CHAPTER 3

MATERIALS AND METHODS

3.1 INTRODUCTION

This chapter presents the experimental methods followed in the present study. The isolation and identification techniques followed in the current study for the marine isolate are presented. The optimization methods using RSM and the extraction procedures followed in the present study and analytical methods followed are explained in detail. Numerous biological applications procedures followed in the current study using the metabolites obtained from marine isolate employed in this study are elaborated.

3.2 CHEMICALS AND REAGENTS

All the chemicals and reagents used in the present research work were purchased from Himedia, Mumbai, India.

3.3 SELECTION OF SAMPLING AREA FOR THE ISOLATION OF *ACTINOMYCETES*

Marine soil samples were collected from seventeen different points and depths covering the parts of Manora Fort situated 65 km (40 miles) away from Thanjavur, Tamil Nadu, India. The fort was situated away from Thanjavur in a place called Sarebendrarajapattinam in shores of Bay of Bengal. The name and geographical position of the study cite were located at latitude of 10.20 °N and longitude of 79.24 °E. The locations of the study

sites were fixed during the time of collection with the help of GPS (Global Position System) and Google map to study the location of Manora Fort.

3.4 CLEANING OF GLASSWARES

All the glassware's were initially soaked in chromic acid cleaning solution containing 10% of potassium dichromate in 25% sulphuric acid and washed thoroughly in tap water. Consequently, they are soaked in detergent solution again, washed thoroughly in tap water and dried in oven (Harrigan 1998).

3.4.1 Sterilization

All the glassware's, culture media, buffers and reagents used in this study were sterilized in an autoclave at 121 $^{\circ}$C at 15 lbs/inch^2 for 20 minutes. The heat liable chemicals such as antibiotics, culture filtrate, crude metabolites, were filter sterilized using Millipore filter of 0.2 µm pore size (Millipore, Molsheim, France).

3.5 COLLECTION OF SOIL SEDIMENT SAMPLES

The collection of soil samples from the sampling site were done during the low tide. About seventeen different soil samples were collected from different depths of seashore, sea deep soil sediments and sea water samples of Manora Fort, Thanjavur, Tamilnadu, India. The soil collection study was carried out for a period of two months from June 2012 to July 2012. Marine deep sea soil samples were collected from the sampling site using mini dredge following sterile standard procedures. The corer is sterilized with alcohol before sampling at each sampling point. The central portion of the top 2 cm sediment sample was removed with the help of a sterile spatula. Since the soil samples collected from 0-6 inch depth

(15.24 cm) on the upper surface of sediments consist of higher microbial load, the soil sediments were collected from a deep sea at depth of five to ten cm.

The marine samples collected were transferred to separate clean polythene bags and sterile containers immediately after collection sealed and labelled as described by Jensen et al in (1991). All the marine samples were brought to laboratory for further processing within ten hours of collection. The collected samples were brought in aseptic condition to the Bioremediation Laboratory, A.C.Tech campus, Anna University for further processing. The samples were aseptically air dried thoroughly in aluminium trays. After thoroughly dried, for about seven to fourteen days the samples were incubated at 55 $^{\circ}$C for 5 min (Balagurunathan 1992, Williams et al 1972) to reduce the number of vegetative bacterial cells. When completely dried, the sediments were crushed and passed through a 2 mm sieve to remove stones, shells and other debris transferred to fresh clean polythene bags, sealed, labelled and stored at 4 °C until used.

3.5.1 Determination of pH

The pH of all the seventeen collected marine samples was measured. 10 g of each marine sediment sample was weighed and suspended in 20 ml of sterile distilled water. It was then allowed to stand for 20 min with intermittent stirring to attain the equilibrium. After being left to settle, the pH of the supernatant solution was measured.

3.5.2 Pretreatment of Samples (Takizawa et al 1993)

Collected marine soil samples were subjected to different pretreatment methods such as antibiotics, dry heat, air dry, wet heat and phenol were carried out in the first 24 hrs after soil sampling.

3.5.3 Selection of Media and their Composition

Different types of media were employed in this study for various purposes such as isolation, screening, optimization, and production of secondary metabolites is shown in Table 3.1. All the media were prepared in natural aged seawater.

Table 3.1 Different types of medium and its composition

S.No	Medium	Media Components (g/L)
1.	**Seed culture medium**	Glucose-10.0 Malt extract-3.0 Yeast extract-5.0 Agar-20.0 pH- 7.0±0.2
2.	**Starch Casein agar**	Starch – 10.0 Vitamin- free casein-0.3 $CaCO_3$-0.02 $Fe_2SO_4.7H_2O$-0.01 KNO_3-2.0 K_2HPO_4-2.0 $MgSO_4.7H_2O$-0.05 NaCl-2.0 Agar- 20.0 pH- 7.0±0.2
3.	**Yeast malt extract agar**	Peptic digest of animal tissue-5.0 Yeast extract-3.0 Malt extract-3.0 Dextrose-10.0 Agar-20.0 pH- 7.0±0.2
4.	**Trace element solution**	$CuSO_4.5H_2O$-0.64 $FeSO_4.7H_2O$-0.11 $MnCl_2$-0.79 $ZnSO_4.7H_2O$-0.15 Natural seawater-100.0 ml

Table 3.1 (Continued)

S.No	Medium	Media Components (g/L)
5.	**Glucose asparagine agar**	L-asparagine 0.5 Glucose-10.0 K_2HPO_4-0.5 Agar-20.0 pH- 7.0±0.1
6.	**Maltose yeast extract agar**	Yeast extract-2.0 Maltose-10.0 Beef extract-1.0 Agar-20.0 pH- 7.0±0.1
7.	**Luria Bertani agar**	Tryptone-10 Sodium chloride-5.0 Yeast extract-5.0 Agar-20.0 pH- 7.0
8.	**Nutrient agar**	Beef extract-1.0 Yeast extract-2.0 Peptone-5.0 NaCl-5.0 Agar-20.0 pH- 7.0±0.2
9.	**Potato dextrose agar**	Potato-200 Dextrose-20.0 Agar-20.0 pH- 7.0±0.2

3.6 ISOLATION OF *ACTINOMYCETES*

About 10 g of marine sediment soil samples were suspended in 95 ml of sterilized seawater and these suspensions were considered as 10^{-1} dilution. SCA (Starch casein agar medium) was prepared in sterilized sea water was used for the isolation of marine *actinomycetes*. The medium was

supplemented with amphotericin B - 50 µg/l and nystatin - 50 µg/l to prevent the bacterial and fungal growth. From 10^{-1} dilution suspension, 1 ml of sediment sample was transferred to 9 ml of sterilized sea water and subsequently serially diluted to 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}. From the required dilution, 0.1 ml of suspension was drawn and plated over the surface of SCA medium. All the plates were incubated at 28±2 °C for 21 days for the growth of the *actinomycetes* isolates.

