ISOLATION AND IDENTIFICATION OF ACTINOMYCETES FROM SOIL OF KANCHANPUR DISTRICT AND ASSESSMENT OF THEIR ANTIBACTERIAL ACTIVITY

A Faculty Research Report

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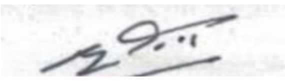
Declaration

I Mr. Krishna Prasad Pant hereby declare that the research work incorporated in this faculty research entitled " Isolation and identification of Actinomycetes from soil of Kanchanpur district and assessment of their antimicrobial activity" is my original work. This research report or any part of it has not been submitted to any institution for an award, degree, or any academic purposes. Wherever necessary, I have appropriately acknowledged the material collected from secondary sources and all sources of information and literature used have been cited in the reference section of the report. I am solely responsible for the originality of the entire content of this research work.



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Abstract

Antimicrobial resistance (AMR) is rapidly increasing in developing countries due to the unsought use of antibiotics. AMR is a global threat, complicating the treatment of bacterial infections and resulting into huge morbidity and mortality. Development/ production of new antimicrobial compound from Actinomycetes is a charming solution.

Soil Actinomycetes were isolated from 30 soil samples from Far Western Nepal's Kanchanpur district to assess their diversity, morphology, biochemical properties, and antimicrobial potential. The pH of the soils ranged from 5.1 to 8.4, and they varied in color, from grey and black to brown. A total of 21 distinct actinomycete colonies were isolated with starch casein agar, mainly from neutral to alkaline soils. All the isolates were found to be Gram-positive filamentous bacteria, matching that of actinomycetes. Various colony pigmentation diversity observed in all isolates were white, yellow, pink, and blue.

Further, the biochemical profiling showed that all tested isolates were catalase positive oxidase, and indole negative, and they exhibited hydrolysis of starch and gelatin. Most of the isolates could use citrate and give a positive urease reaction; only one gave a positive Voges-Proskauer reaction.

Four active isolates were active against human pathogens that inhibited at least one test bacteria (about 19% of total). The most potent was yellow and originated from sample S25 it showed wide-spectrum inhibition with clear zones up to 7–8 mm against Gram-positive bacteria like *Staphylococcus aureus* and *Enterococcus* and even moderate activity (4mm) against Gram-negative bacteria *Pseudomonas*. Other isolates showed inhibitory activity against *S. aureus* but other test bacteria were poorly inhibited.

The isolated Actinomycetes was probably *Streptomyces* species which would be further verified by 16 s rRNA gene sequencing. This Actinomycetes is very diverse and rich in soil of Kanchanpur district and exhibit strong antibacterial activity, mainly to Gram-positive pathogens. This would highlight the bioprospecting potential of actinomycetes in Nepali soil for novel antibiotics. We, therefore, recommend further taxonomic identification of these isolates and optimization of their metabolite production for possible use in pharmaceutical or other industrial applications. The study highlights the need for further research on Actinomycetes to find the novel strain and novel bioactive compound from soil of Kanchanpur district and other parts of Sudurpaschim Province.

Keywords: Actinomycetes, Bioactive compounds, Primary screening, Antimicrobial resistance (AMR)

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Table of Content

List of Tables

List of Figures

LIST OF ACRONYMS AND ABBREVIATIONS

%	Percentage
µg	Microgram
µl	Microliter
16SrRNA	16 Svedberg unit ribosomal Ribonucleic Acid
CFU	Colony Forming Unit
DAP	Diamino Pimelic Acid
DNA	Deoxyribonucleic Acid
G+C	Guanine + Cytosine
GC-MS	Gas Chromatography Mass Spectroscopy
HPLC	High Performance Liquid Chromatography
LC-MS	Liquid Chromatography Mass Spectroscopy
MDR	Multidrug Resistant
MDRSA	Multidrug Resistant *Staphylococcus aureus*
mg	Milligram
MHA	Mueller Hinton Agar
MIC	Minimum Inhibitory Concentration
mm	Millimeter
MR	Methyl Red
MRSA	Methicilin Resistant *Staphylococcus aureus*
NA	Nutrient Agar
ºC	Degree Celsius
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PKS	Polyketide Synthase
RAPD	Random Amplified Polymorphic DNA
Rpm	Revolution per minute
SCA	Starch Casein Agar
SIM	Sulphide Indole Motility
spp.	Species
SPSS	Statistical Package for Social Science
TLC	Thin Layer Chromatography
VRE	Vancomycin Resistant Enterococci
ZOI	Zone of Inhibition

Chapter 1 Introduction

1.1 Background of study

Actinomycetes are unique microbes found in soil, water, plant and degrading materials. They are a link between bacteria and fungi. They are prokaryotes, which means they have specific features that set them apart from other types of cells. One of those characteristics is G+C content more than 55%. This group attracts researchers due to its Gram-positive characteristics and its unique characteristics from other groups (Goodfellow & Williams, 1983; Goodfellow, 1989; Ventura et al., 2007). Actinomycetes, commonly located in soil, are essential for the degradation of complex polymers into simpler compounds. This process is very important for breaking down organic matter in soil and recycling nutrients (Goodfellow & Williams, 1983; McCarthy & Williams, 1992; Stach & Bull, 2005).

They are the most common microorganisms in soil, but the type of soil may influence them. These organisms inhabit different habitats, including soil, water, marine sediments, and hot springs (Anderson & Wellington, 2001). Actinomycetes are generally aerobic but can survive under anaerobic conditions (except for *A. meyeri* and *A. israelii*, which are facultative anaerobes). Actinomycetes possess the ability to produce spores; they are rod-shaped organisms that develop colonies exhibiting a network-like branching mycelium akin to fungi. (Holt et al., 1994).

Initially, Actinomycetes were classified based on their appearance, and their classification was believed to be based on their morphology. However, this approach was insufficient for distinguishing many similar species and genera. Moreover, phylogenetic and molecular methods have substantially impacted when utilized to aid in their categorization processes (Babalola et al., 2009; Hozzein & Goodfellow, 2011). However, advanced molecular techniques have enabled the accurate classification of many previously misclassified organisms (Zhi et al., 2009). The PCR and 16S rRNA for sequencing and phylogenetic analysis have been widely used in species categorization and phylogenetic studies (Wood et al., 2007; Zhi et al., 2009).

They can produce many bioactive compounds including anthracyclines, aminoglycosides, peptides, glycopeptides, polyethers, macrolides, polyenes, beta-lactams, nucleosides, terpenes, alkaloids, tetracyclines etc. which exhibit extensive biological activities (Raja &

Prabhakaran, 2011; Suthindhiran & Kannabiran, 2009). Most of the clinically used antibiotics are either innate products or artificially produced derivatives derived from microbes and their metabolic products (Thakur et al., 2007). Thus, extraction and identification of bioactive compounds from novel Actinomycetes strains is great significance (Rahman et al., 2011). *Streptomyces*, a prevalent genus within Actinomycetes, is mainly present in soil due to its antagonistic activity towards other microbes (Ceylan et al., 2008). *Streptomyces* is renowned for its capacity to produce and release an extensive array of bioactive compounds, particularly antibiotics. Substances derived from soil Actinomycetes demonstrate a diverse array of biological activities like bactericidal, plant growth promoting, anti-cancerous, anti-inflammatory, antifungal, neurotoxic, antiviral properties (Black et al., 1982; Schulz et al., 2009).

Antibiotics are biologically active metabolites that are produced by many microorganisms including Actinomycetes which can be produced either partially or entirely through chemical process. Actinomycetes are highly efficient microorganisms in producing bioactive compounds that contain antibiotics. Among the 22,000 bioactive substances derived from microorganisms, Actinomycetes contribute 45%, fungi 38%, and other bacteria 17%. Actinomycetes produce secondary metabolites such as antibacterials, antifungals, antivirals, immunosuppressants, and antitumor medications. Various studies have demonstrated that these bioactive substances are effective in treating infectious diseases (Berdy, 2005; Farnet & Zazopoulos, 2005). These are the main producers of antibiotics and are important to most naturally occurring antibiotic interactions. Within Actinomycetes, *Streptomyces* being predominant producer of antibiotics and bioactive compounds, followed by *Micromonospora*. *Streptomyces* and *Micromonospora* together producer more than 70% of naturally derived antibiotics (Okami, 1988; Hopwood et al., 2000).

Various studies in Nepal have assessed the antimicrobial nature of Actinomycetes collected from soil in different regions, including Kalapatthar, Mount Everest, hills, and Terai. Researchers have looked at how Actinomycetes are spread at different altitudes in different parts of the country and how well they work against a number of infectious microorganisms (Gurung et al., 2009; Sah & Lekhak, 2017; Rai et al., 2018; Budhathoki & Shrestha, 2020; Gurung & Rai, 2021). Research done in different parts of Nepal identified Actinomycetes that make antimicrobials were isolated, characterized, and found effective against pathogens

(Pandey et al., 2004; Yadav et al., 2008; Gurung et al., 2009; Rai et al., 2016; Lekhak et al., 2016).

