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Screening and Molecular Characterization of Antibacterial Secondary Metabolite Producing Actinomycetes from Soils of Eastern Mountain Regions of Nepal

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Abstract

Antibacterial secondary metabolite is a bioactive compound like antibiotic that can be considered a substance, produced by one microorganism, which in low concentration inhibits the growth of other microorganisms. Actinomycetes, slow-growing gram-positive bacteria, are the major sources of bioactive compounds. This study aimed to screen and identify antibacterial secondary metabolite-producing actinomycetes by sequencing the 16S rRNA method (molecular identification) from the soils of the mountain region of eastern Nepal. Starch casein agar (SCA) medium was used for the isolation of actinomycetes which were confirmed by primary screening and secondary screening. Identification of presumptive genera was done based on macroscopic, microscopic, and biochemical characteristics and confirmed by sequencing their 16S rRNA genes.

The antibacterial compound was produced by culturing the potential isolate in starch casein broth. Using organic solvents such as ethyl acetate, n-butanol, chloroform, dichloromethane, and methanol, the chemical was recovered from the fermented broth. TLC performed the antibacterial substance characterization. Only 9 (13.6%) of the 66 actinomycetes isolates showed antibacterial activity against test microorganisms. Only one of the nine isolates, M_3, had antibacterial activity in primary screening against gram-positive (*Staphylococcus aureus*) and gram-negative (*Escherichia coli, Salmonella* Typhi*, Shigella* spp., and *Pseudomonas aeruginosa*) test bacteria. M_3 was chosen for secondary screening due to its strong antibacterial activity.

The minimum inhibitory concentration (MIC) of crude antibacterial substances was found to be 2.5 mg/mL against test organisms. According to the TLC chromatogram, the isolate produced only one compound with an R_f value of 0.81, completely distinct from the spot formed by gentamicin (standard), which had an R_f value of 0.89. The isolates were considered *Streptomyces* spp., a distinct taxonomic group based on characterization by macroscopic, microscopic, biochemical, physiological, and molecular techniques. This study concluded that Mountain regions are the reservoir of antibiotic-producing actinomycetes. *Streptomyces* is the most common genus.

Key words: Actinomycetes, Secondary metabolite, PCR, 16S rRNA, TLC

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Introduction

Actinomycetes are a diverse collection of prokaryotic bacteria with thread filaments that are gram-positive and distinguished by possessing DNA with a high G+C content (>55%) [1-4] and can produce several bioactive compounds [5]. Most of the secondary metabolites like novel antibiotics were extracted from actinomycetes isolated from soil [6].

Actinomycetes are the source of about 45% of the 22500 active chemicals produced by microorganisms [7]. According to Singh et al. [6], the genus *Streptomyces* is responsible for producing 60% of all antibiotics [8].

The advent of novel multidrug-resistant bacterial infections has increased the need for new antibiotics [9]. Antimicrobial resistance is a serious threat to public health worldwide. MDR bacterial infections continue to be the second biggest cause of death globally [10]. Antibiotic- producing actinomycetes that have been identified from aquatic and terrestrial ecosystems can also meet the growing demand for new antibiotics [11-13].

Twenty-seven actinomycetes having antibacterial activity were isolated from soils of Mount Everest regions [14]. Twenty of the total 54 actinomycetes from Kathmandu Valley showed antibacterial activity. They were all members of the *Streptomyces* genus [15]. *Streptomyces* spp. (T_{18}), a powerful actinomycetes isolate with antibacterial activity against both gram-positive and gram-negative bacteria, was discovered in soils of a harsh environment like Taplejung, a cold district of Nepal [16]. Actinomycetes taxonomy was previously linked to morphology, which is insufficient to distinguish between several species of many genera. Classification methods have greatly benefited from the application of phylogenetic and molecular evolutionary methodologies [17,18]. Currently, 16S rRNA gene and polymerasechain reaction (PCR) sequence studies have become popular methods for identifying species and phylogenies[19,20].

Streptomyces is the most well-known actinomycete genus, although others have also been isolated from soil, including *Norcardia, Microbispora, Micromonospora, Actinomyces, Actinoplanes*, and *Streptosporangium* [21].

Antibacterial secondary metabolites producing actinomycetes were able to be isolated, screened, and identified in this study using soil samples from Nepal's eastern mountain region. Based on this study of the 16S rRNA gene sequence in conjunction with morphological, physiological, and biochemical information, taxonomic characterization was completed.

Materials and methods

Collection of soil samples

Throughout the research period (April 2019–March 2021), the study site was the eastern mountain region of Nepal. Soil samples were collected at several locations in eastern mountain areas of Nepal. Due to conditions created by the coronavirus pandemic, the most potent isolates were also lost when they were not used in additional experiments. Four to five grams of preferably dry soil samples were taken at a depth of four to five centimeters, added to a clean polyethylene bag, and thoroughly mixed [14]. The bag also had about one gram of $CaCO_3$ in it. The soil samples were then dried out for a further three weeks at room temperature. The research was finished at the Department of Microbiology's laboratory on the Central Campus of Technology (CCT), Hattisar, Tribhuvan University, Dharan-14, Nepal.

Isolation of Actinomycetes

After serially diluting soil samples, actinomycetes were isolated using the spread plate technique on starch casein agar [22] plates with nystatin and cycloheximide (each at a concentration of 50 µg/mL of medium). A sterile inoculating loop was used to pick up typical actinomycetes colonies from Starch Casein Agar (SCA) plates and then streak them on another SCA using the quadrant streaking technique. To isolate pure colonies of actinomycetes, a minimum of two rounds of plating was done and the inoculation plates were then kept in an incubator for 5-7 days at 28°C.

