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Antibacterial potential of Actinobacteria from a Limestone Mining Site in Meghalaya, India

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Abstract

This work attempts to assess the antimicrobial potential of actinobacteria isolated from limestone mining sites which hitherto, is an under-explored niche for exploring novel bioactive metabolites. Actinobacteria were selectively isolated from Mawsmai, Meghalaya, India, a limestone mining area, using different pretreatment methods. Forty-seven isolates were obtained, which were identified based on their morphological, biochemical and chemotaxonomical characteristics. S*treptomyces* was the dominant cultivable genera which constituted 76% of the isolates cultivated. All the isolates were screened for antimicrobial activity against three Gram-negative viz. *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae,* and three Gram-positive bacteria viz. *Staphylococcus aureus, Bacillus subtilis* and *Micrococcus luteus* and besides, two candidal species viz. *Candida albicans* and *C. tropicalis*. 19% of the total isolates showed antibacterial activity against at least one of the test bacterial strains used. The identity of the four bioactive isolates viz. LD-21, LD-29, LD-34 and LD-39 was confirmed as *Streptomyces* sp. on the basis of their 16S rDNA sequence and 16S rRNA secondary structure analysis. These isolates showed antibacterial activity against at least two Gram-positive bacteria and all the four harbored at least one of the three biosynthetic gene clusters viz. type-I and type-II polyketide synthases and non-ribosomal peptide synthetase which are related to synthesis of bioactive metabolites.

Keywords: *Streptomyces,* Antimicrobial, Biosynthetic gene clusters, 16S rRNA secondary structure.

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INTRODUCTION

Actinobacteria is a phylum of Gram-positive or Gram-variable bacteria with diverse morphology ranging from cocci to highly differentiated mycelia[1]. They have high G+C content in their DNA ranging from 50-70 mol %[1]. They are widely distributed in all kinds of terrestrial and aquatic environments[1]. Most of them are saprophytic, however, some are pathogenic to plants and animals[1]. Actinobacteria has been one of the important sources of naturally-derived antibiotics for the last 75 years and will remain so in the years to come especially for the discovery of novel antibiotics[2]. Antibiotics have also been discovered from various other natural sources like plants, animals, fungi and bacteria[3]. Naturally-derived antibiotics, even though are more difficult to discover due to complexity in identifying new scaffolds[4], are superior over the synthetic ones because they are products of natural selection and have gone through the long process of evolution and have established compatibility with biological targets[4]. More than two-thirds of clinically used antibiotics were either natural products or their semi-synthetic derivatives[5]. However, in recent years, there has been some lag in discovering new classes of natural product antibiotics and this has put a pressure on the pharmaceutical industry due to the emergence of resistance amongst the pathogens. Reasons like rediscovery of known compounds, complexity of the natural antibiotic scaffolds and their suitability as drugs, their unstability, very low yield and difficulty in purification have constrained pharmaceutical companies to shift their interests to synthetic antibiotics in the 21st century[4] to stay ahead of emerging antibiotic-resistance. Recent advancement in new generation genome sequencing technology, bioinformatics, analytical chemistry, metagenomics and genome-scale metabolic models[4–7] has now impelled the researchers to return to natural products for drug discovery. Search for microbial taxa from under-explored ecological niches increases the possibility of discovering novel molecules from nature[5]. Limestone-rich areas mainly dominated by a $CaCO_3$ environment, are under-explored ecological niches for mining of microorganisms for various metabolic properties. Recent studies have revealed that both hypogean and epigean limestone actinobacteria are potential antibiotics producers[8–14]. The state of Meghalaya located in the north-eastern part of India falls under the Indo-Burma Biodiversity Hotspot[15]. Therefore, mining for antibiotics producing actinobacteria from under-explored ecological niche of limestone mines of diversity-rich state of Meghalaya enhances the possibility of discovery of novel metabolites. This paper, therefore, reports on diversity of actinobacteria having antibacterial activities from limestone mining areas located at Mawsmai, Meghalaya, India.

MATERIALS AND METHODS

Sample collection

The sampling site was from a limestone mining area at Mawsmai near Sohra in Meghalaya, India (N25°15.364' E91°43.885'). Soil samples from around the mining area were aseptically collected in sterilized polythene bags and brought to the lab and stored at 4±1°C until further use.

Determination of soil physico-chemical characteristics

Soil and air temperatures (in °C) were measured in three different locations using a soil thermometer and an ordinary thermometer respectively. Moisture content (%) of the soil was determined gravimetrically[16]. pH was determined using a digital pH meter[17]. Organic carbon was determined following the method of Anderson and Ingram (1993)[17]. Total nitrogen was determined by the Kjeldahl Method[18]. Available Phosphorus was determined by the molybdate blue method[16]. Potassium was determined by the flame photometer method[18].

Soil pretreatment and isolation of actinobacteria

The collected soil samples were air-dried at room temperature for one week and mixed equally into one composite sample. The air-dried samples were then pretreated by (i) dry heating at 120°C for 1 hour, (ii) wet heating at 70°C for 15 minutes in a water bath, (iii) 1.5% phenol at 25±1°C for 30 minutes, (iv) 0.2% humic acid at 25±1°C for 30 minutes and (v) combination of i & iii. The unpretreated air-dried soil samples were also used as inoculum for isolation. 1g pretreated and unpretreated soil samples were suspended in 9ml sterilized distilled water and serially diluted. All the undiluted and serially diluted soil sample suspensions were used as inocula for isolation.

