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METABOLIC PROFILING OF ACTINOMYCETES HAVING ANTIMICROBIAL PROPERTIES

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ABSTRACT

Chemical screening is a simple and an efficient approach for identifying the possible chemical class of compounds present in culture extracts. It includes separation of fractions from crude extract of a microbe by thin layer chromatography (TLC) and subsequent staining with range of chemical reagents. In the present study, eight actinomycetes isolated from diverse ecological habitats including sanitary landfill soil, pesticide contaminated soil, agricultural soil, radiation exposed soil, industrial soil and possessing antimicrobial activity against pathogenic strains, were subjected to chemical screening. Bioactive compounds were extracted either from culture broth or culture plates of the isolates using organic solvents like ethyl acetate and methanol with different polarities. Metabolic pattern/profiling of extracts was observed with help of TLC followed by visualization under UV as well as staining with chemical reagents including anisaldehyde/H_2SO_4, vanillin/H_2SO_4 and methanolic/H_2SO_4. The results depicted diversity of crude culture extracts. On the basis of chemical screening, chemical nature of compounds was tentatively identified. Preliminary study indicated that the compounds may belong to macrolide, anthracycline, polypeptide or xanthone class of antibiotics. Taxonomic status of strains was determined by phylogenetic tree construction based on 16S rRNA gene sequences. Phylogenetic studies indicated that actinomycete isolates belong to the genus *Streptomyces*. The 16S rRNA gene sequences of isolates B.69, L3.41, L3.46, RI.24, RI.30, S.4A and SL.4 were submitted to GenBank nucleotide database provided by the National Centre for Biotechnology Information (NCBI) and assigned following accession numbers KF529978, KF499021, KF529977, KF529979, KF529980, KF529981 and EF424409, respectively.

Keywords: Actinomycetes, Antimicrobial compounds, Metabolic profiling, Thin layer chromatography (TLC), Chemical screening, 16S rRNA gene sequencing.

[I] INTRODUCTION

Microbial natural products are an important source of new antimicrobial compounds [3,40]. A large number of microbial strains are tested in the screening programs for discovery of new antibiotics. Consequently, there is a requirement to adopt methodologies for avoiding strain duplication and for simultaneous selection of metabolically useful strains [36]. Chemical screening is a simple and an efficient approach which can fulfill these requirements. It involves separation/fractionation of metabolites from a microbial strain extract by thin layer chromatography (TLC) followed by staining

with chemical reagents like anisaldehyde/H_2SO_4, orcin-$FeCl_3$, vanillin/H_2SO_4, ninhydrin, naphthoresorcin-sulphuric acid to name a few [39,42,43,53]. Chemical screening of crude extracts was initiated by Zahner and others in the 1980s [53].

Visualization of the complete secondary metabolite pattern of a microbe is known as metabolic fingerprinting or metabolic profiling [53]. Tentative functional groups present in the bioactive compounds can be identified by staining with particular chemical reagents [39,43]. The present study deals with chemical screening of extracts of actinomycete cultures, well known for production of bioactive compounds and assessing their possible functional groups. Genera of isolates were thereafter identified by phylogenetic studies based on 16S rRNA gene sequences [8,28,46].

[II] MATERIALS AND METHODS

Actinomycete cultures isolated from diverse ecological habitats including sanitary landfill soil, pesticide contaminated soil, agricultural soil, radiation exposed soil, industrial soil were tested for antimicrobial activity, if any, against five sensitive strains: *Escherichia coli*, *Bacillus cereus*, *Candida albicans*, *Fusarium oxysporum* and *Staphylococcus aureus*. Primary screening (qualitative screening) of isolates was done by agar plug method. Agar well method was used for quantification of antimicrobial activities during secondary screening [41]. On the basis of results obtained, following eight isolates (Table 1) showing substantial activity against more than one pathogen, were selected for chemical screening and for functional characterization of compounds in their extracts.

2.1. Extraction of antimicrobial compounds

2.1.1. Extraction from culture broth

Actinomycete cultures were inoculated in Nutrient medium [34] and incubated at 28°C on shaker for 7 days. Cultures were centrifuged twice at 8000 rpm and supernantant was filtered through Whatman no.1 filter paper to get rid of cellular components. Culture filtrates were extracted thrice with equal volume of organic solvent. Organic phase was separated from aqueous phase by centrifugation at 8000 rpm for 20 minutes. Organic solvent having antimicrobial compounds was evaporated in a rotavapor at 40°C-45°C to remove organic solvent and get powdered form of extracts. Ethyl acetate extracts were processed by this method [2,42].

2.1.2. Extraction from culture plates

Actinomycete cultures were inoculated on YM medium plates and incubated at 28°C for 10 days for production and diffusion of antimicrobial compounds. Agar medium with culture was cut into small pieces and collected into a flask containing solvent (about 100 ml solvent was used for 4-5 culture plates). Chopped agar pieces with organic solvent were kept on shaker for 4-5 hours. Contents were centrifuged at 4000 rpm for 10 min and filtered through Whatman No. 1 filter paper to get rid of culture and agar pieces. This process was repeated 2-3 times. Crude extracts were obtained by evaporation of solvent. Methanolic extracts were mainly processed by this method [38], although, ethyl acetate extracts could also be processed by this method.

2.2 Chemical Screening

2.2.1. Thin layer chromatography of extracts

Bioactive culture extracts were applied to the TLC plate (Merck silica gel plate 60 F_{254}, 0.2mm). Spots were air dried. Among different solvent systems tested for separation of extract fractions Dichloromethane:Methanol (9:1) and Butanol:Acetic acid:Water (3:1:1) gave best result for ethyl acetate and methanolic extracts, respectively. Chromatograms were therefore

developed using Dichloromethane:Methanol (9:1) as the mobile solvent system for ethyl acetate extracts and Butanol:Acetic acid:Water (3:1:1) for methanolic extracts [7,42].

2.2.2. Chemical screening

Secondary metabolite profile of strains was visualized by observing TLC plates under UV at wavelengths of 254 and 365nm. TLC plates were also stained with a range of staining reagents including anisaldehyde/H_2SO_4, vanillin/H_2SO_4 and methanolic/H_2SO_4. Resolved fractions were marked and their R_f values were calculated [36,42].

