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Isolation and Screening of Antibiotics Producing Actinomycetes from Soil of Khumbu Region above 5000m Altitude

Bishan Rai*, Dhiren Subba Limbu, Kamana Bantawa, Dibhya Adhikari, Khusbu Shahi Thakuri

Central Campus of Technology, Tribhuvan University, Dharan, Nepal

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Correspondence
Bishan Rai
e-mail: bishanrai45@gmail.com

ABSTRACT

Actinomycetes are gram positive bacteria having fungi-like structure, which recently gained a considerable interest because of their importance in not just treatments but also organic matter recycling. This study aimed to isolate and evaluate Actinomycetes from soil samples for their potential to produce antibiotics collected from different places of Khumbu region above altitude of 5000m in Nepal. The isolates were meticulously retrieved from soil samples using the enriching medium of Starch Casein Agar. Primary Screening and secondary screening were conducted employing the perpendicular streak method and the agar well assay technique, each applied in a systematic and precise manner. This research compared the antibacterial efficacy of purified extracts with various test bacteria. The actinomycetes with broad spectrum antibacterial activity was selected for fermentation and was converted into the crude extract. Then crude extracts were partially purified by solvent extraction method. In the preliminary screening, the perpendicular streak technique was employed, only 2 (3%) of the isolates exhibited antibacterial activity against majority of test bacterium among 3 Gram positive bacteria and 5 Gram negative bacteria. S27 displayed the largest zone of inhibition to *Klebsiella pneumoniae* and S34 showed highest zone of inhibition against *MRSA*. Both isolates showed broad spectrum in the secondary screening with higher level of statistical significance, both S34 and S27 having p value of .000 and Chi-square value 119.169[a] and 91.157[a] of respectively.

1. INTRODUCTION

Actinomycetales are an important group of soil-dwelling microorganisms. They belong to the phylum Actinobacteria, which is one of the largest groups in the bacterial world. This phylum includes the actinomycetes and is known for having Gram-positive bacteria with a high amount of guanine and cytosine (G-C) in their DNA. The G-C content can vary, ranging from about 51% in bacteria like *Corynebacterium* to over 70% in *Streptomyces* and *Frankia*(De Simeis & Serra, 2021). Actinomycetes are mostly free-living microbes that exist in many different environments, but soil is their main natural habitat. It's in the soil that they are most active and interact closely with other living organisms(Singh & Dubey, 2018). Essential ecological features, including nutrient availability, aeration, pH, temperature, salinity, moisture, and the presence of organic matter, are crucial in shaping the development and proliferation of Actinomycetes in soil and other substrates. These terrestrial microorganisms are particularly noteworthy for their diverse and remarkable antibacterial capabilities(Mousumi & Dayanand, n.d.). Researchers isolated rare actinomycetes such as Micromonospora, Nonomuraea, Kribbella, Lechevalieria, and Saccharothrix, along with the more common Streptomyces, from limestone quarries in the Deccan Traps region of Gulbarga, Karnataka. The antibiotics produced by these strains were found to be effective against harmful microbes like Bacillus subtilis and the fungus Fusarium solani(Quadri & Agsar, 2012). According to (Sharma & Thakur, 2020) the actinomycetes *Dactylosporangium, Microtetraspora, Saccharomonospora,* and *Micromonospora* were often found in different types of soil,

including forest soils in hilly areas, flat forest land, and pasturelands. Likewise, Actinomycetes found in garden soil from Nasr City in Cairo, Egypt, were shown to produce useful secondary compounds with antibacterial effects. These compounds were effective against several harmful bacteria, including Escherichia coli, Bacillus megaterium, Pseudomonas aeruginosa, and Klebsiella oxytoca(Ghadir E. Daigham, 2020).

Filamentous actinomycetes are recognized for their significant contribution to the production of approximately two-thirds of all known antibiotics. These compounds encompass a broad spectrum of therapeutic agents, spanning antifungal, antitumor, and immunosuppressive classes, showcasing the diverse potential of these microorganisms in the development of essential pharmaceuticals(*Sapkota et al.,2020*.). Some strains of Streptomyces bacteria naturally produce bioactive compounds with strong antibacterial properties. For instance, Streptomyces sp. RAUACT-1 secretes 1,4-dihydroxy-2-(3-hydroxybutyl)-9,10-anthraquinone; S. arenicola produces arenimycin; S. griseus releases frigocyclinone; S. nodosus secretes mitomycin C; S. lincolnensis produces lincomycin; S. pacifica synthesizes pacificanones A and B; and S. pristinaespiralis is known for producing pristinamycin. Additionally, other species of Streptomyces can produce antibacterial agents such as bisanthraquinone, carbomycin, glaciapyrroles, and tirandamycins, which further emphasize the genus's significance in antibiotic discovery(*Harir et al., 2018*).

