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Isolation and Screening of Antibiotic Producing Actinomycetes from Soils of Hills and Plains of Eastern Nepal

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Keywords
- Actinomycetes
- Agar well diffusion
- Antimicrobial activity
- *Streptomyces*

Abstract

Objective: The study was aimed to assess the isolation method for *Actinomycetes*, understand the current status of the presence of antibiotics producing *Actinomycetes* in the soil sample of Hile and Jhapa, and evaluate the different methodology of testing antimicrobial activities.

Methods: Actinomycetes were isolated from five soil samples of Hile and Jhapa on starch casein agar (SCA) media supplemented with amphotericin and rifampicin. Selected cultures were tested for antibiotic-producing properties in primary screening by placing isolates with the plate of test organisms. Antimicrobial activity was tested in secondary screening using agar well diffusion method from solvent extracts of potential *Actinomycetes* produced by submerged fermentation.

Results: Out of seven isolates of *Actinomycetes* from five tested soil samples, two isolates showed some antimicrobial activity against Gram-positive test organisms viz *Staphylococcus* spp. and *Bacillus* spp. A larger zone of inhibition was shown by actinomycetes sample AG_4 against *Staphylococcus* spp when compared with other test organisms.

Conclusion: The findings show the possibility of antimicrobial activity of the *Actinomycetes* isolates in the soil of Hile and Jhapa and highlight the necessity of extensive studies for the isolation and purification of bioactive metabolites. Molecular characterization analysis of isolated *Actinomycetes* spp would help discover novel compounds of commercial value.

ABBREVIATIONS

spp: species; **°C:** Degree Centigrade; **g:** Gram; **SCA:** Starch Casein Agar; **µg/ml:** microgram per milliliter; **MHA:** Muller Hinton Agar; **YMEB:** Yeast Malt Extract Broth; **rpm:** Revolution per minute; **µl:** microliter; **mm:** millimeter

INTRODUCTION

Despite the discovery of thousands of antibiotics over several decades, microbial resistance towards them has been increasing. Because of the production of primary and secondary metabolites of economic significance, *Actinomycetes* are the most economically and biotechnologically valuable prokaryotes [1] which are responsible for the production of antibiotics, antifungal, antiviral, anti-tumor agents, immunosuppressive agents, and enzymes [2,3]. *Actinomycetes* are Gram-positive, generally aerobic, a high percentage of G+C content (about 60-70%) [4]. Several species have complex cell wall structures that make the Gram staining unsuitable [5]. *Actinomycetes* are widely distributed in both terrestrial and aquatic ecosystems [6] and include important plant and animal pathogens [7]. They can degrade biopolymers in soil and litter, produce taste and odor in potable waters, and fix nitrogen in a variety of non-leguminous plants [8–11]. Approximately two-thirds of naturally occurring antibiotics, including many of medical importance, have been isolated from *Actinomycetes* [12]. *Actinomycetes* are useful agents for bioremediation, biodegradation, composting, humus formation, plant growth promotion, pesticide activities, biocontrol tools, and biocorrosion compounds [13,14].

Actinomycetes produce some valuable antibiotics such as Streptomycin (*Streptomyces griseus*), Chloramphenicol (*S. venezuelae*), Neomycin (*S. fradiae*), Nystatin (*S. noursei*), Erythromycin (*S. erythreus*), Amphotericin B (*S. nodosus*), Vancomycin (*S. orientalis*), Kanamycin (*S. kanamyceticus*), etc [15,16]. *Actinomycetes* include a very diverse group of organisms such as *Streptomyces, Nocardioform,* and *Corynebacterium* [17,18]. They impart an "earthy" odor to the soil after rain which is due to the presence of geosmin (volatile organic compound) [19,20].

The availability of antibiotic-producing *Actinomycetes* varied depending on the cultivated lacustrine soil [21]. Acidophilic *Actinomycetes* were isolated from natural soils and mine wastes [22]. Out of the total isolated colonies producing antibiotics, most of them belong to the genus *Streptomyces* [23].

