Material and Methods

Soil Sample Collection

Rhizosphere soil samples were collected from 23 sites of undisturbed forest area of Courtallam region in Western Ghats, Tamilnadu and transferred to sterile containers. The containers were labeled, transported to the laboratory and stored at room temperature for 2 days to minimize the contaminant microbial community.

Determination of pH and Dry Weight of the Soil Sample

Soil pH was measured at a soil: water ratio of 1:2.5 (weight /volume). Air dried soil (10g, with < 2 mm size) and 25 ml of deionised water were shaken together for a minute, left to settle for 30 min and the procedure was repeated. The pH was determined using a pH electrode (Pietri and Brookes, 2008).

Aliquots of freshly sieved soil were accurately weighed and dried at 105°C for 3 days. The samples were then weighed and allowed to cool to room temperature in a desiccator. The factor for conversion of fresh weight to dry weight of soil was calculated and the results were expressed on unit dry weight (Janssen et al., 2002).

Isolation of Actinomycetes

Isolation and enumeration of Actinomycetes present in soil sample was performed by serial dilution and spread plate technique using starch casein nitrate agar. After sterilization of the medium, cycloheximide was added to inhibit the fungal growth at a concentration of 50 μg/ml prior to pouring into the plates. The soil sample were serially diluted up to 10^-6 dilution and from each dilution 0.1ml of the diluted sample was spread onto the starch casein nitrate agar medium using a sterile glass rod. The plates were incubated at room temperature for 7-10 days (Williams and Gross, 1971). After incubation, dry, elevated, earthy odor, white and different colored colonies were taken into account. These colonies were sub-cultured in sterile Bennett's agar plates with 50 μg/ml cycloheximide. The sub culturing proceeded until a pure form of Actinomycetes was obtained and the pure colonies were stored in Bennett's agar slant.
Screening of Actinomycetes for Antimicrobial Activity

The Actinomycetes were screened in two ways viz., primary screening by Giant colony method and secondary screening by submerged fermentation process. In Primary screening, the determination of antimicrobial activities of pure Actinomycetes cultures was performed using giant colony method. In secondary screening, the determination of the antimicrobial activity was determined by agar well method against test organisms.

- **Giant Colony Technique for Antimycobacterial Activity**
  
  Each of the Actinomycetes isolates was streaked in a narrow band across the center of Muller Hinton agar (Tween 80 and Glycerol) plates. The plates were incubated at room temperature for 7-10 days. The bacterial cultures like *Mycobacterium smegmatis* and *Mycobacterium vaccae* were used as target organisms. These organisms were inoculated into the Muller Hinton containing with 0.02 % Tween 80 and 1% Glycerol and incubated at 28°C for 48 hrs. The culture suspension was compared with McFarland Nephelometric standard 0.5 and adjusted with saline. Then the test organisms were streaked from the edges of the plate to the Giant colony without touching the colony. Again the Muller Hinton Agar plates were incubated at 28°C for 2 to 3 days. After incubation, the length of no growth line was measured in millimeter from the edge of the Giant colony to the tip of the growth of the test organism (Lazzarini *et al*., 2000).

- **Giant Colony Technique for Antibacterial activity**
  
  The bacterial cultures like *Staphylococcus aureus*, *Bacillus subtilis*, *Aeromonas hydrophila*, *Escherichia. coli* and *Pseudomonas aeruginosa* were inoculated into the Nutrient broth incubated at 37°C for 24 hrs. The culture suspension was compared with McFarland Nephelometric standard 0.5 tubes and adjusted with saline. (Baron and Finegold, 1990). The test organisms were streaked from the edges of the plate to the Giant colony but not touching the colony. Again the Muller Hinton Agar plates were incubated at 37°C for 24 hrs. After incubation, the length of the length of no growth line was measured in millimeter from the edge of the Giant colony to the tip of the growth of the test organism (Shomura *et al*., 1980).
• **Submerged Fermentation Process**

  Antibacterial activities were tested against different organisms, such as *Aeromonas hydrophila, Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis, Mycobacterium smegmatis, Mycobacterium vaccae* and *Staphylococcus aureus* by agar well plate method.

  The Actinomycetes were inoculated into 100ml of seed medium (Bennett's broth) taken in a 250ml Erlenmeyer flask. The flasks were shaken for 3-7 days in the Orbital shaker at 28°C at 160 rpm. From the seed medium the inoculum was taken and inoculated into Antibiotic production medium and kept in shaker at 160 rpm at 28°C for 11 days. After fermentation the broth and mycelium was extracted.

**Extraction of Antimicrobial Secondary Metabolites**

• **From Fermented Broth**

  Antimicrobial compounds were extracted from the filtrate by solvent extraction method of Kim *et al.* (1999b). After incubation, fermented broth was filtered through a cellulose filter paper. Ethyl acetate was added to the filtrate at equal volume and shaken vigorously for an hour for complete extraction. The ethyl acetate phase containing antibiotic substances was separated from the aqueous phase using a separating funnel. The lower aqueous layer was discarded. The upper solvent layer was retained and concentrated. It was concentrated and used to test the antimicrobial activity against the test organisms.

• **From Mycelia**

  The separated mycelia were suspended in equal volume of distilled water, mixed well and stored at –20°C. It was freeze thawed for 4-5 times to break the mycelia to flush out the intracellular secondary metabolites. The extracted compounds were used for antimicrobial assay.

**Detection of Antibacterial Spectrum by Well Plate Method**

  The antimicrobial activity was determined by agar well plate method (modified producer of Thakur *et al.*, 2007). The partially purified extract was obtained by evaporating the ethyl acetate extract of spent medium and water extract of mycelia. For antibacterial activity, test organisms were inoculated into nutrient
broth and incubated at 37°C for 24 hrs in a shaker. The inoculum was prepared and adjusted to match with McFarland Nephelometric Standard 0.5 \(10^8\) CFU ml\(^{-1}\) tube and swabbed over the surface of Muller Hinton agar plates. The wells were made on the agar plate using a cork borer. The extracts of broth (50 µl) and mycelia (50µl) were added separately to the wells. The plates were incubated at 37°C for 24 hrs. After incubation, the zone of inhibition was measured along with the diameter of the well.

