1. Isolations:

Different types of soil samples were collected from various localities of the Raipur region and nearby places. The samples were air dried, powdered and stored in sterilized polythene bags till used.

Before isolation the samples were treated with 1% phenol and 0.1% pimaricin solution, so that mainly the actinomycetes could grow. Inoculations were made by the dilution plate technique (Warcup, 1960) in plates containing the following media.

i) Czapek’s Agar:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>30.0 gm</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>2.0 gm</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>1.0 gm</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.5 gm</td>
</tr>
</tbody>
</table>
KCl - 0.5 gm

FeSO₄·7H₂O - 0.01 gm

Agar-agar - 15.0 gm

Distilled water - 1000 ml

pH - 6.6

ii) Starch-Agar:

Soluble starch - 2.0 gm

K₂HPO₄ - 0.5 gm

MgSO₄·7H₂O - 0.2 gm

CaCl₂ - 0.05 gm

NaNO₃ - 0.05 gm

Asparagine - 0.05 gm

FeSO₄·7H₂O - In trace

Agar-agar - 20.0 gm

Distilled water - 1000 ml

pH - 7.4

iii) Starch-Casein Agar:

Soluble starch - 10.0 gm

Casein - 0.3 gm

K₂HPO₄ - 2.0 gm

KNO₃ - 2.0 gm

NaCl - 2.0 gm

MgSO₄·7H₂O - 0.05 gm

CaCO₃ - 0.02 gm

FeSO₄·7H₂O - 0.01 gm
### Agar-agar
- 20.0 gm

### Distilled water
- 1000 ml

### pH
- 7.2

The inoculated plates were allowed to incubate at 30°C, and were observed regularly to record visible growth of the actinomycetous colonies. Successive sub-culturing was done to obtain pure cultures of the incident actinomycetes.

Purified cultures were maintained on Oat meal agar medium of the following composition:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oat meal</td>
<td>20.0 gm</td>
</tr>
<tr>
<td>Agar-agar</td>
<td>18.0 gm</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>0.001 gm</td>
</tr>
<tr>
<td>MnSO₄</td>
<td>0.001 gm</td>
</tr>
<tr>
<td>ZnSO₄</td>
<td>0.001 gm</td>
</tr>
<tr>
<td>pH</td>
<td>7.2</td>
</tr>
</tbody>
</table>

### 2. Identification and Taxonomy:

The isolates were subjected to several morphological and biochemical tests for their confirmed taxonomic identification.

i) **Morphological studies:**

Colonies were developed in petridishes to record their gross morphology, colour, texture, aerial mycelia and reverse colouration.
To obtain an illustrative form of the species, slide cultures were prepared for microscopic examination.

Details of sporophores and the spores were taken with visual observations and micrometric measurements.

ii) Biochemical studies.

Biochemical tests were carried out in respect of nitrate reduction, \( \text{H}_2\text{S} \) production, proteolysis and starch hydrolysis.

These were considered useful parameters in delimiting species in the group, besides their morphology.

a) Nitrate reduction test:

The test was performed in the culture filtrate of the organism, by using sulphanilic acid-\( \text{H}_2\text{SO}_4 \) (solution a) and Naphthylamine-\( \text{H}_2\text{SO}_4 \) (solution b).

To 1 ml of the culture filtrate two drops each of the solution a and b were added. Development of a pink or red colour, indicated presence of Nitrite and showed the Nitrate reduction positive test.

Composition of solutions a and b:

Solution a:

- Sulphanilic acid - 0.8 gm
- \( \text{H}_2\text{SO}_4 \) (conc) - 4.8 ml
- Distilled water - 100 ml
Solution b:

<table>
<thead>
<tr>
<th>Substance</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthylamine</td>
<td>-</td>
</tr>
<tr>
<td>H₂SO₄ (conc)</td>
<td>0.8 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

b) H₂S production test:

Isolates were grown in starch-casein broth medium. A strip of filter paper, moistened with saturated solution of lead acetate, was placed in the flask in such a way that its lower end remained about 5 mm above the surface of the medium. On incubation the presence of H₂S was indicated by the blackening of the filter paper strip. It remained unchanged if no H₂S was produced by the organism.

c) Protein hydrolysis (Protease activity):

This test was performed on solid medium following the method of Hankin and Anagnostakis (1975). Observations were taken for the zone formed in the plate, after acid mercuric chloride reaction. The radial size of enzyme diffusion zone represented the relative amount of the enzyme and the capacity for proteolysis.

d) Starch hydrolysis (Amylase activity):

Enzyme activity was measured by using Lugol's iodine solution. A yellow zone, around the colony in an otherwise blue medium, indicated amylolytic activity of the organism. The diameter (mm) of the zone of
activity represented the amount of enzyme produced by the organism.

The identification was made according to the characterization based on the minimum description criteria established by the International Streptomyces project (ISP). Species identification has been done according to the description given by Shirling and Gottlieb (1958, 1959 and 1972); The Bergey's Manual of Determinative Bacteriology 8th edition (1975); and Krassilnikov (1981).

In this study the species of Streptomyces have been systematically classified according to the system of Pridham et al. (1958).

