SCREENING OF POTENTIAL ANTIBIOTIC PRODUCING ACTINOMYCETES EXTRACTED FROM SOIL AND WATER SAMPLES FROM DIFFERENT REGIONS OF KATHMANDU VALLEY

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 **Chandra Kishore Sardar**, Symbol No : 500330080, T.U. registration No: 5-2-33-283-2017, has carried out project work entitled **"Screening of Potential Antibiotic Producing Actinomycetes Extracted from Soil and Water Samples from Different Regions of Kathmandu Valley"** for the requirement to the project work in Bachelor of Science (B.Sc.) degree in Microbiology under my/our supervision in the Department Microbiology, Amrit campus, Institute of Science and Technology (IoST), Tribhuvan University (T.U.), Nepal.

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

He 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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DECLARATION

This project work entitled **"Screening of Potential Antibiotic Producing Actinomycetes Extracted from Soil and Water Samples from Different Regions of Kathmandu Valley"** 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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On the recommendation of Assistant Prof. Suman Rai, this project work is submitted by Mr. Chandra Kishore Sardar, Symbol No: 500330080, T.U. Registration No: 5-2-33-283-2017 entitled **"Screening of Potential Antibiotic Producing Actinomycetes Extracted from Soil and Water Samples from Different Regions of Kathmandu Valley "** 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.

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BOARD OF EXAMINATION AND CERTIFICATE OF APPROVAL

This project work (PRO-406) entitled **"Screening of Potential Antibiotic Producing Actinomycetes Extracted from Soil and Water Samples from Different Regions of Kathmandu Valley"** by Chandra Kishore Sardar, Symbol No: 500330080 and T.U. Registration No: 5-2-33-283-2017 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), 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, Institute of Science and Technology, Tribhuvan University, Nepal for the legal procedure.

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ABSTRACT

Actinomycetes are Gram-positive, aerobic spore forming bacteria that are characterized by aerial and mycelial growth and are chief antibiotic producers. The aim of this study was to screen antibiotic producing actinomycetes and determine its antibiotic activity against ATCC cultures. A total of 60 samples (30 water and 30 soil) were collected from different regions of Kathmandu Valley viz. Kathmandu, Lalitpur and Bhaktapur spanning from the month of March 2022 to May 2022. Spread plate technique was employed to isolate the actinomycetes on Starch M-Protein Agar, and primary and secondary screening techniques were performed via Perpendicular Streak method and Agar-well diffusion respectively for screening their ability to produce antibiotics. The actinomycetes were confirmed by the macro and microscopic examination, biochemical and physiological tests. The crude extract obtained from the submerged state fermentation was filtered and centrifuged; tested against the Standard cultures viz; *Staphylococcus aureus* ATCC 43300, *Escherichia coli* ATCC 35218, *Escherichia coli* ATCC 25922 and *Klebsiella* spp. ATCC 700603 via agar well diffusion method. Out of the 28 (93.3%) isolates obtained from 30 soil samples, only two isolates (7.3%) i.e., NP1 and MI4 showed antimicrobial activity against the ATCC cultures which were presumed to be *Streptomyces*. No actinomycetes were obtained from water samples. ANOVA revealed no significant difference at 5% level of significance (0.535; P>0.05) between the standard streptomycin (100 µg/ml) and NP1.The soil of Kathmandu Valley harbors microbial diversity that encompasses potential antimicrobial producing actinomycetes which in turn can help in booming the economy by enabling the production of indigenous antibiotics.

***Keywords*:** Actinomycetes, Agar Well Diffusion, ATCC cultures, antimicrobial activity, Primary and secondary screening, Sub-merged state fermentation.

शोधसार

एक्टिनोमाईसिटिज ग्रामपोजेटिभ, एरोबिक स्पोर उत्पादन गर्ने ब्याक्टेरिया हो, हवाई र माईसेलेलियाको विकाश यसकोविशेष्ता हो साथै प्रमुख एन्टिबायोटिक उत्पादन गर्ने एक्टिनोमाईसिटिज हो। यस अध्ययनको मुख्य उद्देश्य एन्टिबायोटिक उत्पादन गर्ने एक्टिनोमाईसिटिज छुट्टयाउनु साथै एटिसिसि कल्चर्सहरुलाईमार्ने जीवाणुरोधीगतिविधि क्षमतानिर्धारण गर्नु थियो । कूल ६० नमुनाहरु (३० पानी र ३० माटो) काठमाण्डौ उपत्यकाका विभिन्न स्थान जस्तै: काठमाण्डौ, ललितपुर र भक्तपुरबाट मार्च देखि मई २०२२ मा सङ्कलन गरिएको थियो । एक्टिनोमाईसिटिजलाई स्टार्च एम–प्रोटिन एगारमा स्प्रेड प्लेट विधिबाट छुट्टयाईयो । प्राथमिक र दोस्रो छनौट क्रमश: पर्पेडिक्यूलर सर्ट्रिकिङ्ग तथा एगार ह्वेल डिफ्यूजन विधिद्वारा एन्टिबायोटिक उत्पादन गर्ने एक्टिनोमाईसिटिज छुट्टयाईएको थियो । एक्टिनोमाईसिटिजलाई म्याक्रो तथा माईक्रोस्कोपिक परीक्षण, जैविक-रसायनिक तथा भौतिक परिक्षण पुष्टि गरिएको थियो ।सब–मर्ज फर्मेन्टेसन विधिबाट उत्पादित कच्चापद्धार्थ फिल्टर तथा सेन्ट्रिफ्यूज गरिएको थियो । एगार ह्वेल डिफ्यूजन विधिबाट एटिसिसि कल्चर्सहरु जस्तै; *S. aureus* 43300, *E. coli* 35218, *E. coli* 25922 र *Klebsiella* 700603 को विरुद्धपरिक्षण गरिएको थियो । ३० माटोबाट छुट्टयाएका २८ वटा नमुनाहरु (९३.३%) मध्ये २ वटा मात्रनमुनाहरु NP1 र MI4 (७.३%) ले एटिसिसि कल्चर्सहरुको विरुद्ध जीवाणुरोधीगतिविधिप्रदर्शन गरेको थियो ।जसलाई स्ट्रेप्टोमाईसिस प्रजातीभएकोअनुमानितगरियो। पानीबाट एक्टिनोमाईसिटिज छुट्टयाउन सकिएन। ५% को सिर्क्नफिक्यान्स लेबलको स्तरमा एनोभा तथ्याङ्क अनुसार (०.५३५; पि>०.०५) मानकस्ट्रेप्टोमाईसिन(१०० µg/mL) को तुलनामा NP1 कोजीवाणुरोधीगतिविधि खासै फरक नभएको देखियो । काठमाण्डौ उपत्यकाको विभिन्न स्थानका माटोमा शुक्ष्मजैविकविविधता रहेको छ,जहाँ सशक्त एन्टिमाईक्रोवायल्स उत्पादन गर्ने एक्टिनोमाईसिटिज समेटिएको छ। जसले रैथाने एक्टिनोमाईसिटिजबाट एन्टिबायोटिक उत्पादन गरि आर्थिक वृद्धी गर्न सहयोग गर्दछ ।

***Keywords*:** एक्टिनोमाईसिटिज, एगार ह्वेल डिफ्यूजन विधि, एटिसिसि कल्चर्स, जीवाणुरोधीगतिविधि, प्राथमिक र दोस्रो छनौट, सब–मर्ज फर्मेन्टेसन

LIST OF ACRONYMS AND ABBREVIATIONS

ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
DNA	Deoxyribonucleic acid
GISA	Glycopeptides Intermediate *Staphylococcus aureus*
G+C	Guanine + Cytosine
MHA	Mueller Hinton Agar
mM	Millimolar
Mol	Molecular
MRSA	Methicillin Resistant *Staphylococcus aureus*
NaCl	Sodium chloride
RNA	Ribonucleic acid
Sc	Standard Culture
VRE	Vancomycin Resistant *Enterococcus*
YEMB	Yeast Extract Mannitol Broth
ZOI	Zone of Inhibition

LIST OF SYMBOLS

º C	Degree Centigrade
µ	Micron
β	Beta
%	Percentage
γ	Gamma
<	Less-than sign
±	Plus Minus

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CHAPTER 1

1. INTRODUCTION

1.1 General Introduction

Actinomycetes are Gram-positive, filamentous bacteria, with high G+C content (69-78%) in DNA exhibiting highly differentiated developmental cycle, inhabiting a wide range of habitats. Unlike bacteria, actinomycetes are unique in their morphology with extensive branching substrate and aerial mycelium bearing chain of arthrospores. Of the 22,000 known microbial secondary metabolites, 70% are produced by actinomycetes, and two thirds of them are contributed by the genus *Streptomyces* (Tamang, *et al*., 2017). These are traditionally considered to be transitional forms between bacteria and fungi. Like bacteria, these organisms possess cell wall containing muramic acid, prokaryotic nucleus and are susceptible to antibacterial antibiotics, whereas like fungi they form a mycelial network of branching filaments. They are actually true bacteria related to Corynebacterium and Mycobacterium. These are nonmotile, nonsporing, noncapsulated filaments which usually break up into small bacillary and coccoid elements (Chakraborty, 2018).

