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Isolation of Actinomycetes from Different Soil Samples of Davangere City

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Abstract: ***Actinomycetes have provided many industrially important bioactive compounds having great economic importance. Actinomycetes are widely distributed in natural habitats, hence various methods like pre-treatments, enrichment,combinations of antibiotics, specific isolation media and some novel methods has been adapted for isolation. Actinomycetes have long been recognized as prolific producers of enzymes, antibiotics, anti-cancerous agents and play an important role in recycling of organic matter. Soil actinomycetes were isolated by various methods and found to be important source for secondary metabolites. Rare actinomycetes isolated by providing combinations of methods and with screening methods. Strategy for a range of isolation methods have been mentioned in this article for the discovery of various genera of actinomycetes.***

Keywords: Actinomycetes, antibiotics, Isolation methods, Substrate

1. Introduction

Actinomycetes are useful as producing organisms of antibiotics, enzymes and other bioactive compounds. They are also important resources for the development of industrial products. The compounds they produce are valuable for industrial and pharmaceutical purposes. Actinomycetes are the most widely distributed groups of organisms in nature. They are attractive and charming filamentous gram-positive bacteria having high GC content in excess of 55% DNA and carbon content varies from 12-65%. Some species have hexosamine in the cell wall to the extent of 2-18% and identification of aerobic actinomycetes is based on analysis of whole cell diaminopimelic acid. They make up in many cases, especially under dry alkaline conditions, a large part of the microbial population of the soil (Warren *et al.*,2004). The actinomycetes are also a group of physiologically diverse bacteria. This diversity is seen in both in the production of extracellular enzymes and in the thousands of kinds of metabolic products which they synthesize and excrete. Actinomycetes are wide spread in nature, occuring typically in soil, composts, aquatic habitats and colonizing plants (oskay*et al*.,2004).

The majority of actinomycetes are free living. These microorganisms are found in a wide range of aquatic and terrestrial environment, but some form symbiotic associations and others are pathogenic in man, animals and plants. The organisms are chemoorganotrophs, collectively they can degrade a wide range of substances which includes agar, cellulose, chitin, keratin, paraffin and rubber. In recent years, there has been an increasing interest in discovering new agricultural antibiotics for the protection of our living environments. These actinomycetes are prolific producers of antibiotics and other industrially useful secondary metabolites (Chaudhary *et al*., 2013).

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). 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. Actinomycetes have been shown to be a promising source of wide range of enzymes, enzyme inhibitors, immunomodifers and vitamins. Unlike bacteria, actinomycetes are relatively drought resistant and have an ecological advantage over bacteria in desert soils which tend to be dry and alkaline. The ecological niche of most actinomycetes is probably the aerobic zone of be soil, where they live saprophytically at the expense of a wide variety of organic substrates. Saprophytic actinomycetes are important primary colonizers of soil organic material, the bulk of which is in form of insoluble polymers. Actinomycetes have ability to penetrate and solubilize the polymers. Most of actinomycetes are isolated from fresh and sea water sediments. Actinomycetes have some unique properties that may be related to their ability to survive and grow in soils. They are prolific producers of extracellular enzymes that degrade the complex macromolecules substrates commonly found in soils. Most ecological studies of actinomycetes have been carried out with the dilution plate procedure, which does not differentiate between forms of growth in the natural habitats nor provide a direct assessment of activity. 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*. Somer of the ecological parameters

influences on growth and development of Actinomycetes. 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. The ideal temperature for the growth of the actinomycetes is in the range of 25-30^{o}C. 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).

The selectively of the isolation medium is controlled by its composition, the addition of selective inhibitors, and the period of incubation. Many media formulations have been recommended for the isolation of one or more actinomycetes genera. Recently differences in sensitivity to antibiotics have been used to increase the selectively of media for particular actinomycetes. For example, tetracycline, novobiocin.

The various methods have been innovatively established and successfully used for the isolation of actinomycetes as such. These methods involve the use of specific substrates. The innovative isolation methods were generally established on the basis of some physiological or ecological properties that are common and unique to specific group of genera. (Pettet*et al*., 2004).

The isolation of actinomycetes from the mixed micro flora present in nature is complicated by their characteristic slow growth relative to that of other soil bacteria. This has resolved in the development of selective isolation procedures based primarily on one or both of the following approaches;

- **Nutritional selection:**

In which media are formulated with nutrients which are formulated with nutrients which are preferentially utilised by actinomycetes.

