SCREENING OF ANTIBIOTIC PRODUCING ACTINOMYCETES FROM THE SOIL SAMPLES AND ANTIBACTERIAL ACTIVITY IN VARYING SODIUM NITRATE CONCENTRATION

A PROJECT WORK SUBMITTED TO THE

DEPARTMENT OF MICROBIOLOGY

AMRIT CAMPUS

INSTITUTE OF SCIENCE AND TECHNOLOGY

TRIBHUVAN UNIVERSITY

NEPAL

FOR THE AWARD OF

BACHELOR OF SCIENCE (B.Sc.) IN MICROBIOLOGY

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RECOMMENDATION

This is to recommend that **Swornima Dangol,** (Symbol No: 500330079, T.U registration No: 5-2-33-157-2017), has carried out project work entitled **"Screening of Antibiotic Producing Actinomycetes from the Soil Samples and Antibacterial Activity in Varying Sodium Nitrate Concentration."** For the requirement to the project work in Bachelor of Science (B.Sc.) degree in Microbiology under my supervision in the Department of Microbiology, Amrit campus, Institute of Science and Technology (IoST), Tribhuvan University (T.U.), Nepal.

To my knowledge, this work has not been submitted for any other degree.

She has fulfilled all the requirements laid down by the Institute of Science and Technology (IoST), Tribhuvan University (T.U.), Nepal for the submission of the project work for the partial fulfilment of Bachelor of Science (B.Sc.) degree.

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Assist. Prof. Suman Rai

Department of Microbiology

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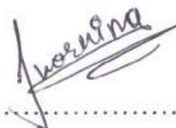
15 June 2022

DECLARATION

This project work entitled **"Screening of Antibiotic Producing Actinomycetes from the Soil Samples and Antibacterial Activity in Varying Sodium Nitrate Concentration."** is being submitted to the Department of Microbiology, Amrit Campus, Institute of Science and Technology (IoST)., Tribhuvan university (T.U), Nepal for the partial fulfillment of the requirement to the project work in Bachelor of Science (B.Sc.) degree in Microbiology. This project work is carried out by me under the supervision of Assist. Prof. Suman Rai in the Department of Microbiology, Amrit Campus, Institute of Science and Technology (IoST), Tribhuvan University (T.U.), Nepal.

This work is original and has not been submitted earlier in part or full in this or any other form to any university or institute, here or elsewhere, for the award of any degree.



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LETTER OF FORWARD

Date: 20 June 2022

On the recommendation of Assist. Prof. Suman Rai, this project is submitted by Ms. Swornima Dangol, Symbol No: 500330079, T.U. Registration No: 5-2-33-157-2017, **"Screening of Antibiotic Producing Actinomycetes from the Soil Samples and Antibacterial Activity in Varying Sodium Nitrate Concentration."** is forwarded by the Department of Microbiology, Amrit campus for the approval to the evaluation committee, Institute of Science and Technology (IoST), Tribhuvan University (T.U.), Nepal.

She has fulfilled all the requirements laid down by the Institute of Science and Technology (IoST), Tribhuvan University (T.U), Nepal for the project work.

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BOARD OF EXAMINATION AND CERTIFICATE OF APPROVAL

This project work (PRO-406) entitled **"Screening of Antibiotic producing Actinomycetes from the Soil Samples and Antibacterial Activity in Varying Sodium Nitrate Concentration."** by (Swornima Dangol), (Symbol No: 500330079 and T.U. Registration No: 5-2-33-157-2017) under the supervision of supervisor Assist prof. Suman Rai in the Department of Microbiology, Amrit campus, Institute of Science and Technology (IoST), Tribhuvan University (T.U), is hereby submitted for the partial fulfillment of the Bachelor of Science (B.Sc.) degree in microbiology. This report has been accepted and forwarded to the controller of examination, Tribhuvan University, Nepal for the legal procedure.

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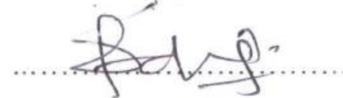

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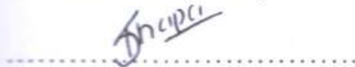

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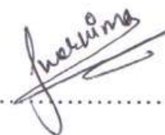

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ABSTRACT

Actinomycetes are gram positive filamentous, slow growing bacteria, best known to produce antibiotic. The aim of this study was to screen antibiotic producing actinomycetes and determine its antibiotic activity against ATCC cultures at different gradient of $NaNO_3$. This study was carried out from March 29 to April 28, 2022. Thirty collected samples were collected and transported and processed in Amrit campus. Primary and secondary screening were performed by perpendicular streaking and agar well diffusion method respectively. Characterization and identification of the isolated actinomycetes were performed. From thirty collected samples, twenty-eight samples were actinomycetes. Only two samples were antibiotic producing actinomycetes MI410 $^{-3}$ and NP110 $^{-2}$ showed antibacterial activity against ATCC cultures viz: *S. aureus* 43300, *E. coli* 35218, *E. coli* 25922 and *Klebsiella* 700603 in primary screening. Antibiotic was produced by sub-merged state fermentation with varying concentration of $NaNO_3$ and secondary screening was done by agar well diffusion against ATCC cultures in comparison to standard streptomycin (100 µg/mL).

In comparison to standard streptomycin (100 µg/mL) extract MI410^{-3} with 1% $NaNO_3$ was effective only against *E. coli* 25922 while NP110^{-2} with 0.5% $NaNO_3$ against *E.coli* 35218 (14.67mm), *E.coli* 25922 (19.67mm) and *S. aureus* 43300 (17mm) whereas NP110^{-2} has also shown antibacterial effect with 1.5% $NaNO_3$ against *E. coli* 35218 (13mm), *Klebsiella* spp. ATCC 700603 (11.5mm) and *S. aureus* 43300 (12.5mm).

Statistically, there is significant difference at 5% level of significance between the sample concentration at 0.5% of $NaNO_3$ (.001, $P<0.05$) and 1.5% of $NaNO_3$ (0.024, $P<0.05$) in antimicrobial activity. However, there is no significant difference at 5% level of significance between the sample concentration at 1% (0.356, $P>0.05$). The isolated actinomycetes was presumed as *Streptomyces* spp. Both MI410^{-3} and NP110^{-2} (7.14%) showed antibacterial activity against cultures in primary screening. The indigenous species of actinomycetes, isolated from various places of Kathmandu valley can be used in industrial production of antibiotics which can help in economic growth of Nepal.

Key words: Agar well diffusion, Antimicrobial activity, Antibiotic, ATCC cultures, $NaNO_3$, *Streptomyces* spp.

शोधसार

एक्टिनोमाईसिटिज ग्रामपोजेटिभ फिलामेन्टस, ढिलो बढने ब्याक्टेरिया हो, जुन एन्टिबायोटिक उत्पादनको लागि उत्कृष्ट मानिन्छ । यस अध्ययनको मुख्य उद्देश्य एन्टिबायोटिक उत्पादन गर्ने एक्टिनोमाईसिटिज छुट्टयाउनुका साथै विभिन्नमात्राको सोडियम नाईट्रेटमा एटिसिसि कल्चर्सहरुलाई मार्ने क्षमता निर्धारण गर्नु थियो । यस अध्ययनको लागि काठमाण्डौ उपत्यकाका विभिन्न स्थानका माटो सङ्कलन गरिएको थियो । यस अध्ययन मार्च २९ देखि अप्रिल २८, २०२२ सम्म गरिएको थियो । तीस नमुनाहरु सङ्कलन गरि अमृत क्याम्पसमा लगियो । प्राथमिक र दोस्रो छनौट पर्पेडिक्यूलर स्ट्रिकिङ्ग तथा एगार ह्वेल डिफ्यूजन विधिद्धारा क्रमशः गरिएको थियो र एक्टिनोमाईसिटिजको विशेषता र पहिचान गरिएको थियो । २८ नमुनाहरु एक्टिनोमाईसिटिज भेटिएको मध्ये दुई वटा एन्टिबायोटिक उत्पादन गर्ने एक्टिनोमाईसिटिज $MI410^{-3}$ र $NP110^{-2}$ ले एटिसिसि कल्चर्सहरु *S. aureus* 43300, *E. coli* 35218, *E. coli* 25922 र *Klebsiella* 700603 को विरुद्ध जीवाणुरोधी गतिविधि प्राथमिक छनौट विधिमा प्रदर्शन गरिएको थियो । एन्टिबायोटिक उत्पादन विभिन्न मात्राको सोडियम नाईट्रेट सहित सब–मर्ज फर्मेन्टेसन र दोस्रो छनौट मानक स्ट्रेप्टोमाईसिन (१००μg/mL) सँग तुलना गरि एगार ह्वेल डिफ्यूजन विधिद्धारा गरिएको थियो । मानक स्ट्रेप्टोमाईसिनको तुलनामा १% $NaNO_3$ सहित $MI410^{-3}$ ले *E. coli* 25922 विरुद्ध प्रभावकारी थियो जबकि ०.५% $NaNO_3$ सहित $NP110^{-2}$ ले *E. coli* 35218 (१४.६ मिमि), *E. coli* 25922 (१९.६ मिमि) र *S. aureus* 43300 (१७ मिमि) को विरुद्ध जीवाणुरोधी गतिविधि देखाएको थियो भने १.५% $NaNO_3$ सहित $NP110^{-2}$ ले *E. coli* 35218 (१३ मिमि), *Klebsiella* spp. 700603 (११.५ मिमि) र *S. aureus* 43300 (१२.५ मिमि) को विरुद्ध जीवाणुरोधी गतिविधि देखाएको थियो । एनोभा (५%) तथ्याङ्कानुसार एटिसिसि कल्चर्सहरुको तुलना गर्दा ०.५% (०.००१; पि<०.००५) तथा १.५% (०.०२४; पि<०.००५) $NaNO_3$ सहित $NP110^{-2}$ कोजीवाणुरोधी गतिविधि फरक देखियेा । यद्यपी, १% (०.३५६; पि>०.००५) $NaNO_3$ सहित $NP110^{-2}$ को जीवाणुरोधी गतिविधि खासै फरक देखिएन । सो एक्टिनोमाईसिटिज स्ट्रेप्टोमाईसिस प्रजाती भएको अनुमानित गरियो । दुवै नमुनाले (७.१४%) एटिसिसि कल्चर्स विरुद्ध जीवाणुरोधी गतिविधि प्राथमिक छनौटमा देखाएको थियो । काठमाण्डौ उपत्यकाको माटोमा पाइने रैथाने एक्टिनोमाईसिटिजबाट एन्टिबायोटिक औद्यौगिक स्तरमा उत्पादन गर्न सकिन्छ जसले देशको आर्थिक वृद्धी गर्न सहयोग गर्दछ ।

मुख्य शब्दहरु : *एगार ह्वेल डिफ्यूजन विधि, जीवाणुरोधीगतिविधि, एन्टिबायोटिक, एटिसिसि कल्चर्स, सोडियम नाईट्रेट, स्ट्रेप्टोमाईसिस. प्रजाती*

LIST OF ACRONYMS AND ABBREVIATION

ATCC	The American type culture collection
ANOVA	Analysis of Variance
G+C	Guanine + Cytosine
MHA	Muller Hinton Agar
YMB	Yeast Mannitol Broth
ZOI	Zone of inhibition

LIST OF SYMBOLS

Symbol	Meaning
^{o}C	Degree Celsius
µl	Microliter
µg	Microgram
ml	Milli liter
%	Percentage
$<$	Greater than
±	Plus, Minus

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CHAPTER 1

1. INTRODUCTION

1.1. General Introduction

Soil is the biologically active, porous medium that has developed in the uppermost layer of Earth's crust. Soil is one of the principal substrata of life on Earth, serving as a reservoir of water and nutrients, as a medium for the filtration and breakdown of injurious wastes, and as a participant in the cycling of carbon and other elements through the global ecosystem. It has evolved through weathering processes driven by biological, climatic, geologic, and topographic influences (Sposito, 2021). Soil is the thin layer of material covering the earth's surface and is formed from weathering of rocks. Soil is dynamic and diverse natural system that lies at the interface between earth, air, water, and life. Soil minerals are divided into three classes- clay, sand, and silt. Soil comprises of mineral particles, organic matters, air, water and living organisms. Most plant gets their nutrient from the soil. The living organisms found in soil are soil microorganisms. Soil microorganisms can be grouped into bacteria, actinomycetes, fungi, algae, protozoa, and nematodes (Balasubramanian, 2017).