In this era, the rising resistance of microorganisms to widely used antibiotics has created a major concern in global healthcare challenge. This escalating antimicrobial resistance among pathogenic microorganisms has spurred an intensified search for innovative antimicrobial agents(Mühlen & Dersch, 2015). Notable examples include methicillin-resistant Staphylococcus aureus (MRSA) and vancomycin-resistant *Staphylococcus aureus* (VRSA), along with vancomycin-resistant *Enterococcus* (VRE) and penicillin-resistant *Streptococcus pneumoniae* (PRSP). In addition, strains of *Escherichia coli* and *Klebsiella pneumoniae* that produce extended-spectrum beta-lactamases (ESBLs) are being reported more often, as are carbapenem-resistant *Enterobacteriaceae* (CRE), which are resistant to some of the most powerful antibiotics available. On top of this, hard-to-treat infections caused by non-fermenting Gram-negative bacteria like multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii* have become common in hospitals. This growing threat has been widely reported in recent scientific studies(Alotaibi, 2019; Kiran et al., 2024; Thabit et al., 2023). So, there is a growing need to explore new strategies and consider alternative therapies to effectively treat stubborn infections caused by antibiotic-resistant microbes. (Talebi Bezmin Abadi et al., 2019). To tackle this growing problem, we need to find new ways to discover fresh antibacterial compounds. One promising strategy is the renewed focus on actinomycetes, often called the "Renaissance of Antibacterial Discovery from Actinomycetes." By exploring unique environments and using improved methods to isolate new types of actinomycetes, scientists can uncover previously unknown genes—potentially leading to the development of entirely new antibiotics(Meenakshi et al., 2024).

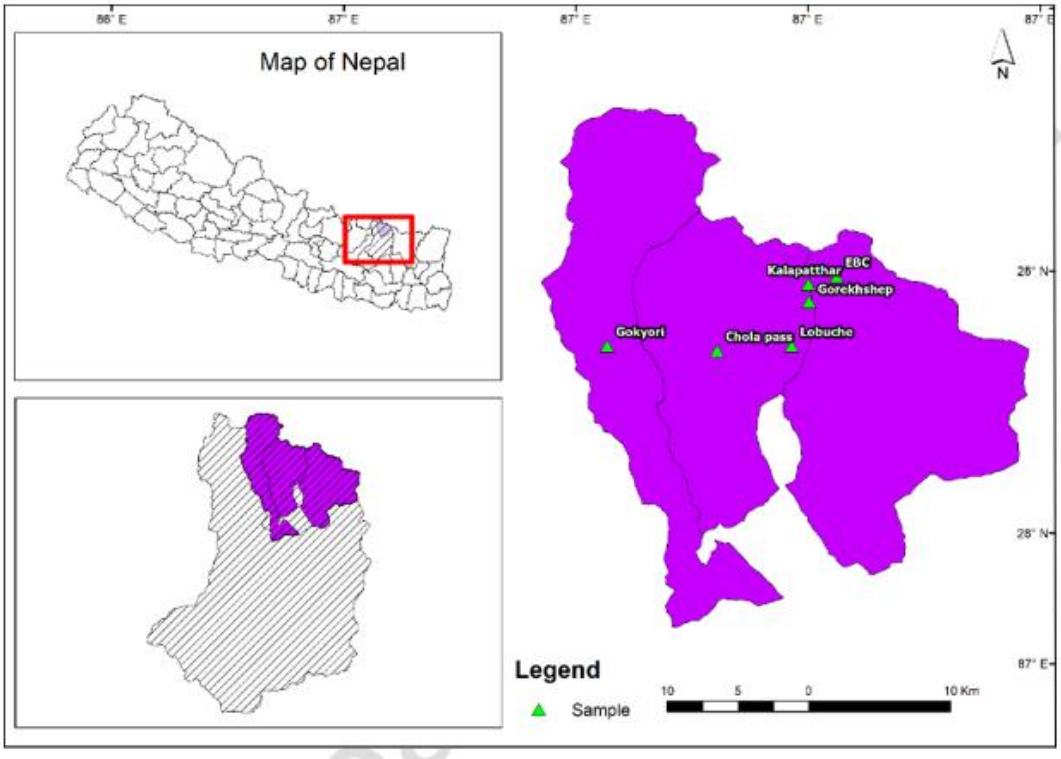

Figure 1
Study Area Map.

