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Research Article

CHARACTERIZATION OF SECONDARY METABOLITES WITH MOSQUITO-LARVICIDAL ACTIVITY EXTRACTED FROM ACTINOMYCETES ISOLATED FROM DIFFERENT ECOLOGICAL NICHE

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ABSTRACT

As mosquito vector has major role in transmission of mosquito borne diseases like malaria, filariasis, dengue etc, mosquito control program has prime importance to restrict mosquito development. The Actinomycetes secondary metabolite possessing mosquito-larvicidal activity can be part of this control program. Actinomycetes are common habitat of soil. During life cycle, it produces various secondary metabolites like antibiotics, growth promoter substances, insecticides. The insecticidal nature of secondary metabolites can be used to kill mosquito larvae. In current study 107 soil samples were collected from different ecological niche like farm area, forest area, open ground area and polluted area of west Maharashtra, India. All these samples were processed by microbiological methods to obtained 197 actinomycetes isolates. In primary screening 16 isolates was found to be possessing mosquito-larvicidal activity. LC_{50} study of secondary metabolite produced by 16 isolates helps in selecting two potent producers. The functional groups of ethyl acetate extracted secondary metabolite were analyzed by FTIR. These two effective actinomycetes isolates were identified by 16S-rRNA technique.

Keywords: Actinomycetes, Secondary metabolite, mosquito-larvicidal activity, 16S-rRNA, FTIR.

INTRODUCTION

Vector borne disease is a major source of illness and death worldwide. Mosquitoes transmit a number of diseases such as malaria (*Anopheles*), filariasis (*Culex*), dengue (*Aedes*) causing millions of deaths every year. Mosquitoes can be controlled by chemical insecticide, plant insecticides but increasing insecticide resistance creates a serious problem and Chemical residues pose environmental hazards and health concerns. Thus microbial insecticides are seen as an alternative means of mosquito larvae control.[1]

Actinomycetes are Gram positive, filamentous, spore forming, aerobic bacteria with cell wall containing L-L diaminopimelic acid and with high G + C content (57–75%) in their DNA.There are certain actinomycetes isolated from soil possessing mosquito larvicidal potential.[2] A number of different types of secondary metabolite from actinomycetes having bioactive property.[3]

The secondary metabolites are low molecular mass products of secondary metabolism, usually produced during the late growth phase (idiophase).[4.] The secondary metabolite responsible for bioactivity can be characterized by using Fourier-transform infrared spectroscopy (FTIR) technique. This method used to identify the functional group of compounds.[5]

The secondary metabolites produced of microbial origin have various mode of action including inhibition of enzyme activity. Acetylcholinesterase, carboxylesterase, alkaline and acid phosphatase enzyme of larvae can be inhibited. Some microbes produce bioactive peptides which are amphiphilic membrane active biosurfactant and peptide antibiotics with potent larvicidal activity.[6]

One of the most attractive potential uses of 16S rRNA gene sequence informatics is to provide genus and species identification for isolates that do not fit any recognized biochemical profiles, for strains generating only a "low likelihood" or "acceptable" identification according to commercial systems, or for taxa that are rarely associated with human infectious diseases. The cumulative results from a limited number of studies to date suggest that 16S rRNA gene sequencing provides genus identification in most cases (>90%) but less so with regard to species (65 to 83%).[7]

Soil is the most complicated biomaterial present on earth. It is composed of a variety of substances and provides a habitat to various organisms. Microorganisms including bacteria, fungi, actinomyces and algae are widely distributed in soil.[8]

The present study explores the processing of soil sample to isolate and identified secondary metabolite producing actinomycetes having mosquito-larvicidal activity and functional group analysis of that secondary metabolite by FTIR.

MATERIAL AND METHODS

Enrichment and Isolation of Secondary Metabolite producer

Total 107 soil samples collected from different ecological niche like polluted area, Farm area, Forest area and Open ground area of west Maharashtra, India. All soil samples weighing about 5gm were collected in small polythene bag. All soil samples were processed by using basic microbiological methods like enrichment and isolation.

Sterile Actinomycetes isolation Broth [AB] was used for enrichment and isolation of Actinomycetes. Two tubes containing

1gm each of soil samples was inoculated in 20 ml of AB. Incubation was carried out at varied temperature conditions like i.e.60^0C and room temperature to obtain thermophiles and mesophiles respectively.