Large numbers of actinomycetes, as many as millions per gram, are present in dry warm soils. The most predominant genera of this group are *Nocardia, Streptomyces,* and *Micromonospora*. These organisms are responsible for the characteristic musty or earthy odor of a freshly plowed field. They are capable of degrading many complex organic substances and consequently play an important role in building soil fertility. They are also noted for their ability to synthesize and excrete antibiotics (Pelczar, *et al*., 2006). Actinomycetes are the most widely distributed group of microorganisms in nature which can be found everywhere including soil and water sources. Different antibiotics can be extracted from Actinomycetes, namely, tetracycline, macrolide, chloramphenicol, nucleosides, and polyenes (Shrestha, *et al*., 2021).

An antimicrobial agent is a chemical that either kills or inhibits the growth of microorganisms. Each class of antimicrobial agents has a unique mode of action

(Sandle, 2016). The advent of synthetic methods has, however, resulted in a modification of this definition and an antibiotic now refers to a substance produced by a microorganism, or to a similar substance (produced wholly or partly by chemical synthesis), which in low concentrations inhibits the growth of other microorganisms (Denyer, *et al*., 2004).

The diversity of terrestrial actinomycetes is of great significance in several areas of medical sciences, particularly in antibiotic production (Magarvey, *et al*., 2004). The number and types of actinomycetes present in soil would be greatly influenced by geographical location such as soil temperature, soil type, soil pH, organic matter content, cultivation, aeration, and moisture content (Manandhar, *et al.*, 2013). The dominant actinomycetes *Micromonospora* can be isolated from aquatic habitats such as streams, rivers, lake mud, river sediments, beach sands, sponge, and marine sediments. Several novel bioactive compounds were discovered from aquatic actinomycetes, for example rifamycin from *Micromonospora*; salinosporamide-A, an anticancer metabolite from a *Salinispora* strain; marinomycins from *Marinophilus* spp; abyssomicin-C from *Verrucosispora* sp. and marinopyrroles from *Streptomyces* spp. (Gebreyohannes, *et al.*, 2013).

Nepal is a landlocked, narrow rectangular country situated in the Himalayas which is surrounded by cold, arid Tibetan plateau in the north and hot humid planes in the north thus making it a transition zone for the two extremes of north and south regions which in turn bestows it a very diverse ecosystem (Baniya, *et al*.,2018). It is a geographically diverse country which is divided into three regions: the mountain region, the hilly region, and the terai region. Based on their variation in altitude and soil type and their contents, there is a possibility of observing similar microflora, which conjectures to vary the distribution of antimicrobial-producing actinomycetes (Sapkota, *et al*., 2020). Due to large geographic variation and unique niche, there is large variation in soil type and their contents in Nepal and hence it is quite likely that the distribution of antibiotic producing actinomycetes is also variable (Pandey, *et al*., 2004). Marine environment contains a wide range of distinct microorganisms that are not present in the terrestrial environment. Though some reports are available on antibiotic and enzyme production by marine actinomycetes, the marine environment is still a potential source for new

actinomycetes, which can yield novel bioactive compounds and industrially important enzymes (Arasu, *et al*, 2013).
The exploration of diverse ecosystems and habitats and the subsequent isolation of diverse genera of actinomycetes from the same are central to the procurement of new antibiotics (Mahajan and Balachandran, 2012).

Actinomycetes are distributed widely, however only a small proportion of actinomycetes have been screened (Agadagba, *et al*., 2014). So, there is a need to screen actinomycetes, a potential antibiotic producer from different habitats for antimicrobial activity with the hope of discovering new strains capable to produce antibiotics against the multi-drug resistant pathogenic microorganisms (Rai, *et al.*, 2016).

Actinomycetes are well known as an inexhaustible source for antibiotics. The produced substances include all important drug classes used in clinics today, such as β-lactams, tetracyclines, macrolides, aminoglycosides, or glycopeptides. However, in the past years the effectiveness of these impressive weapons have become endangered by the rise of resistances of live-threatening pathogenic bacteria. In 2002, the first *Streptomyces* genome sequence was published. This was the genome sequence of the model actinomycetes *Streptomyces coelicolor*. Mining this sequence revealed that *S. coelicolor* harbors 22 secondary metabolite gene clusters but indeed produces only four of the encoded metabolites under standard lab conditions. Currently, more than 625 genome sequences are available only of the genus *Streptomyces*. Genome mining analyses suggest that less than 10% of the genetic potential of antibiotic producers is currently being used, which implicates that there is a huge untapped genetic reservoir waiting to be exploited for drug discovery. In addition to that, metagenomic data indicate that there are far more potential antibiotic producers in nature awaiting isolation and investigation (Mast and Stegmann, 2019).

1.2 Rationale

Nepal has extremely diverse geographic variations due to which variation in soil types as well as water bodies such as ponds, rivers, swamps, streams, etc is amply found in Nepal. While much research has been done on actinomycetes from soil sample, there may have been very less research done on actinomycetes from the water samples. Natural antibiotics extracted from water and soil samples have very promising lethal effects on pathogenic bacteria and therefore their extraction and commercialization can add to the economy of the country and enable health sector better to fight off pathogenic bacteria.

The findings of the study will be beneficial and contributory to the scientific field for further research into the hunt for new noble antibiotics to fight many emerging pathogenic and multi-drug resistant bacteria.

1.3 Objectives

1.3.1 General Objectives

- To screen actinomycetes showing antibacterial activity from the soil and water samples collected from different parts of Kathmandu, Nepal.

1.3.2 Specific Objectives

- To isolate actinomycetes from soil and water samples
- To identify the isolated actinomycetes by microscopic & macroscopic examination, physiological and biochemical tests.
- To screen antibiotic producing actinomycetes by primary screening.
- To carry out submerged state fermentation for the production of antibiotic.
- To screen actinomycetes isolates by secondary screening for antibacterial property against ATCC cultures viz: *Staphylococcus aureus* ATCC 43300, *Escherichia coli* ATCC 35218, *Escherichia coli* ATCC 25922 *and Klebsiella* spp. ATCC 700603 by comparing with Streptomycin (100 µl).

CHAPTER 2

2. LITERATURE REVIEW

2.1 Actinomycetes:

The name "Actinomycetes" was derived from Greek "*atkis*" (a ray) and "*mykes*" (fungus) and has features of both bacteria and fungi. However, they are now generally considered to be more closely related to bacteria. The chemical composition of their cell wall is similar to that of gram-positive bacteria but because of their well-developed morphological (hyphae) and cultural characteristics, actinomycetes have been considered as a group, well separated from other common bacteria (Das, *et al*., 2008).

Actinomycetes are aerobic, spore forming gram-positive bacteria, belonging to the order Actinomycetales characterized with substrate and aerial mycelium growth. It has a high (G+C) ratio of the DNA (>55mol %), which are phylogenetically related from the evidence of 16S ribosomal cataloguing and DNA:rRNA pairing studies (Chaudhary, *et al.*, 2013)

2.2 Habitat

The actinomycetes exist in various habits in nature (George, et al., 2012). Actinomycetes constitute a significant component of the microbial population in most soils and counts of over 1 million per gram are commonly obtained. The soil is also the most prolific source of isolates, which include many found to produce antibiotics and other useful metabolites (Goodfellow and Williams, 1983). The majority of actinomycetes are free living, saprophytic bacteria found widely distributed in soil, water and colonizing plants (Pandey, *et al*., 2004).

2.3 Isolation of Actinomycetes from soil

The isolation of actinomycetes from mixed micro flora found in the nature is difficult because of their features with a slow growth relation to that of the other soil bacteria; however, there are several technologically important options used for

the isolation of actinomycetes (Chavan, *et al*., 2013). Various isolation methods have been used earlier for different genera of actinomycetes and some novel methods has been applied in recent year. Hayakawa and Nonomura developed various methods for isolating desirable rare actinomycetes genera from natural habitats. These methods include a variety of pretreatment techniques in combination with enrichment techniques that appropriately supplement agar media with selective antibacterial agents (Hayakawa *et al*., 2008). Actinomycetes are gram positive bacteria utilize both simple and complex substrate for their growth. General bacterial isolation methods like serial dilution to reduce overcrowd, pour plate, streaking and centrifugation techniques are also applicable for isolation of actinomycetes. Centrifugation of soil sample followed by serial dilution of supernatant can enhance the chances of actinomycetes growth on plate (Rehacek, 1959).

