacterial identification, since it provides information about the phylogenetic placement of species (Brenner et al., 2001). The DNA sequences of the ribosomal genes are highly conserved, but the genes also contain variable regions, which sometimes can be useful for species discrimination (Rosselló-Mora and Amann, 2001). Kataoka et al. (1997) utilized the hyper variable region of the 16S rRNA gene to create an index for *Streptomyces* species identification. However, the 16S rRNA sequence informational one is not sufficient for species identification.

*Streptomyces* can be analyzed at various levels to gain information suitable for constructing databases and effecting identification. The highest level is the genome and its direct expression as RNA. Sequence analysis of various genes provides for stable classification and accurate identification, which have become the cornerstone of modern phylogenetic taxonomy. Nucleic acid hybridization also offers identification possibilities, indeed, chromosomal DNA hybridization forms the basis of the generally accepted species definition in bio systematics, that members of the same species should hybridize more than 70%
with minimal mismatch as displayed by reduction in melting temperature (Wayne et al., 1987).

Some investigators propose ELISA (Krassimira et al., 1995), electrophoretical mobility of total protein extracts and computer programmed analysis of radiolabelled protein binding patterns as new approaches in the biosystematics of *Streptomyces*.

The multilocus enzyme electrophoresis which has been a standard method in the eucaryotic population genetics and systematic for a long time, is applied more widely in the investigations of genetic heterogeneity in bacterial clarifying the intraspecies diversity of the prokaryotes (Goodfellow and O'Donnell, 1994).

The obtained results showed that the method is applicable for pure cultures and the data coincides in a considerable degree with the results obtained by molecular methods such as restriction fragment length polymorphism (RFLP) and sequencing of amplified rRNA. Restriction analysis and DNA/RNA hybridization methods are those suitable for distinguishing of some *Streptomyces* species. The RNA/DNA sequencing analysis is a method which registrates a phylogenetic relationship. The determination of the full 16S rRNA sequencing of *S. lividans* and *S. coelicolor* has given a possibility the 2 species to be classified as *S. violaceoruber*. The registered partial sequencing of 16S rRNA of 14 species belonging to genera *Streptomyces* and *Streptoverticillum* together with the data of the numerical phenotypic analysis and chemotaxonomic properties give a possibility to include genus *Streptoverticillum* in genus *Streptomycetes*. 
The sequence analysis, oligonucleotide probing or PCR diagnosis. For example, nucleotide 929 of 16S rRNA is highly specific for the *Streptomycetes* and this nucleotide together with its flanking regions allows the construction of genus-specific probe. The regions 158 to 203 of 16S rRNA and 1518 to 1645 of 23S RNA were used as diagnostic probes for selection of PCR primers and they possess a great potential for investigation of the species differentiation. Together with 16S and 23S rRNA, 5S rRNA were also used for estimation of the relationship among different prokaryotes including *Streptomycetes*.

DNA hybridization has played a pivotal role in bacterial systematics and provided one of the first molecular methods for identification. If the organism has a particular and specific trait, hybridization reactions are valuable for identifying members of the taxon from environment, food or clinical samples (Stackebrandt and Goodfellow, 1991). The most popular probe target by far is the ssu rRNA molecule or its larger counterpart. These are present in high copy number ($10^4$ to $10^5$ molecules per bacterial cell), thus greatly improving the sensitivity of the hybridization within these genes are regions that are highly variable and differ significantly between species where as other areas are more conserved and suitable for identification at the generic level (Amann and Ludwig, 2000). This technique is the forerunner of the hybridization micro and macro arrays that have now been adopted for bacterial identification.

The classical microbiological and chemotaxonomical methods which form the bias of the present classification of the species of this genus, as well as the modern molecular biological approaches, analyses of protein patterns, Multilocus
enzyme electrophoretical (MLEE) analysis, restriction analysis, analysis of the nucleotide sequences of 16S and 23S RNA, RNA/DNA sequencing, DNA finger printing with different probes or polymerase chain reaction (PCR) primers were described. The profound analysis showed the advantages of the molecular biological methods for *Streptomyces* taxonomy and indicated that none of these methods applied independently could solve the existing problems in the taxonomy of the genus *Streptomyces* (Krassimira et al., 1995).

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

The sequence of chromosomal DNA holds the essential genetic information for an organism. Phylogenetic relationship is often examined using data on nucleotide sequences of ribosomal RNA. DNA (RNA) homologies, which have been applied to various kinds of bacteria, present useful information on bacterial systematics. Homology indices are the relative values used in comparing microorganisms. DNA homology data contribute to the clarification, not only of the relatedness of two organisms, but also of the evaluation of phenotypic characteristics (Komagata and Suzuki, 1987).

3.2. METHODOLOGY
The entire experimental studies on chemo biosystematics and molecular biosystematics were carried out at The Key Laboratory For Microbial Resources Yunnan Institute of Microbiology, Yunnan University, Kunming, Yunnan, China.

3.2.1. CHEMO BIOSYSTEMATICS

The seven prominent isolates of *Streptomycetes* were subjected for the study of chemo biosystematics (Goodfellow and Minnikin, 1985; Stackerbrandt and Goodfellow, 1991 and Goodfellow and O'Donnell, 1993). Cell wall amino acids, cell wall fatty acids, polar lipid content, whole cell sugar and whole cell protein were the major components studied for the chemo biosystematics of *Streptomycetes*. The determination of all these components in brief is as follows.

3.2.1.1. CELL WALL AMINO ACIDS

The biomass production for the determination of amino acids in the cell wall of all seven test isolates of *Streptomycetes* was carried out as per the procedure of Staneck and Roberts (1974). The protocol for the same is as shown below.

