WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.074
Volume 7, Issue 7, 81-102. Conference Article ISSN 2277– 7105

BIOPROSPECTING OF HALOPHILIC ACTINOMYCETES ISOLATED FROM MARINE SEDIMENTS IN CHENNAI SHORES

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Article Received on
13 Feb. 2018,

Revised on 05 March 2018,
Accepted on 26 March 2018,

DOI: 10.20959/wjpr20187-11593

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ABSTRACT

The aim of study was to isolate actinomycetes from marine sediments and to screen their bioactivity, to analyze the qualitative enzyme production, growth in different media and to extract the bioactive metabolites. The sediments collected from various places of Bay of Bengal coast was subjected to Actinomycete isolation. The antibacterial susceptibility of the extracellular extracts of all isolates was tested against antibiotic resistant bacterial strains. The isolates were screened for qualitative enzyme production and their growth, pigmentation was observed in different ISP media. The chosen actinomycete isolate ACT 1 was identified by biochemical characterization and spore morphology. The actinomycete isolates ACT 1 and ACT 8 showed higher inhibition about against tested strains. The isolates grown in Starch Casein medium and ISP 5 medium produced pigmented growth. The ACT 1 isolate posses cellulolytic, amylolytic and proteolytic properties. ACT 8 posses pectinolytic, cellulolytic and proteolytic properties. Through spore morphology and biochemical characterization revealed that the ACT 1 isolate as ***Streptomyces* sp**. This investigation suggests the extracellular extracts of *Streptomyces* sp. ACT1to be a potential broad spectrum antimicrobial compound.

KEYWORDS: Bio prospecting, Marine Actinomycetes, Qualitative enzyme assay, Biochemical characterization.

INTRODUCTION

Infectious diseases are leading health problems with high morbidity and mortality in the developing countries.[4] The development of resistance to multiple drugs is a major problem in the treatment of infectious diseases caused by pathogenic microorganisms. This multidrug resistance is presently an urgent focus of research and new bioactive compounds are necessary to combat these multidrug resistance pathogens. It is indisputable that new drugs, notably antibiotics, are urgently needed to halt and reverse the relentless spread of antibiotic resistant pathogens which cause life threatening infections and risk of undermining the viability of healthcare systems.[50]

Marine environment is the prime reservoir of biological diversity and the marine microorganisms are recognized to be rich sources of novel compounds. In India, about 1000 natural products were derived from marine microbes. Many research efforts over the past decade or so have been directed at assessing the diversity of actinomycetes in the marine environment. Actinomycetes are very widely distributed in the world's oceans and, moreover, truly indigenous marine actinomycetes have now been described[5]. Actinomycetes are aerobic, spore forming gram-positive bacteria, belonging to the order Actinomycetales characterized with substrate and aerial mycelium growth.[21] Actinobacteria are present in various ecological habitats and marine environments[5] and to cope with the environmental stress, marine microorganisms have developed a complex stress management for their survival, which is unrevealed for multiple purposes.[45]

Actinomycetes are a vital cluster of microorganisms, not only as degraders of organic matter within the natural environment, but also as producers of antibiotics and other valuable compounds of commercial interest.[31] Additionally, actinomycetes are important for the production of enzymes, like chitinase (eg. Streptomyces viridificans), cellulases (eg. Thermonospora sp.), peptidases, proteases (Nocardia sp.), Xylanases (Microbispora sp.), ligninases (Nocardia autotrophica), amylases (Thermomonospora curvata), sugar isomerases (Actinoplanes missouriensis), pectinase, hemicellulase and keratinase.[47] Actinomycetes and their bioactive compounds show antibacterial and antimicrobial activity against various pathogens and multi drug resistant pathogens e.g vancomycin resistant Enterococci, methicillin resistant Staphylococcus aureus, Shigella dysenteriae, Klebsiella sp., Escherichia coli, Pseudomonas aeruginosa etc.[34;37;44]

With the efforts of mankind, this natural treasure can become a gainful source of utilization. The best is yet to come as microbes move into the environmental and energy sectors. As stated many years ago by Jackson W. Foster "Never underestimate the power of the microbe," and by David Perlman "If you take care of your microbial friends, they will take care of your future".

In accordance with various previous reports, our work was focused on isolation of potentially bioactive Actinomycetes from marine sediments, screening for their enzyme production, extraction and separation of bioactive compounds.

MATERIALS AND METHODS

Collection of marine sediment

Marine sediment samples were collected from Bay of Bengal Coastal areas including Royapuram (13.1136° N, 80.2961° E), Ennore (13.2146° N, 80.3203° E), Besant Nagar (13.0002° N, 80.2668° E), Thiruvanmiyur (12.9830° N, 80.2594° E) and Covelong (12.7870° N, 80.2504° E) and Pulicat lake estuary (13.5593° N, 80.2098° E) Thiruvallur. Samples were collected at 30 cm depth using plastic borer and transferred to sterile plastic zip lock covers in field and were transported immediately to the laboratory and stored for further study.

Isolation of actinomycetes

Isolation of actinomycetes was achieved by various methods including physical and chemical pretreatment of soil samples, different media sources and various inoculation methods of samples are described below:

1. Pretreatment of samples

The air dried sediment samples were ground aseptically with pestle and mortar, mixed thoroughly and passed through 2 mm sieve filter to remove gravel and debris. Selective pretreatments of the soil samples included dry heat at 55°C for 5 min, 55°C for 15 min, 70°C for 15 min[12; 36] and chemical treatment with 1.5 % phenol for 30 min at 30°C.

2. Pour Plate Method

The pretreated soil samples were diluted 1:10 v/v with sterile 1/4 Ringer's solution (Kui Hong et al., 2009). and serial dilutions prepared down to 10-4. One hundred µL of the 10^{-1} - 10^{-4} suspensions were mixed with partially cooled isolation agar media supplemented with

chloramphenicol and amphotericin (50µg/100mL) and poured into petriplates. These plates were incubated at 28˚C and periodically observed for colony of actinomycetes.

3. Spread Plate Method (Ishita Chakraborty *et al.*, 2015)

The soil samples were serially diluted using sterile 50% sea water up to 10^{-7} dilution and one milliliter of the serially diluted samples were spread plated into media plates. Actinomycetes isolation agar and starch casein agar was used for the isolation of actinobacteria and the media was supplemented with chloramphenicol and amphotericin (100µg/mL) to avoid bacterial and fungal contamination respectively. The plates were incubated at 28°C and monitored periodically up to 30 days of incubation period for actinobacterial growth.