Most antibiotics don't work against pathogenic bacteria because they have become more resistant to them (Bizuye et al., 2013). Antimicrobial resistance is becoming a life-threatening problem for management of infectious diseases. Because bacterial resistance is becoming more common, it is important to keep an eye on the antimicrobial effectiveness of actinobacteria from different environments in order to find new strains that can make novel antibiotics against multidrug-resistant pathogens. In Nepal, a lot of research looked at the antimicrobial properties of Actinomycetes found in soil from different areas, such as Kalapatthar, Mount Everest, the hills, and the Terai. Researchers have looked into the diversity of Actinomycetes at different altitudes in Nepal and tested how well they work against different pathogens. The different types of Actinomycetes that come from different sources work better against different types of pathogenic bacteria (Pandey et al., 2004; Yadav et al., 2008; Gurung et al., 2009; Wahab et al., 2015; Lekhak et al., 2016; Sah & Lekhak 2017; Rai et al., 2016; Rai et al., 2018; Budhathoki & Shrestha, 2020; Gurung and Rai, 2021).

When antibiotics are used incorrectly, they may not work as well as they used to because bacteria become resistant to them (Bizuye et al., 2013). The fact that bacteria can become resistant to antibiotics makes it very hard to stop and treat infectious diseases, which is the main cause of death around the world (WHO, 2002). In this situation, it is not only important but also urgent to study how well Actinomycetes from different habitats kill bacteria. Finding new strains that can make very strong antibiotics against drug-resistant pathogens is very important because bactericidal resistance is becoming more common.

1.2 Statement of problem

The rapid increase in antimicrobial resistance (AMR) is becoming a major issue worldwide because existing antibiotics are less effective due to the rise of drug-resistant pathogens. So, there is an immediate necessity for identifying Actinomycetes synthesizing new antibiotics/antimicrobials from different niches of Kanchanpur for combating the issue of AMR.

1.3 Rationale of the study:

The Kanchanpur district spans 1,610 square kilometers (620 sq mi). Distinguished by its varied ecosystems, which include the Terai and Chure hills, it has an elevation range from 176m to its highest point at 1528m above sea level. The average annual precipitation is 15-20cm, and the typical temperature varies from 0°C to 46.4°C. The district is characterized by tropical and subtropical climate, and the seasonal fluctuations result in challenging environments that can support unique microorganisms. Due to its topography and climate, the Kanchanpur district is rich in biodiversity and is believed to contain a diverse and distinct microbiome, including Actinomycetes.

Moreover, there is a rich abundance of natural resources in the area. Therefore, isolating new strains of Actinomycetes and analyzing the secondary metabolites from different niches in Kanchanpur is advantageous. Extensive studies have not been done on Actinomycetes from the soil of Kanchanpur district. The screening and identification of a unique strain with novel bioactive compounds having antimicrobial properties would support further exploration of its potential use in developing new drugs. As a result, this study will focus on isolating and identifying Actinomycetes from the soil in the Kanchanpur district, as well as evaluating their antimicrobial activity. Regarding current situation, it is essential to identify new antimicrobial compounds to address challenges like AMR, delay in finding new antibiotics, and insufficient detection of actinobacteria regardless of their diverse characteristics.

1.4 Research Questions

- What is the diversity of Actinomycetes in the soil of Kanchanpur, and do they exhibit antimicrobial activity against pathogenic microorganisms?
- What are the optimal conditions for isolating Actinomycetes from the soil of Kanchanpur?
- How can isolated Actinomycetes be morphologically or biochemically characterized?
- Which Actinomycetes strains exhibit significant antimicrobial efficacy against pathogens?

1.5 Objectives

General

Isolation and identification of Actinomycetes from soil of Kanchanpur district and assessment of their antibacterial activity.

Specific

- To isolate and identify Actinomycetes from soil.
- To isolate secondary metabolites from selected antibiotic-producing Actinomycetes.
- To assess their antibacterial activity.

1.6 Limitations

- Soil samples collected from specific locations in Kanchanpur may not fully represent the microbial diversity of the entire region.
- Secondary screening of the Actinomycetes isolates, determination MIC, MBC, carbohydrate utilization tests and substrate hydrolysis test may not be done due to unavailability of culture media and chemicals.
- Characterization of Actinomycetes by molecular method 16S rRNA methods may not be done due to lack of specialized equipment.
- Budgetary and time constraints may limit the scale of experiments, and the number of strains analyzed.
- The characterization of bioactive compounds may not be performed due to lack of sophisticated analytical instruments such as FTIR, LCMS, HPLC, GCMS, NMR etc.
- The findings may be specific to Kanchanpur soil and may not be directly applicable to other regions with different soil and environmental conditions.

Chapter 2 Literature Review

2.1 Actinomycetes

The term "Actinomycetes" comes from two Greek words: "aktis," which means "ray," and "mykes," which means "fungus." This is because of how they look. The taxonomic categorization of these organisms concerning prokaryotes is still unclear. Actinomycetes exhibit a combination of mycoplasma-like and bacterial-like characteristics, including the lack of a nuclear membrane, a narrow filamentous diameter, unique cell wall chemistry, a flagellar structure, and vulnerability to antibacterial agents. A lot of these species make spores, which help them move across water, stay alive in the air, and stay in the soil for a long time (Jeffrey, 2008; Chaudhary, 2013; Rahman 2011).

Actinomycetes are bacteria that are filamentous and gram-positive. They are found all over the place in nature and need oxygen to live. Jeffrey (2008) says that they mostly live in dry, alkaline soil. The fact that they can make spores and mycelial structures makes them distinctive. These microorganisms are important for making several antibiotics. Actinomycetes are the principal category of microorganisms found in natural habitats worldwide (Srinivasan et al., 1991). A range of factors, including soil temperature, type, pH, organic matter content, farming methods, airflow, moisture levels, geographic location, and cultivation conditions, influence the diversity and abundance of Actinomycetes in the soil (Arifuzzaman, 2010).

2.2 Classification of Actinomycetes

Actinomycetes are filamentous bacteria that can be either Gram-positive or Gram-variable. They belong to the Superkingdom Bacteria and are classified under the Firmicutes phylum, within the Actinobacter class and the Actinobacteriaceae subclass. According to Bergey's Manual of Determinative Bacteriology (2000), Actinomycetes are divided into eight families: Mycobacteriaceae, Actinoplanaceae, Frankiaceae, Dermatophilaceae, Nocardiaceae, Streptomycetaceae, Micromonosporaceae (as noted by Holt in 1989), and there are 63 genera within these families (Nisbet and Fox 1991).

Initially, Actinomycetes were categorized based on their morphology. However, later research using chemotaxonomic markers helped to identify specific genera and species across various groups. Actinomycetes are classified into eight categories (types I-VIII) based on their cell wall chemotypes, which include LDAP, Meso-DAP, DABA, aspartic acid, glycine, lysine, ornithine, arabinose, and galactose. Types II to IV specifically have whole-organism sugars such as arabinose, fructose, galactose, madurose, and xylose.

Several taxonomic techniques have been used to define the genera and suprageneric groups of Actinomycetes, but partial sequence analysis of the 16S rRNA is particularly significant. Recent classification using the 16S rRNA scheme has categorized Actinomycetes into eleven suborders: Actinomycineae, Corynebacterineae, Frankineae, Glymcineae, Micrococcineae, Micromonosporineae, Proionibacterineae, Pseudonocardineae, Streptomycineae, Strektosporangineae, along with many Actinomycetes that remain ungrouped.

