Isolation, Molecular Characterization and Identification of Antibiotic Producing Actinomycetes from Soil Samples

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ABSTRACT
A total of 9 actinomycetes (FA1-FA9) isolates were isolated from soil and water samples using starch casein agar and actinomycetes isolation agar. Except FA4, all the isolates showed antimicrobial activity against one or more of the bacteria (Escherichia coli, Enterococcus faecalis, Klebsiella pneumonia and Micrococcus luteus) tested by cross-streak method. The actinomycete isolate FA9 exhibited better antimicrobial efficacy for Gram-negative (E. coli) than Gram-positive organism (E. faecalis) did. Antimicrobial activity of the crude extracts obtained from solid state fermentation showed a better zone of inhibition than those obtained by submerged state fermentation. Minimum inhibitory concentration of the crude extract was 0.51µg/ml for E. Coli and K. pneumonia whereas it was 1.02µg/ml for E. faecalis and M. luteus. The free radical scavenging ability (DPPH) of the ethanolic crude extract recorded IC₅₀ value of 24.384. The FT-IR spectral analyses of extracts showed functional groups such as alcohol (O-H), alkanes (C-H), alkene (C = C) and alkyl halide (C-F). The isolate FA9 was Gram positive and the spore chain morphology was simple rectus with a smooth spore surface. The 16S rRNA gene sequence was closely related to Nocardia alba. The RNA secondary structure predictive of 16SrRNA gene of Nocardia alba showed a free energy of −152.4 kcal/mol, threshold energy of −4.0 with Cluster factor 2, Conserved factor 2 and Compensated factor 4. The 16S rRNA gene of Nocardia alba showed restriction sites Alu I, Hae III, Bst EII, etc. A GC content of 58% and AT content of 42% was obtained based on restriction site analysis.

INTRODUCTION
Actinomycetes are an ubiquitous group of prokaryotes widely distributed in natural ecosystem and a variety of man-made environments (Lam, 2006; Debananda et al., 2009). They are Gram positive branching unicellular bacteria that have unique spore-forming abilities and mycelia structures. The colonies are of pastel colours, soil-like odour, hard and stick to the agar. The term actinomycetes was derived from the Greek words “aktis” (a ray) and “mykes” (fungus) (Das et al., 2008). They have a high guanine (G) and cytosine (C) ratio in their DNA (>55 mol %) (Goodfellow and Williams, 1983). These soil organisms possess characteristics common to both bacteria and fungi and yet have sufficient distinctive features to delimit them into distinct category (Kumar et al., 2005). Actinomycetes produce aerial hyphae and differentiate into chains of spores (Meera et al., 2013). The aerial mass are of various colours such as white, grey, red, green, blue and violet series, and the various colours of the substrate mycelia vary from grey to greyish yellow to orange, moderate yellow, pale red greyish orange pin, bluish grey and white (Amit et al., 2011).

Melanoid pigments are greenish brown, brownish black or distant black. Reverse-side pigments are pale yellow, olive or yellowish brown. Soluble pigments are recorded as red, orange, green, yellow, blue and violet. Spore surface are observed under electron microscope and are characterized as smooth, spiny, hairy and warty (Sivakumar et al., 2011). The culture conditions for the isolation include enrichment, selective media such as ISP media (1-7), starch casein agar, Kuster agar and asparagus-glucose agar (Baskaran et al., 2011). Various traditional biochemical tests (Aparanji et al., 2013) and molecular methods like 16s rRNA sequencing followed by BLAST can be done to identify them.
These microorganisms are active in organic matter (carbon and nitrogen) recycling (Thongchai et al., 2003). Many bioactive compounds such as asavermectin (Perscot et al., 1993), enzymes such as amylase, lipase, and cellulases produced from actinomycetes play an important role in therapeutic medicines, food, fermentation, textile and paper industries (Mustafa et al., 2004). They are used as plant growth promoters, biocontrol agents, biopesticides and also as a source of agro-active compounds (Mukesh, 2014). The current study was undertaken to isolate, characterize and identify antibiotic producing actinomycetes from soil samples of Chennai, Tamil Nadu.