3.6.1 Maintenance and Prevention of *Actinomycetes* Isolates

The marine *actinomycetes* were isolated as pure culture isolates by using the aseptic microbiological methods. All the obtained isolates were maintained on SCA slants. The spores were further preserved and stored in 15% glycerol under low temperature of -20 °C (Williams & cross 1971). The isolates were temporarily designated as SSD01 to SSD08.

3.7 PRIMARY SCREENING AGAINST HUMAN BACTERIAL AND FUNGAL PATHOGENS

About 25 ml of NA (Nutrient agar) medium and PDA (Potato dextrose agar) medium was prepared, sterilized in autoclave and poured into petriplates. It was then allowed to solidify. Each *actinomycetes* isolate maintained in SCA slants was streaked at the left corner of petriplates with 5 mm width and 4 cm length and incubated at 28 °C for 5 days (Cappuccino & Sherman 1999, Souza & Souza 2000, Kokare et al 2004). Six human pathogens was selected and used for the primary screening of the potent marine actinomycetes. Six bacterial pathogens such as *E. coli (Escherichia coli), B. subtilis (Bacillus subtilis), (S. aureus) Staphylococcus aureus, S. epidermidis (Staphylococcus epidermidis), (P. Aeruginosa) Pseudomonas aeruginosa* and *(V. Cholera) Vibrio cholerae* and four fungal pathogens such as (*A. niger) Aspergillus niger, (A. flavus) Aspergillus flavus,* (*A. fumigatus)*

Aspergillus fumigatus and *(C. Albicans) Candida albicans* were used to determine the potent *actinomycetes* colonies against the human pathogens using cross streak technique. The bacterial and fungal pathogens were streaked separately on the NA and PDA plates served as controls. All the plates were incubated at 28±2 °C up to 48 hrs. The zone of inhibition was observed and the plates were measured against the respective pathogens.

3.8 MORPHOLOGICAL CHARACTERISTICS OF MARINE *ACTINOMYCETES* ISOLATES

All the marine *actinomycetes* isolates were sub cultured on SCA medium. The pigmentation for aerial and substrate mycelia, colony morphology, diffusible pigment and exopolysaccharides were observed for all the isolates.

3.9 GROWTH INDIVIDUALITY CHARACTERISTICS OF MARINE *ACTINOMYCETES* ISOLATES

All the marine isolates were grown on SCA medium at 28 °C for 14 to 21 days. All the isolate plates were observed everyday for the growth of marine *actinomycetes*. The isolates, which showed satisfactory growth within four days were considered as fast growers and those showed growth during four to seven days were listed as moderate growers and those isolates took more than seven days were treated as slow growers in growth.

3.10 SELECTION OF POTENTIAL STRAIN

Among the isolated strains, the marine *actinomycetes* isolate SSD08 was selected for further studies due to its superior antimicrobial activity against human pathogens in primary screening.

3.11 IDENTIFICATION OF POTENTIAL *ACTINOMYCETES* STRAIN

3.11.1 Classical Approach

The identification of the potent SSD08 marine *actinomycetes* isolate after primary screening was initially done by classical approach. The morphological, physiological and biochemical characteristics of the isolate SSD08 were done by classical approach. The classical method was performed according to the method described in the identification key by Nonomura in (1974) and Bergey's Manual of Determinative Bacteriology (Buchanan & Gibbons 1974). These distinctive characters have been commonly engaged in routine identification and taxonomy of *actinomycetes* for several years.

3.11.1.1 Colony morphology

Morphological characterization was performed with a magnified lens on *actinomycete*s isolate SSD08 after its growth from 4 to 14 days on SCA medium. Colony morphology was recorded with respect to aerial mass colour, melanoid pigments, reverse side pigments, soluble pigments, spore surface, spore chain morphology were observed under microscope.

3.11.1.2 Aerial mass color

The color of the mature sporulating aerial mycelium of the *actinomycete*s isolate SSD08 was recorded (Shirling & Gottlieb 1966).

3.11.1.3 Melanoid pigments

The production of melanoid pigments by the *actinomycetes* isolate SSD08 on the SCA medium was recorded. The isolate was grouped as melanoid pigment produced (+) and not produced (-).

3.11.1.4 Reverse side pigments

The ability of the strains to produce characteristic pigments on the reverse side of the colony, namely, distinctive positive (+) and not distinctive or none negative (-) was recorded.

3.11.1.5 Soluble pigments

The capacity of the *actinomycetes* isolate SSD08 to produce soluble pigments other than melanin: namely, produced positive (+) and not produced negative (-) was observed.

3.11.1.6 Spore chain morphology using light microscopy and scanning electron microscopy (SEM)

The spore chain morphology in *Streptomyces* sp. were divided into three sections (Shirling & Gottlieb 1966), namely rectiflexibiles (RF), retinaculiaperti (RA) and spirales (S). A cover slip culture technique was adopted for light microscopic studies (Pridham et al 1958). *Actinomycetes* culture plate was prepared and 5 to 6 sterile cover slips were placed at an angle of 45°. The *actinomycetes* culture was slowly released at the intersection of medium and cover slip. The plates were incubated at 28±2 °C for 4-8 days. Afterwards, the cover slips were removed and observed under the high power magnification. The morphological features of spores, sporangia and aerial, substrate mycelium were observed and recorded under Light microscope (40X). Similarly, the spore morphology was examined using SEM (Williams & Davies 1967) initially suspension culture of *actinomycetes* isolates was harvested by centrifugation at 10,000 rpm for 10 min. The cell pellet was continuously washed three times in phosphate buffer (0.1 M and pH 7.2-7.4). Then the cells were fixed with the fixative, formaldehyde, for 3-6 hrs at room temperature. The cell pellets were washed

repeatedly for three times with phosphate buffer at 20 min interval and at 4 °C then dehydrated through a graded series of acetone. The samples were transferred to stub and they were observed under the scanning electron microscope

3.11.2 Physiological and Biochemical Tests

Various physiochemical and biochemical tests were carried out according to the methods of Shirling & Gottlieb in (1966), methods outlined in the Bergy's Manual of Systematic Bacteriology (Williams et al 1989).

3.11.2.1 Gram's staining

Table 3.2 Reagents and medium used for Gram's staining

S.No	Physiochemical Test	Reagents/Medium Used
1.	**Gram's Staining**	**Solution A** Crystal violet -2.0 g Ethanol – 20.0 ml **Solution B** Ammonium oxalate- 0.8 g Distilled water – 80.0 ml **Gram's iodine** Iodine -1.0 g Potassium iodide- 2.0 g Distilled water – 50.0 ml **Safranine** Safranine- 0.25 g Ethanol- 10.0 ml Distilled water -100.0 ml

A thin smear of *actinomycetes* isolate was made air dried and heat fixed in a clean glass slide. The slide was flooded with crystal violet for a minute and rinsed with tap water. Followed by, the addition of gram's iodine solution, kept for a min in the glass slide. It was then rinsed with tap water. The smear was decolorized with gram decolorizer and immediately rinsed with tap water. Finally, the slide was then flooded with safranine for a min, rinsed with tap water, blot dried and observed under a light microscope. The reagents and medium used for Gram's staining is shown in Table 3.2.