Soil microorganisms perform important function in nature, affecting the soil properties. Microorganisms in soil are important because they affect the structure and fertility of different soils (Jose and Jha, 2016). A great number of morphological and physiological types of microorganism can be found in soils. Several microorganisms are known to produce a wide variety of antibiotics that are being developed and used against numerous life-threatening infections and diseases in human, animal, and agriculture. Antibiotics are produced by several groups of microbes such as bacteria, fungi and actinomycetes as their natural defense system against other microbes living in their vicinity. Soil microorganism had always been the primary source of production of antibiotics and disinfectants in medicine, agriculture, and fish culture and their release in environment has given birth to another critical problem of multidrug resistant pathogenic microbes (Kumar, 2010).

Antibiotics are secondary metabolites produced by microorganisms, which have antimicrobial properties and have been used as chemotherapeutic agents against infectious and disease-causing microbes (Prosopopoeial, 2012). Most antibiotics producers used today are the soil microbes. Fungal strains and *Streptomyces* members

are extensively used in the industrial antibiotic production. (Pankaj, 2014). Antibiotics such as Beta lactam, Aminoglycosides, Streptomycin, and Tetracyclines and others; and being produced by soil bacteria and fungi. Fungal antibiotics such as Penicillin, Cephalosporin, Fusaric acid, Griseofulvin and Fumagillin have been obtained by fungal species *Penicillium*, *Cephalosporin*, and *Aspergillus.* Several *Pseudomonas* spp. and *Bacillus* spp. are among the soil bacteria which have been exploited like Gramicidin, Bacitracin, Tyrothricin, Pyocyanin and Pyrrolnitrin (Beardy, 1980). *Streptomyces* spp. is one of the soils actinomycetes which provides the highest number of commercial antibiotics such as Tetracycline, Streptomycin, Viomycin, Kanamycin. Several other commonly used antibiotics Gentamicin and Rifamycin have been isolated from actinomycetes like *Micromonospora*, *Actinomandura* and *Nocardia* spp. (Berdy,1980). Need of new antimicrobial agents is greater than ever because of emergence of multidrug resistance in common pathogens, the rapid emergence of new infections and the use of multidrug resistant pathogens in bioterrorism (Spell Berg, *et al.*, 2004). Resistance of bacteria to the effects of antibiotics has been a major problem in the treatment of diseases. Infectious diseases are still the second leading cause of death worldwide (WHO, 2002).

Actinomycetes are gram-positive filamentous bacteria which are acid fast in nature. Through actinomycetes are recognized as gram-positive bacteria, they differ from other bacteria having their morphological characteristics such as high Cytosine and Guanine content in DNA. The availability of antibiotic producing actinomycetes varies depending upon the texture and cultivation of soil. Actinomycetes are a group of branching unicellular organisms, which reproduce either by fission or by the means of special spores or conidia. They are closely related to bacteria; frequently, they are considered as higher, filamentous bacteria (Selman and Waksman, 1959). Approximately two third of naturally occurring antibiotics have been isolated from actinomycetes (Kumar, 2010). A huge number of currently used antibiotics including Erythromycin, Streptomycin, Rifamycin and Gentamycin are all products isolated from actinomycetes (Jeffery, 2008). The two major groups of soil actinomycetes that serves as important sources of antibiotic are *Streptomyces* and *Micromonospora* (Rai, *et al.*, 2016). It has been stated that *Streptomyces* account for about 80% of the total antibiotic products, while *Micromonospora* closely follows with less than one tenth as much as *Streptomyces* (Arifuzzaman, 2010). They belong to the order actinomycetes (Super

kingdom: Bacteria, Phylum: Firmicutes, Class: Actinobacteria, Subclass: Actinobacteria). They are the best common source of antibiotics and provide approximately two- third of naturally occurring antibiotics, including many of medical importance (Okami and Hotta, 1988). Actinomycetes are the important source for novel antibiotics and hence having a high pharmacological and commercial interest including control of infectious disease (Aghamirian and Ghiasian, 2009). Only few of the actinomycetes have been screened. Actinomycetes are slow growing bacteria. It is the most useful antibiotic producing bacteria and it is commonly found in soil sample. Actinomycetes can be grown by maintaining pH, temperature, carbon source concentration and sodium nitrate concentration.

Actinomycetes are ubiquitous Gram-positive bacteria that constitute one of the largest bacterial phyla with characteristic filamentous morphology and high G+C DNA. The actinomycetes have been recognized as premier source and inspiration for a substantial fraction of antibiotics that play an important role in human health. (Jose and Jha, 2016).

However, in Nepal, most of the research have been focused on accessing the antimicrobial properties of soil derived Actinomycetes from various ecological niches ranging from soils of Kalapatthar, Everest region to Terai region along with other sites like riverbanks, forests, etc. The distribution of Actinomycetes have also been accessed according to the altitude in various parts of Nepal and their potential has been tested against several pathogenic bacteria and fungi (Gurung, *et al.*, 2009; Pandey, *et al.*, 2004; Rai, *et al.*, 2018; Budhathoki & Shrestha, 2020; Sah & Lekhak, 2017).

1.2. Rationale of the study

Antibiotics are best known products of actinomycetes. The actinomycetes produce an enormous variety of antimicrobial compounds. One of the first antibiotics used was streptomycin produced by *Streptomyces griseus.* Most of the antimicrobial compounds from actinomycetes are sorted into Amino glycosides (e.g., Streptomycin and Kanamycin), Annamycin's (e.g., Rifampin), Macrolides (e.g., Erythromycin), Tetracycline's. Most antibiotics producers used today are the soil microbes. Fungal strain and streptomyces members are extensively used in industrial antibiotic production. Bacteria are also reported for their antibiotic production. *Bacillus* species being the predominant soil bacteria because of their resistant endospore formation and production of vital antibiotics like bacitracin etc., have always been found inhibiting the growth of the other organisms. It is advisable to screen antibiotics producing

actinomycetes as they are easy to isolate, culture, maintain and improve. As, actinomycetes are widely found in soil of Nepal, the indigenous actinomycetes can be isolated and can be used in industrial production of antibiotic for commercial benefit.

1.3. Objective of study

1.3.1. General objective:

- To screen the antibiotic producing actinomycetes from the soil samples and antibiotic profiling in varying sodium nitrate concentration.

1.3.2. Specific objective:

- To isolate and identify antibiotic producing actinomycetes.
- To screen antibiotic producing actinomycetes by primary screening.
- To produce antibiotic by sub-merged fermentation.
- To compare the antimicrobial activity with varying $NaNO_3$ concentration in comparison to standard antibiotic.

CHAPTER 2

2. LITERATURE REVIEW

2.1. Actinomycetes:

Actinomycetes are gram-positive bacteria showing a filamentous growth. They are aerobic and widely spread in nature. They are predominant in dry alkaline soil (Jeffrey, 2008). They are unique for their spore forming abilities and formation of mycelia structures. These bacteria have been noted to serve as rich reservoirs of medicinal antibiotics and are therefore extremely relevant to scientists, pharmaceutical industries, and agricultural industries. (Kumar, *et al.*, 2010). Actinomycetes are ubiquitous group of microbes widely distributed in natural ecosystems around the world (Srinivasan, *et al.*, 1991). The richness and diversity of actinomycetes present in any specific soil is influenced by the soil type, geographical location, cultivation, and organic matter amongst other factors. (Arifuzzaman, 2010). The number and types of actinomycetes present in a particular soil would be influenced by geographical location such as soil temperature, soil type, soil pH, organic matter content, cultivation, aeration, and moisture content. Actinomycetes are the most fruitful source for production of bioactive secondary metabolites, actinomycetes total 7899 (100%) compounds have been identified up to 1988; 67% from actinomycetes, 12% from bacteria and 20% fungi (Tanaka and Omura, 1990). Actinomycetes populations are lower than other soil microbes and contain a predominance of *Streptomyces* that are tolerant to acid conditions (Davies and Williams, 1970). An enormous number of currently used antibiotics including Erythromycin, Streptomycin, Rifamycin and Gentamycin are all products isolated from soil actinomycetes. The two major group of soil actinomycetes that serves as important sources of antibiotics are S*treptomyces* and *Micromonospora* (Jeffrey, 2008). Actinomycetes populations are identified as one of the major groups of soil population, which may vary with soil type (Chansiropornchai, *et al.*, 2000). Actinomycetes are prokaryotic organism with filamentous nature, branching pattern, and conidia formation, which are like those of fungi. For this reason, they are also known as ray fungi. They are gram-positive, free-living, saprophytic bacteria (Chaudhary, 2013, Rahman 2011). Actinomycetes are gram-positive bacteria showing a filamentous growth like fungi. They are aerobic and widely spread in nature. They are

predominant in dry alkaline soil (Jeffrey, 2008). Actinomycetes DNA are rich in G+C content with the GC % of 57 - 75% (Lo, *et al.*, 2002).

2.2. Occurrence and habitat:

Actinomycetes are the most abundant free-living saprophytes that form thread-like filaments in the soil. They grow as hyphae like fungi responsible for the characteristically earthy smell of freshly turned healthy soil. The actinomycetes exist in various habits in nature and represent a ubiquitous group of microbes widely distributed in natural ecosystems around the world (Chamikara, 2016). Actinomycetes are widely distributed in soil and ocean. There are many reports for isolation of actinomycetes from terrestrial soils, marine ecosystem, mangrove ecosystem, composts, vermicompost's. Environmental factors also influence the type and population of actinomycetes in soil. They are found both at mesophilic (25- 30 ^{o}C) and thermophilic (40°C) environments (Haseena, *et al.*, 2016). Many mesophilic actinomycetes are active in compost in initial stages of decomposition. However, the capacity for self-heating during decomposition provides ideal conditions for thermophilic actinomycetes (Chavan, *et al.*, 2013). The pH is also a major environmental factor determining the distribution and activity of actinomycetes. Most of the actinomycetes grow at optimum pH around 7 (Vasavada, *et al.*, 2006).

Actinomycetes play a significant role in soil biodegradation and in humus formation and produce numerous volatile substances such as geosmin, which is responsible for the feature wet earth odor (Wilkins, 1996). Numerous bioactive metabolites of actinomycetes have shown to possess antimicrobial, antiviral, cytotoxic, antioxidant, insecticidal, plant growth-promoting, and herbicidal activities (Kumar, 2012). Actinomycetes are widespread in nature and may occur in extreme environments (Meklat, 2011). It has been known that *Streptomyces* species can produce secondary metabolites. Toxic metabolites isolated from *Streptomyces* and *Nocardiopsis* isolates have shown to be mitochondriotoxic in spermatozoa (Peltola, 2001) It is recognized that some isolates of *Streptomyces griseus* produce valinomycin, a toxin that cause mitochondrial swelling, activity loss, and apoptosis in human natural killer cells (Andersson, 1998).

2.3. Structure of actinomycetes:

Actinomycetes are characterized by the formation of normally branching threads or rods. The hyphae are non-septate. The sporulating mycelium may be branching or non-

branching, straight or spiral shaped. The spores are spherical, cylindrical, or oval. (Chamikara, 2016). Morphologically they resemble fungi may be due the adaptation to same habitat their cell wall composition is like that of gram-positive bacteria. Actinomycetes are a group of branching unicellular organisms, which reproduce either by fission or by means of special spores or conidia. They are closely related to bacteria; frequently, they are considered as higher, filamentous bacteria (Selman and Waksman, 1959). Actinomycetes diversity can also be influenced by the diversity of plant species grown on that soil. Since different plants produce different chemical metabolite, so to survive the microbes (Actinomycetes in this case) need to adapt to the environment (Oskay, *et al.*, 2004). The Bergey's Manual of Systematic Bacteriology - 2nd edition for Actinobacteria classification has five volumes, the phylum Actinobacteria is divided into six classes, namely Actinobacteria, Acidimicrobia, Coriobacteriia, Nitriliruptoria, Rubrobacteria, and Thermoleophilia. The class Actinobacteria is further divided into 16 orders that are Actinomycetales, Actinopolysporales, Bifidobacterial, Catenulisporales, Corynebacterial, Frankiales, Glycomycetales, Jiangellales, Kineosporiales, Micrococcales, Micromonosporales, Propionibacteriales, Pseudonocardiales, Streptomycetales, Streptosporangiales, and Incertaesedis.