3. Soil Plate Method: (Kokare *et al.*, 2004)

Pretreated soil sediments (about 0.5g) were directly sprinkled on Actinomycetes Isolation Agar media and Starch Casein Agar media (SCA). After 3 – 7 days of incubation, total number of bacteria, fungi and actinomycetes present on each plate were counted. Plates were observed for 14 days and actinomycete colonies were sub-cultured on SCA.

4. Selective Isolation Method

To enhance the actinomycete's growth, the pretreated sediments were serially diluted 10 fold using sterile 50% sea water (Kim *et al.*,[15]) About 0.1 mL of the diluted samples were spread over the Kuster's agar medium. The plates were incubated at 28±2°C for 14 days. After 5 days, the actinomycetes colonies grown on Petri plates were counted at regular intervals. All the morphologically different actinomycete colonies were sub-cultured on SCA.

5. Isolation by Broth Culture (Selvam, 2011)

One gram of sediment was transferred to 100ml of Starch Casein Broth supplemented with chloramphenicol and incubated at 30˚C for 7 days in shaker at 200rpm. A loopful of inoculum from the starch casein broth was streaked onto the starch casein agar (SCA) supplemented with chloramphenicol and incubated at 30˚C for 7 days. Single separated colonies were selected and the subcultures were maintained on starch casein agar media.

Test microorganisms

Staphylococcus aureus (MRSA)

Staphylococcus aureus (ATCC, 25923)

Escherichia coli (ESBL)

Pseudomonas aeruginosa (MDR)

The cultures were obtained from Sri Ramachandra Medical College and Research Institute, Porur, Chennai-116.

PRELIMINARY ANTIBACTERIAL ACTIVITY

1. Cross streak method: (Remya and Vijayakumar, 2008)

Actinomycetes isolates were streaked on the starch casein agar plates. After incubation at 28˚C for 6 days, 24 hr cultures of *Staphylococcus aureus* ATCC, MRSA *Staphylococcus aureus*, *Escherichia coli* ESBL and *Pseudomonus aeruginosa* MDR were streaked perpendicular to the central strip of actinomycetes culture. All plates were incubated at 30˚C for 24 hrs and zone of inhibition was measured.

2. Agar Well Diffusion Method

The preliminary antibacterial activity was carried out by Kirby Bauer‘s agar well diffusion method. 3 hour cultures of each test organisms were compared with standard Mc-Farland Scale (M.0.5) and the culture was uniformly inoculated on the MHA plate. Culture filtrate of actinomycetes isolates of centrifuged for 10 min at 15,000 rpm were loaded. Appropriate controls were maintained. Plates were incubated at 37ºC for 24 h for bacterial growth. Antimicrobial activity was measured based on zone of inhibition.

Qualitative Enzyme Screening

All the isolates were screened for various enzyme production such as Cellulase, Pectinase, Chitinase, Amylase, Protease and Gelatinase.

Screening of Cellulase (Saowapar Khianngam *et al.*,[32])

Actinomycete isolates were inoculated on 1% Carboxy Methyl Cellulose (CMC) agar plates and incubated for 7 days at 28˚C. The plates were flooded with 0.1% Congo red for 20 min and washed with 1 M NaCl for 15 min. The clear zone formed by the isolates was indicated their cellulase activity.

Screening of Pectinase (Rokade, 2015)

Actinomycete isolates were inoculated on 1% Commercial pectin agar media and 1 % Citrus pectin agar plates, incubated for 7 days at 28 ˚C. The plates were flooded with iodine solution for 15 min. The clear zone formed by the isolates was indicated their pectinase activity.

Screening of Chitinase

1. Preparation of colloidal chitin

Colloidal chitin was prepared from the chitin flakes (Sigma Chemicals Company, USA) by the method of Mathivanan.[24] The chitin flakes were ground to powder and added slowly to 10 N HCl and kept overnight at 4°C with vigorous stirring. The suspension was added to cold 50% ethanol with rapid stirring and kept overnight at 25°C. The precipitate was collected by centrifugation at 10000 rpm for 20 min and washed with sterile distilled water until the colloidal chitin became neutral (pH 7.0). It was freeze dried to powder and stored at 4°C until further use.

2. Qualitative Screening*: (*Shanmugaiah *et al.*, 2008)

The pure colonies of actinomycete isolates maintained on starch casein agar slants were streaked on colloidal chitin agar medium plates (1%) and then selected based on their ability to hydrolyze and grow on chitin plates due to secretion of chitinase. The plates were then kept for incubation at 37°C for 24 hrs. The zone of clearance was measured and the cultures giving significant results were selected for further analysis.

Screening of Amylase (Shyam Sunder Alariya *et al.*, 2013)

Actinomycete isolates were inoculated on 1% starch as sole carbon source and incubated for 7 days at 28˚C. The plates were flooded with iodine solution for 15 min. The presence of amylase was measured by halo zones.

Screening of Protease (Farris *et al.*, 2016)

The isolated colonies were screened for protease production using skim milk agar medium and 1% casein enzyme hydrolysate agar medium. All the isolates were streaked on to plates and incubated for 48 h at 28˚C. the plates were flooded with 10% Gram's Iodine solution. The clear zones around the colonies were evaluated as protease producers.

Screening of Gelatinase (Haider M. Hamza *et al.*, 2006)

Actinomycetes isolates were streaked as a single line across the center of the plate containing 1% gelatin agar medium, after incubation for 2-4 days at 28˚ C; the medium was flooded with a 10 % Gram's Iodine solution. A clear zone around the growth of the isolates was indicated to gelatinase activity.

Characterization of Actinomycetes

Determination of the color of the actinomycete isolates

Morphological characters and color determination of selected isolate was studied on different media such as Casein yeast agar (ISP-1),Yeast extract malt extract agar (ISP-2), Inorganic salts starch agar medium (ISP-4), Glycerol aspargine agar medium (ISP-5), Peptone yeast extract iron agar medium (ISP-6) and Tyrosine agar medium (ISP-7) in accordance with the International Streptomycetes Project (ISP).[42] Color was determined using the color names lists.[28]

Morphological Characterization

The colony characteristics and colours of mature sporulating aerial mycelium, substrate mycelium, macro morphology, diffusible pigment, colony reverse colour, colony texture etc. were recorded (Tresner and Backus,[51]).

Coverslip culture technique

Study of the aerial mycelium and its spore characteristics was identified by inclined cover slip culture technique.