**Protocol for biomass production**

Isolates were streaked on starch casein agar to get well isolated colonies.
Plates were incubated at 28°C for seven days.

Well-isolated colonies were inoculated in 50ml Brain heart infusion broth (Hi media, Mumbai) in 250 ml Erlenmeyer flask and incubated in the incubator shaker (200 rpm) at 28°C for 3 to 4 days.

Cell mass was harvested by centrifugation at 4000g for 10 minutes

Cell mass was washed thrice with distilled water and finally washed with 95% ethanol and dried at 45°C for two days.

The cell wall amino acids from the biomass of the entire test isolates were determined as per the procedure prescribed by Becker et al. (1964). The protocol in brief is as mentioned below.

**Protocol for cell wall amino acid analysis**

Dry cell mass (3mg) was placed in 2.0ml Pyrex test tube, 1.5 ml of 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 content was filtered through Whatmann filter paper No.1. The cell wall extract was dried at 50°C.

Distilled water (300μl) was added to the dried cell extract and again dried at 50°C. This procedure was repeated once again.
The dried cell extract was collected in 300 μl distilled water and stored in microfuge tubes. This was used for amino acid analysis. 5 μl cell extract was applied to the baseline of Whatmann filter paper No. I.

5 μl of DL-DAP (sigma) and glycine mixture (1 μg / μl) was applied to the baseline on the same TLC plate as the standard amino acids.

Ascending chromatography was performed using methanol: distilled water: 6N HCl: pyridine (80:26:10:10 w/v) for approximately 4 hours.

Chromatogram was air dried and developed with 0.2% nin-hydrine in acetone and kept at 100°C for 3 minutes.

### 3.2.1.2. CELL WALL FATTY ACIDS

The cell wall fatty acids from the biomass of all seven test isolates of *Streptomyces* was assessed according to the standard procedure of Jantzen and Bryn (1985) and Kroppenstedt and Kutzner (1980).

#### Protocol for cell wall fatty acids

500 μg freeze dried bacterial cells in a Teflon screw capped tube.

Heated for over night at 85°C in methanolic 2M HCl (1ml).

Concentrate the sample to approximately ½ volume by using N₂ to reduce the HCl concentration.

Add two volumes of half saturated NaCl and three volumes of Hexane, vortex and separate the phase by centrifugation.
The (top) hexane phase is transferred to another tube, and the hexane extraction is repeated.

The combined hexane phase are taken just to dryness with N₂.

Add TFAA (50% in acetonitrile; 250 μl), heat for 2 min with hair dryer, concentrate just to dryness with N₂ and dissolve in 30 μl TFAA (10% in acetonitrile)

Analyze the sample by capillary Gas-liquid chromatography (25X0.2 mm fused silica coated with SE-30, methyl silicone) using a temperature gradient of 70°C (2min) → (20°/min) → 130° → (8°/min) → 280°C.

3.2.1.3. POLAR LIPID CONTENTS

The protocol (Bligh and Dyer, 1959) for the determination of polar lipid contents from the biomass of all seven test isolates is as shown under.

Protocol for polar lipid contents

100mg of dried cell (0.5 gm of harvested and washed cells) Suspend in 2 ml of 0.3% saline

20ml of methanol is added and the mixture is held at 100°C for 5 minutes in a screw cap tubes

After cooling, 10ml of chloroform and 6ml of saline are added to the tube

The mixture is shaken for two to three hours and the debris removed by filtration.
10 ml of each chloroform and saline are added to the mixture to partition the two layers.

Chloroform layer is collected, concentrated to dryness, and redissolve in 100 μl of chloroform-methanol (2:1,v/v) and used as a sample.

10 μl of samples are applied to the silica gel TLC plate [solvent for first development are chloroform-methanol-water (65:25:4,v/v), and for second development, chloroform-acetic acid-methanol-water (80:18:12:5, v/v)]

For comparison of fingerprint pattern of two dimensional chromatograms, the entire lipids are visualized by spraying with 50% H₂SO₄ and charring at 150°C for 5 minutes.

3.2.1.4. WHOLE CELL SUGARS

The whole cell sugars of the test isolates from their biomass was determined as per the protocol prescribed by Lechevalier and Lechevalier (1970).

Protocol for whole cell sugars

Approximately 50mg of dried cells are hydrolyzed with 1 ml of 1N H₂SO₄ at 100°C for 2 h in a screw-capped tube.
After cooling, a saturated solution of Ba (OH)$_2$ is added and the pH is adjusted to 5.2-5.5.

The precipitate is removed by centrifugation and the supernatant is dried with a rotary evaporator.

Small amount of ethanol is added to prevent the foaming during the evaporation.

The residue dissolve in 0.3ml of water is used as a sample solution.

1 μl of the sample is applied on a cellulose TLC plate and developed with the solvent system n-butanol-water-piridine-toluene (10:6:6:1, v/v) for 4 h.

Sugars are visualized by spraying with acid aniline phthalate, and heated at 100°C for 4 minutes. As standard, 1 μl of 0.1% (w/v) solution of sugars is employed.

3.2.1.5. WHOLE CELL PROTEINS

The biomass of all test isolates was processed separately for the determination of the whole cell proteins as per the standard protocol of Russell (1988) and the protocol is as presented below.

**Protocol for whole cell proteins**

Dissolve about 3 mg of dried bacterial cells in 1 ml of sample buffer and incubate at 37°C for 2 hours or heat for 3 minutes at 100°C.

To 100 μl of above solution add 5 μl of 0.05% bromophenol blue solution, 1 drop of glycerol and 1 drop of mercaptoethanol and mix.
For preparation gels (10%), mix 13.5ml of acrylamide solution, 15ml of gel buffer, 1.5 ml of ammonium persulphate solution, and 0.045 ml of TEMED or DMAPN.