3.11.2.2 Acid fast staining

A uniform thin smear of *actinomycetes* isolate was made on a clean slide. It was air dried and heat fixed. The slide was then flooded with concentrated carbol fuchsin and steamed at intervals to prevent the stain. The slide was washed with water and decolorized with 1% sulphuric acid. The slide was again washed with water and flooded with methylene blue counter stain for 2 min. The slide was then rinsed in water, blot dried and observed under light microscope.

3.11.2.3 Motility test

The hanging drop method was used to examine the motility of the *actinomycetes* isolate. The four corner of cover slip, a thin film of vaseline was placed and a loopful of log phase culture of *actinomycetes* isolate was placed in the centre of the cover slip and pressed so that the drop hangs in the cavity slide and motility of the isolate was observed using a light microscope.

3.11.2.4 Endospore staining

Actinomycetes isolate was grown on the above sporulation medium for 7 days. A loopful of sporulated culture was taken, uniform smear was made on a clean slide and heat fixed. The slide was flooded with malachite green reagent and steamed intermittently for 5 min. The slide was washed under running tap water, counter stained with safranine and observed under a light microscope. Table 3.3 shows the reagents and medium used for Endospore Staining.

Table 3.3 Reagents and medium used for Endospore Staining

S.No	Physiochemical Test	Reagent/Medium Used
1.	**Endospore Staining**	Peptone- 5.0 g Beef extract-10.0 g $MnSO_4$-0.005 g Agar- 20.0 g Seawater- 1000.0 ml pH- 7.0 **Malachite green reagent** Malchite green- 5.0 g Distilled water- 100.0 ml **Safranine solution** Safranine -0.25 g Ethanol – 10.0 ml Distilled water- 100.0 ml

3.11.2.5 Indole production

A loopful of *actinomycetes* culture was inoculated on the indole broth and incubated at room temperature for 7 days. The production of indole derivatives by the isolate was determined by the addition of Kovac's reagent. The formation of a red colored ring in the inoculated indole broth tubes indicated a positive reaction. Table 3.4 shows the reagents and medium used for Indole Production.

Table 3.4 Reagents and medium used for Indole Production

S.No	Physiochemical Test	Reagent/Medium Used
1.	**Indole Production**	Peptone – 20 .0 g NaCl- 5.0 g Sea water- 1000.0 ml pH- 7.0 **Kovac's reagent** p- dimethylaminobenzaldehyde- 5.0 g Isoamyl alcohol- 75.0 ml Con. HCl- 25.0 ml

3.11.2.6 MR-VP test

The isolate was inoculated into the tube containing 5 ml of MR-VP medium and incubated for 7 days at room temperature. The volume of the culture in the tube was divided equally between 2 tubes. To one of the tubes , few drops of methyl red reagent was added and to the another , Barrits A and Barrits B (for VP test) were added in the ratio of 6:1 and left open for 30 sec to react with oxygen in air. The appearance of red colour by addition of MR reagent was a positive reaction and indicates the production of acids by *actinomycetes* isolate. Development of crimson's red to ruby pink by addition

of VP reagent indicated a positive reaction for VP test. Table 3.5 shows the reagents and medium used for MR-VP test.

Table 3.5 Reagents and medium used for MR-VP test

S.No	Physiochemical Test	Reagent/Medium Used
1.	**MR-VP test**	**MR- VP medium** Peptone- 7.0 g Dextrose -5.0 g K_2HPO_4- 5.0 g Seawater -1000.0 ml pH- 7.0 **Methyl red reagent** Methyl red- 1.0 g Ethanol- 300.0 ml Distilled water- 200.0 ml Voges Proskauer (VP test) **Barrits A reagent** α- naphthol- 5.0 g Ethanol- 95.0 ml **Barrits B reagent** KOH- 40.0 g Creatine – 0.3 g Distilled water- 100.0 ml

3.11.2.7 Citrate utilization

Actinomycetes isolate was streaked on simmon's citrate agar slants. It was then incubated at room temperature for 48 hrs. The changes in the medium color were observed. The change of color from green to Persian blue

indicates the ability of the *actinomycetes* isolate to utilize the citrate as carbon source. Table 3.6 shows the reagents and medium used for Citrate Utilization.

Table 3.6 Reagents and medium used for Citrate Utilization

S.No	Physiochemical Test	Reagents/Medium Used
1.	**Citrate Utilization**	Simmon's citrate agar NH_4HPO_4- 1.0 g K_2HPO_4-1.0 g NaCl-5.0 g $MgSO_4.7H_2O$-0.2 g Bromothymol blue-0.08 g Sodium citrate-2.0 g Agar-18.0 g Sea water-1000.0 ml pH-6.8

3.11.2.8 Urease production

The production of urease by the *actinomycetes* isolate on Christensen's urea agar medium was tested. Urea solution of 40% was prepared in distilled water and sterilized using filter. About 5.0 ml of the aliquot of urea solution was added to 100 ml of molten medium and poured into test tubes. The culture was streaked on to slants, incubated at room temperature for 48 hrs and the change in the medium color was observed. The change of medium colour from orange to deep pink indicates the production of Urease. Table 3.7 shows the reagents and medium used for Urease Production.

Table 3.7 Reagents and medium used for Urease Production

S.No	Physiochemical Test	Reagents/Medium Used
1.	**Urease Production**	Peptone-7.0 g Glucose-1.0 g NaCl- 5.0 g K_2HPO_4- 2.0 g Phenol red-0.12 g Agar-18.0 g Sea water- 1000.0 ml pH- 6.8

3.11.2.9 Melanin production

The above medium was prepared and poured into petriplates. A loopful of *actinomycetes* isolate culture was streaked on the surface of medium and incubated at room temperature. After 14 days of incubation, the plates were observed for every 12 hrs up to 5 days. Indication of deep brown, greenish brown, greenish black or black color was recorded as melanin positive. Absence of brown to black color, or total absence of diffusible pigment was considered as negative for melanoid pigment. The inoculated tubes were compared with uninoculated controls. Table 3.8 shows the reagents and medium used for Melanin production.

Table 3.8 Reagents and medium used for Melanin production

S.No	Physiochemical Test	Reagents/Medium Used
1.	**Melanin production**	Nutrient agar -100.0 ml Tyrosine- 0.5 g

3.11.2.10 H_2S production

The *actinomycetes* isolate was inoculated into yeast malt extract agar slants for 14 days. Observations for the presence of characteristics of the substrate color were made for every 12 hrs to 4 days and at 24 hours interval up to 15 days.