Screening of Actinomycetes for Antibacterial Activity

Primary screening and secondary screening are used for the selection of actinomycetes having antibacterial activity. The perpendicular streak method was used for the initial screening of actinomycetes isolates on Nutrient Agar [23]. *Escherichia coli, Salmonella Typhi, Shigella* sp., *Pseudomonas aeruginosa*, and *Staphylococcus aureus* were the test microbes. Actinomycetes isolates were subjected to secondary screening using the agar well assay method on Mueller Hinton Agar (MHA) against the test microorganisms used in primary screening [24].

Characterization of Actinomycetes

Macroscopic Characterization

The color of the aerial mycelium and diffusible pigments, as well as other colony features like size, consistency, colony edge, etc., were examined in the isolated colonies of actinomycetes grown on starch casein agar.

Microscopic characterization

By using the coverslip culture method, microscopic characterization was carried out [25]. Then, under a 1000X microscope, they were examined for their mycelial structure, the sporophore's design (conidiospore and arthrospore), and the placement and shape of the spores on the mycelia.

The presumptive identification of the isolates was achieved by comparing the observed morphology of the isolates with the actinomycetes morphology reported in Bergey's Manual of Determinative Bacteriology, Eighth edition [26].

Biochemical characterization

Various biochemical tests were conducted to identify the isolates. Indole and hydrogen sulfide production, nitrate reduction, urea hydrolysis, tween 0 hydrolysis, starch hydrolysis, and esculin hydrolysis tests were among them [25].

Physiological characterization

For physiological characterization, tests such as temperature tolerance, sodium chloride (NaCl) tolerance, and motility were carried out [25].

Fermentation

Based on primary and secondary screening, a single potent isolate (M_3) was utilized for fermentation using the submerged state culture approach. The isolate was added to a 100 mL Erlenmeyer flask containing 25 mL starch casein broth (SCB) and incubated at 28° C at 160 rpm in order to create inoculums for 4 days in a water bath shaker. For 1000 mL fermentation, five sterile Erlenmeyer flasks (500 mL capacity) containing 200 mL of sterile SCB were inoculated with the prepared inoculum, and it was then incubated for 7 days at 28° C and 160 rpm in a water bath shaker [14].

Recovery of Antibacterial Secondary Metabolites from Fermented Broth

The broth was aseptically filtered via Whatman No. 1 filter paper once the fermentation process was finished.

The filtrate was taken for further processing; while the remainder was discarded. To recover the secondary metabolites, solvent extraction was applied to the filtrate. The filtrate broth and ethyl acetate were combined in a separating funnel (1:1, v/v), rapidly agitated for one hour, and then allowed alone for 30 minutes. Similarly, extraction was done by using n-butanol, chloroform, dichloromethane, and methanol.

To get pure antibacterial secondary metabolites, the solvent phase was separated and subjected to evaporation in a water bath at 40° C for 15 hrs. The resulting residue (antibacterial metabolite) was weighed, diluted in phosphate buffer, and utilized for TLC analysis, determining antibacterial activity, and determining minimum inhibitory concentration.

Determination of Antibacterial Activity and Minimum Inhibitory Concentration (MIC) of Antibiotic

The MIC of the antibiotics was calculated by serial dilution method in nutritional broth against *E. coli* [14] and the antibacterial activity of the antibiotics was assessed by the agar cup assay method against the test organisms [24].

Thin layer chromatography

The preparation and activation of silica gel plates (20 cm x 20 cm, 1 mm thick) took place at 80° C for two hours. Ten µL of the reference antibiotic (gentamicin) and the test antibiotic were applied to the TLC plate, and the chromatogram was produced using a solvent system of chloroform: methanol (10:90). The spots were visible in the iodine vapor chamber after the chromatogram was dried at 110° C for 5 min [14].

PCR amplification and sequencing of 16S rRNA gene

The isolates exhibiting potent antibacterial activity were subsequently examined using molecular techniques. To extract the genomic DNA, each isolate was grown as a single colony in 50 mL of International Streptomyces Project Medium (ISP) for 18 to 4 hours at 26 degrees Celsius. After centrifuging the culture for 3 minutes at 2375 g, the supernatant was discarded. At last, 70% ethanol was used to purify the genomic DNA of the lysed cells after it had been precipitated with 0.6 volumes of isopropanol [27].

A set of primers (forward (StF): 5'AAGCCCTGGAAACGGGGT3' and reverse (StR): 5'CGTGTGCAGCCCAAGACA3 ') were used to amplify the 16S rDNA gene fragment [28]. Using Master Mix (Merck), 0.4 µM primer, 40 ng chromosomal DNA, and a final volume of 25 µL, PCR amplification was carried out. Thermo Fisher's thermal cycler was used to carry out the PCR amplification, which went as follows: 94° C for 5 min of initial denaturation, followed by 35 cycles of 94° C for 1 min, 57° C for 1 min, 72° C for 105 sec, and 72° C for 10 min of extension. With the use of gel electrophoresis on 1% agarose (Qiagen, Germany) and a 1 kb DNA ladder (Thermo Fisher Scientific), the PCR results were seen.

Results

Following the serial dilution of soil samples, slow-growing actinomycetes were isolated using the spread plate technique using starch casein agar medium that contained nystatin and cycloheximide (each at a concentration of 50 µg/mL of media) [29]. 42 soil samples from up to 3700 m above sea level in Nepal's eastern Himalayan region were collected, and 66 distinct actinomycetes were ultimately isolated in SCA. Only 9 (13.6%) of the 66 actinomycetes isolates showed antibacterial activity against test microorganisms.