In order of abundance in soils, the common genera of Actinobacteria are *Streptomyces* (nearly 70%), *Nocardia* and *Micromonospora*, although *Actinophages, Micromonospora*, and *Streptosporangium* are also generally found (Ranjani, 2016). At present, the molecular identification is based on 16S rDNA sequences, which is most significant for Actinobacteria.

2.4. Screening of actinomycetes for antimicrobial property:

For isolation of desirable rare actinomycetes genera from natural habitats various methods have been developed (Nonomura and Hayakawa, 1988). These methods include a variety of pretreatment techniques in combination with enrichment techniques that appropriately supplement agar media with selective antimicrobial agents (Hayakawa, 1990). In natural soil habitat, *Streptomyces* are common and are usually a major component of the total actinomycetes population. Therefore, to selectively isolate a particularly rare genus of actinomycetes in natural habitat samples enrichment or elimination undesirable *Streptomyces* and other contaminants from the isolation plate media (Pre-treatment) must be employed (Hayakawa, 2008). Screening and isolation of

promising strains of actinomycetes with potential antibiotics is still a thrust area of search for many years (Hacene and Lefebvre, 1996).

The development of resistance by the pathogens as well as the emergence of new pathogens has led to the necessity for the discovery of new antibiotics / antimicrobial for their infection. Hence, screening of antimicrobial activity of actinomycetes and study of their antimicrobial action against pathogens is an important action against pathogens is an important process for the discovery of an antibiotic (Budhathoki, 2020). The physician and other healthcare workers may prescribe antibiotics. Many patients self-treat with antibiotics, including prior to hospital admission, which can contribute to increased resistance rate. Drug resistance in microorganism has been increasing and this has posed a serious threat for the humankind. Discovery of the novel antimicrobials that can control the growth of these microorganisms is of global importance (Gurung, *et al.*, 2009). In the conventional isolation techniques, several factors must be considered, namely, the choice of screening source, the selective medium, culture conditions and the recognition of candidate colonies in the primary isolation. Furthermore, choosing appropriate media and growth conditions is important and published media are typically associated with a particular microbial genus or species. As with other microbial discovery research, when working with environmental samples harboring communities of novel microbial populations, the media and growth conditions chosen will enrich for certain populations and not others (Schneegurt, 2012).

Muthu, *et al.* (2013) found 5 isolates of total 10 screened samples having antimicrobial activity from Cauvery River soil sample. Five Isolates actinomycetes were identified by 16s rRNA sequencing and were confirmed by using bioinformatics tool as BLAST (*Isoptericola variabilis*). Tyagi, *et al.* (2014) reported that 11 Actinomycetes were isolated from 21 soil samples at two different temperatures 28ºC or 37ºC. These actinomycetes were screened regarding potential against Gram-positive and Gram-negative bacteria. They were evaluated for their inhibitory activities on test organisms like *Streptococcus aureus, E. coli, Staphylococcus aureus* and *Bacillus* strains. These 11 actinomycetes isolates were highly active with an inhibition zone more than 16 mm in 12 diameters. These 11 actinomycetes isolates were isolated mostly from alkaline soil.

2.5. Cultural characteristics:

Actinomycetes show different cultural characteristics when grown on agar surface (Shirling and Gottlieb, 1976) to distinguish and characterize actinomycetes at different

level and have been used by many researchers to isolate actinomycetes from soil and other natural habitats such as starch casein agar (Kusters and Williams, 1964; Duddu, *et al.*, 2016), actinomycetes isolation agar (Patnaik and Reddy, 2012; Pandey, *et al.*, 2011), glycerol asparagine agar (Pridham and Lyons, 1961; Low, *et al.*, 2015), Oatmeal Agar (Low, *et al.*, 2015), yeast malt extract Agar (Mohseni, *et al.,* 2013). They branch and form network of hyphae growing on both on the surface of the agar (aerial mycelium). Their growth is characterized by small compact, soft to hard colonies tenaciously adhering to the medium, the surface being either flat or elevated (Sathi, *et al.*, 2001). The color of the aerial mycelium can range from white, creamy white, chalky, powdery, brown, gray to pinkish and violet and substrate mycelium may vary from brown, yellow to orange (Arifuzzman; Mohseni, *et al.*, 2013; Jeffrey, 2008, Salim, *et al.*, 2017; Amit, 2011). They are found to be pigment producing; blackish brown, yellow to orange, brown, orange, brown, red (Shirling and Gottlieb, 1966; Mohseni, *et al.*, 2013).

2.6. Antibiotics:

Antibiotics are bioactive secondary metabolites that can be obtained from microorganisms and can also be semi-synthesized or chemically synthesized (Balagurunathan & Radhakrishnan, 2010)**.** Antibiotic refers to the chemical compound derived from microorganism or living cells that inhibits or stop the growth of a microorganism. They are used in the treatment of external or internal infections. While some antibiotics are produced by a microorganism, most are now manufactured synthetically (Denyer, *et al.*, 2011). Actinomycetes have been noted to serve as rich reservoirs of medicinal antibiotics and are therefore extremely relevant to scientist, pharmaceutical industries, and agricultural industries (Kumar, *et al.*, 2010). Actinomycetes species synthesize a numerous natural metabolite with diverse biological activity such as antibiotics. Antibiotics of actinomycetes origin evidence a wide variety of chemical structure including Aminoglycosides, Anthracyclines, Beta lactams, Nucleosides, Peptides, Polyenes, Actinomycin and Tetraclyine (Barrios- Gonzalez, *et al.*, 2005). The antibiotics are widely produced by fermentation using free culture to enhance the productivity (Dhananjay, 2010). Need of new antimicrobial agents is greater than ever because of emergence of multidrug resistance in common pathogens the rapid emergence of new infectious and the use of multidrug resistant pathogens in bioterrorism (Spell Berg, *et al.,* 2004). Number of antibiotics have now been isolated from cultures of actinomycetes, such as Actinomycetin, Micromonosporin, Mycelin and

Actinomyces lysozyme etc. have been only partially purified whereas others including Actinomycin, Proactinomycin, Streptothricin, and Streptomycin have been isolated and crystallized. These substances differ greatly chemical structure and their antimicrobial properties, toxicity to animals and in vivo activity (Waksman, *et al.*, 2010). The nature of active agents or the antibiotics produced by actinomycetes depends upon the species frequently upon the strain; the composition of the medium in which it is grown and the conditions of cultivation. The antimicrobial properties of a given actinomycetes culture also depend upon the composition of the medium in which it is grown (Waksman, *et al.,* 2010).

Most of the secondary metabolites are widely recognized from actinomycetes including many antimicrobials such as Streptomycin, Erythromycin, and Tetracycline, with original and ingenious structures and potent biological activities. Thus, actinomycetes are a potent resource for new lead compounds in drug development. In the exploration of marine derived actinomycetes as a source of antitumor compounds (Bailoori, 2020). Actinomycetes are known to produce an assortment of metabolites that are active against antibiotic-resistant bacteria. (Zhang, 2012). Actinomycetes are known as the most biotechnologically valuable prokaryotic microorganisms. They are well known as a source of antibiotics and bioactive molecules. Most of their bioactive molecules have been shown to have Antibacterial (Streptomycin, Tetracycline and Chloramphenicol), Antifungal (Nystatin), Antiviral (Tunicamycin) and Antiparasitic (Ivermectin) properties. Indeed, most of the antimicrobials used today in remedying diseases caused by pathogens have been developed from actinomycetes (Chaudhary, 2013). These organisms are potential sources of pharmaceutically and agriculturally vital bioactive compounds. Approximately two-thirds of naturally occurring antibiotics are provided by *Streptomyces* (Narayana 2007); (Rahman 2012). Many of these secondary metabolites are potent antibiotics, which has made Streptomyces's the primary antibiotic-producing organisms exploited by the pharmaceutical industry. Since the discovery of actinomycin, the first antibiotic from actinomycetes, many commercially important bioactive compounds and anti-tumor agents have been produced using actinomycetes. Nowadays, the control of pathogenic microorganisms by synthetic products is becoming less attracting due to the emergence of resistant strains and because of the undesirable effects of these products on the environment. Therefore, it's necessary to find antagonistic microorganisms that are used as a means of biocontrol (Hassouni, 2019).

2.7. Minerals and its effect on antimicrobial activity:

Essential mineral elements, namely magnesium, sodium, potassium, phosphorus, and calcium are as important as amino acids and vitamins in maintenance of life, wellbeing, and production (Saif, 2003). It is quite interesting to discuss the impact of exogenous supplementation of metal ions as well as complex compounds based on certain specific metal ions minerals, on the growth of microbial organism (bacteria and fungi) as well as upscaling production of antibiotic from organism (Kayode, 2008) and (Mishra, 2009). The adequate supplementation of essential metal ions/ minerals is quite mandatory for microbiologists to work along these notions and objectives in future (Mishra, 2012). According to Mishra the strain improvement by the mutagenic agents and exogenous supplementation of certain specific metal ions/minerals to the microbial culture media probably being of great significance in view of enhancing the antibiotic activity.

CHAPTER 3

3. MATERIALS AND METHODS

3.1. Materials required

All the Materials including glass wares, chemical and microbiological media used in this study are listed in Appendix I.

3.2. Study area

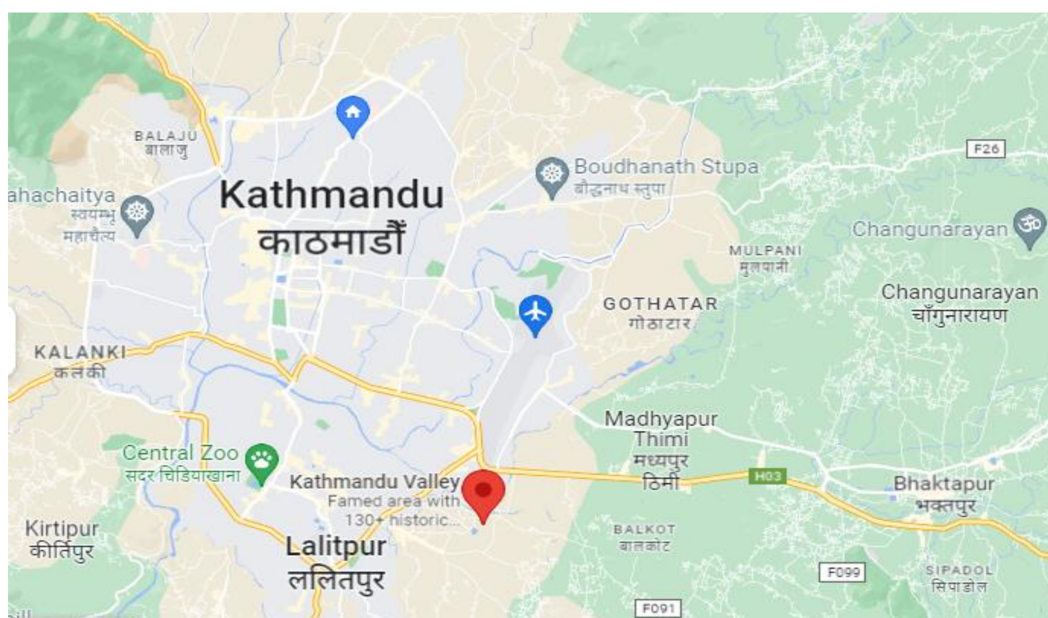


Figure 1: A map of study site

3.3. Research methodology

3.3.1. Research design

This research design was qualitative type. The study was focused on antibiotic producing actinomycetes found in soil of Kathmandu valley and their antibacterial property against test bacterial strains in different concentration of $NaNO_3$.

3.3.2. Population and Sample

For this research work soil sample from different places of Kathmandu valley was collected as large variety of actinomycetes species are found in soil.

3.3.3. Sample Size:

Total 30 different soil samples were collected from different places of Kathmandu valley. the places of soil samples sites are listed in Appendix V.