Streptomyces species have long been the best antibiotic-producing bacteria and several strains have been screened by the pharmaceutical industry. Therefore, the chances of isolating new genera or compounds have considerably reduced. Hence the study was aimed to assess the current status of the presence of antibiotics producing *Actinomycetes* in the soil sample of Hile and Jhapa. The research also tried to assess the knowledge of isolating *Actinomycetes* and different methodology of testing antimicrobial activities.

MATERIALS AND METHODS

Collection of soil samples and sampling sites

In this study, soil samples were collected from the different locations of Hile of Dhankuta district and Jhapa district for the screening of *Actinomycetes* with inhibitory activity against other soil bacteria. Samples were collected from various depths of the earth's surface, ranging from layers just beneath the upper surface to 1 feet depth. A trowel was used to dig the soil. The samples were collected in sterile small plastic tubes and properly labeled indicating the date of collection and depth. Three soil samples from Hile and two soil samples from Jhapa were collected over an extended period of six weeks (Table S1, Supplementary data). Soil samples from Hile were taken from forest areas which contained leaf litter, organic matter, high moisture content, large particle size, and other extraneous matter. The area was not much exposed to man-made chemicals or fertilizers and human encroachment. Soil samples of Jhapa were also from forest areas with low moisture content, small particle size, and organic matter. The sampling site was also far from the residential area.

Drying of soil samples

The collected soil samples were dried in a hot air oven at 60-65 °C for about one hour. This was done to reduce the number of bacteria in the soil other than *Actinomycetes.* This is one of the several approaches for selective isolation of *Actinomycetes* from soil samples. The dried soils were then transferred to respective tubes and were processed in the research laboratory of the Department of Microbiology, Mahendra Morang Adarsh Multiple Campus, Biratnagar. Heating air-dried soil samples (120 °C or 100 °C) reduced the numbers of filamentous bacteria and *Streptomycetes* on isolation plates, resulting in selective isolation of various rare *Actinomycetes* genera [24].

Isolation and purification of actinomycetes

In this study, SCA medium was used as an isolation medium for isolation of *Actinomycetes.* This medium was prepared with several ingredients (Soluble starch 10 g + Casein 0.3 g + Potassium nitrate 2 g + Sodium chloride 2 g + Dipotassium hydrogen phosphate 2 g + Magnesium sulphate 2 g + Calcium carbonate 0.02 g + Ferrous Sulphate 0.01 g + Agar 18 g) mixed in one-liter distilled water. The final pH of the media was set at 7.0 ± 0.1 at 25 °C and was sterilized in an autoclave at 121 °C for 15 minutes. The medium was then allowed to cool at 50 °C and 75 µg/ml amphotericin B and 2.5 µg/ml rifampicin was added to the flask and mixed well to inhibit the growth of fungi and bacteria respectively [25]. Then the media was poured into the petri-plates fairly thick such that they did not dry out during the incubation period. Six sterile test tubes with 9 ml of sterile distilled water were used for serial dilution with one Gram of dried soil sample. Finally, the dilution of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} was obtained.

One hundred microliters of the aliquot from each dilution was transferred onto the respective starch casein agar plates. Immediately after the transfer, each soil sample was spread on the agar medium using a sterile glass spreader. After using the spread plate technique, the plates were incubated at 30 °C for 7 days. After the incubation period, the plates were examined for typical *Actinomycetes* colonies. True bacteria grow quickly with slimy distinct colonies on culture media while *Actinomycetes* colony grows slowly, show powdery, consistency, and stick firmly. *Actinomycetes* form typical round, small, opaque, compact, powdery, frequently pigmented, and appear dull looking. They produce hyphae and conidia/ sporangia like fungi. Certain *Actinomycetes* whose hyphae undergo segmentation resemble bacteria, both morphologically and physiologically [26].