A loop full of spore suspension of actinomycete isolate ACT 1 was dispensed at the intersection of the casein starch agar medium and the cover slip. The plates were incubated at 28°C for 4-8 days. The cover slips were removed at intervals of 2-4 days and were observed under light microscope. Morphology of aerial mycelium, substrate mycelium, length of hyphae, arrangement of sporogenous hyphae and were recorded according to International Streptomyces Project.

Biochemical Characterization

Actinomycetes isolate ACT 1 are characterized using H_2S production, Starch hydrolysis, Gelatin hydrolysis, Casein hydrolysis and Urea hydrolysis, Triple sugar test according to International Streptomycetes Project and Bergey's Manual of Systematic Bacteriology.

1. Hydrogen Sulphide production

SIM agar medium slants were prepared and the actinomycetes isolate ACT 1 was inoculated as stab culture and incubated for 2 days at 28°C. The colour change to insoluble black precipitate seen along the line of the stab inoculation was indicative of H2S production. Absence of precipitate is the evidence of negative reaction.

2. Production of Urease

The christensen′s urea broth 10g urea was prepared and sterilized. The actinomycete isolates were inoculated and incubated at 28˚ C for four days. After growth, pH indicator Phenyl Red was added, the colour change in broth was observed.

3. Triple Sugar Test

To facilitate observation of carbohydrate utilization patterns, the TSI agar slants contain lactose and sucrose in 1% concentrations and glucose in a concentration of 0.1% which permits detection of utilization of this substrate only. The slant is inoculated by means of a stab-and-streak procedure. Change in colour of slant was observed and recorded.

Extraction of Bioactive Compound (Smriti Singh *et al.*,[46])

Actinomycete isolate ACT 1 was inoculated in 500 mL starch casein agar medium pH 8 under sterile conditions. Flasks were kept on the rotary shaker at a speed of 110 rpm at 28°C for 14 days. After fermentation, culture was separated by centrifugation at 10,000 rpm for 10 min. The culture filtrate was extracted with solvents ethyl acetate (1: 1, v/v) and hexane (1:1, v/v), vapoured to dryness and stored in 4°C. The intracellular compounds from cell biomass were extracted by using solvents Acetone and Ethanol. Cell suspension was mixed with sterile glass beads and 50 ml of ethanol and acetone in different conical flasks kept for vigorous shaking for 2 hrs. Later, filtered through whatman no. 1 filter paper and dried to vapour, stored in 4°C for further use.

Bioactivity of Crude Metabolite

The antibacterial activity of secondary metabolites of actinomycete isolate ACT 1 extracted with different solvents was tested by agar well diffusion assay. 3 hour cultures of each test organisms were compared with standard Mc-Farland Scale (M.0.5), adjusted to 1.5×10^{8} colonies in 1 ml and the culture was uniformly inoculated on the medium. 100 µL of crude compounds dissolved in 20% DMSO were loaded in the wells. Appropriate controls were maintained. Plates were incubated at 37ºC for 24 h for bacterial growth. Antimicrobial activity was measured based on zone of inhibition.

Determination of Minimum Inhibitory And Minimum Bactericidal Concentrations

The minimum inhibitory concentration (MIC) of the crude extract of isolate ACT 1 was tested by a twofold broth micro dilution as by the method described by NCCLS. The crude extracts were first dissolved in dimethyl sulphoxide (DMSO) and incorporated into sterile

saline and serially diluted 6 times. A standardized suspension of 100 µL of each tested bacterial pathogens (M.0.5) were transferred to each well in microtitre plate. Appropriate controls were maintained. All tubes were incubated at 37°C for 24 h. Turbidity observation was recorded and all the wells were spotted onto MHA plates and incubated at 37°C for 24 h. The lowest concentration of extract that did not show any visible growth of test organisms was determined as MIC, which was expressed in mg/mL. The lowest concentration of the sample at which complete absence of the growth of bacterial colonies was observed was defined as the minimum bactericidal concentration (MBC).

RESULTS

Isolation of actinomycetes

The Actinomycetes were isolated from the marine sediment sample and which were collected from coastal areas of Ennore, Besant nagar and Thiruvanmiyur (Figure 1). Sediment samples treated in 55˚C in 5 min was considered for effective isolation of actinomycete. Chemical pretreatment method is not as effective as physical method. A total of 8 actinomycetes were isolated. Pure culture of actinomycete isolates were maintained in Starch Casein Agar medium (SCA) stored at 4˚C till further use. The morphology of isolated actinomycetes were shown in Figure 2.