2.3 Taxonomic Classification (Barka et al., 2015)

1. **Micromonosporinea**

Family: *Micromonosporaceae*

Genera: *Micromonospora*, *Actinoplanes*, *Catellospora*, *Couchioplanes*, *Pilimelia*, *Dactylosporangium*

2. **Frankineae**

Families: *Frankiaceae*, *Sporichthyeaceae*, *Geodermatophilaceae*, *Microsphaeraceae*, *Acidothermaceae*

Genera: *Frankia*, *Sporichthya*, *Geothermophillus*, *Blastococcus*, *Microsphera*, *Acidothermus*

3. **Pseudonocardineae**

Family: *Pseudonocardiaceae*

Genera: *Pseudonocardia*, *Actinopolyspora*, *Kibelosporium*, *Kutzneria*, *Saccharomonospora*, *Saccharopolyspora*, *Saccharothrix*, *Streptoalloteichus*, *Thermocrispum*

4. **Streptomycieae**

Family: *Streptomycetaceae*

Genera: *Streptomyces*

5. **Corynebacterium**

Families: *Nocardiaceae*, *Gordoniaceae*, *Mycobacteriaceae*, *Dietziaceae*, *Tsukamurellaceae*, *Corynebacteriaceae*

Genera: *Nocardia*, *Rhodococcus*, *Gordonia*, *Mycobacterium*, *Dietzia*, *Tsukamurella*, *Corynebacterium*, *Turicella*

6. **Actinomyineae**

Family: *Actinomycetaceae*

Genera: *Actinomyces*, *Mobiluncus*, *Acanobacterium*

7. **Propionibacterianeae**

Family: *Propionibacteriaceae*

Genera: *Propionibacterium*, *Luteococcus*, *Microlunatus*, *Propionoferrans*

8. **Streptosporangineae**

Families: *Streptosporangiaceae*, *Thermomonosporaceae*, *Nocardiopsaceae*

Genera: *Streptosporangium*, *Herbidospora*, *Microbiospora*, *Microteraspora*, *Planobispora*, *Planomonospora*, *Thermomonosporia*, *Actinomadura*, *Spirillospora*, *Nocardiopsis*

9. **Glycomycineae**

Family: *Glycomycetaceae*

Genera: *Glycomyces*

2.4 Occurrence and habitat

Actinomycetes are adaptable microorganisms that function as free-living saprophytes, creating thread-like filaments in the soil that contribute to its earthy aroma. They live in many different environments, such as soils, oceans, mangroves, compost, and vermicompost (Chamikara, 2016). They can be found in many different places, such as decaying plant debris, freshwater basins, and ocean sediments. Actinomycetes are important soil organisms (Kuster, 1964) that like alkaline and neutral soils. Their numbers are affected by humidity, pH, temperature, and vegetation.

These organisms thrive in mesophilic (25–30 °C) and thermophilic (40 °C) environments (Haseena et al., 2016). Mesophilic Actinomycetes initiate compost degradation, whereas thermophilic Actinomycetes prosper in the heat produced (Chavan et al., 2013). For best growth, most require a pH of about 7. However, acidophilic strains can thrive between pH 3.5

and 6.5, and alkaliphilic strains can be discovered in soils with a pH of 10 (Jiang et al., 1993). Halotolerant Actinomycetes are prevalent in marine ecosystems (Kushner, 1985).

Actinomycetes are necessary for breaking down organic materials into humus and making geosmin, the chemical that gives wet soil its unique smell (Wilkins, 1996). They also make bioactive substances that can affect a wide spectrum of creatures, such as bacteria, insects, and even cancer cells (Kumar, 2012). Their ability to survive in harsh environments (Meklat, 2011) shows even more how useful they could be in many different ways.

2.5 Structure of Actinomycetes:

Actinomycetes are single-celled creatures that branch out and have hyphae that don't have septa. They can also make spores that are round, cylindrical, or oval (Chamikara, 2016). They look like fungus in terms of shape, but their cell walls are more like those of gram-positive bacteria. They can multiply by splitting in two or by making spores or conidia. People often think of them as filamentous bacteria (Selman & Waksman, 1959).

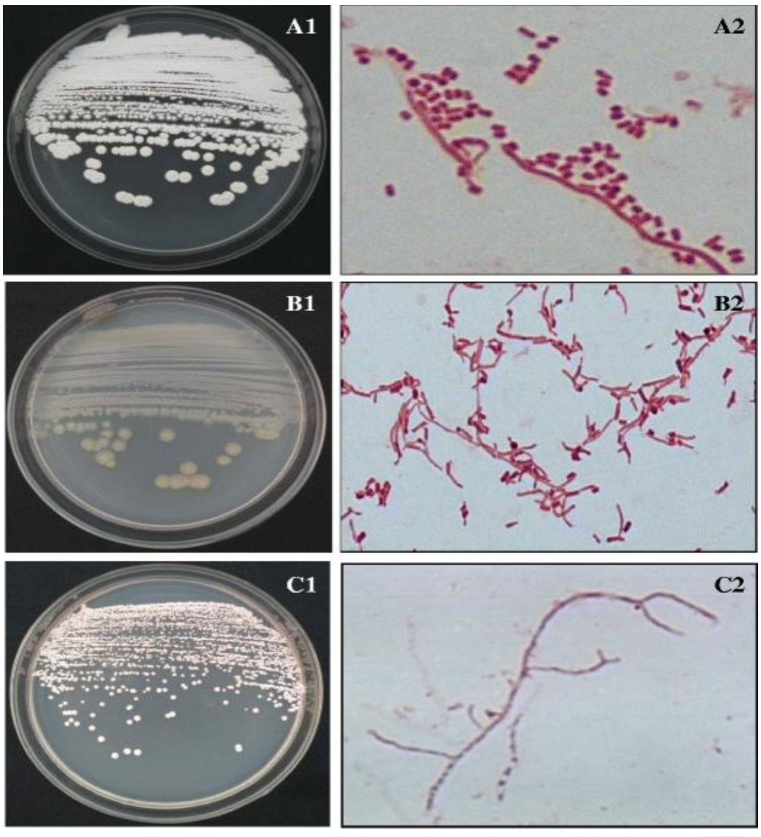


Figure 1 Colony morphology and respective microscopic features (Source Malviya et al., 2013)

Different types of plants make different chemical metabolites, which cause Actinomycetes to change (Oskay et al., 2004). This affects the diversity of plants in the soil. There are six classes in the phylum Actinobacteria, and each class is split into 16 orders, such as Actinomycetales and Streptomycetales. *Streptomyces* (almost 70%) are the most prevalent genera in soil, followed by *Nocardia* and *Micromonospora*. Other genera, such *Actinophages* and *Streptosporangium* are also there (Ranjani, 2016). 16S rDNA sequences are being used for molecular identification, and they are very important for telling Actinomycetes apart.

2.6 Screening of Actinomycetes for antimicrobial property

There are different ways to get rare actinomycete genera out of their natural environments. These methods include pretreatment methods combined with enrichment methods that use certain antimicrobial agents (Hayakawa, 1991). *Streptomyces* are found everywhere in soil ecosystems and may make up most of the Actinomycetes population. To selectively isolate rare Actinomycetes, it's crucial to eliminate undesirable *Streptomyces* and contaminants (Hayakawa, 2008). The search for promising actinomycete strains with antibiotic potential continues to be an important area of research (Hacene and Lefebvre, 1996).

The rise of antimicrobial resistance among pathogens emphasizes the need for new antibiotics (Budhathoki, 2020). This situation is compounded by patients self-medicating with antibiotics, which contributes to increased resistance rates. Discovering novel antimicrobials is vital for public health (Gurung et al., 2009).

In conventional isolation techniques, factors such as the screening source, selective medium, and culture conditions must be considered. The chosen media and conditions can enrich certain microbial populations while excluding others (Schneegurt, 2012).

Muthu et al., (2013) reported five antimicrobial isolates from ten screened samples of Cauvery River soil, identified as *Isoptericola variabilis*. Tyagi et al. (2014) isolated 11 actinomycete strains from 21 soil samples at temperatures of 28°C or 37°C, demonstrating strong activity against various bacteria.

Actinomycetes grown on agar media exhibit distinct cultural characteristics (Shirling and Gottlieb, 1976). Networks of hyphae are formed on the agar, with colony colors varying from white to pink. The colors of the substrate mycelium transition from brown to orange

(Arifuzzman et al., 2010; Mohseni et al., 2013). They are acknowledged for their ability to produce pigments in shades such as blackish brown, yellow, and red (Shirling and Gottlieb, 1966; Mohseni et al., 2013).

2.7 Antibiotics

Antibiotics are bioactive secondary metabolites that come from microorganisms. They can also be made partially or entirely by chemically synthesized (Radhakrishnan et al., 2010). They stop the growth of other bacteria and are necessary for treating infections both inside and outside the body. Some antibiotics are made by nature, but most are now made in a lab (Denyer et al., 2011). Actinomycetes are a great source of medical antibiotics, and scientists and the pharmaceutical industry care a lot about them (Kumar et al., 2010). They make a lot of different natural metabolites, such as antibiotics with different structures, such as aminoglycosides, anthracyclines, and tetracyclines (Barrios-Gonzalez et al., 2005). Fermentation is an important part of making antibiotics since it increases productivity (Dhananjay, 2010).

The rise of multidrug-resistant bacteria shows how much we need new antimicrobial drugs (Spellberg et al., 2004). Actinomycetes have been used to make several antibiotics. Some of these antibiotics have been completely purified, while others are only partially purified (Waksman et al., 2010). The efficiency of these antibiotics relies on the type of bacteria, the strain, and the conditions in which they thrive. Actinomycetes are the source of many well-known antimicrobials, such as streptomycin and erythromycin, which makes them useful for finding new drugs (Bailoori, 2020). Actinomycetes also make chemicals that kill bacteria that are resistant to antibiotics (Zhang, 2012). About two-thirds of the antibiotics that exist naturally come from the species *Streptomyces* (Narayana, 2007). Actinomycetes are still highly valuable in competing infectious diseases since they have been exploited to make several important bioactive chemicals and cancer-fighting medications since actinomycin was discovered. Finding microorganisms that can destroy other bacteria and be utilized for biocontrol is very important (Hassouni, 2019).