Likely, Nepal's diverse geography is divided into the mountain, hilly, and terai regions—features variations in altitude, soil types, and soil composition. These differences suggest that while similar types of microorganisms may exist across these areas, the presence and distribution of actinomycetes that produce antimicrobial compounds are likely to vary from one region to another(Gurung et al., 2009). So, this study was carried out to isolate and characterize the antibiotic producing actinomycetes from soil samples of Khumbu region above 5000m altitude, Nepal.

2. MATERIALS AND METHODS

2.1 Study Area

The research work was mainly focus on actinomycetes present in soil that are known for their ability to produce antibiotics from different places of Khumbu region (Lobuche, Gorakhshep, Everest base camp, Kalapatthar, Chola pass, and Gokyo Ri) above altitude of 5000m as shown in the **Error! Reference source not found.**.

2.2 Collection of Soil Sample

Soil samples were gathered from the different places of Khumbu region above 5000m altitude. Ideally, soil samples in a dry state (4-5g each) were gathered from a deepness of 4-5 cm, transferred in a disinfected polythene bag, and thoroughly mixed with approximately 1g of CaCO3, which had already been added to the bag. All the samples were labeled and transported to the laboratory. Then the soil samples were further dried at room temperature for about 3 weeks.

2.3 Isolation of Actinomycetes

After serial dilution of soil, actinomycetes were isolated using Starch Casein Agar (SCA). Typical actinomycetes by their Colonies exhibiting a dry, tough, and wrinkled appearance were carefully selected from SCA plates using a sterile inoculating loop or wire and streaked onto fresh SCA plates and incubates for 7 days at 28ºC for pure culture.

2.4 Macroscopic and Microscopic Characterization of Actinomycetes

The isolated colonies of actinomycetes on SCA were studied for the color of aerial mycelium, diffusible pigments, and additional colony traits such as size, consistency, and margin of colony. Gram staining of the isolated colonies was performed.

2.5 Biochemical and Physiological Characterization of Actinomycetes

As defined by (Kawato, Mineko and Shinobu, R, 1959), various biochemical assays, including oxidase activity, carbohydrate utilization tests, substrate hydrolysis test catalase test, citrate utilization test, urease, and nitrate reduction) were conducted to aid in the identification of the isolates.

2.6 Primary Screening of Actinomycetes for Antibacterial Activity

The initial screening was directed using the perpendicular streak method on Nutrient Agar(N S Egorov, 1985). Actinomycetes were inoculated in a streak down the center Nutrient Agar plate and subsequently incubated for 1-2 weeks at 28°C. The bacterial strains tested included *Escherichia coli, Salmonella typhi, Shigella spp*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, Staphylococcus aureus, *Bacillus subtilis, Colistin resistant E. coli, Methicillin resistant Staphylococcus aureus*). The plates were kept at 37°C for 24 hours of incubation. The inhibition of bacterial growth along the bacterial streak line if detected, was noted, and sent for the fermentation.

2.7 Fermentation

A broad-spectrum actinomycete was selected for fermentation using a submerged culture method. The inoculum was prepared in starch casein broth, incubated at 28°C with stirring at 160 rpm for 2 days, and then transferred to sterile flasks containing fresh broth. Fermentation was carried out under similar conditions for 4 days. The resulting broth was filtered to remove residues, yielding a crude extract for further analysis(Gurung et al., 2009).

2.8 Partial Purification for Extracts by Solvent Extraction

The filtrate was processed using a solvent extraction method to isolate the antibacterial metabolites in their pure form (Liu et al., 1986). An equal volume of ethyl acetate (1:1, v/v) was mixed with the filtrate and shaken vigorously for 1 hour to ensure complete extraction. The phase exhibiting antibacterial properties was then transferred to a porcelain basin for evaporation. It was evaporated in a water bath at 40ºC, and the resulting residue was weighed and dissolved in phosphate buffer for storage.

