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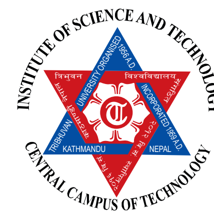


# Screening of Antibiotic Producing Actinomycetes for Antibiosis from Soil of Siraha, Nepal

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**Abstract**

The increasing need of novel antibiotics has provided a pace for the search of antibiotics from actinomycetes. Primary and secondary screenings of antibiotic producing actinomycetes from the soil of Siraha (75-600 m) were performed. The minimum inhibitory concentration of the metabolites was determined against *E. coli*. Macroscopic, microscopic and biochemical characterization were performed for the identification of presumptive genera. Characterization of the antibacterial substances was done by TLC. Among 92 isolates, 22showed antibacterial activity against at least 1 bacterium out of 6 test bacteria used. Microscopy and other characteristics studies revealed that 19 (86.36%) were *Streptomyces* spp., 1 (4.55%) was *Thermomonospora* spp., and 2 (9.09%) were unidenti ed. Five potent isolates were selected for the secondary screening where 2 isolates inhibited Gram negative bacteria with an MIC value of 1.2 mg/mL for each isolate. TLC showed that both antibiotics produced only one spot suggesting the presence of one active compound other than vancomycin (standard) . The active isolates from primary screening were heterogeneous in their overall macroscopic, biochemical, and physiological characteristics. The two potent isolates showing antibacterial activity were found to belong to different distinct taxonomic groups.

**Key words**: actinomycetes, antibacterial activity, minimum inhibitory concentration, thin layer chromatography

## Introduction

Actinomycetes are gram positive bacteria widely distributed in natural and man-made environments. They are found in large numbers in soils, fresh waters, lake, river bottoms, manures, composts, dust as well as on plant residues and food products. However, the diversity of actinomycetes that produce secondary metabolites can be determined by different physical, chemical and geographical factors (Ogunmwonyi et al., 2010). As there is increasing trends of antibiotic resistant microorganisms, the search for novel antibiotics is necessary. In this case, actinomycetes are the best common sources of novel antibiotics (Okami & Hotta, 1988).

The diversity of terrestrial actinomycetes is of great signi cance in several areas of medical sciences, particularly in antibiotic production (Magarvey et al., 2004). Out of 22,500 biologically active compounds obtained from microbes, 45% are from actinomycetes (Berdy, 2005). Need of new antimicrobial agents is greater than before because of the emergence of new multidrug resistance in common pathogens, rapid emergence of new infections, and the use of multidrug resistant pathogens in bioterrorism (Spellberg et al., 2004). Antibiotic resistant bacteria have been great problems in the treatment ofi nfectious diseases which are still the second leading cause of death worldwide (WHO 2002 and Luzhetskyy et al., 2007).

In the search for novel antibiotics, a study in Nepal revealed that twenty seven actinomycetes isolated from soil samples of 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 signi cant antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli* (Kalyani et al., 2012). These all evidences show the abundant presence of actinomycetes having antimicrobial activity.

In this regard, Terai (75-600 m), a warm region of Nepal, is of a signi cant interest. Sun radiations and seasonal variations create extreme environment which likely to harbor unusual microorganisms. This poorly studied habitation increases the chances of nding novel microorganisms. Therefore, the present study was undertaken to isolate and characterize antibacterial actinomycetes from soil samples of Siraha, a warm district of Terai, Nepal.

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## Materials and Methods

**Collection of soil samples**

Soil samples were collected from eastern region of Siraha district in November 2008. Four to ve grams of dry soil samples were collected from a depth of 4-5 cm of paddy elds, bank of river, bank of pond and garden, placed in separate clean polyethylene bags, and mixed well with 1 g of $CaCO_3$, already added to the bags. Then the soil samples were further dried at room temperature for about 3 weeks.

**Isolation and Puri cation of Actinomycetes**

Isolation of actinomycetes was done by spread plate technique following the serial dilution of soil samples on starch casein agar (SCA) plates containing nystatin and cycloheximide (50 µg/mL each) (Williams & Davies, 1965). Typical actinomycetes colonies (dry and tough wrinkled) were picked up from SCA plates using a sterile inoculating loop and streaked on another SCA plates by quadrant streaking technique. The inoculated plates were incubated for 2-4 days at 28 °C to isolate pure colonies.

