Isolation of Lichens associated Actinomycetes: Determining its antibacterial activity against Multidrug resistant *Klebsiella pneumoniae* and Methicillin resistant *Staphylococcus aureus*

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ABSTRACT:

The rapid emergence of drug resistance among pathogenic bacteria, especially multidrug-Resistant bacteria, underlines the need to search for new antibiotics. In the present study,actinomycetes were isolated from lichens collected from acoconut tree. The actinomycetes were investigated for antimicrobial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and *Klebsiella pneumoniae*in order to identify potential antibiotic producer.MIC was determined and the organism was identified using 16s rRNA sequencing. Crude actino extracts showed 11mm and 9mm against MRSA and *Klebsiella pneumoniae*. The isolated actinomycetes was found to be *Streptomyces hygroscopicus*subspecies. GC-MS analysis identified several novel compounds. Thus, the isolated actinomycetes can be used for development of new antibiotics for treatment of MDR infection.

Keywords: Actinomycetes,Multidrug resistant bacteria, *Streptomyceshygroscopicus*,GC-MS, 16s rRNA sequencing,

INTRODUCTION:

The order actinomycetales consists of about eighty genera, almost all from terrestrial soils, in which they stay commonly as saprophytes, water and colonizing flowers displaying marked chemical and morphological variety, however from an awesome evolutionary line.[1]Actinomycetes are gram-effective microorganism with excessive guanine+cytosine content material of over 55% of their DNA, that have been identified as reassets of numerous secondary metabolites, antibiotics, and bioactive compounds that have an effect on microbial increase. Actinomycetes have filamentous nature, branching pattern, and conidia formation, that are much like the ones of fungi. For this reason, they're additionally called ray fungi. Actinomycetes produce branching mycelium which can be of types, viz., substrate mycelium and aerial mycelium. Streptomyces are the dominant of all actinomycetes[2].

Considering antimicrobial resistance problem, marine microorganisms with the bioactivity towards multi-drug resistant (MDR) pathogens have attracted many clinical interests [3].Although the share of methicillin-resistant Staphylococcus aureus (MRSA) reduced among 2011 and 2014, this lower become much less suggested in comparison with the preceding 4-yr period. In 2014, the EU/EEA population-weighted imply MRSA percent remained excessive, with seven out of 29 reporting nations having MRSA percentages >25 %[4].

Since several people began out stricken by illnesses resulting from infectious microorganisms, the hunt for his or her treatments began out and brought about the invention of a massive wide variety of antibiotics from microorganisms along with actinomycetes. Microorganisms have made an first-rate contribution to the fitness and wellbeing of humans all through the world [5]. Actinomycetes are filamentous gram-tremendous microorganism with excessive G + C content material and are the maximum extensively dispensed organization of micro-organisms in nature which in most cases inhabit the soil [6]. Among actinomycetes, the genus Streptomyces has lengthy been identified as a wealthy supply of beneficial secondary metabolites and remains a prime supply of latest bioactive molecules [7]. They are the foundation of a great variety of advertised antibiotics .

Multidrug resistant (MDR) microorganism are well-diagnosed to be one of the maximum critical modern-day public fitness problems. The Infectious Diseases Society of America acknowledges antimicrobial resistance as "one of the best threats to human fitness

international"Several problems underlie the important risk this is posed via way of means of the upward push of MDR microorganism. First and maximum importantly, results in sufferers inflamed with MDR microorganism have a tendency to be worse in comparison with sufferers inflamed with extra prone organisms.As a outcome of antibiotic overuse and misuse, nosocomial infections as a result of multidrug-resistant microorganism constitute a physician's nightmare at some point of the world. The primary multidrug-resistant nosocomial pathogen, are to be had or below investigation. Actinomycete enables to forestall the boom of microorganisms[8].

Lichens are symbiotic association of fungi, inexperienced algae and/or cyanobacteria with an extensive sort of morphologies and international distribution.As pioneers of terrestrial habitats colonization they're discovered from arctic to tropical areas in a big variety of environments growing amongst others on stones, arid soils, or as epiphytes on plant life.Although the symbiotic additives of lichens had been significantly defined collectively with their richness as manufacturers of metabolites with organic activitieslittle is understood approximately the range of the microbial network inhabiting theseenvironments. Antarctic bloodless desolate tract cryptolithic groups ruled with the aid of using lichens were one of the maximum substantially studied endolithic microbial associations. Their exam via way of means of molecular techniques has proven the extreme,microbial variety inhabiting this ecosystem, wherein awesome phylotypes of Actinobacteria were defined amongst others. [9]

Therefore, the present study focuses on solation of actinomycetesfrom lichens. The pathogenic organisms were tested for its drug resistance by drug profiling. The actinomycetes were investigated for antimicrobial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and *Klebsiella pneumoniae*. MIC was determined and the organism was identified using 16s rRNA sequencing. GC-MS analysis was performed to identify the bioactive compounds present in the extract.

MATERIALS AND METHODS

Sample collection and surface sterilization

Lichen samples were attained from the tree barks of the botanical garden, Nilgiris, Tamilnadu. The collected lichen samples were washed in water, followed by surface sterilization with 70% ethanol for 30 s, 4% sodium hypo chloride for 3 min and lastly it was washed with

sterile distilled water (Araújo et al., 2000). After sterilization 3 g of sample was homogenized with 30 ml of sterile distilled water in a surface sterilized motor and pestle.