2.8 History of Antibiotic Development

Three hundred years ago, mold was used as an antibacterial. Ancient Russian peasants treated infected wounds with warm earth. Mold and plants were used in ancient China, Egypt, and Greece to heal illnesses. Sudanese-Nubian civilization used tetracycline antibiotics in 350 A.D. Mediaeval infections were treated with unprocessed plant extracts and cheese curds. Antibiotics were sought in the late 1800s as the germ theory of disease gained popularity. After then, medications to kill the disease-causing microorganisms are studied. This study sought the "magic bullet" that might kill bacteria without harming the patient (Wainwright, 1990).

2.9 Isolation of Actinomycetes from soil

Mixed microflora, distinguished by their distinctive sluggish development, pose considerable obstacles in isolation (Hirsch & Christensen, 1983). These problems have led to the creation of selective isolation processes, which are very important steps forward in microbiological research.

2.9.1 Preservation of soil samples:

One efficient way is to mix air-dried soil with calcium carbonate in a mortar and let the mixture sit for 10 days at 28°C in a closed, sterile petri dish that is turned upside down. Filter discs that are soaked in water assist keep the relative humidity high. This method gives the most Actinomycetes overall, but the least bacteria and fungi. Sodium propionate (Crook et al., 1950), phenol (Lawrence, 1969), and centrifugation are some of the other compounds and methods used.

2.9.2 Nutritional Selection:

General nutrient medium (Kuster & William, 1964; Williams & Davies, 1965; Okazaki & Okami, 1972), chitin medium (Lingappa & Lockwood, 1961), modified Benedict's medium (Porter et al., 1960), soybean meal-glucose medium (Tsao et al., 1960), Gauze's agar medium (Rehacek, 1959), Czapek's agar medium, glucose-asparagine medium (Nomura & Ohara, 1969), and glycerol asparagine medium II (Waksman, 1961) are all commonly used to grow Actinomycetes. Actinomycetes can make exoenzymes that break down important polymers like chitin, cellulose, and lignin. So, choosing the right medium is very important for isolating Actinomycetes successfully. The best sources of carbon are glycerol, starch, and chitin. The finest sources of organic nitrogen are casein, asparagine, and arginine. A lot of media also have inorganic nitrogen sources like nitrate and phosphate.

2.9.3 Selective Inhibition:

This method involves adding antibiotics to the medium to stop germs other than Actinomycetes from growing (Williams & Davies, 1965). This technique uses antibiotics that only kill non-Actinomycete bacteria or fungi, which lets Actinomycetes proliferate without any competition. Different ways have been suggested to separate Actinomycetes from organisms that are closely related to them. It is easy to tell them apart from fungus since their physiological traits are different. For instance, an antibiotic that only works against fungi and not Actinomycetes can be used efficiently. Dulency et al., (1955) utilized cycloheximide, whereas Porter et al., (1960) employed Nystatin and Pimaricin. Williams and Davis (1965) discovered that a combination of Nystatin and cycloheximide (50 µg/ml of medium each) successfully suppressed the majority of soil fungi without negatively impacting Actinomycetes; conversely, the use of cycloheximide alone proved to be less effective. Therefore, the use of antifungal medicines is crucial for the isolation of soil Actinomycetes. It can be harder to tell them apart from real bacteria, though, since some Actinomycetes may be resistant to antibiotics. Adding Penicillin (1 µg/ml) and Polymyxin (5 µg/ml) to the media can help limit the development of germs (Williams & Davis, 1965).

2.9.4 Soil Dilution Plate Method:

The soil dilution plate method is a very important way to separate and count Actinomycetes and other soil microorganisms. Williams & Davis, (1965) elucidate this simple method, commonly employed in ecological research to produce soil plates for the isolation of soil fungus. Usually, sterile water is diluent. For most types of soil, you can make plates by mixing soil with water at a ratio of 10^{-1} to 10^{-6}. The efficacy of this procedure can be affected by the content of the nutrient media and the way of incorporating the diluted soil suspensions into the agar medium.

2.9.5 Alternative Methods:

While numerous researchers have employed the dilution plate approach for soil Actinomycetes, various alternate methodologies exist, especially in ecological study. Adding modest amounts of soil to culture media can help separate soil fungus. Nonomura and Ohara (1960) determined that this method is very efficacious for isolating certain Micromonospora species. Hirsh and Christensen (1983) also described a method that used a membrane filter technology. In this method, a cellulose ester membrane with pores that are 0.2 to 0.45 µm wide is put on top of a nutrient agar medium. Samples, like soil, water, or different types of vegetables, are then put on the membrane's surface. When you inoculate, the branched mycelium of Actinomycetes goes through the filter pores to get to the agar media underneath. Non-Actinomycete bacteria can only grow on the filter surface. This method is especially useful for working with soil samples that have a lot of different microorganisms in them.

2.10 Screening of Actinomycetes for antimicrobial activity

Scientists have found new antibiotics in the past using screening. This method involves collecting a significant quantity of isolated microorganisms from nature that have the potential to create antibiotics. These isolates are used to make pure cultures so that their antagonistic activity can be tested. In this test, a chosen strain of bacteria is put in the same environment as the cultivated isolates, and the zone of inhibition that forms around it is watched. The bacteria selected for testing are often indicative of or associated with pathogenic pathogens.

The cross-streak approach is the traditional way to screen novel microbial isolates for antibiotic synthesis. Alternatively, the organisms may be cultivated in a liquid medium, and the filtered broth can be deposited into wells created on a seeded agar plate, resulting in a zone of inhibition that verifies antibiotic production. There are more than 8,000 recognized antibiotics right now, and every year, hundreds of new ones are found. Microorganisms from genera such as *Streptomyces, Penicillium, and Bacillus* are recognized for their antibiotic synthesis, indicating that numerous additional antibiotics may be identified through the examination of other microbial groups (Berdy, 2005).

Antibiotic discovery research is not simply a scientific pursuit but also an essential undertaking with profound ramifications for public health. Muthu et al. (2013) found that 5 out of 10 samples taken from the Cauvery River showed antibacterial activity. Through 16S rRNA sequencing and the bioinformatics tool BLAST, five isolates of Actinomycetes were identified (*Isoptericola variabilis*). Tyagi et al. (2014) documented the isolation of 11 Actinomycetes from 21 soil samples at temperatures of 28 °C or 37 °C. They tested these Actinomycetes to see how well they worked against both Gram-positive and Gram-negative bacteria. We specifically looked at how well they worked against *Staphylococcus aureus*, *Escherichia coli,* and *Bacillus* strains. The 11 actinomycete isolates exhibited significant activity, with inhibition zones surpassing 16 mm in diameter.

Rotich et al., (2017) isolated a total of 107 Actinomycetes, discovering that only 39 (36.4%) exhibited antimicrobial activity against five of the six test isolates, which included reference strains of *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), and *Candida albicans* (ATCC 90028), as well as three clinical strains: *Trichophyton mentagrophyte*, *Microsporum gypseum*, and Methicillin-Resistant *Staphylococcus aureus* (MRSA). Two of the isolates exhibited action against MRSA, and four shown greater efficacy than the conventional chloramphenicol (30 µg) against *S. aureus*.

2.11 Fermentation and Antibiotic production

Microbial biodiversity is an important resource for the biotechnology sector since it contains many genes and metabolic pathways that may be used to study enzymes, antibiotics, and other useful chemicals (Singh & Agrawal, 2002). Antibiotic research has become more important since Fleming and Chain's discovery in 1928. It now focusses on screening and changing microbes to make them useful.

Fermentation is the most common way to make antibiotics. It is an anaerobic process that doesn't need oxidative phosphorylation but does make Adenosine Triphosphate (ATP) through glycolysis. Actinomycetes are a major source of antibiotics, such as aminoglycosides, β-lactams, and tetracyclines (Okami & Hotta, 1988). Some actinomycete strains make distinct antibiotics, yet strains from different species can make the same ones (Lechevalier, 1975).

When environmental stress causes a metabolic reaction, antibiotics help microorganisms to protect themselves. Co-culturing *Streptomyces* with *T. pulmonis* can stimulate the synthesis of secondary metabolites absent in pure cultures (Garashi, 2005). Sodium azide and ethidium bromide are two chemicals that can induce Actinomycetes to synthesize antibiotics (Bhattarai et al., 2007).

