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Research Article

Isolation and Screening of Antibiotic Secreting Actinomycetes from Soils of Province 1 Nepal

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Abstract

Objective: The study's goal was to evaluate the isolation method for Actinomycetes, identify the current state of the existence of antibiotic-producing Actinomycetes in soil samples from Dharan and Belbari, and evaluate various antimicrobial activity testing methodologies.

Methods: Actinomycetes were recovered from five soil samples from Dharan and Belbari using starch casein agar (SCA) media containing amphotericin and rifampicin. In primary screening, isolates were placed on a plate of test organisms to see if they produced antibiotics. Antimicrobial activity was assessed in secondary screening utilizing the agar well diffusion method with solvent extracts of putative Actinomycetes prepared by submerged fermentation.

Results: Four Actinomycetes isolates from five soil samples shown antibiotic efficacy against Gram-positive pathogens, including Staphylococcus and Bacillus. Actinomycetes sample AG10 demonstrated a greater zone of inhibition against Staphylococcus spp. when compared to other test organisms.

Conclusion: The study suggests that Actinomycetes isolates in Belbari and Dharan may have antibiotic potential. Further research is needed to isolate and purify bioactive compounds from these soils. Molecular characterization of isolated Actinomycetes spp. would aid in the discovery of novel substances with commercial value.

ABBREVIATIONS

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 to them has grown. Actinomycetes are the most commercially and biotechnologically valuable prokaryotes due to their ability to produce significant primary and secondary metabolites [1] which are responsible for the creation of antibiotics, antifungals, antivirals, antitumor drugs, immunosuppressive compounds, and enzymes [2,3]. They are responsible for the production of antibiotics, antifungals, antivirals, anticancer medicines, immunosuppressive chemicals, and enzymes [4]. Several species have complex cell wall features, making Gram staining inappropriate [5]. Actinomycetes are found in both terrestrial and aquatic habitats and have a significant role as plant and animal diseases [6-7]. They can breakdown biopolymers in soil and litter, produce taste and odor in drinkable water, and fix nitrogen in a wide range of non-leguminous plants [8-11]. Actinomycetes are responsible for almost two-thirds of naturally occurring antibiotics, many of which are medically important [12]. Actinomycetes are effective agents for bioremediation, biodegradation, composting, humus formation, plant growth stimulation, pesticide activity, biocontrol tools, and biocorrosion compounds [13,14].

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), and others are produced by actinomycetes [15,16]. Streptomyces, Nocardioform, and Corynebacterium are among the many species classified as actinomycetes [17,18]. They leave an "earthy" odor in the soil after rain due to the presence of geosmin (a volatile organic molecule) [19,20]. The presence of antibiotic-producing Actinomycetes varied according to the cultivated lacustrine soil [21]. Acidophilic Actinomycetes have been identified from both natural soils and mine waste [22]. The genus Streptomyces accounts for the majority of the isolated antibiotic-producing colonies [23].

Streptomyces species have long been the greatest antibiotic-producing bacteria, and various strains have been tested by the pharmaceutical industry. As a result, the possibilities of isolating new genera or chemicals have significantly decreased. As a result, the study sought to analyze the current state of antibiotic-producing Actinomycetes in soil samples from Dharan and

Belbari. The study also attempted to evaluate the expertise of isolating Actinomycetes and various methodologies for testing antibacterial activity.

MATERIALS AND METHODS

Collecting Soil Samples and Sampling Sites

In this work, soil samples were taken from several places in Dharan and Belbari, province 1, to screen for Actinomycetes having inhibitory activity against other soil bacteria. Samples were taken from a variety of depths below the earth's surface, ranging from just beneath the upper surface to one foot deep. A trowel was used to excavate the earth. The samples were collected in sterile tiny plastic tubes and labeled with the date of collection and depth.