2.9 Secondary Screening of Actinomycetes for Antibacterial Activity

Five-millimeter wells were created in Mueller-Hinton Agar plates using a sterile agar borer, and 40 µl of ethyl acetate-extracted antibiotic fractions were introduced into each well. The plates were left at room temperature for 20–30 minutes to allow the antibiotic fractions to diffuse into the agar before being incubated at 37ºC for 24 hours. After incubation, the plates were examined, and the diameter of the inhibition zones around each well was measured (Gopinath et al., 2013).

2.10 Thin Layer Chromatography

A 10 µl volume of each antimicrobial metabolite (at a concentration of 50 mg/ml) and standard antibiotics, vancomycin, and tetracycline, was carefully applied separately to chromatography paper and the chromatogram was developed using butanol: acetic acid: water (40:10:50) as solvent system. The spots were observed within an iodine vapor chamber (Busti et al., 2006).

2 RESULT

2.1 Macroscopic Characterization

Out of 79 isolated actinomycetes, 31(39.240%) showed white, 23(29.113%) showed yellow, 18(22.784%) showed pale, 4(5.063%) showed pale yellow and 3(3.797%) showed red colonies. Likewise, 64(81.012%) colonies showed round, 11(13.924%) colonies showed slightly round, and 44(5.063%) colonies showed irregular shape, 66 having regular and 13 having irregular margin. With, 71 colonies having convex along with 11 tough and buff nature colonies within it and 5 colonies having concave elevation. Two of the active isolated colonies had characteristics shown in Table 1.

Table 1
Macroscopic characteristics of the active actinomycetes isolates on SCA.

S. N	Actinomycetes	Macroscopic Characteristics			
		Color	Shape	Margin	Elevation
1	S27	White	Round	Irregular	Convex (Tough and Buff)
2	S34	White	Slight Round	Irregular	Concave

Table 2
Primary Screening of Actinomycetes against Test Bacteria.

S. N	Actinomycetes	Gram negative bacteria						Gram positive bacteria		
	Isolates	Tb1	Tb2	Tb3	Tb4	Tb5	Tb6	Tb7	Tb8	Tb9
1	S27	×	×	×	×	×	✓	×	✓	×
2	S34	×	×	✓	✓	✓	✓	✓	×	×

Tb1: *Escherichia coli* ATCC 25922, **Tb2:** *Salmonella typhi*, **Tb3:** *Shigella spp*, **Tb4:** *Pseudomonas aeruginosa*, **Tb5:** *Klebsiella pneumoniae* ATCC700603, **Tb6***: Colistin resistant Escherichia coli,* **Tb7***: Staphylococcus aureus* ATCC 25922, **Tb8:** *Bacillus subtilis*, **Tb9:** *Methicillin Resistant Staphylococcus aureus.*

Table 3
Utilization of Carbohydrates Test.

Actinomycetes	Carbohydrates							
	Mannose	Fructose	Maltose	Sucrose	Lactose	Mannitol	Glucose	Xylose
S27	-	+	+	+	+	+	+	+
S34	-	+	+	+	+	+	-	-

+ = Utilized, - = Not utilized

Table 4
Hydrolysis of Substrates.

Actinomycetes	Carbohydrates				
	Urea	Tween 20	Starch	Esculin	Gelatin
S27	+	+	+	+	+
S34	+	+	+	+	+

+ = Hydrolyzed, - = Not hydrolyzed

Table 5
Other Biochemical Test.

Actinomycetes	Biochemical Tests				
	Catalase	Oxidase	Urease	Citrate Utilization	Nitrate Reduction
S27	+	-	+	-	-
S34	+	-	-	-	+

+ = positive, - = Negative

Table 6
Antibacterial activity of partially purified extracts from S34 Isolate.

Concentration	Zone of Inhibition									P value
	Tb1	Tb2	Tb3	Tb4	Tb5	Tb6	Tb7	Tb8	Tb9	
C	16	12	20	10	18	8	0	21	27	.000
1:1	0	24	17	17	12	23	13	19	15	
1:2	9	17	19	11	36	8	25	30	0	
2:1	18	18	13	17	19	8	29	28	10	

Table 7
Antibacterial activity of partially purified extracts from S27 Isolate.

