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Research Article

FERMENTATION AND EXTRACTION OF ANTIBACTERIAL METABOLITE USING *Streptomyces* spp. ISOLATED FROM TAPLEJUNG, NEPAL

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ABSTRACT

Realizing an increasing need for a novel antibiotic, this study was carried out to screen antibacterial metabolites producing actinomycetes from 15 soil samples collected from Taplejung. Antibacterial metabolites producing actinomycetes were confirmed by primary screening and secondary screening. Macroscopic, microscopic, and biochemical characteristics were used for presumptive identification of probable actinomycetes genera. The potential isolate was cultured in starch casein broth for production of possible antibacterial compound. The antibacterial compound was extracted from fermented broth using organic solvents like ethyl acetate, n-butanol, chloroform, dichloromethane, and methanol. Among 24 isolates, only one (T_{18}) showed antibacterial activity against both Gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram-negative (*Escherichia coli*, *Salmonella* Typhi and *Pseudomonas aeruginosa*) test-bacteria. The isolate was considered as *Streptomyces* spp based on microscopy and various biochemical, and physiological characteristics. Extracted antibacterial metabolite showed antibacterial activity with a MIC value of 1.2 mg/mL against *E. coli* (ATCC 25922). The chromatogram in Thin Layer Chromatography showed only one spot exhibited by extract with R_f value 0.87 suggested that the isolate produced a compound that was completely different from the spot with R_f value 0.94 produced by gentamicin (standard). This study revealed the distribution of the potent antibacterial metabolite producing actinomycetes in the soils of Taplejung.

Keywords: Antibacterial metabolite, Fermentation, *Streptomyces* spp., Taplejung, Thin layer chromatography.

INTRODUCTION

Actinomycetes are considered the most invaluable prokaryotes in the medicinal and biotechnology industries because of their ability to produce several bioactive molecules, particularly antibiotic compounds (Golinska & Dahm, 2011). These filamentous bacteria belong to the phyla actinobacteria and the order actinomycetes (Mellouli *et al.,* 2003; Fguira *et al.,* 2005). They are Gram positive characterized with a high G+C content (>55 %) in their DNA (Gonzalez-Franco & Robbles-Hernandez, 2009; Ogunmwonyi *et al.,* 2010). Actinomycetes are found in large numbers in soils, fresh waters, lakes, river bottoms, manures, composts, and dust as well as on plant residues and food products. However, the diversity and distribution of actinomycetes that produce secondary metabolites is determined by different physical, chemical, and geographical factors (Gurung *et al.,* 2009; Ogunmwonyi *et al.,* 2010). Researchers have done numerous studies on the isolation and screening of antimicrobial-producing actinomycetes. It has been propounded that most of the novel antibiotics was found with screening of isolates from soil (Baniya *et al.,* 2018).

There are increasing trends to develop the antimicrobial compound effective against antibiotic resistant bacteria. actinomycetes are the common sources of novel antibiotics (Okami & Hotta, 1988) because of their ability to produce several bioactive molecules, particularly antibiotic compounds (Mellouli *et al.,* 2003). Out of 22500 biologically active compounds from microbes, 45 % are obtained from actinomycetes (Berdy, 2005). The genus *Streptomyces* is responsible for the production of 60 % of the antibiotics (Singh *et al.,* 2005). The demand for new antimicrobial agents is high because of the emergence of new multidrug resistance in common pathogens (Rana *et al.*, 2019), and the potential of use of multidrug-resistant pathogens in bioterrorism (Spellberg *et al.,* 2004). Antibiotic-resistant bacteria have challenged the treatment of infectious diseases which are still the second leading cause of death worldwide (Luzhetsky *et al.,* 2007). In the search for a novel antibiotic compound, a study in Nepal revealed that twenty-seven actinomycetes isolated from soil samples of the Mount Everest region were reported to have antibacterial activity (Gurung *et al.,* 2009).

Likewise, One hundred and seventeen antibiotic-producing actinomycetes were isolated from non-agricultural wasteland, alkaline soils and compost-rich garden soil (Kumar *et al.,* 2010). Twenty species of actinomycetes were isolated from marine soil samples in which three showed significant antimicrobial activity against *Staphylococcus aureus* and *Escherichia*

coli (Kalyani *et al.,* 2012). Fifty-four actinomycetes were isolated from the soil collected from five different parts of Kathmandu valley out of which 20 isolates produced antibiotics; all of them belonged to the genus *Streptomyces* (Rai *et al.,* 2016). *Streptomyces* spp. and *Thermomonospora* spp. among twenty-two isolates from soils of Siraha were the most potent isolates showing antibacterial activities against Gram negative bacteria (Sah & Lekhak, 2017).