Streptomycin and Penicillin were the first antibiotics produced in large quantities during World War II. Today, high-yield strains are grown in big fermentation tanks that are kept at the right temperature and humidity. After fermentation, the microbial cell mass is taken out, and antibiotics are separated from the rest of the cells using filters and other ways. The idiophase is when the concentration of antibiotics is at its highest, hence they are harvested then.

Liu et al. (1986) documented a 5-day fermentation of polyether antibiotics utilizing *Streptomyces* malachite-fuscus, whereas Kumagai (1993) employed a 6-day method employing *Nocardia brasiliensis*. Casidia (1959) delineated three phases of antibiotic synthesis, commencing with rapid mycelium growth, which is subsequently followed by a reduction, despite the continuous generation of antibiotics. It goes up quickly in the second phase. After 7 days of incubation, the manufacturing of the antibiotic stops in the third phase.

2.12 Media Composition

For the production of antibiotics, it requires the right media, which must have important parts like carbon, nitrogen, phosphates, and trace elements. Researchers often utilize glucose as the carbon source and adding it slowly during fermentation can help prevent catabolite suppression (Srinivasan et al., 1991).

Lactose and starch are two other types of carbohydrates that can also be used to stimulate antibiotic production. Ammonium nitrate is the best nitrogen source for making antibiotics, which shows that the kind and amount of nitrogen have a big effect on antibiotic levels. Meat extract was first utilized as a nitrogen source for making streptomycin, but yeast extract has mostly taken its place. Yeast extract supports growth and is a good supply of nitrogen (Egorov, 1985).

Phosphates are very important for synthesizing antibiotics; however, too much phosphorus might slow down development and lower antibiotic production. The best amounts are between 0.04 and 0.07 µg/ml (Egorov, 1985). Iron, zinc, and magnesium are also necessary for the growth of Actinomycetes and the generation of antibiotics. To get the most streptomycin, the best amount of ferrous sulphate ($FeSO4 \cdot 7H2O$) is between 0.0007% and 0.005%. More than that can slow down growth and make it harder to make antibiotics (Egorov, 1985).

2.13 Physiochemical factors

Temperature is a key factor in the process of biosynthesis of antibiotics and growth of Actinomycetes. It has been reported that the best temperatures are normally between 25 and 30 degrees centigrade, with a maximum at 28 degrees centigrade. Egorov (1985) mentioned that a temperature above 30 degrees might stop the biosynthesis of antibiotics. Further, pH affects the production of antibiotics. Casida (1959) reported optimum pH near 7, with formation taking place in the pH range of 7–8.

2.14 Isolation and Characterization of Antimicrobial Compounds

Since fermentation gives minute quantities of antibiotics in the fermentation broth, an effective purification technique is highly required. Basically, any antibiotic, which is soluble in an organic solvent and immiscible in water, can be dissolved into a small volume of solvent like n-butanol (Waksman 1968). Otherwise, it has to be separated from the broth. The main target here is reaping a highly pure crystalline product although some antibiotics are very difficult to crystallize.

Actinomycin was detected by Waksman in 1968 using n-butanol, while Egorov in 1985 concentrated streptomycin through activated carbon adsorption. The antibiotic formation has been quantified by Liu et al. (1986) after extraction of the compounds with ethyl acetate, and then analyzed by HPLC. Macrolide antibiotics were also extracted using acetone and butanol by Makoto in 1995 and thin-layer chromatography analyzed, as done by Ikekawa et al., (1963). On another research, Nabi et al. (2006) developed and characterized antibiotics using TLC after their extraction with ethyl acetate from the supernatant. HPLC has been applied in works by Kumagai et al. (1993) and Saha et al., (2012) to characterize antibiotics that were extracted from the supernatant of isolated Actinomycetes.

2.15 Mechanisms of antibiotic resistance

The multidrug efflux systems increase bacteria's antibiotic resistance. Effective antibiotics against drug-resistant bacteria require molecules that block efflux functions. The abundance of bacterial genetic data suggests several efflux mechanisms. A single bacteria may have efflux transporters from distinct families with overlapping substrate spectra. The MexXY multidrug efflux system is a major cause of aminoglycoside resistance in Psedomonas aeruginosa, according to growing evidence. A timely review by Morital et al., (2012) covered the *P. aeruginosa* MexXY pump and other aminoglycoside efflux pumps in other bacteria. Transcriptional regulators suppress or activate multidrug efflux genes to modulate bacterial multidrug efflux system expression. Usui et al., (2013) and Deng et al., (2013) showed the complexity of multidrug efflux system modulation. Baucheron et al., (2014) found that multi drug efflux systems may not be overestimated for certain antibiotics or organisms. In 2013, Chuma et al. found that Salmonella enterica Serovar Infantis had developed cefotaxime resistance due to beta-lactamase. Watkins et al., (2013) examined new betalactamase inhibitors nearing clinical use. Beta-lactamase inhibitors for combination therapy have been developed, however their varied affinity and the large amount of beta-lactamase produced by resistant organisms make their use difficult. Production of aminoglycoside-modifying enzymes is a key resistance mechanism. Shi et al., (2013) reviewed aminoglycoside kinase's structure and reported on structure-guided phosphotransferase inhibitor finding.

2.16 Confirmation of antibiotic presence

Different methods are used to confirm the presence of antibiotics, including biological, serological, and molecular techniques, along with thin-layer chromatography (TLC) and Basic Local Alignment Search Tool (BLAST) analysis. TLC is the most recognized and effective method for detecting and separating bioactive compounds. About 60% of compounds worldwide are analyzed using TLC, making it essential to understand its basic operations and procedures (Oppong et al., 2010).

Thin-layer chromatography separates non-volatile mixtures (Harry et al., 1989). It uses a sheet of glass, plastic, or aluminum foil coated with a thin layer of adsorbent material like silica gel, aluminum oxide (alumina), or cellulose. This adsorbent layer is called the stationary phase. When the sample is applied to the plate, a solvent moves up the plate through capillary action. Different substances travel at different rates, which leads to their separation (Vogel et al., 1989).

To measure the results, divide the distance traveled by the substance of interest by the total distance traveled by the solvent. This ratio is known as the retardation factor (Rf). Thin-layer chromatography helps monitor reactions, identify compounds in a mixture, and check the purity of a substance (Reich et al., 2007).

2.17 Research Gap

Research on Actinomycetes from soil of Kanchanpur district remains unexplored. Thus, this research may produce important knowledge into the diversity of Actinomycetes of Kanchanpur district and help in search of novel bioactive compounds, effective against pathogens. This research also fulfills the research gap by exploring novel Actinomycetes with novel bioactive compounds, contributing to the issue of antimicrobial resistance.

Chapter 3 Research Methodology

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 1.