**Identification of Antibiotics Produced Based on their Mode of Action**

The selected isolates were analyzed for the types of antibiotics produced following the guidelines of IMTECH (1996) Actinomycetes – Laboratory Manual, India.

- **Detection of MLS (Macrolide-Lincosamide and Streptogramin) Class of Antibiotics**

  Muller Hinton agar plates were prepared and *S. aureus* MLS-16 (MTCC 2940) culture was swabbed on to the plates. In each plates, 8-10 wells were made as described above. Two MHA plates were used, in which one received Erythromycin (Macrolide antibiotic) pre-induction and the other plate without such pre-induction. Paper disc impregnated with 1µg of erythromycin was placed near to the well and incubated for an hour to induce resistance. After the incubation period, the extract of fermented broth and mycelia was added and incubated at 37°C for 24 hrs. The inhibitory zone around the well was analyzed. The changes in the zone morphology with and without pre-induction were analyzed (IMTECH, 1996).

- **Detection of Glycopeptides / Cell-Wall Binding Antibiotics**

  *S. aureus* (MTCC 96) suspension (2%) was heat killed (80°C for 30 minutes) and seeded in Muller Hinton agar plates. Live *Micrococcus luteus* (MTCC 106) (0.5%) was swabbed in both *S. aureus* seeded MHA media and plain MHA plates. Wells were made and 50µl of extracted products were loaded and incubated at 37°C for 24 hrs. After incubation, the zone of inhibition in *S. aureus* seeded MHA plates was compared to plain MHA plates.
• **Detection of Beta-Lactam Antibiotics**

  Parent strain *E. coli* (MTCC 739) and *E. coli* ESS2231 supersensitive to \(\beta\)-lactam class of antibiotics cultures (MTCC 2939) were swabbed on Muller Hinton agar plates separately. Wells were made and loaded with microbial extracts and incubated at 37°C for 24 hrs. After incubation, the zone of inhibition in both plates was compared.

• **Antifungal Activity**

  The antifungal activity was assessed for selected Actinomycetes isolates. Primary screening was done as described for bacteria with slight modification. The isolates were primarily screened against fungi by giant colony method. *C. albicans*, *S. cerevisiae* and *Aspergillus* sp. were utilized as target fungi.

**Spectral Analysis of Actinomycetes Extracts**

  Ethyl acetate and aqueous extracts of Actinomycetes were subjected to spectral analysis in UV-VIS and infrared spectral wavelength. The compounds were analyzed using UV-VIS spectrophotometer (Shimadzu, Japan) and FT-IR spectrophotometer (Shimadzu, Japan). Tabulation of Mohan (2007) for strings versus peaks was used for the detection of various bioactive antibiotic compounds.

**Improvement Strategy for Antibiotic Production (by UV and NTG Treatment)**

  There are two types of agents used to improve the production of antibiotics in wild type Actinomycetes (Smithers and Engel, 1974 & Sanchez and Olmedo, 1974). The agents used were UV radiation and N-methyl-N’-nitro-N-nitrosoguanidine (NTG).

• **Preparation of Spore Suspension**

  Well sporulated plate/agar slant was selected for collecting spores. Required amount of sterile water with wetting agent Tween 80 (0.01%) was added to slant or plate. The surface of growth was gently scraped with an inoculating loop to liberate spores in the water. The suspension was poured into a sterile centrifuge tube and vigorously vortexed for one to two min. The suspension was filtered through
nonabsorbent cotton wool using a filter tube, to remove mycelia and agar pieces. The spore suspension was centrifuged at low speed about 5000 rpm for 20 mins. The supernatant was discarded immediately after the centrifugation to prevent the detachment of pellet from the wall of tubes. The pellet was washed with normal saline and finally suspended in 1ml of sterile distilled water. The spore suspension was stored in the refrigerator for further usage (IMTECH, 1996).

- **UV Treatment**

  The spores of Actinomycetes isolate were collected and stored in distilled water. The spore suspension was counted by viable plate counting method. The spore suspensions were exposed to UV-C irradiation (Philips 30W, India) at a distance of 30cm for 20, 40, 60, 80, 100, 120,140 and 160 seconds (Smithers and Engel, 1974). Precautions were taken to avoid photoreactivation of UV exposed cells as described by Li *et al.* (1992). The survival ratio was calculated by spreading the UV exposed spore suspension and parent strain on Starch casein nitrate agar plates and incubated at 28°C for 5 days in dark. After incubation, the observed colonies were counted, sub-cultured and incubated for 7 days at 28°C.

- **Bioassay of UV Treated Strains**

  The agar plug method of Shirling and Gottlieb (1966) was used to compare the effect of the UV treatment on Actinomycetes and wild type. After incubation, colonies with different morphology were selected for analysis. Selected survivors were patched over the surface of the antibiotic production medium and incubated for 7-9 days at room temperature in dark. After incubation, colonies were lifted along with the agar gel as a disc/agar plug (4mm X 6mm) using a sterile cork borer.

  The agar plugs were placed on to the surface of Muller Hinton agar plates swabbed with *S. aureus* and incubated at 37°C for 24hrs. After incubation, the zones formed by different agar plugs were compared to select efficient mutants.

- **NTG Treatment**

  NTG stock solution was prepared by adding 9mg of NTG in 3ml of distilled water. The stock solution (3ml) was mixed with 27 ml of spore suspension ($10^8$CFU/ml). The time of exposure of spore suspension in NTG was 15, 30, 45, 60,
75, 90, 105, 120, 135, 150 and 165 min. (Sanchez and Olmedo, 1974). At the end of every periodical exposure, 1ml of spore suspension was centrifuged at 10,000 rpm for 10 min aseptically. After centrifugation, pellet was washed in saline, serially diluted, and spread over the starch casein nitrate agar plates and incubated in dark at 28°C for 5 days. After incubation, survival ratio of both viable count of wild type and NTG treated colonies were measured.

