J. Sci. Trans. Environ. Technov. 2009, 2(4): 191-201

Isolation and characterization of antagonistic actinomycetes from Coimbatore soils, Tamil Nadu, India

R. Vijayakumar*[1], V. Seethalakshmi[1], S. Anitha and R. Saravanamuthu[2]

[1]Department of Microbiology, Dr.GR. Damodaran College of Science (Autonomous), Coimbatore-641 014, Tamil Nadu, India
[2]Department of Botany, A.V.C. College (Autonomous), Mannampandal-609 305 Mayiladuthurai, Tamil Nadu, India.

Abstract

A total of 42 actinomycetes were isolated from terrestrial soils of Coimbatore district, Tamil Nadu, South India of which, twelve isolates belonged to the genus *Streptomyces,* ten isolates to *Nocardiopsis*, eight isolates to *Saccharopolyspora,* six isolates to *Nocardia,* four isolates to *Actinopolyspora* and two isolates to *Actinomadura.* Among them, two isolates showed strong antimicrobial activity against *Bacillus subtilis, Escherichia coli, Salmonella typhi, Enterococcus faecalis, Proteus mirabilis, Staphylococcus aureus, Shigella dysenteriae, Candida albicans* and *Cryptococcus neoformans*. Based on the morphological, cultural, biochemical and physiological characteristics the potent antagonistic actinomycetes were identified as *Nocardiopsis* sp. TE1 and *Nocardiopsis* sp. VPA1. Protocols for the optimization of the conditions suitable for the maximal production of antimicrobials are suggested.

Keywords : actinomycetes diversity, antimicrobial activity, disc diffusion method, *Nocardiopsis* spp.

INTRODUCTION

Microorganisms are miniature chemical factories, as they have the capacity to convert a variety of raw materials to a series of commercially valuable products. Actinomycetes are Gram-positive bacteria, which produce branching mycelium of two kinds *viz.*, substrate and aerial mycelium. Actinomycetes are widely distributed in natural and man-made environments, and play an important role in the degradation of organic matter. They are also well known as a rich source of antibiotics and bioactive molecules, and are of considerable importance in industry. Actinomycetes hold a prominent position for their diversity and ability to produce novel substances. The terrestrial soil actinomycetes have potential biotechnological applications, and are a new resource for structurally diverse secondary metabolites (Okami and Hotta, 1988). For the purpose of screening of novel bioactive molecules, several factors must be considered which include: choice of screening source, pretreatment, selective medium, culture conditions, and recognition of colonies on primary isolation plates (Nolan and Cross, 1988). Employing pretreatment of soil, drying and heating, stimulated the isolation of rare actinomycetes (Kim *et al.,* 1995). An alternative approach was to make the isolation procedure more selective by adding chemical such as phenol to the soil suspension (Hayakawa *et al.*, 1991).

Most of the isolates recovered on agar plates by conventional isolation techniques have been identified as *Streptomyces,* which are the dominant actinomycetes in soil (Lechevalier and Lechevalier, 1967; Nolan and Cross, 1988; Iwai and Takahashi, 1992). Rare actinomycetes (non-streptomycetes) have usually been regarded as strains of actinomycetes whose frequency is lower than that of streptomycete strains. As basic knowledge of the habitat, physiology and productivity of molecules of rare actinomycetes increased, ecologically significant properties of actinomycetes assumed significance which made the screening source to expand into uncommon environments.

Thus, it has been emphasized that new groups of microbes from unexplored habitats be pursued as sources of novel antibiotics and other small molecules that are therapeutic agents (Bull *et al.*, 2000). The objectives of the present study was to isolate and identify the high antimicrobial activity producing actinomycetes strains from soils, to characterize them, to determine their antimicrobial efficiency and to optimize the suitable conditions for their growth and antimicrobial compounds production.

MATERIAL AND METHODS

Sample collection and isolation of actinomycetes

The soil samples were collected from four different terrestrial locations of Coimbatore, Tamil nadu, South India at random in sterile polythene bags, brought to the laboratory and stored for further analysis. For isolation of actinomycetes starch casein agar medium (Kuster and Williams, 1964) (g/l: soluble starch 10; casein 0.3; potassium nitrate 0.2; sodium chloride 2; di-potassium hydrogen phosphate 2; magnesium sulphate 0.5; calcium carbonate 0.02; ferrous sulphate 0.07; agar 18; pH 7.2) (Difco chemicals) was used. Streptomycin 40 µl/ml and griseofulvin 50 µl/ml (Himedia) was used

*Corresponding Author
email: *rvijayakumar1979@yahoo.com*

to prevent bacterial and fungal contamination respectively. The collected soil samples were subjected to pretreatment by heating in oven at 100°C for 30 min. Soil samples were serially diluted up to 10^{-7} and 0.1 ml of aliquots were spreaded over the starch casein agar plates. After incubation at 28°C for 7-10 days, actinomycetes colonies were purified by streak plate technique on cultivation medium.

Characterization of actinomycetes

Morphology

The actinomycetes were characterized morphologically following the methods given in the International *Streptomyces* Project (ISP) (Shirling and Gottlieb, 1966). The characters including colony morphology of the strains such as the colour of aerial mycelium, reverse side colour, size of the colony and production of diffusible pigments were observed after incubation at 28°C for 7-10 days on starch casein agar medium. The microscopic morphology of strains such as formation of aerial and substrate mycelium and spore arrangement, which are highly characteristic and useful in the identification of actinomycetes, were observed by cover slip technique (Burkholder *et al*., 1954; Nolan and Cross, 1958) with light microscopy.

Screening of antimicrobial activity of actinomycetes

Preliminary screening

Antimicrobial activity of actinomycetes was subjected to primary screening by cross streak plate technique (Egorov, 1987). Straight-line inoculation of the actinomycetes isolates was made on modified nutrient agar medium (g/l: beef extract 3; yeast extract 3; $NaCl_2$ 2; peptone 5; glucose 20; agar 20; pH 7.0) (Difco chemicals) and incubated at 28° C for 4-5 days. After incubation the test organisms (The test bacterial and fungal cultures were obtained from Institute of Microbial Technology, Microbial Type Culture Collection, Chandigarh, India) namely, Bacillus subtilis MTCC 441, Salmonella typhi MTCC 531 and Cryptococcus neoformans MTCC 4425 were streaked at right angles of actinomycetes culture and incubated at 37°C. Based on the inhibition zone, measured after 24-48 hr the antagonistic actinomycetes were selected for further study.