2.3.1 Selective Isolation Methods:

Non-actinomycetes bacteria prevent the growth of actinomycetes as a pure culture and hence, selective isolation of actinomycetes was developed using six approaches (Kumar and Jadeja, 2016):

(i) Nutritional selection, where media are formulated with nutritional components, which are preferentially utilized by actinomycetes. Several carbon, nitrogen and complex substances have been considered as selective substrates for actinomycetes. Selective media were always preferred for isolation of actinomycetes because sample contains other genera of microorganisms. So, isolation media must be designed to reduce the development of competing microbes without adversely affecting actinomycetes propagules (Cross, *et al*., 1982; Goodfellow, *et al*., 1989).

(ii) Selective inhibition, in which inhibitors such as antifungal agents and antibiotics are incorporated to inhibit non-actinomycetes bacteria. Most antibacterial antibiotics inhibit actinomycetes along with other bacteria which lead to difficulty in suppression of bacteria while allowing growth of actinomycetes. Dulaney *et al*., (1955) recommended a mixture of antibacterial and antifungal antibiotics to allow selective development of actinomycetes.

(iii) Pretreatment of sample, in which soil, marine sample or plant parts were treated with physical or chemical method in order to decrease the number of non-

actinomycetes bacteria or fungi. Pre-treatment of soil can stimulates the isolation of actinomycetes by either promoting growth of actinomycetes or eliminating most unwanted gram negative bacteria (Matsukawa *et al*., 2007 and Hong *et al*., 2009).

(iv) Enrichment method, in which nutrient media can be enriched with certain additional supplements, which favors the growth of actinomycetes or inhibit the growth of other microbes. Enrichment is one of the successful methods in terms of diversity and abundance culturable bacteria.

(v) Membrane filter method, which does not dependent upon pretreatment, specific media or antibiotics. It was described by Hirsch for selective isolation of filamentous actinomycetes from natural mixed microbial populations without relying upon specific media and antibiotics. Nutrient agar medium supports the growth of mix bacterial cultures and also suitable for actinomycetes isolation.

(vi) Integrated method, in which any combination of different approaches can be applied. This is most preferred method for selective isolation of actinomycetes. A combination of physico-chemical method with suitable antibiotics and other selective method promotes desired growth of actinomycetes.

2.4 Antibiotics

An antibiotic was originally defined as a substance, produced by one microorganism, which inhibited the growth of other microorganisms. The advent of synthetic methods has, however, resulted in a modification of this definition and an antibiotic now refers to a substance produced by a microorganism, or to a similar substance (produced wholly or partly by chemical synthesis), which in low concentrations inhibits the growth of other microorganisms (Denyer, *et al*., 2004).

There are three major sources from which antibiotics are obtained.

1) Microorganisms:

For example, bacitracin and polymyxin are obtained from some *Bacillus* species; streptomycin, tetracyclines, etc. from *Streptomyces* species; gentamicin from *Micromonospora purpurea*; griseofulvin and some penicillins and cephalosporins from certain genera (*Penicillium*, *Acremonium*) of the family Aspergillaceae; and

monobactams from *Pseudomonas acidophila* and *Gluconobacter* species. Most antibiotics in current use have been produced from *Streptomyces* spp.

2) Synthesis:

Chloramphenicol is now usually produced by a synthetic process.

3) Semi-synthesis:

This means that part of the molecule is produced by a fermentation process using the appropriate microorganism and the product is then further modified by a chemical process. Many penicillins and cephalosporins are produced in this way (Denyer, *et al*., 2004).

Actinomycetes are considered as the most invaluable prokaryotes in medicinal and biotechnology industries because of their ability to produce number of bioactive molecules, particularly of the antibiotic compounds (Baniya, *et al*., 2018). Almost 80% of the world's antibiotics are known to come from actinomycetes, mostly from the genera *Streptomyces* and *Micromonospora* (Kumar, *et al*., 2010). The produced substances include all important drug classes used in clinics today, such as β-lactams, tetracyclines, macrolides, aminoglycosides, or glycopeptides (Mast & Stegmann, 2019).

2.5 Fermentation:

Fermentation is the technique of biological conversion of complex substrates into simple compounds by various microorganisms such as bacteria and fungi. In the course of this metabolic breakdown, they also release several additional compounds apart from the usual products of fermentation, such as carbon dioxide and alcohol. These additional compounds are called secondary metabolites. Secondary metabolites range from several antibiotics to peptides, enzymes and growth factors. Submerged Fermentation utilizes free flowing liquid substrates, such as molasses and broths. The bioactive compounds are secreted into the fermentation broth. Antibiotics are the most important category of bioactive compounds extracted from microorganisms using fermentation. Now, there are a multitude of antibiotics that have been produced using fermentation. This includes cyclosporins, tetracyclins, surfactins, streptomycin, and cephalosporin (Subramaniyan and Vimala, 2012).

2.6 Antimicrobial resistance:

Infectious diseases today are a leading cause of deaths world-wide, accounting for 25% of all deaths (around 13.3 million) (Selvameenal, *et al*, 2009). According to the Infectious Disease Society two million drug-resistant infections are reported each year, causing great suffering, and costing the health system up to 34 billion U.S. dollars a year (Mahajan and Balachandran, 2012). Resistance to antibiotics can either be naturally occurring for a particular organism and drug combination or acquired resistance can occur. This is where over-use(misuse) of antimicrobials results in a population being exposed to an environment in which organisms that have genes conferring resistance (either spontaneously mutated or through deoxyribonucleic acid (DNA) transfer from other resistant cells) have been able to flourish and spread (Sandle, 2015).

There are a number of resistant organisms causing concern at present. Notable Gram-positive organisms include methicillin resistant *Staphylococcus aureus* (MRSA) and coagulase-negative staphylococci, glycopeptides intermediate sensitivity *S. aureus* (GISA), vancomycin-resistant *Enterococcus* (VRE) species and penicillin-resistant *Streptococcus pneumoniae*. Concerns among the Gram-negative organisms include multidrug-resistant *Pseudomonas aeruginosa, Stenotrophomonas maltophilia* and *Acinetobacter baumannii* and members of the Enterobacteriaceae with extended-spectrum β-lactamases. Multidrug resistance in the acid-fast bacilli *Mycobacterium tuberculosis* and *M. avium* complex pose major health threats worldwide (Denyer, *et al*., 2004).

Resistance can be caused by a variety of mechanisms: (i) the presence of an enzyme that inactivates the antimicrobial agent; (ii) the presence of an alternative enzyme for the enzyme that is inhibited by the antimicrobial agent; (iii) a mutation in the antimicrobial agent's target, which reduces the binding of the antimicrobial agent; (iv) post-transcriptional or posttranslational modification of the antimicrobial agent's target, which reduces binding of the antimicrobial agent; (v) reduced uptake of the antimicrobial agent; (vi) active efflux of the antimicrobial agent; and (vii) overproduction of the target of the antimicrobial agent. In addition, resistance may be caused by a previously unrecognized mechanism. On the other hand, a gene which is not expressed in vitro may be expressed in vivo (Fluit, *et al*., 2001).

CHAPTER 3

3. MATERIAL AND METHODS

3.1 Materials

The various materials and equipment used in this study are listed in Appendix I.

3.2 Methods:

3.2.1 Study site/ Sampling site

This study was conducted in Kathmandu district. The process of sampling and analyzing of the soil and water samples was done from the month of March 2022 to May 2022 in Kathmandu valley. The soil and water samples were collected from different places of Kathmandu, Lalitpur and Bhaktapur.

Figure 1: Map of Study area

3.2.2 Research design

This study was descriptive and quantitative type of research design. The study primarily focused on isolating and screening actinomycetes from the soil and water samples of Kathmandu valley for their ability to produce antibiotics.

3.2.3 Sample size

Thirty each soil and water samples were collected from different parts of Kathmandu Valley.

3.2.4 Sample collection and Transportation

500 grams of moist soil samples were collected from a depth of 8-10 cm in the ground preferably from the land, left barren or organically farmed. The soil samples were collected in clean polyethylene bags and then closed properly and then transported to the laboratory. Likewise, for sampling of water, sterile bottles were used. 200-300 ml of water samples at the depth of 5-8 cm from the surface of water bodies like streams, stone-spouts, rivers, ponds, etc. was collected into the bottles. The samples were processed within 2-3 hours from the time of collection at the sites. The samples were collected by the random sampling method.