After enrichment loopful of enriched broth was streaked on solid plate medium, for solid medium, 1.5% (w/v) agar was used. Plates were incubated at their enrichment temperature. Different colonies were picked and restreeted to obtain pure cultures. The pure cultures were stored at 10°C in the isolation medium slant.

Each isolate was employing for secondary metabolite production in their respective broth media and incubated 15 days at their respective temperature. After incubation the cell biomass were separated by using cooling centrifuge. The supernatant obtained which may contain secondary metabolite in diluted form were used for primary screening.

Primary Screening of isolates by mosquito-larvicidal assay

3rd instar *Culex quinquefasciatus* spp.[9] mosquito larvae were taken in 30 ml of tap water in disposable cups. In that the 10 ml centrifuged supernatant was added. The numbers of mosquito larvae killed in 48 hours were recorded. The isolate that show the 100% killing effect in this primary screening were selected for further study. The screened isolates were allowed to produce secondary metabolite which was extracted by using Ethyl Acetate.

Determination of LC_{50} value of effective isolates

The Lethal concentration 50% [LC_{50}] of secondary metabolite was determined according to WHO guidelines for laboratory testing of mosquito larvicides. Some modification is done in original guidelines. The dried residue was dissolved in 1 ml of Dimethyl Sulfoxide [DMSO] and various 10-fold serial dilutions were prepared.

Culex quinquefasciatus mosquito larvae were taken in disposable cup and metabolite dilution were added. Results were recorded after 48 hours. The numbers of dead larvae were detected.

The secondary metabolite dilution showing 50% dead larvae were considered as LC_{50} of those secondary metabolites. 1% Temephos was used as a positive control whereas distilled water was used as negative control in experimental set-up.[10] The LC_{50} value of the secondary metabolite was calculated by probity analysis.[11]

Fourier-transform infrared spectroscopy [FTIR] analysis of secondary metabolite extract

The actinomycetes secondary metabolite was extracted by analytical grade ethyl acetate. The extract was allowed to air dry at room temperature. The dried extract was analyzed by FTIR technique to determine functional group present in it. (FTIR instrument Model used was Jasco FT/IR-4100.)

Identification of Secondary metabolite Producer by 16S rRNA

The secondary metabolite producer was identified by 16S rRNA technique as follows. Initially the solid medium purified colonies were employed for DNA extraction. The extracted DNA sample then polymerized by Polymerase Chain Reaction [PCR] technique. A 20 base pair forward primer and 22 base pair reverse primer used. The forward primer namely 27F had sequence "AGAGTTTGATCMTGGCTCAG" whereas reverse primer 1492R had sequence "TACGGYTAC CTTGTTACGACTT." The PCR were carried out in three stages as Denaturation, Annealing and Extension. The denaturation step was carried out at 94°C for 3 minutes whereas annealing and extension at 50°C for 60 sec and 72° C for 10 min.

The product formed during PCR amplification was purified as follows. Removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore). The PCR product was sequenced using the 27F/1492R primers. Sequencing reactions were performed using a ABI PRISM®BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems). Later Sequencing of the 16S rRNA gene was carried out by sequencing PCR product. The PCR products were precipitated and denatured with formamide and sequenced by model ABI 3730 automatic sequencer. The alignment of all the 16S rRNA gene sequences was done using CLUSTAL W. The primer used for sequencing PCR were 16F 536, 16F 704, 16R 343, 518F, 800R. The sequence of the PCR product was compared with known 16S rRNA gene sequences obtained from Gen Bank to determine similarity value and distance matrix. The Phylogenetic tree was constructed using 500 base pair aligned sequences by the neighbor joining method using MEGA 7. Obtained partial 16S rRNA sequences were submitted to the gene bank.[12-16]

RESULTS AND DISCUSSION

Amongst the different microorganisms inhabiting in the soil, actinomycetes are ubiquitous microorganisms in the soil. These organism during life cycle produced secondary metabolite when it enters in stationary phase. There are many problems associated with the extensive use of chemical insecticides. Major one is insect acquires resistance to the chemicals, chemical residue poses environmental hazards and health concern. Thus, microbial insecticides are seen as an alternative means of pest control to reduce our dependence on chemical insecticides.