Table 1: Zone of inhibition of active isolates in Primary screening against test bacteria

S.N.	Isolate code	Zone of inhibition (in mm) against test bacteria				
		Gram negative bacteria				Gram positive bacteria
		Tb_1	Tb_2	Tb_3	Tb_4	Tb_5
1	M_3	12	14	16	14	18
2	M_8	–	–	–	–	8
3	M_{19}	–	–	–	–	7
4	M_{39}	–	–	–	–	10
5	M_{42}	–	–	–	–	6
6	M_{47}	–	–	–	–	8
7	M_{56}	–	–	–	–	7
8	M_{59}	–	–	–	–	10
9	M_{63}	–	–	–	–	8

Tb_1, *E. coli*; Tb_2, *Pseudomonas aeruginosa*; Tb_3, *S.* Typhi; Tb_4, *Shigella* spp.; Tb_5, *Staphylococcus aureus*

Primary antibacterial activity of Actinomycetes

By using the perpendicular streak method in nutrient agar, they were exposed to first screening against one gram-positive (*Staphylococcus aureus*) and the four gram-negative bacteria (*Escherichia coli, Salmonella* Typhi, *Shigella* spp., and *Pseudomonas aeruginosa*). Actinomycetes that produce antibiotics with the M_3, M_8, M_{19}, M_{39}, M_{42}, M_{47}, M_{56}, M_{59}, and M_{63} codes were discovered (**Table 1**). M_3 was the most effective isolate of all of them (**Figure 1**).

Secondary Antibacterial Activity of Actinomycetes

One (M_3) of the nine active isolates was chosen and put through submerged culture fermentation in Starch Casein broth. All of the test microorganisms on Mueller

Hinton agar were inhibited by it. Gram-positive and gram-negative bacteria were both shown to be resistant to their antimicrobial effects (**Table 2**).

Figure 1. Zone of inhibition of M_3 isolates in Primary screening against test bacteria Tb1, *Escherichia coli*; Tb2, Pseudomonas aeruginosa; Tb3, Salmonella Typhi; Tb4, Shigella spp.; Tb5, Staphylococcus aureus.

Table 2: Zone of inhibition of active isolates in secondary screening

S.N.	Isolate	Zone of inhibition (in mm) against test bacteria				
		Gram-negative bacteria			Gram-positive bacteria	
		Tb_1	Tb_2	Tb_3	Tb_4	Tb_5
1	M_3	7	8	9	8	12

Tb1, *E. coli*; Tb2, *P. aeruginosa*; Tb3, *S.* Typhi; Tb4,*Shigella* spp.; Tb5, *S. aureus*

Characteristics of active isolates

Macroscopic characteristics

The active actinomycetes isolates produced substrates mycelium in different shades of colors i.e., brown, dark brown, yellow, yellowish white, creamy, white, and buffy white. Out of 9 isolates, 1(11.11%) showed brown-colored substrate mycelium, and 1 (11.11%) showed dark brown-colored substrate mycelium. 2 (22.22%) showed yellow colored and 2 (22.22%) showed yellowish white colored substrate mycelium. 1(11.11%) showed White, 1(11.11%) showed creamy and 1(11.11%) showed buffy white colored mycelium substrate. Out of the 9 isolates, 6(66.66%) produced white aerial mycelium, 1(11.11%) produced buffy white, 1(11.11%) showed buff color, and 1 (11.11%) showed cream color aerial mycelium. Two types of texture i.e., powdery and smooth of the aerial mycelium of the active actinomycetes were observed. The texture of the aerial mycelium powdery in 7(77.77%), smooth 2 (22.22%) of the total active isolates. The diameter of the colony of the active isolates varied from 1-2 mm. Most of them were 1 mm in size and the observed colonies were of entire round margin (**Table 3**).

Table 3. Macroscopic characteristics of the active Actinomycetes isolates on SCA

Isolate code	Macroscopic characteristics		
	Color of substrate mycelium	Color and texture of aerial mycelium	Other colony characteristics
M_3	Brown	White, powdery	Colony entire round, 2 mm diameter
M_8	Yellow	White, smooth	Colony entire round, 1mm diameter
M_{19}	Dark brown	White, powdery	Colony entire round, 1 mm diameter
M_{39}	Creamy	White, Cream color, fluffy	Colony entire round, 1 mm diameter
M_{42} (Pink or purple pigment producing (white)	Yellowish white	White powder	Colony entire round, 2 mm diameter
M_{47} (Yellow pigment producing (cream white)	More yellowish white	White powder (late)	Colony entire round, 2 mm diameter
M_{56} (light buff colony)	Buffy white	Buffy white powder	Colony entire round, 2 mm diameter
M_{59} (Buff colony)	White	Buffy white powder	Colony entire round, 2 mm diameter
M_{63}	Yellow	Cream color	Colony entire round, 2 mm diameter

Table 4: Macroscopic characteristics of the active actinomycetes isolates

S.N.	Isolated code	Microscopic characteristics	Presumptive genera
1	M_3	Mycelium unfragmented, Sporophore morphology: rectiflexible	*Streptomyces*
2	M_8	Mycelium unfragmented, Sporophore morphology:rectiflexible	*Streptomyces*
3	M_{19}	Mycelium unfragmented, Sporophore morphology: rectiflexible	*Streptomyces*
4	M_{39}	Mycelium unfragmented, Sporophore morphology: rectiflexible	*Streptomyces*
5	M_{42}	Mycelium unfragmented, Sporophore	*Streptomyces*

		morphology: rectiflexible	
6	M_{47}	mycelium unfragmented, Sporophore morphology: rectiflexible	*Streptomyces*
7	M_{56}	Mycelium unfragmented, Sporophore Morphology: rectiflexible	*Streptomyces*
8	M_{59}	Mycelium unfragmented, Sporophore Morphology: rectiflexible	*Streptomyces*
9	M_{63}	Mycelium unfragmented, Sporophore Morphology: rectiflexible	*Streptomyces*

Microscopic characteristics

Microscopic characteristics were done by a coverslip method. The mycelium and cellular morphology revealed that all the active isolates were presumably identified as *Streptomyces* spp. All of them had rectiflexible sporophore morphology and unfragmented mycelium (**Table 4**).