3.3 Sample processing :

3.3.1 Isolation of Actinomycetes

Isolation of Actinomycetes was performed in the Microbiology laboratory of Amrit campus by Spread plate technique onto Starch M-Protein Agar. One gram of soil sample was dissolved in 10 ml of sterile distilled water contained in a test tube and it was followed by serial dilution to 10^{-3} for each soil sample. 0.1 ml of aliquot from 10^{-2} and 10^{-3} dilutions each were spread plated on two different Starch M-Protein Agar plates by spread plate technique. The plates were left undisturbed for 15 minutes to dry off the water-vapor and finally incubated at 28ºC for 2-4 weeks (Kaur and Teotia, 2019). Likewise, one ml of water sample was dissolved in 9 ml of sterile distilled water contained in a test tube and it was followed by serial dilution to 10^{-3} for each water sample. 0.1 ml of aliquot from 10^{-2} and 10^{-3} dilutions each were spread plated on two different Starch M-Protein Agar plates by spread plate technique. To isolate pure

colonies of actinomycetes, the typical colonies of actinomycetes characterized by tough, dry and wrinkled nature were picked by a sterile inoculating loop from the master plate and streaked on Starch M-protein Agar plates. The plates were then incubated at 28^{o}C for 4-8 days.

3.3.2 Characterization of Actinomycetes

3.3.2.1 Macroscopic Characterization

The sub-cultured isolates of Actinomycetes were observed macroscopically for the color of the aerial mycelium and diffusible pigments and other colony characteristics such as texture size, elevation, and opacity of the colonies (Majhi 2019).

3.3.2.2 Microscopic characterization

The microscopic characterization was done by Gram staining. They were observed for their mycelial structure and its color under microscope (100X) (Nanjwade *et al*., 2012).

3.3.2.3 Biochemical characterization

Several biochemical tests like Catalase, Oxidase, Methyl-Red, Voges Proskauer, Citrate utilization, Indole and Hydrogen Sulphide production, Urea hydrolysis, Tween 20 hydrolysis, Starch Hydrolysis, Temperature Tolerance, and NaCl Tolerance tests were performed (Appendix X &XI) (Chaudhary *et al.,* 2013).

3.3.3 Primary screening of Actinomycetes for Antimicrobial activity

Primary screening was done by Perpendicular streak method on Mueller Hinton Agar (MHA) (Sah, et al, 2021). A vertical line of *Actinomycetes* was streaked along the diameter of the MHA plate and subsequently incubated at 28 ºC for 4-8 days to get a proper growth. The test microorganisms i.e., ATCC cultures of *Escherichia coli* ATCC 35218, *Escherichia coli* ATCC 25922, *Klebsiella* spp. ATCC 700603 and *Staphylococcus aureus* ATCC 43300 were inoculated in 3 ml of nutrient broth and incubated at 37 ºC for 4 hours to obtain visible turbidity which was matched with 0.5 McFarland Nephelometer Standard in

good light. The aforementioned test microorganisms were streaked perpendicularly keeping 0.5-1mm gap from the growth of Actinomycetes and incubated at 37ºC for 18-24 hours (Manandhar *et al.,* 2013). The zone of inhibition produced by Actinomycetes was measured if there were any and duly noted.

3.3.4 Fermentation

Fermentation was done by Sub-merged state fermentation method in which the Actinomycetes isolates showing antimicrobial activity screened from primary screening was inoculated into 60 ml of Yeast Extract Mannitol Broth contained in the conical flask. It was placed in the water bath maintained at 28 ºC and shaken at 120 rpm for 7-10 days (Gebreyohannes, *et al*., 2013). The confirmation of the growth of the Actinomycetes in the flask was ensured by visible pellets, clumps or aggregates along with turbidity in the broth.

3.3.5 Filtration and centrifugation of antibacterial metabolites

The fermented broths with the Actinomycetes isolates were filtered through Whatman No.1 filter paper after the completion of incubation period. The residue was discarded whereas the filtrate was taken for further processing (Valli *et al*., 2012). The filtrate was centrifuged at 5000 rpm for 15 minutes for further purification by removing cells and debris. The pellet was discarded, and the supernatant liquid was decanted to obtain the crude extract of antimicrobial metabolite in pure form (Palla, *et al*., 2018). This crude extract was then employed for secondary screening.

3.3.6 Secondary Screening of Actinomycetes for Antimicrobial activity

Secondary screening was done by Agar Well Diffusion method on Mueller Hinton Agar (MHA). Bores (cups) were made on the MHA plates which were about 4 mm in depth. The bacterial test cultures of comparable turbidity of 0.5 McFarland Nephelometer Standard were swabbed on the MHA plates with the bores. 100 µl of crude extract was then pipetted into the bores with the help of micropipettes and left undisturbed for few hours to ensure diffusion of the antibacterial extract on the agar medium (Sapkota, *et al*., 2020). The plates were incubated at 37ºC for 24 hours. The zone of inhibition around the bores were measured if there were any and duly noted.

3.3.7 Determination of Antimicrobial Activity

The determination of antimicrobial activity was carried out by Agar well diffusion method. For the same, MHA plates were bored to create bores (cups) of about 4mm depth with the help of cork-borer. The plates were then swabbed with bacterial test cultures having a visual turbidity comparable to 0.5 McFarland Nephelometer Standard. 100 µl of the crude extract was pipetted into the bores with the help of micropipette and incubated at 37 ºC for 24 hours (Fatima, *et al*., 2017). After incubation, it was observed for zone of inhibition around the bores if there were any and measured and noted.

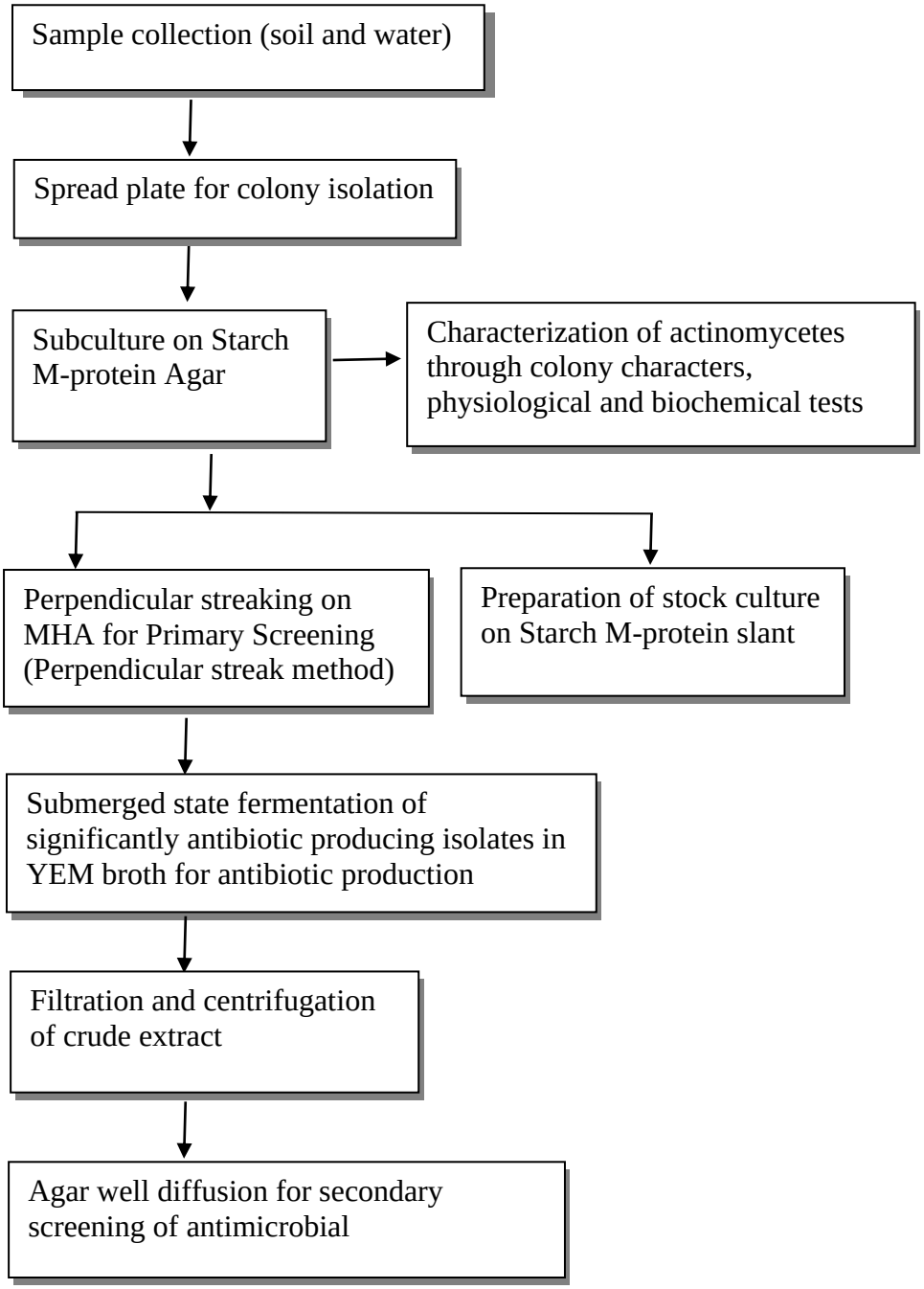


Figure 2: Flow chart for the isolation and screening of Actinomycetes (Sapkota, *et al*., 2020; Gebreyohannes, *et al*., 2013: Sah, *et al*.,2021)

CHAPTER 4

4. RESULTS AND DISCUSSION

4.1 Results

Thirty soil and thirty water samples were collected from different sites of Kathmandu valley. Actinomycetes were not isolated from the water samples of any sites. However, out of 30 soil samples, 28 isolates of actinomycetes were obtained which were confirmed by colony and physiological characteristics, microscopic observation, and biochemical tests. Of these isolates, two showed promising activity against different pathogenic microorganisms.

