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Biochemical Characterization of Actinomycetes Isolated from Different Soil Samples

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Abstract

In the present study, the different soil samples were collected around Davangere city. Actinomycetes strains were isolated in specific media like Starch Casein Nitrate Agar Medium (SCA), Benedict Agar Medium (BA), Glucose Asparagine Agar Medium (GAA), Oat Meal Agar Medium (OMA). Cultural characteristics of actinomycetes are determined based on morphology, production of diffusible pigments, utilization of various carbon and nitrogen sources and by various physiological tests. Actinomycetes strains were performed in biochemical tests such as Fermentation of citrate, starch hydrolysis, triple sugar iron agar, gelatine hydrolysis, casein hydrolysis etc. Strategy for a range of biochemical characterization methods have been mentioned in this article for the discovery of various genera of actinomycetes.

Keywords: Actinomycetes, Biochemical tests, *Streptomyces* sp, *Nocardia* sp. *Saccharopolyspora* sp, *Amycolatopsis* sp

1. Introduction

Actinomycetes, gram positive filamentous organisms are thought to be a transition group between bacteria and fungi. These organisms are distributed widely in nature and are well known for their beneficial properties (Niladevi *et al*., 2005) [23]. Actinomycetes populations forms an important component of the soil microflora. According to the estimate of Alexander, 70-80% of the actinomycetes in virgin and cultivated soils are *streptomyces* species. *Streptomyces* species are also found to occur in fresh water and marine environments (Thakur *et al*., 2007) [33]. Actinomycetes hold a prominent position as targets in screening programmed due to their diversity and their non-antibiotic bioactive molecules of pharmaceutical interest. Since, the discovery of actinomycin, the first antibiotic from an actinomycetes, many commercially important bioactive compounds and antitumour agents have been produced using actinomycetes (Suzuki *et al*., 2001) [32]. Actinomycetes have been shown to be a promising source of wide range of enzymes, enzyme inhibitors, immunomodifiers and vitamins. Actinomycetes play a very supporting role in the degradation of organic matter. Ecological significance of actinomycetes are degradation of lignin, organic matter, formation and stabilization of compost piles, formation of stable humus, production of antibiotics, combine with other soil microorganisms in breaking down tough plant and animal residues. Major groups of actinomycetes are *Streptomycetaceae, Nocardiaceae, Micromonosporaceae, Actinoplanaceae, Dermatophilaceae, Frankiaceae, actinomycetaceae*. Some of the ecological parameters influences on growth and development of Actinomycetes (Suzuki *et al*., 2001) [32]. Which includes alkaline and neutral soils are more favourable for the development of actinomycetes is in the range of 6.5-8.0. They cannot survive in acidic pH. In soils, with pH less than 5.0 they are almost absent, waterlogged soils with 80-90% moisture content is detrimental for the survival of actinomycetes, the percentages of actinomycetes in the total microbial population increases with the depth of soil. However, they are also found in surface soils (Rifaat *et al*., 2005) [30]. The ideal temperature for the growth of the actinomycetes is in the range of 25-30ºC. as such most of the actinomycetes are mesophilic. However, most of the thermophilic actinomycetes play an important role in the transformation of various organic residues inside the compost pits. The most common genera of actinomycetes inhabiting the soil are the *Streptomyces, Nocardia* and *Micromonospora* (Jimenez *et al.,* 2005) [18].

Cultural characteristics of actinomycetes are determined based on morphology, production of diffusible pigments, utilization of various carbon and nitrogen sources and by various physiological tests (Moncheva *et al*., 2002) [21]. All strains grew on a range of agar media showing morphological characteristics of actinomycetes. These strains show tough, leathery colonies on the selective media (Xu *et al*., 2005) [35].