Quickly pipette out gel mixture into 10cm long gel tubes and allow to polymerize under a layer of water. Remove the water completely after polymerization.

Apply the prepared protein sample to the gel and layer 1:1 diluted gel buffer

Similarly, load a few gels with standard marker proteins in the sample buffer.

Carry out electrophoresis with 1:1 diluted gel buffer with the positive electrode in the lower chamber at 8 mA/tube until the tracking dye is almost at the end of the gel.

Rinse and remove the gels using a fine hypodermic syringe and wash with distilled water.

Measure the length of each gel and the distance migrated by the tracking dye.

Immerse the gels in tubes containing staining solution overnight (16-18 hours).

Destain the gels in destaining solution until the background is clear.

Measure the length of the gel after destining and the position of each protein band along with standard markers.

3.2.2. MOLECULAR BIOSYSTEMATICS

All seven test isolates of *Streptomyces* in addition to their chemo biosystematics were also processed for their molecular biosystematics. The
different methods employed, as per the international standards for molecular biosystematics are as follows.

3.2.2.1. GENOMIC DNA

The genomic DNA of the test isolates were extracted by enzymase method as per the protocol prescribed by Marmur and Doty (1962) and the purity was checked on agarose (0.8%) gel electrophoresis (Marmur, 1961).

Protocol for DNA extraction

Take 1-2 gm of bacterial suspension in 40ml centrifuge tube, add 7ml of 1X TE buffer and 0.7 ml of 0.5 M EDTA.

Add 700 μl of 50mg/ml lysozyme, enable the end concentration of lysozyme to 5mg/ml, and shake vigorously.

Keep it for 37°C in incubator for 2 hours, shaking with every 10-15 minutes.

Add 700 μl of 20% sodium dodecyl sulphate (SDS).

Add 50mg/ml Protinease K and enable the end concentration of protinease K to 5mg/ml, mix it uniformly, keep it at 55°C for 30 minutes.

Add equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) to the extracted solution, shake vigorously for 10 seconds, centrifuge at 12000rpm For 10 minutes, remove the supernatant and shifted to a new tube, repeat three times.

Add 1/10th volume of 3M sodium acetate and same volume of isopropyl alcohol, shake gently to mix uniformly.
Use a glass rod to pool out the DNA fiber.

Add 2-5ml of 70% ethyl alcohol to wash DNA, and repeat twice.

Dry at 40°C and add 5ml of TE buffer and preserve at 4°C

3.2.2.2. PCR AMPLIFICATION

Purified DNA was subjected for PCR amplification to obtain 16S rRNA gene for its sequencing. PCR kit containing Primer A.8.27f (5’-CCGTCGACGAGCTC AGAGTTTGATCCTGGCTCAG-3’) and Primer B-1573-1504-R (5’-CCCGGGTACCAAGATT AAGGAGGTGACCAGCGCA-3’) were employed in the amplification. The standard protocol for PCR amplification (Cui et al., 2001) is as shown below.

Protocol for PCR amplification

Denaturation at 95°C for 5 minutes followed by 35 cycles consisting of Denaturation at 95°C for 1 minute,
Primer annealing at 56°C for 1 minute

Primer extension at 72°C for 3 minutes.

At the end of the cycle, the reaction mixture was kept at 72°C for 5 minutes

Cooled to 4°C.

The amplified 1.5kb 16S rRNA gene fragment was separated by agarose gel electrophoresis.

The purified fragments were used for sequencing

3.2.2.3. 16S rRNA GENE SEQUENCING

The purified fragments of DNA molecules containing 16S rRNA genes after PCR amplification were sent to Sino-America Biotechnology Co., Beijing, for 16S rRNA gene sequencing. The entire profile of nucleotide of 16S rRNA gene indicating the complete sequence was availed back from the said company.

3.2.2.4. SEQUENCE ALIGNMENT AND PHYLOGENETIC ANALYSIS

The 16S rRNA gene sequence of five prime isolates of Streptomyces were aligned manually with representative sequence of related Streptomyces obtained from the Gene Bank database (NCBI and DDJB). The evolutionary tree illustrating the phylogenetic relationship was inferred by using neighbor joining method (Thompson et al., 1997). The standard protocol (Felsenstein, 1981,1985) with four main steps used for phylogenetic tree (Saitou and Nei, 1997) preparation are as shown under.
Protocol for phylogenetic tree preparation

Clustal file

Load sequence (from document)

Output format option

Select Clastal W-sequence Number - On

Alignment

Do complete alignment

Clastal-Align-Wait for while

Bio-edit

Open (file)

Check for alignment (ATGC ends)

Save as FASTA format

Close

Mega

Convert to Mega format

File-ok-ok-Close-close
3.3. RESULTS

3.3.1. CELL WALL COMPONENTS

The cell wall components of seven prominent isolates of *Streptomyces* were studied to understand their chemo biosystematics. The cell wall amino acids of *Streptomyces* are illustrated by thin layer chromatogram in Figure-4.
The gas chromatogram illustrating the fatty acid content of the cell wall of all *Streptomycetes* are presented from Figure-5 to 11. The overall major fatty acid profile of all the isolates of *Streptomycetes* is as presented in Table-13. The polar lipids as another important constituent of the cell wall of the *Streptomycetes* are presented in Figure-12 by thin layer chromatography.

3.3.2. WHOLE CELL COMPONENTS

The protein profile and total sugars were the major whole cell components studied for all the seven isolates of the *Streptomycetes*. Figure-13 illustrates whole cell protein profile of all seven isolates of *Streptomycetes* by SDS-PAGE.

As diagnostic sugars were absent in all test isolates, they could not be recorded.

