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Screening of Antibiotic producing Actinomycetes for Antibiosis from soil of Sunsari, Nepal

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Abstract

Actinomycetes, slow-growing gram-positive bacteria, are useful in the search for bioactive compounds. A total of 24 different actinomycete strains were recovered from farming soil samples collected from the Sunsari district. The isolates were then tested against two gram-positive and three gram-negative bacteria. Results showed that 21% of all isolates are antibacterial at least, one of the test organisms, *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Salmonella Typhi*, and *Pseudomonas* spp. According to antibacterial activity and spectrum broadness, one of the isolates (S11) was selected for secondary screening. The minimum inhibitory concentration (MIC) of crude antibacterial substances extracted from the broth culture of the isolate (S11) was found to be 1.3 mg/ml against test organisms. The chromatogram in TLC showed only one spot with an Rf value of 0.87 by the isolate suggesting that the isolate produced only one compound which was utterly different from the spot with an Rf value of 0.94 paid by gentamycin. According to identification by Microscopy (1000X) and overall biochemical, and physiological characteristics, the isolate was considered *Streptomyces* antibioticus, a distinct taxonomic group.

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1. Introduction

A chemical produced by one bacterium that prevents the growth of another microorganism is known as an antibiotic. Today, compounds produced by one bacterium or those made similarly through chemical synthesis that, in small doses, prevents the growth of other microorganisms are referred to as "antibiotics" (Russell, 2004). The pharmaceutical sector, veterinary medicine, and agriculture are just a few industries that have employed antibiotics. Though not entirely, bacteria are the main source of the majority of antibiotics used today in clinical treatment (Russell, 2004). Actinomycetes are gram positive, rod shaped slow growing bacteria (Holt et al., 1994; Isoken et al., 2010; Oskay et al., 2004a). They have high G+C content (>55%) in their DNA and are the best common sources of antibiotics (Roitch et al., 2017). They are widely distributed in both natural and man-made settings, including soil, freshwater, lakes, compost dust, plant wastes, and food products (Isoken et al., 2010). For many years, researchers have been experimenting with various microbes to develop novel antibiotics (Oskay et al., 2004b). Actinomycetes have the capacity to produce a wide variety of biologically active secondary metabolites, including antibiotics, herbicides, insecticides, anti-parasitic, and waste-treatment enzymes like cellulase and xylanase (Oskay et al., 2004b). Actinomycetes also produce and expel melanin or malanoid, which is a dark pigment (Amal et al., 2011).These melanin compounds are irregular, dark brown polymers having radio protective and antioxidant properties that can effectively protect living organisms from ultraviolet radiation (Romero-Martinez et al., 2000). Melanins are commonly utilized in pharmacology, cosmetics, and medicine preparations (Quadri & Agsar, 2012). Actinomycetes and their bioactive compounds exhibit antimicrobial activity against a variety of pathogens, including

multi-drug resistant pathogens (Isoken et al., 2010) like *Shigella dysenteriae*, Methicillin-Resistant *Staphylococcus aureus* (MRSA), Vancomycin-Resistant *Enterococci*, *Klebsiella* spp., and *Pseudomonas aeruginosa*, among others (Bhatnagar & Kim, 2010; Saadoun et al., 1999; Selvameenal et al., 2009; Servin et al., 2008).

According to Bérdy (2005), out of 22,500 biologically active compounds obtained from microbes, 45% of them are obtained from actinomycetes. The variety of terrestrial actinomycetes has a significant impact on the medical sciences, especially in the synthesis of antibiotics (Magarvey et al., 2004). Due to the rapid introduction of new illnesses, the development of new multi-drug resistant pathogens in common pathogens and the use of these pathogens in bioterrorism, the development of new antimicrobial drugs are crucial (Spellberg et al., 2004). The greatest problem is the treatment of antibiotic resistant bacteria for infectious diseases and are still the second leading cause of death worldwide (WHO, 2002). As there is an increasing trend of antibiotic resistant microorganisms, the search for novel antibiotics is necessary. Since actinomycetes are the best common sources of antibiotics (OKAMI, 1988), this study was carried out to isolate and characterize the antibiotic producing actinomycetes from soil samples of Sunsari, Nepal.