2.3. Phylogenetic studies

2.3.1. Small scale DNA isolation from actinomycete isolates

1.5ml of the late stationary phase culture was pelleted and washed with lysozyme buffer. Culture was re-suspended in 500µl of lysozyme mix having 2mg/ml of lysozyme and incubated at 37°C for 30-40 minutes for lyses of mycelia. 250µl of 2% SDS was added to release the cell contents. Thereafter, 250µl of Tris-equilibrated phenol: chloroform was added for precipitation of proteins. Suspension was centrifuged at 10,000 rpm for 10 minutes. The upper aqueous phase having DNA was transferred to a fresh microcentrifuge tube and DNA was precipitated by addition of $1/10^{th}$ volume of 3M Na acetate (pH 4.8). Equal volume of isopropanol was added and tubes were incubated at -20ºC overnight. DNA was pelleted by centrifuging tubes at 10,000 rpm for 20 minutes, dissolved in 300µl of 0.3M Na acetate (containing 10mM $MgCl_2$) and DNA was re-precipitated by adding 700µl of ethanol. DNA was pelleted and washed with 500µl of 70% ethanol to remove any impurities. DNA pellet was finally dissolved in 50-100µl of MQ water [24].

2.3.2. 16S rRNA gene amplification

The 16S rRNA gene of strains were amplified using either a combination of universal primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGTTACCTTGTTACGACTT-3') or of 27F (5'-AGAGTTTGATCCTGGCTCA-3') and 1542R (5' AAGGAG GTGATCCAGCCGCA 3') [14]. A standard PCR reaction mix (100µl) was prepared which consisted of the following reagents: DNA template- 1µl, MQ-71.7µl, 10X PCR buffer (Tris with 15mM $MgCl_2$) -10µl, 10mM dNTP mix (2.5mM each)-6µl, 20µM Forward and Reverse Primers - 5µl each, 3U/µl Taq polymerase-1.3µl. PCR conditions were as follows: initial denaturation (5 min at 95°C) followed by 30 cycles of denaturation (30 sec at 95°C), primer annealing (30 sec at 55°C), primer extension (1.5 min at 72°C) and a final extension step (10 min at 72°C). Amplification was performed in a Mastercycler gradient thermocycler (Eppendorf).

2.3.3. Elution of amplified DNA of 16S rRNA gene

100µl of the PCR reaction mixture was loaded on an agarose gel (0.8% agarose in 100ml of TAE buffer) to visualize the amplification band corresponding to 16S rRNA gene (approximately 1.5Kb) of genomic DNA with the help of standard DNA marker. DNA band of interest was excised from ethidium bromide stained agarose gel. Gel piece was weighed and 2.5 volumes of sodium iodide solution was added to the gel piece. Solution was incubated at 45ºC-55ºC for 2 to 3 minutes to ensure complete solubilization of gel. Contents were mixed thoroughly and incubated for further 5 minutes in the water bath at 45ºC-55ºC. The glass solution (provided in the gel extraction kit of Bangalore Genei) was solubilized by vortexing, till it formed a homogeneous mixture. 15µl of glass solution was

added to the sample containing 5µg or less of DNA. The contents were mixed thoroughly and left at room temperature for 5 minutes, with occasional mixing. It allowed adsorption of DNA molecules to the glass solution. Tubes were centrifuged at 12,000 rpm for 30 seconds and supernatant was discarded. DNA bound to the glass formed a hard pellet. 200µl of wash buffer was added, vortexed, centrifuged at 12,000 rpm for 30 seconds and supernatant was discarded. This step was repeated. Tubes were dried at 55ºC for 10 minutes to remove traces of wash buffer completely. For elution of DNA, 30-40µl of MQ was added to the pellet and pellet was resuspended by mild vortexing and incubated at 45ºC to 55ºC for 5 minutes. DNA got unbound from the glass due to lack of high salt concentration and eluted in water. Tubes were centrifuged at 12,000 rpm for 30 seconds and supernatant was collected in a fresh microcentrifuge tube. Greater part of DNA gets eluted in this step. A second elution was also done for recovery of remaining DNA. Supernatants having eluted amplified DNA were pooled. Finally, traces of glass solution were removed by centrifugation at 12,000 rpm for a few seconds. 3µl of eluted DNA was loaded on the gel and its size was confirmed with the help of marker.

2.3.4. Sequencing of 16S rRNA gene

Sequencing PCR was done using BigDye® terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) and primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3'), 27F (5'-AGAGTTTGATCCTGGCTCA-3'), 341F (5'-CTACGGGAGGCAGCAGTGG G-3'), 786F (5'-GATTAGATACCCTGGTAG-3'), 536R (5'-GTATTACCGCGGCTG CTG-3'), 939R (5'-CTTGTGCGGGCCCCCGTCAATTC-3'), 1492R (5'-TACGGT TACCTTGTTACGACTT-3'), 1542R (5'-AAGGAGGTGATCCAGCCGCA-3') [14]. The sequencing PCR reaction mix was composed of the following contents: Template DNA to be sequenced (150-200ng)-1 µl, 5X BigDye® terminator sequencing buffer-2µl, 20µM sequencing primer-0.5 µl, BigDye® terminator v 3.1- 1µl; MQ- 5.5µl. PCR conditions were as follows: initial denaturation (1 min at 96°C), 25 cycles of denaturation (10 s at 96°C), primer annealing (5 s at 50°C), primer extension (4 min at 60°C) and a final extension step (10 min at 60°C). DNA sequencing was performed using 3100 Avant™ Genetic Analyzer sequencer (Applied Biosystems, USA).

2.3.5. Phylogenetic tree construction

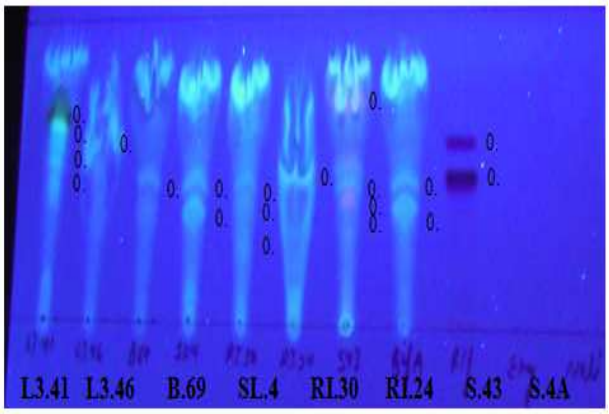
Phylogenetic neighbors of isolates were identified from the Eztaxon database of type strains using 16S rRNA sequences [12]. The best 20 sequences of type strains with highest similarity from EzTaxon database were selected. Sequences of neighbor strains were retrieved from GenBank sequence database. Phylogenetic tree for each strain was constructed by neighbor-joining method using MEGA version 4.1 software package [44] after multiple sequence alignment of data by Clustal_X [45].