3.3.3. GENOMIC DNA

The genomic DNA content of all seven isolates of *Streptomycetes* is illustrated in Figure-14 by agarose gel electrophoresis along with a marker.

---

*Figure- 4. Thin layer chromatogram illustrating cell wall amino acids of *Streptomycetes***

1 2 3 M 4 5 6 7
M-Marker, 1-DAS 69, 2-DAS 131, 3-DAS 139
4-DAS 143, 5-DAS 147, 6-DAS 165, 7-DAS 178
Figure-5. Gas chromatogram indicating the fatty acid content of *Streptomyces* isolate DAS 69
Figure-6. Gas chromatogram indicating the fatty acid content of *Streptomyces* isolate DAS 131
Figure-7. Gas chromatogram indicating the fatty acid content of *Streptomyces* isolate DAS 139
Figure-8. Gas chromatogram indicating the fatty acid content of Streptomycete isolate DAS 143
Figure-9. Gas chromatogram indicating the fatty acid content of *Streptomyces* isolate DAS 147
Figure-10. Gas chromatograms indicating the fatty acid content of Streptomyces isolate DAS 165
Figure-11. Gas chromatograms indicating the fatty acid content of Streptomyces isolate DAS 178
Table-13. Major fatty acid profile (%) of the isolates of *Streptomycetes*

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>DAS69</th>
<th>DAS123</th>
<th>DAS131</th>
<th>DAS135</th>
<th>DAS139</th>
<th>DAS143</th>
<th>DAS147</th>
<th>DAS165</th>
<th>DAS178</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₁₄:₀</td>
<td>--</td>
<td>0.30</td>
<td>0.98</td>
<td>---</td>
<td>0.20</td>
<td>--</td>
<td>0.51</td>
<td>0.87</td>
<td>2.25</td>
</tr>
<tr>
<td>i-C₁₄:₀</td>
<td>3.98</td>
<td>1.27</td>
<td>5.38</td>
<td>1.39</td>
<td>5.88</td>
<td>3.68</td>
<td>15.56</td>
<td>6.14</td>
<td>3.78</td>
</tr>
<tr>
<td>i-C₁₅:₀</td>
<td>10.18</td>
<td>24.35</td>
<td>10.39</td>
<td>25.77</td>
<td>11.97</td>
<td>12.01</td>
<td>11.45</td>
<td>13.59</td>
<td>9.08</td>
</tr>
<tr>
<td>ai-C₁₅:₀</td>
<td>18.59</td>
<td>44.34</td>
<td>25.60</td>
<td>42.74</td>
<td>15.87</td>
<td>19.08</td>
<td>15.56</td>
<td>17.84</td>
<td>16.77</td>
</tr>
<tr>
<td>C₁₆:₀</td>
<td>2.29</td>
<td>2.40</td>
<td>--</td>
<td>3.32</td>
<td>3.72</td>
<td>5.37</td>
<td>1.66</td>
<td>9.64</td>
<td>16.43</td>
</tr>
<tr>
<td>i-C₁₆:₀</td>
<td>29.70</td>
<td>6.18</td>
<td>19.75</td>
<td>6.91</td>
<td>29.78</td>
<td>24.30</td>
<td>31.98</td>
<td>28.11</td>
<td>17.27</td>
</tr>
<tr>
<td>i-C₁₆:₁</td>
<td>4.55</td>
<td>--</td>
<td>2.85</td>
<td>--</td>
<td>7.24</td>
<td>4.81</td>
<td>8.07</td>
<td>2.37</td>
<td>1.50</td>
</tr>
<tr>
<td>i-C₁₇:₀</td>
<td>6.52</td>
<td>0.19</td>
<td>2.90</td>
<td>5.87</td>
<td>3.66</td>
<td>4.75</td>
<td>1.68</td>
<td>3.76</td>
<td>2.52</td>
</tr>
<tr>
<td>i-C₁₇:₁</td>
<td>4.24</td>
<td>--</td>
<td>1.71</td>
<td>0.44</td>
<td>3.98</td>
<td>3.77</td>
<td>2.23</td>
<td>1.65</td>
<td>3.10</td>
</tr>
<tr>
<td>ai-C₁₇:₁</td>
<td>4.61</td>
<td>--</td>
<td>2.83</td>
<td>--</td>
<td>2.55</td>
<td>2.63</td>
<td>1.30</td>
<td>1.64</td>
<td>2.01</td>
</tr>
</tbody>
</table>

i-iso, ai-anteiso
Figure-12. Thin layer chromatogram illustrating polar lipids of *Streptomyces*

M-Marker, 1-

DAS 69, 2-DAS 131, 3-DAS 139
4-DAS 143, 5-DAS 147, 6-DAS 165, 7-DAS 178
Figure-13. SDS-PAGE illustrating whole cell protein profile

M-Marker, 1-DAS 69, 2-DAS 131, 3-DAS 139
4-DAS 143, 5-DAS 147, 6-DAS 165, 7-DAS 178
Figure-14. Agarose gel electrophoresis illustrating the genomic DNA of the *Streptomyces*
3.3.4. PCR AMPLIFICATION AND 16S rRNA GENES

16S rRNA genes of all the test isolates of Streptomyces were detected by PCR amplification under agarose gel electrophoresis and are as illustrated in Figure-15.

3.3.5. SEQUENCE ALIGNMENT AND PHYLOGENETIC RELATION

The complete sequence of 16S rRNA genes of five prominent isolates of Streptomyces obtained is presented in Figure-16, 18, 20, 22 and 24. A phylogenetic relationship of the respective isolates of Streptomyces derived based on the complete 16S rRNA gene sequences are illustrated in Figure-17, 19, 21, 23 and 25.
Figure-15. Agarose gel electrophoretic PCR amplification illustrating 16S rRNA gene of *Streptomyces*

DAS 69, 2-DAS 131, 3-DAS 139
4-DAS 143, 5-DAS 147, 6-DAS 165, 7-DAS 178
Figure-16. Complete 16S rRNA gene sequence of the Streptomycete DAS 131

TCCTTCGGGAGGGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCTTCCTACTCTGGGACAAAGCCTGGGAAAGGAGCTGCCTGGGAGGGGAATTAGTGGCGAACGGGTGAGT...