Figure – 1: COLLECTION OF MARINE SEDIMENT

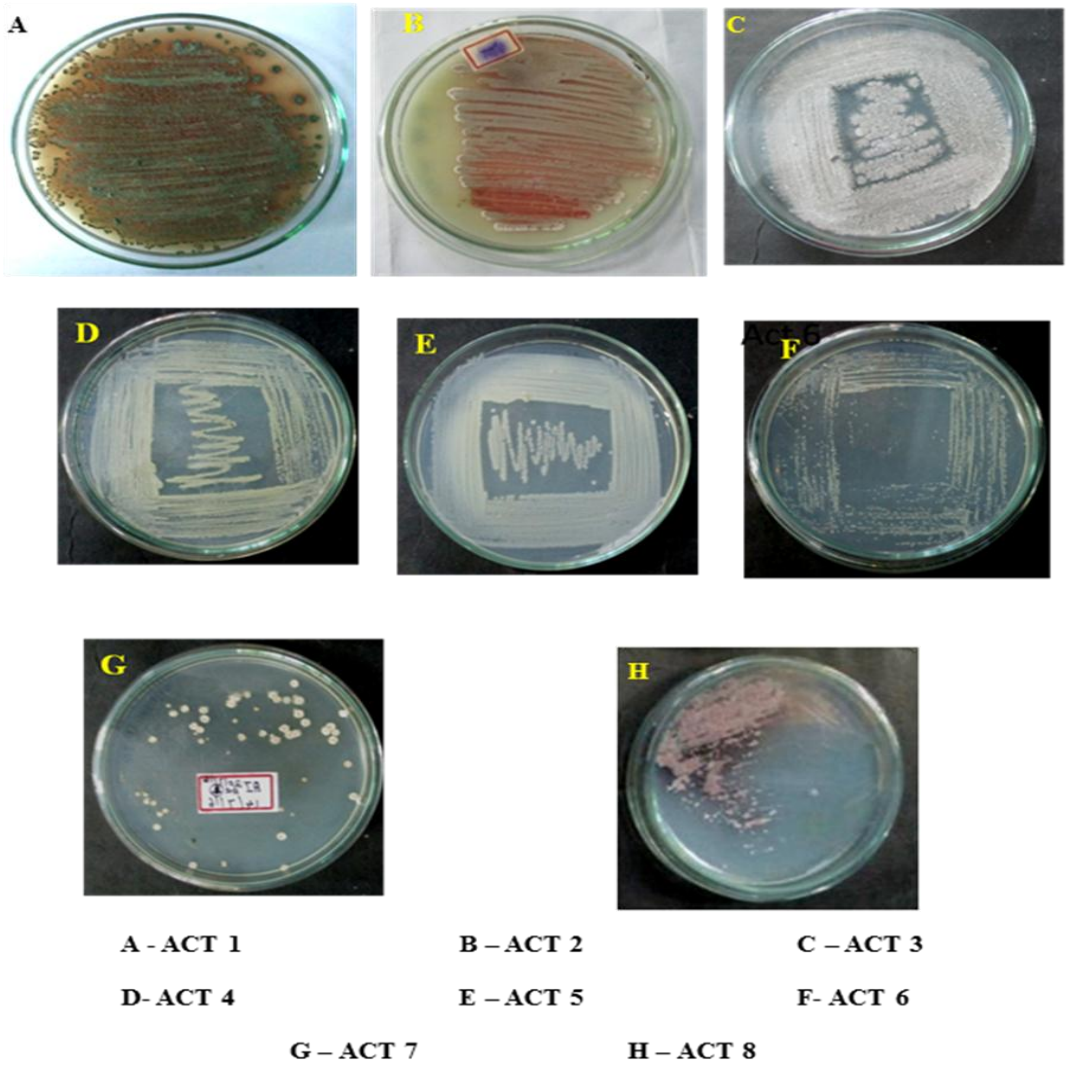
Royapuram

Thiruvotriyur

Pulicat lake

Covelong shore

Figure 2: MORPHOLOGY OF ACTINOMYCETE ISOLATES



A - ACT 1 B – ACT 2 C – ACT 3

D- ACT 4 E – ACT 5 F- ACT 6

G – ACT 7 H – ACT 8

Preliminary Antibacterial Activity

Cross streak Method

In this method, all the bacterial strains didn't produce zone of inhibition against actinomycete isolates. This result revealed that the suppression of drug resistant strains may require adequate amount of active metabolites.

Agar well diffusion method

Among 8 actinomycete isolates, three had significant antibacterial activity against *Staphylococcus aureus* (ATCC), *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* (MDR). All the isolates showed resistance to Escherichia coli (ESBL). The results were tabulated in table 1.

Table1: Preliminary Antibacterial activity of Culture filtrate.

S.No	Test bacteria	Zone of Inhibition (mm)							
		ACT 1	ACT 2	ACT 3	ACT 4	ACT 5	ACT 6	ACT 7	ACT 8
1	ATCC	12	10	-	-	-	9	-	10
2	MRSA	10	-	-	-	-	-	-	9
3	ESBL	-	-	-	-	-	-	-	-
4	MDR	9	-	-	-	-	-	-	9

Screening of enzyme production

All the isolated actinomycetes were screened for cellulase, pectinase, chitinase, protease and gelatinase enzyme activity using their respective substrates. Among them, isolate ACT 1 showed production of cellulase, pectinase, chitinase, amylase and protease enzymes. ACT 2 and ACT 6 isolates showed cellulase, protease and gelatinase enzyme and ACT 8 produced cellulase, amylase and protease enzymes. The results are tabulated in Table: 2.

Table 2: Qualitative Enzyme Screening.

S.NO	ENZYME	ACT 1	ACT 2	ACT 3	ACT 4	ACT 5	ACT 6	ACT 7	ACT 8
1	Amylase	+	+	-	-	-	-	-	++
2	Cellulase	++	+	-	-	-	+	-	++
3	Chitinase	+	-	-	-	+	-	-	-
4	Gelatinase	-	++	-	-	-	+	-	-
5	Protease(skimmed milk)	++	+	-	-	-	++	-	++
6	Protease(caseine)	+	+	-	-	-	-	-	-
7	Pectinase	+	-	-	-	-	-	-	+

(+) Presence of zone of hydrolysis, (-) absence of zone of hydrolysis

Growth in various media

Morphological characters of actinomycete isolates were studied on different media such as SCB, ISP-1, ISP-2, ISP-4, ISP-5, ISP-6, ISP-7. Comparatively faster growths of all the actinomycete isolates were observed in ISP-1 ISP-2 faster ISP-5 and SCB. The isolates grown in SCB and ISP-5 showed effective for pigment production dorsally brown color diffusible pigment ventrally. In ISP-5 medium, ACT 1 produced pink color and ACT 2 produced mauve color, respectively. In SCB, ACT 1exhibited green pigmentation at the dorsal surface of brown diffusible pigmentation ventrally and ACT 2exhibited grayish dorsal pigmentation and deep pink ventral pigmentation. Various colony characteristics were recorded (Table: 3).

Table -3: Determination of The Color Of The Actinomycete Isolates.

S.NO	Name of the medium	Mycelium	ACT 1	ACT 2	ACT 3	ACT 4	ACT 5	ACT 6	ACT7	ACT 8
1	Caseine, yeast, broth Medium(ISP-1)	Aerial mycelium Diffusiable pigment	white	white	white	white	white	white	white	white
2	Malt extract and yeast extract Broth medium (ISP-2)	Aerial mycelium Diffusiable pigment	Green	pink	Brown	white	white	white	white	white
3	Inorganic Salt Starch broth medium (ISP-4)	Aerial mycelium Diffusiable pigment	pink	white	white	Light yellow	brown	white	white	brown
4	Glycerol asparagines broth medium(ISP-5)	Aerial mycelium Diffusiable pigment	White ↓ pink	white ↓ brown	white pink ↓ white	White ↓ Light pink	white	white	white	white
5	Peptone yeast extract iron broth medium (ISP-6)	Aerial mycelium Diffusiable pigment	White ↓ Pink	white	white	yellow	White yellow	Light pink	Light yellow	white
6	Tyrosin broth medium (ISP-7)	Aerial mycelium Diffusiable pigment	Light pink	white	white	white	white	white	white	white
7	Starch, caseine, broth medium(SCB)	Aerial mycelium Diffusiable pigment	Green ↓ brown	White ↓ pink	white	brown	White brown	white	White yellow	white