Collection of MDR pathogen and Drug Profiling

Multidrug resistant *Escherichia coli* and *Klebsiella pneumoniae* was procured from Microtek Laboratories. The Drug profiling of the bacteria is necessary to identify the resistance of the bacteria to drugs. Muller hinton plates were prepared. Overnight cultures of test pathogens were cultured and 0.1% of culture solution of each test organisms was streaked throughout the petri plate with the sterile cotton swab by roating the plate at 60º angle for each streaking. Antibiotic discs were placed on the plates and the plates were incubated at 37ºC for 48h. The zones were measured and compared with the standard zone interpretation chart (Himedia).

Isolation of actinomycetes

The homogenized lichen samples were used for isolation of actinomycetes (Deng et al., 2015). One ml of homogenized sample was serially diluted up to 10^{-5} dilution. 100 µl of sample from each dilution was inoculated into starch casein nitrate (SCN) agar and incubated at 28 °C for 5 days. After incubation, the colonies on SCN agar plates were re-streaked in a fresh plate containing SCN medium to obtain a pure culture.

Extraction of bioactive compounds

Isolated actinomycetes was cultured in starch casein broth for production of antibiotic compounds. Each active actinomycetes isolate was cultured in broth on a rotary shaker at 180 rpm at 30Cup to 4 to 5 days. Then the media was harvested, centrifuged at 5000 rpm for 10 min to remove cell debris and supernatant was filtered by wattsmen filter paper no.1. Ethyl acetate was added to the culture filtrate in the ratio of 1:1 (v/v) and shaken vigorously for one hour. The solvent phase that contains antibiotic was separated from the aqueous phase using separating funnel. Ethyl acetate layer that contain antibiotic was concentrated by evaporation to dryness at 40ºC to obtain residue. Crude antibiotic residue obtained was purified using methanol to give (0.8 g) of brown crude extract of antibiotic (Ahmed, 2007). The crude extracts of isolated actinomycetes were dissolved in the methanol and were tested for antimicrobial activity using agar-well diffusion method. Test organism were inoculated in nutrient broth and incubated for 24 hours at 37 °C. After the Incubation twenty four hours old grown test organism were spread on nutrient agar plate by sterile spreader. The wells (6 mm diameter) were made using

sterile cork borer, 100 µl crude extract were added in wells and incubated at 37 °C for 24 hour. After incubation the zone of inhibition were measured and recorded. (Kumar S, Kannabiran K.2010)

Determining Minimum inhibitory concentration (MIC) of the actinomycetes extract

Minimum inhibitory concentration (MIC) of the actinomycetes extract was determined against the two major UTI causing pathogen *Klebsiella pneumoniae* and *Escherichia coli* by well diffusion method [10]. Nutrient Agar was prepared and sterilized, and poured into plates. (Nutrient agar Composition (for 100ml): Peptone: 0.5g; Yeast extract: 0.5g, Beef extract: 0.3g, Sodium chloride: 0.5g, Agar 1.5 g; Total pH: 7.0 ± 0.2). Overnight cultures of test pathogens were cultured and 0.1% of culture solution of each test organisms was streaked throughout the petri plate with the sterile cotton swab by roating the plate at 60º angle for each streaking. 6mm well borer was used to bore wells on the agar surface of each NA plates. About four concentrations of samples (2.5µl, 5 µl, 7.5 µl & 10 µl) were loaded into the well and the plates were incubated in an incubator at 37ºC for 48h. The antibacterial activity was determined in terms of inhibitory zones around the wells loaded with samples in all the Nutrient Agar plates containing test pathogens. The obtained clear zones were observed and measured in millimetre (mm). The minimum concentration showing inhibitory zones was recorded as MIC

DNA Isolation from Sample (Kit method)

To the culture pellet, add 750µl of homogenizing 1X Buffer vortex and keep it in a dry bath at 65°C for 20 minutes.Add 750 µl of Lysis buffer and mix it well manually.Keep it in the dry-bath for 20 minutes at 65°C and Centrifuge it at 12,000 rpm for 10 minute.Transfersupernatant into 2ml tube.Add same volume of Isopropanol and mix it manually and transfer to the column (750 µl each time).Spin at 12000 rpm for 1 minute and add wash buffer 750 µl and spin at 12000 rpm for 1 minute.Repeat the wash buffer step,dry spin for 2 minutes.Add 50 µlof elution buffer, spin at 12000 rpm for 1minute.The DNA thus obtained has to be quantified.

DNA quantification

DNA concentration in the sample is estimated by recording absorbance at 260 and 280 nm in a UV/ VIS spectrophotometer.10µl of genomic DNA sample taken in a quartz cuvette. The volume made to 2ml with distilled water.The absorbance is measured at 260 and 280 nm using the UV spectrophotometer.The ratios of A260/A280 are calculated.Calculated DNA concentration using the relationship for double stranded DNA, 1 O.D. at 260 nm = 50 g/ ml

PCR conditions:

Initial denaturation 95°C for 2 minutes.Final denaturation 95°C for 30 seconds and annealing 55°C for 30 seconds .Elongation 72 °C for 1 minute and repeat steps 2,3 and 4 for 30 cycles,Final Elongation 72 °C for 10 minutes ,Hold at 4°C forever.