Identification of Actinomycetes

After growth observation on SCA plates, each discrete colony from the selected colonies on SCA plates was inoculated onto another plate of SCA medium to obtain well isolated pure colonies. The plates were incubated at 28 °C for 3 to 4 days by streak method. Isolated colonies were subjected to Gram's staining, catalase test, nitrate reduction test, and carbohydrate fermentation test to identify the strain of *Actinomycetes* based on Bergey's Manual of Determination of Bacteriology [27]. Strains were identified based on their phenotypic and physiological characteristics.

Phenotypic characterization

The classification of actinomycetes was based basically upon morphological observations. Morphology was the only characteristic for the genera which was used in many early descriptions, particularly of *Streptomyces* species in the first few editions of Bergey's Manual. For the characterization of the strains, some basic tests like aerial mass color, reverse side pigmentation, and spore morphology have been suggested.

For the grouping and identification of *Actinomycetes*, the chromogenicity of the aerial mycelium was considered to be an important character. The colors of the mature sporulating aerial mycelium might be white, grey, red, green, blue, and violet. When the aerial mycelium color falls between two colors series, both the colors were recorded.

The strains were divided into two groups according to their ability to produce characteristic pigments on the reverse side of the colony, called as distinctive (+) and not distinctive (-). A color with low chroma such as pale yellow, olive, or yellowish-brown occurs, it was included in the latter group (-). Spore surface features were observed under the microscope. The spore structures in *Actinomycetes* could be reported to be four types: smooth, spiny, warty, and hairy.

Utilization of carbon sources and biochemical tests

The utilization of carbon sources was examined according to the method of Shirling and Gottlieb [28]. Chemically pure carbon sources certified to be free of admixture with other carbohydrates

or contaminating materials were used in the test. Carbon sources used for this test were galactose, fructose, dextrose, arabinose, sucrose, maltose, lactose, and mannitol. No carbon source was used as negative control while D-glucose was used as the positive control. For one liter of phenol red carbohydrate broth media, the formulation was trypticase or proteose peptone No.3 10 g, sodium chloride 5 g, phenol red (7.2 ml of 0.25% phenol red solution or 0.018 g powder), and carbohydrate 10 g. The sterile carbohydrate source was mixed aseptically only after the autoclaving of the broth. Before adding carbohydrate to the broth to give a concentration of approximately 1%, it was cooled to 60 °C. The mixture was then agitated and poured into the sterile test tubes. Results were recorded as follows: Strongly positive utilization (++), Positive utilization (+), Utilization doubtful (+/-), and Utilization negative (-). Strongly positive utilization (++) was used when growth on tested carbon in the basal medium was equal to or greater than growth on the basal medium plus glucose. Positive utilization (+) was indicated when growth on tested carbon was significantly better than on basal medium without carbon but somewhat less than on glucose. Utilization doubtful (+/-) was referred when growth on tested carbon was only slightly better than on the basal medium without carbon and significantly less than with glucose. Utilization negative (-) was indicated when growth was similar to or less than growth on basal medium without carbon. Utilization was recorded as negative if growth was not better than no-carbon control.

Nitrate broth of 5 ml was incubated with a loopful of spores of selected isolates and incubated at 28 °C for 7 days. Controls were also run without inoculation. On the 7th day, the inoculated nitrate broth was tested for the presence of nitrate using α -naphthylamine test solution and sulphanilic acid. To 1 ml of broth under examination and 1 ml of control, two drops of sulphanilic acid followed by two drops of α – naphthylamine solution were added. The presence of nitrate was indicated by a pink, red, or orange color and no color change was considered as nitrate negative.

A catalase test was performed by adding 3% H_2O_2 reagent to the culture. The release of bubbles (free oxygen gas) indicated a positive catalase test.