3.11.2.11 (TSI) Triple sugar iron test

The TSI agar was prepared and *actinomycetes* isolate was inoculated in the TSI slants by stabbing the butt and streaking in the slants. The tubes were incubated at room temperature for 6 days and examined for the production of acid, gas and H_2S. Acid butt, alkaline slant (yellow butt, red slant) indicated glucose fermentation. Alkaline butt, acid slant (red butt, yellow slant) indicate that there was no fermentation occurred. Acid butt, acid slant (yellow butt, yellow slant) indicated the lactose or sucrose fermentation. Blackening of the butt indicated H_2S production and appearance of bubble in the butt indicated gas production.

3.11.2.12 Oxidase test

A loopful of *actinomycetes* isolate culture was rubbed over the oxidase disc with the help of the clean glass rod. Then the color change in the oxidase disc was noted. Indication of color change to blue or purple of the oxidase disc within 5-10 sec indicated the production of oxidase.

3.11.2.13 Catalase test

A loopful of *actinomycetes* isolate culture was transferred from agar plate to a clean glass slide. Immediately a drop of 3% hydrogen peroxide was added onto the culture and quick evolution of air bubbles was noted indicating the production of catalyse by the organism.

3.11.2.14 Nitrate reduction test

Actinomycetes isolate was grown in tryptone nitrate broth for 5 days. About 0.5 ml of sulphanilic acid and α-naphthylamine reagent were added and then observed for the development of red color which indicated the ability of organism to reduce the nitrate. Table 3.9 shows the reagents and medium used for Nitrate reduction test

Table 3.9 Reagents and medium used for Nitrate reduction test

S.No	Physiochemical Test	Reagents/Medium Used
1.	**Nitrate reduction test**	**Tryptone nitrate broth** Tryptone-20.0 g Dextrose-1.0 g KNO_3-1.0 g Agar-1.0 g Sea water- 1000.0 ml pH- 7.2 **Reagents** **Solution A** Sulphanilic acid-0.008 g 5 N Acetic acid-10.0 ml **Solution B** α-naphthylamine-0.005 g 5 N Acetic acid- 10.0 ml

3.11.2.15 Xanthine/hypoxaanthine/esculin/arbutin hydrolysis

The above medium was prepared and poured into the petriplates. A loopful of *actinomycetes* isolate culture was streaked on the surface of medium and incubated at room temperature. After 14 days, the plates were

observed for hydrolysis of different substrates and the results were recorded. Table 3.10 shows the reagents and medium used for Xanthine /Hypoxaanthine /esculin/arbutin hydrolysis

Table 3.10 Reagents and medium used for Xanthine /Hypoxaanthine /esculin/arbutin hydrolysis

S.No	Physiochemical Test	Reagents/Medium Used
1.	**Xanthine/Hypoxaanthine/esculin/arbutin hydrolysis**	Nutrient agar- 100.0 ml Xanthine/Hypoxaanthine/esculin/arbutin -0.5 g

3.11.2.16 Gelatin/casein/cellulose/starch/tween 20/tween 80 hydrolysis

The above medium was prepared and poured into the petriplates. A loopful of *actinomycetes* isolate culture was streaked on the surface of the medium and incubated for 7 days at room temperature. The results were recorded for Gelatin/Casein/Cellulose/Starch/Tween 20/Tween 80 hydrolysis with respective indicators. Table 3.11 shows the reagents and medium used for Gelatin/Casein/Cellulose/Starch/ Tween 20/Tween 80 hydrolysis.

Table 3.11 Reagents and medium used for Gelatin/Casein /Cellulose /Starch/ Tween 20/Tween 80 hydrolysis

S.No	Physiochemical Test	Reagents/Medium Used
1.	**Gelatin/Casein/Cellulose/Starch/Tween 20/Tween 80 hydrolysis**	Peptone- 10.0 g Beef extract- 10.0 g NaCl- 5.0 g Gelatin/Casein/Cellulose/Starch/ Tween 20/Tween 80- 10.0 g Agar- 18.0 g Seawater- 1000.0 g pH- 7.0

3.11.2.17 Chitin/ pectin hydrolysis

A loopful of *actinomycetes* isolate was streaked onto the medium and incubated for 7 days at room temperature. The plate was flooded with methyl red (0.5%) solution for 3- 5 min and observed. The clear zone around the colony against reddish white background indicated the hydrolysis of chitin / Pectin and the results were recorded. Table 3.12 shows the reagents and medium used for Chitin/ Pectin hydrolysis.

Table 3.12 Reagents and medium used for Chitin/ Pectin hydrolysis

S.No	Physiochemical Test	Reagents/Medium Used
1.	**Chitin/ Pectin hydrolysis**	Peptone – 10.0 g Beef extract- 10.0 g NaCl- 5.0 g Colloidal chitin/Pectin- 30.0 g Agar- 18.0 g Sea water-1000.0 ml pH- 7.0

3.11.3 Molecular Approach

The most powerful advanced approach to taxonomy is through the study of nucleic acids. Because these are either direct gene products or the genes themselves and evaluation of nucleic acids give in extensive information about true relatedness. Significance of phylogenetic studies based on 16S rDNA sequences is increasing in the systematics of bacteria and *actinomycetes* (Yokota 1997). Sequences of 16S rDNA have provided actinomycetologists with a phylogenetic tree that allows the analysis of evolution of *actinomycetes* and also provides the basis for identification.

3.11.3.1 16S rRNA sequencing

DNA extraction, PCR (Polymerase chain reaction) and sequencing of 16S rRNA genes were done according to modified procedure of (Kim et al 1998). The specific primers of 493F (5-CAAGCGTTGTCCGGAATTAT-3) and 777 R (5-TAGTTCCCAACGTTTACGGC-3) was used for the amplification of 16S rRNA sequence. The nucleotide sequence of the marine isolate was determined and compare to the similarity level with reference species of *actinomycetes* in the genomic data bank. The BLAST (Basic local alignment searching tool) program was employed to determine the similarity of the sequences and the sequence similarity search was performed using BLAST (Altschul et al 1997). Sequences of closely related taxa were retrieved, aligned using Clustal X programme (Thompson et al 1997).

Multiple sequence alignment of nucleotide sequences was performed using CLUSTAL W software (Thompson et al 1994). Bootstrap analysis was performed to assess the confidence limits of the branching (Felsenstein 1985). Phylogenetic analysis was carried out using MEGA (Molecular Evolutionary Genetics Analysis) software version 6. The distances between the sequences were calculated using Kimura two – parameter model (Kimura 1980) and the phylogenetic tree was constructed using the Neighbour – joining method (Saitou & Nei 1987). The resultant unrooted tree topologies were evaluated by bootstrap analyses based on 1000 replications (Felsenstein 1985) of the Neighbour – joining dataset, using options of the MEGA 6 package (Tamura et al 2007).