Extraction of bioactive compounds

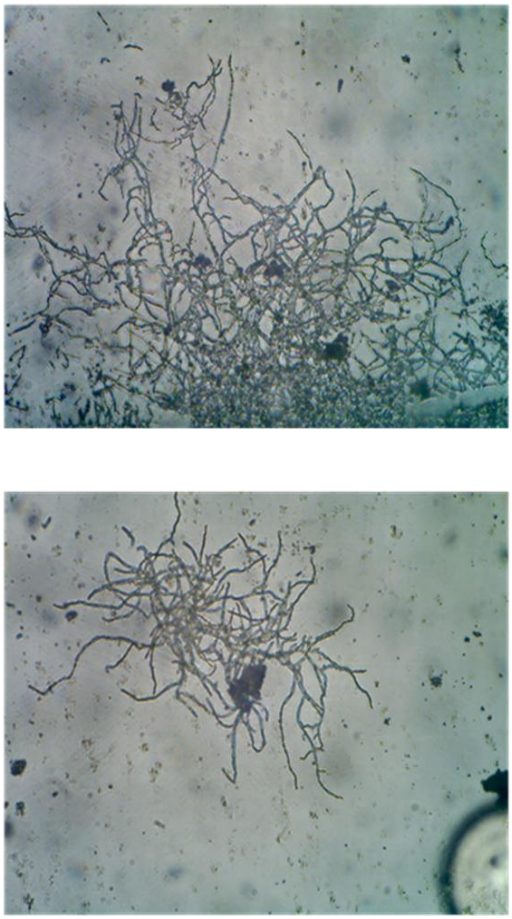
The actinomycete isolate ACT 1 which showed maximum antibacterial activity and enzyme production was chosen for the further study. Intracellular and Extracellular cell extracts were extracted and stored at 4^{o}C.

Characterizations of Actinomycete Isolate Act 1

Morphological Characterization (Slide culture technique)

The mycelial structure of actinomycete isolate ACT 1 were observed from 4^{th} day of growth to 10^{th} day in light microscope 450x and the photographs were taken (Figure - 3).

Figure 3: MORPHOLOGICAL CHARACTERIZATION OF ACTINOMYCETE ISOLATE ACT 1 (COVER SLIP CULTURE TECHNIQUE)



Biochemical Characterization

Biochemical characterizations such as starch hydrolysis, casein hydrolysis, gelatin hydrolysis were tested in ACT 1. Casein and starch show zone of clearance but gelatin showed no show

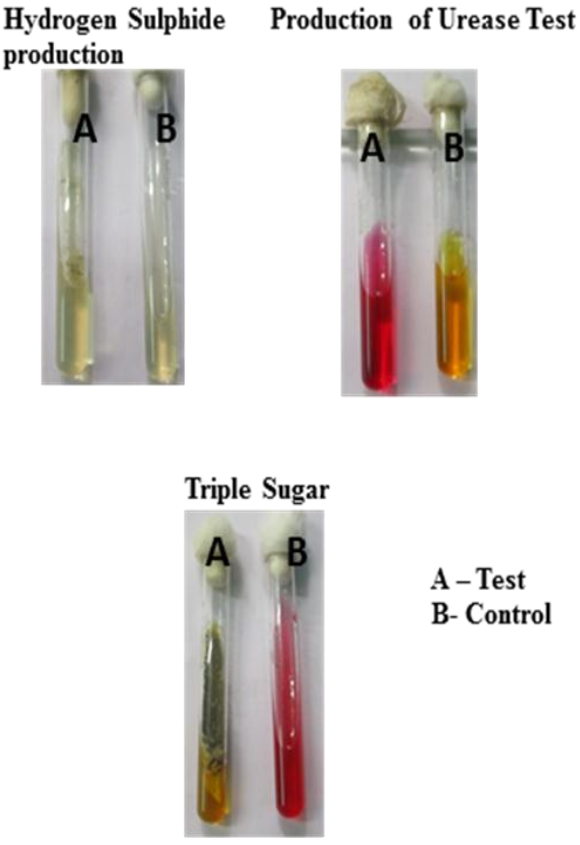
of hydrolysis in ACT 1 (Table 4). In Hydrogen sulphide production test, no change of color was evident from growth of ACT 1 on Sim agar thus reavealing absence of H_2S production (Figure: 4) Change in color of urea broth to pink indicated were production of urease by ACT 1. Change color of the slant to yellow indicated fermentation of sugars leading to acid production. No gas bubbles were observed.

From the above characterization methods, the actinomycete isolate ACT 1 was identified as ***Streptomyces* sp.** and chosen for further study.

Table 4: Biochemical Characterization of Actinomycete Act 1.

S.NO	NAME OF THE TEST	ACTIVITY SHOWN
1	Hydrogen Sulphide production	-
2	Production of Urease Test	+
3	Triple Sugar Test	+
4	Starch hydrolysis	+
5	Gelatin hydrolysis	-
6	Casien hydrolysis	+

Figure 4: BIOCHEMICAL CHARACTERIZATION OF ACTINOMYCETE ISOLATE ACT 1



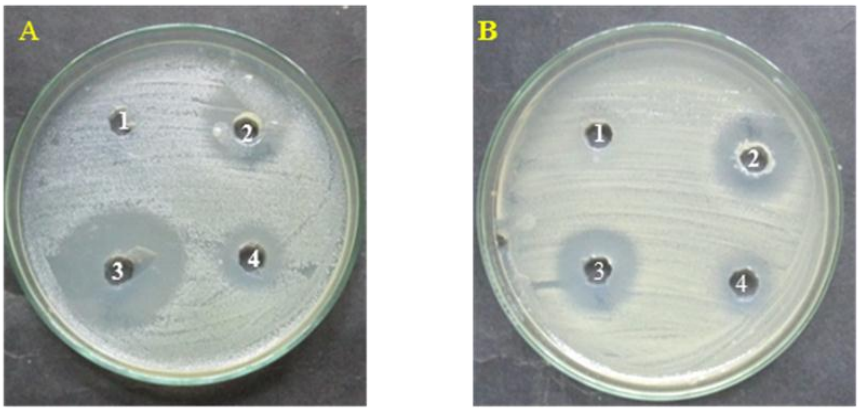
Antibacterial activity of crude compounds

Ethyl acetate and ethanolic extracts of ACT 1 were screened for the antibacterial activity by agar well diffusion method. The extract showed prominent zones of inhibition against all the four tested bacteria. Ethanolic crude extract showed prominent zone of about 30mm against MRSA and pseudomonas sp. All the extracts showed moderate activity against Eshcherichia coli (ESBL). The results were tabulated in table 5 (Figure 5).