The almost complete 16S rRNA sequence of strain DAS 131 (1477 nucleotides) has been deposited in the Gene Bank database. The Accession No. for strain DAS 131 is DQ 317411.
Figure-17. Dendrogram indicating the phylogenetic relation of the *Streptomyces* DAS 131

Phylogenetic dendrogram obtained by distance matrix analysis of 16S rRNA gene sequence, showing the position of strain DAS 131 among its phylogenetic neighbours. Numbers at the branch nodes are boot strap values, expressed as a percentage of 1000 replicates.
Figure-18. Complete 16S rRNA gene sequence of the Streptomycete DAS 139

CGATGACCACCTTCGTTGGGGATTAATGGCGAACGGGTGTGAGTAACACCTGGGCGAACGGGTGTGGGATTAGTGGCGAACGGGTGTGAGTAACACGTGGGCAATCTGCCCTTCACCTCTGGGAAACGGGGGTCTAATACCGGATACGACCTGCCGAGGCATCTCGGTGGGTGGAAAGCTCCGGCGGTGAAGGATGAGGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAACGGCTCACCAAGGGGAAAGCTGGATGCAGC
ACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCT

TTCATACGCGCCACAGCGCCCGGTGCTACAGTGAGGCGCAAGCCTTGTCCGGAATTATGGGCGTAAAGAGACTCGTGAAGCCGGCGTCTGTCACTCGGTGGTGTGAAGACTCATCGGAGTTTTGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGACACCGTGCCAGGAGCACCGGCGAAGGC

GGATCTCTGGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGAACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCTGGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACACCCGAAAGCCGGTGGCCCAACCCC.
Figure-19. Dendrogram indicating the phylogenetic relation of the *Streptomyces* DAS 139

Phylogenetic dendrogram obtained by distance matrix analysis of 16S rRNA gene sequence, showing the position of strain DAS 139 among its phylogenetic neighbours. Numbers at the branch nodes are boot strap values, expressed as a percentage of 1000 replicates.
The almost complete 16S rRNA sequence of strain DAS 143 has been deposited in the Gene Bank database. The Accession No. for strain DAS 143 is DQ 345437.
Figure-21. Dendrogram indicating the phylogenetic relation of the *Streptomyces* DAS 143

Phylogenetic dendrogram obtained by distance matrix analysis of 16S rRNA gene sequence, showing the position of strain DAS 143 among its phylogenetic neighbours. Numbers at the branch nodes are bootstrap values, expressed as a percentage of 1000 replicates.
Figure-22. Complete 16S rRNA gene sequence of the *Streptomycete* DAS 165

AAATAACGGCCGGTGTATTCGAGCTCGGTACCGTAATACGACTCACAATAGGG
GCGACATATGATCGATGATATCCCATGGGCGGCCGCCTGCAGACCAGGTCTC
AGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCA
AGTCGAACGATGAACCACTTTCGGTGAGGAGATTAGTGGCAGGAACGGGTGAGTAAC
ACGTTGGCAATCCTGCCACTCTGGGAACAGCCCTTGGAACAGGGGCTCTAA
TACCCGATACTGACCCCTCAGCAAGCATCTGCGAGGTTCGAAAGCTCCGGCGGT
GCAGGATGAGCCCGCGACGGCCGGCTTTGTTGGTGAGGTAATGGCTCACCAAG
GGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAG
GACACGGCCAGACTCTACGGGAGGCAGATGGGGAATATTCAGCACAATG
GGCGAAAGCTCTGATGCAACGCAGCAGCGCCGCTAGGGATAGGGGACGCTCGGTTG
TAAACCTCTTTCAAGCAGGAAAGACGCAAGTGACGGTACCTGCAAGAAAGA
GCGCCGGCTAATACTAGCTGAGCCAGCCGGCTAATCGTGGGCGAAGGCTAAACT
CAAAGGAATTGACGGGGGCCCGCACAAGCGGCAGATGTGCTTAAATTCC
ACGCAACGCCGAGAACCTTACCAAGCTCGGATACACACGCAAGACGGGCTCT
AGATGGTGCCCCCTTGTGCTGGTGTAGCTTGTTGCGACGCTCGGCTCGTGCAGC
TCGTTGGCAGAATGTTTGAGTCTTGCACACCGCCCAACCCCTGCTCC
GTGGTGCAACGGCCGCTGGTTGCTGGTGCTGGGAGCCGACTACGGAGACCCGGCGG
TCAACTCGGAGAGTGGGAGGACGCTCGTACCTGCAATCATTCAATGGTG.
The almost complete 16S rRNA sequence of strain DAS 165 has been deposited in the Gene Bank database. The Accession No. for strain DAS 165 is DQ 345779.