**Bioassay of NTG Treated Strains of Actinomycetes Isolate**

After treatment, colonies with different morphology were selected for analysis. Selected survivors were patched over the surface of the antibiotic production medium and incubated for 7-9 days at room temperature in dark. After incubation, colonies were lifted and proceeded as described for UV-treatment assay.

**Identification of Selected Actinomycetes**

- **Gram’s Staining**
  A thin smear from isolate was prepared on a clean grease free glass slide, air dried and heat fixed. Crystal violet solution was flooded on smear and allowed for 1min and washed with running tap water. Decolurizer was flooded and washed immediately. Finally Safranin solution was added, allowed for 1min and washed with water. This smear was air dried and viewed under oil immersion objective.

- **Modified Acid Fast Staining**
  Smear was prepared from Actinomycetes culture on a clean dry glass slide, dried and fixed by gentle heat. The smear was covered with strong carbol fuchsin and heated from lower side until white steam rises from the surface of flooded staining solution. The smear was allowed to cool for about 8 to 10mins. The smear was decolourized by 1% sulphuric acid, washed with water and counter stained with 0.05% malachite green for 30 seconds. After washing, the dry smear was observed under oil immersion lens of the light microscope.

- **Cover Slip Culture Technique**
  Cover slip culture technique is used to study the Actinomycetes morphological features such as arrangement of spores and presence of sporangia as well as to distinguish between aerial and substrate mycelium. The isolated Actinomycetes cultures were streaked on SCN Agar plates. Over the streaked lines
3-4 sterile cover slips were inserted separately at an angle of 45° using a sterile forceps. The plates were incubated at room temperature for 4-6 days. After incubation, the cover slips were removed and placed in clean glass slide containing few drops of 0.05% malachite green. Slide was observed under oil immersion lens of the microscope (IMTECH, 1996).

- **Antibiotic Sensitivity Test**
  The susceptibility pattern of Actinomycetes was identified by the method described by Nakamura et al. (1989). Antibiotic sensitivity was analyzed against erythromycin, penicillin G, chloramphenicol, streptomycin, ciprofloxacin, meropenem and gentamicin. The isolates were swabbed on the surface of Muller Hinton agar and antibiotic discs were placed aseptically. Plates were incubated at 28°C for 5 days. To study the antifungal susceptibility, the isolates were swabbed on potato dextrose agar (PDA) plates eventually. Then antifungal antibiotic discs (Himedia, India) Amphotericin-B (100U), Ketoconazole (10mcg) and Nystatin (100 U) were placed on the surface of the agar and incubated aseptically at room temperature for 3-5 days.

- **Lysozyme Test**
  A lysozyme stock solution was prepared by adding lysozyme to 0.01N HCl at 1mg/ml concentration and filter sterilized. From the stock solution, 5ml was added to the 100ml of sterile Bennett's broth. The isolates were inoculated in respective tubes and incubated at 28°C for 5 days. (Pirouz et al., 1999).

- **Carbohydrate utilization test**
  The Ammonium salt agar medium was used for carbohydrate utilization test. \((\text{NH}_4)_2\text{HPO}_4\) was the main source of nitrogen in this medium. Bromocresol purple is the pH indicator. After 5 to 7 days of incubation, carbohydrate utilization is indicated by the formation of yellow color in the tube (Nonomura, 1974).

- **Chemotaxonomy**
  The cell wall amino acid and sugar in Actinomycetes were identified following the procedure of Shirling and Gottlieb (1966).
Determination of Cell Wall Aminoacids

Dried cells (3mg) were hydrolysed in 6N HCl in a screw–capped vial at 100°C for overnight. It was cooled and filtered through Whatman No 1 filter paper and the filtrate was dried with the help of a vacuum evaporator. The residue was washed repeatedly to remove the acid traces. An aliquot of 3µl of 0.01M of 2, 6-Diaminopimelic acid, and LL-DAP were spotted along with the isolates samples on to TLC plate (Merck- Silica Gel with a pore size of 60 Å) separately. The source of LL-DAP was *Streptomyces aureofaciens* (MTCC 325). Chromatogram was run using the solvent system containing methanol: water: 6N HCl: pyridine (80:26:4:10; v/v) for 3 hours. The Chromatogram was visualized by spraying 0.2% (w/v) ninhydrin solution prepared in 80% acetone. The TLC plates were developed by heating at 105°C for few minutes.

Determination of Cell Wall Sugars

About 50mg of dried biomass was solubilized with in 1ml of 1N H₂SO₄ by incubating at 100°C for 2 hours. The tube was cooled and approximately 0.5g of barium hydroxide/Ba(OH)₂ was added to neutralize the solution. The precipitate/cell debris was spun at 10,000 rpm and the supernatant was placed in the evaporator. The dried material was redissolved in 400µl of water and stored at –20°C. Approximately 5 to 10µl of sample was spotted on to TLC plate (Merck- Silica Gel with a pore size of 60 Å). Chromatogram was run using the solvent mixture acetonitrile: water; 92.5:7.5, v/v for 20min and sprayed with acid aniline phthalate and heated for 5 minutes at 100°C. The Aniline-phthalate reagent was prepared by adding aniline (2ml), phthalic acid (3.3g) in water saturated butanol (100ml). Hexoses appeared as yellowish brown spots whereas Pentoses appeared as maroon spots.