2. Materials and Method

2.1 Study site and duration

The study site is Sunsari district of Province no. 1, Nepal which is situated at 71-1430 m in the height from the sea level. The district has three distinct climate types due to the variation in elevation: tropical, subtropical, and temperate. It covers an area of 1257 square km and the annual rainfall is about 114.3 mm and temperatures vary from 10° C to 30° C. This study was carried out from June 2019 to December 2020.

2.2 Collection of sample

A total of 32 soil samples were collected from crop fields, a bank of the river, a bank of ponds, a forest, a hilly area, and a garden inside the Sunsari district. Soil samples were collected from 4-5 cm depth into sterile plastic bags containing 1 g of CaCO3 and mixed well. Then the soil samples were further dried at room temperature for 3 weeks.

2.3. Isolation of Actinomycetes from soil

After serial dilution of soil, actinomycetes were isolated using Starch Casein Agar (SCA) (starch 10 g, casein 0.30g, KNO3 2 g, MgSO4.7H2O 0.05g, K2HPO4 2g, NaCl 2g, CaCO3 0.02 g, FeSO4.7H2O 0.01g, agar-agar 18g) plates containing nystatin and cycloheximide (50µg/mL each) following William and Davies, 1965 (Williams & Davies, 1965). A sterile inoculating loop by quadrant streaking approach was used to cultivate typical actinomycetes colonies (dry and tough wrinkled) into other SCA plates. To isolate pure colonies, the inoculated plates were kept at 27°C for seven days.

2.4. Macroscopic and Microscopic Characterization of Actinomycetes

The separated colonies on SCA were examined for the color of the ethereal mycelium and diffusible pigments, and other colony characteristics such as size, consistency, and the margin of the colony. The microscopic (1000X) characterization of the isolates was done by coverslip culture technique for their mycelial structure, the configuration of sporophore (conidiospore and arthrospore), arrangement and shape of spores on the mycelia following Kawato and Shinobu, 1959 (M. Kawato & R. Shinobu, 1959). The inspected morphology of the colonies was compared with the actinomycetes morphology depicted in Bergey's Manual of Determinative Bacteriology, Eighth version 1974 for the presumptive identification of the isolates.

2.5. Biochemical and physiological Characterization of Actinomycetes

As described by M. Kawato and R. Shinobu (1959) different biochemical tests (oxidase, carbohydrate utilization, citrate utilization, indole and hydrogen sulfide production, nitrate reduction, urea hydrolysis, tween 20 hydrolysis, starch hydrolysis, and esculin hydrolysis) were performed for the identification of the isolates. For physiological characterization, temperature tolerance, NaCl tolerance, and motility tests were carried out (M. Kawato & R. J. N. S. Shinobu, 1959).

2.6. Biochemical and physiological Characterization

of Actinomycetes

For primary screening of antibiotic producing actinomycetes, the perpendicular streak method was carried out along the center of nutrient agar plates as described by Egorov (1985) and incubated at 28°C for 7 days (Egorov, 1985). Test microbes included two gram positive (*Bacillus subtilis* and *Staphylococcus aureus*) and three gram negative (*Escherichia coli*, *Salmonella* Typhi, and *Pseudomonas aeruginosa*). According to Haque et al. (1996a) secondary screening of the isolates was performed after fermentation using the agar well assay method on Mueller Hinton agar (Haque et al., 1996b).

2.7. Fermentation

Five potent isolates were used for the fermentation by the submerged state culture method after the primary screening. 100 mL Erlenmeyer flask containing 25 mL starch casein broth (SCB) and incubated into a shaker water bath at 28°C at 160 rpm for 4 days for inoculum advancement. The prepared inoculum was poured in a sterile 1 L Erlenmeyer flask containing 400 mL sterile SCB and incubated in a shaker water bath at 160 rpm at 28°C for 7 days following Gurung et al (2009) (Gurung, Sherpa, Agrawal, & Lekhak, 2009).