2. Materials and Methods

Structural/Morphological Characteristics

Morphological characteristics are still widely used characterizing genera. The morphology of an actinomycete growing on agar can provide useful and rapid clues to it identify, but viewing isolated colonies can give little worthwhile information. Many actinomycetes will grow on the common bacteriological media used in the laboratory, such as nutrient agar medium. Tough, rough, leathery, white- and buff-coloured colonies were observed on different selective media (Benson, 1994) [6].

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Staining (Gram's Staining)
In this staining procedure, a thin smear was made on a clean glass slide which was air dried and fixed by flaming. Then the thin smear was subjected to gram's staining. The smear was flooded with crystal violet solution for 1 min, decolorized crystal violet with 95% ethanol for a few seconds and then counter stained with saffranine solution. Then, the slide was blot dried and observes under microscope under oil immersion (Benson, 1994) [6].

Motility
When attempting to identify unknown bacteria it is usually necessary to determine whether or not the microorganisms is motile.
In this test, a small amount of Vaseline was placed near each corner of the cover glass with a tooth pick and two loopful of organisms was placed in centre of cover glass. Depression slide was pressed against Vaseline on cover glass and quickly inverted. The completed preparation was examined under oil immersion (Benson, 1994) [6].

Catalase Test
This demonstrates the presence of catalase, an enzyme that catalyzes the oxygen from hydrogen peroxide. One or two drops of 3% hydrogen peroxide was taken on a clean glass slide and one loopful of 24-48 hours old culture was added or mixed with hydrogen peroxide. Observation was made for the emergence of bubbles due to release of oxygen (Collee *et al*., 1996) [8].

Oxidase Test
This test depends on the presence in bacteria of certain oxidases electron donors in the bacteria and a redox dye tetramethyl-p-pheylene diamine. The dye is reduced a deep purple colour. In this test, p-phenylene diamine discs were placed in a clean glass slide and moistened with distilled water and loopful of 24-48 hours old culture was transferred onto the disc. This test was carried out to determine the presence of oxidases that catalyze the transport of electrons between electron donors in the culture to a redox dye tetramethyl-p-phenylene diamine. Observation was made the deep purple colour formation within 1 min (Collee *et al*., 1996) [8].

KOH Solubility Test
In this test, a drop of freshly prepared 3%KOH solution was taken on a clean glass slide and it was mixed with a loopful of 24-48 hours old culture with the help of toothpick. Then, toothpick was raised a few centimetres from the slide and it was observed for the formation of a mucoid thread.

Starch Hydrolysis Test
In this test, starch agar medium was prepared by the addition of 0.2% soluble starch to the nutrient agar, sterilized and poured into sterile Petri plates. Then, the plates were point inoculated with the test isolates, these plates were incubated at 37^0C for 3 days. After incubation, the culture plates were flooded with gram's iodine. Finally, plates were observed for the formation of a clear zone of hydrolysis around the growth (Benson, 1994) [6].

Casein Hydrolysis Test
In this test, 24-48 hours old culture were point inoculated onto skimmed milk agar medium and incubated at 370C for 48 hours and observed for clear zone formation around the colony (Benson., 1994) [6].

Nitrate Reduction Test
This is a test for the presence of the enzyme nitrate reductase which causes the reduction of nitrate, in the presence of suitable electron donors to nitrite, which can be tested for by an appropriate colorimetric reagent. It was tested by the addition of 0.1 ml NR test reagent top the culture and incubated at 37^0C for 96 hours. A red colour development occur within few minutes indicates the presence of nitrite (Collee *et al*., 1996) [8].

ß-Galactosidase or ONPG Test
The ß-galactosidase or ONPG test, which determines the presence of the enzymes ß-galactosidase by utilizing o-nitro-phenyl-ß-D-galacto pyronoside (ONPG), is used to differentiate late lactose fermenting organisms and is of particular use in the identification of *Enterobacteria*. In this test, the culture suspension was prepared in saline using 24-48 hours old cultures. Then, ONPG discs were immersed in culture suspension and incubated for 24 hours at 37^0C. cultures were observed for development of yellow colour (Benson, 1994) [6].