Preparation of PCR reaction mixture:

Each PCR reaction for testing the amplification efficiency and development of multiplex PCR assays for DNA barcode primers contained 1µl DNA template (25 ng), 2µl 10X reaction buffer, 0.5µl MgCl2 (50pM), 1µl dNTPs mix (10mM), 1µl forward primer (10pM), 1µl reverse primer (10pM), 0.5µl Taq polymerase (5 U/pi) and the final volume 25µl will be adjusted with molecular grade water. Primers are standard primers available for 16srRNA gene amplification.

Gel purification protocol

Cut the required DNA band on gel then add 600 µL of Gel solubilization buffer and heat at 55°C until gel dissolves completely.Add 200 µL of Isopropanol, Mix and transfer to column.Spin at 12000 rpm for 1 minute.Add 700 µL of wash buffer, spin at 12000 rpm for 1 min.Dry Spin for 2 minutes and then add 20 µL of Elution Buffer, Spin at 12000 rpm for 1 minute

Sanger Sequencing PCR

Initial denaturation 95°C for 2 minutes .Final denaturation 95°C for 30 seconds.Annealing 50°C for 30 sseconds, ermination 60 °C for 4 minutes and rpeat steps 2,3 and 4 for 30 cycles,Hold at 4°C forever.

Post Sequencing and PCR Purification

Add 125 Mm 2.5 µL EDTA to each well and give a short spin and then add 35µL of Ethanol using multichannel pipette.Vortex for 10 minutes at 2000 rpm, Centrifuge at 3510 rpm for 30 minutes.Using tissue bed decant ethanol at 300 rpm (for 30 seconds invert the plate).Add 40µL of 80% Ethanol to the wells and Centrifuge at 3510 rpm for 12 minutes and repeat the above mentioned invert spin ,Air dry for 30-45 minutes covering the plate with lint free tissue,Add 13µL of HiDiFormamide and give a short spin.Denature at 95°C for 5 minutes.Place the plate in sequencer

Data analysis

Sequencing files obtained will be .AB1 format which can be viewed by using softwares like FinchTV, BioEdit, ChromasLite, SeqScanneretc.Quality of the obtained sequence can be observed through Electropherogrampeaks.Analyse the sequencing data using BLAST server or servers related to specific databases.

GC-MS analysis:

GC-MS analysis of methanol extract was performed with GC clarus 500 Perkin Elmer system and Gas chromatograph interfaced to a Mass spectrometer (GC-MS) equipped with an Elite – 1 fused silica capillary column (30 mm x 0.25 mm 1D x 1 µm df, 37 composed of 100% Dimethyl poly siloxane and the components were separated using Helium as carrier gas at a constant flow of 1 ml/min. The injector temperature was set at 260°C during the chromatographic run. The 1µL of extract sample injected into the instrument the oven temperature was as follows: 60 °C (2 min); followed by 300 °C at the rate of 10 °C min−1; and 300 °C, where it was held for 6 min. Mass spectra were taken at 70 eV, a scan interval of 0.5 seconds and fragments from 45 to 450 Da. The relative % amount of each component was calculated by comparing its average peak area to the total areas, the software adopted to handle mass spectra and chromatograms was a turbomass (Srinivasan et al., 2013). The spectrums of the components were compared with the database of spectrum of known components stored in the GC-MS NIST (2008) library.