Screening for antimicrobial activity

Sterilized Muller Hilton Agar plates were prepared and 12-24 hours broth culture of test organisms was used. The test organisms used in this study were prepared with a turbidity of 0.5 McFarland. The test strains used were *Staphylococcus* spp. and *Bacillus* spp. (Gram-positive) and *Escherichia coli* and *Shigella* spp. (Gram-positive bacteria). The test organisms were highly sensitive against different antibiotics. Screening of *Actinomycetes* was done by observing their antimicrobial activity against test organisms. A preliminary study was done by placing the *Actinomycetes* strain against test bacteria. Six isolated *Actinomycetes* strains were placed on Muller Hinton Agar (MHA) plates which were initially inoculated with test organisms. All the plates were incubated at 28 °C for 5 days. The test organisms like *Escherichia coli, Shigella* spp, *Staphylococcus* spp, and *Bacillus* spp were tested on different antibiotics for their sensitivity and antibiogram. Based on the results of antagonistic activity, the sensitive test organism strains were selected for further studies [29].

Submerged fermentation and Extraction of crude antibiotics

Based on the results of primary screening, potent isolates of *Actinomycetes* were cultivated in 500 ml conical flasks for submerged fermentation in yeast malt extract broth (YMEB) with continuous shaking for about 2 weeks at 28 °C. After incubation, the contents of the flasks were filtered aseptically through sterile muslin cloth followed by sterile Whatman filter paper. The crude culture filtrates were centrifuged at 5000 rpm for 10 min and then were subjected to solvent extraction in a separation funnel using a solvent like ethyl acetate. In the separation funnel, supernatant and ethyl acetate were taken in proportion 1:1 and agitated well for about 30 minutes to ensure proper mixing. The separation funnel was left for about 15 minutes to allow separation of the organic phase (solvent) from the aqueous phase. The solvent layer was separated and the supernatant was again extracted with ethyl acetate. The solvent layers were pooled and evaporated to dryness at 40 °C. The crude solvent extracts were screened for their antibacterial activity. [30].

Antimicrobial activity of solvent extracts

Agar well diffusion method was performed to screen the antimicrobial efficacy of solvent extracts of selected *Actinomycetes*. Test bacteria were added in the culture media after allowing the media to cool and the media was uniformly poured over the plate. Samples of the crude solvent extract were assayed for antimicrobial activity using a modification of the agar well diffusion method. After aseptically pouring the agar (5 mm deep), plates were left to solidify. Then, four well (each of about 7 mm diameter) were cut into four quadrants of each plate. Crude solvent extract (50 µl) was transferred to the well with a micropipette. The plates were incubated at 30 °C for 24 hours and were observed for a zone of inhibition. Plates with uninoculated culture media served as a control.

RESULTS

Isolation of actinomycetes

After processing of the soil samples collected from different areas of Hile and Jhapa, a total of six isolates of *Actinomycetes* (AG_1, AG_2, AG_4, AG_5, AG_7, AG_8) were isolated from five different soil samples. Out of six isolates, four were isolated from Hile and two were isolated from Jhapa. Isolation and enumeration of *Actinomycetes* were done by spread plate technique using Starch Casein Agar (SCA) media. All the six isolates grown on SCA showed typical morphology of *Actinomycetes* (Figure 1A-F). The colonies were slow-growing, aerobic, folded, and with aerial and substrate mycelia of different colors.

Though not adequate to differentiate between genera, morphology has been an important characteristic for the description of taxa. And in fact, it was the only character used in many early descriptions. The color of the substrate mycelium was determined by observing the plates after 7-10 days. It was done only after seeing the heavy spore mass surface (Table 1). The strains were divided into two groups according to their ability