Three soil samples from Dharan and two soil samples from Belbari were obtained over the course of six weeks. Soil samples from Dharan were collected from forest areas that contained leaf litter, organic matter, high moisture content, large particle size, and other foreign material. The environment was relatively free of man-made chemicals or fertilizers, as well as human encroachment. Belbari soil samples were also collected from forests with low moisture content, small particle size, and organic matter. The sampling location was also remote from the residential area.

Drying of Soil Samples

The soil samples were dried in a hot air oven at 60-65 °C for approximately one hour. This was done to limit the number of bacteria in the soil besides Actinomycetes. This is one of numerous ways for selectively isolating Actinomycetes from soil samples. The dried soils were then transported to their appropriate tubes and processed in the Department of Microbiology's research laboratory at Central Campus of Technology, Dharan, Sunsari. Heating soil samples to 120 or 100 °C reduced the quantity of filamentous bacteria and Streptomycetes on isolation plates, resulting in the selective separation of rare Actinomycetes genera [24].

Isolation and Purification of Actinomycetes

In this investigation, SCA medium was employed to isolate Actinomycetes. The medium was made by combining 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), and agar (18 g) in one liter of distilled water. The media had a final pH of 7.0 ± 0.1 at 25 °C and was autoclaved at 121 °C for 15 minutes. To prevent fungi and bacteria development, the medium was cooled to 50 °C and 75 µg/ml amphotericin B and 2.5 µg/ml rifampicin were added to the flask and thoroughly mixed [25].

The media was then placed into the petri plates in a thick enough layer to prevent them from drying out throughout the incubation time. Ten sterile test tubes containing 9 ml of sterile distilled water were used for serial dilution of one gram of dried soil sample. Finally, the dilution of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} was determined. Each dilution's aliquot was applied to the corresponding starch casein agar plates in the amount of 100 microliters. Following the transfer, each soil sample was distributed over the agar medium with a sterilized glass spreader. After employing the spread plate technique, the plates were incubated at 30 °C for 7 days. After incubation, the plates were checked for characteristic Actinomycetes colonies. Actinomycetes colony grows slowly, has a powdery substance, and sticks firmly. Actinomycetes are often spherical, tiny, opaque, compact, powdery, heavily colored, and appear drab. They produce hyphae and conidia/sporangia, just like fungi do. Certain Actinomycetes that segment their hyphae are morphologically and physiologically similar to bacteria [26].

Identification of Actinomycetes

Following growth observation on SCA plates, each distinct colony from the selected colonies was injected onto another plate of SCA medium to produce well-isolated pure colonies. The plates were incubated at 28 °C for 3-4 days using the streak method. To identify the strain of Actinomycetes, isolated colonies were submitted to Gram's staining, catalase test, nitrate reduction test, and carbohydrate fermentation test according to Bergey's Manual of Bacteriological Determination [27]. Strains were identified using their morphological and physiological traits.

Phenotypic Characterization

Actinomycetes were classified mostly on the basis of their morphology. In the first few editions of Bergey's Manual, the only characteristic utilized to describe genera was morphology, notably for Streptomyces species. Some basic tests for strain characterization have been proposed, including aerial mass color, reverse side pigmentation, and spore shape. The chromogenicity of the aerial mycelium was thought to be a crucial characteristic for classifying and identifying Actinomycetes. The adult sporulating aerial mycelium may be white, gray, red, green, blue, or violet. When an aerial mycelium color falls between two color series, both colors are recorded.

The strains were separated into two categories based on their capacity to create specific colors on the colony's reverse side, known as distinctive (+) and non-distinctive. When a color with low chroma, such as pale yellow, olive, or yellowish-brown, appears, it is classified as negative. Spore surface characteristics were examined under a microscope. Actinomycetes' spore structures can be classified as smooth, spiny, warty, or hairy.

Utilization of Carbon Sources and Biochemical Tests

The usage of carbon sources was investigated using the Shirling and Gottlieb technique [28]. The test used chemically pure carbon sources that had been confirmed as free of admixture with other carbohydrates or contaminating contaminants. Carbon sources employed in this test included galactose, fructose, dextrose, arabinose, sucrose, maltose, lactose, and mannitol. No

carbon source was employed as a negative control, whereas D-glucose was used as a positive control.