• Molecular Level Identification of Actinomycetes Isolates

DNA Isolation

DNA isolation was carried out by salting out method (Kieser *et al.*, 2000). The selected organisms were grown in Middle brook 7H9 broth and incubated at 28°C for 4 days. From this medium 2ml of broth was collected and centrifuged at
5000rpm for 15min. The mycelium was suspended in 5ml of SET buffer containing 10μl lysozyme solution and incubated for 30-60 min at 37°C. Proteinase K (140μl) was added and mixed well. About 600μl of 10% SDS was introduced into the tube and mixed by inversion. The mixer was incubated at 55°C for 2 hrs. After incubation, 2ml of 5M NaCl was added and mixed by inversion and the solution was cooled to room temperature. To this, 5ml of chloroform was added, mixed by inversion and incubated for 30 min at 20°C. The solution was centrifuged at 6000 rpm for 10 min at 20°C. The supernatant was collected and 0.6 volume of isopropanol was added and mixed by inversion. After 3min, DNA was collected by spooling onto a sealed Pasture pipette tip and DNA was rinsed in 5ml of 70% ethanol. DNA was air dried and dissolved in 1-2ml of TE buffer at 55°C.

**PCR Amplification**

Molecular identification of Actinomycetes was performed as described by Cook and Meyer (2003). PCR was carried out in 50μl of reaction mixture containing 25μl of 2X Taq Master mix, 5μl of Primer (24F), 5μl of Primer(1492R), 10μl of milliQ water, 5μl of DNA template. The primer set used was helpful in amplifying nearly full length of 16s rRNA gene sequences. The PCR program was initially denaturing at 96°C for 2 minutes with 30 cycles (96°C for 45 seconds), annealing (56°C for 30 minutes), extension (72°C for 2 min) and final extension (72°C for 5 min). The PCR products were electrophoresed on 1% agarose gels, containing ethidium bromide to visualize the amplified DNA. Amplification primers:

27F - AgA gTT TgA TCC TGG CTC Ag
1492R - TAC ggT TAC CTT gTT ACg ACT T

**Agarose Gel Electrophoresis**

Agarose gel electrophoresis was carried out in a horizontal matrix of agarose with 1X TAE Buffer. The DNA samples were diluted with 1/6 vol of 6X loading buffer. Samples were loaded on to agarose gel containing Ethidium Bromide (0.8μg/ml) along with a different size DNA standard marker. Electrophoresis was performed at 5V/cm until the run is complete. After completion of electrophoresis, the gel was visualized under UV Transilluminator.
Purification of DNA from PCR
QIAquick Gel Extraction Kit Protocol- Using microcentrifuge

DNA was purified by eluting DNA from the bands in agarose gel by adapting the QIAquick Gel Extraction Kit Protocol by using a microcentrifuge tubes (Qiagen, Germany). Up to 400mg agarose gel piece can be processed in the microfuge tube.

DNA purification was carried out by the method described by the product manufacture.

- The DNA fragment was excised from the agarose gel with a clean sharp scalpel.
- The gel slice was weighed in a colorless tube. Three volumes of Buffer QG were added to 1 volume of gel (100 mg ~ 100 µl).
- The tubes were incubated at 50°C for 10 min or until the gel slice has completely dissolved with intermitted mixing. After the gel slice has completely dissolved, the mixture retained its QG buffer color (yellow). (If the color of the mixture is orange or violet, 10 µl of 3 M sodium acetate should be added to set the pH 5.0. The color of the mixture turns to yellow). The adsorption of DNA to the QIAquick membrane was efficient only at pH \( \leq 7.5 \). Buffer QG contained a pH indicator which is yellow at pH \( \leq 7.5 \) and orange or violet at higher pH, allowing easy determination of the optimal pH for DNA binding.
- One gel volume of isopropanol was added to the sample and mixed well. QIAquick spin column was placed in a 2 ml collection tube and the sample was added to the QIA quick column and centrifuged for 60 sec.
- After centrifugation, filtrate was discarded. Again QIA quick column was placed in the same collection tube. Buffer QG (0.5ml) was added to QIA column and centrifuged for 60 sec.
- Buffer PE (0.75ml) was added to QIA quick column and centrifuged for 60 sec.
- The filtrate was discarded and the QIA quick column was centrifuged for an additional 60 sec.
QIA quick column was placed into a clean 1.5ml microcentrifuge tube. About 30µl of elution buffer was added to the QIA quick membrane. The column was allowed to stand for 60sec. and centrifuged for a minute. The eluted substance was stored at 4°C.

**Sequencing**

The purified PCR products of approximately 1,400 bp in size were sequenced using the following primers;

518F: CCA gCA gCC gCg gTA ATA Cg

800R: TAC CAg ggT ATC TAA TCC.

Sequencing was performed by Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). The sequencing reaction mixture was prepared by adding 1µl of BigDye v3.1, 2 µl of 5X sequencing buffer and 1 µl of 50% DMSO. Sequencing reaction mix (4µl) was added to 4 pico moles of primer (2µl) and sufficient amount of template DNA. The reaction was denatured at 95°C for 5 minutes. Cycling began with denaturing at 95°C for 30 seconds, annealing at 52°C for 30 seconds and extension for 4 minutes at 60°C and cycle repeated for a total 30 cycles in a MWG thermocycler. The reaction was then purified on sephadex plate (Edge Biosystems, USA) by centrifugation to remove unbound labeled and unlabelled nucleotides and salts. The purified products were resolved by loading on to the 96 capillary Biosystems model 3730XL-automated DNA sequencing system (Applied BioSystems, USA) and electrophoresis was carried out for 4 hrs.

**Sequence Contig**

The forward primer generated sequences and reverse primer generated sequences were contiged using DNA Baser, version 3.2.5 as an offline tool. The sequence assembly project was opened in the tool. Both forward and reverse sequences were given as input. To assemble the sequence as a complete set of scaffold was generated by ‘assemble’ command. As a result contige sequence was obtained (Misbah et al., 2005)
Similarity Search – BLAST

BLAST – Basic Local Alignment Search Tool was used to find the sequence similarity of our desire sequence. BLAST is an online tool. To perform BLAST, the URL of the program is “http://blast.ncbi.nlm.nih.gov/Blast.cgi” was entered in the browser. For nucleotide searching ‘BLAST’ program was selected and Query sequence was given through input box. The parameter for searching similarity was set to search in ‘non redundant database’ and hit BLAST button to get result (Misbah et al., 2005).