3. Biodegradation capacity:

The actinomycetes, being the soil inhabitants, naturally sustain themselves as saprophytes. They elaborate a sufficient capacity for biodegradation of organic matter in their dissimilation. The hydrolytic enzymes in their dissimilation are mainly the cellulases, pectinases, proteinases and amylases.

i) Enzyme preparation:

The isolates were grown in Starch-Casein broth medium at pH 7.2 and incubated at 30°C. The culture filtrate was obtained after 4, 8 and 12 days of incubation, through filtration. The filtered culture
filtrate was subsequently centrifuged at 3,000 rpm for 20 minutes, to yield a cell-free supernatant. This served as the crude enzyme preparation for the assay of enzymes in each case.

ii) **Enzyme assay procedures**

a) **Cellulase.**

Cellulase was assayed by the method of Gascoigne and Gascoigne (1960). The reaction mixture contained: 1 ml of the enzyme preparation and 1 ml of the enzyme substrate (0.55% CMC solution in sodium-citrate buffer of pH 5.5). The mixture was incubated at 30°C for 1 hour.

Liberation of reducing sugar was determined by treating the enzyme substrate mixture with DNS reagent before reading its absorption. The procedure for this was:

1 ml aliquot of the reaction mixture was added to a tube containing 1 ml of DNS reagent and heated over a boiling water bath for 5 minutes. It was then cooled to room temperature over a running water tap and was diluted with distilled water to a final volume of 10 ml.

The absorption of the sample was measured on SpeCol (Carl Zeiss Jena) at 540 nm. The amount of reducing sugars liberated was obtained by referring to a standard curve prepared by using an aqueous solution of D-glucose.
One unit of cellulase enzyme activity is defined as the amount of enzyme required to liberate reducing sugar equivalent to 10 ug of glucose (Olutida, 1976).

**Preparation of D.S. reagent:**

Dinitrosalicylic acid - 1.0 gm
Sodium hydroxide - 1.0 gm
Phenol (crystal) - 0.2 gm
Sodium sulphite - 0.05 gm
Distilled water - 100 ml

b) **Pectinases:**

Determinations were made for the pectinases such as polygalacturonase (PG) and polymethylgalacturonase (PMG) by viscometric method of Bateman (1964).

The enzyme reaction mixtures were placed in Ostwald-Fenske 300 viscometers to determine percentage loss of viscosity in the enzyme substrate due to its hydrolysis by the respective enzymes.

The reaction mixtures for PG and PMG were:

a) **PG:**

1% Sodium polypectate, pH 4.5 (as substrate) - 3.5 ml
McIlvaine's buffer, pH 4.5 - 1.5 ml
Distilled water - 1.5 ml
Enzyme preparation - 1.5 ml
b) PMG:

1.5% Citrus pectin, pH 5.5 - 3.5 ml (as substrate)
McIlvaine's buffer, pH 5.5 - 1.5 ml
Distilled water - 1.5 ml
Enzyme preparation - 1.5 ml

Viscosity readings were taken at 30°C after incubation periods of 30, 60, 90 and 120 minutes. The boiled culture filtrate was used as control.

The loss of viscosity was calculated by the formula:

\[
\% \text{ loss in viscosity} = \frac{E_{To} - E_{Tt}}{E_{To} - E_{Tw}} \times 100
\]

where, \( E_{To} \) = Efflux time at 0 hour,
\( E_{Tt} \) = Efflux time at time t,
\( E_{Tw} \) = Efflux time of water.

c) Protease:

Enzyme assay was done by the modified method of Hayashi et al. (1967) as followed by Mayers and Ahearn (1977).

The reaction mixture contained:

1.5% Casein in 0.1 M citrate - 1.0 ml
buffer of pH 5.0
Sodium-Citrate buffer of pH 6.0 - 0.5 ml
Enzyme preparation - 0.5 ml

The reaction mixture was incubated for 20 minutes at 35°C. The proteolysis of casein was
terminated by the addition of 4 ml of 9% Trichloroacetic acid (TCA) solution. After an hour the solution was centrifuged at 2,000 rpm for 15 minutes to remove precipitated protein. 1 ml of supernatant was mixed with 5 ml of 0.4 M sodium carbonate solution, followed by addition of 0.5 ml phenol reagent (Lowry et al., 1951). The blank consisted of 0.5 ml distilled water, 1 ml of 1% casein dissolved in citrate buffer and 0.5 ml of sodium-citrate buffer.

Liberation of free tyrosine was read over Specol at 560 nm.

The amount of tyrosine thus determined was calculated by multiplying it with 6 and dividing it by 20.

One unit of enzyme activity is defined as the amount of enzyme liberating one micro-mole of tyrosine per minute under the defined condition.

d) Amylase:

It was assayed on solid medium using the Lugol's iodine test. Activity was measured in mm dia. of the clear zone (already described in 2.ii.d).