The formulation for one liter of phenol red carbohydrate broth media was 10 g trypticase or proteose peptone No.3, 5 g sodium chloride, 7.2 ml of 0.25% phenol red solution or 0.018 g powder, and 10 g carbohydrate. The sterile carbohydrate source was combined aseptically only after the soup had been autoclaved. To achieve a carbohydrate concentration of around 1%, the broth was chilled to 60 ℃.

The liquid was then stirred and placed into sterile test tubes. The results were categorized as follows: strongly positive (++), positive (+), questionable (+/-), and negative (-). Growth on tested carbon in the basal medium equaled or exceeded growth on the basal medium plus glucose, resulting in strongly positive consumption (++).

Positive utilization (+) was indicated when growth on the measured carbon was significantly higher than on the basal medium without carbon but slightly lower than on glucose. Utilization dubious (+/-) was defined as growth on tested carbon that was only slightly better than on the basal medium without carbon and much less than with glucose. Utilization was negative (-) when growth was comparable to or less than growth on a carbon-free basal medium. Utilization was marked as negative if growth did not outperform the no-carbon control.

Nitrate broth (5 ml) was cultured with spores of selected isolates for 7 days at 28 ℃. Controls were also conducted without inoculation. On the seventh day, the inoculated nitrate broth was examined for the presence of nitrate with α-naphthylamine test solution and sulphanilic acid. To 1 ml of broth under study and 1 ml of control, two drops of sulphanilic acid and two drops of α-naphthylamine solution were added. A pink, red, or orange tint indicated the presence of nitrate, while no color change was deemed nitrate negative. A catalase test was carried out by adding 3% H2O2 reagent to the culture. The discharge of bubbles (free oxygen gas) indicated a successful catalase test.

Screening for Antimicrobial Activity

Sterilized Muller Hilton Agar plates were made, and test organisms were cultured in broth for 12–24 hours. The test organisms for this study were produced at a turbidity of 0.5 McFarland. The test strains were Staphylococcus spp. and Bacillus spp. (Gram-positive bacteria), as well as Escherichia coli and Shigella spp. The test organisms were very sensitive to various antibiotics. Actinomycetes were screened based on their antibacterial efficacy against test organisms.

A preliminary investigation was conducted by comparing the Actinomycetes strain to test bacteria. Ten separate Actinomycetes strains were placed on Muller Hinton Agar (MHA) plates, which had been previously inoculated with test organisms. The plates were incubated at 28 ℃ for 5 days. Escherichia coli, Shigella spp, Staphylococcus spp, and Bacillus spp were tested on several antibiotics to determine their sensitivity and antibiogram. The sensitive test organism strains were chosen for further study based on their antagonistic activity results [29].

Submerged Fermentation and Extraction of Crude antibiotics

Potent Actinomycetes isolates were cultured in 500 ml conical flasks for submerged fermentation in yeast malt extract broth (YMEB) with constant shaking for about 2 weeks at 28 ℃. Following incubation, the contents of the flasks were aseptically filtered through sterile muslin cloth and Whatman filter paper. The crude culture filtrates were centrifuged for 10 minutes at 5000 rpm before being extracted in a separating funnel using a solvent such as ethyl acetate.

To ensure good mixing, supernatant and ethyl acetate were combined in the separating funnel in a 1:1 ratio and vigorously stirred for about 30 minutes. The separation funnel was allowed for around 15 minutes to separate the organic phase (solvent) from the aqueous phase. The solvent layer was removed, and the supernatant was extracted again using ethyl acetate. The solvent layers were combined and evaporated until dry at 40 ℃. The crude solvent extracts were tested for antibacterial activity [30].