Secondary screening

The selected antagonistic actinomycete isolates were inoculated into starch casein broth, and incubated at 28° C in a shaker (200-250 rpm) for seven to ten days. After incubation the culture broth was filtered through filter papers such as blotting paper, Whatmann no.1 and then through membrane filter, to get cell free extract. To the cell free culture filtrates equal volume of solvents namely, acetone, butanol, chloroform, distilled water and ethyl acetate were added, and centrifuged at 5000 rpm for 10 min to extract the compounds (Sambamurthy and Ellaiah, 1974). The obtained compounds of actinomycetes were tested for their antimicrobial activity by disc diffusion method against nine different test organisms namely, *Bacillus subtilis* MTCC 441, *Escherichia coli* MTCC 294, *Salmonella typhi* MTCC 531, *Enterococcus faecalis* MTCC 2729, *Proteus mirabilis* MTCC 425, *Staphylococcus aureus* MTCC 3103, and *Candida albicans* MTCC 183 and *Cryptococcus neoformans* MTCC 4425, *Shigella dysenteriae* which were obtained from PSG Hospitals, Coimbatore, India. They were swabbed over the Muller-Hinton agar and Sabouraud's dextrose plates for bacterial and fungal organisms, respectively. The sterile discs (Himedia) were dipped in extracts of actinomycetes, placed on pathogen-seeded plates and incubated at 37°C for 24-48 hr. After incubation the diameter of inhibition zone was measured to evaluate the antimicrobial activity of the isolates.

Colony morphology on different media

The selected two antagonistic actinomycetes were cultured on four different culture media namely nutrient agar, actinomycetes isolation agar, glycerol yeast extract agar and starch casein agar, and incubated for 7-10 ten days at 28°C. Colony morphology including colour of aerial mycelium, reverse side colour, size of the colony and production of diffusible pigments of the isolates was recorded as recommended by ISP (Shirling and Gottlieb, 1966).

Physiological and biochemical characterization

The physiological characteristics of the isolates such as, growth at different pH (3, 5, 7, 9 and 11), temperature (10, 20, 30, 40 and 50°C) and NaCl concentration (1, 2, 4, 8 and 16 g/l) were recorded in starch casein broth. The antibiotic sensitivity pattern of the isolates were also recorded for various antibiotics. The biochemical characterizations of the isolates were also studied by the procedures of ISP (Tresner *et al.,* 1968; Lyons and Pridham, 1973; Flowers and Williams, 1977; Shimizu *et al.,* 2000).

Chemotaxonomy Analysis of whole cell sugars

The whole cell sugars of the isolates were also analyzed by the methods of Lechevalier and Lechevalier (1970) and Myertons *et al.* (1988). One ml of 1N sulphuric acid was added to 50 mg freeze dried actinomycete in a vial and heated at 100°C for 2 hr. The mixture was centrifuged at 300 rpm for 10 min. The pH of the supernatant was adjusted to 5 with saturated barium hydroxide, followed by centrifugation at 6000 rpm for 10 min. The supernatant was filtered through Millipore filter, 5 µl of supernatant and 3 µl of standard 1% sugar solutions (galactose, glucose, arabinose and ribose) were spotted separately onto a TLC silica gel plate. The plate was developed in the solvent (acetonitrile: water- 92.5: 7.5 v/v) for 20 min and dried for 2 hr. Then it was sprayed

with aniline phthalate and heated at 100°C for 4 min on a hot plate.

Analysis of cell wall amino acids

One ml of 6 N HCl was added to 50 mg freeze-dried actinomycetes in a vial with a screw cap and heated overnight at 100°C. The mixture was completely dried at 45°C in vacuum. Two ml of distilled water was added to the vial and then dried off. This step was repeated several times to remove HCl. The final dried material was dissolved in 0.2 ml of water. Five ml of the sample and 1 ml of standard diaminopimelic acid solution were separately spotted onto a TLC silica gel plate. The plate was developed in the solvent (methanol: distilled water, 6N HCl, pyridine = 80:26:4:10) for 3 hr, air dried in a chemical hood for 2 hr and sprayed with 0.1% ninhydrin, followed by heating at 120°C for 10 min on a hot plate (Becker *et al*., 1965) .

Optimization of antimicrobial compound production

The optimization of the suitable conditions for the antimicrobial compounds production was carried out in different media (nutrient broth, starch casein broth, yeast extract broth and peptone broth), temperature (10, 20, 30, 40 and 50°C), pH (3, 5, 7, 9 and 11), and NaCl concentrations (1, 2, 4, 8 and 16 g/l) and incubation periods (4-10 days) (Tresner *et al*., 1968; Omura *et al*., 1973; Kokare *et al*., 2004a).

RESULTS AND DISCUSSION

The dominance of other bacteria and fungal contamination inhibited the colonization of actinomycetes on isolation medium. When antimicrobial agents such as streptomycin (40 µl/ml) and griseofulvin (50 µl/ml) were supplemented in to the isolation medium, the number of bacteria and fungi decreased. So, the isolation medium was supplemented with these antibiotics in succeeding experiments. In the present study, a total of 42 isolates of actinomycetes were isolated from four different soil-sampling stations of Coimbatore, and their morphological (size of the colony, colour of aerial mycelium, reverse side colour and production of diffusible pigment) and microscopical (formation of aerial and substrate mycelium and sporophore morphology) characteristics were studied. Based on these characteristics it was established that twelve isolates belonged to the genera *Streptomyces* (28%), ten isolates to *Nocardiopsis* (24%), eight isolates to *Saccahropolyspora* (19%), six isolates to *Nocardia* (14%), four isolates to *Actinopolyspora* (10%) and two isolates to *Actinomadura* (5%) (Table 1; fig. 1). Nineteen isolates produced the ash series colour of aerial mycelium, and sixteen isolates produced white series colour of aerial mycelium on starch casein agar medium, and seven isolates produced diffusible pigments on the same medium. Similar findings were previously reported by Kim *et al.* (1994) and Vijayakumar *et al*. (2007) also.

