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

Fatima Mehaboob Salim[1], S. Aruna Sharmili[1*], J. Anbumalarmathi[1], K. Umamaheswari[2]

[1]Department of Biotechnology, Stella Maris College (Autonomous), Chennai, India.
[2]Department of Biotechnology, University of Madras, Guindy Campus, Chennai India.

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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 antimicrobal activity against one or more of the bacteria (*Escherichia coli*, *Enterococcus faecalis*, *Klebisella 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 IC_{50} 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 16SrRNA gene of *Nocardia alba* showed restriction sites Alu I, Hae III, Bst UI, etc. A GC content of 58% and AT content of42% 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 “atkis” (a ray) and “mykes” (fungus) (Das *et al*., 2008).They have a high guanine (G) and cytosine (C) ratio in their DNA(>55mol %) (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 asparagine–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.

* *Corresponding Author*
Aruna Sharmili. S, Department of Biotechnology,
Stella Maris College (Autonomous), Chennai, Tamilnadu, India.
Email: arsharmilis @ gmail.com

These microorganisms are active in organic matter (carbon and nitrogen) recycling (Thongchai *et al.,* 2003). Many bioactive compounds such 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 $10^{-5}$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 7to 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, Klebisella 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 45ºC 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 ºC, the cover slip was removed and placed upwards on a clean glass slide. The culture was fixed with few drops of methanol for 15min 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 at30°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˚Cfor 18 hr and the turbidity was adjusted to 10^{-5} cells using Mac Farland standard and 100 µl of the same was plated on to the plate. Wells were cut using sterile cork borer and various volumes (50µl, 75µl and 100µl) of extract were added. Streptomycin sulphate (1µg/100µl) 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 200rpm (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 from15-day-old FA9 isolate grown on oat meal agar and the flasks were incubated at 30˚Cwas15 days. The completely fermented rice grains were ground in mortar and pestle and allowed to dry for 24 hours at room temperature. One hundred millilitre 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 1ml 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 submerged 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˚Cfor 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 millilitre of nutrient broth was dispensed into 10 test tubes and 2 ml into 11^{th}test tube (broth control). Two millilitre of the crude extract alone was added in the 12^{th} test tube (crude extract broth). One millilitre of crude extract solution was added to test tube 1 and 2 and serial dilution was done from the 2^{nd} up to the 10^{th} test tube. Finally, 1 ml was discarded from the 10^{th} test tube. One hundred millilitre 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 millilitre 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 IC_{50} 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 IC_{50} value (Sandhya *et al.,* 2016).

%of DPPH radical scavenging activity =

$$\frac{Abs_{control} - Abs_{sample} \times 100}{Abs_{control}}$$

$Abs_{control}$is the absorbance of DPPH radical+ethanol
Abs_{sample} is the absorbance of DPPH radical + crude extract

The procedures were performed in triplicate. IC_{50} (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 and4000 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). The16SrRNA gene fragment was amplified using Universal Primers (Actino Specific Forward Primer -5'-CGTATTACACATGCAAGTCGA-3' and Actino Specific Reverse primer- 5'CGTATTACCGCGGCTGCTGG-3') (Nilson and Strom, 2002). PCR amplifications were carried out in a final volume of 20µl of reaction mixture containing10µlof 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/blst) 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/rna2_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 f 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).

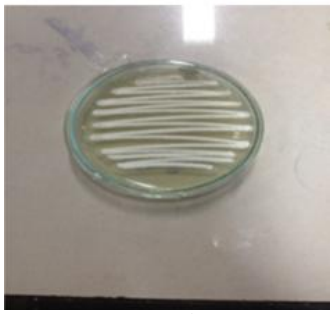

Fig. 1: Morphology of *Nocardia alba* (FA9).

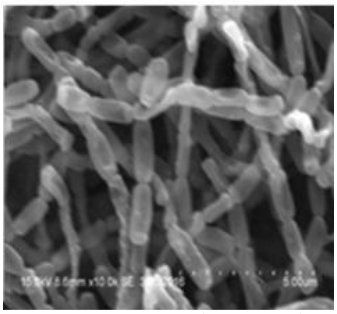

Fig. 2: SEM analysis of Spore Surface Morphology of *Nocardia alba* (FA9).

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).

Table 1: Biochemical Characteristics of *Nocardia alba* (FA9).

Characteristic	Result
Spore chain	Rectus
Indole	-
Methyl Red –VogesProskauer	+
Citrate utilization	+
Starch hydrolysis	+
GelatinLiquefaction	+
Casein hydrolysis	+
Urease production	-
Oxidase	+
Catalase	+
Coagulation of milk	+
Carbon utilization	
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).