- **Selective Inhibition:**

In which compounds such as antibiotics are incorporated into media to selectively inhibit non actinomycetes. The major difficulties with each of these approaches are that neither is strictly selective for actinomycetes and each has the inherent potential to actually inhibit the growth of some actinomycetes.

2. Materials and Methods

a) Chemicals and Media:

Culture media and chemicals are used in this study such as Starch casein nitrate agar medium (SCA), Benedict agar medium (BA), Yeast malt extract agar medium (YMEA), Glycerol casein agar medium (GCA), Soil extract agar medium (SEA), Glucose asparagine agar medium (GAA), Glycerol yeast extract agar medium (GYEA), Oat meal agar medium (OMA), Actinomycetes isolation agar (AIA), Mueller Hilton agar, Nutrient broth, phosphate broth, peptone water, simmons citrate agar, triple sugar iron agar, nalidixic acid etc.

b) Survey and Collection of Samples:

This study was undertaken with an aim of highlighting the presence of actinomycetes in these ecosystems. Totally, eleven (11) different farming soil samples were collected from different areas of Davangere city such as around Shivagangotri campus, market, near pond, garden, coconut field, sugar cane field, jowar field, corn field, sunflower field ragi field etc. Soil samples were collected from 5-10cm depth into sterile bags using sterile spatula. After tacking soil samples is directly transferred into polyethylene bags to minimize moisture losses during transportation. The detailed information of soil samples as shown in Table 2.1.

c) Isolation of actinomycetes

The samples were air dried for one week at room temperature. Isolation and enumeration of actinomycetes were done by serial dilution method and spread plate technique.

- One gram of soil was suspended in 9ml of sterile double distilled water and then the dilution was carried out upto 10-7 dilutions.
- Different aqueous dilutions were applied onto respective medium plates containing 20 ml of sterile selective medium with antifungal agents like cycloheximide or nystatin (100 microgram/l) by spread plate method.
- Then, the plates were incubated at 27^{o}C for 7-14 days. After incubation rough, white, buff-coloured colonies of actinomycetes were observed on selective medium plates.
- Selective actinomycetes colonies were transferred from mixed culture of the plates onto respective plates and incubated at 27^{o}C for 7 days.
- plantsor slants containing pure cultures were stored at 4oC until further examination (Oskay *et al*.,2004).

d) Primary screening of soil actinomycetes:

Totally eight (8) different selective media were used for the isolation and enumeration of actinomycetes, different medium composition were used for each different soil samples.

For the isolation of actinomycetes from soil samples 1, 5, 10 and soil sample 11, Starch casein nitrate agar medium composition were used. For soil samples 2,3,4,6,7,8,9, Benedict agar medium, Yeast extract agar medium, Glycerol casein agar medium, soil extract agar medium, Glucose asparagine agar medium, Glycerol yeast extract agar medium and Oat meal agar medium was used respectively.

Table 2.1: Collection and Characteristics of Soil Samples

Soil sample	Sample collected place	Colour of soil samples	Texture of soil samples
SS- 1	Near Pond	SCNAM	20
SS- 2	Ragi Field	BAM	1
SS- 3	Quaters garden	YMEAM	5

SS- 4	Campus garden	GCAM	11
SS- 5	Pond	SCNAM	No Growth
SS- 6	Coconut field	SEAM	5
SS- 7	Sugarcane field	GAAM	8
SS- 8	Corn field	GYEAM	2
SS- 9	Jower field	OMAM	3
SS- 10	Near Campus Quaters	SCNAM	No Growth
SS- 11	Sunflower field	SCNAM	10
		Total	65 Isolates

Note: SS- Soil Sample

3. Results and Discussion

a) Collection of soil samples:

The details of the samples collected and the isolated microorganisms were shown in Table 3.A.

The primary screening and isolation pattern of actinomycetes presented in Table 3.A. in this study, total 65 strains of actinomycetes were isolated from nine (9) different soil samples collected from different fields, by serial dilution method and spread plate method using different selective media. These are the media supported the growth of actinomycetes. The cultural characteristics of isolated actinomycetes were shown in Table 3.B.