Out of 120 isolates having the antimicrobial property, 4 most potent strains, *Nocardiopsis prasina, Streptomyces violarus, Streptomyces krainskii,* and *Streptomyces tsusimaensis* exhibited both antibacterial and anti-fungal property (Lekhak *et al.,* 2018). Forty-one isolates were obtained from 11 soil samples and identified as *Streptomyces* spp. (70.7 %), *Micromonospora* spp. (19.5 %), and *Nocardia* spp. (9.5 %). 43.34 % of actinomycete isolates were found to be potent antimicrobial producers from the primary screening among which 46.34 % were effective against Gram-positive and 12.19 % against Gram-negative test organisms. Isolate C7 (*Micromonospora* spp.) showed the best broad-spectrum antimicrobial activity during secondary screening (Sapkota *et al.,* 2020). These evidences documented the abundant presence of actinomycetes with antimicrobial activity. In this regard, Taplejung (1441-7000 m), a cold region of Nepal is of significant interest because no study has been carried out from these unexplored areas. Low temperature and seasonal variations due to sun radiations create an extreme environment, which is likely to harbor unusual microorganisms. Expecting such habitat increases the chances of finding novel microorganisms, this study was undertaken to isolate and characterize antibacterial actinomycetes from soil samples of Taplejung, a cold district of Eastern Nepal.

MATERIALS AND METHODS

Collection of soil samples

About 4-5 grams of dry soil samples were collected in separate clean polyethylene bags and mixed well with approximately one gram of $CaCO_3$, already added to the bags, from a depth of 4-5 cm of cultivated fields, riverbanks, and gardens of Taplejung (Lattitude: 27°25′46″N; Longitute: 87°46′3.8″E) of Eastern Nepal between July 2016 and April 2017. Then the soil samples were further dried at room temperature for about 3 weeks and the laboratory work was carried out in the microbiology laboratory of Central Campus of Technology, Tribhuvan University, Dharan, Sunsari, Nepal. Test bacteria such as *Escherichia coli*, *Salmonella* Typhi, *Pseudomonas aeruginosa, Staphylococcus aureus,* and *Bacillus subtilis* obtained from the Department of Microbiology, Central Campus of Technology, Dharan, were used for antibiosis study of antibiotic produced by *Streptomyces* spp. isolated from the soil samples.

Isolation of actinomycetes

Actinomycetes were isolated by spread plate technique following the serial dilution of soil samples, on starch casein agar (SCA) (Williams & Davies, 1965) plates containing nystatin and cycloheximide (each at a concentration of 50 µg/mL of medium). Typical actinomycetes colonies characterized by their dry and tough wrinkled nature were picked with a sterile inoculating loop and streaked on SCA by quadrant streaking technique. The inoculated plates were incubated for 5-7 days at 28° C to isolate pure colonies of actinomycetes.

Screening of actinomycetes for antimicrobial activity

The screening method consists of two steps, primary screening, and secondary screening. The primary screening of actinomycetes isolates was done by the perpendicular streak method (Egorov, 1985) on Nutrient Agar (NA). The test bacteria were *E. coli, S.* Typhi, *P. aeruginosa, B. subtilis,* and *S. aureus.* Secondary screening of actinomycetes isolates was done by agar well assay method (Haque *et al.,* 1992) on Mueller Hinton Agar (MHA) against the test bacteria used in primary screening.

Characterization of actinomycetes

Macroscopic characteristics

The isolated colonies of actinomycetes on starch casein agar were studied for the color of the aerial mycelium and diffusible pigments and other colony characteristics such as size, consistency, margin, etc.

Microscopic characteristics

The microscopic characterization was done by the coverslip culture method (Kawato & Sinobu 1959). The mycelial structure, the configuration of sporophore (conidiospore and arthrospore), and the arrangements and shape of spores on the mycelia were observed under a microscope (1000X). The observed morphology was compared with the actinomycetes morphology described in Bergey's manual of determinative bacteriology (Holt *et al.,* 1994) for the presumptive identification of the isolates.

