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Isolation of Actinomycetes from different soils for analysing the antagonistic activity against pathogens

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Abstract

Actinomycetes are the most widely distributed groups of microorganisms in nature. They are attractive, bodacious and charming filamentous. They make up in many cases, especially under dry alkaline conditions, a large part of the microbial population of the soil based on several studies among bacteria, the actinomycetes are noteworthy as antibiotic producers, making three quarters of all known products. Investigations can possibly reveal actinomycetes species that produce novel antibiotics. It is anticipated that the isolation, characterization and the study on actinomycetes can be useful in the discovery of antibiotics and novel species of actinomycetes. This present study reveals that isolation of actinomycetes from diverse soils samples and its antagonistic activityin contradiction of both the gram positive and negative microorganisms.

Keywords: Actinomycetes, Antibiotics, Antagonistic activity, Microorganisms.

Introduction

Actinomycetes are gram positive, free living, saprophytic bacteria, widely distributed in soil, water, and colonizing plants. Their population has been identified as one of the major group of soil population [1]. The actinomycetes having high G + C content in their DNA. The name Actinomycetes was derived from Greek aktis (a ray) and mykes (funges) and given to these organisms form initial observation of their morphology. Actinomycetes were originally considered to be an intermediate group between bacteria and fungi but now are recognized as prokaryotic organisms. The majority of actinomycetes are free living, saprophytic bacteria found widely distributed in soil, water and colonizing plants. Actinomycetes population has been identified as one of the major group of soil population. Screening of micro-organisms for the production of novel antibiotics has been intensively pursued for many years by scientists. Antibiotics has been used in many fields including agriculture, veterinary and pharmaceutical industry [2]. Actinomycetes have the capability to synthesize many different biologically active. Secondary metabolites such as antibiotics, herbicides, pesticides, anti-parasitic and enzymes like. Cellulase and xylanase used in waste treatment. The soil actinomycetes produce a typical earthy odour caused by the production of geosmin[3].An antibiotic may be defined as a chemical substance produced by microbes. Which has the capacity of inhibiting the growth and even destroying other microbes and are also active in dilute solutions. A new era in the history of medical science was begun in 1927, when Alexander Fleming invented penicillin. The first purified antibiotic to be obtained from an actinomycetes was actonomycin.

Actinomycetes have characteristic biological aspects such as mycelia forms of growth that accumulates in sporulation and the ability to produce an array of secondary metabolites many of which have antibacterial or antifungal properties. Some *Streptomyces sp*produce antibiotics at the same time of cell sporulation [4]. In general, *Streptomycessp* grow best in media containing carbon and nitrogen sources including chitin, starch, glycerol, arginin, asparagine, casein and nitrate [5]. The streptomyces are especially prolific and can produce many types of antibiotics and other class of biologically active secondary metabolites. A large number of pathogenic bacteria and fungi have become resistant to antibiotics in common use. These antibacterial and antifungal resistance are presently an urgent focus of research and new antibiotics are necessary to against these pathogens [5]. Actinomycetes filamentous soil bacteria, are widely recognized as important micro-organisms because of their ability to produce many kinds of secondary metabolites with diverse chemical structures and biological activities such as antibiotics [6]. The discovery of new antibiotics reached a peak in the 1970s then decline in the late 1980s and 1990s due to a decrease in screening efforts rather than an exhaustion of compounds. This studies investigated on the isolation of soil actinomycetes from poorly explored areas having either antibacterial activities against Gram-positive and Gram-negative bacteria or antifungal activities against yeast and mold.

Materials and Methods

Collection of Soil

Soil samples were collected from various locations of Sivanthi Aditanar College Pillayarpuram, Peyankuzhi in Kanyakumari District, during December 2008 to January 2009. The collected soil samples were transferred to sterile polythene bags for the isolation of actinomycetes.

Pretreatment of the Soil Sample

The soil sample were pretreated in order to reduce the proportion of other micro-organisms than actinomycetes. The soil samples were dried for about 50-60^{0}c for 5-10 minutes.