MATERIALS AND METHODS
Collection of soil Samples
Soil samples were collected from Stella Maris College Campus, Adyar River and Marina Beach. All the soil samples were air dried for 10 days to reduce Gram negative bacteria. One gram of Adyar River soil was enriched in 100 ml starch casein broth, 1 gm of Marina Beach soil was added to 100 ml of distilled water and 1 gm of soil collected from Stella Maris College Campus were pre-heated at different temperature (55°C, 65°C, 75°C, 100°C) for 15 min to which 100 ml distilled water was added and left in the shaker for 12 hours for the detachment of the spore chains. Serial dilution was done up to 10⁶ and plated on starch casein agar, actinomycetes isolation agar (prepared with 50% sea water for marine sample) supplemented with streptomycin sulphate 50 µg/ml and nystatin75µg/ml. The plates were incubated at 28°C for 7 to 14 days (Baskaran et al., 2011; Jagan et al., 2013).

Primary Screening
The antibacterial activity of pure isolates was determined by cross-streak method on nutrient agar (Lemos et al., 1985). The isolated actinomycetes strains were streaked as a parallel line on nutrient agar plates and incubated at 28°C for 5 day. After observing a good ribbon like growth, Escherichia coli, Enterococcus faecalis, Klebsiella pneumonia and Micrococcus luteus were streaked at right angles to the original streak of actinomycetes and incubated at 28°C. The inhibition zone was measured after 24-48 hr.

Morphological characterization of the Isolate
Morphological characters such as colony characteristics, colour of aerial and substrate mycelium and pigment production were studied by inoculating the isolate FA9 in various media such as glycerol yeast extract agar, oatmeal agar, nutrient agar and yeast maltagar and incubated at 30°C for 7-15 days (Baskaran et al., 2011). Spore surface features were studied under scanning electron microscope (SEM). Gram staining was carried according to the method of Bailey and Scott, 1966.

Cover Slip Culture Method
The arrangement of spore and sporulating structures was examined microscopically using cover slip culture method (Gebreselema et al., 2013). A sterile cover-slip was inserted at an angel of 45°C in the centre of a nutrient agar plate. A loop full of the culture taken from a seven day old culture plate was inoculated at the insertion place. After 7 days of incubation at 30°C, the cover slip was removed and placed upwards on a clean glass slide. The culture was fixed with few drops of methanol for 15 min and washed with running tap water and flooded with crystal violet for 1 min followed by washing and drying. The cover-slip was finally examined under the microscope (100X).

Biochemical Tests
Biochemical tests such as indole production, methyl red(MR), Voges Proskauer (VP), citrate utilization, starch hydrolysis, gelatin liquefaction, casein hydrolysis, urease production, oxidase test, catalase test and coagulation of milk were carried according to International Streptomyces Project(ISP) (Shirling and Gottlieb, 1996). The results were compared with Bergey’s Manual of Determinative Bacteriology.

Assimilation of Carbon Sources
The ability of the isolate FA9 in utilizing various carbon compounds as source of energy was studied by following the method recommended in ISP using carbon utilization medium. Growth was observed by comparing them with positive and negative control.

Secondary Screening for Antimicrobial Activity
The mature spores of the isolate were inoculated in 100 ml of ISP2 broth and incubated at 30°C in rotary shaker at 200 rpm for 15 days. The fermented broth was centrifuged at 5000 rpm at 4°C for 20 min. The supernatant was collected and the antibacterial activities of E. coli, E. faecalis, K. pneumonia and M. luteus were tested. The test bacterial isolates were cultured in nutrient broth at 37°C for 18 hr and the turbidity was adjusted to 10⁻¹ cells using Mac Farland standard and 100 µl of the same was plated on to the plates. Wells were cut using sterile cork borer and various volumes (50 µl, 75 µl and 100 µl) of extract were added. Streptomycin sulphate (1 µg/100 µl) was taken as the positive control. The plates were incubated at 37°C for 24 hr (Baskaran et al., 2011).

Production of Secondary Metabolite from Crude Extract
Submerged State Fermentation
One hundred millilitre of the ISP2 broth was dispensed into 250 ml conical flask, sterilized, cooled and inoculated with 2 ml suspension of pure FA9 isolate and left in the rotary shaker at 200 rpm (Gebreselema et al., 2013) for 15 days. The fermented broth was centrifuged at 5000 rpm at 4°C for 20 min. The supernatant was collected and equal volume of ethyl acetate was added and shaken vigorously at regular intervals. The solvent phase was separated from aqueous phase using separating funnel and evaporated at room temperature to remove the solvent. The crude extracts obtained were stored for further use.
Solid State Fermentation