3.12 OPTIMIZATION OR STANDARDIZATION OF FERMENTATION CONDITIONS

3.12.1 CCD using RSM

In order to optimize a particular fermentation process, statistical methods provide an alternative methodology by considering the mutual interactions among the variables and to give an estimate of the combined effects of these variables of fermentation process (Aravindan et al 2007, Lim et al 2005). The factors influencing the secondary metabolites production were optimized using RSM with the important class of second order design called CCD. A CCRD (central composite rotatable design) with 5 variables was followed to examine the response pattern and to determine the optimum synergy of variables in the fermentation process. CCD is the most common experimental design used in RSM and the design exhibits equal certainty in all directions from the centre. Optimization studies were carried out by considering the effect of five variables such as, temperature, pH, incubation period, agitation and salinity. Performing the statistically designed experiments, estimating the coefficients in a mathematical model, and predicting the response and checking the adequacy of the model are the steps involved in the optimization (Montgomery 2001, Box et al 1978, Murthy et al 2000, Xin et al 2005). The F-test ANOVA (Analysis of variance) was checked for the statistical significance of model equation. Experimental data was analyzed via RSM in order to fit the following second order polynomial equation. Second order coefficients were generated via regression equation. The quality of the fit of models was evaluated using the coefficients of determination and analysis of variance. CCRD design leading to 50 runs of experiments was performed to determine the effect of these parameters.

The R^2 (coefficient of determination) and adjusted R^2 (Adj R^2) were used to determine the significance of the second-order polynomial model. The

final model was displayed as three-dimensional response surface plots by varying two factor levels while keeping the third one at “0” level.

3.12.1.1 Software and data analysis

The design of experiments, analysis of the results and prediction of the responses were carried out using Design-Expert Software (Version 7.0).

3.12.1.2 Verification and validation of the CCD model

The responses were determined under the recommended conditions of the extraction and performed. Finally, the predicted values were compared with the experimental value in order to determine the validity of the model

3.12.2 Preparation of Inoculum

About 1 ml of spore suspension of *S. cirratus* SRP11 was inoculated into 50 ml of seed culture medium in 250 ml flask. The flask was incubated on a shaker at 150 rpm for 5 days at 28 $^{\circ}$C.

3.12.2.1 Growth and secondary metabolites production in *S. cirratus* SRP11 in different liquid media

Initially, *S. cirratus* SRP11 was grown in 100 ml of four different medium such as SCB (Starch casein broth), NB (Nutrient broth), PDB (Potato dextrose broth), MYEB (Malt yeast extract broth) in 250 ml flasks. Each flask was inoculated with 6% of seed culture and incubated on a rotary shaker with the speed of 150 rpm at 28 $^{\circ}$C. The culture was harvested after 7 days and antimicrobial activity against *E. coli*, *B. subtilis*, *A. fumigatus* and *A. flavus* was tested using culture filtrate. The dry weight of the crude secondary metabolites and respective zone of inhibition was measured.

3.12.2.2 Growth and secondary metabolites production in *S. cirratus* SRP11 in different solid media

Four different media such as glucose aspargine agar, MYEA (Maltose yeast extract agar), PDA and NA were prepared and poured into the petriplates. To each culture, equal volume of $CHCl_3$ (chloroform) was added. Solid media cultures were finely chopped with a spatula to ensure proper mixing. Further, the cultures were shaken overnight (100 rpm) at room temperature and consequently filtered using vacuum. The filtrate was stirred for 1 h with 90 ml of $CHCl_3$ and 100 ml deionized H_2O. This mixture was then relocated to a separate funnel and the bottom organic layer was evaporated to dryness in vaccum. The dry weight of the crude secondary metabolites and respective zone of inhibition was measured against *E. coli, B. subtilis, A. fumigatus* and *A. flavus.*

3.12.2.3 Determination of growth and secondary metabolites production in *S. cirratus* SRP11 in MYEB medium

The culture was grown in 50 ml of MYEB in 100 ml flasks. Each flask was inoculated with 3% of seed culture and incubated on a rotary shaker with a speed of 150 rpm at 28 $^{\circ}$C. For every 24 hrs, the culture was harvested and the antimicrobial activity against was tested against *E. coli, B. subtilis, A. fumigatus* and *A. flavus*. The dry weight of the secondary metabolites and antimicrobial activity against pathogenic strains was estimated up to 10 days.

3.12.2.4 Effect of pH (Flowers & Williams 1978)

MYEB medium was prepared with initial pH from 5, 6, 7, 8 and 9. It was dispensed in separate flasks. About 6% of seed culture of *S. cirratus* SRP11 was inoculated in each flask. The cultures were incubated at room temperature on a rotary shaker at 150 rpm. After six days, the cultures were harvested and filtrate was centrifuged at 10, 000 rpm for 10 min. The cell free

supernatant was filter sterilized using 0.2 µM filter paper (Millipore India Ltd.) and antimicrobial activity was measured against *E. coli*, *B. subtilis*, *A. fumigatus* and *A. flavus.*

3.12.2.5 Effect of temperature (Shimizu et al 2000)

About 50 ml MYEB medium was prepared with the initial pH of 7.0. It was dispensed in separate flasks. About 6% seed culture of *S. cirratus* SRP11 was inoculated in each flask. The culture flasks were incubated at different temperatures between 26, 27, 28, 29 and 30 °C. After six days, the culture filtrate was centrifuged at 10,000 rpm for 10 min. The cell free supernatant was filter sterilized using 0.2 µM filter paper (Millipore India Ltd.) and antimicrobial activity was measured against *E. coli*, *B. subtilis*, *A. fumigatus* and *A. flavus.*

3.12.2.6 Effect of incubation period

About 50 ml MYEB medium was prepared with pH 7.0 in different flasks and inoculated with 3% seed culture of *S. cirratus* SRP11 in each flask. The cultures were incubated at 28 °C with the varied incubation periods of 1, 2, 3, 4 and 5 days. The cell free supernatant was filter sterilized using 0.2 µM filter paper (Millipore India Ltd.) and antimicrobial activity was measured against *E. coli*, *B. subtilis*, *A. fumigatus* and *A. flavus.*

3.12.2.7 Effect of agitation

Initially 50 ml MYEB medium was prepared with pH 7.0 in different flasks and inoculated with 3% seed culture of *S. cirratus* SRP11 in each flask. The cultures were incubated at 28 °C, pH 7 with different varied speeds of rpm of 100, 150, 200, 250 and 300 rpm for four days. The cell free supernatant was filter sterilized using 0.2 µM filter paper (Millipore India

Ltd.) and antimicrobial activity was measured against *E. coli*, *B. subtilis*, *A.fumigatus* and *A. flavus.*