Concentration	Zone of Inhibition									P value
	Tb1	Tb2	Tb3	Tb4	Tb5	Tb6	Tb7	Tb8	Tb9	
C	6	12	0	0	0	0	0	6	5	
1:2	7	0	0	0	7	0	7	7	5	.000
2:1	0	12	11	0	32	7	35	40	44	

C: Concentrated form of extracted antibiotics from isolated actinomycetes, **1:1**: it was 1:1 ratio of the extracted antibiotics from isolated actinomycetes and Phosphate Saline buffer volume by volume, **1:2:** it was 1:2 ratio of the extracted antibiotics from isolated actinomycetes and phosphate saline buffer volume by volume respectively,**2:1:** it was 2:1 ratio of the extracted antibiotics from isolated actinomycetes and phosphate saline buffer volume by volume respectively. **Tb1:** *Escherichia coli* ATCC 25922, **Tb2:** *Salmonella typhi*, **Tb3:** *Shigella spp*, **Tb4:** *Pseudomonas aeruginosa*, **Tb5:** *Klebsiella pneumoniae* ATCC700603, **Tb6***: Colistin resistant Escherichia coli,* **Tb7***: Staphylococcus aureus* ATCC 25922, **Tb8:** *Bacillus subtilis*, **Tb9:** *Methicillin Resistant Staphylococcus aureus.*

Table 8
Thin layer Chromatography of the Antibacterial substances.

Antibacterial substances	Concentration (mg/ml)	Amount of load µl	Solvent system	No of moved spots	Distance travelled by in cm		Rf value
					Solvent front	Antibacterial substance	
S27	C	10	40:10:50	0	14.4	0	0
	50	10	40:10:50	0	14.4	0	0
S34	C	10	40:10:50	0	14.4	0	0
	50	10	40:10:50	0	14.4	0	0
Vancomycin	5	10	40:10:50	0	14.4	0	0
	25	10	40:10:50	1	14.4	3.9	0.272
	50	10	40:10:50	1	14.4	4.2	0.291
Tetracycline	5	10	40:10:50	1	14.4	7	0.486
	25	10	40:10:50	1	14.4	7.9	0.548
	50	10	40:10:50	1	14.4	8.7	0.604

2.2 Microscopic Characterization

79 isolated actinomycetes were determined to be Gram-positive with primary stain retention including the sample 27 and 34.

2.3 Primary Screening of Actinomycetes

Of the 79 actinomycetes isolates, only 2 (3%) demonstrated antimicrobial activity against the test bacterial strains and which is shown in **Error! Reference source not found.**.
Among 2 active isolates, 2(100%) exhibited activity against one Gram-positive bacteria, 2(100%) demonstrated activity against one Gram-negative bacteria and 1(50%) showed activity against more than one Gram-negative bacterium.
Out of the entire lively isolates, 2(100%) showed action counter to Colistin resistant *E. coli*, 1(50%) showed activity against *Shigella spp*, 1(50%) displayed activity in contrast to *Pseudomonas aeruginosa*, 1(50%) presented activity contrary to *Klebsiella pneumoniae* ATCC700603, 1(50%) showed activity against *Staphylococcus aureus* ATCC 25922, 1(50%) showed activity against *Bacillus subtilis*.

2.4 Biochemical and Physiochemical Analysis of Actinomycetes

For the biochemical profiling of the isolates, tests were conducted to assess carbohydrate utilization, substrate hydrolysis, and the presence of catalase, oxidase activity, Urease, Citrate utilization and nitrate reduction tests were carried out.
The results of the carbohydrate utilization test are presented in **Error! Reference source not found.**, the substrate h ydrolysis test in **Error! Reference source not found.**, and the other biochemical tests in **Error! Reference source not f ound.**.