Biochemical and physiological characteristics

Carbohydrate utilization tests

The active actinomycetes isolates were tested for their ability to utilize 5 different carbohydrates namely fructose, maltose, sucrose, lactose, and glucose were utilized by 4(44.44%), 3(33.33%), 3(33.33%), 0(0%) and 0(0%) respectively (**Table 5**).

Table 5. Carbohydrate utilization tests

S. N.	Isolate code	Presumptive genera	Fru	Mal	Suc	Lac	Glu
1	M_3	*Streptomyces*	–	–	–	–	–
2	M_8	*Streptomyces*	–	–	–	–	–
3	M_{19}	*Streptomyces*	–	–	+	–	–
4	M_{39}	*Streptomyces*	–	+	–	–	–
5	M_{42}	*Streptomyces*	+	–	–	–	–
6	M_{47}	*Streptomyces*	+	–	–	–	–
7	M_{56}	*Streptomyces*	+	+	+	–	–
8	M_{59}	*Streptomyces*	+	–	–	–	–
9	M_{63}	Streptomyces	–	+	+	–	–

Substrate hydrolysis tests

The active actinomycetes isolates were tested for their ability to hydrolyze urea, starch, tween 20, esculin, and gelatin. Urea, starch, tween 20, esculin, and gelatin were hydrolyzed by 9 (100%), 9 (100%), 9 (100%), 8 (88.88%), and 9 (100%) respectively whereas potent isolated actinomycetes M_3 hydrolyzed all five substrates (**Table 6**).

Table 6. Substrate hydrolysis tests of active isolates

Isolate code	Presumptive genera	Hydrolysis tests of				
		Urea	Tween 20	Starch	Esculin	Gelatin
M_3	*Streptomyces*	+	+	+	+	+
M_8	*Streptomyces*	+	+	+	–	+
M_{19}	*Streptomyces*	+	+	+	+	+
M_{39}	*Streptomyces*	+	+	+	+	+
M_{42}	*Streptomyces*	+	+	+	+	+
M_{47}	*Streptomyces*	+	+	+	+	+
M_{56}	*Streptomyces*	+	+	+	+	+
M_{59}	*Streptomyces*	+	+	+	+	+
M_{63}	*Streptomyces*	+	+	+	+	+

Other Biochemical tests

The active isolates were also subjected to various other biochemical tests namely oxidase, catalase, H_2S production, citrate utilization, nitrate reduction, and indole production. All of the isolates were catalase positive and oxidase negative, 3(33.33%) of the active isolate was able to produce H_2S on Sulphide indole motility (SIM) medium. Among 9 isolates, 2(22.22%) of the active isolate was able to utilize citrate as the sole source of carbon, and 3 (33.33%) of the active isolates were able to reduce nitrate. None of the active isolates were able to produce indole. The potent antibiotic-producing isolate M_3 was catalase positive, H_2S production positive and citrate utilization positive whereas oxidase and nitrate reduction tests were negative (**Table 7**).

Table 7. Other biochemical tests of active isolates

Isolate code	Presumptive genera	Other biochemical tests				
		Cat	Oxid	H_2S	Citrate	Nitrate
M_3	*Streptomyces*	+	–	+	+	–
M_8	*Streptomyces*	+	–	+	–	–
M_{19}	*Streptomyces*	+	–	–	–	–
M_{39}	*Streptomyces*	+	–	+	–	–
M_{42}	*Streptomyces*	+	–	–	–	+
M_{47}	*Streptomyces*	+	–	–	–	–
M_{56}	*Streptomyces*	+	–	–	–	–
M_{59}	*Streptomyces*	+	–	–	–	+
M_{63}	*Streptomyces*	+	–	–	–	+

Cat= Catalase test, Oxid= Oxidase test, H_2S= H_2S production, Nitrate= Nitrate reduction test

Physiological test

Motility test, temperature tolerance test, and NaCl tolerance test were performed on all active isolates. All the active isolates were found to be non-motile when tested for their motility in SIM medium. Although all the active isolates were able to grow at 15° C and 37° C, none of the active isolates grew at 45° C. Concerning salt concentration, the active isolates were able to grow well at 7%, and 10% whereas unable to grow on 13% NaCl.

The potent antibiotic-producing isolates M_3 showed growth at 5° C, and 37° C but did not show growth at 45° C. It was able to tolerate NaCl 7%, and 10% and was unable to grow on 13% concentrations (**Table 8**).

Table 8. Physiological tests of the active isolates

Isolated code	Presumptive genera	Physiological test					
		Temperature tolerance			NaCl tolerance		
		5°C	37°C	45°C	7%	10%	13%
M_3	*Streptomyces*	+	+	–	+	+	–
M_8	*Streptomyces*	+	+	–	+	+	–
M_{19}	*Streptomyces*	+	+	–	+	+	–
M_{39}	*Streptomyces*	+	+	–	+	+	–
M_{42}	*Streptomyces*	+	+	–	+	+	–
M_{47}	*Streptomyces*	+	+	–	+	+	–
M_{56}	*Streptomyces*	+	+	–	+	+	–
M_{59}	*Streptomyces*	+	+	–	+	+	–
M_{63}	*Streptomyces*	+	+	–	+	+	–

Fermentation

Based on its efficacy against gram-positive and gram-negative bacteria as well as a greater zone of inhibition, isolate M_3 was chosen for fermentation. Ethyl acetate among other solvents was able to extract the antibacterial components more efficiently from the broth. The powerful metabolites could be extracted in a detectable quantity from the fermented broth using ethyl acetate solvents.