3.3.4. Laboratory Set Up and Duration of work

The study was completed at the laboratory of Department of Microbiology, Amrit campus, Kathmandu. The laboratory work was done from March 28 to April 29, (2022).

3.3.5. Data collection procedure and Data analysis:

In this study, the method of data collection was observational and experimental for both qualitative and quantitative data.

The collected data and information's were processed, tabulated, and analyzed quantitatively by using tables and pie chart. Similarly, ANOVA (One way) was inferred using GenStat 12 edition.

3.4. Method

3.4.1. Collection and transportation of Soil samples:

Soil samples were collected from 30 different sites of Kathmandu valley in three consecutive days i.e., March 29, 30 and 31. 500 grams of dry and moist soil samples were collected from a depth of 7-8 cm and were placed in clean polyethene bag and labeled accordingly. The samples were transported to the microbiology lab of Amrit campus as soon as possible maintaining cold chain.

3.4.2. Isolation of Actinomycetes:

Isolation of actinomycetes was done by serial dilution method according to Kaur, et al., (2014). For each sample, 1g of soil sample was added in a test tube containing 10ml distilled sterile water and shaken well. This was then serially diluted up to 10^{-3}. From each tube, only 0.1ml of the aliquot was taken and spread plate technique was performed and the plates were left undisturbed for 15 minutes after which the plated were incubated at $28^{o}C$ for 6-7 days for the isolation of actinomycetes. The colonies were observed after 7th day and were identified according to Rai, *et al.* (2016).

3.4.3. Screening of Actinomycetes for Antimicrobial Activity

3.4.3.1. Primary screening:

Primary screening was performed by perpendicular streak method on Muller Hinton Agar (MHA). Actinomycetes isolates were streaked vertically down the center of Muller Hinton agar and then incubated at 28^oC for 7 days. After the incubation, ATCC cultures viz; *S. aureus* 43300, *E. coli* 35218, *E. coli* 25922 and *Klebsiella* 700603 were streaked 1 cm apart and 2 mm on side of the actinomycetes colony on Muller Hinton agar, perpendicular to the colony and then was incubated at 37 ^oC for 24 hours. After incubation, plates were observed for the zone of inhibition (ZOI) and the zone of inhibition (ZOI) were noted according to Ganesan (2017) and Rahman (2011).

3.4.3.2. Secondary screening

Agar well diffusion method was employed for secondary screening of inhibitory action on Muller Hinton agar against ATCC cultures viz; *S. aureus* 43300, *E. coli* 35218, *E. coli* 25922 and *Klebsiella* 700603. The selected isolates from primary screening were inoculated aseptically in Yeast Mannitol Broth (YMB) where different concentration of $NaNO_3$ (0.5%, 1%, 1.2% and 1.5%) was maintained. The inoculated samples were allowed in sub-merged state fermentation at 120 rpm in 28°C for two weeks using shaker incubator. The pellets and clumping formation indicated the growth of organism in broth. After the completion of incubation, the contents of the flasks were filtered through sterile Whatman no.1 filter paper. Then the filtrate was centrifuged at 3000 rpm for 10 minutes. MHA plates were swabbed with test bacterial broth culture. 100 microliters (µl) of crude extract were pipetted into the well and kept undisturbed for 15-20 minutes. For positive control 100µl standard Streptomycin (100µg/ml) was pipette into the well and for negative control 100µl sterilized distilled water was used separately. The plates were incubated at 37 °C for 24 hours. The secondary screening was done in triplicate. After 24 hours, the average zone of inhibition was measured and noted (Gopinath, 2013).

3.4.4. Characterization of Actinomycetes

3.4.4.1. Macroscopic Characterization

The isolated colonies of actinomycetes on Starch M-protein were studied for color of aerial mycelium and diffusible pigments and other colony characteristics such as size, texture, color, elevation, opacity of the colonies. (Rai, *et al.,* 2016).

3.4.4.2. Microscopic Characteristics

The microscopic characterization was done by Gram staining method (Aneja, 2003). A loopful of isolated colony was smeared and gram staining was performed, and slide was observed under microscope (1000X).

3.4.5. Biochemical Characterization

Various biochemical tests like catalase, oxidase, citrate utilization, indole and hydrogen sulphide production, urea hydrolysis, Tween 20 hydrolysis, starch hydrolysis, temperature tolerance, NaCl tolerance and motility tests were performed which are listed in Appendix X (Singh, *et al.,* 2012).

3.5. Fermentation:

The isolated samples from primary screening were fermented for obtaining the purified form of actinomycetes, for evaluating the antibacterial activity of the product. The isolated actinomycetes which showed zone of inhibition (ZOI) in primary screening were inoculated in Yeast Mannitol Broth (YMB). In YMB, different concentration of sodium nitrate was added (0.5%, 1%, 1.2%, 1.5%) separately. It was fermented by sub-merged fermentation using a shaker incubator at 120 rpm in 28°C for 15 days (Gebreyohannes, 2013).

3.6. Filtration and centrifugation

After fermentation, the respective inoculates samples were filtered and centrifuged. Filtration was done using sterile Whatman no .1 filter paper. The filtrates were then centrifuged at 3000 rpm for 10 minutes. The pellets were discarded, and supernatant were decanted in the respective labeled test tubes (Kumar, *et al.,* 2015).

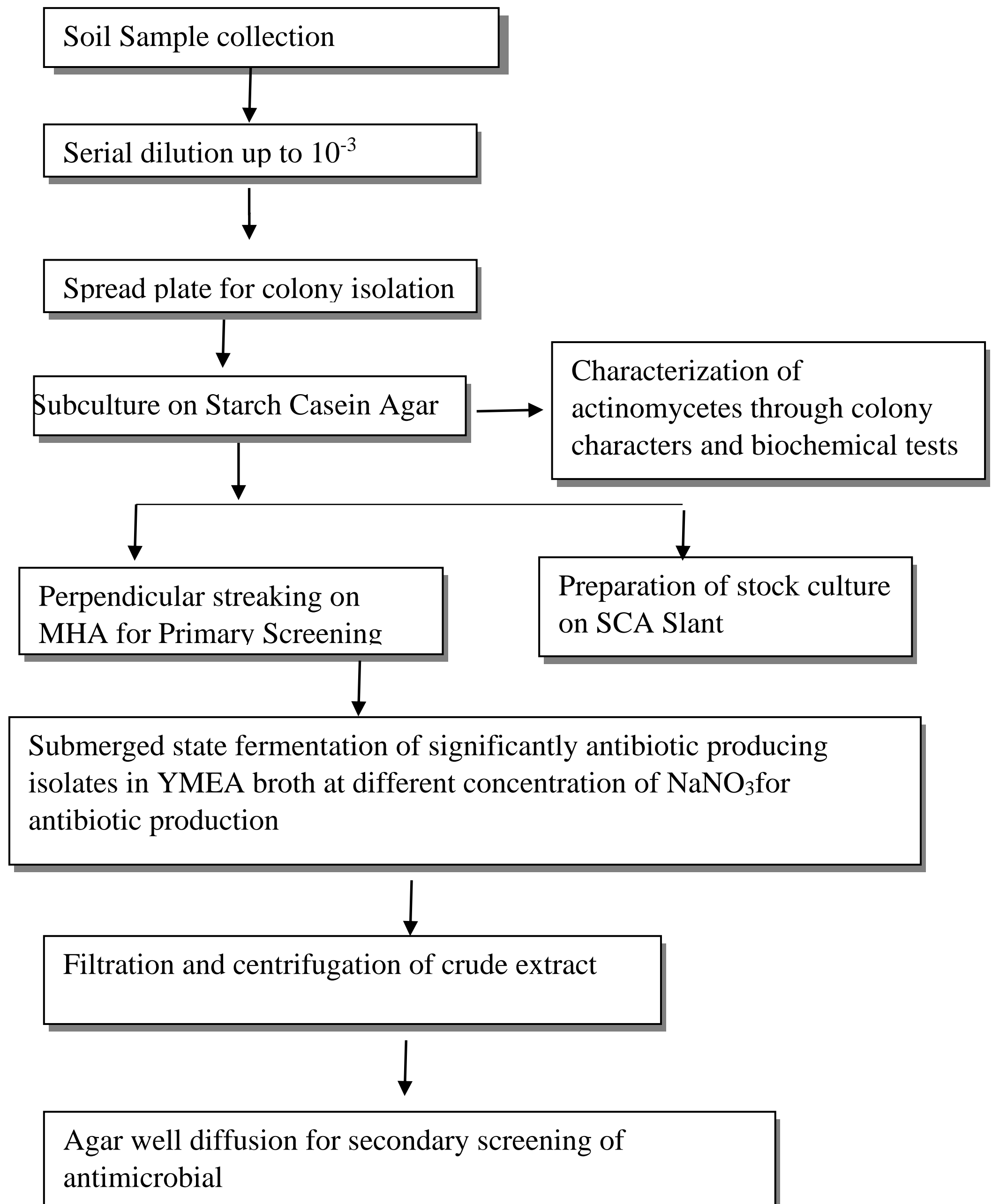


Figure 2: Flow chart for the isolation and screening of actinomycetes. (Sapkota, et al., 2020; Gebreyobannes, 2013; Kumar, et al., 2015)

CHAPTER 4

4. RESULTS AND DISCUSSION

4.1. Isolation of actinomycetes from soil

Thirty soil samples were collected in a sterile plastic bag from 30 sites of Kathmandu which are listed in appendix E. Actinomycetes were isolated from 28 soil samples using Starch M-Protein agar (Aneja, 2018). 28 isolates were found to be actinomycetes which were confirmed by colony and physiological characteristics, microscopic observation, and biochemical tests. Of these isolates, two showed promising activity against different pathogenic microorganisms.

4.2. Characteristics of the antibiotic producing isolates

4.2.1. Colony characteristics

The colonial characteristics of 28 isolated actinomycetes colonies were listed in Appendix-G. The different actinomycetes isolated from the soil sample showed different shades of colors with different pigmentation. Among them, it was revealed that Out of the two isolates, NP110^{-2} (50%) produced Greyish white substrate mycelium whereas MI410^{-3} (50%) produced pale yellow substrate mycelium. Both the isolates (100%) produced Greyish white aerial mycelium. (Table 1).

Table 1: Colony characteristics of the antibiotic producing Actinomycetes isolated on Starch M-protein Agar

Sample code	Texture And elevation	Diameter And opacity	Color	Pigmentation	Presumptive genera
MI410^{-3}	Rough and powdery Raised and crusty	1mm Opaque	Greyish white	Pale yellow	*Streptomyces* spp.
NP110^{-2}	Rough and powdery Raised and crusty	1mm Opaque	Greyish white	No	*Streptomyces* spp.

The texture of both the antibiotic producing isolates of Actinomycetes (100%) were rough and powdery. The average colony diameter of both isolates (100%) was 1mm with rough and crusty elevation and was opaque.

4.2.2. Microscopic characteristics

Microscopic characteristics was done by gram staining method. It was revealed that the isolates $NP110^{-2}$ and $MI410^{-3}$ were found to be Gram positive filamentous rod in appearance with recti flexible and unfragmented mycelium. The mycelium and cellular morphology revealed that all the active isolates were presumable identified as *Streptomyces* spp.

4.2.3. Bio-chemical characteristics

4.2.3.1. Substrate hydrolysis tests:

The substrate (urea, starch, and Tween 20) hydrolysis tests were carried out for 28 isolates to know their ability to hydrolyze urea, starch, and Tween 20 respectively (Appendix -I). Among them, the active actinomycetes isolates were also tested for their ability to hydrolyze urea, Tween 20, starch. It was revealed that urea, starch, and Tween 20 were hydrolyzed by $MI410^{-3}$ (50%), $NP110^{-2}$ (50%) and both $NP110^{-2}$ and $MI410^{-3}$ (100%) respectively. (Table 2).

Table 2: Substrate hydrolysis test of the antibiotic producing Actinomycetes isolates

S. N	Sample code	Hydrolysis tests			
		Urea	Starch	Tween 20	Presumptive genera
1	$NP110^{-2}$	-	+	+	*Streptomyces* spp.
2	$MI410^{-3}$	+	-	+	*Streptomyces* spp.