Multiple Sequence Alignment (MSA)

To perform multiple sequence alignment programs, Multalin - an online tool was used. To perform MSA the URL: http://www.multalin.com was opened. The desire input sequences were saved in a notepad with Fasta format and entire sequences were pasted into the ‘input’ box. The parameter for performing MSA was adjusted. The alignment algorithm ‘Neighbor Joining’ was selected and other parameters were set as default in the tool. After ‘Align’ comment, the results were obtained.

ORF Finder

The URL is ‘http://www.ncbi.nlm.nih.gov/ projects/gorf/orfig.cgi’ for ORF Finder. The desire input search was given in the input box. The parameter for finding ORF in all reading frame was given to obtain the results.

CpG Island Finder

To find the Gene rich G+C content in the sequence, the ‘CpG-Island finder’ an online tool was used. The URL is http://bioinformatics.wistar.upenn.edu/cpg. The desire sequence was given as input. The parameters for detecting C+G rich region was given as default in the tool to predict the regions.
Construction of Phylogenetic Tree

To construct the Phylogenetic tree, the alignment file resulted from Neighbor Joining method of MSA was used as an input. The file obtained from MSA was viewed in the ‘Tree view’ offline tool to know the phylogenetic relationship.

• Effect of Selected Actinomycetes in an in vivo Model

Experimental Animal

Oreochromis mossambicus (Mossambique tilapia), a common fresh and brackish water cichlid fish was obtained from local fisherman and brought to the laboratory in well aerated water tanks. Fish were kept in 300L fiber reinforced plastic (FRP) tanks and were maintained at an ambient, controlled temperature of 28 ± 2°C under natural photoperiod. The water in the FRP tanks was replaced every alternative day to maintain the water quality. The fish were acclimated for 21 days.

Cultivation of Streptomyces flavofungini AM 4

The spores of S. flavofungini AM 4 isolated from soil samples collected from Western Ghats, Tamilnadu, were scraped from the starch casein nitrate agar slant using a sterile saline moistened inoculation loop and were transferred onto 100 ml of Bennett’s broth in a 250 ml Erlenmeyer flask. The flask was incubated at 28°C in an orbital shaker at 200 rpm for five days.

After incubation, 25 ml of inoculum was transferred to 475 ml of Bennett’s broth in 1L Erlenmeyer flask and incubated as previously mentioned for 7 to 10 days. The cells were harvested by centrifugation at 5000rpm for 20 min in a refrigerated centrifuge and washed twice with saline and resuspended in saline. The number of streptomycetes present in saline suspension was determined by counting under Neubauer chamber and later confirmed by serial dilution and spread plate method on Bennett’s agar plates. After 3 days of incubation at 28°C, the numbers of colony forming units were calculated.

Fish Feed Preparation

Normal balanced feed composed of 42g of fish meal, 20g of groundnut oil cake, 15g of tapioca powder, 15g of wheat flour, 5g of blood meal and 3g of mineral-vitamin mixture (per 100g of feed) was used as control diet (protein: 39%,
carbohydrate: 24%, lipid:11% and ash: 9%). All ingredients, except vitamins and minerals were mixed in a container and steam sterilized in an autoclave for 15 minutes. After cooling, vitamin and mineral-mixture were added along with required amount of sterile distilled water before pelletization. The pelletized feed was shade dried. The prepared feed was stored in a moisture free container (Christyabapita, 2007) for further use.

**Safety Testing of *S. flavofungini* AM 4**

To determine the safety nature of Streptomycetes, fish were injected with live suspension of Streptomycetes cells. To test the pathogenicity (if any) of Streptomycetes on *Oreochromis mossambicus* a total of 120 fish (25 ± 5 g fish) were used. The fish were divided among 12 tanks (groups) with 10 fish each. Three tanks were assigned for each dosage of live Streptomycetes challenge. The fish were challenged with 0.2ml of live Streptomycetes at a concentration of either $1 \times 10^7$ or $1 \times 10^8$ or $1 \times 10^9$ CFU through intraperitoneal injection. The control group received 0.2 ml of sterile saline (0.85% NaCl). The mortality rate was recorded daily for 15 days (Aly et al., 2008).

**Preparation of *S. flavofungini* AM 4 Supplemented Fish Feed**

To prepare *Streptomyces* supplemented feed, all ingredients, except vitamins and minerals were mixed and autoclaved 15 minutes at 121°C. After cooling, Streptomycetes cells was mixed at different concentrations of $1 \times 10^7$, $1 \times 10^8$ and $1 \times 10^9$ CFU /g along with mineral-vitamin mixture were added and mixed with required amount of sterile distilled water before pelletization. The pelletized feed was shade dried. The prepared feed was stored in a moisture free container for further use.

**Survival efficacy of *S. flavofungini* AM 4 in Feed**

The experimental feed was analyzed for the presence of Streptomycetes cells by spread plate method after 30 days of feed preparation. Random sample of 1 gram experimental feed from all concentrations ($1 \times 10^7$, $1 \times 10^8$ and $1 \times 10^9$ CFU /g) was subjected to serial dilution. From the diluted sample 0.1ml was spread over the agar plates and incubated at 28°C for 7-10 days (Aly et al., 2008).
**Growth Analysis of Oreochromis mossambicus**

The effect of Streptomyctes on the growth parameters of *Oreochromis mossambicus* was studied using fingerlings weighing 5 ± 0.5 g which were acclimated for 15 days in plastic tank (40 L capacity) with aerator. During acclimation, fish were fed once a day with normal laboratory made fish feed at a rate of 2% of body weight. The fingerlings were kept at ambient, uncontrolled temperature of 28 ± 2º C. The water was replaced on alternative days to maintain recommended water quality parameters. The fingerlings weighing 5 ± 0.5 g were grouped into four groups consisting of 30 fish each per group. Each group received different concentrations (0, 1x10^7, 1x10^8 and 1x10^9 CFU/g) of *Streptomyces flavofungini* AM 4 supplemented feed. Feeding was done for a period of 90 days. At every 15 days interval all fish were weighed to adjust the daily feed rate (to maintain the biomass and feed ratio to 2 % of body weight). The excess feed and faecal materials were siphoned out daily (Eid and Mohamed, 2008).