4.1.1 Characteristics of the antibiotic producing isolates

4.1.1.1 Macroscopic Characteristics

The colonial characteristics of 28 isolated actinomycetes colonies were listed in Appendix VII. Among them, it was revealed that the isolated colonies of antibiotic producing actinomycetes produced greyish white and pale-yellow substrate mycelium. Out of the two isolates, NP1 (50%) produced greyish white substrate mycelium whereas MI4 (50%) produced pale yellow substrate mycelium. Both the isolates (100%) produced greyish white aerial mycelium. The texture of both the antibiotic producing isolates of Actinomycetes (100%) were rough and powdery. The colony diameter of both antibiotic producing actinomycetes (100%) was 1mm in size. The colonies had entire and round margin with rough and crusty elevation and were opaque (Table 1).

Table 1: Macroscopic characteristics of the antibiotic producing Actinomycetes isolated on Starch M-Protein Agar

Isolate code	Macroscopic characteristics			
	Color of substrate mycelium	**Color and texture of aerial mycelium**	**Other colony characteristics**	**Presumptive genera**
NP1	Greyish white	Greyish white, rough, and powdery	entire, round, 1mm diameter, raised and crusty, opaque	*Streptomyces* spp.
MI4	Pale yellow	Greyish white, rough, and powdery	entire, round, 1mm diameter, raised and crusty, opaque	*Streptomyces* spp.

4.1.1.2 Microscopic characteristics

Gram staining was performed to observe the isolates microscopically. It was revealed that the isolates NP1 and MI4 were found to be Gram-positive filamentous thread-like in appearance with rectiflexible and unfragmented mycelium. The mycelium and cellular morphology revealed that all the active isolates were presumably identified as *Streptomyces* spp.

4.1.1.3 Biochemical and physiological characteristics

4.1.1.1.3.1 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 VIII & X). Among them, i.e., NP1 and MI4 were tested for their ability to hydrolyze urea, starch, and Tween 20.

It was revealed that urea, starch, and Tween 20 were hydrolyzed by MI4 (50%), NP1 (50%) and both NP1 and MI4 (100%) respectively (Table 2).

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

Isolate code	Hydrolysis tests			
	Urea	**Starch**	**Tween 20**	**Presumptive genera**
NP1	-	+	+	*Streptomyces* spp.
MI4	+	-	+	*Streptomyces* spp.

4.1.1.3.2 Other Biochemical 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 citrase and other products (Appendix VIII). Among them, the antibiotic producing Actinomycetes isolates were also subjected to numerous other biochemical tests like oxidase, catalase, indole production, Methyl-Red, Voges Proskauer, citrate utilization and H_2S production tests.

It was revealed that NP1 was positive to catalase, oxidase, and indole production and negative to methyl red, Voges Proskauer, citrate utilization and H_2S production tests respectively while MI4 was positive to catalase, oxidase, indole production, methyl red and citrate utilization and negative to Voges Proskauer and H_2S production tests respectively (Table 3).

Table 3: Other biochemical tests of the antibiotic producing Actinomycetes isolates

Isolate code	Other biochemical tests						
	Catalase	**Oxidase**	**Indole**	**Methyl red**	**Voges Proskauer**	**Citrate utilization**	**H_2S production**
NP1	+	+	+	-	-	-	-
MI4	+	+	+	+	-	+	-

4.1.1.3.3 Physiological Tests

Temperature tolerance test, NaCl tolerance test and motility test were performed on the antibiotic producing actinomycetes isolates. It was revealed that NP1(50%) of the two potential isolates was able to grow at 37 ºC. Both the antibiotic producing isolates of actinomycetes i.e., NP1 and MI4 (100%) were able to grow at 15 ºC and 28 ºC respectively.

It showed that both NP1 and MI4 (100%) were able to grow in 5% NaCl whereas both NP1 and MI4 (100%) showed no growth in 10% NaCl respectively.

It was revealed that both NP1 and MI4 (100%) were found to be non-motile when tested in SIM medium for their motility (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%**		
NP1	+	+	+	+	-	-	*Streptomyces* spp.
MI4	+	+	-	+	-	-	*Streptomyces* spp.

4.1.2 Sub-merged state Fermentation

The presence of visible pellets, clumps or aggregates along with turbidity in the broth was observed which indicated that the production of antibiotic was successful.

4.1.3 Screening of Actinomycetes for Antimicrobial activity

4.1.3.1 Primary Screening of Actinomycetes

In total, 30 soil samples were collected from different sites of Kathmandu valley. 28 isolates of Actinomycetes were isolated in Starch M-Protein Agar which are listed in Appendix VI. After colonial and physiological characterization, microscopic examination and bio-chemical tests, the isolates 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 (Rai *et al*., 2016).

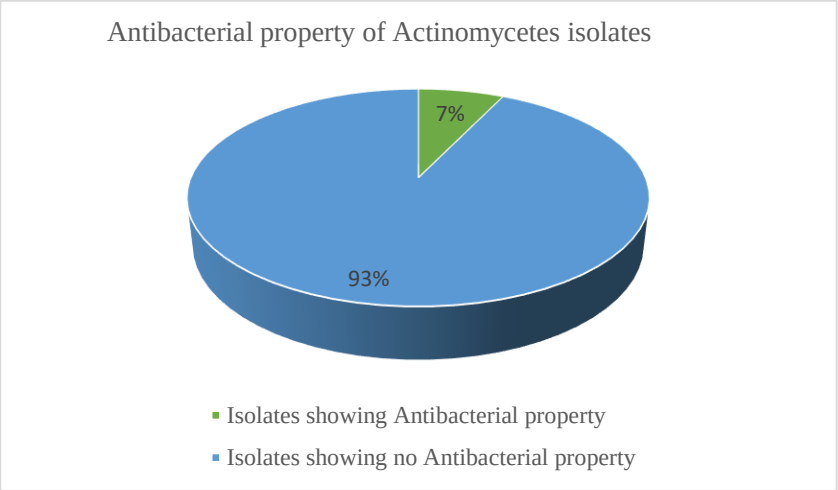


Figure 3: Antibacterial property of Actinomycetes

It was revealed that isolates NP1 and MI4 (7.14%) exhibited potential antimicrobial activity against the aforementioned microorganisms (Figure 3).

4.1.3.2 Secondary Screening of Actinomycetes

The two potential actinomycetes isolates from the primary screening were subjected to submerged state fermentation in YEM broth. The crude extract obtained after filtration and centrifugation showed inhibitory action against the test organism. NP1 isolate showed considerable antibacterial activity against all (100%) the test microorganisms employed for testing whereas MI4 isolate showed antibacterial activity against only one (25%) test organism in Agar well diffusion.