**Primary and Secondary Screenings of Actinomycetes for Antibacterial Activity**

Primary screening was done by perpendicular streak method by streaking along the centre of nutrient agar plates followed by incubation for 7 days at 28 °C (Egorov, 1985). The test bacteria used were *Escherichia coli, Salmonella* Typhi, *Shigella* spp., *Pseudomonas aeruginosa, Bacillus subtilis* and *Staphylococcus aureus*. After fermentation, secondary screening of the isolates was done by agar well assay method on mueller hinton agar (Sen et al., 1993).

**Macroscopic and Microscopic Characterization of Actinomycetes**

The isolated colonies on SCA were studied for the color of the aerial mycelium and diffusible pigments, and other colony characteristics such as size, consistency, margin of colony. The microscopic characterization (1000X) of the isolates was done by cover slip culture method for their mycelial structure, con guration of sporophore (conidiospore and arthrospore), arrangements and shape of spores on the mycelia (Kawato & Sinobu 1959). The observed morphology of the isolates was compared with the actinomycetes morphology described in Bergey's Manual of Determinative Bacteriology, Eighth edition (1974) for the presumptive identi cation of the isolates.

**Biochemical and Physiological Characterization**

For the identi cation of the isolates, different biochemical tests, viz., oxidase, carbohydrate utilization, citrate utilization, indole and hydrogen sulphide production, nitrate reduction, urea hydrolysis, tween 20 hydrolysis, starch hydrolysis and esculin hydrolysis were performed (Kawato & Sinobu, 1959). Tests like temperature tolerance, NaCl tolerance and motility were performed for physiological characterization (Kawato & Sinobu, 1959).

**Fermentation**

Five potent isolates (based on primary screening) were used for fermentation by submerged state culture method.The isolate was inoculated into 100 mL Erlenmeyer ask containing 25 mL starch casein broth (SCB) and incubated in water bath shaker at 28 °C at 160 rpm for 4 days for inoculum development. The prepared inoculum was poured in a sterile 1 L Erlenmeyer ask containing 400 mL sterile SCB and incubated in 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 off ermentation, the broth was ltered through Whatman No. 1 lter paper aseptically.The residue was discarded and the ltrate was taken for further operation. The ltrate was subjected for solvent extraction for antibiotic recovery. Ethyl acetate and the ltrate 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 antibiotic was separated and subjected for evaporation to on water bath at 40 °C for 15 h to obtain pure antibiotic (Liu et al., 1986). The residue (antibiotic) obtained was weighed, dissolved in phosphate buffer and used for the determination of antibacterial activity, minimum inhibitory concentration and for TLC study. Other organic solvents (n-butanol, chloroform, dichloromethane and methanol) were also used to extract the antibacterial substances by the same procedure.

**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 test antibiotic and a reference antibiotic (vancomycin) solutions were applied on the TLC plate and the chromatogram was developed using chloroform: methanol (10:90) as solvent system. The chromatogram was dried and the spots were visualized in the iodine vapor chamber (Gurung et al., 2009).

**Antibacterial Activity and Minimum Inhibitory Concentration (MIC) of Antibiotic**

The antibacterial activity of the antibiotics was determined by agar cup assay method against the test organisms (Sen et al., 1993) and the MIC of the antibiotics was determined by serial dilution method in nutrient broth against *E. coli* (Gurung et al., 2009).

## Results and Discussion

**Primary screening of Actinomycetes**

Out of 92 isolated actinomycetes from 58 soil samples, 22 showed active antibacterial activity against at least one test bacteria. The survival of the microorganisms under such harsh and challenging habitats might be due to their ability for adaptation and spore formation. Isolation of actinomycetes has been most dif cult in comparison to their competitors because of their long incubation period (Williams & Cross, 1971). However, an effort was made to isolate an increased number of actinomycetes by pretreating the soil samples with calcium carbonate and subjecting them to air drying for three weeks.

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Use of SCA medium incorporated with nystatin and cycloheximide antibiotics at a concentration of 50 $g/mL for each was effective in preventing the growth of contaminants. Among 22 active isolates, 20 (90.91%) showed activity against gram positive bacteria, 2 (9.1%) showed activity against gram negative bacteria and 6 (27.27%) showed activity against gram positive and gram negative (Table 1). Out of total active isolates, 8 (36.36%) showed activity against *E. coli*, 3 (13.6%) showed activity against *S.* Typhi, 3 (13.6%) showed activity against *Shigella* spp., 2 (9.1%) showed activity against *Pseudomonas aeruginosa*, 19 (86.36%) showed activity against *S. aureus* and 18 (81.81%) showed activity against *Bacillus subtilis*.