[III] RESULTS

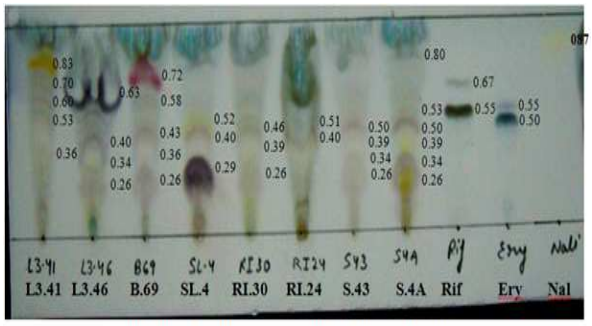
3.1. Chemical Screening

Chemical screening using thin layer chromatography followed by staining with suitable chemical reagents helps to visualize complete secondary metabolite pattern of the isolates. This not only helps in avoiding strain duplication but also indicates types of chemical groups present in culture extracts on the basis of production of specific colored bands after staining with particular chemical reagents. Metabolite profiles of isolates were visualized under UV light (365nm wavelength) and their R_f values were recorded. Secondary metabolic

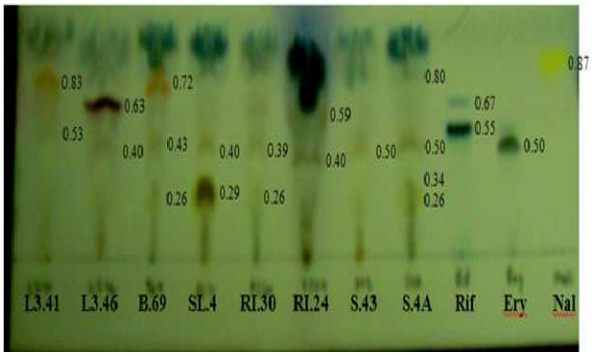
pattern could also be visualized with staining reagents and best results were obtained with vanillin/H_2SO_4 for ethyl acetate culture extracts whereas methanolic extracts showed best results with anisaldehyde/H_2SO_4. The pattern of color bands and their R_f values, after treatment of TLC plates with staining reagents anisaldehyde/H_2SO_4 and vanillin/ H_2SO_4 are shown in Tables 2 & 3 and Figures 1 & 2.

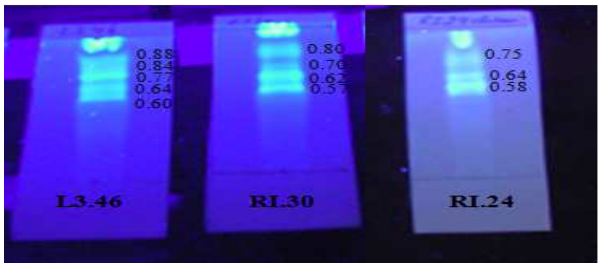


(a) Ethyl acetate extracts under UV at 365nm



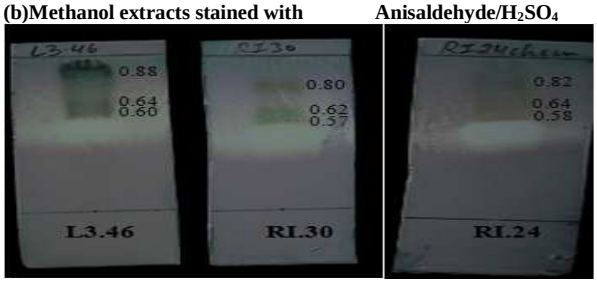
(b) Ethyl acetate extracts stained with Vanillin/H_2SO_4





(a) Methanol extracts under UV at 365nm

(b)Methanol extracts stained with Anisaldehyde/H_2SO_4



(c)Methanol extracts stained with Vanillin/H_2SO_4

Figure 2: Chemical screening of methanolic extracts using TLC followed by visualization under UV at 365nm (a) and staining with anisaldehyde/H_2SO_4(b) and vanillin/H_2SO_4(c)

3.2. Phylogenetic studies

16S rRNA gene of isolates was sequenced and subjected to sequence similarity searches using Eztaxon Database and related strains were identified (Table 4).

Sequences of related strains were retrieved from Eztaxon database and phylogenetic trees were constructed for each isolate (Figures 3-9).

[IV] DISCUSSION

Actinomycete culture extracts were subjected to chemical screening firstly for comparing metabolic profiles of isolates and secondly to ascertain possible chemical moieties present in these

extracts. Bands resolved on TLC showed UV absorption at 254 and 365nm for both ethyl acetate and methanolic extracts. Secondary metabolic pattern was also visualized with a variety of staining reagents and best results were obtained with vanillin/H_2SO_4 in case of ethyl acetate extract and with anisaldehyde/ H_2SO_4 for methanolic extracts. Taddei *et al*., 2006 [42] showed usefulness of chemical screening for avoiding strain duplication on the basis of their metabolic fingerprint and found best results with anisaldehyde/ H_2SO_4 staining. Sajid *et al*., 2009 [36] used chemical screening for selection of potentially useful best actinomycetes on the basis of their secondary metabolite pattern. Chemical screening is thus an important component for natural product discovery.

Taddei et al., 2006 [42] depicted that if particular colors are produced by the metabolite fractions after staining with anisaldehyde/H_2SO_4 and vanillin/H_2SO_4 as shown in Table 5, then the compounds belong to a particular chemical group. In our extracts, brown and grey color fractions were obtained by staining with these two chemicals indicating the presence of sugar moieties in culture extracts whereas yellow, orange, pink and slate color fractions were those which did not have any sugar moiety [42] as given in the following Tables 6-9.

It is well known from literature that antibiotics belonging to aminoglycoside, anthracycline, glycopeptide, macrolide and nucleoside antibiotics contain sugar moieties. However, polypeptide and xanthone antibiotics do not have any sugar moiety [6,10,22,23,25,29,55]. Consequently, in the initial stage with the help of chemical screening, we could predict that our antimicrobial compounds containing sugar moieties may be macrolides or anthracyclines antibiotics whereas others may be polypeptides or xanthones based on physical properties like solubility in solvent used for extraction of these compounds. Further chemical characterization of compounds is underway.

Phylogenetic studies revealed that *Streptomyces atriruber* NRRL B-24165^T (EU812169) [27], *Streptomyces parvulus* NBRC 13193^T (AB184326) [35], *Streptomyces samsunensis* M1463^T (EU077190) [37] and *Streptomyces albogriseolus* NRRL B-1305^T (AJ494865) [19,33] are the closest relatives of isolates B.69, L3.41, L3.46 and SL.4, respectively. *Streptomyces globosus* LMG 19896^T (AJ781330) [30] was relative of RI.30 (KF529980) whereas *Streptomyces rochei* NBRC 12908^T (AB184237) [50] and *Streptomyces enissocaesilis* NRRL B-16365^T (DQ026641) [32,50] were closest relatives of RI.24 and S.4A, respectively. Isolates RI.24 and S.4A shared 100% 16S rRNA gene sequence similarities. 16S rRNA gene of isolate S.43 could not be amplified using standard universal primers inspite of trying out a range of annealing temperatures. This isolate is known to produce a copious pink colored pigment that possibly hindered binding of PCR primers to the DNA and did not allow amplification of gene to occur [13]. 16S rRNA gene sequences of isolates B.69, L3.41, L3.46, RI.24, RI.30, S.4A and SL.4 have been submitted in GenBank sequence database provided by the National Centre for Biotechnology Information (NCBI) and assigned accession numbers: KF529978, KF499021, KF529977, KF529979, KF529980, KF529981 and EF424409 [26], respectively.