In current study 107 soil samples were collected from the different regions of West Maharashtra, India. The four regions were selected for study viz. Farm area, Forest area, Open ground area and Polluted area. Study area covers 3 districts; Kolhapur, Sangli and Ratnagiri. After enriching the 107 soil samples, 191 actinomycetes which comprise 117 mesophiles and 74 thermophiles were obtained. The results are presented in Table 1.

Table 1: Soil samples collected from different ecological niche

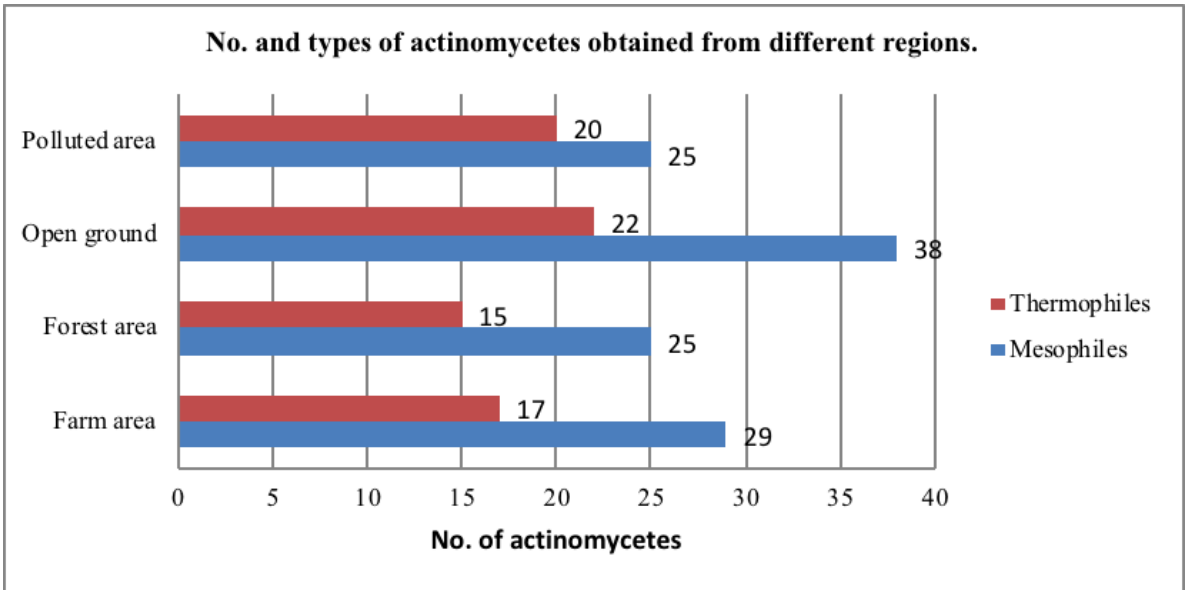
Region	No of soil sample collected	No of isolates obtained
Farm area	27	46
Forest area	25	40
Open ground	30	60
Polluted area	25	45
Total	107	191

Table 2: List of effective isolates having mosquito-larvicidal potential

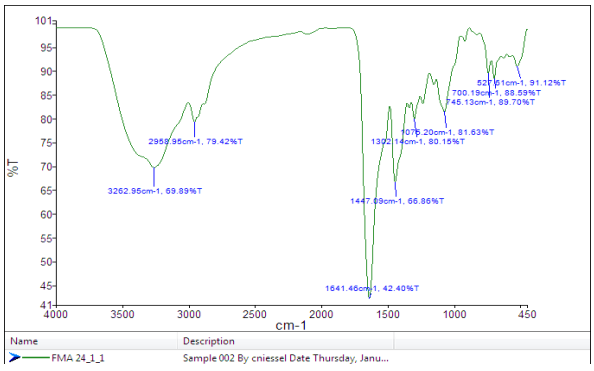
Sr. No.	Isolate	Type	Place	Region
1	FMA 21	Mesophiles	Kolhapur	Farm area
2	FMA 23	Mesophiles	Kolhapur	Farm area
3	FMA 24	Mesophiles	Kolhapur	Farm area
4	FMA 28	Mesophiles	Sangli	Farm area
5	FMA 31	Thermophiles	Kolhapur	Farm area
6	FMA 32	Thermophiles	Kolhapur	Farm area
7	FMA 39	Thermophiles	Kolhapur	Farm area
8	FMA 41	Thermophiles	Kolhapur	Farm area
9	FTA 05	Mesophiles	Ratnagiri	Forest area
10	FTA 29	Mesophiles	Sangli	Forest area
11	FTA 30	Mesophiles	Sangli	Forest area
12	FTA 32	Mesophiles	Ratnagiri	Forest area
13	FTA 37	Thermophiles	Sangli	Forest area
14	FTA 39	Thermophiles	Ratnagiri	Forest area
15	POA 02	Mesophiles	Ratnagiri	Polluted area
16	POA 34	Mesophiles	Kolhapur	Polluted area