To one hundred gram of the substrate (red rice grains) 100 ml of distilled water was added and boiled until the rice grains softened. Mineral salt solution was added to the flask and autoclaved at 121°C for 15 minutes (Gebreselema et al., 2013). It was cooled and inoculated with 2 ml of the culture suspension from 15-day-old FA9 isolate grown on oat meal agar and the flasks were incubated at 30°C was 15 days. The completely fermented rice grains were ground in mortar and pestle and allowed to dry for 24 hours at room temperature. One hundred milliliter of ethyl acetate and 100 ml of methanol were added to the extract and placed on rotary shaker at 120rpm for 12 hours. The extract was filtered through Whatman filter paper and the solvent phase was removed by evaporation at room temperature. The crude extract was stored for further studies.

Antimicrobial Analysis of the Crude Extract

The crude extract obtained was weighed and resuspended in 1 ml methanol and a stock solution of known concentration was prepared and used for antimicrobial analysis (Polpass and Solomon, 2013). Antibacterial activities of the crude extract obtained from submersed state and solid state fermentation were tested using agar well diffusion method. Bacterial cultures (E. coli, E. faecalis, K. pneumonia, M. luteus) were cultured in nutrient broth at 37°C for 18 hr. The turbidity of the test bacterial cultures were adjusted to 10^5 cells using MacFarland standard and 100 µl was plated on to the plate. Wells were cut using sterile cork borer and different volumes (50 µl, 75 µl, 100 µl) of crude extracts were added. Streptomycin sulphate (1µg/100µl) was taken as the positive control and sterile distilled water as negative control.

Determination of Minimum Inhibitory Concentration (MIC)

E. coli, E. faecalis, K. pneumonia, M. luteus were used for determination of MIC. One milliliter of nutrient broth was dispensed into 10 test tubes and 2 ml into 11th test tube (broth control). Two milliliter of the crude extract alone was added in the 12th test tube (crude extract broth). One milliliter of crude extract solution was added to test tube 1 and 2 and serial dilution was done from the 2nd up to the 10th test tube. Finally, 1 ml was discarded from the 10th test tube. One hundred milliliter of inoculum was added into test tubes 1 to 10 and incubated at 37°C for 18-24 hours. After overnight incubation, the lowest concentration inhibiting the visible growth of the microorganism was considered as the MIC value (Gebreselema et al., 2013).

Estimation of Radical Scavenging Activity (RSA) by DPPH Assay

The crude extracts of solid state fermentation of actinomycete isolate FA9 were taken at various concentrations (20-80 µg/ml) and make up to 1 ml using methanol. One milliliter of 0.1mM DPPH was added to all the test concentrations and kept in the dark at room temperature for 30 min. The absorbance of the solution was read at 517 nm in UV spectrophotometer. The percentage inhibition and IC50 values were calculated with ascorbic acid as the reference and DPPH as the control. The concentration of dry weight material per millilitre of solvent (µg/ml) that inhibits the formation of DPPH radicals by 50% is defined as IC50 value (Sandhya et al., 2016).

\[
\% \text{ of DPPH radical scavenging activity} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100
\]

Abscontrol is the absorbance of DPPH radical+ethanol
Abssample is the absorbance of DPPH radical + crude extract

The procedures were performed in triplicate. IC50 (concentration providing 50% inhibition) value was calculated graphically using a calibration curve versus percentage of inhibition.

Characterization of Crude Extract by Infrared Spectroscopy (FT-IR)

The infrared (IR) spectra of the crude ethyl acetate extract of the actinomycete isolate FA9 was measured (as KBr discs)) between 400 and 4000 cm⁻¹ on Perkin Elmer 2000 FT-IR spectrophotometer (Arulappan et al., 2012). The functional groups and types of vibrations were assessed based on the peak values.

DNA Isolation and Amplification

The actinomycete FA9 was grown in ISP2 broth for 3 days and the total genomic DNA was extracted according to the procedure of Sambrook et al. (2011). The 16SrRNA gene fragment was amplified using Universal Primers (Actino Specific Forward Primer -5’-CGTATTACACATGCAAGTCGA-3’ and Actino Specific Reverse primer- 5’CGTATTACCGCGCCTGCTGG-3’) (Nilson and Strom, 2002). PCR amplifications were carried out in a final volume of 20µl of reaction mixture containing 10µl of master mix, 1µl of each primer (20 pmol/µl), 1µl (approximately 200 ng) of template DNA and 7µl of sterile Millipore water. Amplification was for 30 cycles (94°C for 5min, 94°C for 40 sec, 52°C for 1 min and 72°C for 90 sec and final extension at 72°C for 10 min). 2µl of the PCR-amplified products was confirmed on 2% agarose gel and the molecular size of the amplified product was determined using a standard 1-kb DNA ladder.