Antibiotic production profile of isolates B.69, L3.41, L3.46, RI.24, RI.30, S.4A, S.43 and SL.4 were compared with those of their clade members (Table 10). Although, there were considerable dissimilarities between chemical structures and antimicrobial activities of isolates and their respective clade members. However, biosynthetic gene clusters of all belonged to either the PKSs

(Polyketide synthases) or NRPS (Nonribosomal peptide synthetases) systems.

[V] CONCLUSION

Metabolic profiles of actinomycete isolates were compared by chemical screening. Chemical screening was done by TLC followed by staining with different chemical reagents. The results depicted diversity of crude culture extracts. Chemical nature of compounds was tentatively identified on the basis of chemical screening. It had been found preliminary that compounds may belong to different classes of antibiotics including macrolides, anthracyclines, polypeptides or xanthones. The taxonomic status of the isolates was determined by phylogenetic tree construction based on 16S rRNA gene sequences. Phylogenetic studies revealed that all isolates belong to genus *Streptomyces*. 16S rRNA gene sequences of isolates B.69, L3.41, L3.46, RI.24, RI.30, S.4A and SL.4 have been submitted in GenBank sequence database (NCBI) and assigned accession numbers KF529978, KF499021, KF529977, KF529979, KF529980, KF529981 and EF424409, respectively.

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Fig 3-9 and Tables:

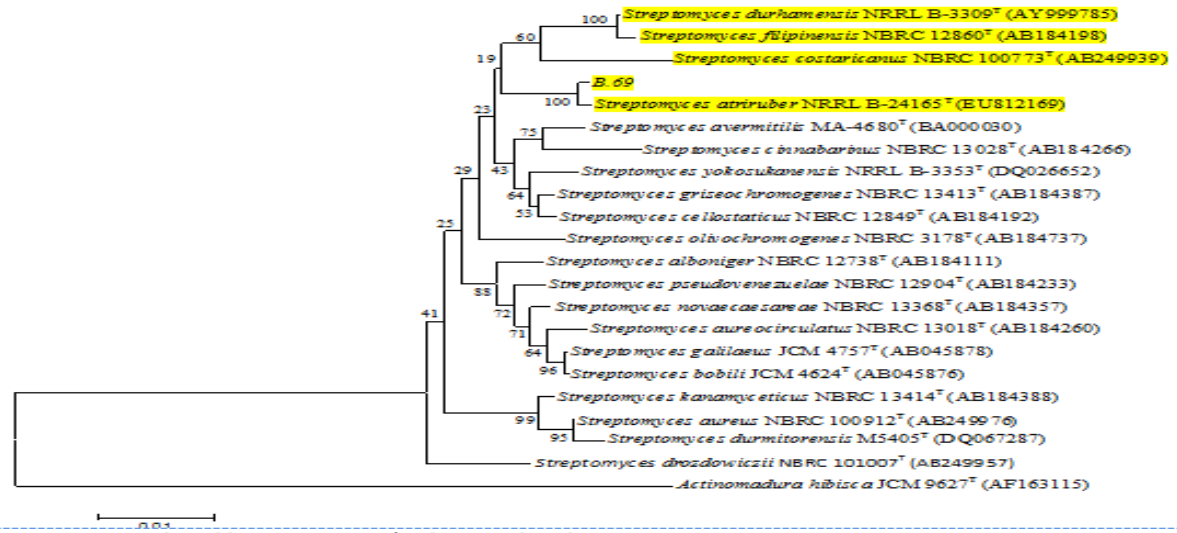
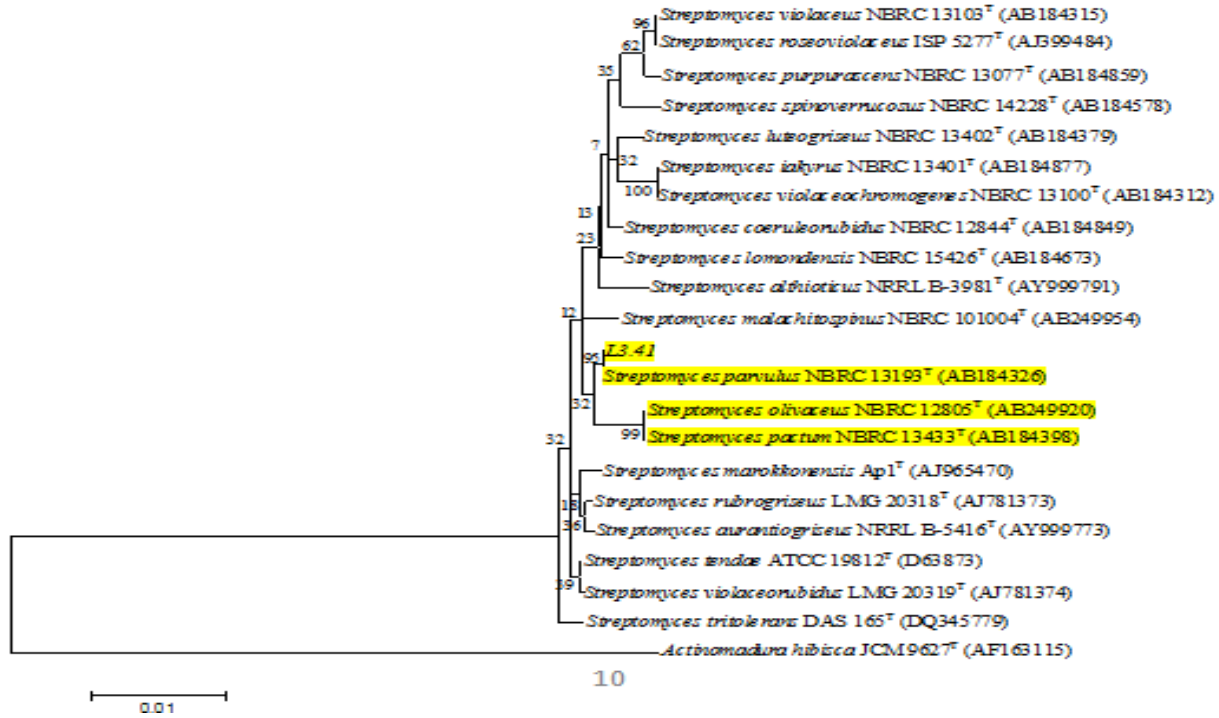


Figure 3: Rooted neighbor joining tree of isolate B.69 based on 16S rRNA gene sequences, showing the relationship between strain B.69 and related representative species of the genus *Streptomyces*. The sequence of the 16S rRNA gene of *Actinomadura hibisca* JCM 9627^{T} (AF163115) was used as an outgroup. Numbers at the nodes indicate the levels of bootstrap support based on 1000 resampled data sets. The scale bar indicates 0.01 substitutions per nucleotide position.