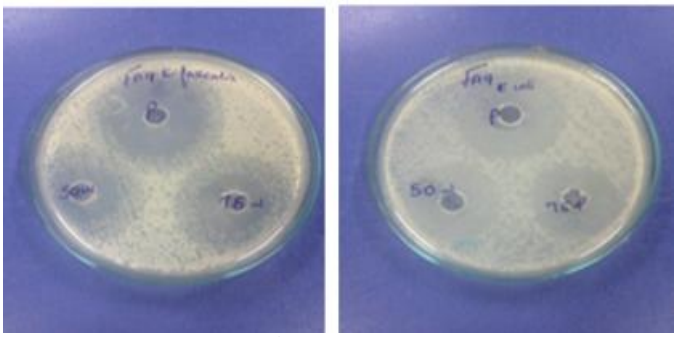

Fig. 3: Antimicrobial activity of crude extract *Nocardia alba* (FA9) (A) submerged state fermentation (B) solid state fermentation.

Tara *et al*. (2009) reported similar antibacterial activity against *E.coli* ATCC-49132, *Proteus mirabils*ATCC-49132, *P. vulgaris*, *K. pneumonia*, *K. oxytoca*, *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).

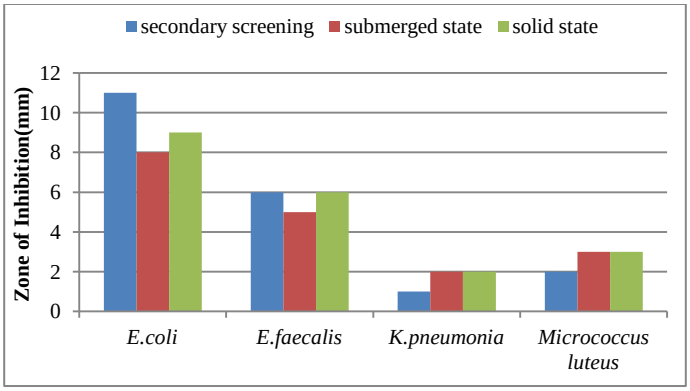

Fig. 4: Antibacterial Activities of *Nocardia alba* (FA9).

Minimum Inhibitory Concentration (MIC)

MIC of the crude extract FA9was 0.51 µg/µl against *E. coli* and *K. pneumonia*, 1.02µg/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_{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.

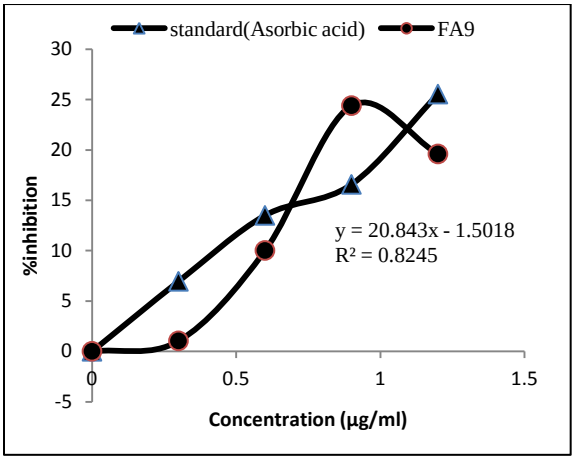

Fig. 5: FT-IR of Crude Extract of Submerged State Fermentation of *Nocardia alba* (FA9).

Characterization of Crude Extracts by Infrared spectroscopy

The FT-IR spectrum of antimicrobial substance produced by FA9 showed peaks at 3359.18cm^{-1},2927.10 cm^{-1},1647.2 cm^{-1}and 1033.89 cm^{-1},corresponding to the following functional groups such as alcohol (O–H), alkanes (C–H), alkene (C=C), alkyl halide (C–F), respectively (Figure 6, Table 2). Similarly, Iman (2014) reported absorbance ranging from 3529 to 3468 cm^{-1} for the isolate streptomyces KGL-13, corresponding to amine group. Arulappan *et al*. (2012) also reported a peak of3398 cm^{-1}that is characteristic of hydroxyl (O-H) and 3 common vibrational peaks between 2899 and 2977 cm^{-1}that are characteristic of a υ (C-H) symmetrical vibration of saturated hydrocarbon in their study.