RESULTS AND DISCUSSION

Collection, isolation and characterization ofactinomycetes

Figure-1: Collection of Lichens

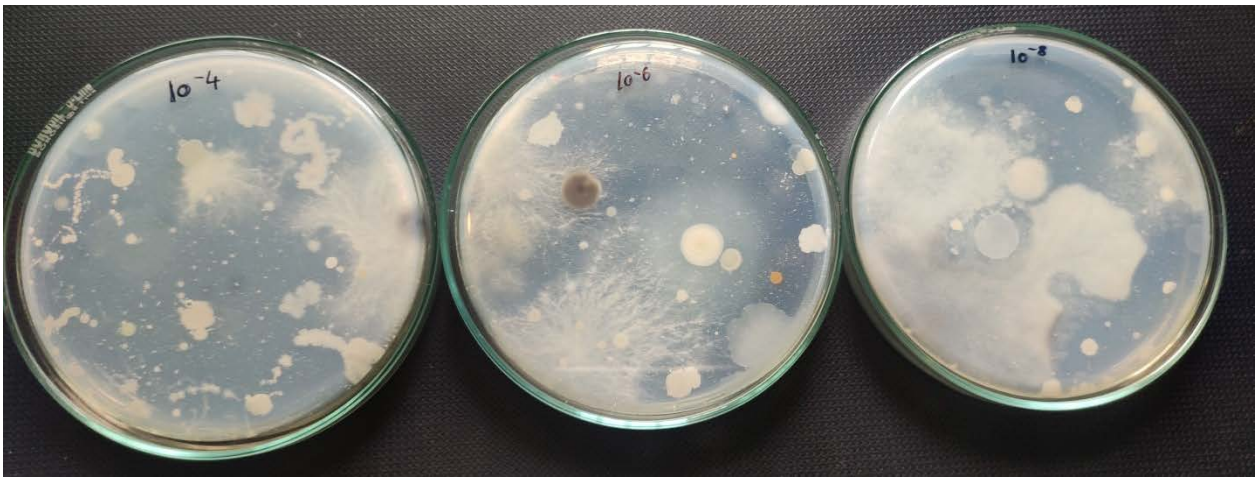

Figure-2: Serial dilution

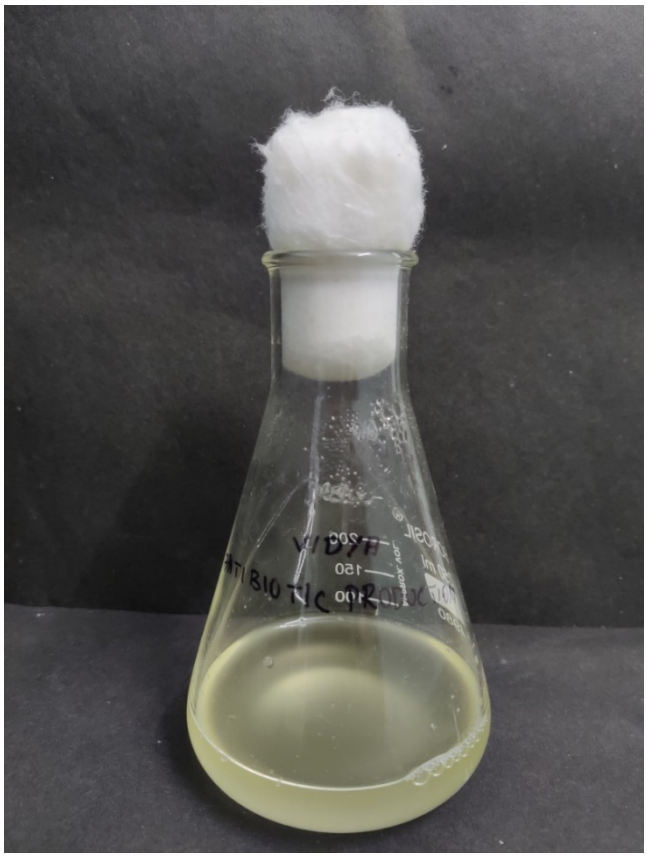

Figure-3: Cultivation of predominant actinomycetes

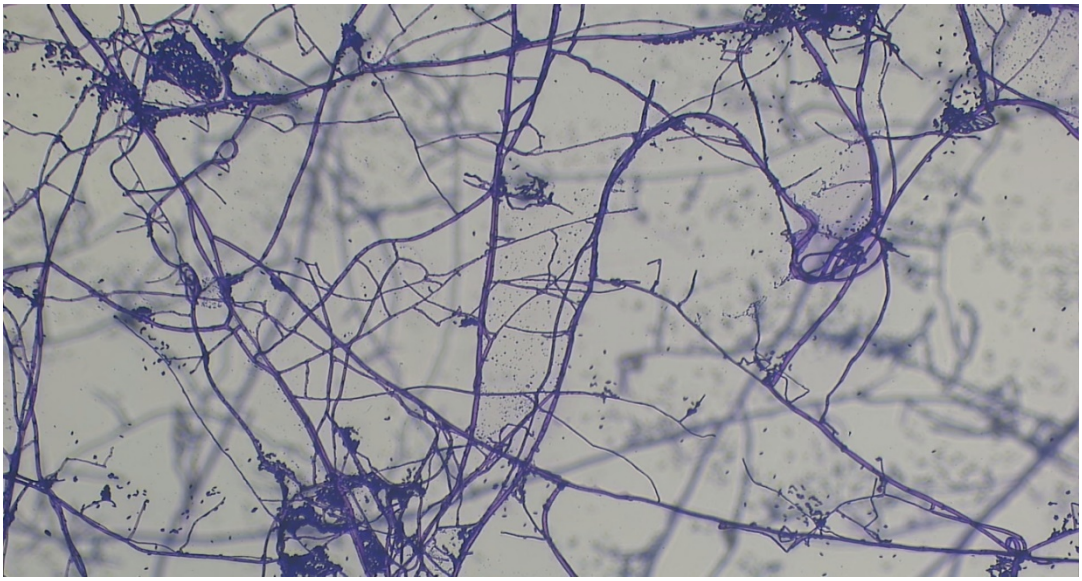

Figure-4: Gram staining of isolated actinomycetes

The lichens were collected from an infected coconut tree (Figure-1). Since lichen is an association of microalgae and fungi, there are possibilities for presence of other bacteria too. The lichens were serially diluted and plated on starch casein nitrate agar (Figure-2). The predominant actinomycetes was isolated and cultivates (Figure-3). The isolated strain was subjected for staining and from staining analysis the strain was found to be long rods (Figure-4).