Five selective media were used for isolation viz. Actinomycete Isolation Agar (AIA), Bennett's Agar (BA), Starch Casein Agar (SCA), Streptomyces Agar (SA) and Humic acid Vitamin Agar (HVA)[19]. The above media were amended with Nystatin (50µg ml^{-1}) and Rifampicin (20µg ml^{-1}). 100µl of the soil sample suspensions were inoculated on the above selective media and incubated at 28±1°C for up to four weeks. Based on colony morphology, colonies were picked from the culture plates, sub-cultured and maintained in Bennett's agar medium. The recovery of actinobacterial colonies from different pretreated samples and media were recorded as actinobacterial population number in cfu g^{-1} soil. All observations were recorded in triplicates and expressed as sample mean ± standard error. Multiple comparisons of sample means were done using ANOVA with Duncan's test at $p \leq 0.05$. Statistical analysis was performed with XLSTAT software.

Morphological characterization

The selected colonies were grown in ISP3 (oat meal agar) medium by coverslip culture method[20] and incubated at 28±1°C for 7 days. The coverslip cultures were then placed on a glass slide and observed under the microscope (Olympus CX21i) at 400X magnification for presence of aerial and substrate mycelia, spore chains and other reproductive structures.

Biochemical and chemotaxonomic characterization

Biochemical tests viz., casein, xanthine, hypoxanthine and tyrosine hydrolysis were performed following the methods of Berd (1973)[21]. Chemotaxonomic analyses viz. cell wall chemotype of 2,6-diaminopimelic acid (DAP) and whole cell sugars were performed by thin-layer chromatography following the methods of Hasegawa *et al.* (1983)[22]. The scheme for preliminary identification of actinobacteria was followed according to Berd (1973)[21] and Staneck and Roberts (1974)[23].

Phylogenetic analysis of 16S rDNA sequence

Genomic DNA of the selected bioactive isolates were extracted by enzymatic method[24] followed by PCR of 16S rDNA. The primers used for amplification were 27F (5'-AGAGTTTGA-TCMTGGCTCAG-3') and A3R (5'-CCAGCCCCACCTT-CGAC-3')[25]. PCR reactions were carried out in an Eco96™ thermal cycler (Himedia, India) in 25µl reaction mixture containing PCR buffer (Promega) with 1.5mM $MgCl_2$, 0.2mM of each dNTP (Promega), 0.5µM of each primer (IDT), 0.625U of Taq DNA polymerase (Promega) and 5µl of extracted DNA as template DNA. Amplification parameter was an initial denaturation of 95°C for 5 minutes, 35 cycles of 95°C for 45 seconds, 56°C for 60 seconds, 72°C for 90 seconds and final extension of 72°C for 7 minutes. The amplified products were analyzed by agarose gel electrophoresis (with 1.2% w/v agarose added with 1µl/10ml v/v LabSafe™ Nucleic Acid Stain, G-Biosciences). A 100bp DNA ladder was used for size reference. The amplified products were purified and sequenced at Xcelris Labs Ltd., Ahmedabad, India. The partial 16S rDNA sequences obtained were matched with type strains from EzBioCloud database[26] for determining sequence similarities. Phylogenetic relationships of the selected bioactive isolates along with thirty-two related type strains were determined using MEGA version 7[27] and phylogenetic tree was constructed using the Maximum Likelihood method based on the Tamura 3-parameter model[28]. A discrete Gamma distribution was used to model evolutionary rate differences among sites (+G). The rate variation model allowed for some sites to be evolutionarily invariable (+I). Bootstrap analysis[29] was done with 1000 replicates. The partial 16S rDNA sequences were deposited in GenBank.

Phylogenetic analysis of predicted 16S rRNA secondary structure

The use of 16S rRNA secondary structures for phylogenetic analysis of actinobacteria was understood from Ghosh *et al.* (2019)[30]. The 16S rDNA sequences obtained were converted into 16S rRNA sequences and subsequently the 16S rRNA secondary structures with their minimum free energy (MFE) were predicted using the RNAfold server[31] at the ViennaRNA Web Services (http://rna.tbi.univie.ac.at/). The obtained 16S rRNA secondary structures of the selected bioactive isolates along with their MFEs were then compared with the related type strains from EzBioCloud database (which were used for phylogenetic analysis based on 16S rDNA sequence) to confirm their identity. Consensus 16S rRNA secondary structures were also generated using the RNAalifold server[32] at the ViennaRNA Web Services between the bioactive isolates and

the related type strains with the closest MFEs to analyze for conserved regions. The selected 16SrDNA sequences with the closest MFEs were aligned using ClustalX 2.1 (http://www.clustal.org/download/2.1/) and sequence overhangs trimmed before using the aligned file for generating consensus 16S rRNA secondary structure.

Screening of isolates for antimicrobial activity

Isolates were screened for antimicrobial activity against *Escherichia coli* MTCC 1669, *Pseudomonas aeruginosa* MTCC 4673, *Klebsiella pneumoniae* MTCC 10309, *Staphylococcus aureus* MTCC 9886, *Bacillus subtilis* MTCC 1305, *Micrococcus luteus* MTCC 1538, *Candida albicans* MTCC 7253 and *C. tropicalis* MTCC 184. The above test microorganisms were obtained from Microbial Type Culture Collection and Gene Bank, CSIR-Institute of Microbial Technology, Chandigarh, India. Culture filtrates used for antimicrobial assays were prepared by first culturing the isolates in 5ml Bennett's broth supplemented with 0.05% humic acid, pH 7.5[33] in 15ml tubes and incubated at 28±2°C for 15 days. The broth cultures were then filtered using an ordinary filter paper followed by sterilization using membrane filters (PVDF 0.2µm). Uninoculated media were used as negative-controls while amikacin (30µg), vancomycin (30µg) and amphotericin-B (20µg) discs were used as positive-controls. Antimicrobial screening was performed by agar-well diffusion method[34]. Antimicrobial activity was assessed as zone of inhibition (mm) by subtracting the diameter of the agar-well from the diameter of inhibition. All the culture filtrates were prepared and tested in triplicates.