The number of colonies was counted on all plates as the growth of the actinomycetes. The average number of colonies on the triplicate plates was recorded. The highest total Cfu was found on soil extract agar medium and on starch casein nitrate agar medium. Highest Cfu per gram of the soil (7x104) were observed in soil sample 1 using SCNAM but least (1x104) were observed in soil sample 3,7,11 from SCNAM and in soil sample 4,7,9 from SEAM and soil sample 7,10 from GCAM. In this investigation, the number of actinomycetes refers to Cfu per gram of the soil samples were shown in Table 3.C. This result agrees with the findings of Tang *et al*., (2002) reported the highest Cfu per gram of soil on SCNAM was9*104 and the highest number of bacteria refers to Cfu per gram of the soil (9x104) were observed in soil sample-3 from SCNAM, in soil sample-6 from SEAM (30x104), in soil sample-2 GCAM (13x104). And viable count of bacteria and actinomycetes in soil samples after plating on to selective agar media were recorded as shown in Table 3.D. This result correlates with the findings of Tang *et al*.,2002. Reported the highest bacteria per gram of soil 37x104. From the plates, representatives of each of the different colony types were subculture on selective media slants, allowed to grow at room temperature for 7 days and stored at 40^{o}C for final identification to species level. Each species isolated from one of the samples was considered an isolate.

Starch casein nitrate agar medium (SCNAM) were used for the isolation of actinomycetes by serial dilution method (Pettet *et al*., 2004). 20 actinomycetes strains were isolated using SCNAM by serial dilution method from 5 soil samples (Niladevi *et al*., 2005). 30 actinomycete strains were isolated from 2 different soil samples using SCNAM by serial dilution method. More number of actinomycetes strains Cfu per gram of soil sample were isolated by using selective media like SCNAM, SEAM, GCAM and a smaller number of strains were recorded on BAM, YMEAM, compare to their results, our results were more significant in the isolation of actinomycetes. Our results found similar with the findings of Niladevi *et al*., (2005) reported aisolation of actinomycetes from SCNAM by serial dilution method.

Table 3 (A): Isolation of actinomycetes from different soil samples.

Soil sample	Location of sample collected	Media used for Isolation	No. of Isolates
1	Near Pond	SCNAM	20
2	Ragi Field	BAM	1
3	Quaters garden	YMEAM	5
4	Campus garden	GCAM	11
5	Pond	SCNAM	No Growth
6	Coconut field	SEAM	5
7	Sugarcane field	GAAM	8
8	Corn field	GYEAM	2
9	Jower field	OMAM	3
10	Campus Quaters	SCNAM	No Growth
11	Sunflower field	SCNAM	10
		Total	65 Isolates

Table 3 (B): Cultural characteristics of Isolated actinomycetes.

Media	Growth	Colony Morphology	Pigmentation	Reverse Side
SCNAM	+++	White and light buff coloured colonies	-	Brown
BAM	+	White coloured dotte colonies	-	Brown
YMEAN	+	Buff coloured colonies	-	Brown
GCAM	++	White and buff coloured colonies	-	Pink
SEAM	++	Buff coloured colonies	-	Brown
GAAM	++	Buff coloured colonies	-	Brown
GYEAM	+	White coloured colonies	-	Brown

OMAM	+	Margin light buff colour, Middle dark buff coloure colony	-	Brown

Note: +++: Maximum
++: Moderate
+: Minnimum

Table 3 (C): Colony Forming Unit (Cfu) of actinomycetes.

Colony Forming Unit (Cfu) actinomycetes on SCNAM

Soil sample	Cfu/ ml
SS- 1	7 X 10^4
SS- 2	2 X 10^4
SS- 3	1 X 10^4
SS- 4	2 X 10^4
SS- 5	-
SS- 6	2 X 10^4
SS- 7	1 X 10^4
SS- 8	3 X 10^4
SS- 9	2 X 10^4
SS- 10	-
SS- 11	1 X 10^4

Colony Forming Unit (Cfu) actinomycetes on SEAM

Soil sample	Cfu/ ml
SS- 1	3X 10^4
SS- 2	3 X 10^4
SS- 3	2 X 10^4
SS- 4	1 X 10^4
SS- 5	5 X 10^4
SS- 6	2 X 10^4
SS- 7	1 X 10^4
SS- 8	-
SS- 9	1 X 10^4
SS- 10	5 X 10^4
SS- 11	6 X 10^4

Colony Forming Unit (Cfu) actinomycetes on GCAM

Soil sample	Cfu/ ml
SS- 1	5 X 10^4
SS- 2	5 X 10^4
SS- 3	4 X 10^4
SS- 4	3 X 10^4
SS- 5	-
SS- 6	2 X 10^4
SS- 7	1 X 10^4
SS- 8	5 X 10^4
SS- 9	4 X 10^4
SS- 10	1 X 10^4
SS- 11	7 X 10^4