Figure-23. Dendrogram indicating the phylogenetic relation of the *Streptomyces* DAS 165

Phylogenetic dendrogram obtained by distance matrix analysis of 16S rRNA gene sequence, showing the position of strain DAS 165 among its phylogenetic neighbours. Numbers at the branch nodes are boot strap values, expressed as a percentage of 1000 replicates.
Figure-24. Complete 16S rRNA gene sequence of the Streptomycete DAS 178

AGCTTGACCATGCTGACGATTAGAGTCTACGCTGACGCGA
ACGCTGGCCGGCGTCTATACACATGAGTCGAAGCATGAAACCAGTTCCGCTG
GGGATTAGTGTCGAGTACAGCTGCACTGACGGTACGGGCTGAGGAC
AGGGCGACCGCCAGGGCTAGGACTGACACCAGGCGCCAGCTCTTACGCC
GGCCACCTAAATATGGAGGAACCCGAGGCGAAGCAGG
CCATCTCTGACCAGTACGCTGAGGAGCCAGACGGCGACCGCCAGCCGA
AGGATTAGATACCCCTGAGTCCAGCGCCAACCCGGTGAAGTAGGTTGG
GGCAGATCACTCCAGGCGTCGCAGGTGCGTCGTCAGCTCGTCGGCAGT
TGACATTCCAGTGATAGGCGTACGAGTTGATGGAGTGAGTGGAGTGG
AGGGATACCCGACCGCGCAAGGCTTTAAACTCCAAAGGATACGGCGGATTGC
AAACGAGACGCAGTTGTCAGGAGTTGCTCCTAACTGGAAGGTGGGGTC
CCGCGACAGCGGAGTGGTCTGACATGGCCTGTCGGTCTAGGGTGCTGACG
GCTGGGACGCGGCGATCAGGAGTTGCTCCTTACGCCGCTGACG
TCCGACGCGGTAGGAGTTGCTCCTTACGCCGCTGACG
GGGGACGCGGCGATCAGGAGTTGCTCCTTACGCCGCTGACG
TGCTGGGACGCGGCGATCAGGAGTTGCTCCTTACGCCGCTGACG
TCGGGGTTCGGGTTGGAGGAGTAGTGCTACGGGCTGACG
CTGGGGTTCGGGTTGGAGGAGTAGTGCTACGGGCTGACG
GCATCTGACGCTGAGGAGTTGCTCCTTACGCCGCTGACG
GGGATACCCGACCGCGCAAGGCTTTAAACTCCAAAGGATACGGCGGATTGC
AAACGAGACGCAGTTGTCAGGAGTTGCTCCTAACTGGAAGGTGGGGTC
CCGCGACGCGGCGATCAGGAGTTGCTCCTTACGCCGCTGACG
GCTGGGACGCGGCGATCAGGAGTTGCTCCTTACGCCGCTGACG
TCGGGGTTCGGGTTGGAGGAGTAGTGCTACGGGCTGACG
CTGGGGTTCGGGTTGGAGGAGTAGTGCTACGGGCTGACG
GGGATACCCGACCGCGCAAGGCTTTAAACTCCAAAGGATACGGCGGATTGC
AAACGAGACGCAGTTGTCAGGAGTTGCTCCTAACTGGAAGGTGGGGTC
CCGCGACGCGGCGATCAGGAGTTGCTCCTTACGCCGCTGACG
GCTGGGACGCGGCGATCAGGAGTTGCTCCTTACGCCGCTGACG
TCGGGGTTCGGGTTGGAGGAGTAGTGCTACGGGCTGACG
CTGGGGTTCGGGTTGGAGGAGTAGTGCTACGGGCTGACG
GGGATACCCGACCGCGCAAGGCTTTAAACTCCAAAGGATACGGCGGATTGC
AAACGAGACGCAGTTGTCAGGAGTTGCTCCTAACTGGAAGGTGGGGTC
CCGCGACGCGGCGATCAGGAGTTGCTCCTTACGCCGCTGACG
GCTGGGACGCGGCGATCAGGAGTTGCTCCTTACGCCGCTGACG
TCGGGGTTCGGGTTGGAGGAGTAGTGCTACGGGCTGACG
CTGGGGTTCGGGTTGGAGGAGTAGTGCTACGGGCTGACG
GGGATACCCGACCGCGCAAGGCTTTAAACTCCAAAGGATACGGCGGATTGC
AAACGAGACGCAGTTGTCAGGAGTTGCTCCTAACTGGAAGGTGGGGTC
CCGCGACGCGGCGATCAGGAGTTGCTCCTTACGCCGCTGACG
GCTGGGACGCGGCGATCAGGAGTTGCTCCTTACGCCGCTGACG
TCGGGGTTCGGGTTGGAGGAGTAGTGCTACGGGCTGACG
CTGGGGTTCGGGTTGGAGGAGTAGTGCTACGGGCTGACG
GGGATACCCGACCGCGCAAGGCTTTAAACTCCAAAGGATACGGCGGATTGC
AAACGAGACGCAGTTGTCAGGAGTTGCTCCTAACTGGAAGGTGGGGTC
CCGCGACGCGGCGATCAGGAGTTGCTCCTTACGCCGCTGACG
GCTGGGACGCGGCGATCAGGAGTTGCTCCTTACGCCGCTGACG
TCGGGGTTCGGGTTGGAGGAGTAGTGCTACGGGCTGACG
CTGGGGTTCGGGTTGGAGGAGTAGTGCTACGGGCTGACG
GGGATACCCGACCGCGCAAGGCTTTAAACTCCAAAGGATACGGCGGATTGC
AAACGAGACGCAGTTGTCAGGAGTTGCTCCTAACTGGAAGGTGGGGTC
CCGCGACGCGGCGATCAGGAGTTGCTCCTTACGCCGCTGACG
GCTGGGACGCGGCGATCAGGAGTTGCTCCTTACGCCGCTGACG
TCGGGGTTCGGGTTGGAGGAGTAGTGCTACGGGCTGACG
CTGGGGTTCGGGTTGGAGGAGTAGTGCTACGGGCTGACG
GGGATACCCGACCGCGCAAGGCTTTAAACTCCAAAGGATACGGCGGATTGC
AAACGAGACGCAGTTGTCAGGAGTTGCTCCTAACTGGAAGGTGGGGTC
CCGCGACGCGGCGATCAGGAGTTGCTCCTTACGCCGCTGACG
GCTGGGACGCGGCGATCAGGAGTTGCTCCTTACGCCGCTGACG
The almost complete 16S rRNA sequence of strain DAS 178 has been deposited in the Gene Bank database. The Accession No. for strain DAS 178 is DQ 345438.