Bile Esculin Disc Test
Bile esculin discs were recommended for rapid detection of esculin hydrolysis in presence of bile. In this test, one bile esculin disc was taken in a sterile test tube and 0.1ml of sterile nutrient broth was added and inoculated with 24-48 hours old cultures and tubes were incubated at 37^0C for 4 hours. Then, tubes were examined every hour for brown or black colour development (Benson, 1994) [6].

Malonate Utilization Test
In this test, utilization of malonate and determination of phenylamine can be combined. 24-48 hours old culture were inoculated onto malonate broth and incubated at 370C for 24 hours and observed for colour change from green to blue (Collee *et al*., 1996) [8].

Citrate Utilization Test
This is a test the ability of an organism to utilize citrate as the sole carbon and nitrogen source for growth and an ammonium salt as the sole source of nitrogen. Simmon's citrate agar medium was used. The medium was inoculated with 24-48 hours old culture and incubate at 37^0C for 96 hours. An observation made for the colour change from green to blue was due to change of pH in the medium (Collee *et al*., 1996) [8].

Lipase and Lecithinase Test
Egg yolk indicates both lipase and lecithinase reactions of bacteria. On solid media containing egg yolk, lipolysis is shown by the formation of a thin, iridescent pearly layer overlying the colonies and a confirmed opalescence in the medium underlying them. Lecithinase is shown by wide zones of opalescence around colonies, more intense and larger than the zones caused by lipolysis. Egg yolk agar medium was point inoculated with 24-48 hours old culture and incubated for 48 hours at 37^0C. Observation was made for the formation of clear zone around the colony (Benson, 1994) [6].

Litmus Milk Test
Milk indicates both saccharolytic and proteolytic properties of

bacteria by detecting whether they ferment lactose or digest casein. Lactose fermenters in litmus milk form acid and cause it to become pink. Large amounts of acid will precipitate the casein as a clot and if gas is formed during coagulation, the clot will be disrupted it (stormy clot). Proteolytic bacteria may decompose milk proteins to a transparent solution of soluble products. In litmus milk, this shows as a clear dark purple solution, usually taking several days and preceded by the formation of a soft, easily disintegrated clot.

In this test, 24-48 hours old culture was inoculated on to sterile litmus milk broth tubes and incubated at 37^0C for 48 hours. Then, observation was made for the formation of coagulation, peptonisation, acid formation, alkaline formation, litmus reduction and for ropiness (Collee *et al*., 1996) [8].

Triple Sugar Iron Agar (TSIA) Test

This medium was originally designed as a multi test medium. It provides a low degree of sensitivity for hydrogen sulphide production. The medium is used principally as a standard test for hydrogen sulphide (Cappuccino *et al*., 2002) [7].

Gelatin Hydrolysis Test

A stab culture was made with a 24-48 hours old culture and incubated at 37^0C for 24 hours. Liquefaction of medium was tested at different time intervals by removing the nutrient gelatin cultures from the incubator and holding them in cool temperature by keeping in refrigerator for 30 min or ice cubes, which are capable of producing the exoenzymes, gelatinases, hydrolyse the gelatin. Then, observations were made for liquefaction of the medium after cooling (Collee *et al*., 1996) [8].

Hydrogen Sulphide Production Test

Hydrogen sulphide test demonstrated by its ability to form black insoluble ferrous sulphide. Hydrogen sulphide can be produced at least in small amounts from sulphur containing amino acids by a large number of bacteria. Sulphide indole motility agar medium was inoculated with 24-48 hours old culture and incubated at 37^0C for 28 hours and observed for the formation of black colour (Cappuccino *et al*., 2002) [7].

Indole Test

This test was conducted to test the ability of bacteria to decompose the amino acid tryptophane to indole, which accumulates in the medium. Tryptone broth inoculated with 24-48 hours old culture and incubated for 48 hours at 37^0C. Indole production was then tested by the coloured reaction with the addition of Kovac's reagent. The red colour in the alcohol layer indicates a positive reaction (Collee *et al*., 1996) [8].