Antimicrobial Activity of Solvent Extracts

The antibacterial effectiveness of solvent extracts from selected Actinomycetes was tested using the agar well diffusion method. After cooling, test bacteria were added to the culture media, which was then uniformly poured over the plate. The crude solvent extract was tested for antibacterial activity using a modified agar well diffusion method.

After aseptically pouring the agar (5 mm deep), the plates were allowed to harden. Then, four wells (each measuring roughly 7 mm in diameter) were cut into four quadrants of each plate. A micropipette was used to deposit the crude solvent extract (50 µl) into the well. Plates were incubated at 30 ℃ for 24 hours and checked for a zone of inhibition. Plates containing uninoculated culture material served as controls.

RESULTS

Isolation of Actinomycetes

After processing soil samples taken from various locations in Dharan and Belbari, a total of ten Actinomycetes isolates (AG_1, AG_2, AG_4, AG_5, AG_7, AG_8, AG_9, AG_3, AG_6, and AG_{10}) were isolated from five separate soil samples. Six of the ten isolates came from Dharan, and four from Belbari. Actinomycetes were isolated and counted using the spread plate technique on Starch Casein Agar (SCA) medium. All ten isolates grown on SCA exhibited typical Actinomycete morphology.

The colonies grew slowly, were aerobic, folded, and had various colored aerial and substrate mycelia.

Although morphology is insufficient to distinguish between

genera, it is a significant feature for taxonomic description. In fact, it was the only figure mentioned in many early descriptions. The color of the substrate mycelium was evaluated by examining the plates after 7-10 days. It was only done after noticing the dense spore density on the surface [Table 1]. The strains were separated into two groups based on their ability to develop pigments on the colony's reverse side: distinctive (1) and non-distinctive or none (0). The colors observed for distinguishing were yellow, whereas not distinctive were pale yellow [Table 1]. The strains were inspected under a microscope, and the spore chain morphology was noted (Figure 1). The majority of the strains exhibited a flexible or open-loop spore chain.

Characteristics

Table 2 shows how different Actinomycetes strains can use a variety of carbon molecules as an energy source. After comparing growth to negative and positive controls, it was observed that most Actinomycetes strains absorbed the greatest carbon sources, mannitol and sucrose, while fructose was the least digested. Only four of the ten strains (AG2, AG3, AG8, and AG9) have positive nitrate reduction test results. Except for AG4 and AG7, all strains were positive for catalase.

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

S.N.	Isolates	Aerial Mass Color	Reverse Pigmentation
1.	AG1	Gray and white	0
2.	AG2	White	0
3.	AG4	White	0
4.	AG5	White	1
5.	AG7	Creamy and white	0
6.	AG8	Gray and White	0
7.	AG_9	White	0
8.	AG_3	White	1
9.	AG_6	Creamy and white	0
10.	AG_{10}	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	AG_9	AG_3	AG_6	AG_{10}
Galactose	-	+	+	-	-	-	+	-	-	-
Fructose	-	+	-	-	-	-	-	-	-	-
Dextrose	+	+	-	-	+	+	-	-	+	+
Arabinose	+	-	+	+	+	+	+	+	+	+
Sucrose	-	+	+	+	+	+	+	+	+	+
Maltose	-	+	+	+	+	+	+	+	+	+
Lactose	+	+	-	+	-	-	-	+	-	-
Mannitol	-	+	+	+	+	+	+	+	+	+

(+) indicate fermentative process. (-) indicates non-fermentative process.

Figure 1: Fermentation of yeast malt extract broth by isolates AG8

Primary Screening of the Antimicrobial Activity

Only four of the ten isolates shown antibacterial activity against a variety of Gram-positive and Gram-negative bacteria (Figure 2). Four isolates (AG_5, AG_8 from Belbari, and AG_4, AG_{10} from Dharan) demonstrated antibacterial activity against certain test organisms. The antibiogram profile of test organisms was studied using the Kirby-Bauer Disc method. However, four strains demonstrated little activity. These strains displayed zones of inhibition ranging from 3 to 20 mm.