Biochemical characterization

Biochemical tests performed for identification were oxidase, carbohydrate utilization, citrate utilization, indole and hydrogen sulfide production, nitrate reduction, urea hydrolysis, tween 20 hydrolysis, starch hydrolysis, and esculin hydrolysis tests (Kawato & Sinobu, 1959).

Physiological characterization

Tests like temperature tolerance, Sodium chloride (NaCl) tolerance, and motility tests were performed for physiological characterization (Kawato & Sinobu, 1959).

Fermentation

The single potent isolate (T_{18}) based on primary and secondary screening was used for fermentation by the submerged state culture method. The isolate was inoculated into a 100 mL Erlenmeyer flask containing 25 mL starch casein broth (SCB) and incubated in a water bath shaker at 28° C at 160 rpm for 4 days for inoculums development. The prepared inoculum was poured in a sterile 500 mL Erlenmeyer flask containing 200 mL sterile SCB and incubated in a water bath shaker at 160 rpm at 28° C for 7 days (Gurung *et al.,* 2009).

Recovery of antibiotics from fermented broth

After the completion of fermentation, the broth was filtered through Whatman No. 1 filter paper aseptically. The residue was discarded and the filtrate was collected. The filtrate was subjected to solvent extraction for antibiotic recovery. Ethyl acetate and the filtrate broth were taken in a separating funnel (1:1, v/v), vigorously shaken for 1 h, and left undisturbed for half an hour. The solvent phase containing antibacterial metabolite was separated and subjected to evaporation on a water bath at 40° C for 15 h (Liu *et al.,* 2020). The residue obtained was weighed, dissolved in phosphate buffer, and used for the determination of antibacterial activity, minimum inhibitory concentration, and TLC study. Other organic solvents (n-butanol, chloroform, dichloromethane, and methanol) were also used to extract the antibacterial substances by the same procedure.

Determination of antibacterial activity and minimum inhibitory concentration (MIC) of antibiotic

The antibacterial activity of the extract was determined by the agar cup assay method against the test organisms (Rajbhandari & Lindequist, 2020) and the MIC of the antibiotics was determined by the serial dilution method in nutrient broth against *E. coli* (Sapkota *et al.*, 2019)

Thin-layer chromatography

Silica gel plates (20 cm × 20 cm, 1 mm thick) were prepared and activated at 80° C for 2 h. Ten µL of the extract and a reference antibiotic (gentamicin) solutions were applied on the TLC plate and the chromatogram was developed using chloroform: methanol (10:90) as a solvent system. The chromatogram was dried at 110° C for 5 mins and the spots were visualized in the iodine vapor chamber (Gurung *et al.,* 2009).

RESULTS

A total of 24 different actinomycetes were isolated in SCA from 15 soil samples collected from Taplejung, an eastern mountain region of Nepal. Out of 24 actinomycetes isolates, only 1 (4.2 %) showed antibacterial activity against test bacteria from brown moist soil samples (S_{12}) (Fig. 1).

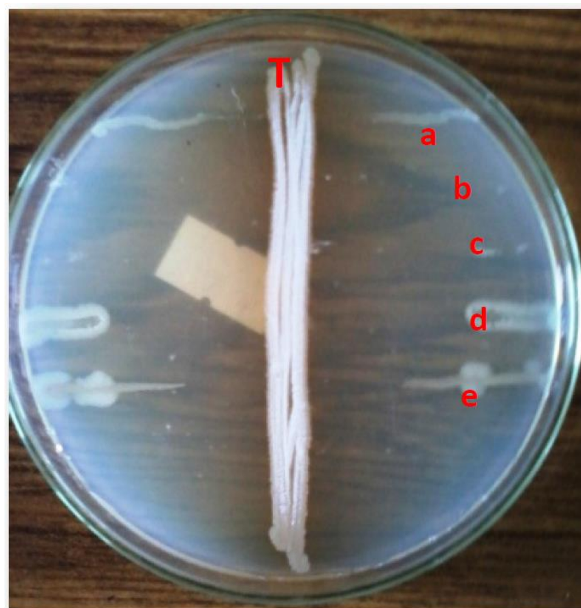

Fig. 1. The antibacterial activity shown by potential actinomycetes isolates in primary screening. T: T_{18} isolate, a: *Escherichia coli*; b: *Salmonella* Typhi; c: *Pseudomonas aeruginosa*; d: *Staphylococcus aureus*; e: *Bacillus subtilis*

Antibacterial activity in primary screening

In primary screening, the isolated actinomycetes (T_{18}) showed antibacterial activity against both Gram-positive and Gram-negative test bacteria. The zone of inhibition shown by the potential isolate against *E. coli, S.* Typhi*, P. aeruginosa, S. aureus,* and *B. subtilis* were 9 mm, 30 mm, 14 mm, 26 mm, and 26 mm respectively.