Figure 4: Rooted neighbor joining tree of isolate L3.41 based on 16S rRNA gene sequences, showing the relationship between strain L3.41 and related representative species of the genus *Streptomyces*. The sequence of the 16S rRNA gene of *Actinomadura hibisca* JCM 9627^{T} (AF163115) was used as an outgroup. Numbers at the nodes indicate the levels of bootstrap support based on 1000 resampled data sets. The scale bar indicates 0.01 substitutions per nucleotide position.



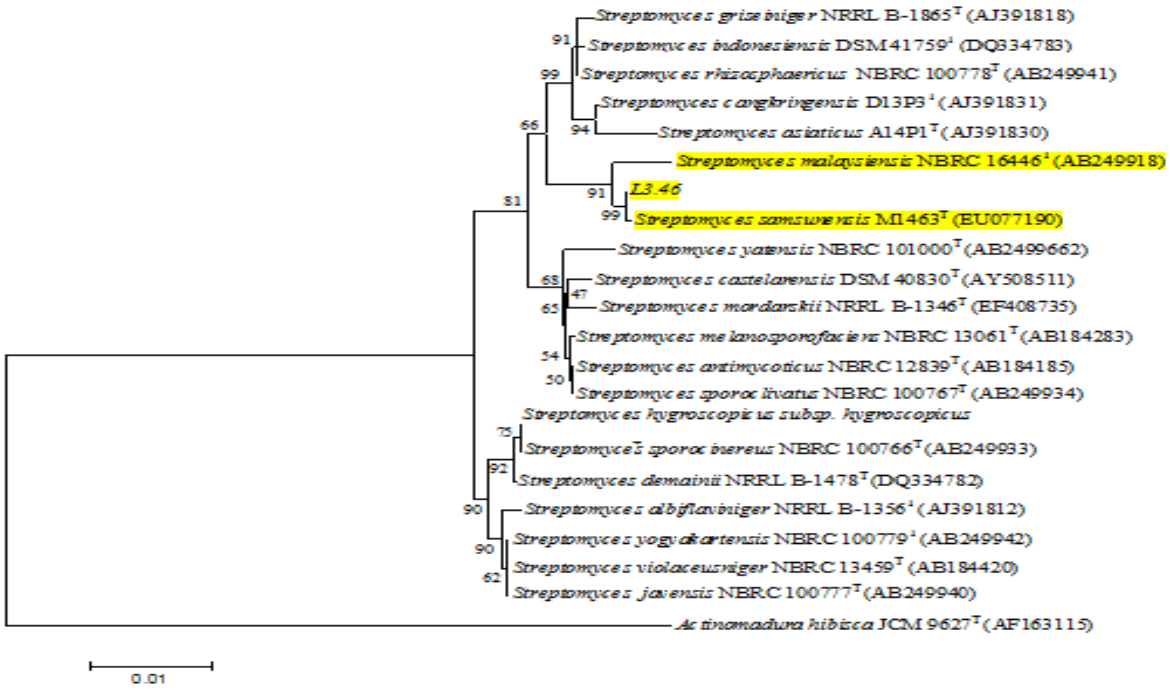
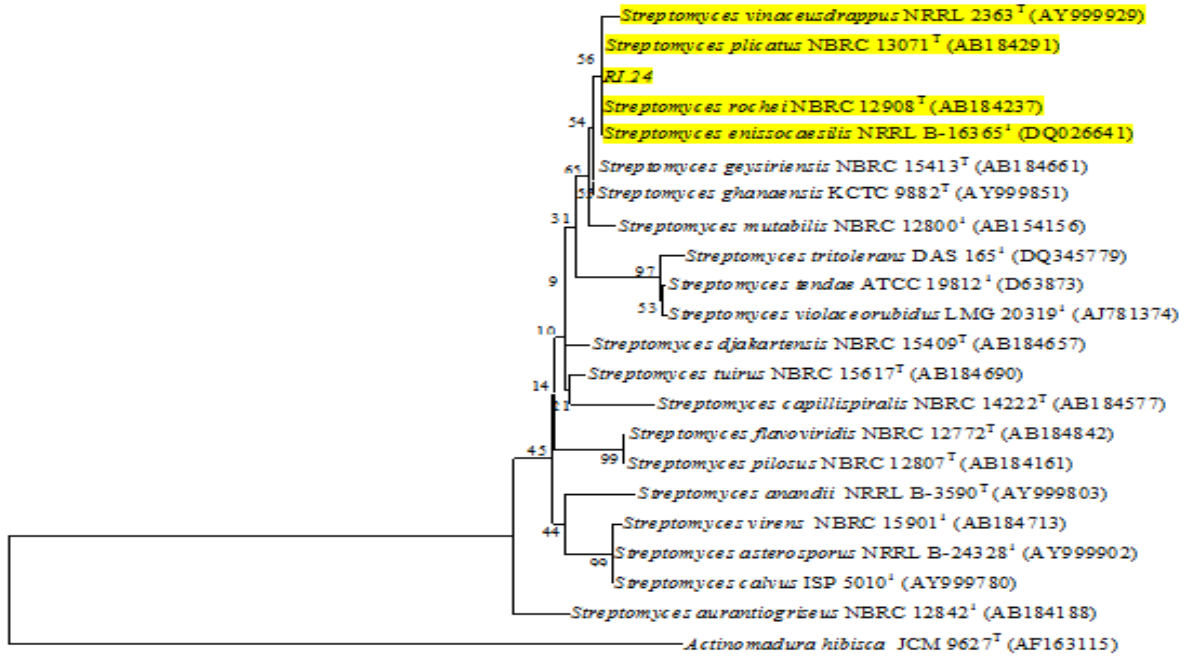


Figure 5: Rooted neighbor joining tree of isolate L3.46 based on 16S rRNA gene sequences, showing the relationship between strain L3.46 and related representative species of the genus *Streptomyces*. The sequence of the 16S rRNA gene of *Actinomadura hibisca* JCM 9627^T (AF163115) was used as an outgroup. Numbers at the nodes indicate the levels of bootstrap support based on 1000 resampled data sets. The scale bar indicates 0.01 substitutions per nucleotide position.