Table 3: LC_{50} value of 16 isolates against *Culex quinquefasciatus* mosquito larvae

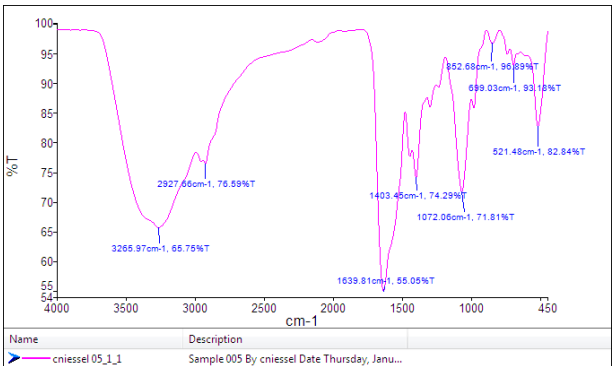
Isolate	Coeffcient	Intercept	Std error of coefficient	Std error of Intercept	Chi-Square goodness of fit	Significance	LD_{50}	Expt. LD_{50}	LD_{90}
FTA 29	0.001	-1.272	0.000	0.088	57.718	0.000	1084	1000	2177
FMA 24	0.001	-0.838	0.000	0.078	57.121	0.000	818	1000	2070
FMA 41	0.001	-1.395	0.000	0.084	84.913	0.000	2784	2000	5341
FMA 31	0.001	-1.420	0.000	0.084	75.120	0.000	2538	2000	4830
FMA 39	0.000	-1.517	0.000	0.090	62.926	0.000	3887	-	7171
FMA 32	0.000	-1.718	0.000	0.099	39.995	0.000	4063	-	7095
FTA 37	0.000	-1.550	0.000	0.091	72.266	0.000	3797	-	6935
FTA 39	0.000	-1.463	0.000	0.088	66.558	0.000	4065	-	7626
FTA 30	0.001	-1.148	0.000	0.076	89.297	0.000	2210	2000	4678
POA 34	0.001	-0.938	0.000	0.072	89.903	0.000	1695	2000	4012
FTA 32	0.001	-1.113	0.000	0.075	69.927	0.000	1924	2000	4140
FMA 28	0.001	-1.020	0.000	0.073	89.722	0.000	1751	2000	3952
POA 02	0.001	-1.403	0.000	0.083	41.061	0.000	2554	2000	4887
FMA 23	0.001	-1.450	0.000	0.085	74.673	0.000	2511	2000	4731
FMA 21	0.001	-1.317	0.000	0.081	95.088	0.000	2334	4000	4607
FTA 05	0.000	-1.292	0.000	0.080	95.389	0.000	2740	4000	5457



Graph 1: Number and types of actinomyvetes isolated from soil of different regions