Table 5: Antibacterial activity of crude extracts of *Streptomyces* sp. ACT 1.

S.no	Pathogen	Zone of inhibition (mm)					
		Negative control (20% dmso)	Culture filtrate	Intracellular filtrate		Extracellular filtrate	
				Acetone	Ethanol	Ethyl acetate	Hexane
1	ATCC	-	20	12	19	22	12
2	MRSA	-	25	-	30	12	15
3	ESBL	-	-	-	9	-	9
4	Pseudomonas	-	-	-	10	9	11

Figure 5: BIOACTIVIY OF CRUDE COMPOUNDS OF ACTINOMYCETE ISOLATE ACT 1



A-Staphylococcus aureus (ATCC)

1. Control

3.Intracellular Extract

B - **Staphylococcus aureus (MRSA)**

2.Supernatent filtrate

4. Extracellular Extract

Determination of Mic And Mbc Values

The ethanolic, ethyl acetate and hexane crude extracts of the Actinomycete isolate *Streptomyces* sp. were analysed for the MIC and MBC by broth micro dilution method and results tabulated in table 6. The Ethyl acetate extract showed lowest MIC ranging from 0.165-

0.33 mg/ml. From the results, the ethyl acetate extract of ACT 1 isolate were chosen for further characterization.

Table 6: MIC and MBC value of crude extracts of *Streptomyces* sp. ACT 1.

Extract	MIC / MBC	Concentration of crude extracts (mg/ml)			
		ATCC	MRSA	ESBL	MDR
Ethanol	MIC	0.93	≥3.75	1.87	≥3.75
	MBC	1.87	-	3.75	-
Ethyl acetate	MIC	**0.165**	**0.33**	**0.33**	**0.33**
	MBC	**0.33**	**0.66**	**0.66**	**0.66**
Hexane	MIC	0.90	0.90	>1.80	>1.80
	MBC	1.80	1.80	-	-

DISCUSSION

Kumar and Kannabiran[16] isolated 52 strains of actinomycetes at the Bay of Bengal from the shores 8 of Covelong to offshore Nagapattinam. They adopted selective screening of marine sediment samples among the four different media, among them Starch Casein Agar (SCA) proved to be effective for isolation. Jagan mohan et al.,[14] have isolated 93 marine actinomycetets from different locations of the Bay of Bengal starting from Visakhapatnam to Singarayakonda. They used pretreatment of sediments and broth culture for effective isolation. In their study, pretreatment of samples was at 55˚c for 15 min was reported as effective method to obtain isolates Sudha and Masilamani[48] isolated 52 actinomycetes from the shores of Pulicat, Ennore, Muttukadu, and Veerampattinam. We isolated 8 actinomycetes strains from the shores of Ennore, Royapuram, Pulicat lake, Besant nagar, Thiruvanmiyur and Covelong by pretreatment of sediments at 55˚C for 5 mins in (SCA) Starch Casein Agar medium.

Sathiyaseelan and Stella[33] worked on antimicrobial potential of marine actinomycetes by cross streak method against *Escherichia coli*, *Salmonella typhi*, *Bacillus cereus* and *Klebsiella pneumonia*. Among the 52 isolates, 30 isolates shows activity against *Escherichia coli*. Dileep et al.,[7] have reported their work on antibacterial activity against *Staphylococcus aureus*. Sharon et al.,[40] also reported the activity of actinomycetes by means of cross streak method. In our study, the antibacterial activity by cross streak method did not prove efficient. This may due to the fact suppression of bacterial strains does not take place by means of direct contact.

Marine microorganisms were proven already to have many beneficial bioactivities such as productions of industrial enzymes[6,23] of Marine actinomycetes are potential producers of a variety of biologically active enzymes. Previous studies show that actinomycetes have gained enormous importance since they possess a capacity to produce and secrete a variety of extracellular hydrolytic enzymes[34] reported that 80.3% isolates possessed amylolytic activity and 94.1% isolates had proteolytic activity. In the present study, all the isolated strains were screened for their cellulase, pectinase, chitinase, amylase, gelatinase and protease activities. Enzymatic activities of the isolated strains ACT 1 showed production of cellulase, pectinase, chitinase, amylase and protease enzymes. ACT 2 and ACT 6 isolates showed presence cellulase, protease and gelatinase enzyme, ACT 8 produced cellulase, amylase and protease enzymes.

Morphology plays an important role in characterization of *Streptomyces* from other sporing actinomycetes. The life cycle of a *Streptomycete* provides 3 features for microscopic characterization viz., vegetative mycelium, aerial mycelium bearing chains of spores and the characteristics of spores themselves. The latter two features produce most diagnostic information.[1,49] In the present study, cultural and microscopic characteristics of the isolate ACT 1 were consistent with its classification as a member of the genus *Streptomyces* sp.

As long as the major challenges in biotechnology and biomedicine remain (e.g. emerging diseases, established diseases, antibiotic resistance, environmental pollution and need for renewable energy) microbial resources will be of interest to mankind providing sustainable and environmentally friendly solutions.[44]

In our study, the ethyl acetate extract and ethanolic extract of *Streptomyces* species (ACT 1) showed remarkable inhibition against MRSA and ATCC *Staphylococcus aureus*. It was found that Gram positive bacteria show more susceptibility than Gram negative bacteria. Similar results were observed in the earlier studies.[11,2,52,17] The low antibacterial activity of ethyl acetate extract against the gram negative bacteria could be ascribed to the presence of an outer membrane that possess hydrophilic polysaccharides chains and forms an additional barrier for extract as well as antibiotics.[22,25]

The MIC and MBC varied among the tested isolates against *S. aureus* ATCC and MRSA. The lowest MIC values were ranging from 0.165-0.33 mg/ml. These results were similar with the report of Sibanda et al.,[43] Joseph Selvin et al.,[38] has reported the MIC of *Streptomyces*

sp.BTL7-ECP for *M. luteus* was 44 µg protein/ml and the MBC was 88 µg protein/ml. The MIC and the MBC of BTL7-ECP for *P. aeruginosa* was determined as 704 and 1408 µg protein/ml. Therefore, crude extracts could be a potent source for antibiotic production, which leads to the development of novel drugs for the treatment of infectious diseases.