4.2.3.2. Other bio-chemical tests

Other bio-chemical tests were carried out for 28 isolates to identify the isolates and their ability to produce enzymes i.e., catalase, oxidase, tryptophanase and citrate and other products (Appendix J). Among them, the antibiotic producing Actinomycetes isolates were subjected to various biochemical tests namely oxidase, catalase, sulphur, indole production, motility, methyl red, Voges Proskauer, citrate, urease.

The result showed that NP110^{-2} was positive to catalase, oxidase, and indole production but negative to methyl red, Voges Proskauer, citrate utilization, H_2S production and urease tests respectively while MI410^{-3} was positive to catalase, oxidase, indole production, methyl red, citrate utilization and urease tests while negative to Voges Proskauer and H_2S production tests respectively. (Table 3).

Table 3: Biochemical test of the antibiotic producing Actinomycetes isolates

Isolate code	Other biochemical tests						
	Catalase	**Oxidase**	**Indole**	**Methyl red**	**Voges Proskauer**	**Citrate utilization**	**H_2S production**
NP110^{-2}	+	+	+	-	-	-	-
MI410^{-3}	+	+	+	+	-	+	-

4.2.3.3. Physiological characteristics

Temperature tolerance test, NaCl tolerance test and motility tests were performed on the antibiotic producing actinomycetes isolates. NP110^{-2} (50%) of the two potential isolates was able to grow at 37°C. Both the isolates i.e., NP110^{-2}and MI410^{-3} (100%) were able to grow at 15°C and 28 ºC respectively. NP110^{-2}and MI410^{-3} (100%) were able to grow in 5% NaCl whereas NP110^{-2} and MI410^{-3}(100%) showed no growth in 10% NaCl respectively. Similarly, both the isolates (100%) were found to be non-motile while tested in SIM. (Table 4).

Table 4: Physiological tests of the antibiotic producing Actinomycetes isolates

Isolate code	Physiological tests						
	Temperature tolerance			**NaCl tolerance**		**Motility test**	**Presumptive genera**
	15º C	**28º C**	**37º C**	**5%**	**10%**		
NP110^{-2}	+	+	+	+	-	-	*Streptomyces* spp.
MI410^{-3}	+	+	-	+	-	-	*Streptomyces* spp.

4.3. Sub-merged state fermentation

The visible pellets and clumps along with turbidity was observed in the broth indicating the production of antibiotic.

4.4. Screening of actinomycetes for antimicrobial activity

4.4.1. Primary screening of actinomycetes

A total 28 samples were isolated in Starch m-protein agar and were subjected to primary screening against a gram-positive bacterium (*Staphylococcus aureus* ATCC 43300) and three-gram negative bacteria (*Escherichia coli* ATCC 35218, *Escherichia coli* ATCC 25922 and *Klebsiella* spp. ATCC 700603) by perpendicular streaking method on Mueller Hinton Agar (Ganesan, 2017). It was revealed that the samples MI4 10^{3} and NP110^{-2} showed positive result for the antibiotic producing actinomycetes. Out of 28 isolates of actinomycetes, only MI410^{-3} and NP110^{-2} (7.14%) showed antibacterial activity against test bacteria. (Figure 3).

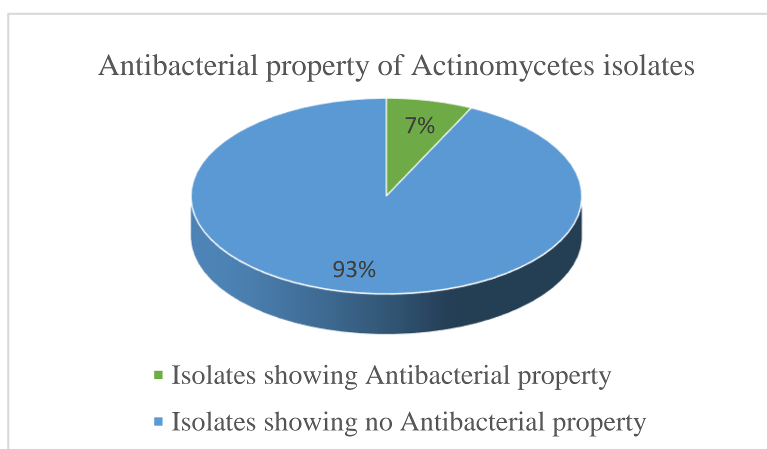


Figure 3: Percentage of Antibiotic producing actinomycetes

4.4.2. Secondary Screening

4.4.2.1. Antibacterial effect of extract at different concentration of $NaNO_3$

It was revealed that the extract MI410^{-3} with 1% $NaNO_3$ had the most effective antibacterial effect against *Escherichia coli* ATCC 25922 in comparison to standard Streptomycin (100µg/mL). Similarly, NP110^{-2} has shown antibacterial effect with 0.5% $NaNO_3$ against *Escherichia coli* ATCC 35218 (14.6mm), *Escherichia coli* ATCC 25922 (19.6mm) and *Staphylococcus aureus* ATCC 43300 (17mm) whereas NP110^{-2} has also shown antibacterial effect with 1.5% $NaNO_3$ against *Escherichia coli* ATCC 35218 (13mm), *Klebsiella* spp. ATCC 700603 (11.5mm) and *Staphylococcus aureus* ATCC 43300 (12.5mm). This indicated that extract MI410^{-3} with 1% $NaNO_3$ was effective

only against *Escherichia coli* ATCC 25922 in comparison while NP110^{-2} with 0.5% and 1.5% $NaNO_3$ was effective against the ATCC cultures. Sterile water was used as negative control and streptomycin (100µg/mL) was used as positive control. (Table 5).

Table 5: Agar well diffusion of the antibiotic producing Actinomycetes isolates

S. N	Sample code	Zone of inhibition (mm)			
		Sc1	Sc2	Sc3	Sc4
1.	MI410^{-3}(0.5%)	-	-	-	-
	MI410^{-3}(1%)	-	35.3	-	-
	MI410^{-3}(1.2%)	-	-	-	-
	MI410^{-3}(1.5%)	-	-	-	-
	Streptomycin (100 µg/mL)	14.5	15	16	17.5
2.	NP110^{-2}(0.5%)	14.6	19.6	-	17
	NP110^{-2}(1%)	-	-	-	-
	NP110^{-2}(1.2%)	-	-	-	-
	NP110^{-2}(1.5%)	13	-	11.5	12.5
	Streptomycin (100 µg/mL)	14.5	15	16	17.5

Note:

Sc: Standard Culture

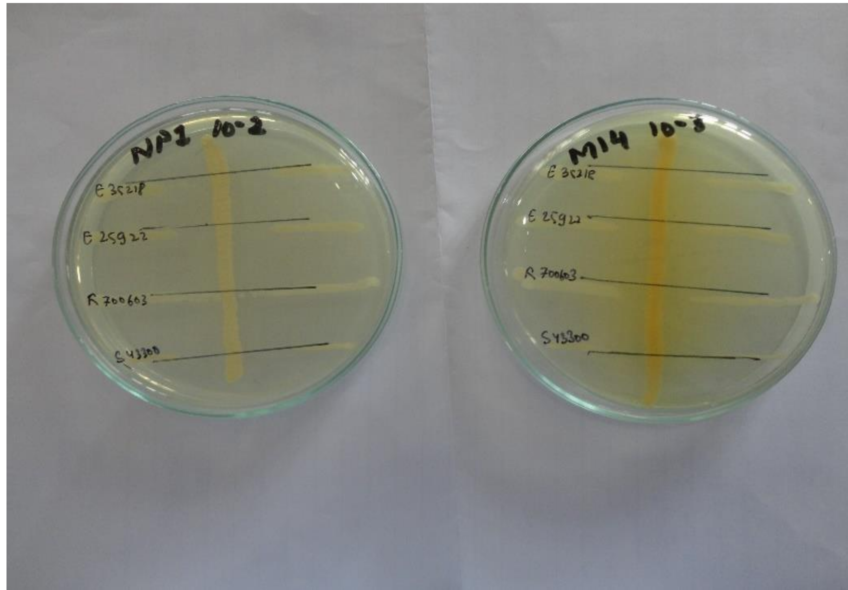
Sc1: *E. coli* ATCC 35218; **Sc2:** *E. coli* ATCC 25922

Sc3: *Klebsiella* ATCC 700603**; Sc4:** *Staphylococcus aureus* ATCC 4330

Statistically, there is significantly difference at 5% level of significance (.001, $P<0.05$) between the sample concentration at 0.5% of $NaNO_3$ in antimicrobial activity. However, there is no significant difference at 5% level of significance between the sample concentration at 1% (0.356, $P>0.05$) and 1.5% of $NaNO_3$ (0.024, $P<0.05$). i.e., Sample MI410^{-3} showed maximum zone of inhibition at 1% $NaNO_3$ concentration against the

test microorganism Sc2, which indicated that the isolate was potential antibiotic producing actinomycetes showing greater activity as compared with standard streptomycin (100 µg/mL) whereas NP1 10^{-2} showed similar zone of inhibition to that of standard streptomycin (100 µg/mL) at 0.5% and 1.5% $NaNO_3$ concentration against the test organism Sc1, Sc2, Sc3 and Sc4, which indicated that the isolate showed similar activity as standard streptomycin (100 µg/mL). Therefore, NP1$10^{-2}$ was found to be potential antibiotic producing actinomycetes.

PHOTOGRAPHS



Photograph 1: Primary screening of sample NP1$10^{-2}$ and MI4$10^{-3}$

Photograph 2: Secondary screening of sample MI4$10^{-3}$

Photograph 3: Secondary screening of sample NP110^{-2}

Photograph 4: Subculturing of the isolates in the Microbiology laboratory of Amrit Campus

4.5. DISCUSSION

Actinomycetes are gram positive filamentous antibiotic producing microorganism mainly found in the soil. Actinomycetes is one of the most important microbes with potential bioactive compounds and antibiotic which is also supported by quotes of Sunthindhiran, *et al.,* (2009).

Streptomyces are prolific and can produce antibiotic effectively along with the potential bio-active compounds. *Streptomyces* covers around 80% of total antibiotic products as mentioned by Hopwood, *et al.,* (2000). With reference to Moncheva, *et al.,* (2002), in the 60's and the 70's of the twentieth century (75-85%) of the discovered antibiotics were derived from order actinomycetes, mainly from *Streptomyces* spp. and it is still one of the promising antibiotic producers.

In this study despite of challenges as it has dual characteristics of both bacteria and fungi; a good number (28 of 30) of actinomycetes were isolated from the soil of Kathmandu valley. All the isolates were slow- growing aerobic with diverse colored with substrate mycelia. Most of them produced pigments namely brown, pink, purple, yellow, green, red on starch M-protein agar (SCA). The texture and color of colonies varied according to different sites. Actinomycetes generally take one to two weeks to develop matured aerial mycelium with spores.

Different environmental constrains and growth factors influence the growth and diversity of actinomycetes, temperature being one of them. The higher number to actinomycetes isolates were found from agricultural and forest land according to the report of Ghorbani, *et al.,* (2013). Abundance of actinomycetes isolates decreases from irrigated cultivated land to pastures. According to Sapkota, *et al.,* (2019), it has profound effect in the physiological, morphological, sporulation, biochemistry, and antimicrobial metabolite production of organisms. In the present study, the optimum temperature for the growth of actinomycetes was found to be in mesophilic range. Similarly, the antibiotic producing actinomycetes can grow at 5% salt concentration indicating that the isolates have mild salt tolerance capacity. Therefore, the isolated actinomycetes were presumed to be *Streptomyces* spp. as per their colonial characteristics, morphological, bio-chemical tests, physiological tests etc.

Currently, the incidence of multidrug resistance organism is increasing and compromising the treatment of growing number of infectious diseases (Okami, 1988)

The present study is similar with Tamamura, *et al.* (1985) as detecting the antibiotic producing actinomycetes from Kathmandu valley will be a fruitful in pharmacological sector and obviously it might fulfill the urgent need for the development of new antibiotic.