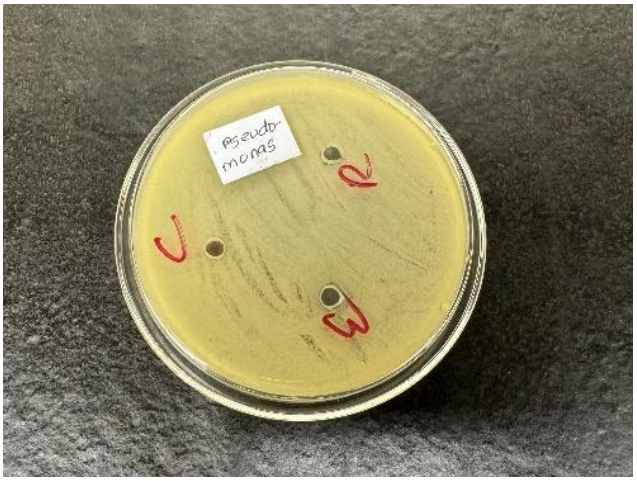

Figure 2
Secondary screening of the S27 isolate on Tb4: *Pseudomonas aeruginosa*.

2.5 Secondary Screening of Actinomycetes

Out of two active isolates, S34 worked against almost all the test bacteria, whilst S27 worked against eight out of nine test bacteria excluding *Pseudomonas aeruginosa* shown in **Error! Reference source not found.Error! Reference so urce not found.**. Antibiotics extracted from active isolates were divided into four groups in S34 and three groups in S27 and secondary screening were performed as shown in the **Error! Reference source not found.** and **Error! Reference so**

urce not found.. Since, P value of the S34 is .000 with Chi-Square value of 119.169[a] which shows that there is statistically significant association between secondary extract concentration and bacterial inhibition. Similarly, in S27 P value is .000 with Chi-Square value of 91.157[a] which also shows there is significant relationship between secondary extract concentration and zone of inhibition for the bacteria tested.

2.6 Thin Layer Chromatography for Antibacterial Activity

Thin Layer chromatography was performed from the extracted antibiotics and compared with the standard antibiotics i.e., Vancomycin and Tetracycline. Both isolates did not move, and the spots weren't seen as shown in **Error! R eference source not found.Error! Reference source not found.** . Vancomycin concentration 25mg/ml and 50mg/ml showed 0.272 and 0.291 respectively. Followed by tetracycline 5mg/ml showed 0.291, 25mg/ml showed 0.486 and 50mg/ml Rf value. But extracted antibiotic didn't show any spots and its Rf value is considered zero. As, shown in the **Error! Reference source not found.**.

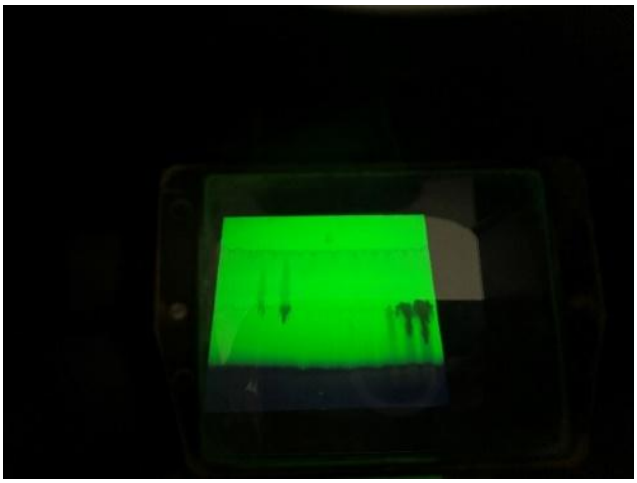

Figure 3
Thin Layer Chromatography on Iodine Vapour Chamber.

3 DISCUSSION

Actinomycetes are well known for producing useful compounds that can fight bacteria, cancer, fungi, and more. The most common way to find new antibacterial substances from them is still through traditional isolation methods(El Karkouri et al., 2019a). Microorganisms that thrive in extreme conditions have drawn significant attention because they create unique natural substances and have developed specialized ways to survive in such harsh environments(Tang et al., 2002). So, this study was carried out to find the antibiotic producing actinomycetes from the extreme temperature and high altitude above 5000m of the Khumbu region soil. A total of 36 soil samples were studied. The total number of isolated actinomycetes was similar in our present study with reported (Gurung et al., 2009), but In the present study, the samples were gathered from six diverse places around the Khumbu region rather (Gurung et al., 2009) collected samples from the one similar places from the Khumbu region, where total 16 actinomycetes colonies were isolated from that similar places in this study. Comparing the previous study of the Actinomycetes by(Limbu et al., 2023) from the soil sample of the different place of Sunsari district, the number was near to the triple. Actinomycetes were evaluated for antibacterial activity through both primary and secondary screening methods. The initial screening was employed to select the isolates and determine the spectrum of microorganisms sensitive to the antibiotic (Limbu et al., 2023) (Gurung et al., 2009). The perpendicular streak method allows testing of several organisms on the same plate where actinomycetes are cultured. It's an effective way to visually assess the activity of an isolate against test organisms in a qualitative manner (Haque et al., 1992).