Table 5: Zone of inhibition (in mm) of potential actinomycetes in secondary screening

Isolate code	Gram negative bacteria			Gram positive bacteria
	Sc1	Sc2	Sc3	Sc4
NP1	14.6	19.6	11.5	17
MI4	0	17.6	0	0
Streptomycin (100 µg/ml)	14.5	15	15.7	17.2

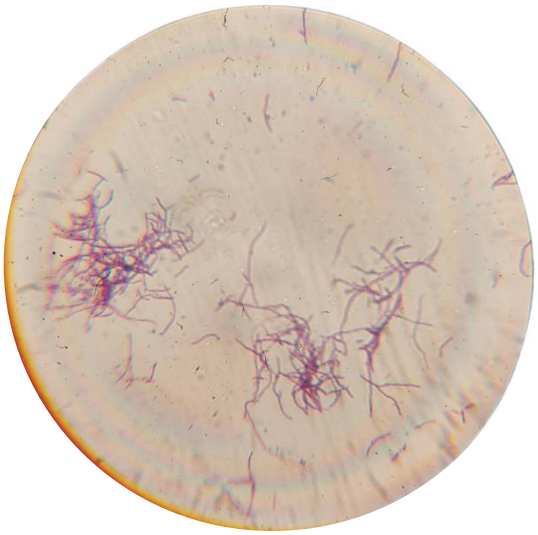
Note:

Sc: Standard culture

Sc1: *E. coli ATCC*35218; **Sc2**: *E. coli ATCC*25922; **Sc3***: Klebsiella* spp. ATCC 700603; **Sc4**: *Staphylococcus aureus ATCC*43300

It was revealed that NP1 has greater antibacterial effect against *E. coli* ATCC 35218 and *E. coli* ATCC25922 in comparison to standard Streptomycin (100 µg/ml) while moderately effective against *Staphylococcus aureus* ATCC43300 but has quite low effect against *Klebsiella* spp. ATCC 700603 respectively. Similarly, it showed that MI4 is only effective against *E. coli* ATCC25922 but is resisted by the rest; in comparison to standard Streptomycin (100 µg/ml). Sterile water was used as negative control and Streptomycin (100 µg/ml) was used as positive control.

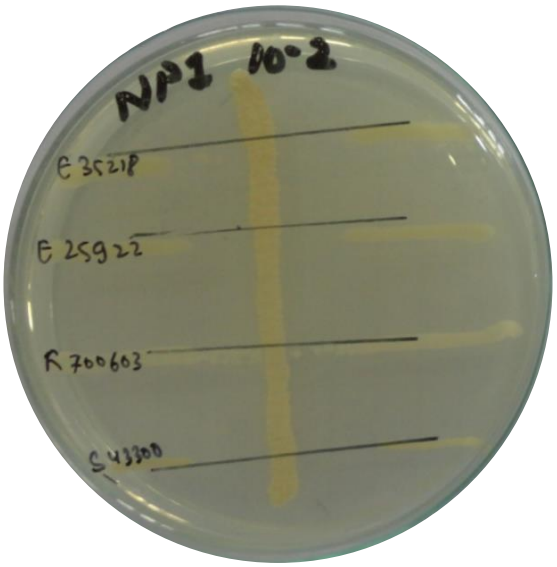
PHOTOGRAPHS



Photograph 1: Gram-positive, filamentous rods of Actinomycetes isolate seen under oil immersion (100X)

Photograph 2: Stock culture of Actinomycete isolates on slants of Starch M-protein Agar

Photograph 3: Biochemical test result of NP1 actinomycetes isolate. (Left to right: Urease- negative, Citrate utilization-negative, MR – Negative, VP- Negative, SIM-H_2S-Negative ,Indole positive & Motility negative)



Photograph 4: Primary screening of the isolate by Perpendicular streak method against Standard bacteria (*Escherichia coli* ATCC 35218, *Escherichia coli* ATCC25922, *Klebsiella* spp. ATCC 700603 and S*taphylococcus aureus* ATCC 43300)

Photograph 5 : Agar well diffusion of the potential antibiotic producing actinomycete isolate

Photograph 6 : Subculturing of the isolates in the Microbiology laboratory of Amrit Campus

4.2 Discussion

This study was undertaken for the purpose of isolating and screening Actinomycetes for antimicrobial property from the soil samples. For the same, 30 soil samples and 30 water samples were collected from different parts of the Kathmandu Valley viz: Kathmandu, Lalitpur and Bhaktapur districts.

The present study was targeted towards the screening of antibiotic producing actinomycetes from the soil of Kathmandu valley in accordance with Goodfellow and Haynes (1984). According to Goodfellow and Haynes (1984), only 10% of the actinomycetes are isolated from nature and according to Kumar *et al.,* (2010), although soils have been screened by the pharmaceutical industry for about 50 years, only a small fraction of the surface of the globe has been sampled. Actinomycetes are the most biotechnologically valuable prokaryotes responsible for the production of about half of the discovered bioactive secondary metabolites, notably antibiotics, antitumor agents, immunosuppressive agents and enzymes.

The present study might unravel the discovery of a novel class of antibiotics for future prospective which is accordance with De Simeis and Serra (2021) i.e., the discovery of new useful, clinical drugs is slow, for sure, and particular attention must be kept using antibiotics.

The present study was carried out in the month of March 2022 to April 2022 since Kathmandu valley has different ecological habitats with different topography which is also supported by Singh and Agrawal (2002). Microbial diversity is a vast frontier and potential goldmine for the biotechnology industry because it offers countless new genes and biochemical pathways to probe for enzymes, antibiotics and other useful molecules. Different ecological habitats with different topography with emphasis on organic farms and places with less human intervention were selected as suitable sites for the collection of soil and water samples aimed at the isolation of actinomycetes.

In the present study, actinomycetes was not isolated from the water samples which may be due to the water pollution and effluents which was also studied by Gurung and Oh (2012) and Manandhar, (2013). However, it was contrary to the study conducted by Shrestha *et al.,* (2021) in which actinomycetes were isolated from water samples. 28 out of 30 soil samples gave characteristic colonies of

actinomycetes with small, white, rough, glabrous, and chalky white, which was similar to study done by Oskay, *et a*l., (2004).

The isolated colonies were slow growing, aerobic, glabrous, or chalky, heaped, folded and with aerial and substrate mycelia of different colors which is also similar and in accordance with Ramazani *et al*., (2013) and additionally, all colonies possessed an earthy odor. Also, all the isolates were Gram positive with mycelial branching ranging from low to moderate to high which was also reported by Gautham, *et al.,* 2012. Therefore, the presumptive identification of the potent antibiotic producing strains revealed that the two isolates belonged to the genus *Streptomyces.* This was in accordance with the findings obtained by Pandey, *et al.,* (2004). According to Procópio *et al*., (2012) today, 80% of the antibiotics are sourced from the genus *Streptomyces*, actinomycetes being the most important.

The typical colony traits and microscopic features of the actinomycetes isolates obtained from all of the samples are shown in Appendix VII & IX respectively. In this study, the majority of aerial and substrate mycelium was found to be white, followed by gray, pink, blue, purple, and orange. The hue of the scattered pigment around each colony was examined and recorded as the colony's pigmentation. The majority of the isolates had pigmentation ranging from brown to yellow to purple to bluish, with a few showing brownish pigmentation which matched with findings obtained by Sapkota, *et al*., (2020).

The biochemical characterization of the isolates showed diverse results. Similar findings were obtained by the study done by Kekuda, *et al.,* (2012). All the isolates were catalase positive. The results of the different biochemical tests carried out, are listed in the Appendix VIII. Various biochemical tests performed were oxidase, indole, methyl red, Voges Proskauer, citrate utilization, H_2S production, starch hydrolysis, Tween 20 hydrolysis, urea hydrolysis, and the physiological test included motility, temperature tolerance (15^{o}C, 28^{o}C and 37^{o}C) and NaCl tolerance (at 5% and 10 %).

Primary Screening exhibited antagonistic interaction, observed as zone of no growth of test organisms around the actinomycetes isolates as described by Kekuda, *et al.,* (2012). Only two (7%) of the total 28 isolates showed antibacterial property i.e., zone of inhibition (ZOI) against the test organism which was close i.e., (8.53% of the isolates showing antibacterial property) to the study conducted

by Shrestha, *et al.,* (2021). The remaining isolates were not further analyzed. According to Jiang, *et al.,* (2016), fermentation is extremely important procedure for the discovery of new drug leads. Different strains need different fermentation conditions, including components, concentration, and pH of broth, and time, temperature, and aeration of fermentation. Based on the results from the primary screening, the two potent isolates were then subjected to sub-merged fermentation using Yeast Extract Mannitol Broth (YMEB). Visible pellets, clumps, or aggregates along with turbidity in the broth confirmed the growth of the actinomycetes in the flask.

In the present study, Secondary Screening showed that sample NP1 and MI4 exhibited antibacterial activity against the test organisms which was similar to the study by Al-Ansari, *et al*., (2019).

The one-way ANOVA revealed that there is no significant difference between the effect of sample NP1 and standard streptomycin (100 µg/ml) against the ATCC (0.535; P>0.05). Therefore, NP1 has greatest antimicrobial effect which may be the potential strain for the production of antibiotic in coming future.

Antimicrobial resistance is a problem that is growing more difficult to solve with each passing year. Antibiotic resistant bacteria can cause serious health problems, including death. According to WHO (2019), if no action is taken - drug-resistant diseases could cause 10 million deaths each year by 2050 and damage to the economy as catastrophic as the 2008-2009 global financial crisis and by 2030, antimicrobial resistance could force up to 24 million people into extreme poverty. This problem has been creeping for many years, but it has recently come to light in a big way due to the spread of antibiotic-resistant infections like tuberculosis and *Staphylococcus aureus*. Antibiotic resistance can lead to more infections and may even result in more deaths from preventable illnesses. There are many ways to combat antimicrobial resistance, but it will require coordinated efforts from governments, pharmaceutical companies, and other organizations.