The initial and final body weight as well as average total gain was calculated following the method of Tolan and Sherif (2007) where as average daily weight gain and Relative Growth Rate (RGR) was estimated following Castell and Tiews (1980).

- Total weight gain (g) = \( Wt_1 - Wt_0 \) (\( Wt_1 \) - final body weight (g); \( Wt_0 \) - initial body weight (g))
- Average daily gain (g) = \( Wt_1 - Wt_0 / T \) (T is the experimental period (day))
- Relative Growth Rate (RGR) = \( \frac{\text{Final mean mass} - \text{initial mean mass}}{\text{Initial mean mass}} \times 100 \)
- Specific Growth Rate (%/d) = \( \frac{(\ln Wt_1 - \ln Wt_0 / T) \times 100}{(\ln - \text{natural logarithm of final and initial body weight respectively; } T - \text{experimental period (day) according to Pouomogne and Mbongblang (1993).}}

**Haematology**

The use of haematological parameters as fish health indicators was proposed by Hesser (1960). Haematology is used as an index of fish health status in a number of fish species to detect physiological changes following different environmental conditions. Fish weighing 25 ± 5 g were used for all haematological studies. The fish were randomly divided into four equal experimental groups with 8 fish each \( \{i.e. \text{ four treatments: Control (Group I), Experimental group II (1x10}^7 \text{ CFU/g of feed)}, \)}
Experimental Group III (1x10^8 CFUg^-1 of feed) and Experimental Group IV (1x10^9 CFUg^-1 of feed). Blood samples were collected from all groups after 7, 14, 28, 35 and 42 days of experimental period.

**Haemoglobin Content**

The Sahli’s method was used to measure the concentration of haemoglobin in the blood as described by Cavill *et al.* (1981). Fish were anesthetized in water buffered with sodium bicarbonate and the anesthetic MS-222 (Sigma, USA) at a concentration of 25mg l^-1 (Green, 1979). The fish became quiescent in about 20 minutes. The right operculum and the gills were lifted using small rod to expose the common cardinal vein. About 100μl of blood was drawn from the common cardinal vein using 1 ml tuberculin syringe moistened with heparin (5000 U/ml) and fitted with 24G needle (Michael *et al.*, 1994). The blood was transferred into a small microfuge tube.

The graduated Sahli tube was filled with 0.1N HCl up to the 2g mark. Fish blood was sucked with the special Sahli pipette to the 20 µl mark (black ring). The blood was expelled immediately below the surface of the 0.1N HCl in the Sahli graduated tube. The content was mixed thoroughly by drawing the suspension into the pipette and by expelling it several times. The tube was placed in the haemoglobinometer and the set up was incubated at room temperature for 5 minutes allow the breakdown of red blood cells and for haemoglobin release into solution. The colour formed in test sample was compared with the colour of the standard on the haemoglobinometer. Distilled water was added drop by drop until the colour of the tube content matches the colour of the standard. The reading was recorded from the graduated tube. The weight of haemoglobin per 100 ml of blood was calculated.

**Packed Cell Volume (PCV)**

The packed cell volume or haematocrit (Hct) is the volume of packed red blood cells found in 100 μl of blood, recorded as percentage. Centrifuging blood causes the cellular elements to spin to the bottom of the tube, with plasma forming the top layer. Since the blood cell population is primarily red blood cells, the packed cell volume is generally considered equivalent to the red blood cell volume. Often a thin whitish layer can be seen between the clear plasma and red cell mass. This
represents the leukocyte fraction and is called the buffy coat. The Hct was determined by the method described by Korzhuev (1964).

The microfuge tube containing the blood was gently inverted 2-3 times (to avoid blood cells settling at the bottom of the tube). One end of the heparinized capillary tube was slightly inserted into the surface of the blood and allowed to enter into the tube to fill at least three-fourths full by capillary action. The blood-containing end was sealed by pressing it into the plasticine. The capillary tubes were centrifuged for 5 minutes at 10,500 rpm in a micro-haematocrit centrifuge.

Hct was calculated by using the formula of Korzhuev (1964).

\[
\text{Hct} = \frac{\text{Height of column composed by the formed elements (mm) \times 100}}{\text{Height of the original column of whole blood (mm)}}
\]

**RBC Count**

Blood samples were drawn from the common cardinal vein using syringe moistened with heparin (5000 U/ml). While performing red blood cell count, the blood was drawn to the 0.5 mark and the dilution fluid (38g sodium citrate, 20mL formalin (37 %), 0.2g Toluidine blue, 1000 mL distilled water as described by Oliveira-Junior et al. 2009) was drawn to the 101 mark. The mixing chamber holds 100 volumes of fluid (blood and diluting fluid). Therefore, 0.5 volumes of blood and 99.5 volumes of diluting fluid results in a dilution of 0.5 volume of blood in 100 volumes of total fluid, written as 0.5:100 or 0.5/100. The standard expression of this dilution is 1/200 or 1:200. Therefore the normal dilution factor for a red cell count is 200. The red blood cells count (RBC) was performed using Neubauer haemocytometer. The cell count was then corrected for dilution to express the results in cells per cubic millimeter of blood. Mean cell haemoglobin concentration (MCHC), Mean cell haemoglobin (MCH), and Mean cell volume (MCV) were calculated using the formulae of Dacie and Lewis (2001) as given below.

\[
\text{MCHC} \% = \frac{\text{Hb}}{\text{Hct}} \times 100
\]

\[
\text{MCH (pg)} = \frac{\text{Hb}}{\text{RBC}} \times 10
\]

\[
\text{MCV (fl)} = \frac{\text{Hct}}{\text{RBC}} \times 10
\]
The mean cell haemoglobin concentration (MCHC) is the haemoglobin concentration per 100 ml of packed erythrocytes.

Mean cell Haemoglobin (MCH) content is the average weight of haemoglobin in individual erythrocyte, expressed in (pg).