In the present study, both the primary and secondary steps were used to screen antibiotic producing actinomycetes. Here, the first screening was done by streaking all the 28 isolates perpendicularly against the standard ATCC cultures namely, *Staphylococcus aureus* ATCC 43300, *Escherichia coli* ATCC 35218, *Escherichia coli* ATCC 25922 and *Klebsiella* spp. ATCC 700603 to determine the antibiotic producing actinomycetes among the 28 isolates and to know the range of antimicrobial effect of actinomycetes against the standard four different ATCC cultures. Out of 28 isolates, 2 isolates showed antimicrobial activity against the test organism. The similar study was carried out by Budhathoki, *et al.,* (2020), where out of 41 pure isolates, 19 isolates showed antimicrobial activity against test organism. The isolates showed greater activity against gram-negative test organism than gram-positive. Unlike Sapkota, et al., 2020 more activity was shown in gram positive test organism. Similarly, the difference in sensitivity might be due to the morphological difference between gram positive and gram-negative bacteria, which is supported by Shirling, *et al.,* (1966) study.

All the isolates were streaked on MHA plate by perpendicular streaking method to determine their antimicrobial property according to Sapkota, *et al.,* (2020) against gram-positive bacteria (*Staphylococcus aureus* ATCC 35218) and three gram- negative bacteria (*Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 35218 and *Klebsiella* spp. ATCC 700603). Out of 28 isolates only two (7%) showed antibacterial property i.e., zone of inhibition (ZOI) against test organism. In study carried out by Budhathoki, *et al.,* 2020, When 19 isolates were subjected to primary screening, 13 isolates showed zone of inhibition. One isolate ($MI10^{-2}$) showed activity against *Escherichia coli* and another isolate ($NP110^{-3}$) showed activity against *Escherichia coli* and *Staphylococcus aureus*. One isolate showed antimicrobial activity against more than one test organism, whereas, in study conducted by Budhathoki et al., 2020, 7 isolates showed antimicrobial activity against more than one test organism. the variation in antimicrobial activity can be observed due to the variation in sampling sites and sampling numbers. Furthermore, the successful antibiotic producing isolates were fermented in yeast extract mannitol broth (YEM broth) with different concentration of $NaNO_3$ (0.5%, 1%, 1.2% and 1.5%)

in shaker incubator at 28°C for 15 days. The crude extract obtained after filtration and centrifugation were then allowed for secondary screening in Muller Hinton agar by Agar well diffusion against the ATCC cultures in accordance with Mishra, *et al.* (2012).

Likewise, the secondary screening method with varied $NaNO_3$ concentration was done mainly to determine the effect of $NaNO_3$ concentration in antibiotic production as mentioned in study of Mishra, *et al.* (2012). Sodium nitrate due to its antimicrobial property (Zemke, 2014), was used for determination of potential antibiotic producing actinomycetes. The different concentration of sodium nitrate used showed different activities against the test organism. The present study revealed that the isolated actinomycetes showed zone of inhibition which determined its antibiotic property against the test organisms. The isolate $NP110^{-3}$ showed maximum activity against *Escherichia coli*, *Klebsiella* spp. and *Staphylococcus aureus* with zone of inhibition 19.6mm, 17mm and 14.6mm. In similar study carried out in Kumar, *et al.,* 2015. The maximum antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumonia* with a zone of inhibition of 17mm, 19.8mm and 8.2mm respectively. The different concentration of $NaNO_3$ affected the antibiotic property of the isolated *Streptomyces* spp. According to Mishra, *et al.* (2009), sodium nitrate in increasing concentrations was found to have an enhancing effect on the bacterial antibiotic activity, but high concentration that is more than 1.2% was found detrimental. At 1.4% the activity was found less inferring the effect of high concentration of sodium nitrate in antibiotic production. Unlike, the isolate $MI410^{-3}$ with 1% $NaNO_3$ (35.33 mm) had the most effective antibacterial effect against *Escherichia coli* ATCC 25922 in comparison to standard Streptomycin (100 µg/mL). In the study carried out by Mishra, et al., (2009) with 1% $NaNO_3$ 11mm zone of inhibition was observed. $NP110^{-2}$ with 0.5% and 1.5% $NaNO_3$ was effective against the ATCC cultures which showed similar activity as standard streptomycin. Therefore, the isolated sample $MI410^{-3}$ showed greater activity than standard streptomycin which concludes that the isolate was potential antibiotic producing actinomycetes. This is also proven statistically i.e., there is significantly difference at 5% level of significance between the sample concentration of $NP110^{-2}$ at 0.5% of $NaNO_3$ (.001, $P<0.05$) and 1.5% of $NaNO_3$ (0.024, $P<0.05$) in antimicrobial activity. However, there is no significant difference at 5% level of significance between the sample concentration of $MI410^{-3}$ at 1% (0.356, $P>0.05$).

CHAPTER 5

5. CONCLUSION AND RECOMMENDATION

5.1. Conclusions

Actinomycetes isolated from different places of Kathmandu valley revealed antimicrobial properties. Among 30 soil samples, 28 isolates of actinomycetes were obtained. The isolated actinomycetes were presumed to be *Streptomyces* genera. The indigenous actinomycetes can be used to produce antibiotics on an industrial scale, which can benefit the nation's economy. At low concentration (0.5%) and high concentration of $NaNO_3$ (1.5%) enhance the efficacy of $NP110^{-2}$ as potent antibiotic producers.

5.2. Novelty and national prosperity aspect of project work

Soil harbor large number of actinomycetes isolates which exhibit inhibitory activity against bacterial strain. Due to geographic variation in Nepal, it is quite likely that there is high probability of the presence of antibiotic producing actinomycetes especially *Streptomyces*. The isolation and subsequent screening of antibiotic producing microbes such as *Streptomyces* form diverse habitats especially from a virgin country like Nepal might lead to discovery of many novel and useful secondary metabolites. The production of novel antibiotics can help in economic growth of nation.

5.3. Limitations of work

1. Spores produced by actinomycetes were not studied due to the lack of time.
2. Protein profiling for crude extract couldn't be done due to time limitation.
3. Molecular characterization was not carried during the study.
4. Time and Budget limited the study.

5.4. Recommendations

1. Actinomycetes from different unexplored places can be isolated to obtain novel antibiotic.
2. Protein profiling for crude extract can be done for obtaining the pure form of antibiotic.
3. Extraction of novel antibiotic might be tested for revealing multi drug resistance bacteria.

4. Data and information from different research can be used for the development and commercial production of antibiotic.
5. Discovery of novel antimicrobial producing strains is important as the pre-existing drugs have failed due to the development of resistance among the microorganisms.

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APPENDICES

APPENDIX I

Materials used

Equipment's

1. Autoclave (life, India)
2. Incubator (leader, UK)
3. Incubator (Memmert, Germany)
4. Hot air oven (Ambassador)
5. Binocular microscope (COSLAB, India)
6. Refrigerator (LG, India)
7. Electronic weighing balance (Phoenix instrument, Germany)
8. Bunsen burner
9. Shaker incubator
10. Centrifuge

Glass-wares/ Plastic- wares:

1. Beaker
2. Sampling bottle
3. Conical flakes
4. Petri plates
5. Pipettes
6. Measuring cylinders
7. Test tube
8. Micropipette
9. Microtips
10. Cork borer

Media (Hi Media Laboratories Pvt. Ltd)

1. Starch M- protein agar
2. Muller Hinton agar
3. Nutrient agar

4. Nutrient broth
5. Sulphite indole motility media
6. Urea agar base
7. Simmons's citrate agar
8. Hugh and Leifson's media
9. MR/VP medium
10. Triple sugar iron agar

Chemicals

1. Crystal violet
2. Dehydrated alcohol
3. Gram's iodine
4. Safranin
5. Catalase reagent
6. Oxidase reagent
7. Kovac's reagent
8. Methyl red reagent
9. Voges-Proskauer reagent

Miscellaneous

1. Inoculating loop and inoculating needle
2. Forceps
3. Pipette filler
4. Paraffin oil
5. Labelling tag
6. Cotton
7. Aluminum foil
8. Tissue paper
9. Tray
10. Test tube rack
11. Record book and pencils

Test organism

1. *E. coli* ATCC 25922
2. *E. coli* ATCC 35218
3. *Staphylococcus aureus* ATCC 43300
4. *Klebsiella* spp. ATCC 700603

APPENDIX II

COMPOSITION AND PREPARATION OF DIFFERENT STAINING REAGENTS

1. Gram staining reagent

a) Crystal violet (Hucker's modification) Composition

Solution A

Ingredients	gms/liter
Crystal violet (90% dye content)	2.0gm
Ethanol (90%)	20.0ml
Solution B	
Ammonium oxalate	0.8gm
Distilled water	80.0 ml

Preparation

In 20 mL ethyl alcohol, 2 gm of crystal violet was dissolved, and 0.8 gm of ammonium oxalate was dissolved in 80 mL distilled water. Both the solutions A and B were mixed and was transferred to clean reagent bottle.

b) Gram's Iodine Composition

Ingredients	gms/liter
Iodine	1.0gm
Potassium iodide	2.0gm
Distilled water	300.0 mL

Preparation

To 300 mL distilled water, 1gm of iodine and 2gm of potassium iodide was added and mixed well to dissolve and was transferred to clean reagent bottle.

c) Acetone-alcohol decolorizer Composition

Ingredients	gms/liter
Acetone	500mL
Ethanol (absolute)	475mL
Distilled water	25mL

Preparation

25 mL distilled water was mixed with 475 mL absolute ethanol. then, 500 mL acetone was added immediately to the alcohol solution and mixed well and was transferred to the clean reagent bottle.

d) Safranin Composition

Ingredients	**gms/liter**
Safranin (2.5% solution in 95% ethyl alcohol)	10.0 mL
Distilled water	100.0 mL

Preparation

10 mL safranin was added to 100mL distilled water and mixed well. Then it was transferred to clean reagent bottle.

2. Catalase reagent Composition

Ingredients	**gms/liter**
Hydrogen peroxide (6%)	50mL
Distilled water	50 mL

Preparation

To 50 mL distilled water, 50 mL hydrogen peroxide (6%) was added and mixed well.

3. Oxidase reagent Composition

Ingredients	**gms/liter**
Tetra methyl paraphenylenediamines dihydrochloride	5.0gm
Distilled water	50mL

Preparation

This reagent was prepared by dissolving 5.0 gm of reagent in 50 mL of distilled water. To that solution stripe of Whatman No.1 filter paper was soaked and drained for about 30 sec. then these stripes were completely dried and stored in dark bottle tightly sealed with a screw cap.

4. Kovac's reagent Composition

Ingredients	**gms/liter**
p-dimethyl amino benzaldehyde	5.0gm

Amyl alcohol	75.0 mL
Concentrated HCL	25.0 mL

Preparation

In 75 mL of amyl alcohol, 5 gm of reagent was dissolved in clean brown bottle. Then, to it, 25 mL of concentrated HCL was added and mixed well.

5. Methyl red reagent Composition

Ingredients	**gms/liter**
Methyl red	0.04 gm
Ethanol (absolute)	40mL
Distilled water	60mL

Preparation

0.04 gm of methyl red was dissolved in 40 mL of ethanol. Then, 60 mL distilled water was added and mixed well.

6. Voges- Proskauer reagent (Barritt's reagent) Composition

a) VP reagent A

Ingredients	**gms/liter**
Alpha- naphthol	5gm
Ethanol (absolute)	100mL

Preparation

To 28 mL distilled water, 5 gm of alpha naphthol was dissolved and transferred to a clean brown bottle. then final volume was made 100 mL by adding distilled water.

b) VP reagent B

Ingredients	**gms/liter**
Potassium hydroxide	40gm
Distilled water	100mL

Preparation

40 gm potassium hydroxide was dissolved and transferred to a clean brown bottle. then, final volume was made 100 mL by adding distilled water.

(Source: Aneja 2003, Cheesbrough 2006).

APPENDIX III

a. COMPOSITION AND PREPEARTION OF DIFFERENT CULTURE MEDIA

1. Starch M- protein Composition

Ingredients	gms/liter
Sodium starch	10.00
M-protein	0.300
Potassium nitrate	2.000
Sodium chloride	2.000
Dipotassium hydrogen phosphate	2.000
Magnesium sulphate, heptahydrate	0.050
Ferrous sulphate, heptahydrate	0.010
Calcium carbonate	0.020
Agar	15.00
Final pH(25°C)	7.2±0.2

Preparation:

31.35 grams was suspended in 1000mL distilled water containing 5 mL glycerol. It was boiled to dissolve the medium completely. then, sterilized by autoclaving at 15 lbs. pressure for 15 minutes and cooled to 40-50°C then it was mixed well and poured into sterile petri plates.