Among the 42 isolates, only 16 (38.1 %) showed antimicrobial activity, and among them 4 (9.5 %) isolates were antagonistic against Gram-positive bacteria and 5 (11.9 %) isolates against Gram-negative bacteria. Three (7.1 %) isolates were antagonistic against both Gram-positive and Gram-negative bacteria, and 3 (7.1 %) isolates were antagonistic against bacteria and fungi. In a similar study, Oskay *et al*. (2004) reported, 50 isolates of actinomycetes from 10 farming soil samples collected in Manisa Province, Turkey. Approximately 34 % (17) of the isolates produced broad and narrow spectrum antibiotics, 16 % (8) of the isolates produced antibacterial substance that were active against only Gram-positive bacteria 6 % (3) of the isolates were active against Gram-negative bacteria and 12 % (6) of the isolates were active against both Gram-positive and Gram-negative bacteria. On the basis of antimicrobial activity and the spectrum of activity broadness, two antagonistic isolates namely *Nocardiopsis* sp. TE1 and *Nocardiopsis* sp. APA1 were selected as experimental isolates using cross streak plate and disc diffusion method. Then antimicrobial efficacy of the isolates was tested by using 5 different solvents namely, acetone, butanol, chloroform, distilled water and ethyl acetate. Among them, ethyl acetate extract produced maximum antimicrobial activity against all the pathogens tested followed by butanol, acetone, distilled water and chloroform extracts. Ethyl acetate extract of isolate (TE1) showed maximum activity against *Shigella dysenteriae* (24 mm) followed by *Enterococcus faecalis* (23 mm), *Bacillus subtilis* (22 mm), *Stapylococcus aureus* and *Cryptococcus neoformans* (20 mm), *Escherichia coli* (18 mm), *Salmonella typhi* (17 mm), *Candida albicans* (15 mm), *Pseudomonas aeruginosa* (14 mm) and *Proteus mirabilis* (10 mm). Similarly, another antagonistic isolate APA1 also produced maximum antimicrobial compound against all the pathogen tested in the extracts of ethyl acetate solvent followed by, chloroform, acetone, butanol and distilled water. The maximum activity of ethyl acetate extract of isolate APA1 was against *C. neoformans* (26 mm) followed by, *C. albicans* (21 mm), *E. coli* (19 mm), *P. miraiblis* (18 mm), *B. subtilis, S. typhi* and *Ps. aeruginosa* (16 mm), *E. faecalis* and *S. dysenteriae* (15 mm) and *S. aureus* (13 mm) (Table 2).

Similarly, different solvents such as n-butanol (Sahin and Ugur, 2003; Augustine *et al*., 2005a) ethyl acetate (Bordoloi *et al*., 2001; Augustine *et al*., 2005a; Taeehowisan *et al*., 2005; Kathiresan *et al*., 2005; Illic *et al*., 2005; Saisivam and Kishan, 2006); methanol (Illic *et al*., 2005); n-hexane (Beran and Zima, 1993); petroleum ether, chloroform, benzene (Thangadurai *et al*., 2004) and xylene (Panchenkov and Korosteleva, 1971) were used for the extraction of antibiotics from actinomycetes.

The potent antibiotic producing strains TE1 and APA1 were characterized following the procedures recommended by International *Streptomyces* Project (ISP). The strain TE1 grew well on starch casein medium and produced gray colour aerial mycelium, yellow white on

Table 1. Cultural characteristics of actinomycetes isolated from the soils of Coimbatore, South India

Name of the isolates	Colour of aerial mycelium	Reverse side colour	Diffusible pigment	Size of the colony (mm)
Streptomyces sp. TS1	White	White	-	3.5
Streptomyces sp. TS2	Dark ash	Fluorescent	Green	3.0
Streptomyces sp. TS3	Ash	Dark pink	-	3.0
Saccharopolyspora sp. TS4	Light ash	Dark pink	-	2.0
Saccharopolyspora sp. TS5	Ash	Dull brown	-	1.0
Nocardiopsis sp. TS6	Light ash	Dark pink	Pink	6.5
Nocardiopsis sp. TS7	Dark ash	Brown	Fluorescent	4.0
Actinomadura sp. TS8	White	Light yellow	-	1.0
Streptomyces sp. TS9	Pale white	Light yellow	-	1.5
Streptomyces sp. TS10	Dull white	Yellow	-	3.0
Nocardia sp. TS11	Dull white	White	-	3.5
Nocardia sp. TS12	Green-ash	Black	-	7.5
Saccharopolyspora sp. TS13	White	Yellow	-	5.5
Saccharopolyspora sp. TS14	White	Yellow	-	7.0
Actinomadura sp. TS15	Dark ash	Dull black	-	2.5
Actinopolyspora sp. TS16	Ash	Light ash	-	3.0
Streptomyces sp. TS17	Ash	Dull black	-	3.5
Nocardiopsis sp. TS18	Dark ash	Light ash	-	2.5
Nocardiopsis sp. TE1	Gray	Yellow	-	3.0
Nocardiopsis sp. TE2	White	Dark ash	-	3.5
Nocardia sp. TE3	Ash	Dull ash	-	6.0
Streptomyces sp. TE4	Gray	Yellow	Brown	6.5
Nocardia sp. TE5	Gray	Brown	Brown	2.0
Streptomyces sp. TE6	Gray	Red-brown	Red-brown	6.0
Nocardiopsis sp. TE7	Ash	Cream	-	4.0
Saccharopolyspora sp. TE8	Ash	Cream	-	3.0
Nocardiopsis sp. TE9	Gray	Cream	-	2.0
Saccharopolyspora sp. TE10	Ash	Cream	-	4.0
Nocardiopsis sp. APA1	Dark gray	Yellow-white	-	6.0
Streptomyces sp. APA2	White	Cream	-	2.5
Streptomyces sp. APA3	White	Cream	-	9.0
Saccharopolyspora sp. APA4	White	Cream	-	3.0
Nocardia sp. APA5	Ash	Cream	-	4.0
Nocardiopsis sp. APA6	Ash	Cream	-	3.6
Actinopolyspora sp. APA7	Ash	Cream	-	2.5
Streptomyces sp. APA8	White	Cream	-	5.2
Actinopolyspora sp. APA9	White	Brown	Brown	3.0
Nocardia sp. APA10	Cream	Cream	-	2.0
Streptomyces sp. APA11	White	Cream	-	3.4
Saccharopolyspora sp. APA12	White	Cream	-	2.0
Actinopolyspora sp. APA13	White	Cream	-	5.0
Nocardiopsis sp. APA14	Cream	Cream	-	3.0