Carbohydrate Fermentation Test

The fermentation ability of carbohydrates such as glucose, sucrose and lactose by cultures was carried out in a fermentation tube that contains Durham's tube placed in an inverted position for the detection of gas production. The observations were made for any colour change in the medium due to change in the pH (Collee *et al*., 1996) [8].

3. Results and Discussion

3.1. Characterization of Actinomycetes

Sixty-five isolates were isolated from different soil samples collected from ragi, jower, coconut, sugar cane, corn, sunflower field, campus garden and near pond. After that, identification of actinomycetes were made by motility, gram staining by microscopy and by various physiological tests like catalase test, oxidase test, KOH solubility test, starch hydrolysis test, casein hydrolysis test, nitrate reduction test, ß-galactosidase or ONPG test, bile esculin test, hydrogen sulphide test, malonate utilization test, citrate utilization test, litmus milk test, lipase and lecithinase test, gelatin hydrolysis test, triple sugar iron agar test, indole test, carbohydrate fermentation test (Augustine *et al*., 2004) [4]. Results were observed in Figure 1

Same results were observed in physiological tests from three times of examination. The identification of *Streptomyces* strain using oat meal agar medium was followed Wu *et al*., 1995 and *Nocardia, Micromonospora, Actinomadura, Saccharopolyspora* based on physiological tests (Warren *et al*., 2004). 235 strains were identified as *Streptomyces* species from 20 different soil samples (Basilio *et al*., 2003). The same method was followed and the isolated actinomycetes were identified as 4 genera, which include *Streptomyces* species, *Nocardia* species, *Saccharopolyspora* species and *Amycolatopsis* species.

65 actinomycetes strains were isolated from 9 different soil samples. Out of 65 isolated actinomycetes 31 isolates were identified as *Streptomyces* species. Among 31 *Streptomyces* species, S-5 strain was identified as *S.phaecochromogenes* based on morphological and physiological tests, compared to other physiological tests, KOH solubility test and milk peptonization test shows negative results in all 31 *Streptomyces* species were shown in Figure 2.

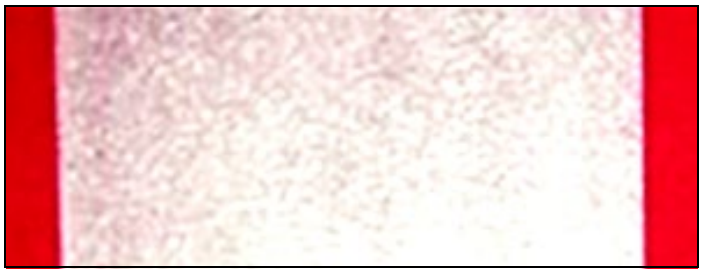
25 isolates were identified as *Nocardia* species. Among 25 *Nocardia* species, S-2strain was identified as *N.amarae*, S-8 as *N.asteroids*, S-22 as *N.carnea*, S-1 as *N.brasiliensis* and strain S-7 and S-16 as *N.brevicatena*. KOH solubility test, milk peptonisation test and casein hydrolysis test show negative results compared to other physiological test results, in all 25 *Nocardia* species. The detailed information was shown in Figure 3.

3 isolates were identified as *Amycolatopsis* species. Among 3 *Amycolatopsis* species, strain S-12 strain was identified as *A.sulphurea* and strain M-8 as *A.rugosa,* compared to other physiological test results, KOH solubility test, hydrogen sulphide test and nitrate reduction test shows negative results in all 3 *Amycolatopsis* species. The detailed information was shown in Figure 4.

4 isolates were identified as *Saccharopolyspora* species. Out of 4 *Saccharopolyspora* species, S-24 strain was identified as *S.rectivirgala*. KOH solubility test, citrate utilization test and nitrate reduction test show negative results compared to other physiological test results, in all 4 Saccharopolyspora species. The detailed information was shown in Figure 5.