Drug profiling

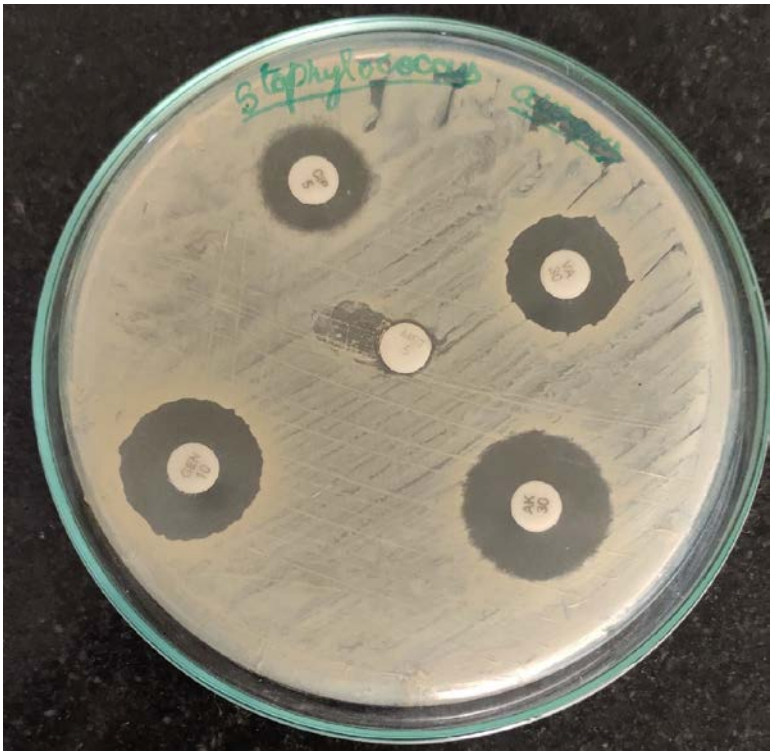

Figure-6: Drug profile of the MRSA

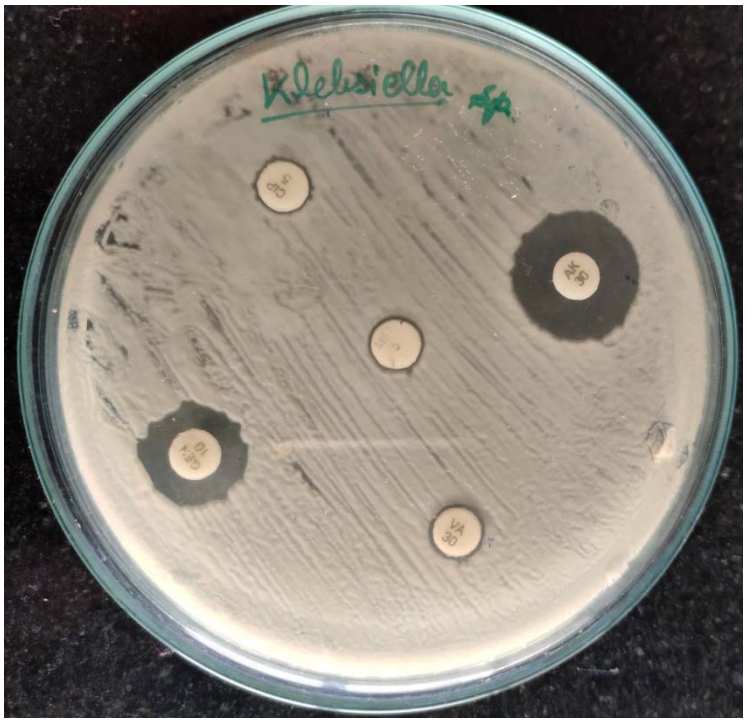

Figure-7: Drug profile of *K. pneumoniae*

Table-1: Drug profiling

S. No	Antibiotics	Inhibitory zones	
		MRSA	***Klebsiella pneumoniae***
1	Methicillin	- (Resistant)	- (Resistant)
2	Ciprofloxacin	14mm (Resistant)	18mm (Sensitive)
3	Gentamycin	16mm (Sensitive)	- (Resistant)
4	Vancomycin	18mm (Sensitive)	13mm (Intermediate)
5	Amaikacin	19mm (Sensitive)	- (Resistant)

Drug profiling was performed to determine the resistance of the pathogens to several antibiotics. MRSA and *Klebsiella pneumoniae* were found to be resistant to Methicillin. Further MRSA was resistant to Ciprofloxacin. *Klebsiella pneumoniae* was found to be resistant to Gentamycin and Amaikacin, intermediate for Vancomycin.

Determining MIC of the extract

Figure-8:MIC determination against MRSA

Figure-9: MIC determination against MDR*Klebsiella pneumoniae*

Table-2: MIC of actinmycetes extract against Multidrug resistant bacteria

S. No	Samples	Inhibitory zones (mm)	
		MRSA	*Klebsiella pneumoniae*
1	Methicillin	0	0
2	25 µl	0	0
3	50 µl	8	0
4	100 µl	11	9

Minimum inhibitory concentration (MIC) is the lowest concentration required to inhibit the bacteria. 25µl of actino extract showed no inhibitory zones. 50µl showed 8mm against *Escherichia coli*and no zone against *Klebsiella pneumoniae.* 100µl of actino extract showed 11mm against *Escherichia coli*and 09mm against *Klebsiella pneumoniae.* Therefore, the MIC of the actino extract against *Escherichia coli*and *Klebsiella pneumoniae*was found to be 50µl and 100µl.

PCR amplification and species identification

From PCR amplification, the amplified product was found to be 850bp. *Figure-9* shows the bands of agarose gel electrophoresis. Further the amplified 16s rRNA gene was subjected for sequencing. The identified sequence was shown below and the chromatogram was shown in Figure-10.