**Table 1:** Zone ofi nhibition of active isolates in primary screening

| SN | Isolate code | Zone ofi nhibition (in mm) against test bacteria | | | | | |
|---|---|---|---|---|---|---|---|
| | | Gram negative (4) bacteria | | | | Gram positive (2) bacteria | |
| | | Tb1 | Tb2 | Tb3 | Tb4 | Tb5 | Tb6 |
| 1 | $R_1a$ | 12 | 8 | 10 | 16 | - | - |
| 2 | $R_1b$ | 12 | 9 | 9 | 16 | - | - |
| 3 | $R_3b$ | - | - | - | - | 7 | 6 |
| 4 | $R_4d$ | 5 | - | - | - | 7 | 9 |
| 5 | $R_5a$ | - | - | - | - | 16 | 17 |
| 6 | $R_6c$ | - | - | - | - | 18 | 16 |
| 7 | $R_8b$ | 4 | - | - | - | 8 | 7 |
| 8 | $R_{12}a$ | - | - | - | - | 16 | 14 |
| 9 | $R_{19}b$ | 4 | - | - | - | 6 | 7 |
| 10 | $R_{21}c$ | 5 | - | - | - | 8 | 7 |
| 11 | $R_{25}g$ | 8 | - | - | - | 7 | 6 |
| 12 | $R_{31}a$ | 5 | 7 | 7 | - | 11 | 8 |
| 13 | $R_{33}c$ | - | - | - | - | 16 | 5 |
| 14 | $R_{36}d$ | - | - | - | - | 13 | - |
| 15 | $R_{38}a$ | - | - | - | - | 14 | 16 |
| 16 | $R_{42}c$ | - | - | - | - | - | 10 |
| 17 | $R_{45}a$ | - | - | - | - | 10 | - |
| 18 | $R_{47}d$ | - | - | - | - | 20 | 4 |
| 19 | $R_{48}a$ | - | - | - | - | 15 | 10 |
| 20 | $R_{52}a$ | - | - | - | - | 7 | 6 |
| 21 | $R_{56}c$ | - | - | - | - | 8 | 7 |
| 22 | $R_{58}b$ | - | - | - | - | 9 | 10 |

$Tb_1$: *Escherichia coli*, $Tb_2$: *Salmonella* Typhi, $Tb_3$: *Shigella* spp., Tb4: *Pseudomonas aeruginosa* $Tb_5$: *Staphylococcus aureus* and $Tb_6$: *Bacillus subtilis*

Among 22 isolates, 5 were selected for secondary screening. All of them showed inhibitory action against the test bacteria. Two isolates inhibited gram negative bacteria while remaining three inhibited gram positive bacteria as shown in the secondary screening (Table 2).

**Table 2**: Zone ofi nhibition of active actinomycetes isolates in secondary screening

| SN | Isolated code | Gram negative bacteria (n=4) | | | | Gram positive bacteria (n=2) | |
|---|---|---|---|---|---|---|---|
| | | $Tb_1$ | $Tb_2$ | $Tb_3$ | $Tb_4$ | $Tb_5$ | $Tb_6$ |
| 1 | $R_1a$ | 12 | 5 | 8 | 13 | - | - |
| 2 | $R_1b$ | 12 | 5 | 7 | 12 | - | - |
| 3 | $R_5a$ | - | - | - | - | 12 | 12 |
| 4 | $R_6c$ | - | - | - | - | 16 | 15 |
| 5 | $R_{12}a$ | - | - | - | - | 11 | 12 |

$Tb_1$: *Escherichia coli*, $Tb_2$: *Salmonella* Typhi, Tb3: *Shigella* spp., $Tb_4$: *Pseudomonas aeruginosa,* Tb5: *Staphylococcus aureus* and $Tb_6$: *Bacillus subtilis*

The rst screening was used to select the active isolates and determine the range of test bacteria that were sensitive towards the antibiotics produced by them. The secondary screening method was important to select the isolates for further studies. The screening may be qualitative or quantitative in its approach. The qualitative approach is used to determine the range of the microorganisms that are sensitive to a potential antibiotic while the quantitative approach provides the information about the yield of antibiotic that can be expected when the organism is grown in different media (Gurung et al., 2009).