Secondary Test of Actinomycetes Isolates for Antibacterial Activity

The recovered crude antibiotics underwent secondary screening for antagonistic activity using the agar gel diffusion method. However, no conclusive evidence of the inhibitory zone was observed. Culture AG_4, AG_5, AG_8, and AG_{10} were utilized in submerged fermentation to ferment broth and produce antibiotics (Figure 3).

The solvent extract was extracted using a centrifuge machine and then tested against the test organisms. Actinomycetes AG_4, AG_8, AG_5 and AG_{10} extracts were only hostile to Gram-positive bacteria (Staphylococcus spp. and Bacillus spp). The bioactive chemicals produced by these four Actinomycetes interfered with Gram-positive bacteria. Due to a paucity of resources, the type of antibiotics could not be determined.

DISCUSSION

A wide range of species inhibit soil. Many soil bacteria are beneficial because they create bioactive compounds, including clinically relevant antibiotics. SCA media was selected since it is one of the most regularly used media for the isolation of Actinomycetes, specifically Streptomyces [31,32]. As per the previously released article [33-35], when soil samples were air-dried, the number of Actinomycetes isolated increased. The dried soil samples were preprocessed at 60 °C for 1 hour to remove moisture, prevent bacterial and fungal growth, and isolate Actinomycetes. Contamination with other bacteria and fungi, in particular, inhibits Actinomycetes' colonization [36]. As a result, heat treatment is used as a pretreatment before sample processing to lower the quantity of Gram-negative bacteria that are commonly found in soil samples.

Four approaches were used to successfully isolate Actinomycetes: air drying, heat treatment, starch casein agar, and the application of antibiotics amphotericin and rifampicin. Using these approach, we discovered an abundance of Actinomycetes colonies on the isolation plates. However, a few bacterial and