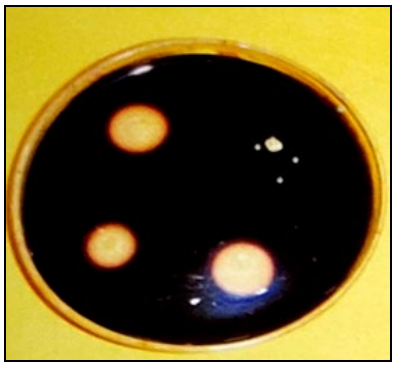
Remaining 2 isolates were unidentified. Results are more similar in the isolation and identification of *Streptomyces* species and other actinomycetes strains compared to results in the identification of actinomycete species (Warren *et al*., 2004) [34]. The morphological structure of identified *Streptomyces*, *Nocardia*, *Amycolatopsis* and *Saccharopolyspora* were shown in Figure 6.

Catalase Test

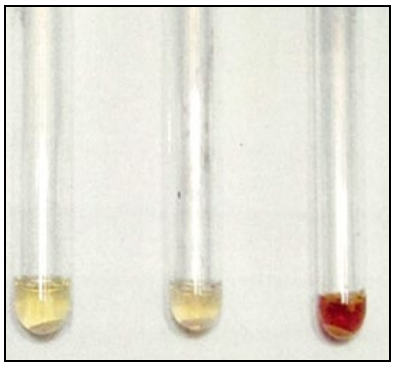


Oxidase Test

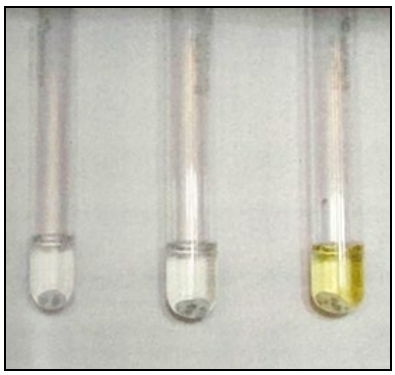
KOH Solubility Test