A preliminary examination revealed that only 2 isolates could show antibacterial property out of 79 isolates of actinomycetes that have been isolated from the soil of various region of Khumbu region above 5000m, which is far more low comparing with the study of the (Gurung et al., 2009) in which the number of antibiotic producing actinomycetes were reported to be 27 only from the soil samples of the Kalapatthar region of the Khumbu region. But it was more than the study of the (Limbu et al., 2023), where only 1 out of 27 isolates had shown the antibacterial property against the two Gram-positive and two Gram-negative bacteria. One of the two isolates (S34), shows the broad spectrum working against the 4-gram negative (*Shigella spp*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* ATCC700603*, Colistin resistant Escherichia coli)* and 1-gram positive (*Staphylococcus aureus* ATCC 25922) bacteria. (S27) isolates exhibited activity against one Gram-positive and one Gram-negative bacterium. Of the nine bacterial samples, eight of the samples were clinical samples provided by the BPKIHS except *Colistin resistant Escherichia coli* which was isolated from the meat sample.

According to the primary screening, both isolates (S27 and S34) were subjected into the fermentation according to their antibacterial effectiveness against a variety of Gram-positive and Gram-negative bacteria. The antimicrobial compounds from the fermented broth were isolated through extraction using the solvent extraction method with the organic solvent ethyl acetate. Ethyl acetate effectively extracted the active metabolites from the fermented broth in significant amounts. This could be due to the metabolites' higher solubility in ethyl acetate than in other solvents(Sah & Lekhak, 2017).

In the primary screening, these two active isolates couldn't show the proper antibacterial activity, but it showed proper antibacterial activity against the bacterial strains tested. In initial screening, actinomycetes may not show significant antimicrobial activity because the standard conditions might not induce the full production of secondary metabolites. These metabolites are often responsible for antimicrobial properties. Secondary screening, on the other hand, involves optimized conditions (e.g., nutrient-rich media, longer incubation periods, or stress factors), which stimulate the production of bioactive compounds, resulting in stronger antimicrobial activity(El Karkouri et al., 2019b). Of the 2

active actinomycetes selected following the primary screening process, only 1 (S34) shows the antibacterial efficacy against the entire range of test bacterial strains and (S27) work against 8 of the test bacteria and couldn't work against the (*Pseudomonas aeruginosa*). Comparing both isolates the highest antibacterial activity on test organisms was showed by isolate (S34) (18 mm against on *E. coli*, 24 mm against on *Salmonella* typhi, 20mm against on Shigella. spp, 17mm against on *Pseudomonas aeruginosa*, 23mm against on *Colistin Resistant E. coli*) and (S27) shows 36mm against on *Klebsiella pneumoniae*, 35 mm against on *S. aureus*, 40 mm against on *Bacillus subtilis* and 44 mm against on *MRSA*).

In our study, extracted antibiotic after the partial purification from solvent extraction method were divided into four parts into S34 and three parts into S27 and mixed with Phosphate Saline Buffer(PSB) with different ratio like 1:1, which was 1:1 ratio of the extracted antibiotics from isolated actinomycetes and Phosphate Saline buffer volume by volume, 1:2, which was 1:2 ratio of the extracted antibiotics from isolated actinomycetes and phosphate saline buffer volume by volume respectively,2:1, which was 2:1 ratio of the extracted antibiotics from isolated actinomycetes and phosphate saline buffer volume by volume respectively and extracted antibiotics were considered as concentrated (C). The motive of this type of technique is to find the correct concentration of PSB to be used for the better preservation of the antibiotics, which was randomly used in the study of the (Gurung et al., 2009). In the isolate (S27), out of 8 of the test bacteria in which 3 were gram-positive and 5 were Gram-negative. C demonstrated antimicrobial properties against four, 2 Gram-negative and 2Gram-positive bacteria. 1:2 demonstrated antimicrobial properties against five, 3 Gram-negative and 2 Gram-positive bacteria and 2:1 demonstrated antimicrobial properties against 5 Gram-negative and 3 Gram-positive bacteria. 2:1 showed highest zone of inhibition and consistent antibacterial property against the 8 of test bacteria, all 3 of the Gram-positive bacteria even had highest zone of inhibition as shown in Figure 1, which could be due to the Gram-positive bacteria lacking the outer polysaccharide membrane that contains the structural lipopolysaccharide components (Nikaido & Vaara, 1985), which also suggests that it could show antibacterial property against other Gram-positive bacteria, due to which 2:1 can be considered as the better preserver of the properties of the extracted antibiotics in this case.