The minimum inhibitory concentration of active compound

The filtrate of the fermented broth was dried off in a porcelain bowl at 40° C. The residue was flaky and greasy in texture, and it was brownish. 252 µg of the isolate M_3 residue was present per 100 mL of the broth. The minimum inhibitory concentrations (MIC) for each of the five test microorganisms (*E. coli, P. aeruginosa, S.* Typhi, *Shigella* spp., and *S. aureus*) were determined after the residue was dissolved in a small amount of phosphate buffer. It was discovered that the MIC for the antibacterial secondary metabolite was 2.5 mg/mL (**Figure 1**).

Characteristics of the Antibacterial Substances

The crude extract of isolate M_3 was dissolved in phosphate buffer to make a concentration of 30 mg/mL. 10 µL of each extract along with 10 µL of 1% of gentamicin prepared in distilled water, a standard was made to run thin layer chromatography using chloroform: methanol (10:90) as solvent system. Only one spot was detected from the extract in iodine vapor near the solvent front. The retention factor (R_f) was 0.81 for the M_3 extract (**Figure 2**).

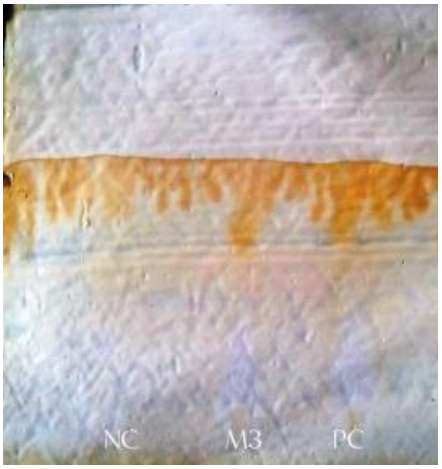

Figure 2: TLC of extracted metabolite from M_3 isolate Note: NC: Negative Control; M_3: Secondary metabolite from M_3 isolates; PC: Positive Control (gentamicin)

Table 9. 16S rRNA sequence analysis of active isolates through BLAST in NCBI databases

S.N.	Sample number	Sample code	Matched with databases			
			QC(%)	PI (%)	Accession code	Scientific Name
1	M_3	>H220124-010_A01_S1_27F.ab1 1322	85	96.29	LT576255.1	Uncultured *Streptomyces* sp
		>H220124-010_C01_S1_1492R.ab1 1453	74	97.88	KF996505.1	*Streptomyces* sp. ess_amH1
2	M_8	>H220124-010_E01_S2_27F.ab1 1341	85	95.46	LT576255.1	Uncultured *Streptomyces* sp.
		>H220124-010_G01_S2_1492R.ab1 1301	89	98.63	KF996505.1	*Streptomyces* sp. ess_amH1
3	M_{19}	>H220124-010_I01_S3_27F.ab1 1215	91	95.41	GU358071.1	*Streptomyces* sp. VITTKGB
		>H220124-010_K01_S3_1492R.ab1 1272	89	98.86	GU358071.1	*Streptomyces* sp. VITTKGB
4	M_{39}	>H220124-010_M01_S4_27F.ab1 1341	59	97.25	LT576255.1	Uncultured *Streptomyces* sp.
		>H220124-010_O01_S4_1492R.ab1 1337	75	95.57	KF996505.1	*Streptomyces* sp.ess amH1
5	M_{42}	>H220124-010_A03_S5_27F.ab1 1181	75	96.1	LT576255.1	Uncultured *Streptomyces* sp.
		>H220124-010_C03_S5_1492R.ab1 1443	74	97	KF996505.1	*Streptomyces* sp. ess_amH1
6	M_{47}	>H220124-010_E03_S6_27F.ab1 1235	80	92.73	AB914463.2	*Streptomyces* sp. VEL17
		>H220124-010_G03_S6_1492R.ab1 1199	96	94.24	AB914463.2	*Streptomyces* sp. VEL17
7	M_{56}	>H220124-010_I03_S7_27F.ab1 1359	85	97.39	KF996505.1	*Streptomyces* sp. ess_amH1
		>H220124-010_K03_S7_1492R.ab1 1346	74	97.54	LT576255.1	Uncultured *Streptomyces* sp.
8	M_{59}	>H220124-010_M03_S8_27F.ab1 1399	61	98.15	GU358071.1	*Streptomyces* sp. VITTKGB
		>H220124-010_O03_S8_1492R.ab1 1299	82	98.51	GU358071.1	*Streptomyces* sp.VITTKGB
9	M_{63}	>H220124-010_A05_S9_27F.ab1 1338	86	97.5	AB914463.2	*Streptomyces* sp. VEL17
		>H220124-010_C05_S9_1492R.ab1 1361	84	98.86	AB914463.2	*Streptomyces* sp. VEL17

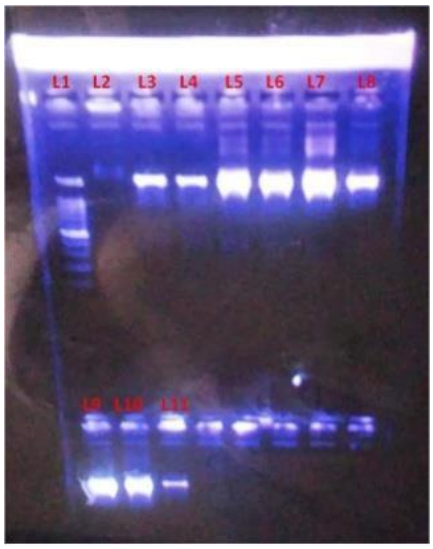

Figure 3: Gel documentation after electrophoresis of PCR products of 16S rRNA gene of presumptive *Streptomyces* genera Note: L1: DNA ladder; L2: Negative control; L3: M_3; L4: M_8; L5: M_{19}; L6: M_{39}; L7: M_{42}; L8: M_{47}; L9: M_{56}; L10: M_{59}; L11:M_{63}