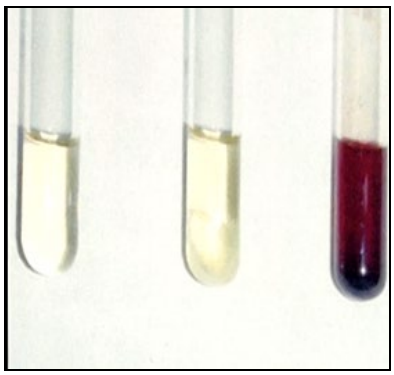
Starch Hydrolysis Test



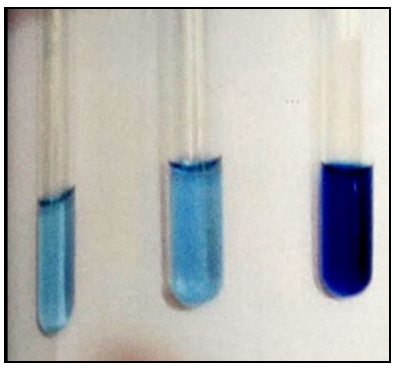
Nitrate Reduction Test



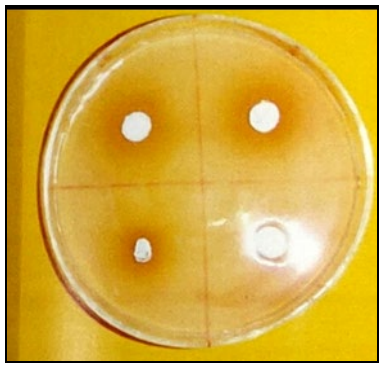
ß-Galactosidase/Onpg Test



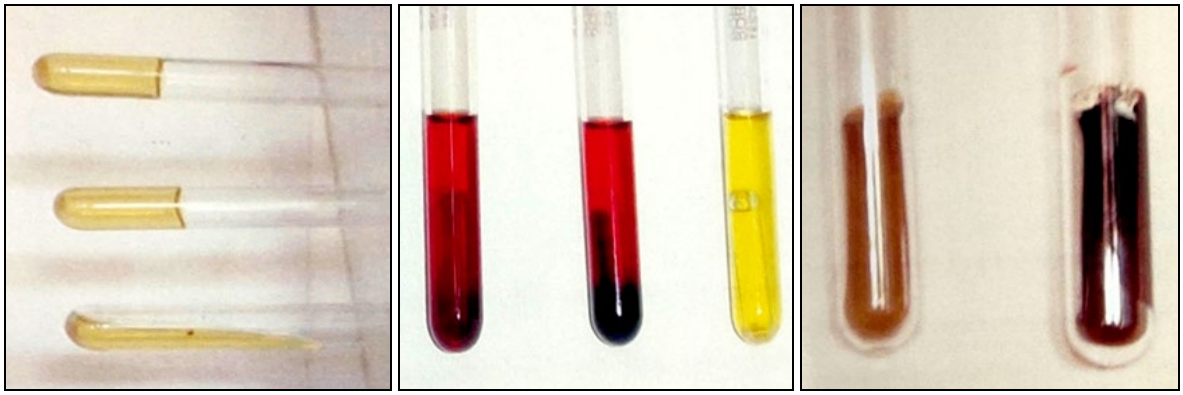
Bile Esculin Test



Malonate Utilization Test



Lipace and Licithinase Test



Gelatin Hydrolysis Test **Hydrogen Sulphide Production Test** **Carbohydrate Fermentation Test**

Fig 1: Physiological Test Results

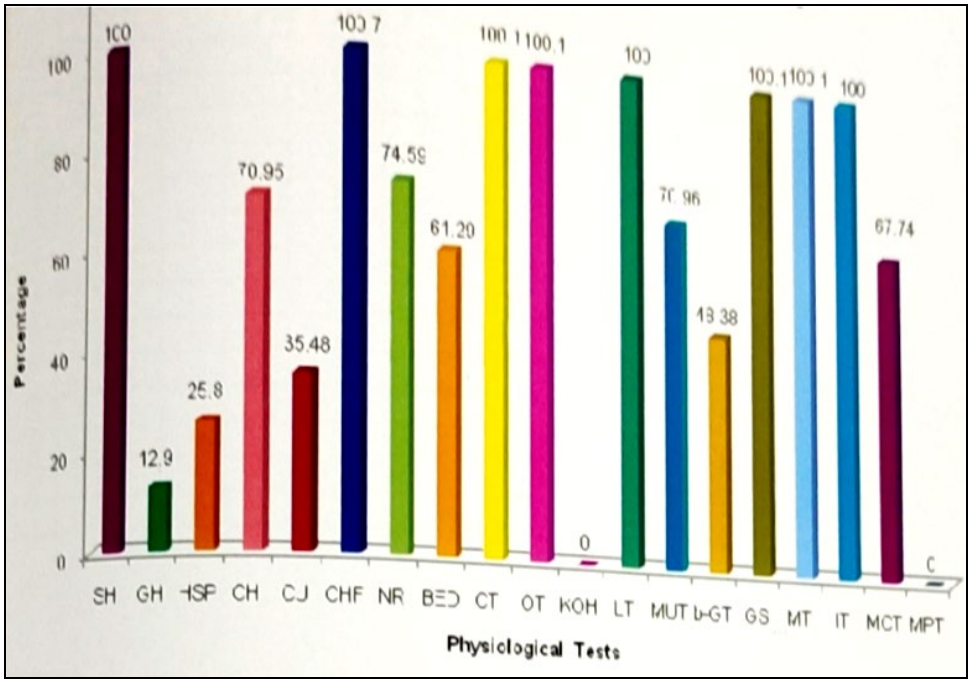


Fig 2: Showing Physiological Tests Percentage in *Streptomyces* and its species.