'-' indicates no pigment

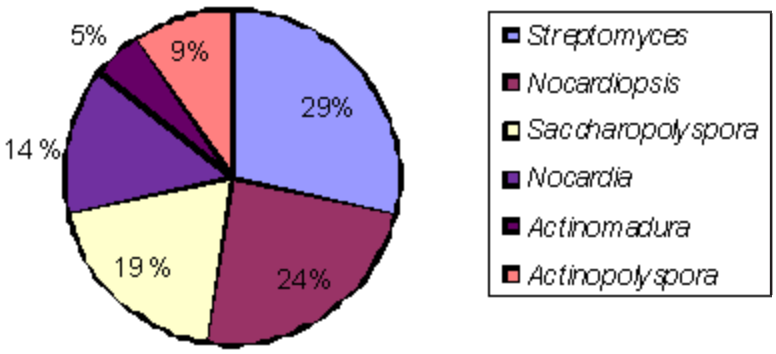

Figure 1. Percentage frequency of actinomycetes in soil samples from Coimbatore, South India.

Table 2. Antimicrobial efficacy of the antagonistic actinomycetes selected in the present study

Name of the test organisms	Zone of inhibition (mm)									
	Nocardiopsis sp. TE 1					*Nocardiopsis* sp. APA 1				
	1	2	3	4	5	1	2	3	4	5
Bacillus subtilis	11	12	22	11	10	13	10	16	10	11
Staphylococcus aureus	12	14	20	07	07	16	11	13	12	09
Enterococcus faecalis	11	18	23	06	04	12	13	15	10	11
Escherichia coli	15	17	18	07	06	12	15	19	16	13
Salmonella typhi	17	19	17	08	06	14	12	16	11	09
Proteus mirabilis	10	11	10	10	11	13	09	18	15	12
Shigella dysenteriae	15	20	24	08	09	14	10	15	14	09
Pseudomonas aeruginosa	12	11	14	07	06	12	09	16	12	04
Candida albicans	06	12	15	04	08	07	09	21	12	11
Cryptococcus neoformans	07	13	20	05	06	08	12	26	13	10

1-5 different solvent extracts (1 – acetone; 2 – butanol; 3 – ethyl acetate; 4 – chloroform; 5 – distilled water)

Table 3. Colony morphology of antagonistic actinomycetes selected for the present study on different media.

Characteristics	*Nocardiopsis* sp. TE 1				*Nocardiopsis* sp. APA 1			
	1	2	3	4	1	2	3	4
Growth	Moderate	Good	Good	Excellent	Moderate	Good	Good	Good
Aerial mycelium	Cream	Cream	White	Grey	White	Grey	White	Dark grey
Substrate mycelium	Grey	Ash	Yellow	Yellow	Yellow	Grey	Yellow	Yellow-white
Diffusible pigment	-	-	Brown	-	-	-	Yellowish brown	-
Melanin pigment	-	-	-	-	-	-	-	-

'-' indicates no pigment

1– Nutrient agar; 2–Actinomycetes isolation agar; 3-Glycerol yeast extract agar;
4- Starch casein agar

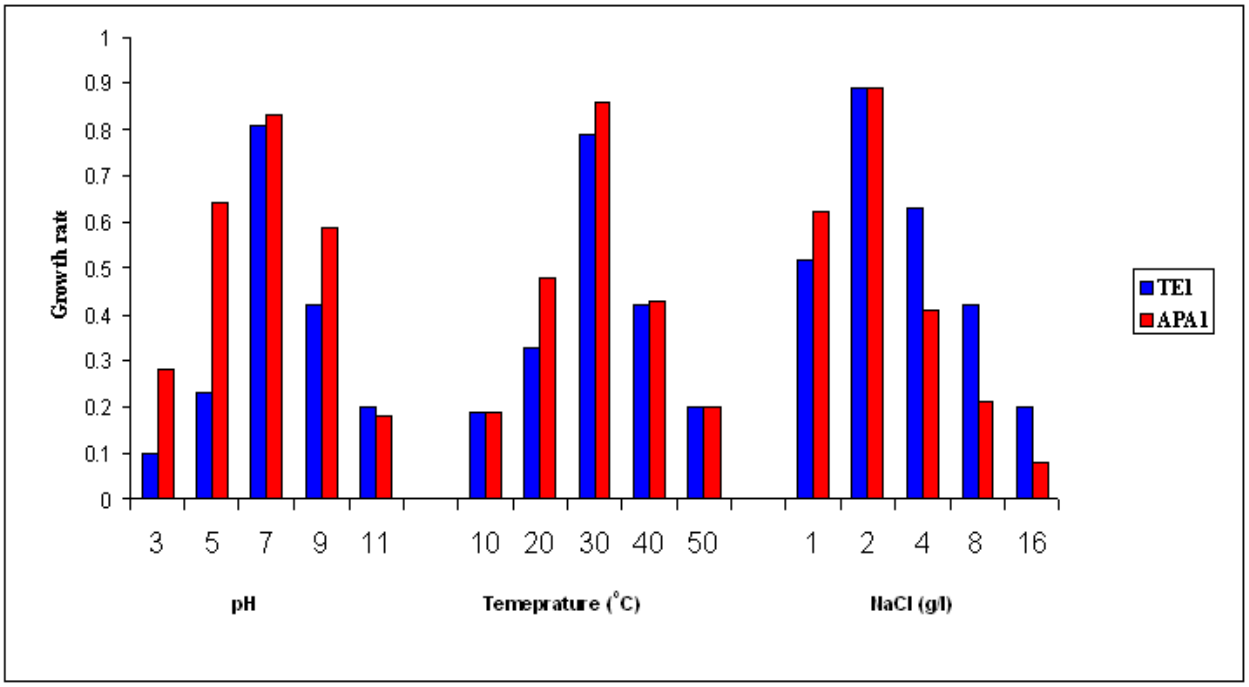

Figure 2. Effect of physico-chemical parameters on the growth of actinomycetes selected in the present study. TE1 = *Nocardiopsis* sp. TE1; APA1 = *Nocardiopsis* sp. APA1.

starch agar medium and brown colour diffusible pigment on glycerol yeast extract agar medium. The strain APA1 developed dark gray coloured of aerial mycelium, yellow white coloured of substrate mycelium on starch casein agar medium and yellow brown coloured diffusible pigment on glycerol yeast extract agar medium (Table 3). Both strains TE1 and APA1 were Gram positive and non-acid fast. The strain TE1 produced well developed and branched irregular vegetative hyphae. Aerial mycelium developed on agar media produced straight/ curved/irregularly spiral spore chains. Similarly, the strain APA1 developed vegetative and aerial mycelium and the aerial mycelium was totally sporulated (Bergey's Manual of Systematic Bacteriology). In the present study both the isolates contained meso-diaminopimelic acid in their cell wall and also there was no sugars found in the both isolates. Thus the present study is in confirmity with the findings of Meyer (1976) who reported that all the species of the genus *Nocardiopsis,* contain meso-diaminopimelic acid in their peptidoglycan and no characteristic sugars in whole cell hydrolysates (wall chemotype III).