3.12.2.8 Effect of salinity or NaCl tolerance test (Tresner et al 1958)

S. cirratus SRP11 was grown in 50 ml of MYEB medium with different concentration of NaCl (2, 4, 6, 8 and 10) at pH 7, 28 °C and 200 rpm on a rotary shaker for four days. The cell free supernatant was filter sterilized using 0.2 µM filter paper (Millipore India Ltd.) and antimicrobial activity was measured against *E. coli, B. subtilis, A. fumigatus* and *A. flavus.*

3.13 GROWTH CURVE OF *S. cirratus* SRP11

The growth pattern of the *S. cirratus* SRP11 was studied to have an idea about the growth pattern of the isolate before the fermentation process. The fermentation growth curve was prepared so that the day in which the culture reaches its stationary phase (phase produce maximum yield of secondary metabolites) of growth is well known. Initially, for plotting the growth curve 50 ml of *actinomycetes* growth medium was prepared containing (Beef Extract 10 g/l, Yeast extract 5 g/l, Tryptose 10 g/l, casein enzyme hydrolysate 4 g/l, Dextrose 5 g/l, L-cysteine hydrochloride 1 g/l, Starch 1 g/l, NaCl 5 g/l, KH_2PO_4 15 g/l, Ammonium sulphate 1 g/l, CaCl 0.02 g/l, $MgSO_4$ 0.20 g/l pH 7.2). It was autoclaved and cooled to room temperature. About 1 ml of 24 hour old grown broth culture of *S. cirratus* SRP11 at pH 7, 28 °C, 200 rpm and 8% was inoculated into 50 ml of *actinomycetes* medium. The flask containing medium alone was maintained as blank. For every 24 hrs the absorbance of inoculated flask was read at 600 nm against the uninoculated flask. A graph was plotted between time in days and absorbance at 600 nm.

3.14 EXTRACTION OF EXTRACELLULAR METABOLITES FROM MARINE *S. cirratus* SRP11

3.14.1 Culture Conditions

S. cirratus SRP11 seed culture (2ml) was inoculated separately into 250 ml erylenmeyer flasks, each containing 50 ml of MYEB. The cultures were incubated on a rotary shaker (200 rpm) at pH 7, 28 °C±2 °C with 8 % NaCl for 5 days. The cultures were harvested, filtered through four layers of cheese cloth centrifuged at 12,000 g for 15 min and the supernatants were collected. The cell free supernatants and the mycelia biomass were used for the extraction of metabolites using organic solvents such as chloroform and ethyl acetate.

3.14.2 Extraction of Extracellular Metabolites

Extraction of extracellular metabolites was carried out by adding chloroform and ethyl acetate to the cell free supernatant of *S. cirratus* SRP11 in the ratio of 1:1(v/v) followed by vigorous shaking for 1 hr and kept overnight at 4 °C. The organic phase was separated and collected in a conical flask and the process was repeated thrice. The solvent layer was separated and preserved. The separated organic phase was dried in rotoevaporator at 40 °C (50 rpm) under vacuum. When the organic phase was dried completely, powdered residue substance was stored in screw capped container at 4 °C.

3.15 CHARACTERIZATION OF CRUDE BIOACTIVE SECONDARY METABOLITES OF *S. cirratus* SRP11

3.15.1 UV and FTIR Spectrophotometric Analysis

The UV spectroscopic analysis was performed for active crude chloroform secondary metabolites extract using U-3900 spectrophotometer. It

was scanned over a range of wavelength (100-350 nm). The spectra plotted as percentage absorbance versus wave length. The FTIR spectrum of the crude chloroform secondary metabolites extract was also detected using spectrum RX-1(Perkin Elmer). The spectrum was scanned in the range of 400 to 4000 cm^{-1}. The spectra plotted as percentage transmittance versus the wave number.

3.15.2 GC-MS Spectrometric Analysis

The powdered, crude bioactive secondary metabolites extract was dissolved in chloroform till it dissolves completely and analyzed by GC-MS-5975C (agilent) using the following condition: Column oven temperature - 70 °C, Injector temperature– 250 °C, injection mode - split, split ratio - 10:1, flow control mode – linear velocity, column dimension - 30 m X 250 m, carrier gas – helium (99.9995% purity) moves at 1 ml/min through the column. MS program: Ion source temperature - 230 °C , Interface temp – 240 °C , Scan range : 40 – 700 m/z , Solvent cut time – 3 min, MS start time - 3 min, MS end time – 35 (min), Ionization - EI (-70ev), Scan speed - 2000. The individual constituents showed by GC were identified by comparing their MS with standard compounds of Nist library.

3.16 BIOLOGICAL ACTIVITIES OF SECONDARY METABOLITES OBTAINED FROM POTENT MARINE *S. cirratus* SRP11

3.16.1 Antimicrobial Studies

3.16.1.1 TLC (Thin layer chromatography)

The crude chloroform extract of *S. cirratus* SRP11 was used for primary analysis of presence of antibacterial and antifungal substances. The separation of antibacterial and antifungal components formulated in the crude

chloroform extract was done. It was performed by thin layered chromatography on silica coated TLC plates by using methanol-chloroform (7:3, v/v) as solvent system. The chromatogram was observed using iodine vapours. The experiment was repeated in triplicates.

3.16.1.2 TLC- contact bioautography

TLC plate was thoroughly dried and was kept in an empty sterile petri plate. About 20 ml of LB agar was prepared, autoclaved and poured on the petriplate. Then, the pathogenic bacterial strain (*M. smegmatis) Mycobacterium smegmatis* was swabbed over the LB agar. In the contact bioautography, the TLC plate was placed on the inoculated agar surface for 60 min to 3 hrs to allow diffusion. Then the plate was removed and the agar layer is incubated at 37 °C for 24 h. After incubation, zone of inhibition around the spot was observed. The sterile zone on the media proved the presence of active antibacterial components in the studied samples (Holt 1994). The R_f values of antibacterial compounds were determined and compared with the reference antibiotics. Similarly the experiment was performed to detect active antifungal components in the chloroform extract of *S. cirratus* SRP11. *C. tropicalis (Candida tropicalis)* was used for the investigation of the antifungal activity of the chloroform extract of *S. cirratus* SRP11. The experiment was repeated in triplicates. The R_f values of the eluted antifungal compounds were determined and compared with the reference antibiotics.

3.16.1.3 Evaluation of chloroform extract of *S. cirratus* SRP11 treated medical gauze cloth for antibacterial and antifungal activity by agar diffusion method

The validation of the antibacterial and antifungal activity of chloroform extract of *S. cirratus* SRP11 was tested in medical gauze cloth.