Isolation of Actinomycetes

Each soil sample (1g) was suspended in 100ml sterile distilled water, then homogenized by vortex mixing, mixtures were allowed to settle and serial tenfold dilutions up to 10^{-4} were prepared by using sterile distilled water. Isolation was carried out on Actinomycetes Isolation agar plates by spreading. The plates were incubated at 25^{0}c for 10 days. After attaining a powdery growth, the colonies were observed. Based on the color and Morphological differences, colonies were selected and restreaked in aActinomyces Isolation Agar medium to get axenic culture. The spore stocks were prepared from the culture grown on Actinomyces isolation medium and stored in refrigerator for further antagonistic studies.

Screening of Actinomycetes for Antimicrobial Activity

The screening method consists of two steps: primary screening and secondary screening.

Primary Screening

The antagonistic activity was tested by following cross streak assay method. Single streak (4-6 mm diameter) of the actinomycetes isolated were streaked on the surface of the Modified Nutrient Agar plates and incubated at room temperature (28 ± 2^{0}C) for 5 – 7 days. On obtaining a ribbon like growth, selected bacteria were streaked at perpendicular to the original streak of actinomycetes and incubated at 28±2^{0}C for 24 hours. The inhibition of growth of each organisms was noted. A control plate was also maintained without inoculating actinomycetes to assess the normal growth of bacteria and fungi. All the actinomycetes isolated from the different soil samples were assayed for the antagonistic activity and those isolates which showed prominent and broad spectrum activity were taken for further studies.

Secondary Screening

Antibiotic Production

Four actinomycetes strains SAC1, SAC2, PK1 and PK2 which showed higher promising antagonistic activity were selected for the mass cultivation and for the extraction of required quantity of antimicrobial metabolites. A loopful inoculum of selected actinomycete strains were further inoculated with 250 ml conical flask containing 100 ml of glucose Soyabean medium and kept at 28^{0}C for 72 h with continuous shaking. Twenty milliter of each broth culture was then transformed to 200 ml of Soyabean mean broth in 500 ml conical flask and incubated for 7 days under continuous shaking.

Isolation of Antibacterial Metabolites

Antibacterial compound was recovered from the filtrate by solvent extraction method following the process described. Ethyl acetate was added to the filtrate in the ratio of 1:1 and shaken vigorously for 1 hour for complete extraction. The ethyl acetate phase that contains antibiotic was separated from the aqueous phase. It was evaporated to dryness in water bath at 80^0-90^0c and the residue obtained was weighted. Thus obtained compound was used to determine antimicrobial activity, minimum inhibitory concentration and to perform bio autography.

Determination of the Antimicrobial Activity

The antimicrobial activity was determined by agar well method. The partially purified extract obtained by the evaporation of the ethyl acetate extract was dissolved in 1ml 0.2ml phosphate buffer. Then 100ml of it was loaded into well be red and test organism. Swabbed Muller Hinton agar plates. The plates were incubated at 37^0c for 18-24 hrs, and examined. The diameter of the zones of complete inhibition was measured to the nearest whole millimeter.

Test Organisms

The antibiacterial activities were tested for *in vitro* against human pathogenic bacteria that included *Staphylococcus aureus*, *Psudomonas* spp ,*Klebsiella E.coli*, *Bacillus* spp *and Streptococococcus* species were obtained from the Vivek Lab, Nagercoil and maintained on Nutrient agar at 4^0C.

Agar Diffusion Method

Cotton swabs with bacterial suspension were inoculated on Muller Hinton plates were spread evenly over the surface of the Muller Hinton Agar plates. Wells of 5 mm diameter were aseptically cut and filled with the diluted extracts. The plates were incubated at 37^0 C for 24-48 hours.

Effect of Different Nitrogen Sources on Antibiotic Production by Sac1, Sac2, Pk1, and Pk2

In the selected culture broth the nitrogen source was replaced by other nitrogen sources such as peptone and potassium nitrate. The inoculated media were incubated at room temperature for 5 days. The culture filtrates were than assayed.

Effect of Different Carbon Sources on Antibiotic Production by Sac1, Sac2, Pk1, and Pk2

In the selected culture broth the carbon source was replaced by other carbon sources such as sucrose, fructose and manitol. The inoculated media were incubated, at room temperature for 5 days. The culture filtrates were than assayed.