CHAPTER 5

5. CONCLUSION AND RECOMMENDATION

5.1 Conclusions

This study showed that the water samples used for the study harbored no potential antibiotic producing Actinomycetes but on the contrary the soil samples were found to have potential antibiotic producing Actinomycetes. Therefore, indigenous actinomycetes strains can be studied further for the discovery of new antibiotics that have antibacterial activity against the pathogens of clinical concern. Also, these indigenous actinomycetes can be harbored to produce antibiotics on industrial scale which in turn can help the nation grow economically.

5.2 Novelty and National Prosperity aspect of Project work

Antibiotics are critically important for treating infections. A different geographical and topographical variation in Nepal harbors microbial communities that are very diverse. Hence, the natural sources like soil and sample harbor potential antimicrobial producing actinomycetes. Its screening and proper laboratory tests can be used to treat many antimicrobial resistant pathogens therefore utilized commercially to boost the national economy.

5.3 Limitations of the work

1. Microbial diversity has been shown to be affected by water pollution. In Kathmandu Valley, urban and agricultural wastewater effluents are discharged into rivers and other water bodies. Due to this actinomycetes were not isolated from water samples and hence a full comparative analysis couldn't be done.
2. Protein profiling couldn't be done for the crude extract and hence its analysis was limited.
3. Time and budget were factors in limiting the study.

5.4 Recommendations for further work

1. Protein profiling of the crude extract can help in the identification and analysis of the antimicrobials extracted.
2. Crude extract can be purified by other advanced methods for further analysis and research and can be stored.
3. Molecular analysis can be helpful in identification up to genus level and to determine shifts in the community due to environmental changes.

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APPENDICES

APPENDIX I

LIST OF MATERIALS

Equipments:

1. Conical flasks	BOROSIL, India
2. Refrigerator	Express Cool, LG
3. Pipette	BOROSIL, India
4. Glass tubes and pipette	BOROSIL, India
5. Hot Air oven	Ambassador
6. Autoclave	Life
7. Petri plate	BOROSIL, India
8. Microscope	COSLAB
9. Electric balance	PHOENIX
10. Incubator	Memmert

Glass-wares/ Plastic- wares:

1. Beaker
2. Cork borer
3. Conical flasks
4. Measuring cylinders
5. Micropipettes
6. Microtips
7. Sampling bottles
8. Test tubes
9. Microscopic slides
10. Bent glass rod

Chemicals:

1. Lysol
2. Ethanol
3. Concentrated HCl
4. Distilled water
5. Crystal violet
6. Safranin
7. Gram's Iodine
8. 3% H_2O_2
9. Oxidase reagent
10. Kovac's reagent
11. Methyl red reagent
12. Barritt's reagent A & B

Test organisms:

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

APPENDIX II

COMPOSITION AND PREPARATION OF MICROBIOLOGICAL MEDIA (HI MEDIA)

A. Basal Media

1. Nutrient agar Composition

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

Preparation

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

2. Nutrient Broth Composition

Ingredients	**gms/liter**
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 ºC)	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 sterilized by autoclaving at 15lbs pressure (121ºC) for 15 minutes.

B. Selective and Differential Media

1. Starch M-Protein Agar Composition

Ingredients	**gms/liter**
M-protein powder	1.00 gm
Starch	10.00 gm
Sea water	37.00 gm
Agar	15.0 gm
Final pH (at 25 ºC)	7.2±0.2

Preparation

63.0 grams was suspended in 1000 ml distilled water. It was heated to dissolve the medium completely. The medium was sterilized by autoclaving at 15lbs pressure (121 ºC) for 15 minutes. It was cooled to 45-50°C. It was mixed well and poured into sterile petri plates.

2. Yeast Extract Mannitol Broth Composition

Ingredients	**gms/liter**
Yeast extract	1.00 gm
Mannitol	10.00 gm
Dipotassium hydrogen phosphate	0.500 gm
Magnesium sulphate	0.200 gm
Sodium chloride	0.100 gm
Final pH (at 25 ºC)	7.0±0.2

Preparation

11.80 grams was suspended in 1000 ml distilled water and heated to dissolve the medium completely. It was then dispensed into the flasks. The medium was sterilized by autoclaving at 15lbs pressure (121 ºC) for 15 minutes.

C. Other Media

1. Mueller Hinton Agar Composition

Ingredients	**gms/liter**
Beef infusion	300.00 gm
Acid of casein hydrolysate	17.50 gm
Starch	1.50 gm
Agar	17.0 gm
Final pH (at 25 ºC)	7.3±0.1

Preparation

38.0 grams was suspended in 1000 ml distilled water. It was heated to dissolve the medium completely. The medium was sterilized by autoclaving at 15lbs pressure (121 ºC) for 15 minutes. It was cooled to 45-50°C. It was mixed well and poured into sterile petri plates.

2. Tween 20 Agar Composition

Ingredients	**gms/ml**
Peptone	10 gm
Sodium chloride	5 gm
Calcium chloride	0.1 gm
Tween 20	10 ml
Agar	20 gm
Final pH (at 25 ºC)	7.0±0.2

Preparation

28.0 grams was suspended in 1000 ml distilled water. It was heated to dissolve the medium completely. The medium was sterilized by autoclaving at 15lbs pressure (121 ºC) for 15 minutes. It was cooled to 45-50°C. It was mixed well and poured into sterile petri plates.

3. Starch Agar Composition

Ingredients	**gms/liter**
Starch (soluble)	20 gm
Peptone	5 gm
Beef extract	3.0 gm
Agar	15.0 g
Final pH (at 25 ºC)	7

Preparation

25.0 grams was suspended in 1000 ml distilled water. It was heated to dissolve the medium completely. The medium was sterilized by autoclaving at 15lbs pressure (121 ºC) for 15 minutes. It was cooled to 45-50°C. It was mixed well and poured into sterile petri plates.

D. Biochemical Media

1. Simmons Citrate Agar Media 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 pH (at 25 ºC)	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 in tubes and sterilized by autoclaving at 15 lbs pressure (121 ºC) for 15 minutes.

2. SIM Media 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 ºC)	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 ºC) for 15 minutes. The tubes were allowed to cool in the upright position.

3. MR-VP Media Composition

Ingredients	**gms/liter**
Buffered peptone	7.00 gm
Dextrose	5.00 gm
Dipotassium phosphate	5.00 gm

Final pH (at 25 ºC)	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 is then dispensed in tubes and sterilized by autoclaving at 15 lbs pressure (121 ºC) for 15 minutes.

4. Urease Agar Media Composition

Ingredients	**gms/liter**
Peptic digest of animal tissue	1.00 gm
Dextrose	1.00 gm
Sodium chloride	5.00 gm
Mono potassium phosphate	1.20 gm
Phenol red	0.012 gm
Agar	15.00 gm
Final pH (at 25 ºC)	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 50ml sterile 40% urea solution was added aseptically to it and mixed well. The medium was dispensed in sterile tubes and allowed to set in the slanting position.

APPENDIX III

COMPOSITION AND PREPARATION OF DIFFERENT STAINING REAGENTS

1. Crystal Violet solution Composition

Solution A

Ingredients	**gms/liter**
Crystal violet (90% dye content)	2.0gm
Ethanol (90%)	20.0ml

Solution B

Ammonium oxalate	0.8 gm
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.

2. Gram's Iodine Composition

Ingredients	**gms/liter**
Iodine	1.0 gm
Potassium iodide	2.0 gm
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.

3. Acetone Alcohol Composition

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

Preparation

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

4. 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. It was then transferred to clean reagent bottle.

5. Catalase reagent (3% Hydrogen Peroxide solution) 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.

6. Oxidase reagent (1% Tetramethyl p-phenylene diamine dihydrochloride)

Composition

Ingredients	**gms/liter**
Tetra methyl paraphenylenediamaine dihydrochloride	5.0 gm
Distilled water	50 ml

Preparation

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

7. Kovac's reagent Composition

Ingredients	**gms/liter**
p-dimethyl amino benzyldehyde	5.0 gm
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. Toit, 25 ml of concentrated HCL was added and mixed well.

8. Voges- Proskauer reagent (Barrit's reagent) Composition

a) VP reagent A

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

Preparation

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

b) VP reagent B Composition

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

Preparation

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

9. Methyl red reagent Composition

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

Preparation

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

APPENDIX IV

METHODOLOGY OF BIOCHEMICAL TEST FOR THE IDENTIFICTION 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. Sulphide production and Indole & Motility 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.