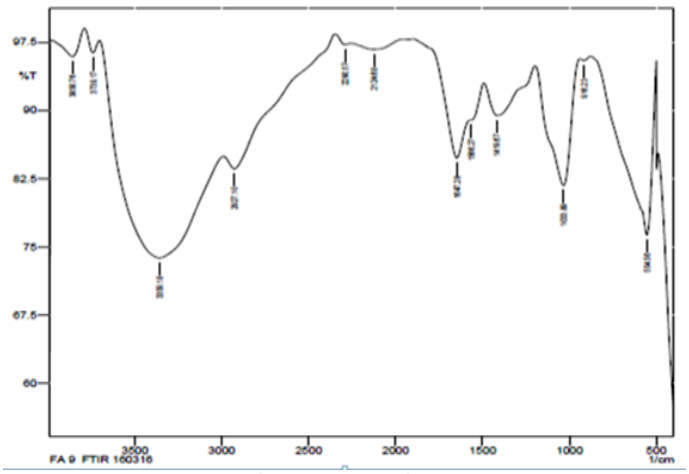

Fig. 6: FT-IR of Crude Extract of submerged state.

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

Peak Value (Cm^{-1})	Intensity	Functional Group	Types Of Vibration
3359.18	Strong Broad	O-H (Alcohol)	Stretching,H-Bonded
2927.10	Strong	C-H (Alkane)	Stretching
1647.28	Variable	C=C (Alkene)	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).

Fig. 7: Phylogenetic tree based on 16S rRNA gene sequence comparison of FA9 (*Nocardia alba*).

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).

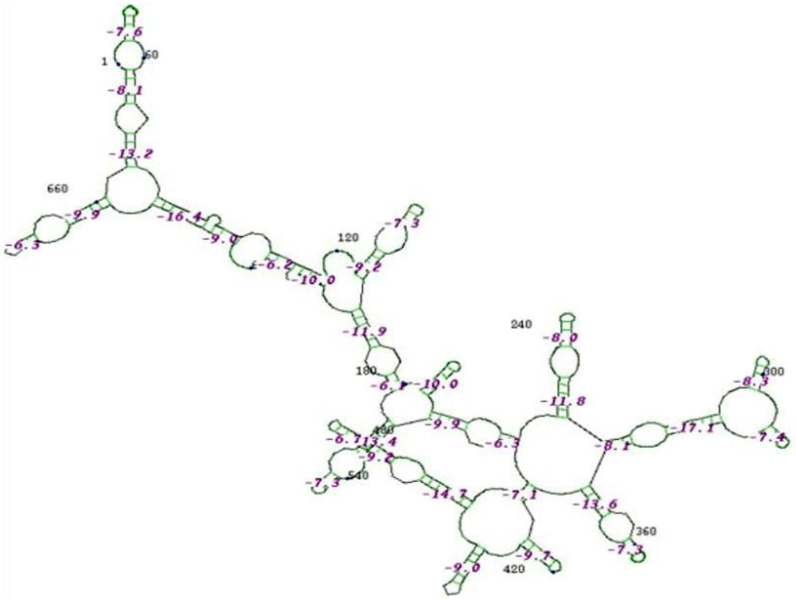

Fig. 8: RNA Secondary structure of *Nocardia alba* (FA9).

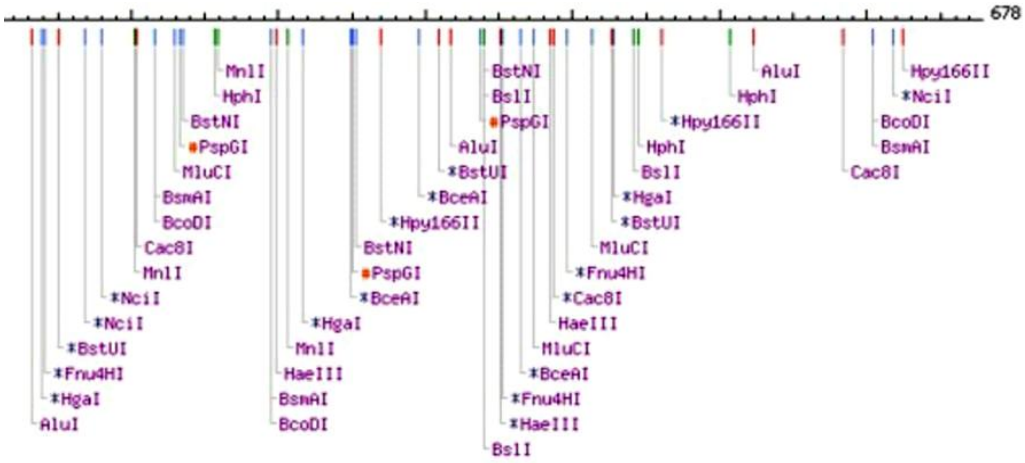

Fig. 9: Restriction sites prediction of *Nocardia alba* (FA9).

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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