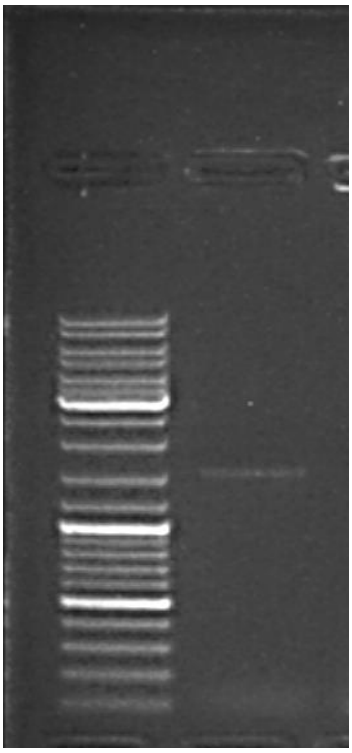

Figure-10: PCR amplification of the 16s rRNA gene

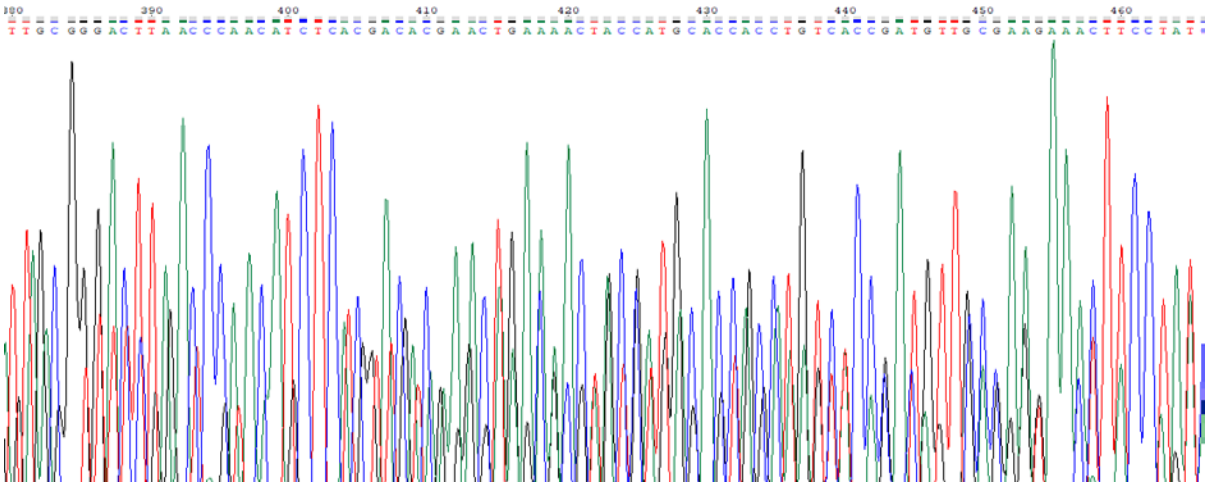

Figure-11: Sequencing result

Sequence of the isolated Actinomycetes:

TGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAACCGGTTTCGGCCGGGGATT
AGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCC
CTGGAAACGGGGTCTAATACCGGATATGACTGCCGACCGCATGGTCTGGTGGTGGA
AAGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTGATG
GCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGG
ACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATG
GGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAA
CCTCTTTCAGCAGGGAAGAAGCGCAAGTGACGGTACCTGCAGAAGAAGCGCCGGCT
AACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTAT
TGGGCGTAAAGAGCTCGTAGGCGGCTTGTCGCGTCGGATGTGAAAGCCCGGTCCGT
ACGTAAGTCGGATTCGGACCGGTTAAAAAAAACCCCTTTCCGGGATTCACGTGCAAC
GTGCAACGTGCATGCACAGTGCATGTCACGTACGTAGCGCTGACCGTAGCTAGCTGA
CGTAGCGTAGCCGATGCCAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGG
GGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTT
ACCAAGGCTTGACATACATCGGAAACCTCTGGAGACAGGGGCCCCCTTGTGGTCGG
TGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCC
CGCAACGAGCGCAACCCTTGTCCTGTGTTGCCAGCATGCCCTTTGGGGTGATGGGGA
CTCACAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCAT
CATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCACGTGCGTAGTGCACG
GTACAATGAGCTGCGAAGCCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGT
TCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGAT
CAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCAC
GAAAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCTTGTGGGGGGCCGTCGAAGG
TGGGACTGGCGATTGGGACGAAGTCGTAACAAGGTAGCCGTACCGGAAGG

BLAST analysis

The similarity was determied between standard sequence and DNA base sequence by BLAST technique and the Query cover was displayed which indicates the similarity percentage of about 89% with *Streptomyces hygroscopicus* subsp. Since the similarity percentage is low, there is a high chance for the new strain. Figure-11 and Figure-12 shows the BLAST results and BLAST graphic summary result.