Table 3 (D): Viable Counts of Bacteria and actinomycetes

Viable Counts of Bacteria and actinomycetes on SCNAM

Soil sample	Cfu/ ml of Soil sample	
	Bacteria	Actinomycetes
SS- 1	3 X 10^4	7 X 10^4
SS- 2	20 X 10^4	2 X 10^4
SS- 3	15 X 10^4	1 X 10^4
SS- 4	11 X 10^4	2 X 10^4
SS- 5	-	-
SS- 6	5 X 10^4	2 X 10^4
SS- 7	3 X 10^4	1 X 10^4
SS- 8	3 X 10^4	3 X 10^4
SS- 9	5 X 10^4	2 X 10^4
SS- 10	-	-
SS- 11	5 X 10^4	1 X 10^4

Viable Counts of Bacteria and actinomycetes on SEAM

Soil sample	Cfu/ ml of Soil sample	
	Bacteria	Actinomycetes
SS- 1	5 X 10^4	2 X 10^4
SS- 2	5 X 10^4	3 X 10^4
SS- 3	9 X 10^4	1 X 10^4
SS- 4	12 X 10^4	5 X 10^4
SS- 5	15 X 10^4	3 X 10^4
SS- 6	30 X 10^4	1 X 10^4
SS- 7	9 X 10^4	1 X 10^4
SS- 8	-	-
SS- 9	3 X 10^4	5 X 10^4
SS- 10	5 X 10^4	2 X 10^4
SS- 11	8 X 10^4	5 X 10^4

Viable Counts of Bacteria and actinomycetes on SEAM

Soil sample	Cfu/ ml of Soil sample	
	Bacteria	Actinomycetes
SS- 1	9 X 10^4	3 X 10^4
SS- 2	13 X 10^4	4 X 10^4
SS- 3	5 X 10^4	5 X 10^4
SS- 4	9 X 10^4	5 X 10^4
SS- 5	-	1 X 10^4
SS- 6	5 X 10^4	1 X 10^4
SS- 7	2 X 10^4	5 X 10^4
SS- 8	4 X 10^4	3 X 10^4
SS- 9	3 X 10^4	2 X 10^4
SS- 10	5 X 10^4	1 X 10^4
SS- 11	7 X 10^4	1 X 10^4

b) Isolation of actinomycetes

Actinomycetes colonies growing on agar media characteristically extend on to and well beneath the agar surface. A total of 65 different actinomycetes strains were recovered from different soil samples collected from different fields.

Actinomycetes are of considerable importance in industry. Several factors must be considered: choice of screening source, pre-treatment, selective medium, culture condition and recognition of colonies on a primary isolation plate (Seong *et al*., 2001).

Isolation and enumeration of actinomycetes were performed by soil dilution plate technique (Oskay*et al*., 2004).

Serially diluted soil suspensions were spread on to selective isolation medium and incubated for four weeks at 25^{o}C. Starch casein nitrate agar medium (SCNAM), Benedict agar medium (BA), Humic acid vitamin agar medium (HVA) were used for the selective isolation of actinomycetes from soil samples collected from Korea, Chonnam. Preliminary designation of actinomycetes colonies were done by microscopic observation (Seong*et al*.,2001).

c) Plate assay for screening of actinomycetes

Sixty-five actinomycete strains were screened by using different selective media and subjected for characterization.

d) Characters of actinomycetes

After serial dilution method, white, powdery, sticky, rough colonies were observed on respective selective medium plate with soil odour. White and buff with brownish background colonies were observed on SCNAM. Compare to other selective media, good growth was recorded on SCNAM and moderate growth were recorded on GCAM, GAAM,SEAM and less number were observed on BAM, YMEAM, GYEAM and OMAM. The results are more significant in the isolation of white, rough, chalky colonies in SCNAM and good growth on SCNAM with the findings of Thakur *et al*.,2007. The morphological characteristics were shown in Table 3.E. the detailed information of isolated actinomycetes characteristics from spread and streakplate method were shown in Figure 3.F. and Figure 3.G. when dilution plating was used, streptomyces were isolated from all the test soil samples as the most abundant group. Surviving *Streptomycetes* were detected in large numbers and outnumbered by non *streptomycetes*, including *Nocardia, Saccharopolyspora and Amycolatopsis*. While these streptomyces grew quickly on the selective isolation media plates. The same method was followed in the isolation of actinomycetes, results are more significant in the isolation of streptomyces species (Hayakawa *et al*.,2004). Then, these selected actinomycete strains were streaked onto selective medium slants and stored at 4^{o}C for further examination, were shown in Figure 3.H.