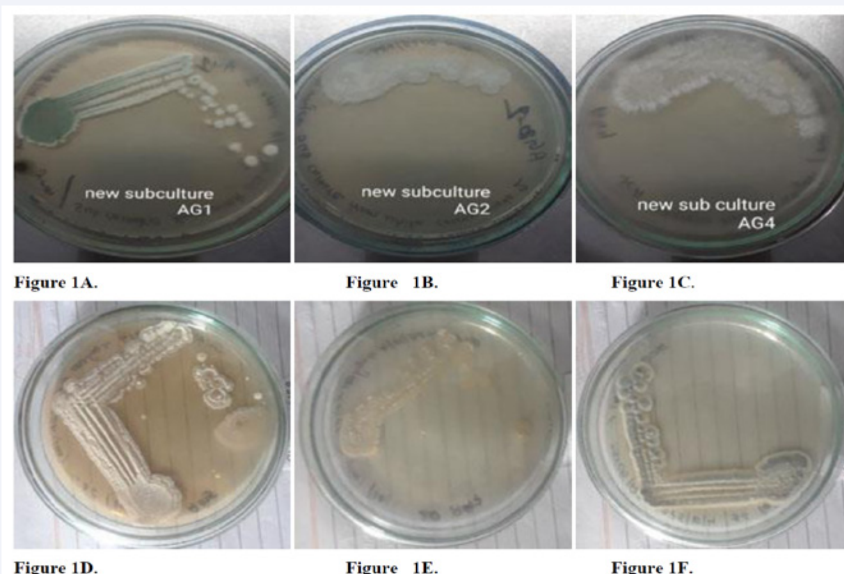

Figure 1 A-F: Subculture of isolates AG_1, AG_2, AG_4, AG_5, AG_7, and AG_8 on starch casein agar (SCA) plates as shown in Figure A-F respectively.

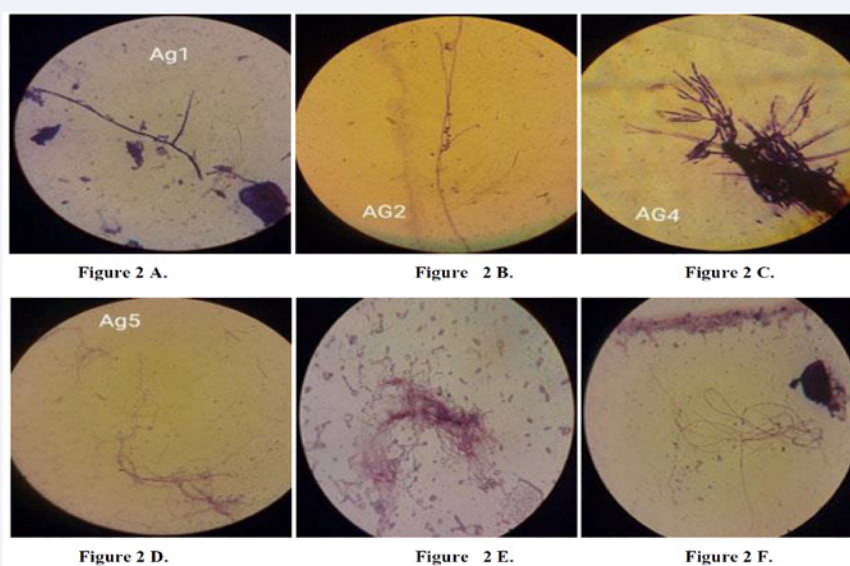

Figure 2 A - F: Gram staining of isolates AG_1, AG_2, AG_4, AG_5, AG_7, and AG_8 as shown in Figure 2 A, Figure 2 B, Figure 2 C, Figure 2 D, Figure 2 E, and Figure 2 F respectively.

to produce pigments on the reverse side of the colony, namely distinctive (1) and not distinctive or none (0) for reverse side pigmentation. The colors observed for distinctive were yellow and not distinctive were pale yellow (Table 1).

All the strains were examined under the microscope and accordingly the spore chain morphology was observed (Fig 2 A-F). Most of the strain showed a flexible or open-loop spore chain.

Species-specific physiological and biochemical characteristics

The ability of different *Actinomycetes* strains in utilizing various carbon compounds as a source of energy was shown in (Table 2). After comparing growth with the negative and positive control, it was observed that mannitol and sucrose were the most assimilated carbon source by most strains of *Actinomycetes* while fructose was the least assimilated carbon source. Among six strains only two strains AG_2 and AG_8 show nitrate reduction test positive. All the strains except AG_4 and AG_7 were catalase positive.