The results of the primary screening revealed that many isolates were active against gram positive bacteria than gram negative bacteria. This might be due to the differences in their cell wall composition. Gram negative bacteria have an outer lipopolysaccharide membrane; hence their cell wall is impermeable to lipophilic solutes, while porins (beta barrel proteins) constitute a selective barrier to the hydrophilic solutes (Nokaido & Vaara, 1985). The gram positive bacteria have only peptidoglycan layer which does not provide effective barrier for the permeation of antibiotics.

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According to the results of primary and secondary screenings, two isolates ($R_1$a and $R_1$b) had shown the largest zone of inhibition against gram negative bacteria and hence could be regarded as the most potential actinomycets producing antibiotic. It is very dif cult to screen such actinomycetes which can inhibit only gram negative bacteria. Since most of the pathogens are gram negative bacteria, they were chosen for fermentation.

**Characteristics of active isolates**

**Macroscopic characteristics**

The active isolates produced substrate mycelium of six different colors. Out of 22 isolates, 9 (40.9%) produced brown, 7 (31.8%) produced creamy, 3 (13.6%) produced yellow, 1 (4.54%) produced pink and 1 (4.54%) produced white colored substrate mycelium. Similarly, the isolates produced aerial mycelium in having four different colors and they were white aerial mycelium by 12 (54.54%), creamy by 5 (22.73%), brown by 4 (18.18%) and pink by 1 (4.54%) active isolates.

Three types of texture of the aerial mycelium namely powdery, uffy and smooth were observed. Of the total 22 isolates examined, 10 isolates had smooth texture, 11 had powdery texture and 1 had uffy texture. The colony diameter of active isolates varied from 1-3 mm in with most of them being 2 mm in size. Four isolates had irregular margin while the rest had entire margin.

**Microscopic characteristics**

19 out of 22 active isolates, were presumable identi ed as *Strptomycetes* spp. where 3 had retinaculiapetri type sporophore morphology and 16 had recti exibles type sporophore morphology. One of the active isolates was presumable identi ed as *Thermomonospora* spp., while the remaining two were unidenti ed.

**Biochemical and physiological characteristics**

Carbohydrate utilization tests indicated that xylose, glucose, mannose, fructose and maltose were utilized by 5, 8,5,13 and 4 actinomycetes isolates respectively. Similarly, mannitol, inulin and salicin were utilized by 1, 2 and 4 active isolates respectively. But none of the isolates utilized lactose. Of the two potential antibiotic producing actinomycetes so found, xylose and fructose were utilized by $R_1$a, while only glucose was utilized by $R_1$b isolate.

**Table 3:**Carbohydrate utilization tests

| S.N. | Carbohydrates Utilized | Pos n |
|---|---|---|
| 1. | Xylose | 5(22.7%) |
| 2 | Glucose | 8 (36.36%) |
| 3 | Mannose | 5 (22.7%) |
| 4 | Fructose | 13 (59.1%) |
| 5 | Maltose | 4 (18.18%) |
| 6 | Sucrose | 4 (18.18%) |
| 7 | Lactose | - |
| 8 | Mannitol | 1 (4.5%) |

Based on the results of substrate utilization tests, it was found that urea, starch, esculin, tween 20 and gelatin were utilized by 19, 21, 20, 15 and 20 isolates respectively. Of the two potent isolates, $R_1$a hydrolyzed all ve substrates, while $R_1$b hydrolyzed all substrate except esculin (Table 4).

**Table 4:**Substrate hydrolysis tests

| S.N. | Substrate | Pos n |
|---|---|---|
| 1 | Urea | 19 (86.36%) |
| 2 | Starch | 21 (95.45%) |
| 3 | Esculin | 20 (90.90%) |
| 4 | Tween 20 | 15 (68.18%) |
| 5 | Gelatin | 20 (90.90) |

Biochemical tests showed that catalase, hydrogen sul de production, citrate utilization, nitrate reduction tests were shown positive by 21, 3, 5 and 8 isolates. But none of the isolates showed positive oxidase and indole tests. Of the two potential isolates ($R_1$a and $R_1$b), both were oxidase negative and catalase positive. Isolate $R_1$a showed positive hydrogen sulphide production test and negative citrate utilization test, while isolate $R_1$b showed positive for hydrogen sul de production and citrate utilization tests (Table 5).