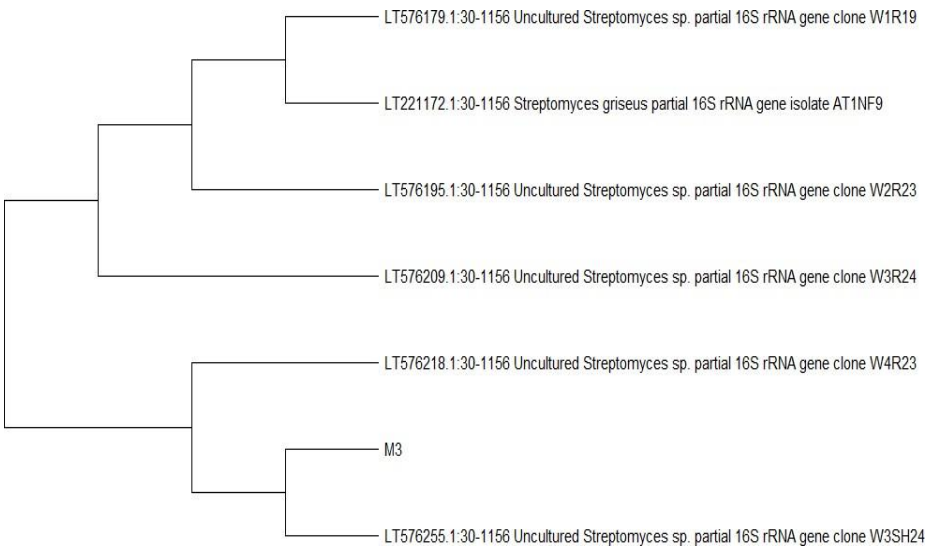

Figure 4: Phylogenetic tree of the most potent isolate (M_3) on the basis of 16S rRNA gene sequences

PCR amplification and sequencing of 16S rDNA gene

All nine of the isolates that displayed antibacterial activity were subjected to further molecular testing. After 35 cycles of PCR, Electrophoresis was run for the amplified gene products. In the eight/eight lane made in gel, lane 1 was loaded with DNA ladder and Lane was loaded with negative control. Lanes 3, 4, 5, 6, 7, 8, 9, 10, and 11 were loaded with PCR products of all 9 active isolates respectively in order M_3, M_8, M_{19}, M_{39}, M_4, M_{47}, M_{56}, M_{59}, and M_{63}. Using gel electrophoresis on 1.0 % agarose (Qiagen, Germany), the PCR results were observed and compared to a DNA ladder (Thermo Fisher Scientific). The 16S rRNA gene measured 1100–1500 nucleotides in length (**Figure 3**).

According to the manufacturer's recommendations, PCR products from high-yield isolates were purified using a PCR purification kit from Qiagen in Germany. MacroGen Inc. (Seoul, Korea) sequenced the purified products. The determined sequences were also compared with the sequences of other active actinomycetes species' 16S rRNA genes using the BLAST software. Analysis of the 16S rRNA gene showed that all nine isolates were substantially similar to different species of *Streptomyces* genera (**Table 9**).

Bioinformatics analysis of the obtained 16S rRNA gene sequences

By using MEGA 11 software, two phylogenetic trees were constructed based on 16S rRNA gene sequences. According to a phylogenetic tree of isolate M_3 was found to be close to uncultured *Streptomyces* sp. (**Figure 4**).

In another phylogenetic tree, Isolate M_{19} and M_{63} are so close to each other followed by M_{47} which is closer to *S. griseus* strain NBRC 15744(standard). Similarly, *S. griseus* strain NBRC 15744(standard) was closer to M_3 than M_8 followed by M_{56}, M_{39}, M_{59} and M_{42} (**Figure 5**)

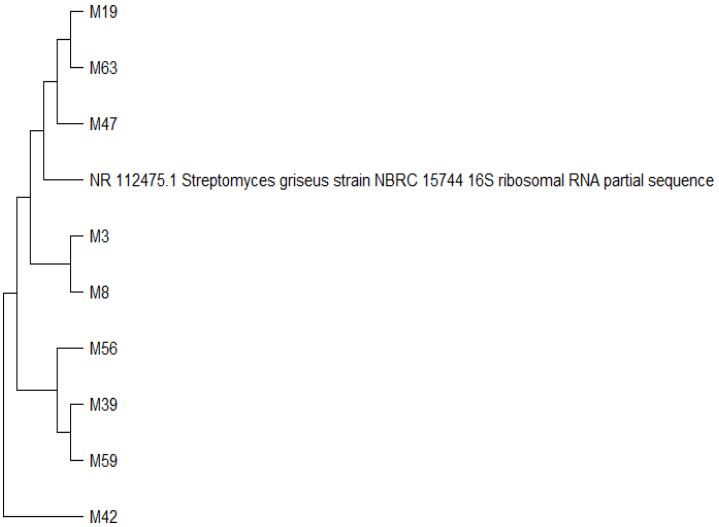

Figure 5. Phylogenetic tree showing relationship among all 9 active isolates belonging *Streptomyces* genera including *Streptomyces griseus* strain NBRC 15744 (as standard) on the basis of 16S rRNA gene sequences.

Discussion

According to Pudi et al., [2016], filamentous prokaryotes called actinomycetes create a wide range of secondary metabolites with unique biological properties, such as antibiotics [30]. Although antibiotic resistance among pathogenic bacteria is a serious threat to therapy, modern medical treatment is heavily dependent on antibacterial secondary metabolites like antibiotics [31]. To combat the issues with infectious infections, a new antibiotic must be found. The post-antibiotic era is quickly approaching on a global scale. While there are still some antibiotics

available, it is vital to look at other therapeutic options [6].