Detection of biosynthetic gene clusters

PCR was performed on the genomic DNA of the selected bioactive isolates for checking the presence of three biosynthetic gene clusters (BGCs) related to synthesis of bioactive metabolites viz. type I polyketide synthase (PKS-I), type II polyketide synthase (PKS-II) and Non-ribosomal peptide synthetase (NRPS)[8,35]. Three pairs of primers viz. K1F (5'-TSAAGTCSAACATCGGBCA-3') – M6R (5'-CGCAGGTTSCSGTACCAGTA-3'), KSαF (5'-TSGCSTGCTTGGAYGCSATC-3') – KSαR (5'-TGGAANCCGCCGAABCCGCT-3') and A3F (5'-GCSTACSYSATSTACACSTCSGG-3') – A7R (5'-SASGTCVCCSGTSCGGTAS-3') were used respectively[8]. PCR was performed in final volume of 25µl containing PCR buffer (Promega) with 1.5mM $MgCl_2$, 0.2mM each dNTP (Promega), 0.4µM of each primer (IDT), 0.5U of Taq DNA polymerase (Promega), 10% DMSO and 5µl of extracted DNA as template DNA. Multiplex PCR for PKS-I and PKS-II and simple PCR for NRPS were performed and amplifications were carried out in an Eco96™ thermal cycler (Himedia, India) in the following cycling parameter: initial denaturation of 95°C for 5 mins, 35 cycles of 95°C for 30 secs, 55°C (for PKS-I and PKS-II) / 57°C (for NRPS) for 2 mins, 72°C for 4 mins and final extension of 72°C for 10 mins.

RESULTS AND DISCUSSION

Soil physico-chemical characteristics

Soil and air temperatures of the study site at the time of sample collection (July) were 21°C and 19°C respectively akin to a sub-tropical climate as Mawsmai, Meghalaya, India falls under such climatic zone[36]. Moisture content of the soil was 16.64±3.85%. The average soil pH was slightly alkaline (7.12±0.9). Soil organic carbon was 2.46±0.05%. Total soil nitrogen, available phosphorus and potassium were 0.08±0.01%, 3.39±0.2µg g^{-1} and 1.35±0.65µg g^{-1} respectively. Moisture content of the soil was higher than that reported by Lamare and Singh (2017)[37] who conducted study on changes in soil quality in a limestone mining area in East Jaintia Hills from the same state of Meghalaya, India. They reported soil moisture content which ranged from 5.55% to 6.86%. The soil moisture content from our study site was higher because samples were collected on a rainy day. Soil moisture content from limestone mining sites is usually low due to presence of a mixture of granular, aggregated and blocky soil structure lowering the water holding capacity and also as a result of low organic matter[37]. Lamare and Singh (2017) also reported slightly alkaline nature of limestone soil[37] and such soils are generally characterized by alkaline pH[38]. Soil organic carbon from our study site was also higher than that reported by Lamare and Singh (2017) who recorded 0.23% to 0.47% of soil organic carbon from intensively mined areas and the reason was probably our soil samples was a composite of samples collected from intensively mined and sparsely mined areas. Total soil nitrogen, though slightly higher than that reported by Lamare and

Table 1. Biochemical and chemotaxonomic profile of actinobacterial isolates from the limestone mining site at Mawsmai, Meghalaya

Isolate	Biochemical profile				Chemotaxonomic profile		Identified genera
	Casein hydrolysis	Xanthine hydrolysis	Hypoxanthine hydrolysis	Tyrosine hydrolysis	Cell wall DAP	Whole cell sugars	
LD-01	+	-	+	+	LL	Ara	*Streptomyces*
LD-02	-	+	+	+	LL	Nd	*Streptomyces*
LD-03	-	+	+	+	LL	Nd	*Streptomyces*
LD-04	+	+	+	+	LL	Nd	*Streptomyces*
LD-05	+	+	+	+	LL	Nd	*Streptomyces*
LD-06	+	+	+	+	LL	Nd	*Streptomyces*
LD-07	+	+	+	+	LL	Nd	*Streptomyces*
LD-08	+	+	+	+	LL	Nd	*Streptomyces*
LD-09	-	-	+	+	meso	Gal, Ara	*Nocardia*
LD-10	-	-	+	-	meso	Gal, Ara	*Nocardia*
LD-11	-	-	+	-	meso	Gal, Ara	*Nocardia*
LD-12	+	+	+	+	LL	Gal	*Streptomyces*
LD-13	-	-	+	+	meso	Gal, Ara	*Nocardia*
LD-14	+	+	+	+	LL	Nd	*Streptomyces*
LD-15	+	+	+	+	LL	Nd	*Streptomyces*
LD-16	+	-	+	-	meso	Gal, Ara	*Nocardia*
LD-17	-	-	-	+	meso	Gal, Ara	*Nocardia*
LD-18	-	+	+	+	LL	Gal	*Streptomyces*
LD-19	+	+	+	+	LL	Gal	*Streptomyces*
LD-20	-	-	-	-	meso	Gal, Xyl	*Nocardia*
LD-21	+	+	+	+	LL	Nd	*Streptomyces*
LD-22	+	+	+	+	LL	Nd	*Streptomyces*
LD-23	+	+	+	+	LL	Gal, Xyl	*Streptomyces*
LD-24	+	+	+	+	LL	Nd	*Streptomyces*
LD-25	+	+	+	+	LL	Xyl	*Streptomyces*
LD-26	+	+	+	+	LL	Nd	*Streptomyces*
LD-27	+	-	+	+	LL	Ara	*Streptomyces*
LD-28	+	+	+	+	LL	Nd	*Streptomyces*
LD-29	+	+	+	+	LL	Nd	*Streptomyces*
LD-30	-	+	+	+	LL	Nd	*Streptomyces*
LD-31	-	+	+	+	LL	Nd	*Streptomyces*
LD-32	+	+	+	+	LL	Nd	*Streptomyces*
LD-33	+	+	+	+	LL	Nd	*Streptomyces*
LD-34	+	+	+	-	LL	Nd	*Streptomyces*
LD-35	+	+	+	+	meso	Nd	*Actinomadura*
LD-36	-	-	+	-	LL	Nd	*Streptomyces*
LD-37	+	+	+	+	LL	Nd	*Streptomyces*
LD-38	+	+	+	+	LL	Nd	*Streptomyces*
LD-39	+	+	+	-	LL	Nd	*Streptomyces*
LD-40	+	+	+	+	LL	Nd	*Streptomyces*
LD-41	+	+/-	-	+	LL	Nd	*Streptomyces*
LD-42	+	+	+	+	LL	Nd	*Streptomyces*
LD-43	+	+	+	+	LL	Nd	*Streptomyces*
LD-44	-	-	-	-	meso	Ara	*Rhodococcus*
LD-45	+	+	+	+	LL	Nd	*Streptomyces*
LD-46	-	-	+	+	meso	Nd	*Nocardia*
LD-47	-	-	+	+	meso	Nd	*Nocardia*