Primary screening of the antimicrobial activity

Among six isolates, only two were found to have antibacterial activities against a range of Gram-positive and Gram-negative bacteria (Figure 3 A-C). Two isolates (AG_5 from Jhapa and AG_4 from Hile) showed antibacterial activity against some test organisms. The antibiogram profile of test organisms was

Table 1. Reading of aerial mass color and reverse side pigment of Actinomycetes.

S.N.	Isolates	Aerial Mass Color	Reverse Pigmentation
1.	AG_1	Gray and white	0
2.	AG_2	White	0
3.	AG_4	White	0
4.	AG_5	White	1
5.	AG_7	Creamy and white	0
6.	AG_8	Gray and White	0

Table 2. Assimilation of carbohydrate sources.

Carbon sources	Strain					
	AG_1	AG_2	AG_4	AG_5	AG_7	AG_8
Galactose	-	+	+	-	-	-
Fructose	-	+	-	-	-	-
Dextrose	+	+	-	-	+	+
Arabinose	+	-	+	+	+	+
Sucrose	-	+	+	+	+	+
Maltose	-	+	+	+	+	+
Lactose	+	+	-	+	-	-
Mannitol	-	+	+	+	+	+
(+) indicate fermentative process. (-) indicates non-fermentative process.						

investigated by the Kirby-Bauer Disc method and tabulated in Table S2 (Supplementary data). However, two strains showed weak activity. The zone of inhibition exhibited by these strains was in the range of 3 - 20 mm. The result of the initial screening is given in (Table 3).

Secondary test of Actinomycetes isolates for their antimicrobial activity

Secondary screening by agar gel diffusion method for the extracted crude antibiotics was performed for their antagonistic activity. However, conclusive evidence of the inhibitory zone was not found. Culture AG_4 and AG_5 were used in submerged fermentation to ferment broth and produce antibiotics (Figure 4). The solvent extract was extracted using a centrifugation machine and was tested against those test organisms. Both extracts of *Actinomycetes* AG_4 and AG_5 were antagonistic to Gram-positive bacteria (*Staphylococcus* spp and *Bacillus* spp) only. The bioactive compounds produced by these two *Actinomycetes* were interfering with Gram-positive bacteria. Due to a lack of resources, the nature of antibiotics couldn't be established.

DISCUSSION

Soil is inhibited by a large number of organisms. Many of the soil microbes are useful as they produce several bioactive metabolites, including clinically important antibiotics. SCA media was used as it has been one of the most commonly used media for the isolation of *Actinomycetes* particularly *Streptomyces* [31,32]. As per the previously published article [33–35], when the soil samples were air-dried, the number of *Actinomycetes* isolation were increased. The dried soil samples were preprocessed at 60 °C for 1 hour to remove moisture and, thereby, avoid bacterial and fungal growth and effectively isolate *Actinomycetes.* Especially the contamination of other bacteria and fungi inhibit the colonization of *Actinomycetes* [36]. Hence heat treatment is done as a pretreatment for sample processing to reduce the number of Gram-negative bacteria generally present in the soil samples.

For the successful isolation of *Actinomycetes*, four procedures such as air drying, heat treatment, starch casein agar, and use of antibiotics amphotericin and rifampicin were tried out. By these strategies, we observed the isolation plates in abundance with *Actinomycetes* colonies. However, few bacterial and fungal colonies were also found. Most of the *Actinomycetes* need more than one week of incubation for their growth. [37] and [38] reported that *Actinomycetes* were slow growers and hence they eliminated the growth of colonies observed on the first and second days of incubation. The distribution of *Actinomycetes* was dependent on the depth of sample collection [39]. In our study, the maximum number of *Actinomycetes* was obtained from samples collected from the Hile which is a hilly region with a cold climate. The aerial mass color of almost all strains was whitish except two strains AG_1 and AG_8 showing gray color. [40] and [41] have also reported that the white color series of *Actinomycetes* were the dominant forms.