The cotton gauze cloth with dimension of 7.0×7.0 cm was washed, dried and sterilized before use. The crude chloroform extract of marine isolate was coated in cotton gauze cloth by dip and dry method for 1 hr. Followed by, immersion of gauze cloth in the extract and kept in shaker for 1 hr at 200 rpm. The cotton gauze cloth was air dried and qualitatively evaluated against clinical bacterial and fungal pathogens such as *M. smegmatis* and *C. tropicalis*. The cotton gauze cloth finished with the above preparations was tested for bactericidal and fungicidal activity in agar plate previously swabbed with the bacterial and fungal pathogens. After 24 hrs of incubation, the agar plates were observed for the zone of clearance around the fabric cotton gauze cloth. The clear interrupted area of growth, absence of growth in the cloth underneath and along the size of the finished cotton fabric gauze cloth indicates the antibacterial and antifungal activity of the crude chloroform extract of marine isolate.

3.16.2 Cytotoxicity Studies

3.16.2.1 Determination of cell viability by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay on NIH 3T3 (Mouse embryonic fibroblast) Cell line

About 25 µl cell suspension (5 x 10^3 cells/well) were seeded in each 96 well and incubated at 37 °C for 48 hrs with 5% CO_2 for the formation of confluent monolayer. The monolayer of the cells in the plate was exposed to various dilutions of chloroform and ethyl acetate extract of *S. cirratus* SRP11. The cell viability was measured using MTT assay with MTT (5 mg/ml) and 10% DMSO (Dimethyl sulfoxide) by Igarashi & Miyazawa in (2001). The tetrazolium salt was metabolically reduced by viable cells to yield a blue insoluble formazan product measured at 570 nm spectrophotometerically. Controls were maintained throughout the experiment (untreated wells as cell control and diluents treated wells as diluents control). The assay was

performed in triplicates. The mean value of cell viability was compared to the control to determine the effect of the extract on cells and percentage of cell viability was plotted against concentration of the *S. cirratus* SRP11. The cytotoxicity of crude chloroform extract on normal cells cells were expressed as $IC_{50.}$ The formula used for percentage viability (%) calculation is shown in Formula 3.1.

$$\% \text{ viability} = \text{Absorbance of treated cells / Absorbance of control cells} \times 100 \quad (3.1)$$

3.16.2.2 MTT assay for assessment of cell viability in cancer cell lines

The cancer cell viability to the crude chloroform extract of marine isolate was assessed in HT-29 (Human colorectal adenocarcinoma *cell line*) and HeLa cell lines using MTT assay. Briefly, cells were seeded at a number of 2 X 10^4 per well onto 96-well plates (200 µl/well) in triplicates, allowed to attach and grow for 24 hrs and subsequently exposed to different concentration of crude extract of *S. cirratus* SRP11 µg/ml. At the end of the treatment, the medium was removed and cells were incubated with 20 µl of MTT (5 mg/ml in PBS) in fresh medium for 4 h at 37 °C. After four hours, formazan crystals were formed by mitochondrial enzyme reduction of MTT, were solubilized in DMSO (150 µl/well) and the absorbance was read at 570 nm after 10 min incubation on the iMark Microplate Reader (BioRad, USA). The cytotoxicity of crude chloroform extract on cancer cells were expressed as IC_{50}. The formula used for percentage viability (%) calculation is shown in Formula 3.2.

$$\% \text{ viability} = \text{Absorbance of treated cells / Absorbance of control cells} \times 100 \quad (3.2)$$

3.16.2.3 LDH (Lactate dehydrogenase) leakage measurement

The incubation of HT-29 and HeLa cancer cell lines for 24 hrs in the presence of crude extract of *S. cirratus* SRP11 with different concentrations (10, 50, 100 and 250 µg/ml) was then centrifuged after incubation for 15 min in room temperature. Evaluation of cell pellets of both cancer cell lines for LDH activity was determined. To test LDH activity in cancer cells, cell pellets were washed once with PBS (Phosphate buffered saline) by means of centrifugation at 10,000 rpm for 10 min at 4 °C. The resulting supernatants were discarded and pellets resuspended in a lysis buffer holding a specific protease inhibitor cocktail. After 15 min of incubation, lysed cells were again centrifuged at 10, 000 rpm for 15 min at 4 °C. The protein-containing supernatants were used for LDH activity measurement as described by Comin- Anduix et al in (2002). The assay medium contained 50 mM Tris– HCl (Tris Hydrochloric acid), pH 8, 0.2 mM β-NADH, and 5 mM pyruvate. The oxidation of NADH (Nicotinamide adenine dinucleotide dehydrogenase) was observed as a reduction in 340 nm absorbance at 37 °C. Protein concentration in cell lysates was measured using the Bradford method (Bradford 1976). The percentage of LDH released from the cells was determined using the IU/mg of protein.

3.16.2.4 Caspase-3 activity evaluation

The evaluation of caspase-3 activity in HT-29 and HeLa cancer cell lines were done using the colorimetric kit from Biovision (Milpitas, CA, USA) in agreement with the manufacturer's instructions. The assay is based on the pNA (para nitroaniline) chromophore cleavage from the labeled substrate DEVDpNA by caspase-3. Protein concentration in the cytosolic extracts was measured using the Bradford method using spectrophotometric detection at 405 nm (Bradford 1976).

3.16.2.5 Detection of apoptotic DNA fragments by DNA fragmentation

HT-29 and HeLa cancer cells were grown in the presence or absence of crude chloroform extract of *S. cirratus* SRP11 250 µg/ml up to 72 hrs. Followed by the counting and washing of cells, the cells were harvested for DNA extraction. The DNA samples obtained were cautiously resuspended in TE (Tris-EDTA) buffer. About 2 µg of each sample was loaded onto the respective lanes of 1.5% TAE (Tris-acetate-EDTA) agarose gel. DNA laddering was visualized on a UV transilluminator using ethidium bromide staining.

3.16.2.6 HIF-1α Measurement in HT-29 and HeLa cells

HIF-1**α** (Hypoxia-inducible factor 1-alpha) quantification was performed in HT-29 and HeLa cancer cell lines using enzyme -linked immunosorbent assay kit from Abcam (Cambridge, UK) in accordance with the manufacturer instructions. Protein concentration in the cell extract was measured at 450 nm using Bradford method (Bradford 1976).

3.16.3 Antioxidant Activities

3.16.3.1 DPPH scavenging activity

Antioxidant activity of crude chloroform extract of *S. cirratus* SRP11 was determined by DPPH scavenging assay (Miliauskas et al 2004). Different concentrations of the extract (10, 20, 40, 60, 80 and 100 µg/ml) were dissolved in water, taken in tubes separately. Ascorbic acid was also taken in different concentrations (10, 20, 40, 60, 80 and 100 µg/ml) and taken as a reference standard. 0.002% DPPH was freshly prepared in methanol. About 2 ml DPPH was added to each tube containing different concentrations of extracts (2 ml) and to the standard solution (2 ml). It was then shaken

vigorously. It was then allowed to stand for 30 minutes at room temperature in dark place. The control was prepared without any extracts. Methanol was used for base line corrections in optical density (O.D) of sample measured at 517 nm. The experiments were repeated in triplicates. The Formula 3.3 is used to interpret the value of the percentage of DPPH scavenging activity of crude chloroform extract of *S. cirratus*.