DAP, 2,6-diaminopimelic acid; LL and meso are two different isomers of DAP; Ara, Arabinose; Gal, Galactose; Xyl, Xylose; +, positive; -, negative; +/-, uncertain; Nd, Not detected

Singh (2017)[37], was low due to loss of vegetation cover due to mining activities accompanied with loss of nitrogen-fixing microorganisms[37]. Available phosphorus and potassium were comparatively much lesser to that reported by Lamare and Singh (2017)[37]. Other workers have also reported low levels of phosphorus and potassium from limestone mining areas[39,40]. Lamare and Singh (2017) however, reported no significant changes in phosphorus and potassium levels of limestone-mined and unmined areas[37]. Generally, soil organic carbon, nitrogen, available phosphorus and potassium are relatively lower in concentration in limestone mining areas[41].

Table 2. Detection of biosynthetic gene clusters (BGCs) in bioactive isolates by PCR

BGC	PCR product length (bp)	Bioactive isolates			
		LD-21	LD-29	LD-34	LD-39
PKS-I	1200-1400	-	+	+	+
PKS-II	600	-	-	-	-
NRPS	700-800	+	+	+	+

PKS-I, Type I polyketide synthase; PKS-II, Type II polyketide synthase; NRPS, Non-ribosomal peptide synthetase; +, PCR product detected; -, PCR product not detected.

Isolation of actinobacteria

Highest actinobacterial population number was recorded from un-pretreated and humic acid pretreated soil samples i.e. respectively 5.6±2.13x10^2 and 4.0±1.56x10^2 cfu g^{-1} soil. Statistically insignificant difference was recorded in population number of these two methods at $p<0.05$ (Fig.1). The differences in population numbers between wet heat pretreated and humic acid pretreated samples were also statistically insignificant at $p<0.05$ (Fig.1). The three pretreatment methods were significantly better than the other pre-treatment methods. Minimum number of Actinobacteria was recovered using dry heating and phenol pretreatment methods. This may be because most actinobacteria are heat killed when samples are exposed to 100°C for a duration of one hour[42] and phenol has been reported to reduce the growth of actinobacteria[43]. Among the selective media used, humic acid vitamin agar (HVA) and Streptomyces agar (SA) statistically gave the highest actinobacterial population number at 3.78±1.95x10^2 and 3.11±1.88x10^2 cfu g^{-1} soil respectively and were significantly better than the other three media used at $p<0.05$ (Fig.1). Humic acid present in HVA serves the sole carbon and nitrogen sources and has been observed to activate spore germination in many actinobacterial species[19]. SA on the other hand is one of the routinely used media for selective

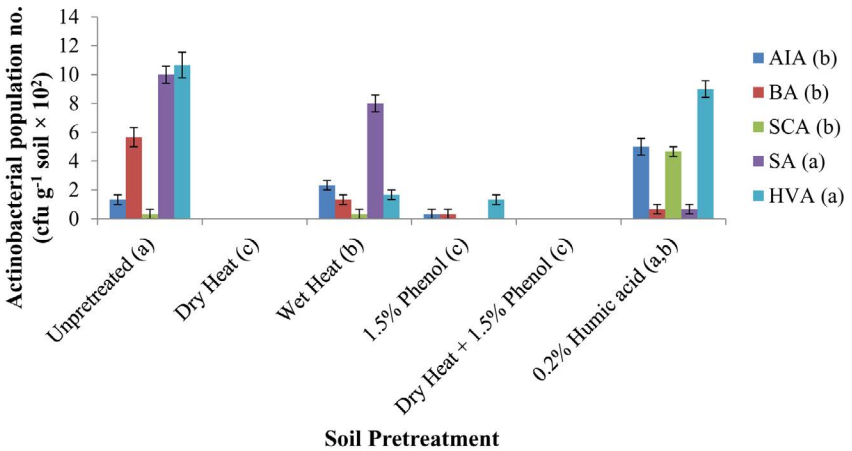

Fig.1. Recovery of actinobacteria from different pretreated soil samples on different selective media. (AIA, Actinomycete Isolation Agar; BA, Bennett's Agar; SCA, Starch Casein Agar; SA, Streptomyces Agar; HVA, Humic acid Vitamin Agar. Different lower case letters within parenthesis were significantly different among different pretreatments and among different selective media at $p<0.05$. Vertical bars represent standard error of mean.)

isolation and maintenance of actinobacteria. The un-pretreated samples recorded highest actinobacterial population when inoculated on HVA (10.67±0.88x10^2 cfu g^{-1}soil) and SA media (10.00±0.58x10^2 cfu g^{-1} soil). The difference was, however, statistically insignificant at $p<0.05$ (Fig.1). The actinobacterial population number recorded from humic acid pretreated sample inoculated on HVA medium (9.00±0.58x10^2 cfu g^{-1} soil) was also not significantly different from the unpretreated sample inoculated on SA medium (10.00±0.58x10^2 cfu g^{-1} soil).