Graph 2: Infrared spectra of compound isolated from FMA 24



Graph 3: Infrared spectra of compound isolated from FTA 29

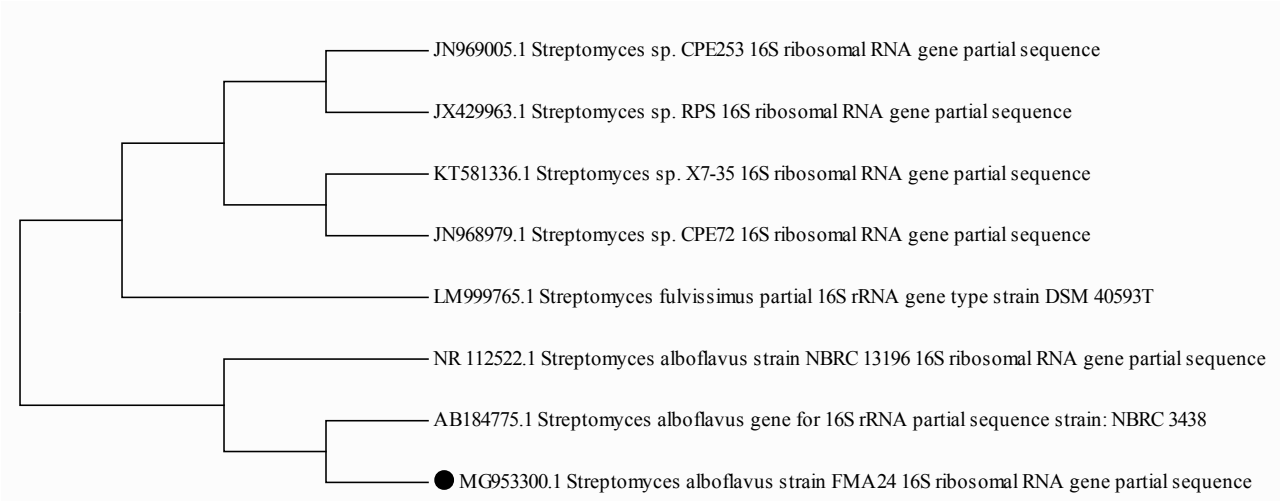


Figure 1: Molecular phylogenetic analysis by maximum likelihood method for FMA24

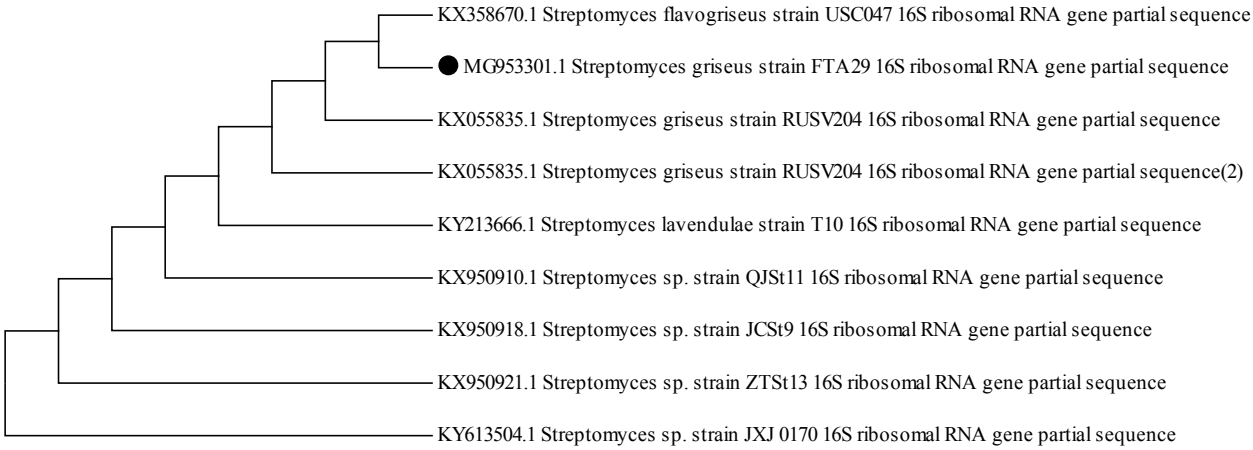


Figure 2: Molecular phylogenetic analysis by maximum likelihood method for FTA29

The highest numbers of actinomycetes isolates were obtained from open ground region; 60 isolates, out of which 38 were mesophiles and 22, were thermophiles. The lowest number of isolates i.e. 45 was obtained from polluted area which comprises 25 mesophiles and 20 thermophiles. Farm area and forest area soil samples processing gave 46 and 40 actinomycetes respectively.