2. Muller Hinton agar Composition

Ingredients	gms/liter
Beef infusion B	300.000
Casein acid hydrolysate	17.500
Starch	1.500
Agar	17.000
Final pH (at 25°C)	7.3± 0.1

Preparation

38.0 grams was suspended in 1000mL of purified distilled water. It was boiled until it dissolves completely. Then sterilized by autoclaving at 15lbs pressure (121°C) for 15minutes and cooled to 40-50 °C then it was mixed well and poured in sterile petri plates.

3. Nutrient Agar Composition

Ingredients	**gms/liter**
Peptic digest of animal tissue	5.0gm
Sodium chloride	5.0gm
Beef extract	1.50 gm
Yeast extract	1.50 gm
Agar	15.00 gm
Final p^H (at 25°C)	7.4 ± 0.2

Preparation

28 grams was suspended in 1000ml distilled water and was boiled to dissolve the medium completely. then, sterilized by autoclaving at 15 lbs. pressure (121 °C) for 15 mins and cooled to 45-50°Cthen it was mixed and poured into sterile Petri plates.

4. Nutrient broth Composition

Ingredients	**gm/l**
Peptic digest of animal tissue	5.00 gm
Sodium chloride	5.00 gm
Beef extract	1.50 gm
Yeast extract	1.50 gm
Final pH (at 25°C)	7.4 ±0.2

Preparation

13.0 grams was suspended in 1000 mL distilled water and heated to dissolve the medium completely. The medium was then sterilized by autoclaving at 15lbs pressure (121°C) for 15 minutes.

b. COMPOSITION AND PREPARATION OF DIFFERENT BIOCHEMICAL MEDIA

1. Hugh and Leifson's medium Composition

Ingredients	gms/liter
Casein enzymic hydrolysate	2.00gm
Sodium chloride	5.00 gm
Dipotassium phosphate	0.30 gm
Bromo thymol blue	0.08 gm
Agar	2.00 gm
Final pH (at 25°C)	6.8 ± 0.2

Preparation

1.38 grams were suspended in 1000 mL distilled water and heated to boiling to dissolve the medium completely. 100mL amounts were dispensed and to the first 100 mL of sterile basal medium, 1gm of dextrose solution was added aseptically then medium was sterilized by autoclaving at 110°Cfor 15 minutes. all the solution was mixed and was dispensed in 5 mL amounts in sterile tubes in duplicate for aerobic and anaerobic fermentation.

2. Sulphide Indole Motility Medium Composition

Ingredients	gms/liter
Peptic digest of animal tissue	30.00 gm
Beef extract	3.00 gm
Peptonized iron	0.20 gm
Sodium thiosulphate	0.025 mL
Agar	3.00 gm
Final pH (at 25°C)	7.3± 0.2

Preparation

36.23 grams of medium was suspended in 1000ml distilled water and heated to boiling to dissolve the medium completely. The medium is then dispensed in tubes and sterilized by autoclaving at 15 lbs. pressure (121°C) for 15 minutes. The tubes were allowed to cool in the upright position.

3. MR-VP medium Composition

Ingredients	gms/liter
Buffered peptone	7.00 gm
Dextrose	5.00 gm
Dipotassium phosphate	5.00 gm
Final pH (at 25°C) 6.9 ± 0.2	

Preparation

17.0 grams was suspended in 1000 mL of distilled water and heated (If necessary) to dissolve the medium completely. The medium was then distributed in 100 amounts in test tubes and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

4. Simmons citrate agar Composition

Ingredients	gms/liter
Ammonium dihydrogen phosphate	1.00 gm
Dipotassium hydrogen phosphate	1.00 gm
Sodium chloride	5.00 gm
Sodium citrate	2.00 gm
Magnesium sulphate	0.20 gm
Bromothymol blue	0.80 gm
Agar	15.00 gm
Final p^H (at 25°C)	6.8 ± 0.2

Preparation

24.28 grams was suspended in 1000 mL distilled water and was heated to dissolve the medium completely. The medium was then dispensed as desired in tubes or flasks and sterilized by autoclaving at 15 lbs. pressure (121°C) for 15 minutes.

5. Triple sugar iron agar Composition

Ingredients	gms/liter
Peptic digest of animal tissue	10.00
Casein enzymatic hydrolysate	10.00
Yeast extract	3.00
Beef extract	3.00

Lactose	10.00
Sucrose	10.00
Dextrose	1.00
Sodium chloride	5.00
Ferrous sulphate	0.20
Sodium thiosulphate	0.30
Phenol red	0.024
Agar	12.00
Final pH (at 25 °C)	7.4 ± 0.2

Preparation

64.52 grams was suspended in 1000 mL distilled water and heated to boiling to dissolve the medium completely. The medium was mixed well and was distributed into test tubes and sterilized by autoclaving at 15 lbs. pressure (121°C) for 15 minutes. The medium was allowed to set in sloped form with a butt about 1 inch long.

6. Urea agar base Composition

Ingredients	gms/liter
Peptic digest of animal tissue	1.00
Dextrose	1.00
Sodium chloride	5.00
Mono potassium phosphate	1.20
Phenol red	0.012
Agar	15.00
Final pH (at 25 °C)	6.8±0.2

Preparation

24.0 grams was suspended in 950 ml distilled water and heated to boiling to dissolve the medium completely. The medium was then sterilized by autoclaving at 10 lbs. Pressure (115 ºC) for 20 minutes. The medium was cooled to 50 ºC

and 47.5 ml sterile 40% urea solution aseptically and mixed well. The medium was dispensed in sterile tubes and allowed to set in the slanting position.

7. Tween 20 agar Composition

Ingredients	**gm/l**
Peptone	10gm
Sodium chloride	5gm
Calcium chloride dihydrate	0.1gm
Agar	20gm
Tween 20	10ml
Distilled water	1000ml
Final pH	7.0 ±0.2

Preparation:

First four ingredients were suspended in 1000 ml distilled water and heated to boiling to dissolve the medium completely. The medium was then sterilized by autoclaving at 15 lbs. Pressure (121°C) for 15 minutes. And 10ml sterile tween 20 was added in the mixture and aseptically poured in sterile Petri plates.

8. Starch agar Composition

Ingredients	**gm/l**
Starch (soluble)	20.0gm
Peptone	5.0 gm
Beef extract	3.0 gm
Agar	15.0 gm
Distilled water	1000.0ml

Preparation:

25.0 grams was suspended in 1000 ml distilled water and heated to boiling to dissolve the medium completely. The medium was then sterilized by autoclaving at 15lbs.pressure (121°C) for 15 minutes.

APPENDIX IV
METHODOLOGY OF BIOCHEMICAL TEST FOR THE IDENTIFICATION OF ACTINOMYCETES

A. Catalase test

This test was performed by picking up a few colonies with applicator stick and mixing it with a drop of 3% h_20_2 on a clean glass slide. Positive test result was indicated by the appearance of gas bubbles.

B. Oxidase test

This test was done by picking up a few colonies with applicator stick and rubbing it on the oxidase paper (Whatman No 1% tetra methyl-para-phenylene-diamine dihydrochloride). Positive test result was indicated with the development of intense deep purple color on the oxidase paper.

C. Citrate utilization test

This test was performed by streaking the in the slant of Simmons's Citrate Agar tubes with the actinomycetes colonies and incubating the tubes at 28 ^oC for 48 hours. Citrate ultization was detected by change of color of medium from dark green to Prussian blue color.

D. Indole, motility, and hydrogen sulphide production test

These two tests were done by stabbing sulphide indole motility (SIM) medium tube with the actinomycetes colonies and incubating the tubes at 28 for 48 hours. Indole production was detected by the production of cherry red color at the interface upon the addition of Kovac's reagent. H_2S production was detected by blackening of medium. Fuzzy growth on the stab line indicated that the organism was motile.

E. Urea hydrolysis test:

Urea agar slant was streaked with actinomycetes colonies and incubated at 28 ^{o}Cfor 48 hours. Positive test was indicated by the change of color of the slant from orange to pink.

F. Starch hydrolysis test.

Starch agar plates were incubated with the isolates and incubated at 28 ^{o}C for 2 weeks. Starch hydrolysis was confirmed by flooding the plates with iodine solution to observe the clear zone of hydrolysis around the colonies.

G. Tween 20 hydrolysis test

The isolates were streaked on solidified twee 20 agar plates and incubated at 28 degree for 4 weeks. Positive test was indicated by the appearance of clear zone around the colony.

H. Temperature tolerance test:

The isolates were streaked in starch m-protein agar and incubated at 15, 28 and 37 ^{o}C for 2 weeks. Positive test was indicated by the growth of isolates.

I. NaCl tolerance test:

Nutrient agar plates with 5% and 10% NaCl were inoculated with the isolates and incubated at 28 ^{o}C for 2 weeks. Positive test was indicated by the growth of the isolates.

APPENDIX V

PLACES OF SOIL SAMPLE SITE

Sample code	Sample site	Isolated actinomycetes	Antibiotic producing actinomycetes
LH5	Lolang height	+	-
NT1	Nepaltar	+	-
DS2	Dharmasthali	+	-
MJ2	Maharajgunj	+	-
BB6	Basbari	+	-
BK2	Budhanilkantha	+	-
SP1	Shivapuri	+	-
MP8	Muhanpokhari	-	-
KH2	Kapan Height	+	-
BG2	Bhangal Height	+	-
TK11	Tokha	-	-
SY3	Swayambhu	+	-
BH3	Balaju Height	+	-
TH3	Thamel	+	-
RB1	Ranibari	+	-
PP2	Pepsicola	+	-
MH1	Manohara	+	-
NP1	New Planning	+	+
KP6	Kritipur	+	-
ST2	Sano Thimi	+	-
LK6	Lower Khokana	+	-
UK3	Upper khokana	+	-
CV2	Chovar	+	-
CG2	Chovar Gate	+	-
UN3	UN park	+	-
SK1	Sankhamul	+	-
SG4	Sallaghari	+	-
BP2	Bhaisepati	+	-
MI4	MadhyapurThimi	+	+
MG3	Magar Gau	+	-

Note: (+) = present, (-) = absent

APPENDIX VI

GRAM STAINING RESULT

S. N	Sample code	Observation
1	LH5	Gram positive, filamentous Rod-shaped bacteria
2	NT1	Gram positive, filamentous Rod-shaped bacteria
3	DS2	Gram positive, filamentous Rod-shaped bacteria with mix culture.
4	MJ2	Gram positive, filamentous Rod-shaped bacteria with mix culture.
5	BB6	Gram positive, filamentous Rod-shaped bacteria
6	BK2	Gram positive, filamentous Rod-shaped bacteria
7	SP1	Gram positive, filamentous Rod-shaped bacteria
8	KH2	Gram positive, filamentous Rod-shaped bacteria
9	BG2	Gram positive, filamentous Rod-shaped bacteria
10	SY3	Gram positive, filamentous Rod-shaped bacteria
11	BH3	Gram positive, filamentous Rod-shaped bacteria
12	TH3	Gram positive, filamentous Rod-shaped bacteria
13	RB1	Gram positive, filamentous Rod-shaped bacteria
14	PP2	Gram positive, filamentous Rod-shaped bacteria with mix culture.
15	MH1	Gram positive, filamentous Rod-shaped bacteria
16	NP1	Gram positive, filamentous Rod-shaped bacteria
17	KP6	Gram positive, filamentous Rod-shaped bacteria
28	ST2	Gram positive, filamentous Rod-shaped bacteria
19	LK6	Gram positive, filamentous Rod-shaped bacteria with mix culture.
20	UK3	Gram positive, filamentous Rod-shaped bacteria
21	CV2	Gram positive, filamentous Rod-shaped bacteria
22	CG2	Gram positive, filamentous Rod-shaped bacteria
23	UN3	Gram positive, filamentous Rod-shaped bacteria
24	SK1	Gram positive, filamentous Rod-shaped bacteria
25	SG4	Gram positive, filamentous Rod-shaped bacteria
26	BP2	Gram positive, filamentous Rod-shaped bacteria with mix culture.
27	MI4	Gram positive, filamentous Rod-shaped bacteria
28	MG3	Gram positive, filamentous Rod-shaped bacteria