Purification of PCR Products and Sequencing

Amplified DNA products were purified by PCR clean-up kit (Bangalore Genei) before sequencing. Automated sequencing was carried out according to the dideoxy chain termination method (Sanger and Coulson, 1975).

Sequencing Similarities and Phylogenetic Analysis

The complete DNA sequence of the 16SrRNA was analysed and compared using the Entrez search engine (http://www.ncbi.nlm.nih.gov/nuccore/). The BLAST program (www.ncbi.nlm.nih.gov/blast) was employed in order to assess the degree of DNA similarity. Multiple sequence alignment and molecular phylogeny were evaluated using MEGA6. The
phylogenetic tree was displayed using the TREEVIEW program. The 16S rRNA sequences were submitted to the GenBank, NCBI, USA.

Secondary Structure Prediction and Restriction Site

The RNA secondary structure of the isolate FA9 was predicted using Gene BEE online software (http://www.genebee.msu.su/services/ma2_reduced.html) by Greedy method and the restriction sites of the DNA of the strain were analysed by NEB cutter online tool version 2.0 (http://tools.neb.com/NEBcutter2/).

RESULTS AND DISCUSSION

Actinomycetes Isolation and Primary Screening

A total of nine actinomycetes were isolated from the sampling sites. Primary screening results indicated that all the isolates FA1-FA9 showed antibacterial activity against at least one of the bacteria tested, except FA4 which had no activity. Bacterial isolate FA9 exhibited broad-spectrum activity against E. coli, E. faecalis, K. Pneumonia and M. luteus and hence chosen for further studies. Aparanji et al. (2013) also reported that all the 42 actinomycete strains tested had antibacterial activity similar to the present study. The actinomycete isolate FA9 had white colonies on all types of agar used. Ariel mass was white while the substrate mycelium was yellow and no pigments were produced (Figure 1). It stained Gram positive and the spore chain morphology was simple rectus. The spore surface was smooth as identified by SEM (Figure 2). A similar report of branched, filamentous and microspore chain colonies with smooth spore surface morphology has been reported by Imran et al. (2012).

Biochemical Characterization and Assimilation of Carbon Sources

The actinomycete isolate FA9 showed positive for MR-VP, citrate utilization, starch hydrolysis, gelatin liquefaction, casein hydrolysis, urease, oxidase, catalase, coagulation of milk, and negative for indole. Excellent growth was observed in basal media with 1% of maltose (strongly positive (++)) followed by fructose plates (doubtful (±)) whereas sucrose and xylose was not utilized (Table 1).

| Characteristic | Result |
|---------------|--------|
| Spore chain   | Rectus |
| Indole        | -      |
| Methyl Red – Voges-Proskauer | + |
| Citrate utilization | + |
| Starch hydrolysis | + |
| Gelatin Liquefaction | + |
| Casein hydrolysis | + |
| Urease production | - |
| Oxidase | + |
| Catalase | + |
| Coagulation of milk | + |

Carbon utilization

| Carbon | Result |
|--------|--------|
| Maltose | ++ |
| Fructose | ± |
| Xylose | - |
| Sucrose | - |

(+ Positive; - Negative; ± Doubtful).

Antimicrobial Activities

Secondary screening of FA9 showed antibacterial activity against E. coli (11mm), E. Faecalis (6mm), K. pneumonia (1mm), Micrococcus luteus (2mm). In the present study, Gram-negative bacteria were highly susceptible to the crude extracts compared to Gram-positive bacteria, which is in contrast to the reports of Stephen (2014) and Gebreselema et al. (2013). FA9 had a zone of 8 mm against E. coli, 5 mm against E. faecalis, 2 mm against K. pneumonia and 3 mm against Micrococcus luteus at a concentration of 500 µg/ml of crude extracts obtained from submerged state fermentation (Figure 3a).

Fig. 1: Morphology of Nocardia alba (FA9).

Fig. 2: SEM analysis of Spore Surface Morphology of Nocardia alba (FA9).

Fig. 3: Antimicrobial activity of crude extract Nocardia alba (FA9) (A) submerged state fermentation (B) solid state fermentation.