The cell free supernatant was used to screen the mosquito-larvicidal positive isolates. About 16 potent isolates were obtained having mosquito larvicidal potential. The secondary metabolite was extracted from the 16 effective isolates given in table 2.

The LC_{50} assay was conducted to estimate the mosquito-larvicidal concentration of secondary metabolite. Bioassay was carried out using secondary metabolite in the range of 4000 ppm to 62.5ppm. The LC_{50} and LC_{90} of the secondary metabolite against 3rdinstar *Culex quinquefasciatus* mosquito larvae showed five metabolites having LC_{50} below 2000ppm and three having

LC_{90} below 4000ppm. PROBIT model: PROBIT (p) = Intercept + BX100

By considering minimum LC_{50} for mosquito-larvicidal activity two isolates were selected for further study. These two isolates are FMA24and FTA 29. They are having LC_{50}values against *Culex quinquefasciatus* mosquito larvae are 818 and 1084 ppm respectively.

The FTIR technique was used for determination of functional group present in secondary metabolite compound. The spectral analysis for secondary metabolite compound isolated from FMA 24 and FTA 29 is given in graph 2 and 3 respectively.

The number of peaks obtained for Secondary metabolite of FMA24, It is observed that1641 cm^{-1} is a sharp strong band showing carbonyl as well as C= C stretching. The broad medium peak spectra obtained at3262 cm^{-1} due to –OH group. 2958 cm^{-1} is a medium band showing Alkyl C-H bond stretching cyclopentane whereas 1447 cm^{-1} is a medium sharp band due to ring mode of benzene ring. C-H bounds of benzene ring out of plane-wagging. The locality of band in fingerprinting area is as follows.745 cm^{-1} weak band CH2 stretching whereas weak band is located at 1392 cm^{-1}which is of sulfonide group. At 1075 cm^{-1} weak band of S=O stretching is observed.

The different peak observed for Secondary metabolite of FTA 29. The prominent sharp narrow peak obtained at 1639 cm^{-1} showing benzene, C=C, C=O stretching. The presence of ring mode is analyzed due to peak at 1403 cm^{-1} which is medium sharp peak. The broad peak noticed at3265 cm^{-1} is a having OH stretching. Medium small peak observed at 2927 cm^{-1} which may have CH stretching. The spectra in fingerprint region are quite noticeable. 1072 cm^{-1} medium sharp peak C-X-C stretching whereas 521 cm^{-1} peak having C-H out of plane bending.

The identification of efficient isolates was done by 16S-rRNA technique. The sequence of the PCR product obtained was compared with known 16S-rRNA gene sequence from gene bank to determine similarity value and distant matrix it is found that the FMA24 belongs to *Streptomyces alboflavus* strain and FTA29 belongs to *Streptomyces griseus* strain. After constructing phylogenetic tree with the help of Maximum Likelihood algorithmit, it was found that the isolate *Streptomyces alboflavus* strain FMA24 is very closer to *Streptomyces alboflavus* NBRC 3438 and next close to *Streptomyces alboflavus* NBRC 13196. Phylogenetic tree shows it is far distant to *Streptomyces sp.* CPE253 whose accession number is JN969005. Similarly *Streptomyces griseus* strain FTA29 is closer to *Streptomyces flavogriseus* strain USC047 and next close to *Streptomyces griseus* strain RUSV204. It is far distant from Streptomyces sp. strain JXJ0170 with accession number KY613504.

CONCLUSION

Soils of four different ecological niches from west Maharashtra, India were processed to obtain 191 Actinomycetes isolates. Secondary metabolites were extracted from all the isolates. The mosquito-larvicidal activity was checked against *Culex quinquefasciatus* spp larvae to screen the efficient isolates on the basis of its efficiency of lysis of larvae. The 16 efficient isolates were studied for its LC_{50} assay and probit analysis.

Two best isolates namely FMA24 and FTA29 were selected for further studies. By using 16S rRNA technique, both these isolates were successfully identified as *Streptomyces alboflavus* strain FMA24 and *Streptomyces griseus* strain FTA 29. The Fourier-transform infrared spectroscopy [FTIR] analysis of secondary metabolite extract was done to reveal its functional groups present in it. Thus current work has enlightened the Characterization and study of mosquito-larvicidal potential of secondary metabolites from soil Actinomycetes.

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