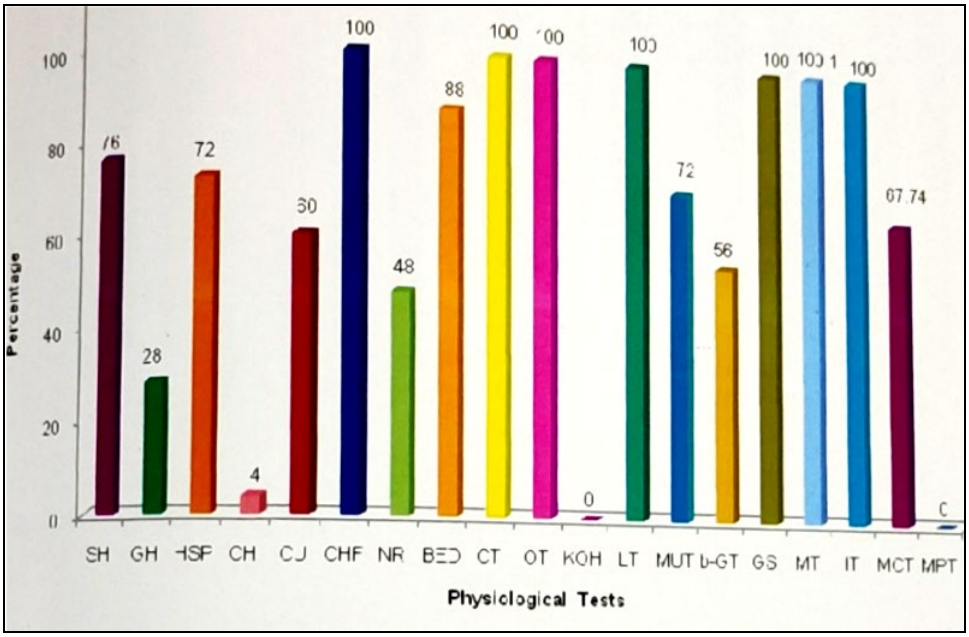


Fig 3: Showing Physiological Tests Percentage in *Nocardia* and its species.

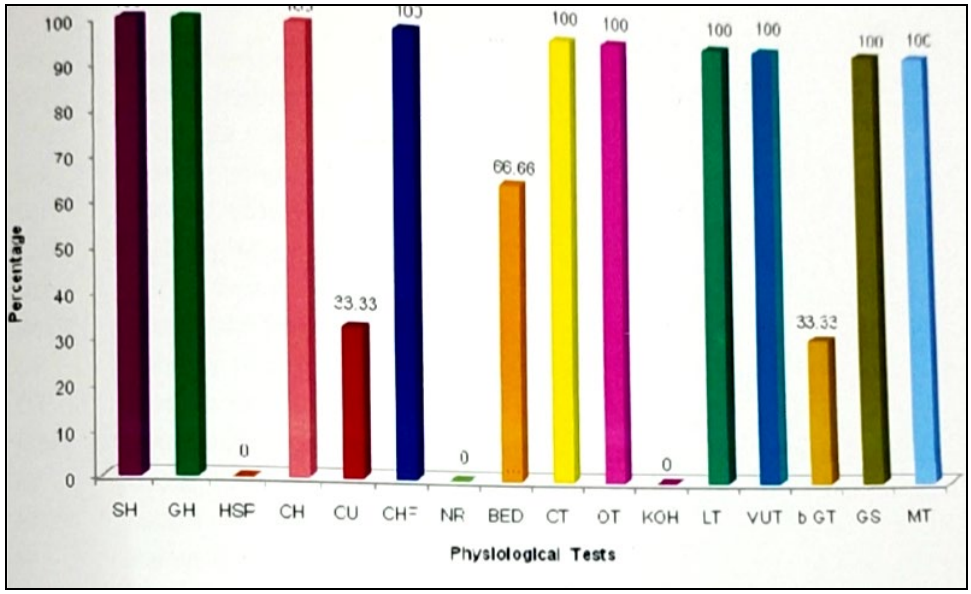


Fig 4: Showing Physiological Tests Percentage in *Amycolatopsis* and its species.