Figure 1
Secondary screening of S27 isolate on Gram positive bacteria Tb7*: Staphylococcus aureus* ATCC 25922, Tb8: *Bacillus subtilis*, Tb9: *Methicillin Resistant Staphylococcus aureus.*

Likewise, in isolate (S34), C, 1:1 and 1:2 showed zone of inhibition against eight except *Staphylococcus aureus* ATCC 25922, *Escherichia coli* ATCC 25922 and *Methicillin Resistant Staphylococcus aureus* respectively. Here, 2:1 shows antibacterial property against all the test bacteria. As it only shows the highest zone of inhibition against three out of nine of the isolates but on average it shows greater consistency of the antibacterial property on another remaining bacterium than other ratio. Even though, some of the other concentration ratio like (1:2 showed 36mm zone of inhibition against *Klebsiella pneumoniae*, C showed 27mm of zone of inhibition against *Methicillin Resistant Staphylococcus aureus* and 1:1 showed 24mm zone of inhibition against *Salmonella* typhi) which was greater zone of inhibition against some of the bacteria but lacks the consistency to another bacterium. This could be due to the various factors like rate of diffusion on agar, bacterial strain variability, compound property and solvent effects. Also, environmental factors like pH, temperature etc. had significant effect. Due to this result in S34 2:1 preserve the properties of the extracted antibiotics in best way.

The correlation between the concentration of the secondary extract and the inhibition zone against the test bacteria was assessed. A statistical analysis was performed and the P value of the S27 and S34 isolates were significantly lower in comparison the level of significance which concludes that a significant correlation exists between secondary extract concentration and zone of inhibition for the bacteria tested. It considers that statistically both isolates are profound.

In the TLC both isolates were run but it couldn't create spots. It was compared with the standard Vancomycin as study of the (Gurung et al., 2009) (Limbu et al., 2023) and tetracycline. This could be the possibility of the novel antibiotics and should be taken into the next steps of the identification. According to (Pandey et al., n.d.) to accurately identify the antimicrobial extracts, it is essential

to obtain them in pure form. This requires a series of purification processes and various chemical analyses, including HPLC, spectroscopy, and other advanced techniques.

Actinomycetes that produce antibacterial substances have been discovered in the soils of Khumbu region above 5000m altitude. S27 and S34 were the most effective actinomycetes isolates, with antimicrobial activity against both Gram positive and negative bacteria. Hence present investigation clearly indicates the spreading of antibiotic generating bacteria actinomycetes in Khumbu region above 5000m altitude.

4 CONCLUSION

An attempt was made to isolate several strains of actinomycetes with antibacterial activity from soil samples collected from Khumbu region above 5000m altitude. Using 36 soil samples, 79 isolates were obtained. Out of the total isolates, 2 of the isolates demonstrated antibacterial activity against one or more of the test bacteria during the primary screening. S27 shows largely inhibited the growth on *Staphylococcus aureus* ATCC 25922, Bacillus *subtilis* and Methicillin *Resistant Staphylococcus aureus* accompanied by the inhibition zone more than 20mm during secondary screening. S34 shows largely inhibited the growth on *Staphylococcus aureus* ATCC 25922, *Bacillus subtilis* with the zone of inhibition more than 20mm during secondary screening. The broad-spectrum activity from secondary screening was shown by both isolates S27 and S34, even though S27 showed low antibacterial property in the primary screening. S27 was isolated from the sample 112 which was from the Kalapatthar and S37 was isolated from the sample 114 which was from Gorakhshep region. Comparing the relationship between the secondary extract and zone of inhibition to the test bacteria, statistically both isolates show greater level of significance. As a conclusion, the prevalence of antibiotic-producing actinomycetes in the soils of Kalapatthar and Gorakhshep is clearly revealed in this study.

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