Actinomycetes were the source of around 80% of valuable bioactive chemicals with high commercial value. As a result, the isolation of new and novel bioactive chemicals, such as antibiotics, is ongoing [32]. This study looked for a new strain of actinomycetes in the soils of Nepal's eastern mountain ranges.

The ability of the formation of spores can adapt to such harsh and challenging environments. Other bacteria and fungi are common contaminants in the isolation of actinomycetes [33]. First, the soil samples were pretreated with calcium carbonate and allowed to air dry for three weeks. Second, Sah et al., [2011] found that the use of starch casein agar as a selective medium combined with antibiotics, nystatin (50 µg/mL), and cycloheximide (50 µg/mL) were effective methods for preventing the growthof impurities [34].

Physical, chemical, and geographic factors all affect the diversity and distribution of actinomycetes and their secondary metabolites [4, 14]. According to Barka et al. [16], the actinomycetales order is mostly known for its aerobic actinomycetes [1], which are easily separated from soil, freshwater, lakes, river bottoms, manures, composts, dust, plant residues, and food products. Additionally, almost 90% of actinomycetes genera have been isolated from soil, making them safe for use in a variety of industries, including the pharmaceutical and industrial sectors [35]. According to Brown-Elliott et al. [2006] and Rahdar et al. [2017], actinomycetes can also beisolated from a variety of environmental sources, such as soil, water, decaying plants, and animals [36, 37]. Actinomycetes can also produce different colors on the media, including red, green, yellow, brown, and black [38].

Actinomycetes can typically produce bioactive secondary metabolites, which may be an immunosuppressive, anticancer, antibiotic, antiviral, antifungal, enzyme, or other helpful substances for industry [39-41]. MDR gram-negative bacteria have lately been ranked as one of the top priorities for battling antibiotic resistance by both the World Health Organisation (WHO) and the U.S. Centres for Disease Control and Prevention (CDC) [42, 43].

By screening soil isolates, researchers have discovered new antibiotics [6]. The development of multidrug-resistant bacteria, however, has posed a significant challenge in the management of infectious diseases affecting sizable populations in the community. To control the current issue, new innovative antibiotics must be discovered [6, 44, 45]. Nearly 40% of the estimated 50 million fatalities per year worldwide are attributed to infectious diseases, although in many poor nations, microbial infections are the leading cause of demise [46].

The synthesis of structurally diverse secondary metabolites with biological activities that are relevant to the pharmaceutical industry comes from a remarkable source, making it crucial to screen for microbial species [7]. In contrast to its rivals, other bacteria, and fungi, actinomycetes have traditionally been difficult to isolate, possibly as a result of their lengthy incubation period. The samples were given a three-week pretreatment in dry air to enhance the actinomycetes isolation ratio.

Three regions make up the geographically diversified nation of Nepal: the alpine region, the hilly region, and the Terai region. According to their different altitudes, soil types, and contents, there may be a similar microflora present, which hypothesizes to change the distribution of actinomycetes that produce antimicrobials [47]. The most effective isolates demonstrating antibacterial properties against gram-negative bacteria among the twenty-two isolates from Siraha soils were *Streptomyces* spp. and *Thermomonospora* spp [34]. Four of the most powerful strains of the 10 isolates with antimicrobial capabilities—*Nocardiopsis prasina, Streptomyces violarus, Streptomyces krainskii,* and *Streptomyces tsusimaensis*—exhibited both antibacterial and antifungal properties [48].

Primary and secondary procedures were employed for the isolation's antibacterial activity screening. The screening process can take a quantitative or qualitative approach. The quantitative technique exposes the yield of antibiotics in various media, whereas the qualitative approach focuses on the variety of microorganisms that are sensitive to a prospective antibiotic [14].

The screening result showed that the isolate had greater activity against gram-positive bacteria than gram-negative bacteria. This might be because the two groups of bacteria have different cell wall compositions. In contrast to porins, which operate as a selective barrier to hydrophilic solutes, gram-negative bacteria's outer lipopolysaccharide membrane renders their cell wall impermeable to lipophilic solutes [49]. Only a peptidoglycan layer, which does not function as a reliable permeability barrier for the antibacterial agents, is present in gram-positive bacteria.

By using the solvent extraction method, the antimicrobial metabolites from the fermented broth were extracted in several organic solvents (n-butanol, chloroform, dichloromethane, ethyl acetate, and methanol]. The powerful metabolites from the fermented broth could only be extracted in detectable amounts using the ethyl

acetate solvent. This may be due to the metabolites' higher solubility in ethyl acetate solvent than in other solvents [34].

Actinomycetes were screened for antibacterial activity using both primary and secondary screening techniques. To choose the antibacterial isolates and identify the range of microorganisms that were antibiotic-sensitive, the first screening was used. To choose the isolates for additional research, the secondary screening approach was essential. The screening process can take a quantitative or qualitative approach. The quantitative strategy offers information on the yield of antibiotics that may be anticipated when the organism is grown in different media, whereas the qualitative approach is utilized to ascertain the range of bacteria that are sensitive to a prospective antibiotic [14].

In the secondary screening, only 9 active actinomycetes (M_3, M_8, M_{19}, M_{39}, M_4, M_{47}, M_{56}, M_{59}, and M_{63}] chosen from the initial screening had antibacterial activity out of a total of 66. This variation may be the result of actinomycetes having different morphologies when grown in solid and liquid media, respectively, as filamentous mycelia and fragmenting mycelia [50], or it may be the result of the active chemicals being chemically altered so that they are rendered inactive in broth culture. 11 soil samples overall, collected from sites ranging in altitude from 1500 to 4380 masl, were investigated. *Streptomyces* (70.7%), *Nocardia* (19.5%), and *Micromonospora* (9.5%) were the three genera represented by the 41 actinomycete strains that were isolated and identified [51].