Mean Corpuscular Volume (MCV) is the average volume of individual erythrocyte in mm$^3$, expressed in femtoliter (fl).

**Total White Blood Cell Count**

The method of Blaxhall and Daisley (1973) was used for total cell count. A 1:50 dilution of blood was made with WBC dilution solution (Natt-Herrick's Stain (NH). Round shaped leukocytes stained uniformly dark violet. The nucleus of the smaller elliptical thrombocyte stained light violet with faint cytoplasm. A cover slip was placed over a Neubauer haemocytometer. Approximately 10µl of dilute blood solution was drawn into a Pasteur pipette and allowed to touch the tip of the pipette to the edge of the cover slip. Due to the capillary action the diluted blood sample is drawn into the chamber. The haemocytometer was placed under the microscope. The leucocytes occurring in the four corner squares (W) on the grid (a total area of 0.1 mm$^3$) was counted. The dilution was 1:50. The number of cells occurring per mm$^3$ was calculated as shown below.

$$\text{Total WBC} = \text{Number of cells counted in 0.1mm}^3 \times 10 \times (\text{area counted}) \times 50 \times \text{(dilution factor)}.$$ 

**Spleen-Somatic Index (SSI)**

Fish weighing 25 ± 5 g were used for all somatic response studies. After 60 days of feeding with experimental feed, the fish were euthanized and total body weight and spleen weight were typically measured within 30 min. Care was taken to remove fat and connective tissue attached to the spleen. Spleen indices were calculated on total body weight (g). The index is used to assess the immunomodulatory function of streptomycetes (Hadidi *et al.*, 2008).

$$\text{Spleen somatic index (SSI)} = \frac{\text{Spleen weight}}{\text{body weight}}.$$
• **Hepatosomatic Index (HSI)**

  Hepatosomatic Index (HSI) is defined as the ratio of liver weight to body weight. It provides an indication on status of energy reserve in an animal. HSI was calculated by weighing the liver and expressing on total body weight (Everaarts et al., 1993).

  \[
  \text{Hepatosomatic Index (HSI)} = \frac{\text{liver weight}}{\text{body weight}}.
  \]

• **The Specific Immune Responses**

  Fish weighing 25 ± 5 g were used for all immunological assays. The fish were randomly divided into four equal experimental groups with 8 fish each {i.e. four treatments: Control (Group I), Experimental group II (1x10^7 CFU/g of feed), Experimental Group III (1x10^8 CFU/g of feed) and Experimental Group IV (1x10^9 CFU/g of feed)}. A total of 2 set of experimental set up were maintained and one was utilized for anti-SRBC antibody titration and another one was used for Lysozyme assay, scale allograft rejection and differential WBC count experiments. Blood samples were collected from all groups after 7, 14, 28, 35 and 42 days of experimental period.

**Anti SRBC-Antibody Titration**

  For preparation of SRBC antigen, sheep blood was collected from butchers market and immediately transferred into freshly prepared Alsever’s solution at a ratio of 1:3.(Blood:Alsever’s solution). The diluted blood was stored in aliquots of 50 ml and stored at 4ºC. SRBC stored at 4ºC in Alsever’s solution (pH 6.1) were washed thrice in isotonic NaCl solution (0.15 N) and resuspended to the required concentration.

**Immunization**

  The experimental fishes (8/group) were administered intraperitoneally with 0.2 ml of 5% of SRBC on the onset of experiment (priming dose). After priming, on the 3\textsuperscript{rd} day, an augmenting dose of 0.2ml of 25% SRBC was administered intraperitoneally. For secondary antibody response, fish were administered with the same priming and augmenting doses of the antigen on the 60\textsuperscript{th} day after immunization. All groups of fish were provided with the same type of feed throughout the experimental period.
Bleeding and Serum Collection

For bleeding, each fish was individually caught using a dip net and were bled from common cardinal vein using 1 ml tuberculin syringe fitted with 24 gauge needle (Michael et al., 1994). About 200 µl of blood was drawn and collected in serological tubes. The clot was stored at 20ºC overnight. The clot was then spun down at 3000rpm for 10 min. The serum was then incubated at 47ºC for 30 min in a water bath (Sakai, 1981) for complement inactivation. The serum collected was stored in sterile tubes at –20ºC. The fish were bled at regular intervals of 7 days after immunization.

Haemagglutination Assay

For detecting anti-SRBC antibodies, antibody titration was performed in 96 well "V" bottom microtitre plate using agglutination assay (Karunasagar et al., 1997). Fifty microlitres of serum was added to the first well and two fold serial dilutions were made with physiological saline (PS). A volume of 50µl of SRBC suspension (1% SRBC) was added to each well. The microtitre plate was gently shaken for efficient mixing of the reagents and was incubated at 37ºC for 1 hour. The highest dilution of serum sample that showed detectable macroscopic agglutination was recorded and expressed as \( \log_{2} \) antibody titre of the serum.

Scale Allograft Rejection

Scale allograft rejection technique was performed as previously described by Kikuchi and Egami (1983) The fish weighing 25 ± 5g were used for scale allograft rejection and divided into four groups (n=8). Allograft rejection in vertebrates is considered as an example of cell-mediated immune reaction, since T cells have the major role in the reaction. The present procedure involves reciprocal allografts among many fish at a time, with autografts as control. Scale transplantation is performed by inserting scale in the dermal scale pockets after removal of the original scales. Plucking of a scale from its pocket involve the removal of the dermis beneath the epidermis and its capillaries, chromatophores, the osteogenic and fibrogenic cells that invest the scale plates, the scale plate and guanophores lying beneath it.
After 60 days of experimental period, fish were placed in the anesthetic (MS222) solution at a concentration of 25mg/l of water. The fish became quiescent in about 20 minutes.

A shallow watch glass with saline was used to keep one scale briefly during reciprocal grafting.

For allograft, two or three scales are plucked from the dorsal pigmented region of the donor fish and kept in saline.