Percentage of DPPH Scavenging Activity (%) = [(Control O.D – Sample/Standard O.D)/Control O.D] × 100 (3.3)

3.16.3.2 Assay of total antioxidant activity

The total antioxidant activity of crude chloroform extract of *S. cirratus* SRP11 was determined according to the method of Prieto et al in (1999). Briefly, 0.3 ml of the crude extract was mixed with 3.0 ml reagent solution containing (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Reaction mixture was incubated at 95 °C for 90 min under water bath. Similarly, all the sample mixtures with different concentrations were measured in the absorbance at 695 nm.

3.16.3.3 FRAP assay

The antioxidant capacity of Starch casein broth (SCB) medium, MYEB, culture filtrate, ethyl acetate extract and crude chloroform extract of *S. cirratus* SRP11 was estimated according to the procedure described by Pulido et al in (2000). 900 µl FRAP reagent was freshly made and was incubated at 37 °C. It was then properly mixed with 90 µl of distilled water and 30 µl of test sample. The same procedure was followed for reagent blank using methanol. The test samples and blank were incubated at 37 °C for 30 min in a water bath. The final strength of the test sample in the reaction mixture was about 1/34. The FRAP reagent containing 2.5 ml of 20 mmol/l

TPTZ (Tripyridyltriazine)solution in 40 mmol/l HCl with 2.5 ml of 20 mmol/l $FeCl_3.6H_2O$ and 25 ml of 0.3 mol/l acetate buffer (pH 3.6). After incubation, the absorbance were measured at 593 nm using a spectrophotometer. Methanolic solutions of known Fe(II) concentration, ranging from 100 to 2000 µlmol/l ($FeSO_4.7H_2O$) were used for the preparation of the calibration curve. The values are expressed as µlmol Fe(II)/ml for the culture filtrates or mg extract.

3.16.3.4 Metal chelating activity

The chelating effect of ferrous ions by crude chloroform extract of *S. cirratus* SRP11 was measured according to the method described by Dinis et al in (1994). Brifly, 200 µl different concentration of the extract (10, 20, 40, 60, 80 and 100 µg/ml) and 740 µl methanol were added into the 20 µl $FeCl_2$ (2 mM). Addition of 40 µl ferrozine (5 mM) into the mixture. It was followed by vigorously shaken well and allowed to stand in room temperature for 10 min. The absorbance was measured at 562 nm. The percentage of inhibition of ferrozine – Fe^{2+} complex formation was calculated using the Formula 3.4.

Percentage of metal chelating activity (%) = [(Control O.D – Sample/Standard O.D)/Control O.D] × 100 (3.4)

3.16.3.5 ABTS $^{*+}$ assay

The antioxidant activity of the crude extract of *S. cirratus* SRP11 was measured by ABTS radical cation decolorization assay according to the method described by Re et al in (1999). The reaction mixture containing 7mM ABTS aqueous solution with 2.4mM potassium persulfate in the dark for 12- 16 h at room temperature were prepared for the production of ABTS. Initially, this solution was diluted in ethanol (1:89 v/v) and equilibrated at 30 $^{\circ}$C to give an absorbance at 734 nm. The addition of 1 ml of diluted ABTS

solution to 10 µl of test sample in ethanol was done. Similarly it was followed for different concentration of the extract (10, 20, 40, 60, 80 and 100 µg/ml). The absorbance was measured at 30 °C accurately 30 min after careful mixing. EDTA was used as standard. The inhibition percentage was calculated for the blank absorbance at 734 nm. The inhibition percentage of free radical by the sample extract was calculated using the Formula 3.5.

Percentage of ABTS radical scavenging activity (%) = [(Control O.D – Sample/Standard O.D)/Control O.D] × 100 (3.5)

3.16.3.6 Assay of superoxide radical scavenging activity

Superoxide radical scavenging activity was measured and determined according to Martinez et al in (2001) for determination of superoxide dismutase. Briefly, 3 mL of reaction mixture containing 50 mM sodium phosphate buffer, pH 7.8, 13 mM methionine, 2 µM riboflavin, 100 µM EDTA (Ethylenediaminetetraacetic acid), NBT (Nitroblue tetrazolium) (75 µM) and 1 ml of the crude extract of *S .cirratus* SRP11 of different concentrations (10, 20, 40, 60, 80 and 100 µg/ml). The final production of blue formazan was followed by observed the raise in absorbance at 560 nm after a 10 min illumination from a fluorescent lamp. The whole reaction set up was covered in a box lined with aluminium foil. The percentage inhibition of superoxide anion production was calculated using the following Formula 3.6.

Percentage inhibition of superoxide anion generation (%)
= [(Control O.D – Sample/Standard O.D)/Control O.D] × 100 (3.6)

3.16.3.7 Assay of nitric oxide-scavenging activity

Briefly, sodium nitroprusside (10 mM), in phosphate-buffered saline was mixed with different concentrations (10, 20, 40, 60, 80 and 100 µg/ml) of

crude extract of *S. cirratus* SRP11 dissolved in water and incubated at room temperature for 150 min. Subsequently, after incubation period, 0.5 ml of Griess reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. The absorbance was read at 546 nm. Ascorbic acid was used as positive control.

3.17 STABILITY OF THE CRUDE SECONDARY METABOLITES EXTRACT OF *S. cirratus* SRP11

3.17.1 Effect of Thermal Stability

To determine the thermal stability of secondary metabolites, 100 µg/ml of crude metabolites extract in water was kept at the temperature of 20, 40, 60, 80 and 100 °C for 1 hr in the water bath. It was cooled and then checked for antimicrobial activity against *M. smegmatis* and *C. tropicalis* by agar well diffusion method.

3.17.2 Effect of pH

To verify the stability of bioactive metabolites in different pH, 100 µg/ml of extract was mixed with 1 ml of 0.1 M phosphate buffer of varied pH (5-13) in different test tubes, incubated for 1 h at 28 °C and the residual was tested for antimicrobial activity against *M. smegmatis* and *C. tropicalis* by agar well diffusion method.

3.17.3 Effect of Shelf Life

The effect of storage time of metabolites extract was determined by storing the dried crude extract at 4 °C for 1, 2, 3, 6 and 12 months. After a specific storage period, 100 µg/ml from each tube was tested for antimicrobial activity against *M. smegmatis* and *C. tropicalis* by agar well diffusion method (Munimbazi & Bullerman 1998).