Diversity of actinobacteria

On the basis of micromorphological characteristics, forty-seven isolates were identified as actinobacteria and all of them showed Gram-positive reaction. Based on biochemical and chemotaxonomic characteristics[21,23], thirty-six isolates belonged to *Streptomyces* genera and the remaining eleven isolates were found to be non-streptomycetes, out of which nine were identified as *Nocardia,* one as *Actinomadura* and one as *Rhodococcus* (Table 1). From the cultivated isolates, it showed a dominance of *Streptomyces* genera which comprised 76% of the total number of isolates. Recent reports on actinobacteria from limestone niches have also shown *Streptomyces* to be the dominant genera[8,13,44,45]. A number of novel actinobacterial strains have also been reported from other limestone niches. Nimaichand *et al.* (2012)[46] reported *Streptomyces manipurensis* sp. nov., a

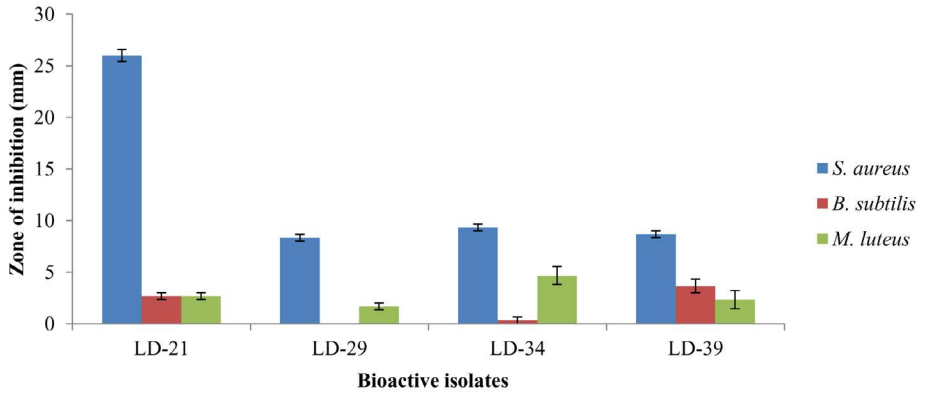

Fig.2. Antibacterial activity of culture filtrates prepared from the bioactive isolates by agar-well diffusion assay. (All the above bioactive isolates were identified as *Streptomyces. S. aureus, Staphylococcus aureus* MTCC 9886; *B. subtilis, Bacillus subtilis* MTCC 1305; *M. luteus, Micrococcus luteus* MTCC 1538. Vertical bars represent standard error of mean.)

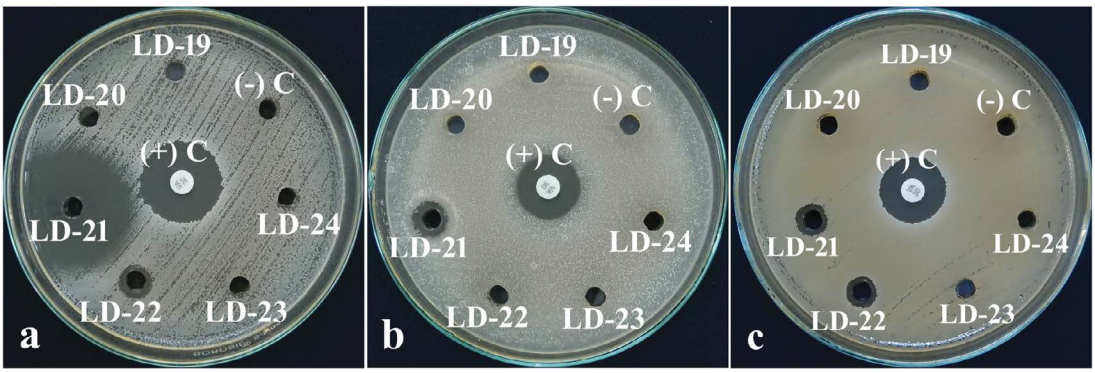

Fig. 3. Antibacterial activity of culture filtrate of bioactive isolate *Streptomyces* LD-21 and other isolates on a lawn of (a) *Staphylococcus aureus,* (b) *Bacillus subtilis* and (c) *Micrococcus luteus.*
(+) C: Positive Control (Vancomycin disc 30µg); (-) C: Negative Control (uninoculated broth).

novel actinomycete, isolated from a limestone deposit site in Manipur, India. Li *et al.*(2014)[47] reported *Streptomyces canchipurensis* sp. nov., isolated from a limestone environment at Hundung, Manipur, India. Cao *et al.* (2015)[48] reported *Lentzea guizhouensis* sp. nov., a novel lithophilous actinobacterium isolated from limestone from the Karst area of Guizhou, China. Hezbri *et al*. (2015)[49] reported *Geodermatophilus sabuli* sp. nov., a γ-radiation-resistant actinobacterium isolated