Studies of on colony morphology of experimental isolates on four different culture media namely nutrient agar, actinomycetes isolation agar medium, glycerol agar and starch casein agar showed that both of the experimental isolates grew well on starch casein medium (Table 3) and also produced differently coloured aerial and substrate mycelia, besides diffusible pigments. Thus it is concluded on the basis of the present and previous studies that the nutrient composition of the medium greatly influenced the morphology of organisms (Gordan *et al.,* 1974; Gesheva and Gesheva, 1993). Several biochemical characteristics, utilization of carbon sources, antibiotic sensitivity patterns and physiological properties are very significant for the identification of actinomycetes at genus level but not helpful for the identification at species level. However, they can be used at least as markers by which an individual strain can be recognized (Balagurunathan and Subramanian, 2001). It is also evident that different physiological characteristics are influencing the growth rate of the actinomycetes (Hasegawa *et al.,* 1978; Kim *et al.,* 1999; Shimizu *et al.,* 2000). In the present study also the physiological characteristics of the strains TE1 and APA1 were assessed on the basis of the key of ISP (Shirling and Gottlieb, 1966) (Table 4). Both isolates grew well at temperature 30°C, pH 7.0 and NaCl concentration 2 g/l (Fig. 2). Biochemical and physiological characteristics and antimicrobial susceptibility patterns of the actinomycetes vary from organism to organism. Thus the present investigation concludes that the physiological characteristics of actinomycetes varied depending on the available nutrients in the medium and the physical conditions as it is evident that the growth of the actinomycetes was influenced by the environmental factors such as pH, temperature, inhibitory compounds and the availability of nutrients.

Production of antimicrobial metabolites is often influenced by the components of medium and cultural conditions,

such as, aeration, agitations, pH, temperature, salinity, time course, nitrogen and carbon sources and suitable media, which often vary from organism to organism. Optimization of antibiotic production necessitates the complete knowledge on optimal fermentation conditions for the strain used (Iwai and Omura, 1982; Pape and Rehm, 1985; Venkateswarlu *et al.*, 2000; El-Tayeb *et al.*, 2004; Augustine *et al.*, 2005a). So in the present study the required conditions have been optimized for the productions of antimicrobial compounds have been justifiably optimized, using the terrestrial actinomycetes. It has been reported that *Actinopolyspora* sp. (AH1) grown on tyrosine agar showed good antibacterial activity against *Staphylococcus aureus* compared to maltose yeast extract agar, starch casein agar and glucose asparagine agar. However, species AH1 grown on glycerol glycine agar and glycerol asparagine agar did not show any antimicrobial activity. Strain AH1 grown on glucose asparagine agar exhibited antimicrobial activity against *Trichoderma* sp. AH1 sp. grown on maltose yeast extract agar showed good antifungal activity against *Trichoderma* sp. compared to SCA and tyrosine agar (Kokare *et al.*, 2004b). In the present study, both isolates of actinomycetes showed good antimicrobial activity, when the culture was grown on starch casein medium against all the pathogens tested (Table 5). Thus the present study and previous findings support and conclude that the antimicrobial activity depends on the medium in which the culture is grown.

Temperature is an important factor that greatly influence the diversity and distribution of biota. It has profound effect in the physiology, morphology, sporulation, biochemistry and also antimicrobial metabolite production of organisms (Waksman and Schatz, 1945; Dawes and Ribbons, 1962). In the present study the actinomycetes was grown on starch casein medium and incubated at 10, 20, 30, 40 and 50°C, and found that 30°C is optimum for the antimicrobial activity. However, both isolates showed poor or no antimicrobial effect at 10°C and 50°C (Table 5). The culture filtrates of three antagonistic *Streptomyces* spp had its highest antifungal activity at 30°C against phytopathogenic fungi such as *Helminthosporium oryzae* and *Fusarium solani* (Kathiresan *et al.*, 2005). Similar findings have also been reported by Yoshida *et al.* (1972) and Augustine *et al.*, (2004). It is also clear from the present study that temperature plays an important role on the production of antimicrobial compounds.

The change in pH of the culture medium induces production of new products that adversely affect antibiotic production (Omura *et al.*, 1973). It is a well-known factor that each particular microorganism has

Table 4. Biochemical and physiological characteristics of antagonistic actinomycetes selected for the present study

Name of the tests	*Nocardiopsis* sp. TE 1	*Nocardiopsis* sp. APA 1
Biochemical characters		
Indole	-	+
Methyl red	-	+
Voges-Proskaur	-	+
Citrate utilization	+	+
Triple sugar iron	+	+
H_2S production	-	-
Starch hydrolysis	+	+
Gelatin hydrolysis	-	-
Casein hydrolysis	+	+
Urease	-	-
Catalase	+	+
Carbon source utilization		
Lactose	+	+
Galactose	-	-
Mannitol	+	+
Fructose	+	+
Sucrose	+	+
Dextrose	-	+
Starch	+	+
Cell wall sugar	Absent	Absent
Meso-di aminopimelic acid	Present	Present

- negative; + positive

Table 5.Optimization of antimicrobial activity of actinomycetes selected for the present study