**Figure-25. Dendrogram indicating the phylogenetic relation of the *Streptomyces* DAS 178**

Phylogenetic dendrogram obtained by distance matrix analysis of 16S rRNA gene sequence, showing the position of strain DAS 178 among its phylogenetic neighbours. Numbers at the branch nodes are boot strap values, expressed as a percentage of 1000 replicates.
3.4.1. CELL COMPONENTS OF STREPTOMYCETES

Systematics is defined as the scientific study of the kinds and diversity of organisms and all relationships among them (Goodfellow and O'Donnell, 1993). The subject encompasses the related but quite distinct processes of classification, nomenclature and identification. Sound classification is a prerequisite for stable nomenclature and accurate identification. Classification is the ordering of organisms into groups (taxa) to produce an orderly arrangement of strains such that knowledge of the properties of one strain allows the properties of other members of the group to be inferred. Biosystematics is, therefore, essential in the development of microbiology and to exploit microbial diversity (Bull et al., 1992). It is possible to view all systematic as chemosystematics since morphology, pigmentation, serology and the biochemical properties of microorganisms reflect their chemical composition. However, given the recognition of molecular and numerical taxonomic methods
as independent approaches to the classification and identification of microorganisms, it is perhaps more useful to restrict the scope of chemosystematics to the study of the distribution of specific chemical components such as lipids, cell wall amino acids, sugars and protein amongst microbial taxa.

Amino acids, fatty acids and polar lipids of the cell wall components, and whole cell proteins and diagnostic sugars of the selected actinomycetes were detected using suitable advanced techniques of chromatography and electrophoresis at Yunnan Institute of Microbiology, which is a Key laboratory for Microbial Resources. On the analysis of amino acids from the whole organism hydrolysate of the seven selected isolates of actinomycetes, the major content of amino acids was detected to be L-diaminopimelic acid (L-DAP). This illustrates that, all selected actinomycetes belong to the genus *Streptomyces*. Majority of the actinomycetes studied for their classification by several investigators (Clarence and Leo, 1968). The results on the analysis of the methyl esters of whole organism hydrolysate of the selected actinomycetes revealed the typical pattern of *Streptomyces* fatty acid profile, including major proportions of iso and anteiso branched components and small quantum of unsaturated fatty acids. This clearly indicates that, all the test isolates belong to the genus *Streptomyces*. The predominant diagnostic phospholipids found in the hydrolysate of all selected test isolates, again proved to be the genus *Streptomyces*. The major phospholipids recorded in all the isolates were Diphosphatidylglycerol (DPG), phosphatidylinositolmannosides (PIM), phosphatidylglycerol (PG) and
phosphatidylethanolamine (PE). The similar observations were recorded with regard to fatty acid methyl ester profiles, while studying the biosystematics of novel *Streptomyces* obtained from soil.

The whole cell protein profile of all the test isolates of actinomycetes showed approximately 24 discrete bands within 14 to 97 kD molecular weight range. Differences in the patterns observed on visual examination were clarified based on computerized cluster analysis (Atalan *et al.*, 2000). None of the selected test isolates of actinomycetes have shown the presence of diagnostic sugars in their whole cell hydrolysates, indicating their belongingness to the genus *Streptomyces*.

Identification of *Streptomyces* below the genus level is notoriously difficult though it is possible to identify unknown, cultivable *Streptomyces* to species, species groups and putatively novel species based on bioinformatics. The species concept is still a difficult issue in *Streptomyces* systematics due to the taxonomic complexity of the genus and the absence of accepted minimal standards for the delineation of species. However, it is evident that *Streptomyces* species should be circumscribed using judicious combinations of phenotypic and genotypic data (Labeleda and Lyons, 1991 *a, b*; Labeleda, 1993; Labeleda *et al.*, 1997 and Kim *et al.*, 1999) though the emphasis in such studies has tended to center on the use of DNA-DNA relatedness data. Comparative biosystematic studies are needed to find rapid and reliable phenotypic methods for delineating potentially novel species of *Streptomyces*.

**3.4.2. GENOMIC COMPONENTS OF STREPTOMYCETES**
Biosystematics has undergone spectacular changes in recent years by taking full advantage of developments in chemistry, molecular biology, and computer science to improve the understanding of the relationship between microorganisms and the underlying genetic mechanisms on which they are based. A relatively large set of techniques are being used routinely for microbial classification. However, it is of primary importance to understand at which level these methods carry information. The kind of information that each technique retrieve is directly related to its resolving power and the correct use of this information is essential to guarantee the adequate classification of a taxon (Rossello-Mora and Aman, 2001).

In the present study the genomic DNA of all the seven selected test isolates of actinomycetes were extracted. The prominent bands indicating the genomic DNA of all the test isolates showed that the DNA is about 7750 bp after running on agarose gel with \( \lambda - \text{EcoT 14 I} \) digest marker. The quantum of the genomic DNA of all the test isolates is very close, exhibiting their belongingness to one genus of actinomycetes. Further the G+C content of the genomic DNA was determined to be about 66 to 76 mol %. This finding is similar to the observation of Williams and Vickers (1988) indicating 69 to 78% GC content of genomic DNA of the genus *Streptomyces*.