Figure 6: Rooted neighbor joining tree of isolate RI.24 based on 16S rRNA gene sequences, showing the relationship between strain RI.24 and related representative species of the genus *Streptomyces*. The sequence of the 16S rRNA gene of *Actinomadura hibisca* JCM 9627^T (AF163115) was used as an outgroup. Numbers at the nodes indicate the levels of bootstrap support based on 1000 resampled data sets. The scale bar indicates 0.01 substitutions per nucleotide position.



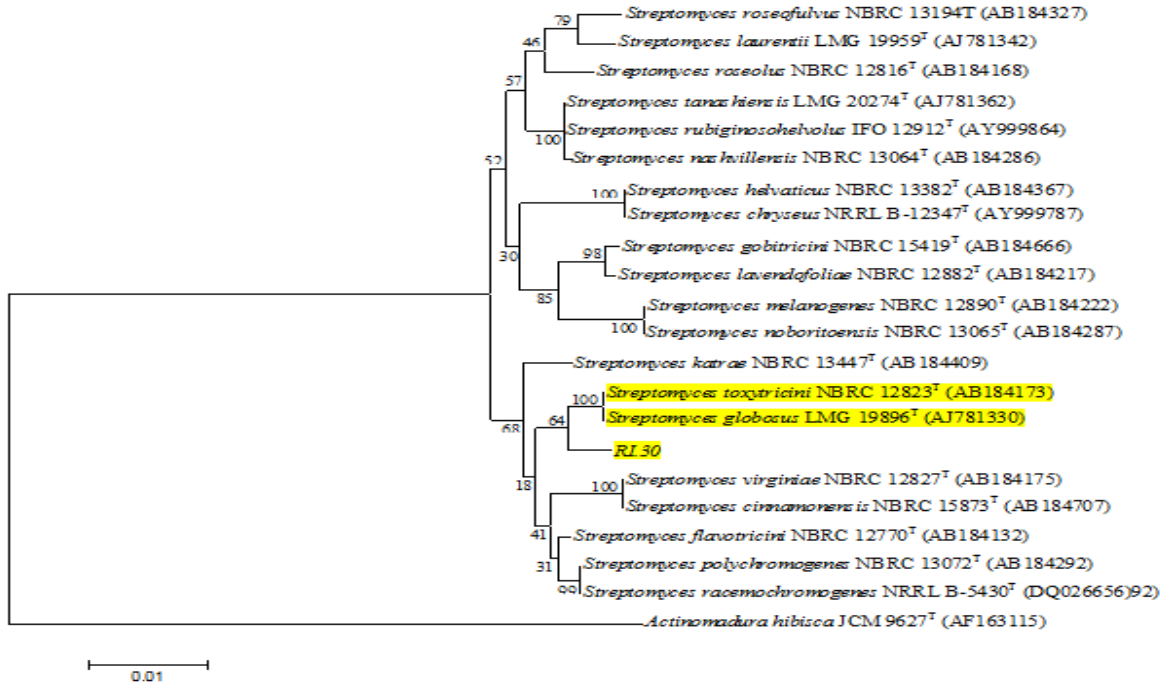
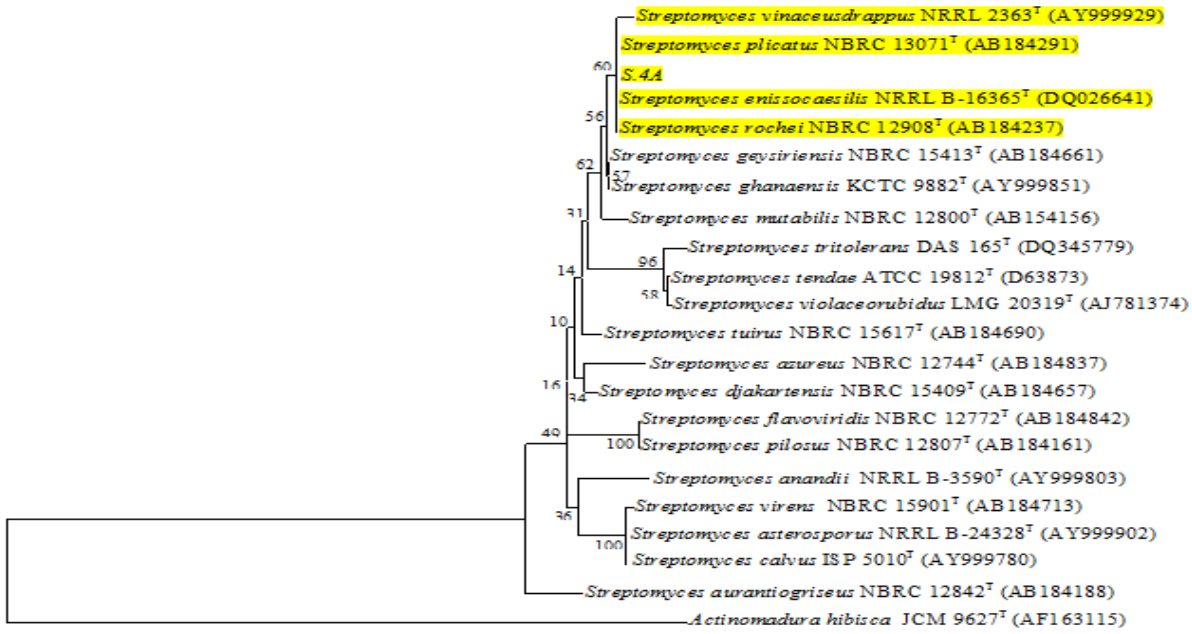


Figure 7: Rooted neighbor joining tree of isolate RI.30 based on 16S rRNA gene sequences, showing the relationship between strain RI.30 and related representative species of the genus *Streptomyces*. The sequence of the 16S rRNA gene of *Actinomadura hibisca* JCM 9627^{T} (AF163115) was used as an outgroup. Numbers at the nodes indicate the levels of bootstrap support based on 1000 resampled data sets. The scale bar indicates 0.01 substitutions per nucleotide position.

Figure 8: Rooted neighbor joining tree of isolate S.4A based on 16S rRNA gene sequences, showing the relationship between strain S.4A and related representative species of the genus *Streptomyces*. The sequence of the 16S rRNA gene of *Actinomadura hibisca* JCM 9627^{T} (AF163115) was used as an outgroup. Numbers at the nodes indicate the levels of bootstrap support based on 1000 resampled data sets. The scale bar indicates 0.01 substitutions per nucleotide position.