Isolates name	Name of the pathogen	Zone of inhibition (mm)																	
		Temperature (° C)					pH					NaCl (g/l)					Media			
		10	20	30	40	50	3	5	7	9	11	1	2	4	8	16	NB	SCB	YEB	PB
Nocardiopsis sp. TE 1	*Bacillus subtilis*	4	6	10	10	4	-	6	10	7	-	-	12	9	4	-	7	13		9
	S. aureus	-	4	7	6	-	-	4	18	6	-	4	13	6	5	-	9	14	12	8
	Enterococcus faecalis	3	7	16	5	2	-	3	18	4	-	3	10	4	3	-	6	17	10	8
	Escherichia coli	2	5	14	3	-	-	7	18	3	-	-	18	3	2	-	7	18	12	10
	Salmonella typhi	3	5	13	6	2	-	9	17	8	-	-	15	7	4	2	5	19	15	12
	Proteus mirabilis	-	4	8	2	-	-	6	11	5	-	-	12	6	2	2	13	16	12	12
	Shigella dysenteriae	-	3	17	4	2	-	7	19	6	-	5	17	8	6	4	8	19	13	10
	P. aeruginosa	2	5	10	5	2	2	5	13	7	-	4	8	2	-	-	6	12	9	9
	Candida albicans	3	10	12	10	7	4	6	15	10	-	6	19	6	4	-	8	18	10	13
	Cryptococcus neoformans	4	13	18	13	5	3	5	18	9	-	8	20	7	3	-	9	17	12	11
Nocardiopsis sp. APA 1	*Bacillus subtilis*	5	13	18	12	7	4	8	17	12	9	9	14	12	9	7	10	14	12	10
	S. aureus	7	10	16	11	5	5	9	15	11	7	8	16	12	9	5	15	16	14	6
	Enterococcus faecalis	6	9	15	10	8	6	10	18	12	8	10	15	11	7	-	10	14	12	7
	Escherichia coli	4	8	18	12	9	7	11	17	10	7	12	18	14	7	5	12	16	13	9
	Salmonella typhi	-	7	16	9	5	6	12	18	13	10	11	16	13	6	-	14	18	11	6
	Proteus mirabilis	5	10	18	13	7	8	12	19	14	10	10	14	12	9	6	12	17	12	7
	Shigella dysenteriae	6	9	15	10	6	6	9	16	10	8	14	18	15	10	7	15	20	13	8
	P. aeruginosa	4	8	16	9	5	6	8	15	14	10	9	12	8	5	-	12	14	11	9
	Candida albicans	4	6	16	12	7	-	4	15	8	3	12	18	4	3	-	8	16	11	9
	Cryptococcus neoformans	5	8	20	14	6	3	8	18	10	4	16	21	10	4	-	10	20	14	7

NB – nutrient broth; SCB – starch casein broth; YEB – yeast extract broth; PB – peptone broth
'-' indicates no inhibition

an optimum, minimum and maximum pH. Actinomycetes are marked by relative intolerance of acidity usually prefering neutral or slightly alkaline soil for the growth and antagonistic activity (Waksman and Curtis, 1918; Flaig and Kutzner, 1960). In the present study, the actinomycetes were grown on starch casein broth at different pH such as 3, 5, 7, 9 and 11 for 7 days, and pH 7 was found to be optimum for maximum antibiotic production for both isolates. All the isolates showed minimal antimicrobial effect up to pH 11 (Table 5). Thus it is concluded that the antimicrobial metabolite production is greatly influenced by pH.

The ability of microorganisms to tolerate high concentrations of NaCl in media is well known. Likewise certain halophilic bacteria are known to develop in pickling brines containing 20 to 30% NaCl (Tresner *et al.,* 1968). The strain *Actinoployspora* sp AH1 showed good growth in the medium containing 10 to 15% (w/v) NaCl, and hence the strain has been considered as a halophilic (Kokare *et al.,* 2004b). Among the various salinity levels tested, 17.5 ppt showed the highest antifungal activity of marine actinomycetes against phytopathogenic fungi, followed by 5 ppt. However 30 ppt showed the least activity (Kathiresan *et al.,* 2005). In the present study six different salinity levels (1, 2, 4, 8 and 16 g/l of NaCl) were tested on the antimicrobial compound production by actinomycetes, and found that the optimal NaCl concentration condition for the antimicrobial compound production was found to be at 2 g/l. All the isolates showed minimal or poor antimicrobial effect up to 1-16 g/l of NaCl concentration (Table 5). Thus it is obvious from the present findings that the salts play a vital role on the production of antimicrobial compounds in the actinomycetes.

The antagonistic actinomycetes were incubated in the broth culture for its production of antibiotics at different incubation periods such as 4, 5, 6, 7, 8, 9 and 10 days. It was found that the inhibition zone increased with the increase in the incubation period in the production medium and the maximum inhibition was found with strain TE1, which was incubated for more than 7 days. However, strain APA1 produced maximum inhibition zone after 9th day onwards (Table 6). Similar type of

Table 6. Effect of incubation period on the antimicrobial activity of actinomycetes selected for the present study

Isolates name	Name of the pathogen	Zone of inhibition (mm) Incubation period (days)						
		4	5	6	7	8	9	10
Nocardiopsis sp. TE1	*Bacillus subtilis*	-	5	10	13	13	12	8
	Staphylococcus aureus	4	6	12	14	14	13	8
	Enterococcus faecalis	3	4	10	17	16	13	10
	Escherichia coli	-	3	12	18	17	16	12
	Salmonella typhi	-	5	14	19	18	17	12
	Proteus mirabilis	-	4	12	16	16	14	10
	Shigella dysenteriae	5	8	13	19	18	16	11
	P. aeruginosa	4	6	9	12	13	12	11
	Candida albicans	6	6	10	18	18	17	11
	Cryptococcus neoformans	5	9	12	17	18	16	10
Nocardiopsis sp. APA1	*Bacillus subtilis*	9	10	12	12	13	14	9
	Staphylococcus aureus	8	9	14	13	15	17	13
	Enterococcus faecalis	6	9	11	12	12	14	14
	Escherichia coli	8	10	13	13	13	17	10
	Salmonella typhi	7	8	11	14	14	18	13
	Proteus mirabilis	6	9	12	13	16	19	15
	Shigella dysenteriae	4	10	13	13	15	20	14
	P. aeruginosa	4	8	11	14	15	18	10
	Candida albicans	4	8	11	13	14	20	13
	Cryptococcus neoformans	6	10	14	1	20	21	12

'-' indicated no inhibition

observation i.e., a declining trend in the development of inhibition zone due to increase in incubation period (at 168 hrs) was reported by De *et al.,* (1992), Kathiresan *et al.,* (2005) and Augustine *et al.* (2005b). Thus the present study reports the required optimum number of days as well for the production of antimicrobial compounds and the development of inhibition zone.