From the recipient fish 2 to 3 scales from the ventral unpigmented region were removed.

One by one the scales from the saline were inserted into the empty dermal scale pockets in the ventral region of the recipient fish.

Rejection of scale allograft can be observed 4 days after the transplantation. The grafted scales were observed under microscope and the changes were noted.

**Non Specific Immunity**

**Lysozyme Assay**

For Lysozyme assay fish weighing 25 ± 5 g were divided into four groups (control and three experimental groups with 8 fishes in each group). Blood samples were collected from all groups after 0, 7, 14, 28, 35 and 42 days of experimental period. Serum lysozyme activity was estimated using the method of Hutchinson and Manning (1996) in 96-well microplate. *Micrococcus lysodeikticus* (Sigma, Germany) of 0.3mg/ml was suspended in 0.05 M sodium phosphate buffer (pH 6.2), an aliquot of 250µl cell suspension was mixed with 10µl serum to read the absorbance at 490nm soon after mixing. Final reading was recorded after 4 min. The decrease in absorbance was used to calculate the lysozyme activity. One unit of Lysozyme activity was defined as a reduction in the absorbance of 0.001 min⁻¹ (Parry et al., 1965). Difference in OD for 10µl per 4 minutes was converted to 1ml per minute using the following formula:

\[
\text{Difference in OD} \times \frac{1000\mu l \times 1\text{min}}{10\mu l \times 4\text{min}} = \text{Difference in OD} \times 25 \text{ units / ml}
\]
**Differential (WBC) Count**

Whole blood (without anticoagulant) was drawn from the common cardinal vein of *O. mossambicus* with a 1 ml syringe. Immediately after extraction, a tiny drop of blood was carefully placed on a clean defatted slide. Thin uniform film was taken for analysis. The films were allowed to dry and then immersed in methanol for 3 min for fixation. After fixation the film was allowed to air dry and thereafter stained with Giemsa stain. The slide was decanted and the remainder was rinsed by dipping the slide into a glass beaker filled with tap water. The film was left to dry, at room temperature. The stained blood film was examined under oil immersion (x 100) in a microscope. To prevent error arising from uneven distribution of leucocytes, the slide was divided into four segments and 50 leucocytes per segment were counted. Leucocytes were counted in a parallel row commencing from the outside edge of the slide to the inside. A total of 200 leucocytes counted per slide were classified according to their general form and affinity to the dye, identified and recorded in a table as a specific cell type (for example, a lymphocyte or monocyte). By dividing the sum of each type of leucocyte by two, the percentage of each cell type was obtained (Wahbi et al., 2004).

**Overall Disease Resistance Test**

**Virulence assessment of fish pathogen *Aeromonas hydrophila***

Diseased fish were collected from local area to isolate *A. hydrophila*. Fish liver and other internal organs were dissected out and crushed with saline. The grained material was streaked on the sterile Starch Ampicillin Agar surface. The plates were incubated at room temperature for 24 hours. After incubation, yellow colonies were taken for further analysis. The colonies were sub cultured on Nutrient agar (NA) and for confirmation on Aeromonas agar plates. The colony on NA was used for Gram staining and oxidase test. Growth of dark green with black centered colonies which are oxidase positive, Gram negative rods confirm the identity of *A. hydrophila* (Kaper et al., 1979).
A. *hydrophila* was inoculated in nutrient broth and incubated at room temperature. Overnight culture of *A. hydrophila* was centrifuged at 5000rpm for 15 min. The packed cells were washed and the required dose was prepared in phosphate buffered saline. Fish were injected intraperitoneally with $10^3$, $10^4$, $10^5$, $10^6$, $10^7$, $10^8$, $10^9$, and $10^{10}$ CFU of virulent *A. hydrophila* / fish. The minimal dosage exhibiting 75 to 80% mortality was selected for challenge experiments (Ellis, 1998).

**Disease Resistance Test**

Overall disease protection ability of streptomycetes was investigated by challenging the fish with 75 to 80% infective dose ($10^3$ CFU/ fish) of *A. hydrophila*. The fish approximately weighing 25g ± 5 g were randomly divided into four equal experimental groups {four treatments: Control Group I, Experimental group II ($1 \times 10^7$ CFU /g of feed), Experimental Group III ($1 \times 10^8$ CFU /g of feed) and Experimental Group IV ($1 \times 10^9$ CFU /g of feed); each treatment consisted of three groups of 30 fish each}. After 60 days of feeding with experimental feed, the fish were experimentally infected with live suspension of *A. hydrophila* ($10^3$ CFU/ fish) was injected through I.P route. Cumulative mortalities were noted daily for 15 days. The cause of death due to *A. hydrophila* infection was confirmed by re-isolating the organism from liver of dead fish using starch-ampicillin agar (Himedia, India). Relative percent survival (RPS) was calculated by the following formula of Ellis (1998).

$$RPS= \left\{ 1 - (\% \text{ mortality in treated group} / \% \text{ mortality in untreated group}) \right\} \times 100$$

**Wound Healing Experiment**

Fish weighing 25 ± 5 g were used for wound healing experiment. After 60 days of feeding with experimental feed, fish were wounded experimentally and the responses of wound closure were observed for another 3 weeks by feeding the basal and experimental feed respectively. Wound infliction consisted of a small incision measuring 1.5 ± 0.2cm in length and 0.3cm in depth made on each lateral side of the
fish with a scalpel and a plastic guide. The incision penetrated the epidermis, dermis and underlying musculature. Wound from individual fish was monitored from the onset of wounding. Wound healing was calculated by determining the area of the wound in experimental groups compared to control. Measurement of wound closure was as per earlier descriptions (Lim et al., 2004).

**Statistical Analysis**

The results were expressed as arithmetic mean ± standard deviation. Group difference was tested by one-way analysis of variances (ANOVA). Multiple comparisons were made using online Tukey test. All statistical calculations were performed using online statistical tool ‘http://vassarstats.net/anova1u.html’. The level of significance was expressed as $p < 0.05$. 