APPENDIX VII

MORPHOLOGICAL CHARACTERISTICS

Sample symbol	Colony color	Texture	Diameter (in mm)	Pigmentation	Elevation	Opacity
MH1	Grey	Rough, powdery	3	No	Raised, crusty	Opaque
UK3	White	Rough, powdery	1	No	Raised, crusty	Opaque
BP2	White	Rough, powdery	1	No	Raised, crusty	Opaque
DS2	White	Rough, powdery	1	No	Raised, crusty	Opaque
CG2	White	Rough, powdery	2	No	Raised, crusty	Opaque
BG2	White	Rough, powdery	2	Greenish black	Raised, crusty	Opaque
NP1	White	Rough, powdery	1	No	Raised, crusty	Opaque
SY3	White	Rough, powdery	1	No	Raised, crusty	Opaque
LH5	Blackish Grey	Rough, powdery	1	Dark green	Raised, crusty	Opaque
MI4	White	Rough, powdery	1	Pale yellow	Raised, crusty	Opaque
TH3	White	Rough, powdery	1	No	Raised, crusty	Opaque
ST2	White	Rough, powdery	1	No	Raised, crusty	Opaque
PP2	White	Rough, powdery	1	Brown	Raised, crusty	Opaque
BB6	White	Rough, powdery	1	Brown, red	Raised, crusty	Opaque

SK1	White	Rough, powdery	3	Purple red	Raised, crusty	Opaque
MG3	Grey	Rough, powdery	1	Greenish black	Raised, crusty	Opaque
CV2	White	Rough, powdery	1	No	Raised, crusty	Opaque
RB1	White	Rough, powdery	2	Pale yellow	Raised, crusty	Opaque
KP6	Greyish white	Rough, powdery	1	Brownish black	Raised, crusty	Opaque
UN3	White	Rough, powdery	2	Greenish brown	Raised, crusty	Opaque
LK6	White	Rough, powdery	1	No	Raised, crusty	Opaque
BK2	Grey	Rough, powdery	1	Green	Raised, crusty	Opaque
SP1	Pale white	Smooth	1	No	Raised	Opaque
BH3	Pinkish white	Rough, powdery	2	Pale yellow	Raised, crusty	Opaque
SG4	Pinkish white	Rough, powdery	2	Pale yellow	Raised, crusty	Opaque
KH2	Dark grey	Rough, powdery	1	No	Raised, crusty	Opaque
MJ2	White	Rough, powdery	1	No	Raised, crusty	Opaque
NT1	White	Rough, powdery	1	No	Raised, crusty	Opaque

APPENDIX VIII

PHYSIOLOGICAL TESTS

Sample code	**Physiological test**					
	Temperature tolerance			**NaCl tolerance**		**Motility test**
	15°C	**28°C**	**37°C**	**5%**	**10%**	
MH1	+	+	+	+	-	-
UK3	+	+	+	+	-	-
BP2	+	+	+	+	+	-
DS2	+	+	+	+	+	+
CG2	+	+	+	+	-	-
BG2	+	+	+	+	-	-
NP1	+	+	+	+	-	-
SY3	+	+	+	+	-	-
LH5	+	+	+	+	-	-
MI4	+	+	-	+	-	+
TH3	+	+	+	+	-	+
ST2	+	+	+	+	-	-
PP2	+	+	+	+	+	-
BB6	+	+	+	+	-	+
SK1	+	+	+	+	-	-
MG3	+	+	+	+	+	+
CV2	+	+	-	+	-	-
RB1	+	+	+	+	+	+
KP6	+	+	+	+	+	-
UN3	+	+	+	+	+	-
LK6	+	+	+	+	+	-
BK2	+	+	+	-	-	-
SP1	+	+	+	-	-	-
BH3	+	+	-	+	-	-
SG4	+	+	-	+	-	-
KH2	+	+	-	+	-	-
MJ2	+	+	+	+	-	+
NT1	+	+	+	+	-	-

Note: (+) = Positive, (-) = Negative

APPENDIX IX

HYDROLYSIS TEST

Sample symbol	Hydrolysis test of		
	Urea	Starch	Tween 20
MH1	-	-	+
UK3	+	+	+
BP2	+	+	+
DS2	+	+	+
CG2	-	+	+
BG2	+	-	+
NP1	-	+	+
SY3	+	-	-
LH5	-	+	+
MI4	+	-	+
TH3	+	-	+
ST2	-	+	+
PP2	-	-	+
BB6	-	+	+
SK1	+	-	+
MG3	-	-	+
CV2	+	-	+
RB1	+	+	+
KP6	+	+	+
UN3	+	-	+
LK6	+	+	+
BK2	+	-	+
SP1	+	+	+
BH3	-	-	+
SG4	-	-	-
KH2	+	+	+
MJ2	+	-	-
NT1	+	+	+

Note: (+) = Positive, (-) = Negative

APPENDIX X

BIOCHEMICAL TEST

Sample code	Catalase	Oxidase	SIM (H_2S production, Indole production, Motility)	MR	VP	Citrate test	Urease test
MH1	+	-	+ - -	-	-	-	-
UK3	+	+	+ - -	+	-	-	+
BP2	+	-	+ - -	+	-	+	+
DS2	+	-	+ - +	-	-	-	+
CG2	+	-	+ - -	-	-	-	-
BG2	+	-	+ - -	-	-	-	+
NP1	+	+	+ - -	-	-	-	-
SY3	+	-	- - -	+	-	-	+
LH5	+	+	+ - -	-	-	-	-
MI4	+	+	+ - +	+	-	+	+
TH3	+	+	+ - +	-	-	-	+
ST2	+	+	+ - -	-	-	-	-
PP2	+	+	+ - -	-	-	-	-
BB6	+	-	+ - +	-	-	+	-
SK1	+	+	+ - -	-	-	+	+
MG3	+	+	- - +	-	-	-	-
CV2	+	-	+ - -	-	-	+	+
RB1	+	+	+ - +	+	-	-	+
KP6	+	+	+ - -	-	-	-	+
UN3	+	-	+ - -	-	-	+	+
LK6	+	-	+ - -	-	-	-	+
BK2	+	-	+ - -	-	-	-	+
SP1	+	-	+ - -	-	-	-	+
BH3	+	-	+ - -	-	-	-	-
SG4	+	-	+ - -	-	-	-	-
KH2	+	-	+ - -	-	-	-	+
MJ2	+	+	+ + +	-	-	+	+
NT1	+	-	+ - -	-	-	+	+

Note: (+) = Positive, (-) = Negative

APPENDIX XI

Procedure of 0.5 McFarland Nephalometer Standard

1.A solution of 1% anhydrous barium chloride ($Bacl_2$) was prepared.

2.A solution of 1% sulphuric acid (H2SO4) was prepared.

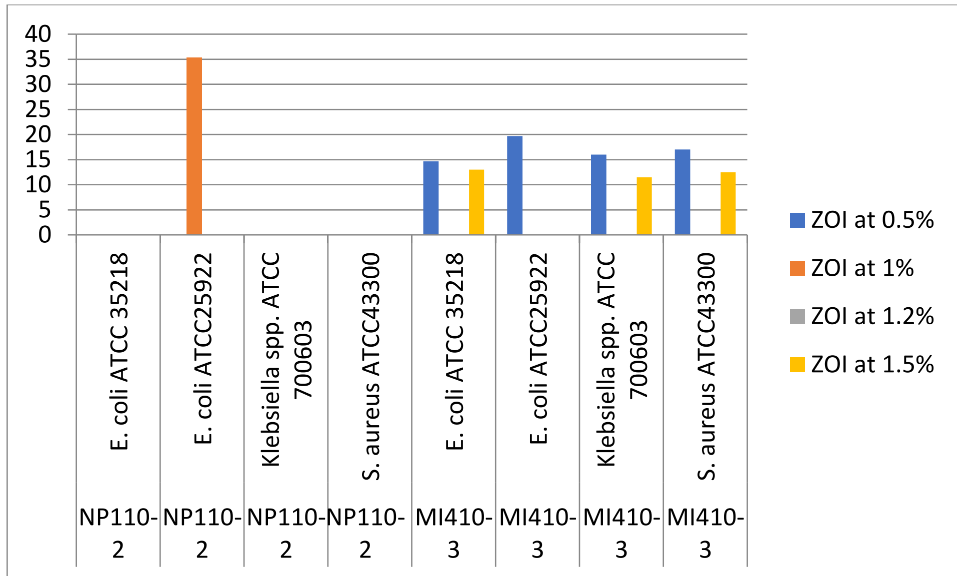
3.Solutions of Barium chloride (0.5ml) and Sulphuric acid (99.5ml) were mixed to form a turbid suspension.

4.The resulting mixture was placed in a foil-covered screwcap tube for the test.

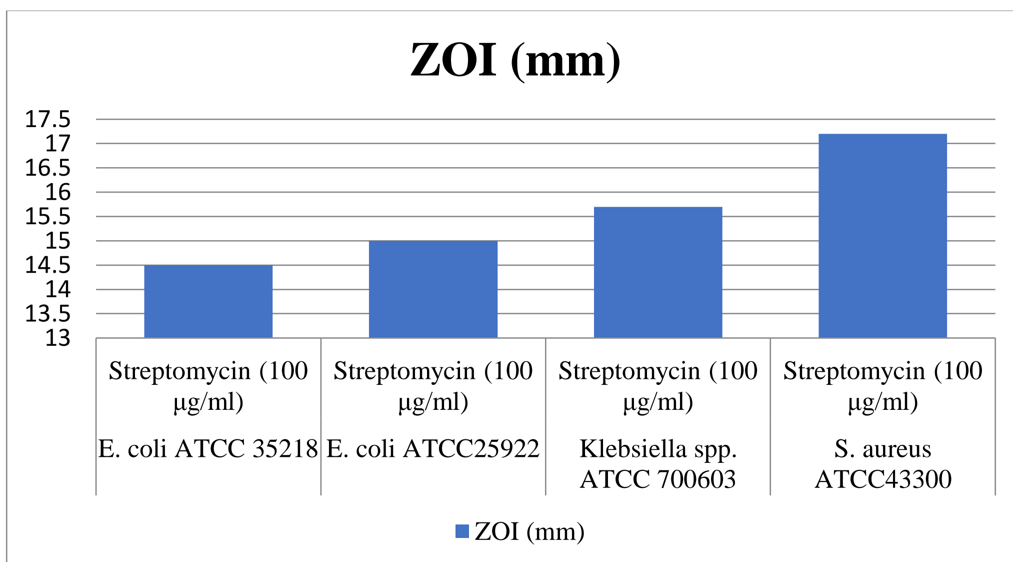
APPENDIX XII

ANOVA CHARTS

1. **Zone of Inhibition (ZOI) by NP110^{-2} and MI410^{-3} at different $NaNO_3$ concentration against ATCC cultures**



2. **Standard Streptomycin (100 µg/ml) and Zone of Inhibition (ZOI)**



Standard Streptomycin (100 µg/ml) and ZOI

APPENDIX XIII

ANOVA TABLES

One way ANOVA of sample

Analysis of variance

Variate: ZOI at 0.5%

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Sample	1	566.834	566.834	252.88	<.001
Residual	6	13.449	2.241		
Total	7	580.283			

Least significant differences of means (5% level)

Table	Sample
rep.	4
d.f.	6
l.s.d.	2.590

Analysis of variance

Variate: ZOI at 1%

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Sample	1	156.0	156.0	1.00	0.356
Residual	6	936.2	156.0		
Total	7	1092.2			

Least significant differences of means (5% level)

Table	Sample
rep.	4
d.f.	6
l.s.d.	21.61

Analysis of variance

Variate: ZOI at 1.2%

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Sample	1	0.	0.		
Residual	6	0.	0.		
Total	7	0.			

Least significant differences of means (5% level)

Table	Sample
rep.	4
d.f.	*
l.s.d.	*

Analysis of variance

Variate: ZOI at 1.5%

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Sample	1	171.12	171.12	8.91	0.024
Residual	6	115.25	19.21		
Total	7	286.38			

Least significant differences of means (5% level)

Table	Sample
rep.	4
d.f.	6
l.s.d.	7.58