3.2 Study Area

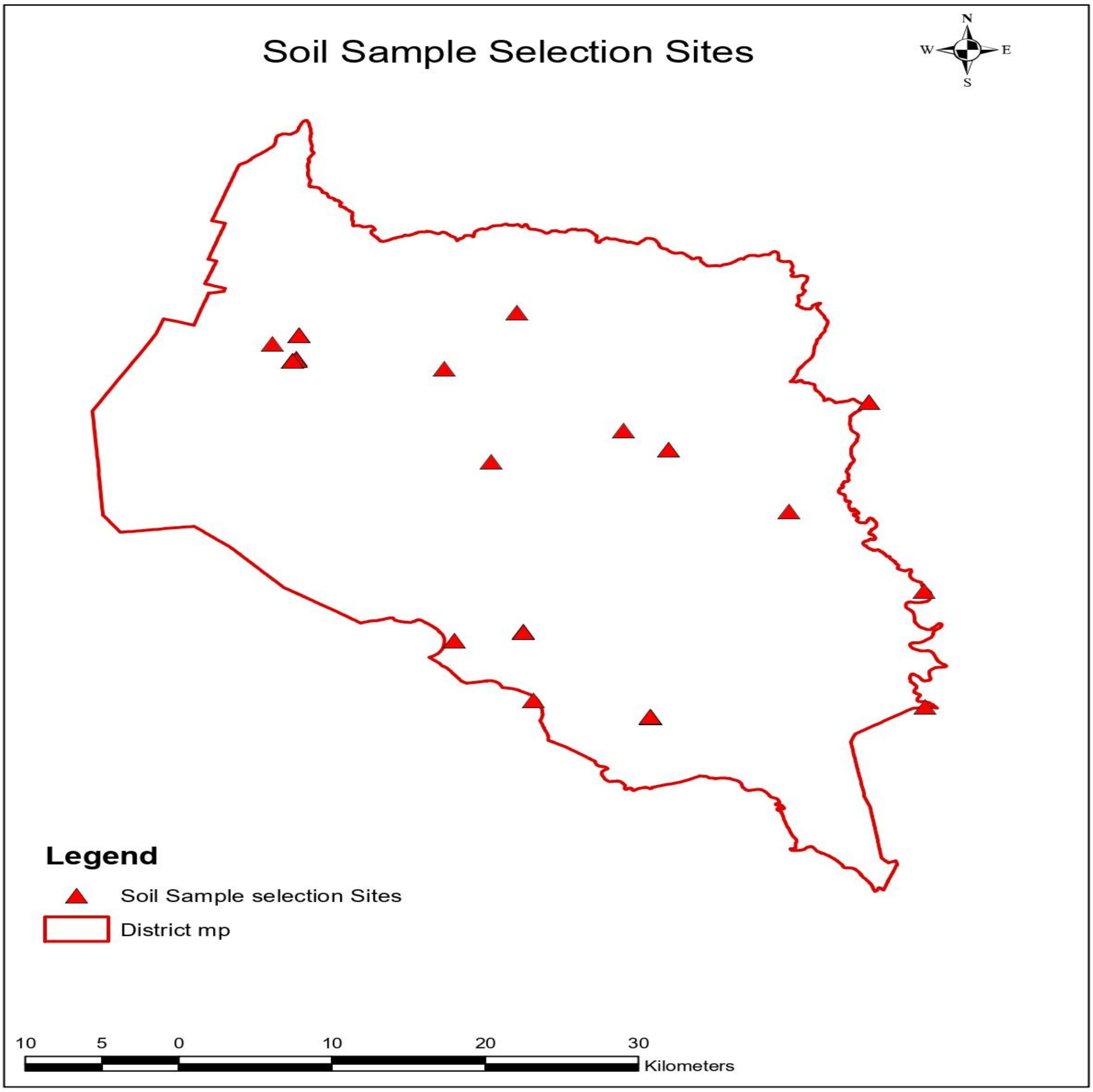


Figure 2 Study Area Map

3.3 Research Design

The study followed a laboratory-based prospective and exploratory design**,** aiming to isolate, identify, and assess antimicrobial activity of actinomycetes from soil samples. This study was started in August 2024. The sample collection and laboratory work were conducted from August 2024 to December 2024. The data validation and report writing was completed in August 2025. The Gantt Chart was listed in Appendix 6 and Budget Framework was listed in appendix 7.

3.4 Research Method

Soil samples were serially diluted and cultured on Starch Casein Agar (SCA) for selective isolation of actinomycetes (Bergey's & Holt, 2000). Purified isolates underwent morphological, Gram staining**,** and biochemical characterization (e.g., starch/casein hydrolysis, citrate/urease activity). Promising strains were screened for antimicrobial activity using perpendicular streak and well-diffusion methods against standard pathogens like *S. aureus* and *E. coli.*

3.5 Research Tools

Tools included SCA and Mueller-Hinton Agar, biochemical reagents, Gram staining kits, and ethyl acetate for metabolite extraction. Lab equipment included incubators, microscopes, autoclaves, laminar hoods, and rotary evaporators etc.

3.6 Sample and Sampling Design

A total of 30 soil samples were collected from diverse locations across Kanchanpur district**,** ranging from agricultural fields to forest areas, at altitudes of 147–671 m. Soils varied in pH (5.1–8.4) and moisture. Air-dried samples were processed for actinomycete isolation. The places of soil samples sites are listed in Appendix 4.

3.6.1 Study Variables

Geography, altitude, soil type, soil pH, soil temperature. The dependent variable is Actinomycetes sps, bioactive compounds and the independent variables are geography, altitude, soil type, soil pH, and soil temperature.

3.6.2 Collection of soil samples

The soil samples were collected from different parts of the Kanchanpur district. Approximately 10 g of dry soil was collected from 10 cm below the earth's crust, kept in a separate sterile polythene bag containing 1g $CaCO_3$ (Gurung et al., 2009). The samples were labeled and transported to Far Western University's microbiology laboratory. They were kept at room temperature for three weeks to dry and reduce bacteria and fungi.(Gurung et al., 2009). The flow chart of the laboratory procedure was listed in Appendix 5.

3.6.3 Isolation of the Actinomycetes

The spread plate technique was employed to isolate Actinomycetes. Soil samples were diluted in a series of steps using sterile distilled water, up to 10^{-2}. To prepare a 10^{-1} dilution, combine 1 g of soil with 9 mL of sterile water. To prepare a 10^{-2} dilution, add 1 ml of this dilution to 9 ml of sterile water. Agitating the soil in a vortex facilitated the release of Actinomycete spores. Then 0.1 mL aliquot from the 10^{-1} dilution was inoculated onto starch casein agar (SCA) containing 20 mg/l of amoxicillin and 30 mg/l of ketoconazole with bent glass rod. The plates were then left for five minutes and incubated at 28°C for seven days (Budhathoki & Shrestha, 2020).

3.6.3.1 Isolation of pure culture of Actinomycetes

The Actinomycetes exhibiting dry, powdery, chalky colony morphology were selected and streaked onto new SCA plates and incubated at 28°C for seven days to obtain pure colonies. The pure cultures were subculture on SCA broth by adding 15% glycerol at 28°C for seven days and stored at -20°C. Sub-culturing was performed every 2 to 3 weeks to maintain the viability of the cultures. The isolated Actinomycetes will be identified based on colony characteristics, morphology, and microscopically (Budhathoki & Shrestha, 2020).

3.6.4 Screening of Actinomycetes for antimicrobial activity

Actinomycetes will be screened for antimicrobial activity through primary and secondary screening.

3.6.4.1 Primary screening

The Actinomycetes isolates were first screened using the perpendicular streak method on Muller Hinton agar (MHA) plates. Actinomycete isolates were streaked in a line down the middle of an MHA plate and let them grow at 28°C for about seven days. The broth culture of the test organism was prepared by growing them on nutrient broth (NB) at 370C for 2 to 4 hours and then comparing the turbidity to the 0.5 McFarland standard. The broth of the test organisms was streaked across the fully grown Actinomycetes growth line on both sides. The plates were kept at 37°C for 24 hours, and the zone of inhibition around the test organisms' colonies was measured to see if they were antagonistic (Pandey et al., 2004; Sah & Lekhak, 2017; Budhathoki & Shrestha, 2020; Gurung & Rai, 2021). For the first screening, the test organisms were *Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Acinetobacter baumannii.*

3.6.5 Characterization of screened Actinomycetes

Morphological, biochemical, and physiological methods will characterize the Actinomycetes isolates selected from the primary screening as described in Bergey's Manual of Determinative Bacteriology, Ninth edition (2000).

3.6.5.1 Phenotypic Characterization

3.6.5.2 Macroscopic Examination

The isolated actinomycete colonies on SCA were examined for the coloration of the aerial mycelium and diffusible pigments, as well as other colony features including size, consistency, margin, and texture, which ranged from waxy and fuzzy to powdery, among others.

3.6.5.3 Microscopic Examination

The microscopic analysis was conducted by performing Gram's staining of the Actinomycetes isolates. The mycelia were examined under a microscope (1000X) to assess their mycelial structure, configuration of sporophores (conidiospore and arthrospore), and the organization and morphology of spores on the mycelia. The morphology of the isolates was compared to the Actinomycetes morphology outlined in Bergey's Manual of Determinative Bacteriology, Ninth edition (2000), for primary identification of the isolates.

3.6.6 Biochemical characterization

A series of biochemical tests such as catalase, oxidase, citrate utilization, methyl red (MR), Voges Proskauer (VP), triple sugar iron agar (TSIA), indole, urease, starch hydrolysis, casein hydrolysis, and gelatin hydrolysis were conducted to identify potent screened Actinomycetes

(Holt et al., 1994; Muiru et al., 2008; Gebreyohannes et al., 2013; Sowmya & Ramalingappa, 2022).

3.6.6.1 Catalase Test

The process was done by selecting an isolated colony using a sterile glass rod and combining it with a drop of 3% H_2O_2 solution on a clean glass slide. The emergence of gas bubbles indicates a positive test.

3.6.6.2 Oxidase Test

The process was done by selecting an isolated colony of Actinomycetes using a sterile glass rod and applying it to a paper strip that has been treated with oxidase reagent (1% tetramethyl para phenylene diamine dihydrochloride). Development of a bright deep purple color on the paper strip within one minute indicates positive test.

3.6.6.3 Starch Hydrolysis Test

The starch hydrolysis test was done by inoculating starch agar plates with a pure culture of the Actinomycetes colony, followed by incubation at 30°C for a duration of 2 weeks. The detection of starch hydrolysis was done by applying iodine solution to the plates. A distinct area of hydrolysis surrounding the colonies indicates a positive result.

3.6.6.4 Gelatin Hydrolysis Test

The gelatin hydrolysis test was done by inoculating gelatin agar plates with the isolated pure culture of Actinomycetes, followed by incubation at 30°C for a duration of two weeks. The detection of gelatin hydrolysis was achieved by applying a mercuric chloride solution to the plates. A distinct area of hydrolysis surrounding the colonies represents a positive result.