Effect of pH on Antibiotic Production by the Extracts of Sac1, Sac2, Pk1 and Pk2

To study the effect of pH on antibiotic production, the pH values of the selected medium were adjusted before sterilization by adding Hydrochloric acid. The values were ranging from 5, 7 and 9. It was incubated for 5 days at room temperature and assayed.

Results

In order to isolate Actinomycetes from soil, the various soil samples were serially diluted and plated on KenknightMunaier's Medium, Actinomycetes isolation Agar, glucose soyabean Agar. Among the different medium tested, Actinomycetes isolation Agar showed confluent growth of powdery colonies. (Table.1) The growth was obtained before 3-4 days than other medium.

Table: 1 Cultural Characteristics of Actinomycetes Strains Sac1, Sac2, Pk1and Pk2on Actinomycetes Isolation Agar, Glucose Soya beans Agar, And Kenknight and Munaier's Medium.

Strain	Actinomycetes Isolation Agar		Glucose Soya bean Agar		Kenknight and munaier's medium	
	Growth	Aerial colour of mycelium	Growth	Aerial Mycelium	Growth	Aerial Mycelium
SAC1	++	Gray	+	Grayish White	+	Grayish White
SAC2	++	White	+	White	+	White
PK1	++	White	+	White	+	Light Yellow
PK2	++	Gray	+	Grayish white	+	Gray

++: very good, +: good

Activity Shown By Actinomycetes in Primary Screening

Out of 26 isolates. Only 10 isolates. Showed the activity against test organisms out of 16 isolates 12 were active against *Bacillus spp.* 6 were active against *E coli.* 6 against *Staphylococcus aureus.* 4 against *Pseudomonas spp*. Among the 15 isolated colonies 4 showed good inhibitory effect and they were selected for further study (Fig 1). Table 2,3,4,5 revealed the effect of incubation period on antibiotic biosynthesis by selected four actinomycetes. The antibacterial activity was detected starting from the second day and reach the maximum activity at sixth day of incubation.The pH of the medium is an important for antibiotic production. Table 6, 7, 8 show antibiotic production by the isolated actinomycetes strains at 72 hours fermented broth on comparing pH5, pH7 and pH9. pH7 show maximum antibiotic effect (34mm/50ml) by Staphylococcus aureus, 31mm in *Bacillus sp* 23 mm in *E.coli* and 19mm in *Pseudomonas sp.*

Table: 2 Effect of incubation period on antibiotic biosynthesis by SAC1.

Strain	Incubation period (days)	Assay Organisms			
		Staphylococcus aureus	***Bacillus* sp.**	***E.coli***	***Pseudomonas* spp**
	Zone of inhibition (mm)				
SAC1	2	R	R	R	R
	3	5	R	6	R
	4	12	3	10	R
	5	30	14	20	R
	6	36	23	21	R
	7	40	26	22	R

Table: 3 Effect of incubation period on antibiotic biosynthesis by SAC2.

Strain	Incubation period (days)	Assay Organisms			
		Staphylococcus aureus	***Bacillus* sp.**	***E.coli***	***Pseudomonas* spp**
	Zone of inhibition (mm)				
SAC2	2	-	-	-	-
	3	12	12	11	-
	4	20	19	17	-
	5	24	21	21	-
	6	27	20	23	-
	7	19	17	19	-

Table: 4 Effect of incubation period on antibiotic biosynthesis by PK1.

Strain	Incubation period (days)	Assay Organisms			
		Staphylococcus aureus	***Bacillus* sp.**	***E.coli***	***Pseudomonas* spp**
	Zone of inhibition (mm)				
Pk1	2	R	R	R	R
	3	7	R	4	R
	4	16	3	8	R
	5	20	8	12	2
	6	23	11	17	4
	7	31	19	23	9

Table: 5 Effect of incubation period on antibiotic biosynthesis by Pk2

Strain	Incubation period (days)	Assay Organisms			
		Staphylococcus aureus	***Bacillus* sp.**	***E.coli***	***Pseudomonas* spp**
		Zone of inhibition (mm)			
Pk2	2	-	-	-	-
	3	14	15	13	-
	4	22	21	19	-
	5	26	23	23	13
	6	29	23	25	16
	7	29	21	21	16