**Table 5:**Other biochemical tests

| S.N. | Biochemical Test | Pos n |
|---|---|---|
| 1 | Catalase test | 21 (95.45%) |
| 2 | Oxidase test | - |
| 3 | Hydrogen sulphide production test | 3 (13.6%) |
| 4 | Citrate utilization test | 5 (22.7%) |
| 5 | Nitrate reduction test | 8 (36.36%) |
| 6. | Indole test | - |

All the active isolates showed growth at 15 °C and 37 °C temperatures and tolerated 5%, 7% and 10% NaCl. None of the isolates showed growth at 45 °C and positive motility. With the help of the result obtained from macroscopic, microscopic, biochemical and physiological characteristics, the $R_1$a and $R_1$b were identi ed as *Streptomyces* spp., and *Thermomonospora* spp., respectively. The microscopic observations of the actinomycetes were determined as according to Bergey's Manual of Determinative Bacteriology (1974) and the result is shown in table 6.

**Table 6:**Physiological tests

| S.N. | Physiological Test | Pos n |
|---|---|---|
| 1. | Temperature tolerance at 15°C | 22 (100%) |
| 2 | Temperature tolerance at 37°C | 22 (100%) |
| 3 | Temperature tolerance at 45°C | - |
| 4 | NaCl Tolerance 5% | 16 (72.72%) |
| 5 | NaCl Tolerance 7% | 14 (63.64%) |
| 6 | NaCl Tolerance 10% | 12 (54.55%) |
| 7 | Motility | - |

**Fermentation**

The residue obtained from isolate $R_1$a was white and had aky/greasy consistency while that from isolate $R_1$b was white aky consistency. Results from fermented broth are tabulated in table 7.

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The amount of residues from the isolates $R_1$a and $R_1$b were 1 and 1 g per 100 mL of the broth respectively. Among ve solvents, only ethyl acetate could extract the potent metabolites in detectable level from the fermented broth. This might be because of the higher solubility of the metabolites in ethyl acetate solvent than in others.

**Table 7**: Concentration of antibiotic substances

| Isolate code | Volume of extracted solvent (ml) | Amount of residue (mg) | Color of residue | Consistency of residue |
|---|---|---|---|---|
| $R_1$a | 100 | 1005 | White | Sticky/greasy |
| $R_1$b | 100 | 1001 | White | Flaky |

**Minimum inhibitory concentration (MIC) of active compound**

The MIC of antibiotics extracted from $R_1$a and $R_1$b against *E. coli*was found to be 1.2 mg/mL. Since these metabolites were obtained by the evaporation of ethyl acetate solvent; it is likely to have this high MIC value. Similar ndings were also reported by Gurung et al., (2009).

TLC showed only one spot produced by each antibiotic solution, indicating the presence of a single compound. The two spots were close to solvent front with $R_f$ value of 0.88 and 0.90 for the compound extracted from active isolates $R_1$a and $R_1$b respectively (Ta). Vancomycin, however, produced one tailed spot with $R_f$ value of 0.09. Similar ndings were also reported by Gurung et al., (2009).

**Characteristics of the antibacterial substances**

**Table 8:**Thin layer chromatography of antimicrobial substances

| Antibacterial substance (s) code | Concentration (mg/mL) | No. of moved Spot | Distance travelled by | | $R_f$ Value |
|---|---|---|---|---|---|
| | | | Solvent front (cm) | Antibacterial Substances (cm) | |
| $R_1$a | 30 | 1 | 16 | 14 | 0.88 |
| $R_1$b | 30 | 1 | 16 | 14.5 | 0.90 |
| Vancomycin | 10 | 1 | 16 | 1.45 | 0.09 |

## Conclusions

Most potent actinomycetes isolates like *Streptomyces* spp. ($R_1$a) and *Thermomonospora* spp. ($R_1$b) which showed antibacterial property against gram negative bacteria were isolated from soils of extreme area like Siraha, a warm district of Nepal. Hence, the present study clearly reveals the distribution of antibiotic producing actinomycetes in the Terai region (75-600m) of Nepal.

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