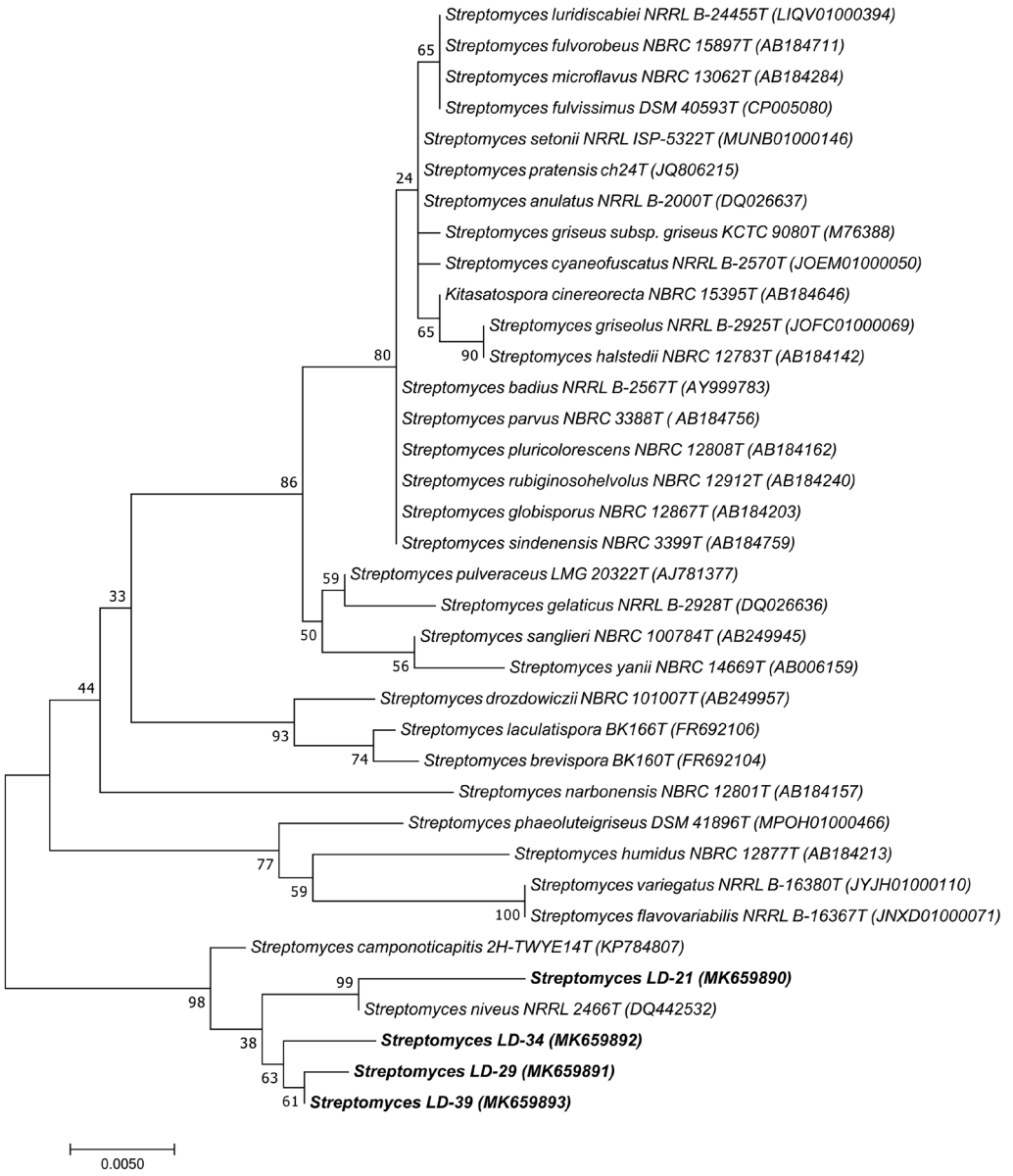

Fig.4. Maximum Likelihood Tree of the bioactive isolates (boldfaced) *Streptomyces* LD-21, *Streptomyces* LD-29, *Streptomyces* LD-34 and *Streptomyces* LD-39 along with related type strains based on 16S rDNA sequence. (Alpha-numeric characters within parenthesis are GenBank accession numbers of the corresponding strains. The numbers at the nodes are bootstrap percentages based on 1000 replicates. Horizontal bar equals 0.5% sequence divergence.)

from a limestone collected in the Sahara desert of Tunisia. Take *et al.* (2018)[50] reported *Streptomyces boninensis* sp. nov., isolated from the soil of a limestone cave in the Ogasawara Islands of Japan. Dominance of *Streptomyces* from the cultivated isolates of this work signifies the adaptation of

Table 3. Minimum free energy (MFE) in kcal mol^{-1} of the predicted 16S rRNA secondary structures of the bioactive isolates along with thirty-two related type strains from EzBioCloud database. Five outgroups have also been included for comparison. The MFEs have been arranged in ascending order