Table: 6 Effect of pH5 on antibiotic production by the actinomycetes SAC1, SAC2, PK1 and PK2

	Assay of organism			
Strain	***Staphylococcus aureus***	***Bacillus* sp.**	***E.coli***	***Pseudomonas* spp**
	Zone of inhibition (mm)			
SAC1	17	24	17	-
SAC2	19	25	19	-
PK1	18	23	19	
PK2	16	23	19	-

Table: 7 Effect of pH7 on antibiotic production by the actinomycetes SAC1, SAC2, PK1 and PK2

	Assay of organism			
Strain	***Staphylococcus aureus***	***Bacillus* sp.**	***E.coli***	***Pseudomonas* spp**
	Zone of inhibition (mm)			
SAC1	34	31	23	19
SAC2	30	30	21	18
PK1	31	29	20	16
PK2	29	28	18	12

Table: 8 Effect of pH 9 on antibiotic production by the actinomycetes SAC1, SAC2, PK1 and PK2 at 72 hrs fermented broth.

	Assay of organism			
Strain	***Staphylococcus aureus***	***Bacillus* sp.**	***E.coli***	***Pseudomonas* spp**
	Zone of inhibition (mm)			
SAC1	29	26	21	20
SAC2	26	24	20	18
PK1	22	18	16	13
PK2	24	19	14	15

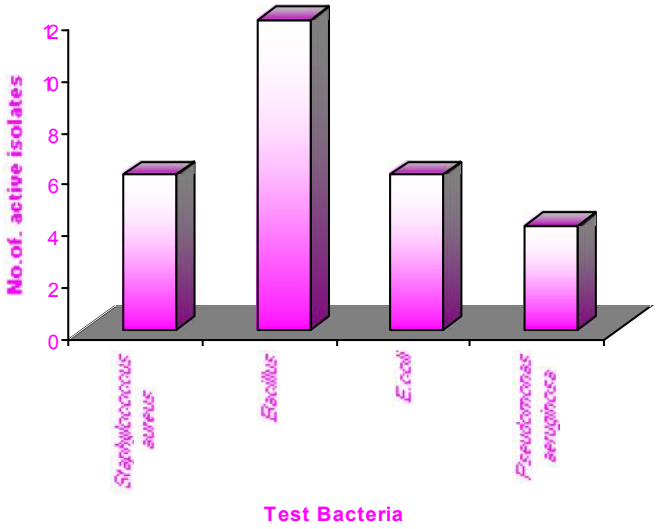

Fig.1 Activity shown by Actinomycetes in primary screening

When compared to PH7, PH5 and pH9 shows minimum activity. Table 9, 10, 11 showed that the isolates SAC1, SAC2, PK1, PK2 was able to grow in all tested carbon sources. However maximum antibacterial biosynthesis was obtained in medium supplemented with sucrose followed by fructose and minimum antibacterial biosynthesis was obtained in medium supplemented with mannitol. Influence of nitrogen sources utilization without casein was shown in Table 12, 13. The actinomycetes strains SAC1, SAC2, PK1, and PK2 showed maximum antimicrobial effect against *Staphylococcus aureus, E.coli, Bacillu spp.* The isolated organisms showed minimum antibacterial effect against *Pseudomonas sp***.**

Table: 9 Effect of mannitol on antibiotic production by actinomyces strains SAC1, SAC2, PK1 and PK2

	Assay of organism			
Strain	***Staphylococcus aureus***	***Bacillus* sp.**	***E.coli***	***Pseudomonas* spp**
	Zone of inhibition (mm)			
SAC1	-	25	24	-
SAC2	-	23	19	-
PK1	-	25	21	-
PK2	-	16	9	-

Table: 10 Effect of Sucrose on antibiotic production by actinomyces strains SAC1, SAC2, PK1 and PK2