Antibacterial activity in secondary screening

A single active isolate (T_{18}) showed inhibitory activity against all the test bacteria in agar well assay method on Mueller Hinton agar (MHA). The zone of inhibition shown by crude extract of the potential isolate against *E. coli, S.* Typhi*, P. aeruginosa, S. aureus,* and *B. subtilis* were 6 mm, 18 mm, 9 mm, 16 mm, and 16 mm, respectively.

Macroscopic characteristics of active isolate

This potential isolate T_{18} produced cream-colored substrate mycelium and white-colored aerial mycelium, which later changed to brownish on the central region of colonies. It had a powdery texture and did not produce any diffusible pigments. The colony diameter was approx 1 mm with an entire margin.

Microscopic characteristics of active isolate

The rectiflexibles type sporophore morphology exhibited by the potential isolate (T_{18}) presumably identified it as *Streptomycetes* spp. (Fig. 2).

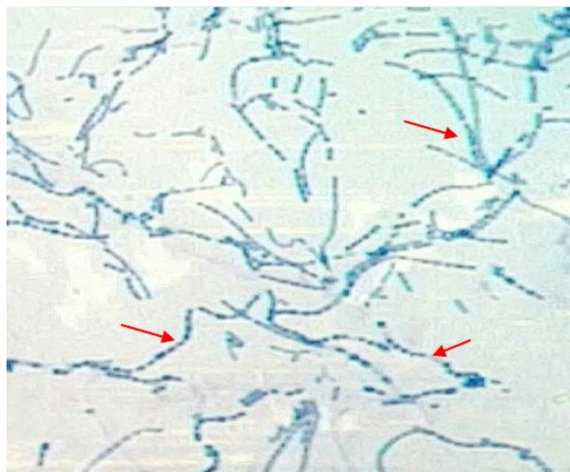

Fig. 2. Microscopic photograph (X1000) in which arrow indicates the filamentous structure of potential isolate (T_{18})

Biochemical and physiological characteristics of T_{18} isolate

The carbohydrate utilization tests indicated that the isolate (T_{18}) utilized all substrates except lactose and maltose. Similarly, the isolate hydrolyzed all the five substrates. Biochemical tests such as catalase, citrate utilization, and hydrogen sulfide production tests were shown positive while nitrate reduction and indole production tests were negative. The motility test confirmed that the isolate was nonmotile. Although the isolate showed growth at 15° C and 37° C, it did not at 45° C. It tolerated 5 %, 7 % and 10 % NaCl (Table 1). According to results obtained from macroscopic, microscopic, biochemical, and physiological characteristics, the potential isolate (T_{18}) was identified as *Streptomyces* spp.

Fermentation

The residue obtained from the filtrate of fermented broth was light brownish-white in color and had flaky and greasy consistency. The amount of the residue from the isolate T_{18} was one gram per 100 mL of the broth. Among five solvents only ethyl acetate could extract the potent metabolites at a detectable level from the fermented broth.

Minimum inhibitory concentration (MIC) of active compound

The Minimum inhibitory concentration of metabolite extracted fromT_{18} against *E. coli* was 1.2 mg/mL.

Characteristics of the antibacterial substances

The TLC showed only one spot produced by the extracted antibacterial metabolite with R_f value of 0.87. The control antibiotic namely gentamicin also produced only one spot with R_f value of 0.94 suggesting that the extracted antibacterial metabolite was different but closely related to gentamicin (Fig. 3).