The screening's outcome showed that the isolate was effective against both gram-positive and gram-negative bacteria. Isolate M_3 was chosen for fermentation to create an antibacterial secondary metabolite because, based on the results of primary and secondary screening, it was determined to be the best strain. It had a significant zone of inhibition against both gram-positive and gram-negative bacteria. We tried using the solvent extraction method to extract the antibacterial metabolites from the fermented broth in the organic solvent ethyl acetate. The strong metabolites could be extracted from the fermented broth at quantities that could be detected using the solvent ethyl acetate. This could be a result of the metabolites' higher solubility in ethyl acetate solvent than in other solvents [16].

The antibacterial metabolite isolated from M_3 had a minimum inhibitory concentration (MIC) of 2.5 mg/mL. This metabolite may have this MIC value because it was created by the solvent ethyl acetate being evaporated.

Thin layer chromatography (TLC) was used to analyze the extract on silica gel with gentamicin used as a reference antibiotic and a solvent system of chloroform: methanol (10:90). The extract only formed one spot on the chromatogram when it was seen under iodine vapor, indicating that just one component was present.

The location had an Rf value of 0.81 for the extract M_3 and was close to the solvent front. Similarly, to that, one spot with an R_f value of 0.89 was created by gentamicin. When Gurung et al. [2009] announced their findings, the Rf value was 0.88 [14]. Bergey's Manual of Determinative Bacteriology was used to determine the microscopic observations of the actinomycetes [26]. The *Streptomyces* species (presumptive) was recognized in the active isolates.

Through sequencing of the amplified 16S rRNA gene, the isolates in this investigation were effectively identified molecularly. The 16S rRNA gene sequencing information was compared on blast at NCBI, confirming that all of the isolates were *Streptomyces* spp. The examination of the isolates' 16S rRNA sequences revealed that every isolate has a 92–99% similarity to one species of the genus *Streptomyces*. All 9 isolates' 16S rRNA gene PCR products ranged in size from 1181 to 1453 bp. The 16S rRNA gene's function has not changed throughout time, and its size is sufficient for use in informatics [52]. The 16S rRNA gene sequence is made up of nine variable areas and nine largely conserved regions, which together allow for a wide range of taxonomic classifications and taxonomic discrimination, respectively [53].

The *Streptomyces* strains identification was aided by the 16S rRNA gene amplified region [54]. Actinomycetes species have 16S rRNA genes that are around 1250 base pairs long, but *Streptomyces* only has 600 base pairs, as determined by actinomycetes-specific primers and 16S rRNA gene amplification [55]. 16S rRNA sequencing confirms genus identification in more than 90% of cases. According to research by Guo et al. [2008], analysis of 16S rRNA gene sequence amplification is a promising technique for studying the phylogeny of microorganisms [56]. The 16S rRNA gene sequence is the most frequently used technique since it is found in practically all bacteria and because the 16S rRNA gene's function is conserved [57].

Based on morphological, biochemical, and 16S rRNA sequencing data, *Streptomyces coelicolor* Strain SU6 (JQ88940) was found to be the most successful strain. According to the study's findings, *Streptomyces* isolated from marine settings may one day be used to produce brand-new antibiotics for the treatment of bacterial illnesses in people [58]. *Streptomyces* spp. accounted for

70.7% of the isolates, *Nocardia* spp. for 19.5%, and *Micromonospora* spp. for 9.5%. The primary screening revealed that 43.34% of actinomycete isolates were strong makers of antimicrobials, of which 46.34% were effective against gram-positive and 12.19% against gram-negative test organisms. During the secondary screening, *Micromonospora* spp. isolate C7 showed the greatest broad-spectrum antibacterial activity [51].

There are various drawbacks to the 16S rRNA gene sequencing approach for bacterial species molecular identification. The 16S rRNA gene sequencing can only be used to distinguish between strains of *Streptomyces* that are only distantly related [56]. Multiple copies of the 16S rRNA are reportedly rare and less likely to have an impact on the phylogenetic study ofthe species, as their sequences are typically totally or nearly identical [59]. Others have demonstrated, though,that as the number of multiple copies of 16S rRNA gene grows, so does the sequence variation [60]. Ten *Streptomyces* strains isolated from three different lichens were found to have similar 16S rRNA gene sequences [61, 62].

Multiple copies of the 16S rRNA were examined in various strains of *Nocardia*, which shows that only BLAST analysis was unable to identify the species present in the isolates [63]. As a result, sequencing multiple copies of 16S rRNA gene does not appearto be helpful for identify species [53]

A special type of diagram is made for phylogenetic tree which a diagram that reveals the evolutionary ancestry of various species, organisms, or genes from a common ancestor. Phylogenies are helpful for classifying organisms, organizing information about biological diversity, and gaining understanding of evolutionary processes [64]. In this study, relationship among all 9 isolates was clearly showed through phylogenetic tree.

Conclusion

Streptomyces genera that produce antibacterial compounds were detected in the soils of the mountainous area. Thus, this study indicates the distribution of actinomycetes that produce antibacterial secondary metabolites in the soils of Nepal's mountainous areas. Multiple copies of the 16S rRNA gene cannot be identified at the species level using molecular sequencing. Fatty acid methyl ester (FAME) studies, metabolite profiling, and DNA-DNA hybridization are required for *Streptomyces* species identification.

Author's Contribution

Conception, molecular work, data analysis, interpretation of data, and drafting were done by Shiv Nandan Sah. Sampling, Screening, and identification of isolates were done by Pradip Pratap Dhakal. Critical revision and Final approval of the manuscript were done by both authors.

Competing Interests,

The authors declare that they have no competing interests.

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