	Assay of organism			
Strain	***Staphylococcus aureus***	***Bacillus* sp.**	***E.coli***	***Pseudomonas* spp**
	Zone of inhibition (mm)			
SAC1	28	26	28	-
SAC2	27	25	28	-
PK1	28	26	29	-
PK2	12	11	-	-

Table: 11 Effect of Fructose on antibiotic production by actinomyces strains SAC1, SAC2, PK1 and PK2

Strain	Assay of organism			
	Staphylococcus aureus	***Bacillus* sp.**	***E.coli***	***Pseudomonas* spp**
	Zone of inhibition (mm)			
SAC1	23	21	19	-
SAC2	21	20	17	-
PK1	23	21	20	-
PK2	16	10	9	-

Table: 12 Effect of Potassium Nitrate on antibiotic production by actinomyces strains SAC1, SAC2, PK1 and PK2

Assay of organism				
Strain	***Staphylococcus aureus***	***Bacillus* sp.**	***E.coli***	***Pseudomonas* spp**
	Zone of inhibition (mm)			
SAC1	25	22	21	-
SAC2	24	25	23	-
PK1	26	28	25	-
PK2	27	32	27	-

Table: 13 Effect of Peptone on antibiotic production by actinomyces strains SAC1, SAC2, PK1 and PK2

Assay of organism				
Strain	***Staphylococcus aureus***	***Bacillus* sp.**	***E.coli***	***Pseudomonas* spp**
	Zone of inhibition (mm)			
SAC1	26	25	21	-
SAC2	22	24	23	-
PK1	27	29	26	-
PK2	15	14	-	-

Discussion

The result of primary and secondary screening revels that most of the active isolates were active against gram positive bacteria than gram negative bacteria. The reason for different sensitivity between gram positive and gram negative bacteria could be ascribed to the morphologica differences between these microorganisms remarked that ability of antibiotic production is not consistent, but could be increased or decreased remarkably under different cultural conditions[7]. The isolate had different antibacterial activities against the test bacteria grown on solid media and in liquid media. The cultural characters of the Actinomycetes strain SAC1, SAC2 PK1 PK2 on Actinomycetes Isolation Agar, glucose soyabean casein Agar and KenknightMunaier's medium. Were studied It was noticed that Actinomycetes Isolation Agar favoured the rapid growth of powdery colonies within 3-4 days. It may be due to the presence of L-aspargine present in the medium stimulate the growth [8] reported that clear elucidation of the antagotonistic properties is largely influenced by the quality of the medium and types of organisms.

The biosynthesis of antibiotic substances was found to be largely influenced, both quantitatively and qualitatively, by the composition of the medium [9]. The incubation period is another important factor to be considered. In the present study it was noticed that the peak time of maximum antibiotic accumulation was on the 6thday [10]stated that the initial pH of 7.2, incubation time of 96 hours was found to be optimal. In the present study maximum activity was observed in the pH 7. The maximum antibiotic biosynthesis by *Streptomyces* isolate J12 was obtained in medium

supplemented with 10g/1 starch as a sole carbon source and 2.5g/1 potassium nitrate in addition to 0.3g/1 casien as nitrogen sources at pH 7.2 after six days of incubation [11]. In the present study it was noted that maximum antibiotic activity was shown against Bacillus (26-mm/50ml) and E *coli* 29mm/80ml on the 7 day of incubation with different nitrogen sources. Among the four strains tested SAC1 was found to be most promising strain for maximum antibiotic production against Bacillus. The carbon sources have a regulatory role in many antibiotic fermentation. In the present study it was observed that the 4 Actinomycete strain were capable of utilizing, mannitol, sucrose fructose, but the maximum antibiotic accumulation was noticed with sucrose.

Conclusion

Actinomycetes are the most economically and biotechnologically valuable prokaryotes. They are responsible for the production of about half of the discovered bioactive secondary metabolites notably antibiotics, anti-tumor agents, immunosuppressive agents and enzymes. Because of the excellent track record of actinomycetes in this regard, a significant amount of effort has been focused on the successful isolation of novel actinomycetes from terrestrial sources for drug screening programs in the past fifty years. Recently, the rate of discovery of new compounds from terrestrial actinomyctes has decreased, whereas the rate of re-isolation of known compounds has increased.

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