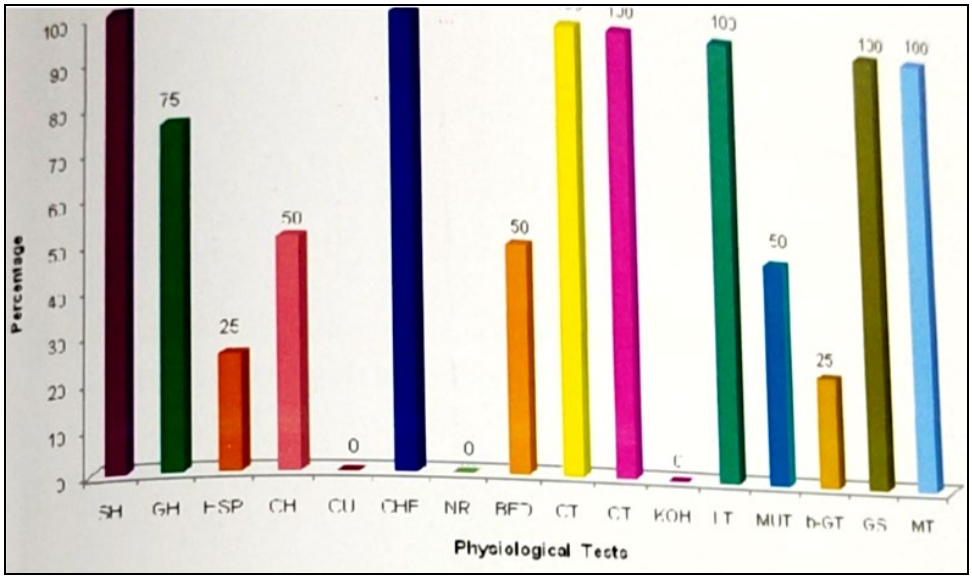


Fig 5: Showing Physiological Tests Percentage in *Saccharopolyspora* and its species.

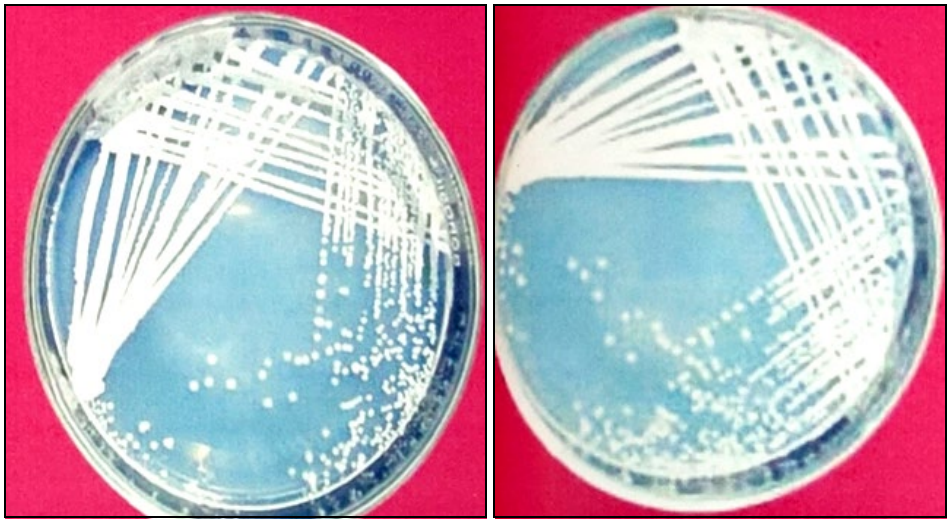


Fig 6: Showing Morphological structure of identified actinomycetes.

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