D. Citrate utilization test

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

E. Urea hydrolysis test

Urea agar slant was streaked with actinomycetes colonies and incubated at 28 degree for 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 ºC 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. Temperature tolerance test

The isolates were streaked in starch M-protein agar and incubated at 15 ºC and 37 ºC for 2 weeks. Positive test was indicated by the growth of isolates.

H. NaCl tolerance test

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

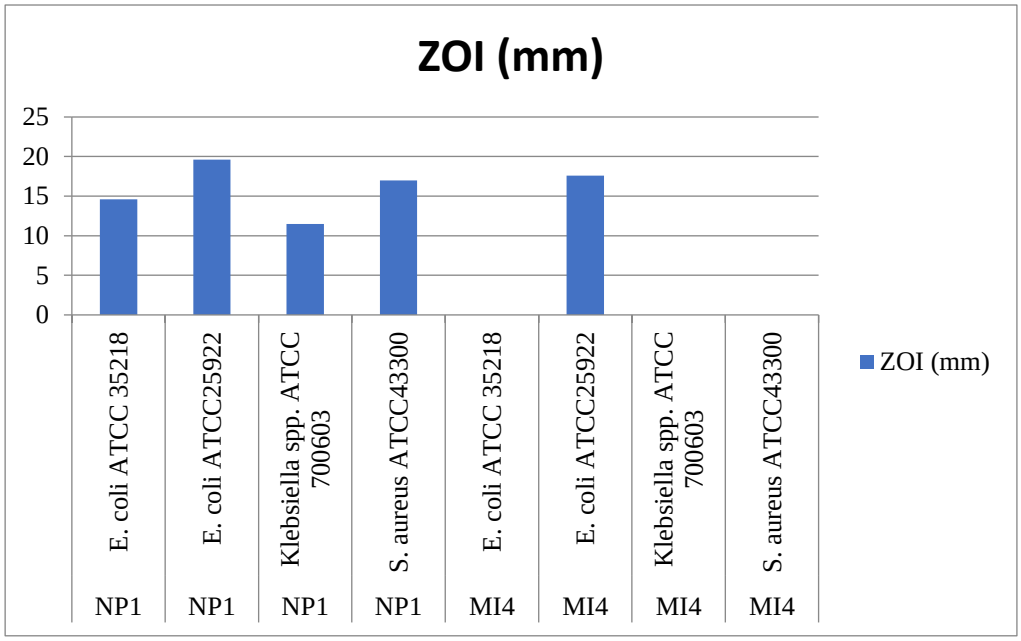
I. Tween 20 hydrolysis test

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

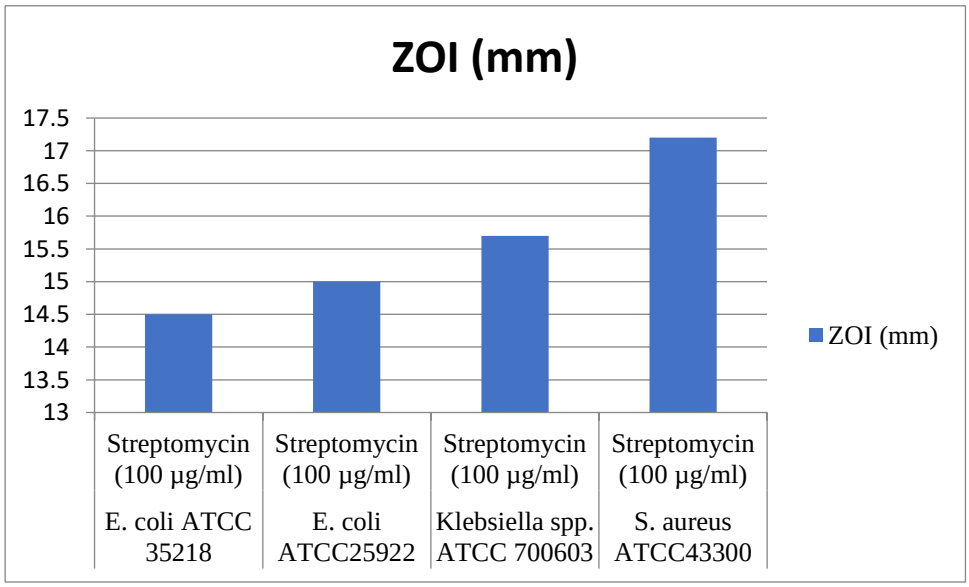
APPENDIX V

ANOVA TABLE

1. Zone of Inhibition (ZOI) by NP1 and MI4 against ATCC cultures



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



3. Analysis of Variance (One way)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
ATCC cultures	3	203.11	67.70	0.85	0.535
Residual	4	319.20	79.80		
Total	7	522.32			

4. Least significant differences of means (5% level)

Table	ATCC cultures
rep.	2
d.f.	4
l.s.d	24.80

APPENDIX VI

SAMPLE SITE

Sample symbol	Sample site	Isolated actinomycetes
MH1	Manohara	+
UK3	Upper Khokhana	+
BP2	Bhaisepati	+
DS2	Dharmasthali	+
CG2	Chovar gate	+
BG2	Bhangal height	+
NP1	New planning	+
SY3	Swayambhu	-
LH5	Lolang height	+
MI4	Madhyapurthimi	+
TH3	Thamel	-
ST2	Sanothimi	+
PP2	Pepsicola	+
BB6	Basbari	+
SK1	Sankhamul	+
MG3	Magar gau	+
CV2	Chovar	+
RB1	Ranibari	+
KP6	Kritipur	+
UN3	UN park	+
LK6	Lower Khokhana	+

BK2	Budhanilkantha	+
SP1	Shivapuri	+
BH3	Balaju height	+
SG4	Sallaghari	+
KH2	Kapan height	+
MJ2	Maharajgunj	+
NT1	Nepaltar	+

APPENDIX VII

MORPHOLOGICAL CHARACTERS OF THE ISOLATES

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,	1	Green	Raised,	Opaque

		powdery			crusty	
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

BIOCHEMICAL RESULTS OF THE ISOLATES

Sample code	Catalase	Oxidase	SIM (Indole production, H_2S 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 IX
GRAM STAINING RESULTS OF THE ISOLATES

Sample symbol	Observation
MH1	Gram positive, filamentous Rod-shaped bacteria
UK3	Gram positive, filamentous Rod-shaped bacteria
BP2	Gram positive, filamentous Rod-shaped bacteria
DS2	Gram positive, filamentous Rod-shaped bacteria
CG2	Gram positive, filamentous Rod-shaped bacteria
BG2	Gram positive, filamentous Rod-shaped bacteria
NP1	Gram positive, filamentous Rod-shaped bacteria
SY3	Gram positive, filamentous Rod-shaped bacteria
LH5	Gram positive, filamentous Rod-shaped bacteria
MI4	Gram positive, filamentous Rod-shaped bacteria
TH3	Gram positive, filamentous Rod-shaped bacteria
ST2	Gram positive, filamentous Rod-shaped bacteria
PP2	Gram positive, filamentous Rod-shaped bacteria
BB6	Gram positive, filamentous Rod-shaped bacteria
SK1	Gram positive, filamentous Rod-shaped bacteria
MG3	Gram positive, filamentous Rod-shaped bacteria
CV2	Gram positive, filamentous Rod-shaped bacteria
RB1	Gram positive, filamentous Rod-shaped bacteria
KP6	Gram positive, filamentous Rod-shaped bacteria

UN3	Gram positive, filamentous Rod-shaped bacteria
LK6	Gram positive, filamentous Rod-shaped bacteria
BK2	Gram positive, filamentous Rod-shaped bacteria
SP1	Gram positive, filamentous Rod-shaped bacteria
BH3	Gram positive, filamentous Rod-shaped bacteria
SG4	Gram positive, filamentous Rod-shaped bacteria
KH2	Gram positive, filamentous Rod-shaped bacteria
MJ2	Gram positive, filamentous Rod-shaped bacteria
NT1	Gram positive, filamentous Rod-shaped bacteria

APPENDIX X
PHYSIOLOGICAL TEST RESULTS OF THE ISOLATES

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 XI

HYDROLYSIS TEST RESULTS OF THE ISOLATES

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 XII

PROCEDURE OF 0.5 McFARLAND NEPHALOMETER STANDARD

1. 1% v/v solution of Sulphuric acid was prepared by adding 1 ml of concentrated sulphuric acid to 99 ml water.
2. 1% w/v solution of barium chloride was prepared by dissolving 0.5g of dihydrate barium chloride ($BaCl_2.2H_2O$) in 5o ml of distilled water.
3. 0.6 ml of the barium chloride solution was added to 99.4 ml of the sulphuric acid solution and mixed.
4. A small volume of the turbid solution was then transferred to a foil-covered screw capped bottle.