Thus, the present investigation envisages that the cultural conditions having starch casein medium, pH 7.0, temperature 20-40°C, NaCl2 g/l and 7-9 days of incubation period are the most suitable conditions for the production of maximal antimicrobial compounds by three isolates of *Nocardiopsis* spp. They have every possibility to be used against human pathogenic organisms.

REFERENCES

Augustine, S.K., Bhavsar, S.P. and Kapadnis, B.P. 2005a. Production of a growth dependent metabolite active against dermatophytes by *Streptomyces rochei* AK 39. *Indian J. Med. Res.,* 121: 164-170.

Augustine, S.K., Bhavsar, S.P. and Kapadnis, B.P. 2005b. A non-polyene antifungal antibiotic from *Streptomyces albidoflavus* (PU23). *J. Biosci.,* 30: 201-211.

Augustine, S.K., Bhavsar, S.P., Baserisallhi and Kapadnis, B.P. 2004. Isolation, characterization and optimization of antifungal activity of actinomycetes of soil origin. *Indian J. Exp. Biol.,* 42: 928-932.

Balagurunathan, R. and Subramanian, A. 2001. Antagonistic *Streptomyces* from marine sediments. *Adv. Biosci,* 20: 71-76.

Becker, B., Lechevalier, M.P. and Lechevalier, H.A. 1965. Chemical composition of cell wall preparations from strains of various form genera of aerobic actinomycetes. *Appl. Microbiol.*, 13: 236-243.

Beran, M. and Zima, J. 1993. Determination of monensins A and B in the fermentation broth of *Streptomyces cinnamonensis* by high performance liquid chromatography. *Chromatographia*, 35: 206-208.

Bordoloi, G.N., Kumarim, B., Guha, A., Bordoloi, M., Yadav, R.N., Roy, M.K. and Bora, T.C. 2001. Isolation and structure elucidation of a new antifungal and antibacterial antibiotic produced by *Streptomyces* sp. 201. *Biosci. Biotechnol. Biochem.*, 65: 1856-1858.

Bull, A.T., Ward, A.C. and Goodfellow, M. 2000. Search and discovery strategies for biotechnology: the Paradigin shift. *Microbiol. Molecular. Biol. Rev.,* 64: 573-606.

Burkholder, P.R., Sun, S.H., Ehrlich, J. and Anderson, L. 1954. Criteria of speciation in the genus *Streptomyces. Ann. New York Acad. Sci.,* 60: 102-123.

Chakraborthy, D., Mondal, B., Pal, S.C. and Sen, S.K. 1995. Characterization and identification of broad-spectrum antibiotic producing *Streptomyces hygroscopicus* D1.5. *Hind. Antibiot. Bull.,* 37: 37-43.

Dawes, E.A. and Ribbons, Q.E. 1962. The endogenous metabolism of microorganisms. *Ann. Rev. Microbiol,* 12: 241-264.

De, K., Gupta, M.K. and Pande, A. 1992. Effect of physiological and physical factors on antifungal activities of *Streptomyces* sp. *J. Ind. Bot. Soc.,* 72: 157-159.

Egorov, N.S., 1985. Antibiotics, A scientific approach. Mir Publishers, Moscow.

El-Tayeb, O.M., Hussein, M.M.M., Salama, A.A. and El-Sedawy, H.F. 2004. Optimization of industrial production of rifamycin B by *Amycolatopsis mediterranei.* IV. Production in the fermenter. *African J. Biotecnol.*, 3: 432-440.

Flaig, W. and Kutzner, H.J. 1960. Beitrag zur oekologie der gattung *Streptomyces* Waksman et Henrici. *Arch. Mikrobiol.*, 35: 207-208.

Flowers, T. H. and Williams, S.T. 1977. The influence of pH on the growth rate and variability of neutrophilic and acidophilic streptomycetes. *Microbes,* 18: 223-228.

Gesheva, V. and Gesheva, R. 1993. Structure of the *Streptomyces hygroscopicus* 111-81 population and characteristics of its variants. *Actinomycetes,* 4: 65-72.

Gordon, R. E., Barnett, D. A., Handerhan, J. E. and Pang, C. H. N. 1974. *Nocardia coeliaca*, *Nocardia autotrophica*, and the nocardin strain. *Int. J. Syst. Bacteriol.,* 24, 54-63.

Hasegawa, T., Yamano, T. and Yoneda, M. 1978. *Sterptomyces inusitatus* sp. nov. *Int. J. Syst. Bacteriol.,* 28: 407-410.

Hayakawa, M., Sadakata, T., Kajiura, T. and Nonomura, H. 1991. New methods for the highly selective isolation of *Micromonospora* and *Microbispora* from soil. *J. Ferment. Bioeng.,* 72: 320-326.

Ilic, S.B., Kontantinovic, S.S. and Todorovic, Z.B. 2005. UV/VIS analysis and antimicrobial activity of *Streptomyces* isolates. *Facta Universitatis Med. Biol.,* 12: 44-46.

Iwai, Y. and Omura, S. 1982. Culture conditions for screening of new antibiotics. *J. Antibiot.,* 35: 123.

Iwai, Y. and Takahashi, Y. 1992. Selection of microbial sources of bioactive compounds. *In*: The search for bioactive compounds from microorganisms S. Omura, (ed.) New York, P. 281-302.

Kathiresan, K., Balagurunathan, R. and Selvam, M.M. 2005. Fungicidal activity of marine actinomycetes against phytopathogenic fungi. *Indian J. Biotechnol.*, 4: 271-276.

Kim, C.J., Lee, K.H., Shimazu, A. and Yoo, I.D. 1994. Re-isolation frequency of soil actinomycetes on multiple isolation media (Korean). *Kor. J. Appl. Microbial. Biotechnol.,* 22: 329-331.

Kim, C.J., Lee, K.H., Shimazu, A., Kwon, O.S. and Park, D.J. 1995. Isolation of rare actinomycetes on various types of soil. *K. J. Appl. Microbiol., Biotechnol.,* 23: 36-42.