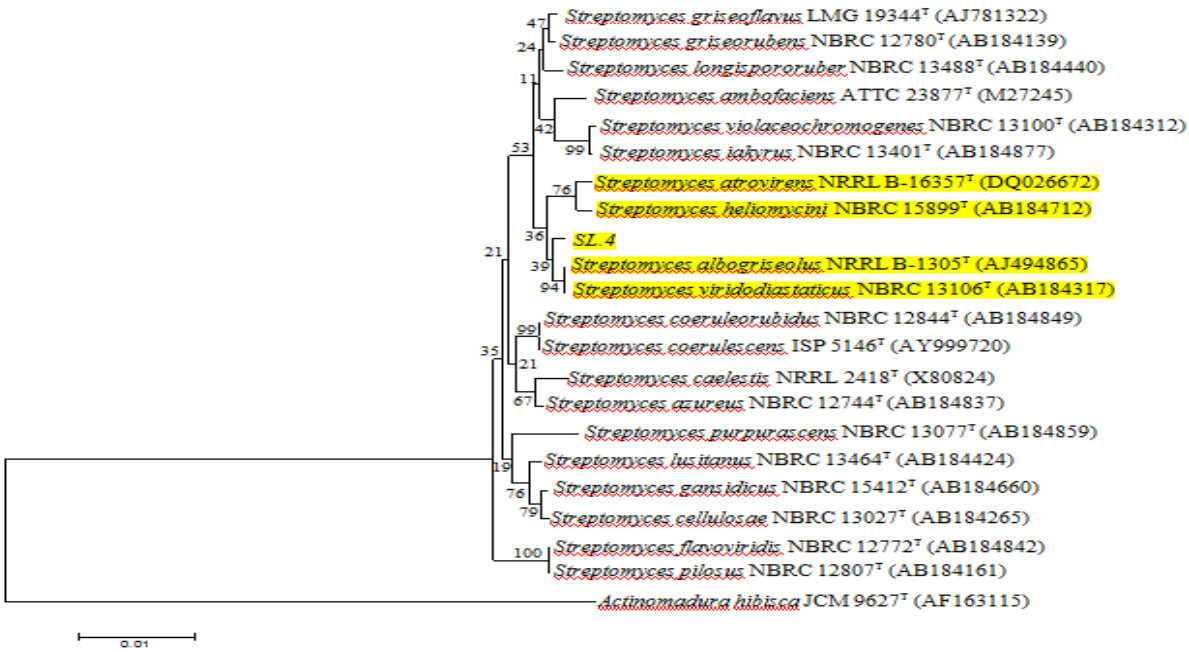


Figure 9: Rooted neighbor joining tree of isolate SL.4 based on 16S rRNA gene sequences, showing the relationship between strain SL.4 and related representative species of the genus *Streptomyces*.

The sequence of the 16S rRNA gene of *Actinomadura hibisca* JCM 9627^{T} (AF163115) was used as an outgroup. Numbers at the nodes indicate the levels of bootstrap support based on 1000 resampled data sets. The scale bar indicates 0.01 substitutions per nucleotide position.

Table 1: Isolates selected for chemical screening and phylogenetic studies

Strain	Ecological Habitat	Primary Screening					Secondary Screening				
		Activity against					Activity against				
		B.c	C.a	E.c	F.o	S.a	B.c	C.a	E.c	F.o	S.a
B.69	Agriculture field	+++	-	++	-	++	+	-	-	-	-
L3.41	Pesticide contaminated	+++	-	-	+++	-	+++	-	-	+	-
L3.46	Pesticide contaminated	+++	++	-	+++	+/-	+++	++	-	+	-
RI.24	Radiation exposed soil	++	-	+++	-	+++	++	-	++	-	++
RI.30	Radiation exposed soil	-	++	-	-	-	-	++	-	-	-
S.43	Soap industry	+++	-	-	-	-	++	-	-	-	-
S.4A	Soap industry	-	-	+++	-	+++	ND	ND	ND	ND	ND
SL.4	Sanitary landfill	+	-	+/-	-	-	+	-	-		-

Table 2: R_f values of ethyl acetate extract fractions of isolates

(a) Under UV at 365nm

L3.41	L3.46	B.69	SL.4	RI.30	RI.24	S.43	S.4A	Rif	Ery	Nal
0.83	0.63	0.47	0.52	0.46	0.51	0.85	0.50	0.67	-	-
0.70			0.40	0.39		0.50	0.39	0.55		
0.60				0.26		0.43				
0.53						0.39				

- Bands of Erythromycin and Nalidixic acid were not visible under UV

(b) After vanillin/H_2SO_4 staining

L3.41	L3.46	B.69	SL.4	RI.30	RI.24	S.43	S.4A	Rif	Ery	Nal
0.83	0.63	0.72	0.52	0.46	0.51	0.50	0.80	0.67	0.55	0.87
0.70	0.40	0.58	0.40	0.39	0.40	0.39	0.53	0.55	0.50	
0.60	0.34	0.43	0.29	0.26		0.34	0.50			
0.53	0.26	0.36				0.26	0.39			
0.36		0.26					0.34			
							0.26			

(c) After anisaldehyde /H_2SO_4 staining

L3.41	L3.46	B.69	SL.4	RI.30	RI.24	S.43	S.4A	Rif	Ery	Nal
0.83	0.63	0.72	0.40	0.39	0.59	0.50	0.80	0.67	0.50	0.87
0.53	0.40	0.43	0.29	0.26	0.40		0.50	0.55		
		0.26					0.34			
							0.26			

Table 3: R_f values of methanol extract fractions of isolates

(a) under UV at 365nm

L3.46	RI.30	RI.24
0.88	0.80	0.75
0.84	0.70	0.64
0.77	0.62	0.58
0.64	0.57	
0.60		

(b) After Anisaldehyde/H_2SO_4 staining

L3.46	RI.30	RI.24
0.88	0.80	0.82
0.77	0.62	0.75
0.64	0.57	0.64
0.60	0.53	0.58
		0.55
		0.40

(c) After Vanillin/H_2SO_4 staining

L3.46	RI.30	RI.24
0.88	0.80	0.82
0.64	0.62	0.64
0.60	0.57	0.58

Table 4: Phylogenetic relatives of isolates showing maximum 16S rRNA gene sequence similarity

Sequences of related strains were retrieved from Eztaxon database and phylogenetic trees were constructed for each isolate (Figures 3-9).