4. Physiological characterisation:

In their biology the actinomycetes show specific responses towards the temperature gradient and the antibiotics. The isolated species of the Streptomycetes were subjected to these tests.
1) **Responses to Incubation Temperatures:**

Cultures were raised on Starch-Casein broth medium and incubated at temperatures of 20°C, 25°C, 30°C, 35°C, 40°C, 45°C, 50°C and 55°C for 8 days, in three replicates, for each of the isolates. Determinations were made for total biomass (dry weight) production and the cellulolytic, amylolytic and proteolytic activities.

The biomass was determined by recording the dry weight of the harvested cellular mass on a pre-weighed filter paper (Whatman No. 42).

Enzymes were assayed as per the procedures described earlier. However, the amylase was assayed here by determining the amount of reducing sugar liberated from starch on enzyme hydrolysis. The procedure followed was:

The reaction mixture consisted of:

- Enzyme preparation: 5.0 ml
- 1% soluble starch solution: 5.0 ml
- 1M acetic acid sodium acetate buffer of pH 6.0: 10.0 ml

It was incubated at 37°C for 24 hours. Amount of reducing sugar was determined after treating an aliquot of the reaction mixture with DNS reagent from 'Specol' at 540 nm. Calculations were made from the standard curve, as done in other cases i.e. cellulase.
ii) **Antibiotic sensitivity:**

It was tested according to the method adopted by Williams (1967).

The cultures were made on Glycerol-nitrate agar medium (Shinobu, 1953) with spore-suspension inoculations of the medium before plating. After plating and solidification of the medium, antibiotic impregnated biodiscs were placed upon the surface of the solidified seeded agar and incubated at 30°C.

The antibiotic sensitivity was determined against all the isolates for the antibiotics, Chloramphenicol, Erythromycin, Gentamycin, Kanamycin, Neomycin, Penicillin-G, Polymyxin-B, Streptomycin, Tetracyclin and Vancomycin (Mfd. by Pasteur Biological Labs (India), Gujarat).

Data for antibiotic sensitivity etc. was recorded by measuring the diameter of the zone of growth inhibition around the biodiscs. Sensitivity was compared with the data given for each of the antibiotic by the manufacturers of biodisc.

5. **Evaluation of the antibiotic potential:**

Twenty one isolates belonging to the species *Streptomyces bikiniensis*, *S. gougeroti*, *S. fradiae*, *S. goedicolor*, *S. levendulae*, *S. griseus*, *S. hygroscopicus* and *S. rameus* were taken for testing their antibiotic potential.
The cultures were done on Starch-Casein broth medium of pH 7. Cell free culture filtrate was obtained by filtration through millipore bacteriological filters (Sartorius, No. 113 06) after 8 days of incubation. The cell free filtrate was used for the antibiotic assays using Escherichia coli as the test organism. The assay was made in three different ways.

a) *food-poison technique*;

To about 10 ml molten sterilized nutrient agar medium was added 2 ml of the culture filtrate and thoroughly mixed. It was then seeded with 1 ml of *E. coli* suspension (1x10^4 cells), plated and incubated at 37°C. Medium without culture filtrate was taken as control. After 2-3 days the growth of *E. coli* was compared in test and control plates by taking the colony count.

b) *Paper-disc method*.

Here blank filter paper biodiscs were impregnated with the culture filtrate (antibiotic preparation) dried under aseptic conditions, and used for plating over the seeded agar. Observations were recorded for the zone of inhibition formed against the test organism *E. coli*. 
c) Ager-well method:

The culture filtrate (antibiotic preparation) was placed in a cavity made in the seeded-agar plate and allowed to incubate. The diffusion of the culture filtrate formed a clear growth free zone around the well, if the culture filtrate was having antibiotic activity. The test organism was *E. coli* in this case also.

5. Amino acid Profile Determination:

The cellular amino acid profile was determined in the isolates of the following species: *Streptomycetes bikiniensis*, *S. gougeroti*, *S. fradiae*, *S. coelicolor*, *S. lavendulae*, *S. griseus*, *S. hygroscopicus* and *S. rameus*.

For preparing the protein hydrolysate one gram dried cellular mass was refluxed in Soxhlet's apparatus with petroleum ether (60°-80°C) for 8 hours to remove fatty substances. The fat-free mass was then extracted with 80% ethanol to remove sugars and free amino acids, etc. Afterwards the residue was refluxed with 25 ml 3N HCl for 24 hours to obtain acid hydrolysate of the cellular protein.

The hydrolysate was subsequently concentrated by evaporation over water-bath, and redissolved in small amount of distilled water before being again concentrated to a volume of about 1-2 ml in each case. This
concentrated hydrolysate was finally redissolved in 3 ml of 70% ethanol and taken as the amino acid preparation for chromatographic analysis.

Chromatographic analysis was made for the amino acid composition of each of the hydrolysates by paper and thin-layer chromatographic techniques. Both the single and two-dimensional chromatographic analysis was done to obtain amino acid identifications with co-chromatograms.

The solvent systems and detecting reagents used were:

**Solvent systems:**

1. n-Butanol Acetic acid-Water
   (4:1.5 v/v)
   Single dimension and the first dimension of the two-dimensional.

2. Phenol Water
   (80:20 w/v)
   Second dimension of the two-dimensional.

**Detection:**

0.3% ninhydrin in acetone.