Table 1. Biochemical and physiological tests

Tests	Results
Carbohydrate hydrolysis	
Glucose	+
Fructose	+
Sucrose	+
Lactose	−
Maltose	−
Mannose	−
Mannitol	+
Substrate hydrolysis	
Starch	+
Urea	+
Tween 20	+
Gelatin	+
Esculin	+
Biochemical test	
Catalase	+
Oxidase	−
H_2S production	+
Citrate utilization	+
Nitrate reduction	−
Indole test	−
Temperature tolerance	
15° C	+
30° C	+
45° C	−
Sodium chloride tolerance	
NaCl 5%	+
NaCl 7%	+
NaCl 10%	+

DISCUSSION

Actinomycetes, a potential source of antibiotics, produce a variety of secondary metabolites with novel biological activities (Pudi *et al.,* 2016). Antibiotics are an indispensable part of modern medicine but the emergence of antibiotic resistance among pathogenic bacteria is a major threat in the treatment of respective diseases caused by such bacteria. Hence for the continuance of the modern medicine novel antibiotics are needed to be introduced (Coates & Hu, 2007). Therefore, it is necessary to search for a new antibiotic for solving the problems of infection

due to resistant bacteria. The world is facing an ever-increasing problem of antibiotic-resistant bacteria and we are rapidly heading towards the post-antibiotic era. There is an urgent need to investigate alternative treatment options while there are still a few antibiotics left. New resistance mechanisms emerge and spread globally threatening the treatment of common infectious diseases, resulting in more deaths and disabilities (Baniya *et al.,* 2018).

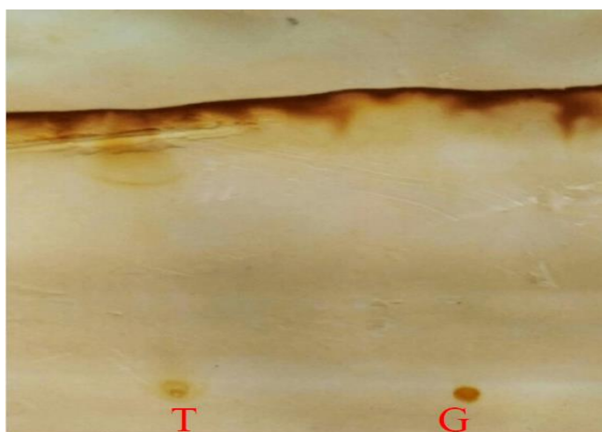

Fig. 3. TLC plate showing chromatogram of extract and gentamicin (standard) (T: Test antibacterial compound extracted from the potential isolate (T_{18}); G= Gentamicin)

Many important bioactive compounds of high commercial value (about 80 percent) were obtained from actinomycetes and the screening for new bioactive compounds especially novel antibiotics active against resistant organisms continued (Velayudham & Murugan, 2012). The diversity of terrestrial actinomycetes are of extraordinary significance in several areas of science and medicine, particularly in antibiotic production (Magarvey *et al.,* 2004). Actinomycetes are considered of special commercial importance because they produce alkaloids with antibiotic activity (Pudi *et al.,* 2016).

This study attempted to search for a novel strain of actinomycetes from soils of Taplejung (altitude: 3700 m), an extreme environment of Nepal. The survival of the microorganisms in such harsh and challenging habitat might be due to their ability to adaptat diverse environment and produce spores. Isolation of actinomycetes has always been faced with difficulties in comparison to their competitors such as, other bacteria and fungi (Williams & Cross, 1971). This may be due to their long incubation period. However, an effort was made to increase the rate of isolation by pretreatment of the samples with calcium carbonate and subjecting them to air dry for three weeks. The use of starch casein agar as a selective medium supplemented with antibiotics (Alferova *et al.,* 1989), nystatin (50 µg/mL), and cycloheximide (50 µg/mL) prevent the growth of contaminating bacteria and fungi. Further, this medium is very specific for the isolation of actinomycetes, as only organisms (mostly actinomycetes) capable of degrading the polymers in the medium will be able to grow (Velayudham & Murugan, 2012).

The first primary screening was done to select the antibacterial isolates and determine the range of microorganisms that were sensitive to the antibacterial metabolites. The secondary screening was crucial to select the isolates for further studies. A qualitative approach of screening determine the range of the microorganisms that are sensitive to a potential antibiotic while the quantitative approach provides information about the yield of antibiotics that can be expected when the organism is grown in different media (Gurung *et al.,* 2009). Two most potent actinomycetes isolate like *Streptomyces* spp. (R_1a) and *Thermomonospora* spp. (R_1b) isolated from soils of Siraha, Nepal, showed antibacterial property against Gram-negative bacteria (Sah & Lekhak, 2017).