The isolated strains of *Actinomycetes* had undergone screening of antimicrobial activity. Mainly strains AG_4 and AG_5 had shown the zone of inhibition against Gram-positive *Bacillus* spp and *Staphylococcus* spp. *Actinomycetales*, an order of filamentous bacteria, especially *Streptomyces* strains have a unique capacity to produce novel antibiotics [40]. In our current study, screening of antimicrobial activity showed better antagonistic activity against Gram-positive than Gram-negative bacteria which agreed with the earlier findings [42,43] that most of the metabolites extracted

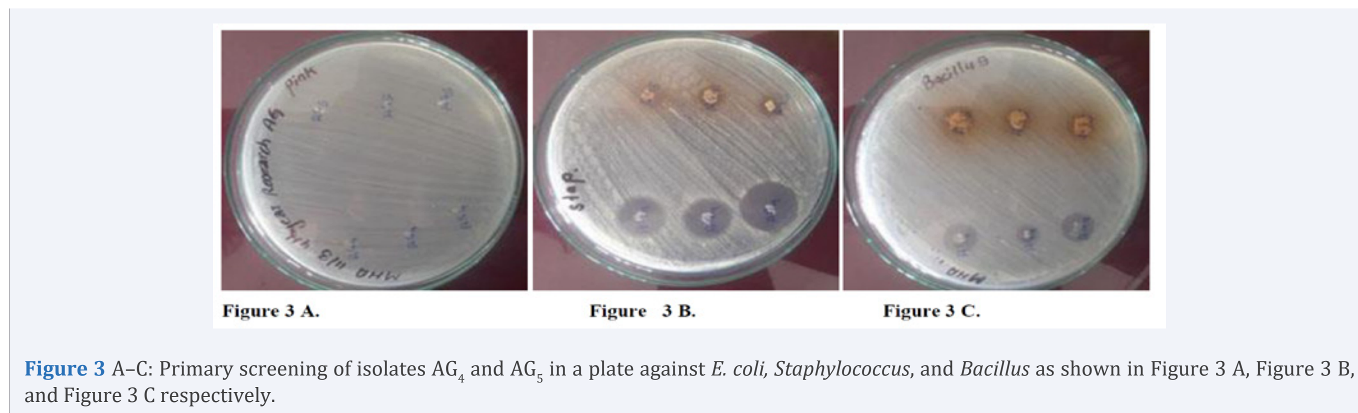

Figure 3 A–C: Primary screening of isolates AG_4 and AG_5 in a plate against *E. coli, Staphylococcus*, and *Bacillus* as shown in Figure 3 A, Figure 3 B, and Figure 3 C respectively.

Figure 4 Fermentation of yeast malt extract broth by isolates AG_4 and AG_5 in two conical flasks.

from *Actinomycetes* inhibit the growth of Gram-positive bacteria, but are ineffective against Gram-negative bacteria due to double membrane barrier and transmembrane efflux mechanism.

CONCLUSION

Through the study, the possibility of the presence of antimicrobial activity of *Actinomycetes* in the soil of Hile and Jhapa was observed. An extensive study would be needed for the identification of bioactive compounds as well. Molecular characterization analysis of isolated *Actinomycetes* spp would help discover novel compounds of commercial value.

RECOMMENDATION

Since it is becoming increasingly clear that our soil is a very rich source of *Actinomycetes*, the probability of finding novel *Actinomycetes* producing newer bioactive compounds cannot be ruled out. Hence, the continued effort and focus in screening such organisms from un- and under-explored habitats for new bioactive compounds have to be in our national policy and compel pharmaceutical industries to invest in the extensive research programs. A nationwide extensive study for the purification of bioactive metabolites has to be done.

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