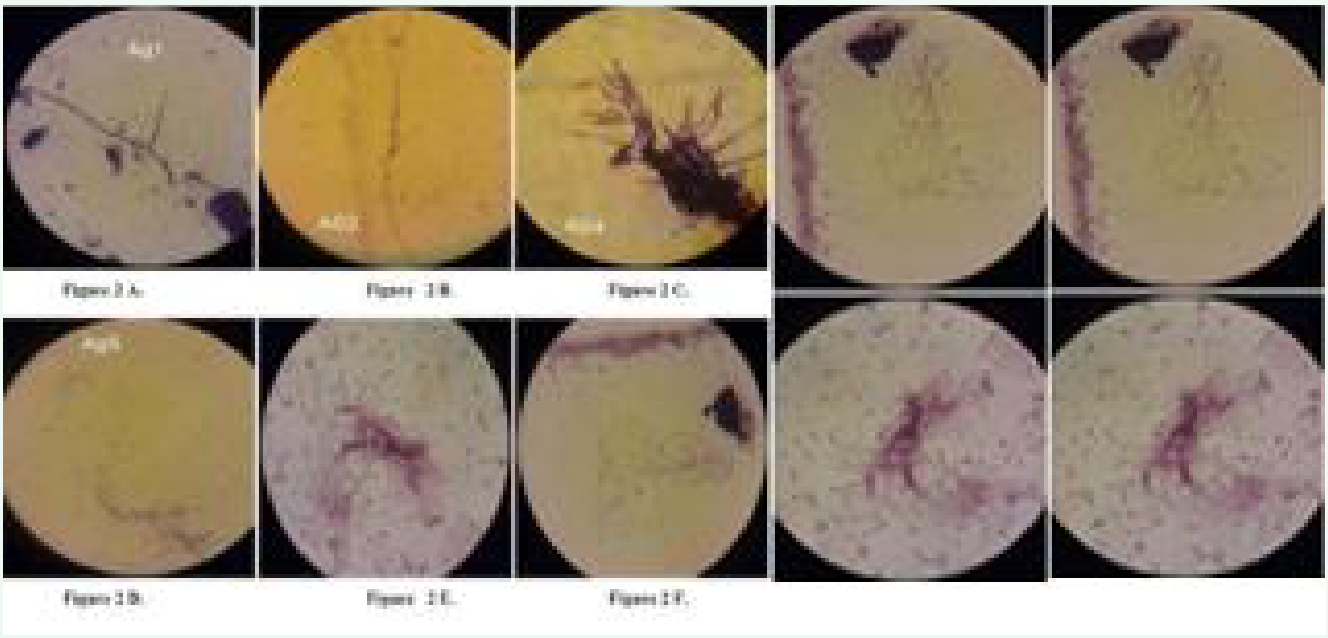

Figure 2: A - F: Gram staining of isolates Species-specific physiological and biochemical

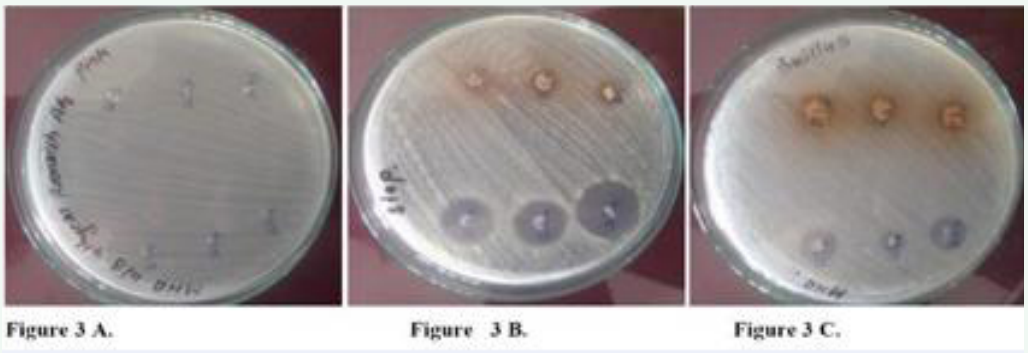

Figure 3

fungal colonies were also discovered. Most Actinomycetes require more than a week of incubation for their growth. According to [37] and [38], Actinomycetes were slow growers, which abolished the growth of colonies observed on the first and second days of incubation. The distribution of Actinomycetes varied according to the depth of sample collection [39]. In our investigation, the most Actinomycetes were found in samples collected from the Dharan, Sunsari area.

The aerial mass of almost all strains was whitish, with the exception of two strains, AG_1, AG_8 and AG_{10}, which were gray. According to [40] and [41], white-colored Actinomycetes were the most prevalent.

The isolated Actinomycetes strains were tested for antibacterial activity. Strains AG_4, AG_5, AG_8 and AG_{10} exhibited the zone of inhibition against Gram-positive Bacillus and *Staphylococcus* spp.

Actinomycetales, an order of filamentous bacteria, particularly Streptomyces strains, possess a unique ability to create new antibiotics [40]. Our current study found that most Actinomycetes metabolites inhibit the growth of Gram-positive bacteria but are ineffective against Gram-negative bacteria due to the double membrane barrier and transmembrane efflux mechanism, which is consistent with previous findings [42,43].

CONCLUSION

The study found that Actinomycetes may have antibacterial

activity in the soil of Dharan and Belbari. Identifying bioactive chemicals would also necessitate substantial research. Molecular characterization of isolated Actinomycetes spp. would aid in the discovery of new substances with commercial value.

RECOMMENDATION

Since it is becoming increasingly obvious that our soil is a highly rich source of Actinomycetes, the possibility of discovering unique Actinomycetes creating newer bioactive chemicals cannot be discounted. As a result, our national policy must include ongoing efforts and an emphasis on screening such creatures from unexplored and underexplored environments for new bioactive substances, requiring pharmaceutical companies to invest in significant research projects. A comprehensive nationwide investigation on the purification of bioactive metabolites is required.

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