Table 3 (E): Morphological characteristics of isolated isolated actinomycetes

Isolates No.	Colony Morphology		
	Colour	Shape	Texture
S- 1	Buff	Round	Smooth
S- 2	White	Concave	Rough
S- 3	Buff	Dotted	Tough
S- 4	White	Dotted	Smooth
S- 5	White	Round	Powdery
S- 6	Buff	Concave	Rough
S- 7	White	Round	Smooth
S- 8	Dark Buff	Dotted	Rough
S- 9	Buff	Round	Tough
S- 10	Buff	Concave	Rough
S- 11	Buff	Concave	Smooth
S- 12	Buff	Dotted	Tough
S 13	White	Round	Rough
S- 14	Buff	Dotted	Tough
S-15	White	Concave	Smooth
S- 16	Buff	Round	Rough
S- 17	White	Concave	Smooth
S- 18	Buff	Irregular	Smooth
S- 19	White	Dotted	Powdery
S-20	Buff	Irregular	Smooth
S- 21	Buff	Dotted	Powdery
S- 22	White	Irregular	Powdery
S- 23	Buff	Concave	Powdery
S- 24	White	Dotted	Smooth
S- 25	Buff	Round	Powdery
S- 26	Buff	Irregular	Powdery
S- 27	Buff	Round	Powdery
S- 28	White	Concave	Powdery
S- 29	White	Concave	Powdery
S- 30	Buff	Dotted	Powdery

Morphological characteristics of isolated actinomycetes on GCAM

Isolates No.	Colony Morphology		
	Colour	Shape	Texture
O- 1	White	Round	Smooth
O- 2	White	Concave	Powdery
O- 3	Buff	Dotted	Tough
O-4	Buff	Dotted	Tough
O-5	White	Round	Powdery
O-6	Buff	Concave	Rough
O- 7	White	Round	Smooth
O- 8	Dark Buff	Dotted	Rough
O- 9	Dark Buff	Dotted	Tough
O- 10	Buff	Round	Powdery
O- 11	Buff	Concave	Smooth
O- 12	Buff	Dotted	Tough
O- 13	White	Dotted	Smooth

Morphological characteristics of isolated actinomycetes on SEAM

Isolates No.	Colony Morphology		
	Colour	Shape	Texture
W- 7	White	Round	Smooth
W- 8	Dark Buff	Dotted	Rough
W- 9	Buff	Round	Tough
W-10	Buff	Concave	Rough
W-11	Buff	Concave	Smooth