S. No	Isolate	Length of 16S rRNA gene sequence	Accession no.	Clade Relatives	% Similarity
1.	B.69	1426 nucleotides	KF529978	*Streptomyces atriruber* NRRL B 24165^T (EU812169) *Streptomyces durhamensis* NRRL B 3309^T (AY999785) *Streptomyces coastaricanus* NBRC 100773^T (AB249939) *Streptomyces filipinensis* NBRC 12860^T (AB184198)	99.71% 98.16% 97.94% 97.93%
2.	L3.41	1439 nucleotides	KF499021	*Streptomyces parvulus* NBRC 13193^T (AB184326) *Streptomyces olivaceus* NBRC 12805^T (AB249920) *Streptomyces pactum* NBRC 13433^T (AB184398)	100% 99.29% 99.29%
3.	L3.46	1410 nucleotides	KF529977	*Streptomyces samsunensis* M1463^T (EU077190) *Streptomyces malaysiensis* NBRC 16446^T (AB249918)	99.85% 99.43%
4.	RI.24	1420 nucleotides	KF529979	*Streptomyces enissocaesilis* NRRL B-16365^T (DQ026641) *Streptomyces plicatus* NBRC 13017^T (AB184291) *Streptomyces rochei* NBRC 12908^T (AB184237) *Streptomyces vinaceusdrappus* NRRL 2363^T (AY999929)	100% 100% 100% 99.85%
5.	RI.30	1423 nucleotides	KF529980	*Streptomyces globosus* LMG 19896^T (AJ781330) *Streptomyces toxytricini* NBRC 12823^T (AB184173)	99.36% 99.36%
6.	S.4A	1423 nucleotides	KF529981	*Streptomyces enissocaesilis* NRRL B-16365^T (DQ026641) *Streptomyces plicatus* NBRC 13017^T (AB184291) *Streptomyces rochei* NBRC 12908^T (AB184237) *Streptomyces vinaceusdrappus* NRRL 2363^T (AY999929)	100% 100% 100% 99.85%
7.	SL.4	1429 nucleotides	EF424409 [26]	*Streptomyces albogriseolus* NRRL B-1305^T (AJ494865) *Streptomyces viridodiastaticus* NBRC 13106^T (AB184317) *Streptomyces atrovirens* NRRL B-16357^T (DQ026672) *Streptomyces heliomycini* NBRC 15899^T (AB184712)	99.65% 99.65% 99.58% 98.80%

Table 5: Key for identification of chemical moiety [42]

Staining reagent	Typical color	Susceptible compounds or chemical function
Anisaldehyde/H_2SO_4	Violet, Blue, Red, Grey/ Green	Sugars, steroids, terpenes
Vanillin/H_2SO_4	Red-brown, Grey, Seldom Yellow	Alcohols, phenols, steroids

Table 6: Color of ethyl acetate extract fractions stained with anisaldehyde/ H_2SO_4

	L3.46	RI.30	RI.24
Color produced	Brown	Light brown Grey	Light Brown
Chemical groups	Alcohols, Phenols, Steroids	Alcohols, Phenols, Steroids	Alcohols, Phenols, Steroids

Table 7: Color of methanol extract fractions stained with anisaldehyde/ H_2SO_4

	L3.46	RI.30	RI.24
Color produced	Brown **Slate color**	Brown Grey	Brown Grey
Chemical groups	Sugars, steroids, terpenes	Sugars, steroids, terpenes	Sugars, steroids, terpenes

Table 8: Color of ethyl acetate extract fractions stained with vanillin/H_2SO_4

	L3.41	L3.46	B.69	SL.4	RI.30	RI.24	S.43	S.4A
Color produced	**Yellow-orange** Grey	Brown Light Grey	Grey	Grey **Light-Yellow**	Grey	**Violet**	**Pink-Light Yellow**	Grey
Chemical groups	Alcohols, phenols steroids	Alcohols, phenols steroids	Alcohols, phenols steroids	Alcohols, phenols steroids	Alcohols, phenols steroids	No Alcohols, phenols steroids	No Alcohols, phenols steroids	Alcohols, phenols steroids

Table 9: Color of methanol extract fractions stained with vanillin/H_2SO_4

	L3.41	**L3.46**	**B.69**	**SL.4**	**RI.30**	**RI.24**	**S.43**	**S.4A**
Color produced	**Yellow-orange**	Brown Grey	**Orange** Grey	Light brown	Light brown	Violet	**Pink**	Grey
Chemical groups	No sugar moiety	Sugars, steroids, terpenes	Sugars, steroids, terpenes	Sugars, steroids, terpenes	Sugars, steroids, terpenes	Sugars, steroids, terpenes	No sugar moiety	Sugars, steroids, terpenes

Table 10: Antibiotics produced by respective clade members of isolates.

Clade members of B.69				
Strains	***Streptomyces atriruber***	***Streptomyces durhamensis***	***Streptomyces filipinensis***	***Streptomyces coastaricanus***
16S rRNA similarity	99.71%	98.16%	97.93%	97.94%
Compounds	Not known	Durhamycin, Pentane, Antifungal [20]	Filipin, Polyene, Antifungal [5]	Antinematodal and antifungal compound [16]

Clade members of L3.41			
Strains	***Streptomyces parvulus***	***Streptomyces olivaceus***	***Streptomyces pactum var. pactum***
16S rRNA similarity	100%	99.29%	99.29%
Compounds	Actinomycin D, Polypeptide, Antibacterial, Antifungal, Antitumor [51]	Kanchanamycins, 36 membered polyol macrolide, Antibacterial, Antifungal [18]	Pactamycin, Aminocyclitol, Antitumor [49]

Clade members of L3.46		
Strains	***Streptomyces samsunensis***	***Streptomyces malaysiensis***
16S rRNA similarity	99.85%	99.43%
Compounds	Not known	i) Azalomycin F complex, Macrocyclic lactone, Antifungal [9] ii) Mitomalcin, Protein, Antileukemic [31]

Clade members of RI.24, S.4A				
Strains	***Streptomyces vinaceusdrappus***	***Streptomyces plicatus***	***Streptomyces rochei***	***Streptomyces enissocaesilis***
16S rRNA similarity	99.85%	100%	100%	100%
Compounds	Amicetin, Nucleoside, Antibacterial Antiviral [54]	i) Amicetin, Nucleoside, Antibacterial, Antiviral [21] ii) Plicacetin, Nucleoside, Antibacterial [21] iii) Norplicacetin, Nucleoside, Antibacterial [17]	Borrelidin, 18 membered macrolide, Antibacterial, Antimalarial [4]	Not known

Clade members of RI.30		
Strains	***Streptomyces globosus***	***Streptomyces toxytricini***
16S rRNA similarity	99.36%	99.36%
Compounds	2-deoxystreptamine, Aminocyclitol aminoglycoside, Antibacterial, Antifungal [15]	Lipstatin, β lactone with hydrocarbon, Inhibitor of pancreatic lipase [48]

Clade members of SL.4				
Strains	***Sreptomyces atrovirens***	***Streptomyces albogriseolus***	***Streptomyces viridodiastaticus***	***Streptomyces heliomycini***
16S rRNA similarity	99.65%	99.65%	99.64%	98.80%
Compounds	Benzaldehyde , Antibacterial [11]	Amphomycin group, Antibacterial[19]	Not known	Heliomycin, Polyphenol, Antibacterial, Vasoconstructive [1,47]