2.8. Recovery of Antibiotics from Fermented Broth

Recovery of antibiotic was done by following LIU et al. (1986) in which the fermented broth was aseptically filtered by Whatman No. 1 filter paper. The filtrate was set aside for solvent extraction in order to recover antibiotics, and the residue was thrown away. Then, filtrate broth and ethyl acetate were taken in a separating funnel at a ratio of 1:1 by volume, agitated vigorously for one hour, and then left undisturbed for 30 minutes. The antibiotics containing solvent phase was extracted and subjected to evaporation at 40°C for 15 hr. in the water bath to obtain pure antibiotics (LIU et al., 1986). The residues obtained were measured, dissolved in the phosphate buffer, and used for determination of antibacterial activity, minimum inhibitory concentration and Thin Layer Chromatography (TLC).

2.9. Determination of Antibacterial Activity

Antibacterial activity was performed according to Haque et al. (1996b) , nutrient agar was used to inoculate test bacteria and the agar cup plate technique was used to assess antimicrobial activity against test organisms by following (Haque et al., 1996a).

2.10. Determination of Minimum Inhibitory Concentration

Determination of MIC was done by following Gurung, Sherpa, Agrawal and Lekhak (2009) in which the extracted antibiotic was serially diluted and tested against E. *coli* (Gurung, Sherpa, Agrawal, Lekhak, et al., 2009).

2.11. Thin Layer Chromatography

TLC was carried out by following Gurung, Sherpa, Agrawal, and Lekhak (2009), who prepared and activated silica gel plates (20 cm × 20 cm, 1 mm thick) at 80oC for 2 hours. On the TLC plate, 10 µL of the test antibiotic and the reference antibiotic (Vancomycin) solutions were added and the chromatogram was formed using chloroform: methanol (10:90) as the solvent system. The dried chromatogram spots were visualized in the iodine vapor chamber (Gurung, Sherpa, Agrawal, Lekhak, et al., 2009).

3. Result and Discussion

3.1. Macroscopic and Microscopic characteristics of isolates

The active isolates developed 4 different colors of mycelium substrates. Out of 24 isolates, 13 (54.16%) produced brown, 8 (33.33%) creamy, 2 (8.33%) yellow, and 1 (4.16%) white colored substrate mycelium. Likewise, 14 (58.33%) isolates produced white, 6 (25%) creamy, 3 (12.5%) brown, and 1 (4.16%) pink aerial mycelium. Similarly, 15 isolates had powdery and 9 had the smooth texture of aerial mycelium. The 18 active isolates were whole, 6 had irregular margins and had an average of 2.5 mm diameter of the colony. Of 5 active isolates, 5 isolates were presumable identified as Streptomyces spp.

3.2. Primary Screening of Actinomycetes

Out of 24 isolated actinomycetes, 5 (21%) demonstrated active antibacterial activity against at least one of the test bacteria in primary screening. Among these 4 (80%) were active against gram positive bacteria only, 2 (40%) against gram negative bacteria only, and 2 (40%) against both gram positive

and gram negative bacteria. Among them 2 (40%) showed activity against *E. coli,* 1 (20%) showed activity against *Salmonella* Typhi, 1 (20%) showed activity against *Pseudomonas* spp, 3(60%) showed activity against *Staphylococcus aureus* and 5 (100%) showed activity against *Bacillus subtilis* (Table 1).

Table 1: Zone of inhibition of active isolates in primary screening

Actinomycetes isolates	**Zone of inhibition (mm) against test bacteria**				
	Gram negative bacteria			Gram positive bacteria	
	E. coli	*Salmonella* Typhi	*Pseudomonas* spp.	*S. aureus*	*B. subtilis*
S2.a	-	-	-	-	-
S2.b	-	-	-	-	-
S2.c	-	-	-	-	-
S5	-	-	-	-	-
S7	-	-	-	-	-
S8	-	-	-	-	-
S9	-	-	-	-	-
S11	14	30	14	26	28
S13	-	-	-	10	17
S15.a	-	-	-	-	-
S15.b	-	-	-	-	-
S18	-	-	-	-	-
S20	8	-	-	9	14
S21	-	-	-	-	-
S22.a	-	-	-	-	-
S22.b	-	-	-	-	-
S25	-	-	-	-	-
S26	-	-	-		9
S27.a	-	-	-	-	-
S27.b	-	-	-	-	-
S29	-	-	-	-	6
S30	-	-	-	-	-
S31.a	-	-	-	-	-
S31.b	-	-	-	-	-