The use of molecular techniques over the past 20 years has shown that, so far only an extremely small fraction of the microbial diversity has been cultivated from all habitats investigated. There is still an enormous unexplored reservoir of natural compounds of potentially large structural diversity that could
be used as a resource for the development of new secondary metabolites (Vicente et al., 2003).

Given the changing role of systematics and the emergence of the new bacterial systematics (Stackebrandt and Goodfellow, 1991; Goodfellow and O’Donnell, 1993), it is timely to review current developments in biosystematics. Molecular systematics was a relatively new area of taxonomic research, which offered, for the first time, the possibility of conducting research on microbial systematics within a phylogenetic framework. There can be no doubt that molecular systematics as fulfilled its early potential and additional effort is now needed to expand the phylogenetic framework (O’Donnell et al., 1993).

3.4.3. GENE SEQUENCE AND PHYLOGENETIC RELATIONSHIP

It is important that the relevance of free ranging actinomycetes from nature’s diverse gene pool is not only forgotten in a perverse scramble to create genetically engineered organisms for microbial technology. Indeed, genetic engineering like microbial systematics should be seen as a means to an end and not as an end in itself. It should also be remembered that actinomycetes are talented engineers and excellent chemists. They are also good friends with many secrete to share (Goodfellow and Cross, 1984). *Streptomyces* systematic has become increasingly objective due to the application of chemotaxonomic, molecular systematic and numerical taxonomic methods (Goodfellow et al., 1992; Manfio et al., 1995). Nevertheless, the subgeneric classification of the genus *Streptomyces* in Bergey’s Manual of Systematic Bacteriology (Williams et al.,
1989) is based on the extensive numerical classification generated by Williams et al. (1983).

The application of genetic methods, such as DNA-DNA reassociation (Labeda, 1992 and Kim et al., 1999) and 16S rRNA gene sequence analysis (Gladek et al., 1985; Stackerbrandt et al., 1992; Kim et al., 1996; Takeuchi et al., 1996 and Hain et al., 1997) has partly confirmed the phenotypic classification, but this approach has also provided new information.

The application of molecular biological techniques has had a profound effect on microbial systematics (Woese, 1992). The most spectacular development, which marked a historical turning point in microbial systematics, was the realization that, related microorganisms have in their respective nucleic acids, record of the changes that have occurred since their divergence from a common ancestor over 3 billion years ago (Zuckerkandl and Pauling, 1965). Subsequent studies on conserved regions of nucleic acids, mainly ribosomal RNA, have shown prokaryotic diversity to be vast in comparison to that of eukaryotes. Further advances in microbial systematics can be anticipated as procedures for nucleic acid sequencing are simplified. Current methods have already reached the stage where it is relatively straightforward to use the polymerase chain reaction to amplify and sequence nucleic acids from small amounts of biomass (Embley and Finlay, 1993 and Hutson et al., 1993).

Currently, prokaryote taxonomists agree that a reliable classification can be achieved only by the exploration of the internal diversity of taxa by a wide range of techniques is generally known as the polyphasic approach in the
reinterpretation of Vandamme et al. (1996). Great attention has been payed to the application of molecular approach in taxonomic investigation in order to solve this problem. As compared with the phenotypic, chemical, enzymatic and serological characteristics, which cover only 5-10% of the genome, the molecular genetic methods deals directly or indirectly with the genome polymorphism. Such methods as protein pattern, multilocus enzyme electrophoresis (MLEE) analysis, plasmid pattern, restriction analysis, analysis of the nucleotide sequences of 16S rRNA and 23S rRNA, DNA fingerprinting with different probes or PCR primers, give a possibility for estimation of the real genetic similarity among species.

The five potential isolates of the *Streptomyces* were subjected for their 16S rRNA gene sequence and their phylogenetic relationship was established. The 16S rRNA gene was amplified by PCR and 1.5 kb amplified 16S rRNA fragment was separated. The purified fragments were sequenced. The almost complete sequence of 16S rRNA genes of all five potential strains of *Streptomyces* was obtained. A neighbor joining tree based on 16S rRNA gene sequence was constructed to show the relationship between potential strains of *Streptomyces* and other related *Streptomyces* species. The comparative analysis of the 16S rRNA gene sequence and the estimation of phylogenetic relationship showed that, strain DAS 131 fall in the same subclade in the tree and showed closest level of sequence similarity (99.4%) with *Streptomyces venezualae* ISP 5230ᵀ. The strain DAS 139 formed a separate subclade in the tree and showed closest level of sequence similarity (99.6%) with *Streptomyces scabiei* KACC
20194\textsuperscript{T}, the strain DAS 143 also formed a separate subclade in tree and showed closest level of sequence similarity (99.1\%) with \textit{Streptomyces stramineus} NRRL1222\textsuperscript{T}. The phylogenetic relationship showed that strain DAS 165 forms a same subclade in tree and showed closest level sequence similarity (99.6\%) with \textit{Streptomyces tendae} ATCC 19812\textsuperscript{T}. The strain DAS 178 is found totally out grouped in the phylogenetic relationship with a closest level of sequence similarity (99.8\%) with \textit{Streptomyces shandosensis} 24#.

\textit{Streptomyces} phylogenetic classification is based on sequence analysis of the small subunit (ssu) 16S rRNA molecule or its genes. Over 20000 ssu RNA gene sequence have now been deposited either in general nucleic acid sequence databases or in specialist ribosomal RNA database such as the rRNA database project [RDP (Maidak et al., 2001)]. These databases provide the most comprehensive opportunities currently available for identification, and their exploitation for identification has been termed ribosequencing or phylotyping. Once the sequence has been obtained, it is submitted to a BLAST search to one of the publicly available websites such as the site for the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). A 99\% to 100\% similarities with an entry in the database can be accepted as a strong indication of identification (Valente et al., 1999; Cappa and Cocconcelli, 2001).