CONCLUSION

The study was successful in the isolation of marine actinomycetes from sediments collected at different shores in chennai. The isolate ACT 1 was identified as a species of the genus *Streptomyces* sp. based on microscopic, cultural and biochemical characterization. However, the fractionated extract (ethyl acetate, ethanolic, acetone and hexane fraction), especially ethyl acetate fraction, showed varied biological activities such as antimicrobial activity and enzyme production. The extract was found to contain bioactive principles which are to be separated and subjected for TLC. The actinomycete isolate *Streptomyces* sp. can be a potential candidate for the development of therapeutic agents. Further studies on characterization of the isolate and the purified bioactive components in the solvent extract are under progress.

REFERENCES

1. Anderson AS, Wellington EMH. The taxonomy of *Streptomyces* and related genera. Int. J. of Sys. Evo. Micro, 2001; 51: 797-814.
2. Anansiriwattana W, Tanasupawat S, Amnuoypol S, Suwanborirux K. Identification and antimicrobial activities of actinomycetes from soils in Samed Island, and geldamycin from strain PC4-3. Thai J. of Phar. Sci., 2006; 30: 49-56.
3. Balagur. With reference to α, β - unsaturated γ - lactone type of antibiotic from *Streptomyces griseobrunneus*. Ph. D. Thesis, Annamalai University, India, 88.
4. Black RE, Brown KH, Becker S, Yunus M. Longitudinal studies of infectious diseases and physical growth of children in rural Bangladesh. I. Patterns of morbidity. Amer. J. of Epi, 1982; 115: 305–314.
5. Bull AT, Stach JEM, Ward AC, Goodfellow M. Marine actinobacteria: perspectives, challenges and future directions. Antonie van Leeuwenhoek, 2005; 87: 65–79.
6. Chatellier MA, Jacky Bhagat, Karthik K, Jaffar Hussain A and Jayaprakashvel M. Deproteination of shrimp shell wastes using immobilized proteolytic marine bacteria. In: The Abstracts book of national level students seminar on biotechnology in daily life

organized by department of biotechnology, AMET University Chennai on 10th March 2011.

7. Dileep N, Syed junaid, Rakesh KN, Prashith Kekuda TR, Onkarappa R. Antibacterial Activity of three *Streptomyces* species Isolated From Soil of Shikaripura, Karnataka, Ind. J. of Bio. Sci. Opi, 2013; 1(3).
8. Farris MH, Kara A, Ford, Richard C Doyle. Qualitative and Quantitative Assays for Detection and Characterization of Protein Antimicrobials. J Vis Exp, 2016; 110: 53819.
9. Goodfellow M, Williams ST. Ecology of actinomycetes. Annu. Rev. Microbiol., 1983; 37: 189–216.
10. Haider M Hamza, Sinaa M Ali, Hamid G Hassan. Partial Purification of Gelatinase Enzyme from Local Isolate of *Brevibacillus laterosporus*. National Journal of Chemistry, 2006; 23: 437-442.
11. Hassan MA, El-Naggar MY, Said WY. Physiological factors affecting the production of an antimicrobial substance by *Streptomyces violatus* in batch cultures. Egyp. J. of Bio, 2001; 3: 1-10.
12. Hayakawa M, Nonomura H. Efficacy of artificial humic acid is a selective nutrient in HV agar used for the isolation of Actinomycetes. J. of Ferment. Tech., 1987; 65: 609-616.
13. Ishita Chakraborty, Priyanka Redkar, Minki Munjal SR, Sathish Kumar and Bhaskara Rao KV. Isolation and characterization of pigment producing marine actinobacteria From mangrove soil and applications of bio-pigments. Der Pharmacia Lettre, 2015; 7(4): 93-100.
14. Jagan Mohan, Sirisha, R Haritha, T Ramana. Selective Screening, Isolation and Characterization of Antimicrobial Agents From Marine Actinomycetes. Int. J. of Phar & Pharmac. Sci., 2013; 5(4): 443-44.
15. Kim CM, Lec KH, Kwon OS, Shimazu A, Yoo ID. Selective isolation of Actinomycetes by physical pre-treatment of soil sample. J. of Appl. Env. Microb. Biotech, 1994; 22: 222-225.
16. Kumar S, Kannabiran K. Diversity and Optimization of Process Parameters for the Growth of *Streptomyces* VITSVK9 spp. Isolated from Bay of Bengal, Indi. J. Nat. Env. Sci., 2010; 1(2): 56-65.
17. Gunda MM, Charya MAS. Physiological factors influencing the production of antibacterial substance by freshwater actinobacteria. J. of Rec. Adv. App. Sci., 2013; 28: 55-62.

18. Selvam K, Vishnupriya B, Subhash Chandra Bose V. Screening and Quantification of Marin Actinomycetes Producing Industrial Enzymes Amylase, Cellulase and Lipase from South Coast of India. Int. J. of Pharm. & Bio. Archiv, 2011; 2(5): 1481-1487.
19. Kokare CR, Mahadik KR, Kadam SS, Chopade BA. Isolation, characterization and antimicrobial activity of marine halophilic *Actinopolyspora* sp. AH1 from the west coast of India. Curr. Sci., 2004; 86(4): 594-597.
20. Kui Hong, An-Hui Gao, Qing-Yi Xie, Hao Gao, Ling Zhuang, Hai-Peng Lin, Hai-PingnYu, Jia Li, Xin-Sheng Yao, Michael Goodfellow, Ji-Sheng Ruan. Actinomycetes for Marine Drug Discovery Isolated from Mangrove Soils and Plants in China. Mar. Drugs, 2009; 7(1): 24-44.
21. Lechevalier H, Lechevalier MP. Introduction to the order Actinomycetales. In: Starr (MP, Stolp H, Trüper HG, Balows A, Schlegel HG, Editors). The Prokaryotes. Germany: Springer-Verlag Berlin, 1981; 2: 1915-22.
22. Lodhia MH, Bhatt KR, Thaker VS. Antibacterial activity of essential oils from Palmarosa, Evening Primrose, Lavender and Tuberose. Indian. J. Pharma. Sci., 2009; 71(2): 134-136.
23. Manasi N, Kaarthikeyan C, Karthik K, Jaffar Hussain A and Jayaprakashvel M. Screening of bacteria isolated from marine environment for industrially important enzymes. In: National conference on marine explorations of the natural bioactive compounds from the marine resources during Feb 10 and 11th organized by Department of Marine Science, Bharathidasan University, 2011; 47-48.
24. Mathivanan N. Studies on extracellular chitinase and secondary metabolites produced by Fusarium chlamydosporum, an antagonist to Puccinia arachidis, the rust pathogen of groundnut. Ph. D. thesis, University of Madras, Chennai, India, 1995.
25. Nalubega R, Kabasa JD, Olila D, Kateregga J. Evaluation of Antibacterial Activity of Selected Ethnomedicinal Plants for Poultry in Masaka District, Uganda. Res. J. of Pharm, 5(2): 18-21.
26. NCCLS-National Committee for Clinical Laboratory Standards. Performance standard for anti-microbial susceptibility testing: eleventh informational supplement. Wayne, PA, USA, 2001; Document M100-S11.
27. Prasantha Kumari, Mantada, Girija Sankar G, Prabhakar T. Isolation and Characterization of Potent Antibiotic Producing Marine Actinomycetes from Tiruchendur and Kulasekarapattinam, Tamilnadu. Global Journals Inc. (US), 2013.