Tara et al. (2009) reported similar antibacterial activity against E. coli ATCC-49132, Proteus mirabilis ATCC-49132, P. vulgaris, K. pneumonia, K. oxytoxa, Staphylococcus aureus ATCC-29213, Bacillus subtilis, Shigella species, Salmonella typhi and Salmonella paratyphi A. FA9 had a zone of 9 mm against E. coli, 6 mm against E. faecalis, 2 mm against K. pneumonia and 3 mm against Micrococcus luteus at a concentration of 500 µg/ml of crude extract obtained from solid state fermentation (Figure 3b). In the current investigation, the antibacterial activity of the crude extract produced in solid state fermentation was high in comparison to the submerged state fermentation (Figure 4).
Fig. 4: Antibacterial Activities of Nocardia alba (FA9).

Minimum Inhibitory Concentration (MIC)

MIC of the crude extract FA9 was 0.51 µg/µl against *E. coli* and *K. pneumonia*, 1.02 µg/µl against *E. Faecalis* and *M. luteus*. Tara *et al.* (2009) reported that isolate K.6.3 had an MIC of 1mg/ml and isolates K.14.2 and K.58.5 had an MIC of 2mg/ml against *S. aureus* ATCC 29213. Hotam *et al.* (2013) recorded an MIC of 2.5 mg/ml against *E. coli* by the isolates AS23, AS33 and AS34.

Estimation of Radical Scavenging Activity (RAS) by DPPH Assay

The percentage inhibitory effect of crude extract (1000 µg/µl) against DPPH was calculated. In the crude extract of FA9, the percentage of inhibition was 24%, with the IC\text{50} value of 24.384±12.95 (Figure 5). In a similar work, Arulappan *et al.* (2012) reported a dose dependent scavenging activity of the ethyl acetate extract.

![FT-IR of Crude Extract of Submerged State Fermentation of Nocardia alba (FA9).](image)

**Table 2:** FT-IR analysis of crude extract of submerged state fermentation of Nocardia alba (FA9).

| Peak Value (Cm\textsuperscript{-1}) | Intensity | Functional Group                  | Types Of Vibration         |
|------------------------------------|-----------|-----------------------------------|----------------------------|
| 3359.18                            | Strong Broad | O-H (Alcohol)                     | Stretching, H-Bonded        |
| 2927.10                            | Strong     | C-H (Alkanes)                     | Stretching                  |
| 1647.28                            | Variable   | C=C (Alkenes)                     | Stretching                  |
| 1033.89                            | Strong     | C-F (Alkyl Halide)               | Stretching                  |

Analysis of PCR Products and Sequencing

The 16S rRNA gene sequence from the strain FA9 was closely related to *Nocardia alba* and was retrieved from GeneBank database using BLAST. The sequence was submitted in the GeneBank and accession number MF063067 was obtained. Phylogenetic tree constructed using Clustal Omega based on 16S rRNA gene sequence in comparison to FA9 showed it was related members of the genus *Nocardia* (Figure 7).

![Phylogenetic tree based on 16S rRNA gene sequence comparison of FA9 (Nocardia alba).](image)

Prediction of Secondary Structure and Restriction Sites Analysis

The RNA secondary structure predictive of 16S rRNA gene of *Nocardia alba* showed a free energy of −152.4 kcal/mol, threshold energy of -4.0 with Cluster factor 2, Conserved factor 2 and Compensated factor 4 (Figure 8). The 16SrRNA gene of *Nocardia alba* showed restriction sites for Alu I, Hae III, Bst UI, etc. and had a GC content of 58% and AT content of 42% (Figure 9).
CONCLUSION

In the present study, Gram negative organism (E. coli) was efficiently inhibited compared to the Gram-positive E. faecalis, as seen from the results of secondary screening and the activity of the crude extracts from solid and submerged state fermentation. Antimicrobial activity of the crude extracts obtained from solid state fermentation showed a better zone of inhibition from those obtained by submerged state fermentation. The MIC value of the Gram-negative E.coli was 0.51µg/ml when compared to Gram-positive E. faecalis (1.02µg/ml). Results of the present investigation indicate that the actinomycete isolate FA9 can be used for the isolation of bioactive compound that can be used as a drug.

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Fig. 8: RNA Secondary structure of Nocardia alba (FA9).

Fig. 9: Restriction sites prediction of Nocardia alba (FA9).

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