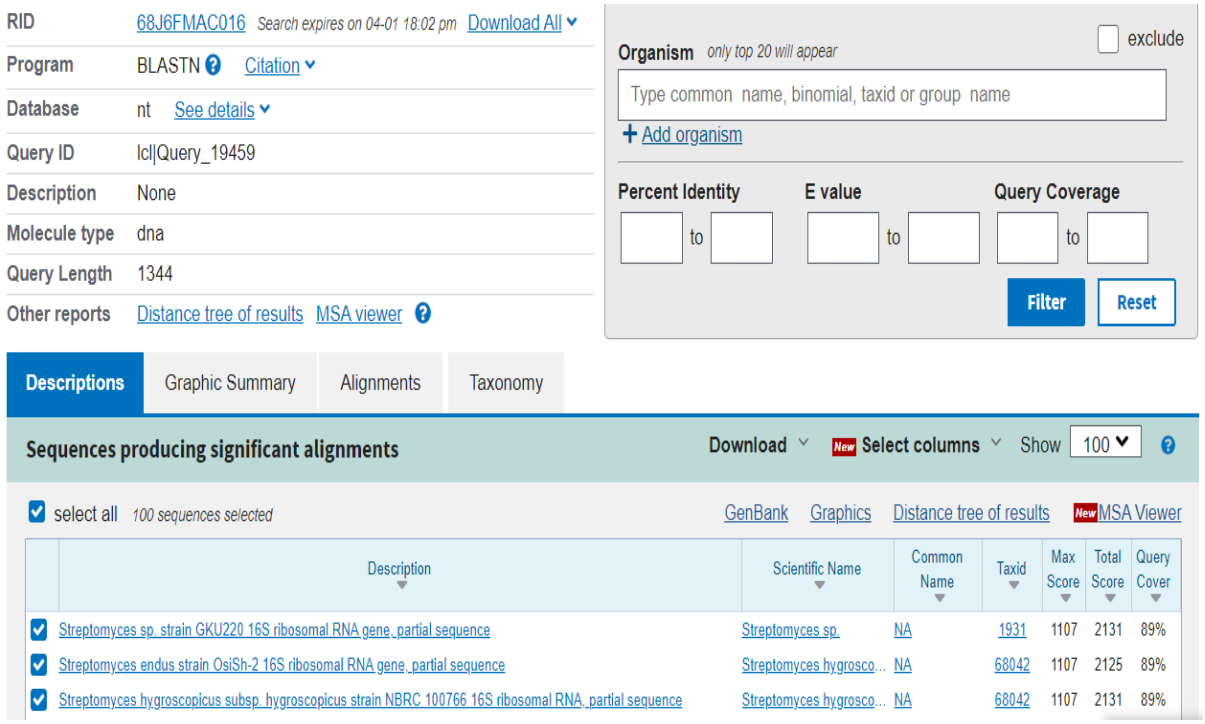

Figure-11: BLAST results

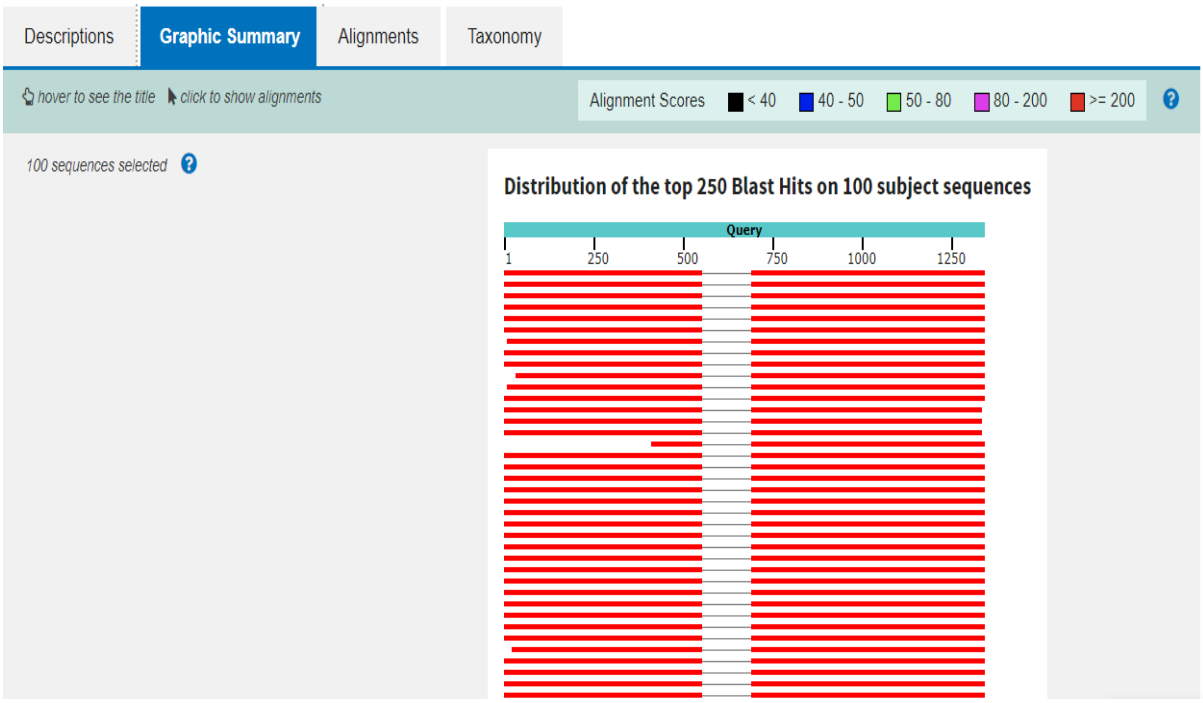

Figure-12:BLAST graphic summary result

GC-MS analysis:

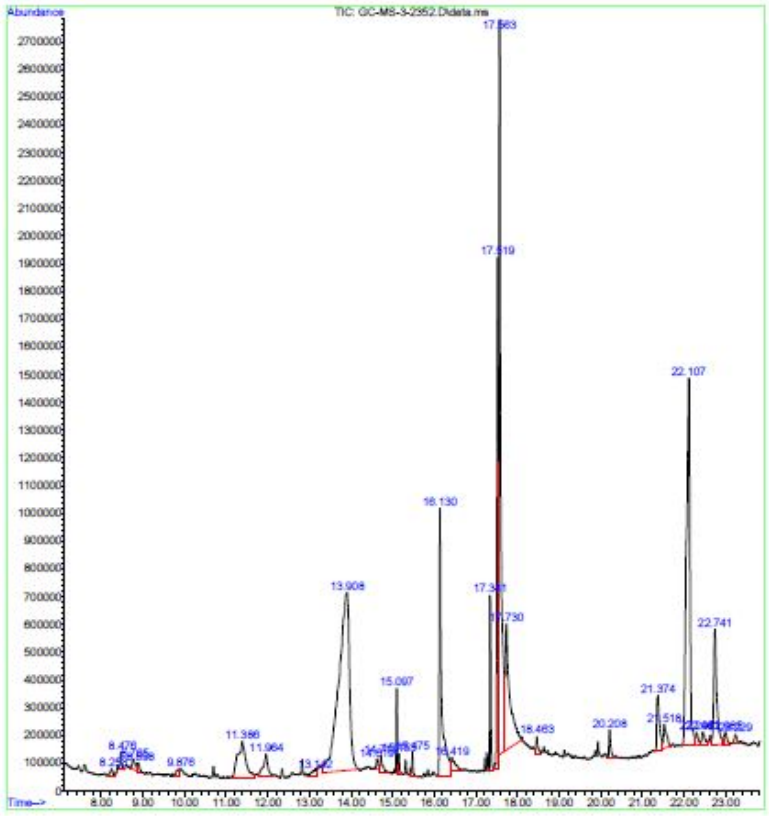

Figure-13: GC-MS chromatogram of crude extract

Table-3: Compounds identified from GC-MS analysis

S. No	Compounds identified	RT
1	1,2-Cyclooctanedione	8.253
2	Cholestan-5-en-3-ol piperidinomethyl ether	8.476
3	Benzofuran, 2,3-dihydro-	8.765
4	Pentylamine, N-ethyl-N-nitroso-	9.876
5	2-Isopropoxyethyl propionate	11.396
6	3,4-Altrosan	11.964
7	d-Glycero-l-gluco-heptose	13.142
8	4-O-Methylmannose	13.908
9	Methoprene	14.619
10	Cyclotetradecane	14.719
11	Trichloroacetic acid, undec-10-enyl ester	15.097

The GC-MS end result proven withinside the figure-thirteen and The spectrums of the additives had been in comparison with the database of spectrum of recognized additives saved withinside the GC-MS NIST (2008) library. Figure-13 shows the GC-MS chromatogram of actino extract and Table-3 shows the compounds identified.

DISCUSSION:

Actinomycetes are the most biotechnologically valuable prokaryotes responsible for the production of about half of the discovered bioactive secondary metabolites including antibiotics [1]. Theyare the main source of clinically important antibiotics, most of which are too complex to be synthesized by combinatorial chemistry; thus, microbial natural products still appear as the most promising sources for developing future antibiotics.,

The potent actinomycete strains isolated from the sediment of Caspian Sea have been identified as Streptomyces using 16S rRNA sequencing followed by BLAST analyzing. Identification via genetic approaches with a good speed is widely developed. Among molecular techniques used, 16S rRNA sequencing is a “gold standard” option due to the possessing the proper gene size, and availability of a large number of sequences in databases for comparison [11]

The isolated actinomycetes showed antibacterial activity against some MDR pathogens such as MRSA and VRE. Sujatha*et al*. observed that marine Streptomyces species possess efficient antagonistic activity against MRSA [12], while Mercy Rajan and Kannabiran reported the ability of some marine Streptomyces isolates to produce antibacterial agents active against VRE [13].

Sibanda et al. reported that crude extracts from the liquid culture of marine actinomycetes inhibited the Gram-negative bacteria more than Gram-positive bacteria strains [14]. Conversly, the antibacterial activity of the strains isolated in the present study was higher against Gram-positive MDR pathogens. Bioactivity against MRSA and VRE is valuable due to many health problems raised from MRSA and VRE. These results correlates with the above results and the strain isolated from Lichens associated Actinomycetes are active against Multidrug resistant bacteria. Actinomycetes have DNA with a high GC content. A simple technique to enrich for

high GC content DNA is to extract the DNA from the soil and then subject it to ultracentrifugation [15].

Conclusion:

In this study we have explored the use of lichen associated actinomycetes as annovel source for antibiotics.Crude actino extracts showed 11mm and 9mm against MRSA and *Klebsiella pneumoniae.* The isolated actinomycetes was found to be *Streptomyces hygroscopicus*subspecies. GC-MS analysis identified several novel compounds. Thus, the isolated actinomycetes can be used for development of new antibiotics for treatment of MDR infection.

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