28. Pridham TG. Color of Steptomyces: Report of an international work shop on determination of Color of Steptomyces. Appl. Microbiol, 1964; 13: 43-61.
29. Remya M, Vijayakumar R. Isolation and Characterization of marine antagonistic Actinomycetes from West coast of India. Facta Universitatis, 2008; 15: 13-19.
30. Rokade DD, Vaidya SL, Rehman Naziya MA, Dixit PP. Screening of Pectinase Producing Bacteria, Isolated From Osmanabad Fruit Market Soil. Int. J. of Interdis. Multidisc. Stud., 2015; 2(6): 141-145.
31. Saugar I, E Sanz, MA Rubio, JC Espinosa and A Jimenez. Identification of a set of genes involved in the biosynthesis of the aminonucleoside moiety of antibiotic A201A from *Streptomyces capreolus*. European J. Biochem., 2002; 269: 5527- 35.
32. Saowapar Khianngam, Yupa Pootaeng-on, Taweesak T, Somboon T. Screening and identification of cellulase producing bacteria isolated from oil palm meal. J. of Appl. Pharm. Sci., 2014; 4(04): 090-096.
33. Sathiyaseelan K, Stella D. Isolation, Identification and Antimicrobial Activity of Marine Actinomycetes Isolated from Parangipettai. Rec. Res. Sci. Tech., 2011; 3(9): 74-77.
34. Saadoun I, Hameed KM, Moussauui A.Characterization and analysis of antibiotic activity of some aquatic actinomycetes. Microbios, 1999; 99: 173-179.
35. Saadoun I, Rawashdeh R, Dayeh T, Ababneh Q, Mahasneh A. Isolation, characterization and screening for fiber hydrolytic enzymes- producing *streptomycetes* of Jordanian forest soils. Biotechnology, 2007; 6(1): 120-128.
36. Seong C H, Choi JH, Baik KS. An improved selective isolation of rare actinomycetes from forest soil. J. of Microbiol, 2011; 17: 23-39.
37. Selvameenal L, Radhakrishnan M, Balagurunathan R. Antibiotic pigment from desert soil Actinomycetes; biological activity, purification and chemical screening. Indian J Pharm Sci., 2009; 71: 499–504.
38. Selvin J, Lipton AP. *Dendrilla nigra*, a marine sponge, as potential source of antibacterial substances for managing shrimp diseases. Aquaculture, 2004; 236: 277–283.
39. Shanmugaiah V, N Mathivanan, N Balasubramanian, PT Manoharan. Optimization of cultural conditions for production of chitinase by *Bacillus laterosporous* MML2270 isolated from rice rhizosphere soil. Afr. J. Biotechnol., 2008; 15: 2562-2568.
40. Sharon G, Garg N, Debelius J, Knight R, Dorrestein PC, Mazmanian SK. Specialized metabolites from the microbiome in health and disease. Cell Metab, 2014; 20(5): 719-30.
41. Shyam SA, Sonia Sethi, Sakhsam Gupt, Lal Gupta. Amylase activity of a starch degrading bacteria isolated from soil. Archiv. of Appl. Sci. Res., 2013; 5(1): 15-24.

42. Shirling EB and Gottlieb D. Methods for characterization of *Streptomycetes* species. Int. J. Syst. Bac, 1996; 16: 313-340.
43. Sibanda, Timothy Olaniran, Ademola Okoh, Anthony. *In vitro* antibacterial activities of crude extracts of Garcinia kola seeds against wound sepsis associated *Staphylococcus* strains. J of Med. Plants Research, 2010; 4: 710-716.
44. Singh S, Kumar P, Gopalan N, Shrivastava B, Kuhad RC, Chaudhary HS. Isolation and partial characterization of actinomycetes with antimicrobial activity against multidrug resistant bacteria. Asian Pac J Trop Biomed, 2012; 1147–50.
45. Sigrid H, Espen F, Kjell DJ, Elena I, Trond EE, Sergey BZ. Characterization of *Streptomyces* sp. Isolated from the Sea surface microlayer in the Trondheim fjord, Norway. Mar. Drugs, 2008; 6: 620–635.
46. Smriti Singh, Pramod Kumar, N Gopalan, Bhuvnesh Shrivastava, RC Kuhad, Hotam Singh Chaudhary. Isolation and partial characterization of actinomycetes with antimicrobial activity against multidrug resistant bacteria. Asian Pac. J. of Trop. Biomed., 2012; 1147-1150.
47. Solans M, G Vobis. Saprophytic actinomycetes associated to the rhizosphere and rhizoplane of *Discaria trinervis*. Ecologia Australian, 2003; 13: 1199-1202.
48. Sudha S, Masilamani Selvam M. *Streptomyces cavourensis* sp. SU 3 Nov., a novel marine *Streptomyces* isolated from sea shore sediment in Chennai. Advanced Biotech, 2011; 32–36.
49. Taddei A, Rodriguez MJ, Marquez-Vilchez E, Castelli C. Isolation and identification of *Streptomyces* sp. From Venezuelan soils: Morphological and biochemical studies. I. Microbiol. Res., 2006; 161: 222-231
50. Talbot GH, Bradley J, Edwards JE, Jr., Gilbert D, Scheld M, Bartlett JG. Bad bugs need drugs: an update on the development pipeline from the Antimicrobial Availability Task Force of the Infectious Diseases Society of America. Clin. Infect. Dis., 2006; 42: 657–68.
51. Tresner HD and Backus EJ. System of Color Wheels for *Streptomycete* Taxonomy. Appl. Microbiol, 1963; 335-338.
52. Valli S, Suvathi SS, Aysha OS, Nirmala P, Kumar VP, Reena A. Antimicrobial potential of Actinomycetes species isolated from marine environment. Asian Pac. J. of Trop. Biomed., 2012; 2(6): 469-473.