3.6.6.5 Indole and Hydrogen Sulphide (H_2S) Production Test

These tests were done by stabbing sulfide indole motility (SIM) agar tube with the pure culture of Actinomycetes followed by incubation of the tubes at 30°C for a duration of 2 weeks. The presence of indole was indicated by the formation of a cherry red color at the interface after the addition of Kovac's reagent. The presence of H_2S was indicated by the darkening of the medium.

3.4.7.6 Citrate Utilization Test

The citrate utilization test involves streaking the slant of Simmon's citrate agar tubes with a pure culture of Actinomycetes colonies. The tubes were incubated at 30°C for a duration of 2 weeks. The detection of citrate utilization was indicated by a color change in the medium from dark green to Prussian blue.

3.6.6.7 Urea hydrolysis

A pure culture of Actinomycetes colony was inoculated on urea agar slants and incubated at 30°C for a duration of 2 weeks. The formation of a pink color on the slant, turning from orange, indicates a positive test result (Gebreyohannes et al., 2013).

3.6.6.8 Motility Test

This test involves inoculating a sulphide indole motility (SIM) agar tube with a pure culture of Actinomycetes colony. The tubes were incubated at 30°C for a duration of two weeks. Motility was indicated by the diffuse growth of Actinomycetes extending from the stab line in the medium.

3.7 Method of Data Collection

Field data included location, altitude, soil pH, and characteristics. Lab data included colony morphology, Gram reaction, enzyme activity, and zones of inhibition against test bacteria. Observations were recorded in structured formats.

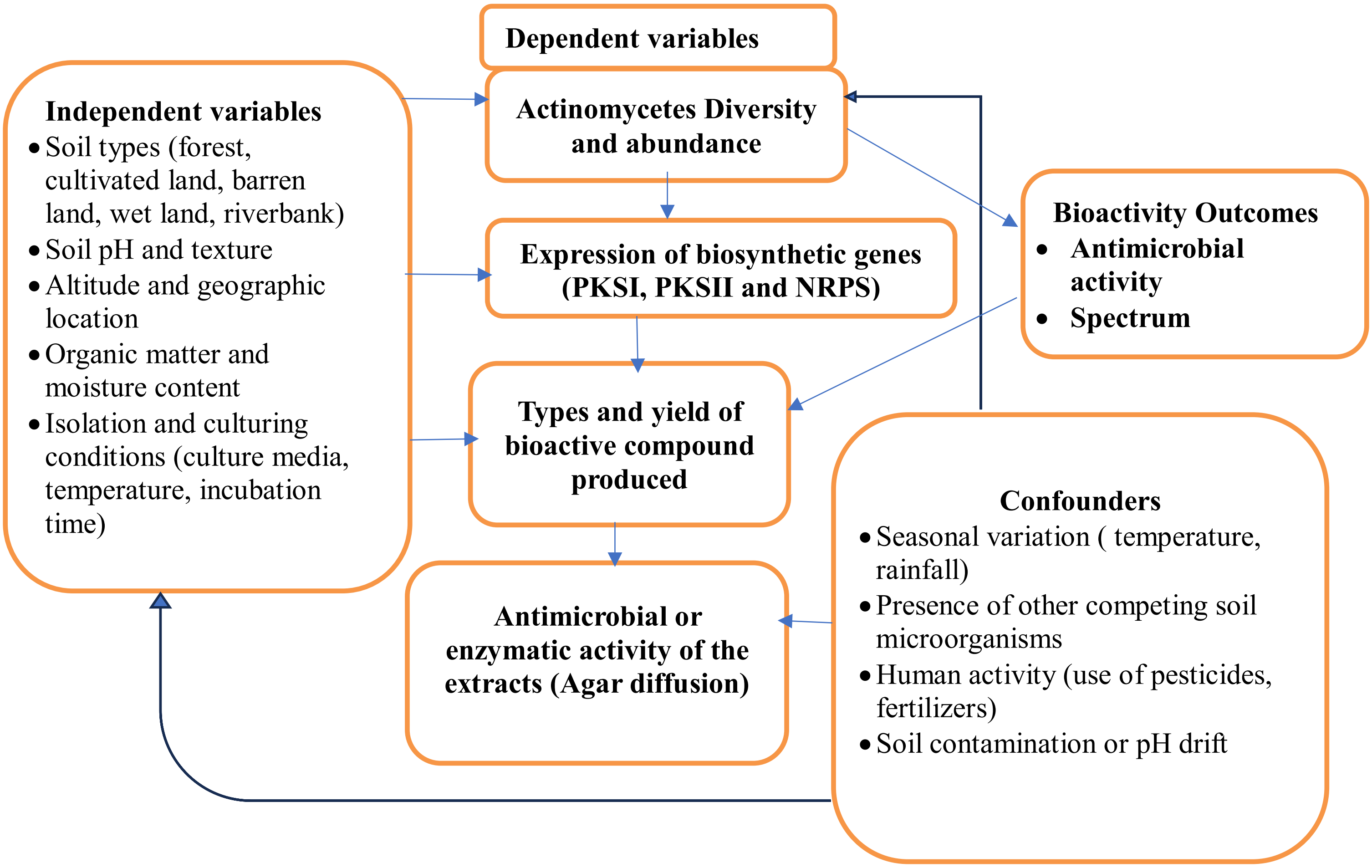
3.8 Data Analysis Procedure

Data were analyzed using descriptive statistics**,** with means and frequencies calculated for isolate characteristics and antimicrobial activity. Correlation tests examined the relationship between soil pH and actinomycete abundance or bioactivity. All data were entered into Excel sheet and then SPSS version 23 was used for statistical analysis. If the P-value is less than 0.05, it will be considered statistically significant.

3.9 Ethical Consideration

All work followed Biosafety Level 2 standards. Soil was collected with permission from the landowners, Ministry of Land Management, Agriculture and Cooperatives, Sudurpaschim Government, Dhanghadi Kailali, appendix 2 and Department of National Parks and Conservation, Babarmahal, Kathmandu, appendix 3 and microbial cultures were handled and disposed of responsibly. No human or animal subjects were involved, and departmental ethical approval was obtained.

3.10 Conceptual Framework



Chapter 4 Result and Discussion

4.1 Isolation and Distribution of Actinomycetes in Soils

Out of the 30 soil samples collected, actinomycete colonies were successfully isolated from 10 samples (33%), yielding a total of 21 isolates. These isolates were linked to soil pH levels, with all Actinomycetes recovered from soils having pH values between 6.4 and 8.3. No isolates were obtained from more acidic samples (pH 5.1 to 5.6). Statistical analysis indicated a significant relationship between higher pH levels and Actinomycetes presence, with an average pH of 7.6 in positive samples compared to 6.6 in negative samples ($p < 0.01$). Our results are consistent with those of Budhathoki and Shrestha (2020), who found that antibiotic-producing Actinomycetes were most frequently present in soils with pH values between 7.4 and 8.4.

Actinomycetes were also isolated from soils under forest, agriculture, and riverbanks, mostly gray or black soils; Actinomycetes were not isolated from soils of brown color. Our results align with those showing the black soils have high organic material and hence are more conducive to the growth of antibiotic-producing Actinomycetes (Karki and Ale Magar, 2024).

The moisture of the soil had a minimal effect on the isolation frequency; 39% and 25% of the dry and moist samples, respectively, yielded Actinomycetes. In this line, the normally moist contents could offer support to their growth while excess water hinders that support. Our results argue about the principal factor for the distribution of Actinomycetes being the pH of the soils, with quite some luck on neutral to alkaline and well-aerated ones.

This moderate recovery of 21 isolates is definitely much lower than that of most other studies. For example, Budhathoki and Shrestha (2020) were able to recover as many as 121 strains from only 15 samples. This lower recovery could possibly be attributed to the acidic sites in our sampling or the use of different methodological approaches. Also, the recovery fell short of the 43–48% ranged bioactive isolates, which were gained in larger surveys, and connected with the trends of one third to one half of all Actinomycetes isolated basically showing antimicrobial activity in primary screenings.

Four bioactive Actinomycete isolates were recovered from three different soil samples: S12, S24, and S25. In all cases, bioactive isolates were derived from a grey type of soil having neutral to alkaline pH values ranging from 7.1 to 8.1. Two of the bioactive isolates were recovered from moist soil at Bhimdatta 6, Aithpur, and two from dry soil at Krishnapur Bank and Bhimdatta 6, Aithpur, indicating that bioactivity might even occur under drier conditions. From the results obtained, it seems that bioactive actinomycetes were predominant in slightly neutral to alkaline pH soil, either moist or humid.