In this study, the result of the screening revealed that the isolate was more active against Gram positive bacteria than Gram negative bacteria; however, its activity was the highest against *S.* Typhi. This might be due to differences in their cell wall composition between those two types of microorganisms. Gram-negative bacteria have an outer lipopolysaccharide membrane; hence their cell wall is impermeable to lipophilic solutes, while porins constitute a selective barrier to the hydrophilic solutes (Nokaido & Vaara, 1985). Hydrophobic molecules can pass through the cell wall of Gram positive bacteria easily compared to Gram negative bacteria because Gram positive bacteria have only a peptidoglycan layer which is not an effective permeability barrier for the antibacterial agents (Ababutain, 2011).

According to results of primary and secondary screening, only one actinomycete coded as T_{18} was found to be the best strain among 24 different isolates. Hence, it was chosen for fermentation for the production of antibacterial metabolite for antibiosis and TLC.

The microscopic observation presumably identified the potential isolate T_{18} as *Streptomyces* spp. Different environmental constraints and conditions, such as temperature, influence the growth and diversity of actinomycetes has a profound effect on the physiology, morphology, sporulation, biochemistry, and also antimicrobial metabolite production (Sapkota *et al.,* 2020).

In this study, *Streptomyces* spp., the potential isolate, produced various enzymes such as amylase, caseinase, gelatinase, urease, and lipase. In a similar study conducted by Sapkota *et al.*, (2020), approximately 78.04 % of the isolates were showed more than one enzymatic activity. The potential of actinomycetes to secrete broad-range

enzymes may have resulted from the natural selection to survive in a competing environment (Boroujeni *et al.,* 2012).

The antibacterial metabolites from fermented broth were extracted in different organic solvents (n-butanol, chloroform, dichloromethane, ethyl acetate, and methanol), however, only ethyl acetate solvent extracted the potent metabolites at a detectable level from the fermented broth. This might be because of the highest solubility of the metabolites in ethyl acetate solvent (Sah & Lekhak, 2017).

The minimum inhibitory concentration (MIC) of extract from T_{18} against *E. coli* (ATCC 25922) was 1.2 mg/mL. In this study, *E. coli* was selected for MIC because it showed the lowest zone of inhibition among the test bacteria in both primary and secondary screening. AST could be performed in 5-6 hours using a fast-growing laboratory strain of *E. coli* ATCC 25922 as a bacterial model because oxygen consumption and optical density measurements provide a more holistic insight into the metabolic state and the evolution of bacterial biomass (Liu *et al.,* 2020).

Since these metabolites were obtained by the evaporation of ethyl acetate solvent; it is likely to have this high MIC value. When the TLC chromatogram was visualized under iodine vapor, each extract produced only one spot indicating the presence of a single compound. The spot was near the solvent front with an R_f value of 0.87 for the extract T_{18}. Similarly, gentamicin also produced a single spot with an R_f value of 0.94. Similar findings were reported by Gurung *et al.,* (2009).

Thin-layer chromatography is a sensitive and highly reliable method for the qualitative and quantitative detection of antibiotic residues. As TLC is less time-consuming, low cost, and can be performed with a less complicated technique it has a wide application in various analyses (Bhandari *et al.*, 2020)**.** TLC can be successfully used, in the preliminary screening of pharmaceutical substances. In modern analysis, TLC is usually used as a separation method, which establishes the presence or absence of antibiotics above a defined level of concentration (Wall, 2005). Gentamicin is one of the broad-spectrum antibiotics inhibiting both Gram positive and Gram negative bacteria (Timsina *et al.*, 2019; Bulger *et al.,* 1963), hence, gentamicin was chosen as a standard reference antibiotic for TLC.

According to this study, the produced antibacterial metabolite can be easily extracted by using a suitable solvent like ethyl acetate. Similarly, the R_f value of such antibiotic can be determined by using known standards through TLC. Hence this study revealed the distribution of broad-spectrum antibacterial metabolite producing *Streptomyces* spp. in Taplejung, the eastern mountain region (1441-7000 m) of Nepal. Such antibacterial metabolite should be further analyzed for their molecular structures and identification of compound.

CONCLUSIONS

The potent actinomycetes isolate like *Streptomyces* spp. (T_{18}) that showed antibacterial property against both Gram positive and Gram negative bacteria, was isolated from soils of an extreme area like Taplejung, a cold district of Nepal. Hence this study reveals the distribution of antibacterial metabolites producing actinomycetes in Taplejung (1441-7000 m), mountain regions of Nepal. The antibiogram results indicated that the mountain soils are a source for actinomycetes antagonistic against the test bacteria.

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