- = No zone of inhibition

Out of 5 isolates subjected for the secondary screening, only 1 (20 %) showed potent antibacterial activity against all the tested bacteria (Table 2).

Table 2: Potent actinomycetes against all tested bacteria

Actinomycetes isolates	**Zone of inhibition (mm) against test bacteria**				
	Gram negative bacteria			Gram positive bacteria	
	E. coli	*Salmonella* Typhi	*Pseudomonas spp.*	*S. aureus*	*B. subtilis*
S11	14	22	16	24	26

3.2. Biochemical and physiological characteristics of isolates

For biochemical characterization of isolates, utilization of carbohydrates, substrate hydrolysis, catalase, oxidase, H_2S production, citrate utilization and nitrate reduction tests were performed.

The result of utilization of carbohydrates test is summarized in table 3, substrate hydrolysis test in table 4, temperature and NaCl tolerance in table 5, and other biochemical tests in table 6.

Table 3: Utilization of carbohydrates test

Samples	Mannose	Fructose	Maltose	Sucrose	Lactose	Mannitol	Glucose	Xylose
S11	-	+	+	+	+	+	+	+
S13	-	+	+	+	+	+	-	-
S20	-	-	+	+	+	+	-	+
S26	+	-	+	+	+	-	+	+
S29	-	+	-	-	-	+	+	+

+ = Utilized, - = Not utilized

Table 4: Hydrolysis of substrates

Actinomycetes	Substrates				
	Urease	**Tween 20**	**Starch**	**Esculin**	**Gelatin**
S11	+	+	+	+	+
S13	+	+	+	+	+
S20	+	+	+	+	+
S26	+	+	+	+	+
S29	–	–	+	+	+

+ = Hydrolized, - = Not hydrolyzed

Table 5: Temperature tolerance and NaCl tolerance

Actinomycetes	Temperature tolerance			NaCl tolerance		
	15°C	37°C	45°C	5%	7%	10%
S11	+	+	-	+	+	+
S13	+	+	-	+	+	+
S20	+	+	-	+	+	+
S26	+	+	-	+	+	+
S29	+	+	-	+	+	+

+ = Tolerance, - = Not tolerance

Table 6: Other Biochemical Test

Actinomycetes	Catalase	Oxidase	H_2S production	Citrate utilization	Nitrate reduction
S11	+	–	+	–	–
S13	+	–	–	–	+
S20	+	–	–	+	–
S26	+	–	–	–	–
S29	+	–	–	–	+

+ = positive, - = Negative

3.3. Fermentation

After fermentation, isolate S11 produced a light brownish white residue with greasy consistency. Results from fermented broth are summarized in (table 7).

Table 7: Concentration of antibiotic substance

Actinomycetes				
S11	100 ml	300 g	Light brownish white	Greasy

3.4. Minimum inhibitory concentration (MIC) of active compound

Only S11 was chosen for fermentation. For the antibacterial metabolites extracted from S11, the minimum inhibitory concentration against *E. coli* was 1.3 mg/ml. According to the results of morphological, biochemical, physiological, and other biochemical tests, isolate S11 might be *Streptomyces antibioticus* (table 8).