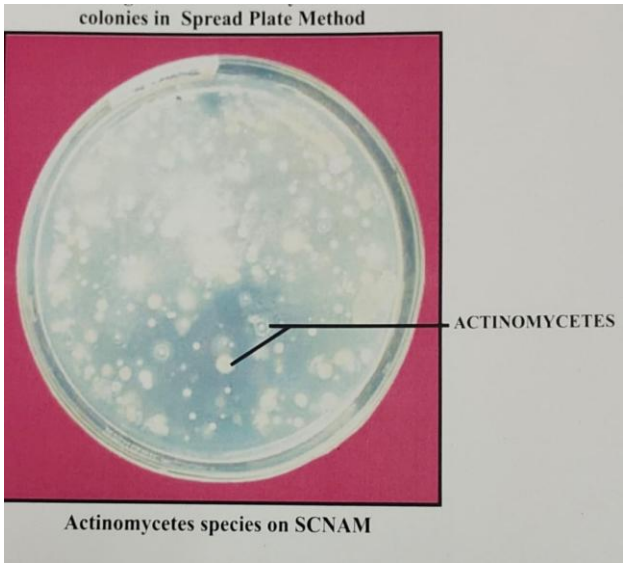

Figure 3 (F): Isolation of Actinomycetes

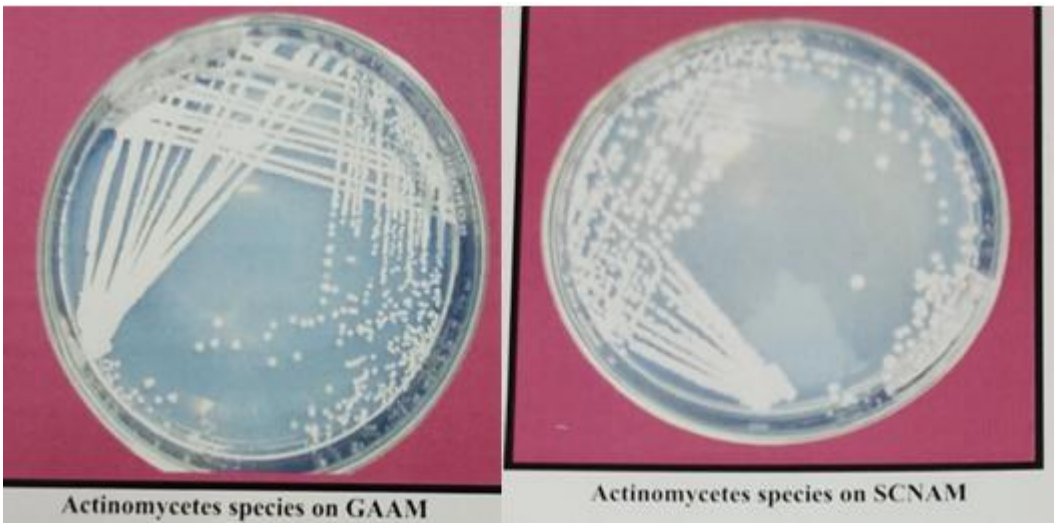

Figure 3 (G): Actinomycete Colonies in Streak Plate Method

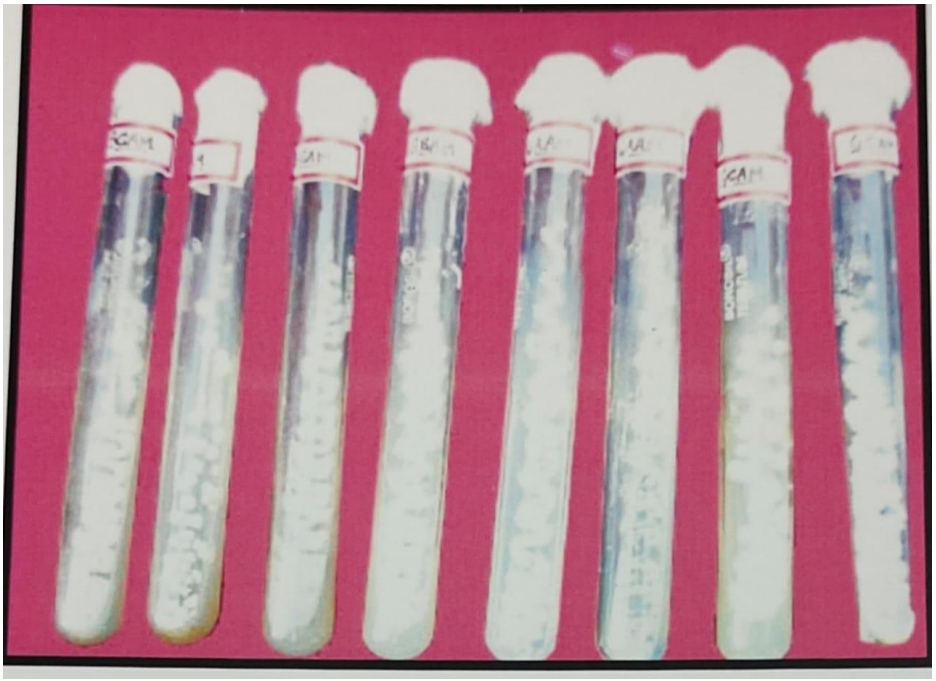

Figure 3 (H): Pure Cultures of Actinomycetes

4. Conclusion

These findings indicated that the soil samples of Davangere region have actinomycetes with metabolites that control bacterial pathogens. This study is a very useful advancing step on the field of diagnostic area. Antimicrobial resistance is a global problem which demands for novel antimicrobial structure against pathogenic microbes. Actinomycetes are famous for antibiotic production and continued to be explored in hope of getting novel antibiotics. Different generalized and advanced methods have been adopted to isolate rare actinomycetes from various sources like soil, water etc. these methods include pre-treatment, enrichment, different media compositions and integration for the isolation of novel genera of the actinomycetes from soil. The actinomycetes most commonly found in soil are *Streptomyces* species, *Nocardia* species, etc.

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