Strain	MFE (kcal mol^{-1})
Micromonospora viridifaciens DSM 43909^T (X92623)$^{\$}$	-553.1
Nonomuraea sp. GW 12687^T (FN356742)$^{\$}$	-552.1
Streptomyces niveus NRRL 2466^T (DQ442532)	-542.7
Actinomadura darangshiensis DLS-70^T (FN646682)$^{\$}$	-539.9
Streptomyces variegatus NRRL B-16380^T (JYJH01000110)	-537.9
Streptomyces flavovariabilis NRRL B-16367^T (JNXD01000071)	-537.9
Streptomyces pulveraceus LMG 20322^T (AJ781377)	-536.3
Streptomyces gelaticus NRRL B-2928^T (DQ026636)	-535.9
Streptomyces sanglieri NBRC 100784^T (AB249945)	-535.1
Streptomyces narbonensis NBRC 12801^T (AB184157)[a]	-534.7
Streptomyces LD-21 (MK659890)[a#]	-534.3
Kitasatospora cinereorecta NBRC 15395^T (AB184646)	-532.9
Streptomyces badius NRRL B-2567^T (AY999783)[b]	-532.8
Streptomyces setonii NRRL ISP-5322^T (MUNB01000146)[b]	-532.8
Streptomyces parvus NBRC 3388^T(AB184756)[b]	-532.8
Streptomyces camponoticapitis 2H-TWYE14^T (KP784807)[b]	-532.8
Streptomyces pluricolorescens NBRC 12808^T (AB184162)[b]	-532.8
Streptomyces rubiginosohelvolus NBRC 12912^T (AB184240)[b]	-532.8
Streptomyces pratensis ch24^T (JQ806215)[b]	-532.8
Streptomyces anulatus NRRL B-2000^T (DQ026637)[b]	-532.8
Streptomyces LD-39 (MK659893)[b#]	-532.3
Streptomyces drozdowiczii NBRC 101007^T (AB249957)[b]	-531.8
Streptomyces humidus NBRC 12877^T (AB184213)	-531.7
Streptomyces phaeoluteigriseus DSM 41896^T (MPOH01000466)[c]	-530.8
Streptomyces LD-29 (MK659891)[c#]	-530.7
Streptomyces globisporus NBRC 12867^T(AB184203)	-530.4
Streptomyces fulvissimus DSM 40593^T (CP005080)	-530.4
Streptomyces microflavus NBRC 13062^T (AB184284)	-530.4
Streptomyces luridiscabiei NRRL B-24455^T (LIQV01000394)	-530.4
Streptomyces cyaneofuscatus NRRL B-2570^T (JOEM01000050)	-530.2
Streptomyces griseolus NRRL B-2925^T (JOFC01000069)	-530.2
Streptomyces halstedii NBRC 12783^T (AB184142)	-530.2
Streptomyces sindenensis NBRC 3399^T (AB184759)	-529.8
Streptomyces brevispora BK160^T (FR692104)	-529.3
Streptomyces griseus subsp. *griseus* KCTC 9080^T (M76388)[d]	-528.5
Streptomyces LD-34 (MK659892)[d#]	-528.5
Streptomyces laculatispora BK166^T (FR692106)	-528.3
Streptomyces fulvorobeus NBRC 15897^T (AB184711)	-528
Mycolicibacterium fortuitum subsp. *acetamidolyticum* NCH E11620 (NR_104775)$^{\$}$	-526.1
Nocardia brasiliensis ATCC 19296^T (X80591)$^{\$}$	-524.8
Streptomyces yanii NBRC 14669^T (AB006159)	-522.3

a,b,c,d, Bioactive isolates along with their closest related type strains on the basis of MFEs; #, Bioactive isolates from this work; $, Outgroups

this genus to thrive on a wide range of nutrients including the synthetic media used during isolation and the genus can also grow faster than the other slower-growing actinobacterial genera thereby establishing its micro niche much earlier than the others[9].

Antimicrobial activity

Antimicrobial screening results from the agar well diffusion assay revealed that 19% of the isolates showed antibacterial activity against at least one of the bacterial strains tested. The screening results also showed stronger antibacterial activity against Gram-positive than Gram-negative bacteria. The isolates, however, did not show anti-candidal activity. Other recent reports on antimicrobial activity of actinobacteria from limestone niches also showed stronger anti-Gram-positive than anti-Gram-negative bacteria. Nimaichand *et al.* (2015)[8] reported on antimicrobial activity of actinobacteria from limestone deposit sites in Hundung, Manipur, India where eighteen actinobacterial isolates showed antibacterial activity against Gram-positive *Bacillus subtilis* and five isolates showed antibacterial activity against Gram-negative *Escherichia coli*. They however, also reported on isolates with anti-fungal activity including anti-candidal activity. Maciejewska *et al*. (2016)[9] also reported on antibacterial activity of moonmilk actinobacterial isolates from a limestone cave in Belgium where the isolates displayed stronger antibacterial activity against Gram-positive bacteria (94% of the phylotypes) than Gram-negative bacteria (71% of the phylotypes). They also reported on anti-fungal activity of the moonmilk isolates where 94% showed activity against a range of fungal strains including *Candida albicans.* Four isolates from this study viz. LD-21, LD-29, LD-34 and LD-39 showed clear antibacterial activity against at least two Gram-positive bacteria (Fig.2). Isolate LD-21 showed the highest antibacterial activity against *Staphylococcus aureus* (26±0.58 mm). Antibacterial activity of culture filtrate of LD-21 is shown in Fig.3. The other three isolates viz. LD-29, LD-34 and LD-39 showed moderate antibacterial activity against *S. aureus* with zone of inhibition of 8.33±0.33, 9.33±0.33 and 8.67±0.33 mm respectively. Isolates LD-21 and LD-39 showed low but clear antibacterial activity against *Bacillus subtilis* with zone of inhibition of 2.67±0.33 and 3.67±0.67 mm respectively. All the above four isolates also showed low but clear antibacterial activity against *Micrococcus luteus* with zone of inhibition of 2.67±0.33, 1.67±0.33, 4.67±0.88 and