Table8: Thin layer chromatography of antimicrobial substances

Anti - bacterial Substances (s)	Concentration (mg/ml)	Amount of load (µl)	Solvent System (M: C)	No of moved spot			**F_f** value
					Solvent front	Antibacterial substance	
S_{11}	30	10	90:10:00	1	9.4	8.2	0.87
Gentamycin	30	10	90:10:00	1	9.4	8.8	0.94

Five types of actinomycetes were isolated from the soils of Sunsari District. Actinomycetes are found in general to harsh environments; which may be due to their ability to develop resistance structures like spore. However, the isolation of actinomycetes has always been challenged because of other bacteria and fungi (Williams & Cross, 1971). This may be due to their long duration of incubation. However, the isolation ratio of Actinomycetes was improved by pretreating the samples with calcium carbonate and exposing them to dry air for three weeks. A critical inhibition of contaminating microorganisms was the use of selective media (starch casein media) containing antibiotics, cycloheximide (50µg/ml), and nystatin (50µg/ml).

Only 1 (20%) of the 5 active actinomycetes that were chosen from primary screening showed antibacterial activity during secondary screening. In solid and liquid media actinomycetes are produced filamentous mycelia and fragmented mycelia, respectively (Pickup, Nolan, Bushell, & bioengineering, 1993). Alternatively, the active chemicals may have undergone a chemical change to render them inactive in broth culture.

The screening results showed that 4 (80%) isolates were active against Gram positive bacteria, 2 (40%) were active against Gram negative bacteria and 2 (40%) were active against both Gram positive and Gram negative bacteria. Similar types of results were obtained by Gurung, Sherpa, Agrawal, Lekhak, et al. (2009). They reported 69.97% of actinomycetes were found to be active against Gram positive bacteria whereas only 11.11% of actinomycetes were active against Gram negative bacteria. This might be because of the morphological variances between those two types of microorganisms. An outer membrane of Gram negative bacteria has lipopolysaccharide; hence their cell wall is impermeable to lipophilic solutes, while porins constitute a selective barrier to the hydrophilic solutes. (Nikaido, 2003).However, the Gram positive bacteria have only a peptidoglycan layer and are not an effective permeability barrier for the antibacterial agents.

The most antibacterial activity on test organisms was shown by isolate S11 (14 mm against E.coli, 30 mm against *Salmonella* Typhi, 14 mm against *Pseudomonas* spp, 26 mm against *S. aureus* and 28 mm against *Bacillus subtilis*). According to

antibacterial activity, 5 isolates were selected and identified. Out of them, the potent isolate was only 1. Morphological examination of all 5 isolates clearly indicates that these belong to the *Streptomyces* genera and Streptomycetaceae family (spore chain with coiling and branching) (Cross, 1989). Additionally, a comparison of both physiological and biochemical characteristics among isolates indicated that they are closely related to the *Streptomyces* genera and the Streptomycetaceae family. Among them, isolate S11 was closely related to *Streptomyces* antibioticus. As a result of primary and secondary screening, only single isolate S11 was found to be the best strain as they had shown broad spectrum activity with a large diameter of zone of inhibition (Average 22.4 mm). It is probable that the isolates develop more than one antibacterial metabolites that have rendered both of them effective inhibitors Gram positive and bacteria with Gram negative. Thus only S11 was chosen for fermentation. For the antibacterial metabolites extracted from S11, the minimum inhibitory concentration against E. coli was 1.3 mg/ml. Since the evaporation of crude filtrate was used to produce these metabolites, it was likely to have a high MIC value. Similar findings were also reported by Gurung, Sherpa, Agrawal, Lekhak, et al. (2009) and Sah et al. (2017). TLC showed only one antibiotic solution created the spot, indicating the existence of a single compound. The R value of S11 and Gentamycin was found 0.87 and 0.94 respectively. This finding is consistence with the findings of Sah et al. (2017).

4. Conclusions

Actinomycetes, which produce the most potent antibiotics, are found in the soils of the Sunsari district. *Streptomyces antibioticus* which showed antibacterial properties against both Gram positive and negative bacteria were isolated from soils of a tropical, subtropical; and temperate area. Hence, this study clearly reveals the distribution of antibiotic producing actinomycetes in the Terai and Mahabharat regions (71-1430 m) of Nepal.

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Conflicts of Interest

The authors declare that there is no conflict of interest.

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