Kim, B.S., Sahin, N., Minnikin, D.E., Screwinska, J.Z., Mordarski, M. and Goodfellow, M. 1999. Classification of thermophilic streptomycetes, including the description of *Streptomyces thermoalcalitolerans* sp. nov. *Int. J. Syst. Bacteriol.,* 49: 7-17.

Kokare, C. R., Mahadik, K.R., Kadam, S.S. and Chopade, B.A. 2004a. Isolation of bioactive marine actinomycetes from marine sediments isolated from Goa and Maharashtra coastlines (West Coast of India). *Indian J. Mar. Sci.,* 33: 248-256.

Kokare, C.R., Mahadik, K.R., Kadam, S.S. and Chopade, B.A. 2004b. Isolation, characterization and antimicrobial activity of marine halophilic *Actinopolyspora* species AH1 from West Coast of India. *Curr. Sci.,* 86: 593-597.

Kuster, E. and Williams, S.T. 1964. Production of hydrogen sulphide by *Streptomyces* and methods for its detection. *Appl. Microbiol.*, 12: 46-52.

Lechevalier, H.A. and Lechevalier, M.P. 1967. Biology of actinomycetes. *Ann. Rev. Microbiol.*, 21: 71-100.

Lechevalier, M.P. and Lechevalier, H. 1970. Chemical composition as a criterion in the classification of aerobic actinomycetes. *Int. J. Syst. Bacteriol.*, 20: 435-443.

Lyons, A.J. and Pridham, T.G. 1973. Standard antimicrobial spectra as aids in characterization and identification of actinomycetales. *Develop. Indus. Microbiol.,* 14: 205-211.

Meyer, J. 1976. *Nocardiopsis*, a new genus of the order actinomycetales. *Int. J. Syst. Bacteriol.,* 26: 487-493.

Myertons, J.L., Labeda, D.P., Cote, G.L. and Lechevalier, M.P. 1988. A thin layer chromatographic method for whole cell sugar analysis of *Micromonospora* species. *Actinomycetes*, 20: 182-192.

Nolan, R. and Cross, T. 1988. Isolation and screening of actinomycetes. *In*: Actinomycetes in Biotechnology M. Goodfellow, S.T. Williams & M. Mordarski (eds.). Academic Press, London, P. 1-32.

Okami, Y. and Hotta, K. 1988. Search and discovery of new antibiotics. *In*: Actinomycetes in Biotechnology, Goodfellow, M., Williams, S.T. and Mordarski, M.(eds) Academic Press, London, P. 33.

Omura, S., Nakagawa, A., Yamada, H., Hata, T., Furusaki, A. and Watanabe, T.C. 1973. Structure and biochemical properties of kanamycin A, B, C and D. *Chem. Pharm. Bull.,* 21: 931-934.

Oskay, M., Tamer, A.U. and Azeri, C. 2004. Antibacterrial activity of some actinomycetes isolated from farming soils of Turky. *African J. Biotechnol.,* 3: 441-446.

Panchenkov, G. M. and Korosteleva, A. I. 1971. Extraction of p-xylene from mixtures of xylenes and ethyl benzene by means of liquid-phase clathration. *Chem. Technol. Fuels Oils,* 7: 258-260.

Pape, H. and Rehm, H.J. 1985. Microbial products. *In: Biotechnology* VCH[Rehm, H.J. and Reed, G.(eds)] , P. 436-457.

Pridham, T.G. and Tresner, H.D. 1974. Streptomycetaceae. *In: Bergey's Manual of Determinative Bacteriology* (8th edn), The Williams and Wilkins Co,. Baltimore, U.S.A., P. 747.

Sahin, N. and Ugur, A. 2003. Investigation of the antimicrobial activity of some isolates. *Turk. J. Biol.,* 27: 79-84.

Saisivam, S. and Kisan, V. 2006. Taxonomy, fermentation and biological activities of a new strain of *Sreptomyces luridus* from Indian soil. *Ind. J. Microbiol.,* 46: 153-160.

Sambamurthy, K. and Ellaiah, P. 1974. A new streptomycete producing Neomycin (B and C) complex *S. marinensis* (Part–I). *Hind. Antibiot. Bull.*, 17: 24-28.

Shimizu, M., Nakagawa, Y., Sato, Y., Furumai, T., Igarashi, Y., Onaka, H., Yoshida, R. and Kunch, H. 2000. Studies on endophytic actinomycetes (1) *Streptomyces* sp. Isolated from *Rhododendron* and its antimicrobial activity. *J. Gen. Pl. Pathol.,* 66: 360-366.

Shirling, E. B. and Gottlieb, D. 1966. Methods for characterization of *Streptomyces* species. *Int. J. Syst. Bacteriol.,* 16: 312-340.

Taechowisan, T., Lu, C., Shen, Y. and Lumyong, S. 2005. Secondary metabolites from endophytic *Streptomyces aureofaciens* eMUAc130 and their antifungal activity. *Microbiology,* 151: 1651-1695.

Thangadurai, D., Murthy, K.S.R., Prasad, P.J.N. and Pullaiah, T. 2004. Antimicrobial screening of *Decalepis hamiltonii* Wight and Arn. (Asclepiadaceae) root extracts against food-related microorganisms. *J. Food Safety,* 24: 239–245.

Tresner, H. D., Hayes, J. A. and Bakus, E. J. 1968. Differential tolerance of streptomycetes to sodium chloride as a taxonomic aid. *Appl. Microbiol.,* 16: 1134- 1136.

Venkateswarlu, G., Murali, P.S., Sharma, G. and Venkateswarlu, R. 2000. Improvement of rifamycin B production using mutant strains of *Amycolatopsis mediterranei. Bioprocess Eng.,* 23: 315-318.

Vijayakumar, R-., Muthukumar, C., Thajuddin, N., Panneerselvam, A. and Saravanamuthu, R. 2007. Studies on the diversity of actinomycetes in the Palk Strait region of Bay of Bengal, India, *Actinomycetologica,* 21: 59-65.

Waksman, S.A. and Curtis, R.E. 1918. The actinomycetes. *Soil Sci.,* 1: 99-134.

Waksman, S.A. and Sohatz, A. 1945. A strain specificity and production of antibiotic substances. *Proc. Natl. Acad. Sci.,* 4: 74-79.

Yoshida, N., Tani, Y. and Ogate, T.C. 1972. Cryomycin. A new polypeptide antibiotic produced only at low temperature. *J. Antibiot.,* 25: 653.