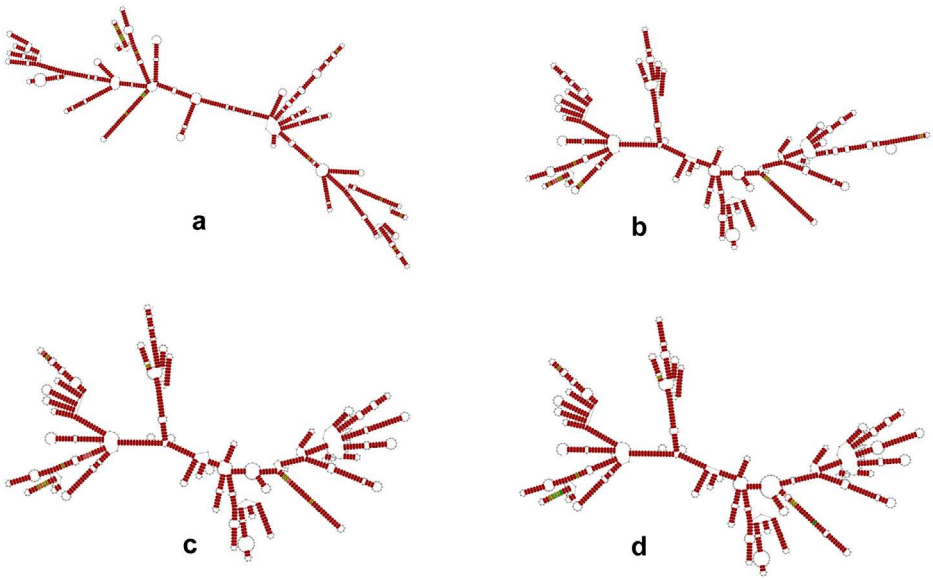

Fig.5. RNAalifold predicted consensus 16S rRNA secondary structures of **a** *Streptomyces* LD-21, **b** *Streptomyces* LD-29, **c** *Streptomyces* LD-34 and **d** *Streptomyces* LD-39 with closely-related type strains based on minimum free energy. Regions shaded in brown denote highest degree of conservation with a single number of base pair type.

2.33±0.88 mm respectively. All the above four bioactive isolates also showed antibacterial activity against Gram-negative bacterium *Pseudomonas aeruginosa* but the zone of inhibition of their culture filtrates on the agar well diffusion assay were not as apparent as the positive control (Amikacin disc 30µg) (data not shown) and there were no antibacterial activity against the other Gram-negative bacteria tested. The above four bioactive isolates also possessed at least one of the three biosynthetic gene clusters (BGCs) viz. PKS-I, PKS-II and NRPS (Table 2). Based on the antibacterial activity profile and BGCs, isolates LD-29, LD-34 and LD-39 seemed to have similar metabolic pathways and metabolite clusters. The antimicrobial screening results from this study recovered bioactive isolates with tendency of antimicrobial activity only towards Gram-positive bacteria and failed to recover isolates with broad-spectrum antimicrobial activity. Yet this find is still significant considering the present era where more emphasis are being undertaken to develop narrower spectrum antimicrobial drugs (due to emergence of antimicrobial drug resistance) and recent advancements in rapid and more accurate diagnostic techniques[5]. It would therefore be interesting to test the above bioactive isolates against more Gram-positive indicator strains and multi-drug resistant clinical isolates. Optimization of the physico-chemical conditions for growth of the bioactive isolates for maximum production of bioactive metabolites[51] is currently under way in the hope of finding novel bioactive metabolites.

Identification of the bioactive isolates

Based on morphological, biochemical and chemotaxonomic profiles (Table 1), all the four bioactive isolates were found to belong to *Streptomyces* genera. Phylogenetic analysis of their 16S rDNA sequences also confirmed to be *Streptomyces.* Isolate LD-21 was closest to *Streptomyces niveus* NRRL2466^T (DQ442532) with 99.47% sequence similarity and the other three isolates LD-29, LD-34 and LD-39 were closest to *Streptomyces camponoticapitis* 2H-TWYE14^T (KP784807) with sequence similarity of 99.26%, 99.41% and 99.48% respectively. The generated phylogenetic tree of the bioactive isolates along with the related type strains is shown in Fig.4. GenBank accession numbers of the partial 16S rDNA sequences of the bioactive isolates LD-21, LD-29, LD-34 and LD-39 were assigned from MK659890 to MK659893 respectively. Therefore, the bioactive isolates are now identified as *Streptomyces* LD-21 (MK659890), *Streptomyces* LD-29 (MK659891), *Streptomyces* LD-34 (MK659892) and *Streptomyces* LD-39 (MK659893). The minimum free energy (MFE) in kcal mol^{-1} of the predicted 16S rRNA secondary structures of the bioactive isolates also placed them under *Streptomyces* genera (Table 3). However, on the basis of MFEs, the *Streptomyces* species with the closest match have changed. The consensus 16S rRNA secondary structures of the bioactive isolates and their closely-related type strains (based on MFE) also showed high degree of conserved regions (Fig.5) confirming that the bioactive isolates belonged to *Streptomyces* genera. It was also noted that the consensus 16S rRNA secondary structure of *Streptomyces* LD-21 looked different from the other three bioactive isolates *Streptomyces* LD-29, *Streptomyces* LD-34 and *Streptomyces* LD-39 while these three were similar in their consensus 16S rRNA secondary structures (Fig. 5) which agrees with the maximum likelihood tree (Fig. 4). Thus, it could be inferred that *Streptomyces* LD-29, *Streptomyces* LD-34 and *Streptomyces* LD-39 were phylogenetically closer to one another and they could even belong to the same strain and probably share very similar metabolic pathways and metabolite clusters as already mentioned which is evident from the antibacterial activity of their culture filtrates (Fig.2) and their BGC profiles (Table 2).

CONCLUSION

In present work, actinobacteria were isolated from a limestone mining site. The soil environment at the sample collection site was having minimal nutritional conditions (oligotrophic). Based on morphological, biochemical and chemotaxonomic characteristics, *Streptomyces* was found to be the dominant cultivable actinobacterium isolated from this environment. This, however, also suggests the use of more specific methods of isolation to recover the rarer genera of actinobacteria. Four bioactive isolates all identified as *Streptomyces* showed considerable antibacterial activity against Gram-positive bacteria and they were also found to harbor at least one of the three biosynthetic

gene clusters viz. PKS-I, PKS-II and NRPS which are related to synthesis of bioactive metabolites. More work is needed to assess their activity over a range of the Gram-positive bacterium including multi-drug resistant strains. Optimization of bioactive metabolites production by the bioactive isolates is under way. This under-explored niche from Meghalaya is important for mining of actinobacteria for obtaining novel bioactive metabolites that might have applications in various fields.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors have made substantial, direct and intellectual contribution to the work and approved it for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the research work.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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