Table 1 Description of soil samples along with the total number of isolates and bioactive isolates

Soil sample	Altitude(m)	Location	Physical characteristics of soil			No. of Actinomycete Isolates	No. of bioactive Actinomycete isolates
			pH	Color	Consistency		
S1	224	Shuklaphanta National Park, Beldandi	5.5	Grey	Dry	0	0
S2	204	Beldadi 1	6.5	Brown	Moist	0	0
S3	205	Belauri-9	6.4	Black	Moist	2	0
S4	205	Belauri-9	7.8	Grey	Dry	2	0
S5	147	Belauri-8, Kalkatta	7.9	Grey	Moist	0	0
S6	153	Belauri-3, Laxmipur	7.4	Brown	Moist	0	0
S7	156	Belauri-4 shreepur	7.5	Brown	Moist	0	0
S8	156	Belauri-4, Shreepur	6.8	Black	Moist	2	0

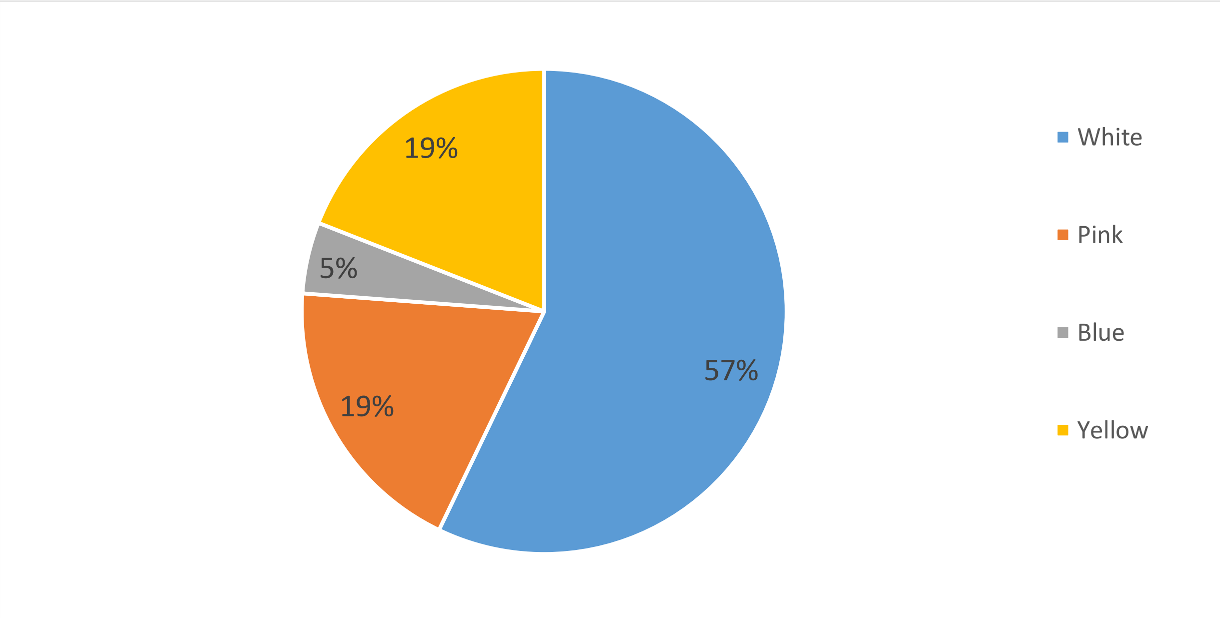
S9	155	Punarvas-11, Dokebazar	8	Grey	Dry	0	0
S10	184	Krishnapur-6, Shantipur	8.3	Grey	Dry	2	0
S11	225	Krishnapur-Gularia	5.5	Grey	Dry	0	0
S12	225	Krishnapur-, Bank	7.7	Grey	Dry	3	1
S13	208	Shuklaphanta , Kaluwapur	7.1	Black	Moist	0	0
S14	215	Shuklaphanta National Park, Arjuni	5.4	Brown	Dry	0	0
S15	667	Bedkot-3, Chhure hill	5.4	Grey	Dry	0	0
S16	671	Bedkot-3, Chhure hill	5.6	Grey	Dry	0	0
S17	507	Bedkot-3, near lake	5.1	Grey	Dry	0	0
S18	458	Bedkot-3, Chella	5.1	Grey	Dry	0	0
S19	206	Bedkot-, Daiji	7.8	Black	Dry	3	0
S20	206	Bhimdatta-4, Gobariya	7.6	Grey	Dry	2	0

S21	199	Bhimdatta-18, Katan	8.4	Black	Moist	0	0
S22	208	Bhimdatta-18, Katan	6.9	Brown	Moist	0	0
S23	208	Bhimdatta-18, Katan	6	Brown	Dry	0	0
S24	200	Bhimdatta-6, Aithpur	8.1	Grey	Dry	1	1
S25	200	Bhimdatta-6, Aithpur	7.1	Grey	Moist	3	2
S26	200	Bhimdatta-6, Aithpur	7.9	Black	Dry	1	0
S27	208	Bhimdatta-15, Nimbukheda	7.4	Grey	Dry	0	0
S28	208	Bhimdatta-15, Nimbukheda	7.6	Brown	Dry	0	0
S29	208	Bhimdatta-15, Nimbukheda	7.4	Brown	Moist	0	0
S30	208	Bhimdatta-15, Nimbukheda	7.5	Black	Moist	0	0

4.2 Characteristics of the bioactive compound producing isolates

The 21 isolates showed great diversity with regard to their morphology on starch casein agar (Figure 2). The white colonies accounted for nearly 57% of total colonies and appeared chalky or creamy; their aerial mycelium was usually powdery. Yellow and pink pigmented colonies were frequent, but one peculiar dark blue pigment was shown by a single isolate. The characteristic property linked to agar from Actinomycetes is mostly related to the pigment (figure 1). Many Actinomycetes, in turn, produce differing earthy pigments or colors at times. Sapkota et al., 2020 found yellow to be the most common pigment in Actinomycetes in Nepal. However, in our collection, white was predominant. The difference between them might reflect the difference in the species composition white colonies are usually related to Streptomyces species, which developed high aerial hyphae and give a whitish color, bright colored or dark colonies which could be from other genera or even from pigmented actinobacteria. Each soil sample was found to have 2–3 unique colony types of Actinomycetes.

Figure 2 Types of pigment produced by isolates on starch casein agar



4.2.1 Colony characteristics

Colony size on agar ranged from 2 mm to 5 mm in diameter after 7–14 days incubation (Table 3). Most colonies were small (2–4 mm) and slow-growing, consistent with the nature of Actinomycetes. A few isolates (a pink isolate from sample S20) formed relatively larger colonies (5 mm). All colonies were tough, adhering strongly to the agar, and many had dry or folded surface features typical of *Streptomyces* and related genera which form filamentous mats. Microscopic examination of colony smears after Gram staining confirmed that all isolates are Gram-positive and exhibit filamentous, branching hyphae that fragment into rod-shaped or beaded elements. This Gram-positive, fungus-like morphology is a hallmark of Actinomycetes (Budhathoki and Shrestha, 2020).

Table 3 Colony characteristics of the isolates

S. No	Sample	Color of colony	Size of colony (mm)
1	S3	White	3
		White	4
2	S4	White	3
		Pink	4
3	S8	White	3
		Yellow	3
4	S10	White	3
		White	4
5	S12	Yellow	3
		White	2
		Blue	2
6	S19	Pink	3
		Yellow	3
		White	3
7	S20	Pink	5
		White	3
8	S21	White	3
9	S25	White	4
		Pink	3
		Yellow	3
10	S26	White	3

4.2.2 Microscopic characteristics

In Gram staining (Table 4) some isolates showed long, branching filaments with occasional spore chains (appearing as bead-like segments), while others showed shorter rod/coccus-like fragments (possibly due to breakage of mycelium). Such pleomorphic, "ray-fungus" morphology is well documented for actinomycetes (Budhathoki and Shrestha, 2020). No acid-fast branching bacteria (e.g. *Nocardia*) were specifically noted; all retained the crystal violet stain, indicating typical actinomycetal cell walls rich in peptidoglycan. Detailed genus-level morphological identification such as spore chain arrangement or melanoid pigment production tests were not conducted. The observed traits strongly suggest that many isolates belong to the genus *Streptomyces* which are Gram-positive, aerobic filamentous bacteria known for earthy odor and powdery colonies. This supposition is further upheld by corresponding research in Nepal. One study indicated that 70 to 71% of soil samples actinomycete isolates were identified as *Streptomyces* spp., with the rest of the isolates in the study pertaining to other genera like *Nocardia* and *Micromonospora* (Sapkota et al., 2020). The isolates also contains a mixture of *Streptomyces* and possibly some rare Actinomycetes which would be confirmed by molecular identification. Regardless of genus, all the isolates conform to the general Actinomycetes morphology and staining characteristics, reinforcing the success of our selective isolation approach.

Table 4 Gram staining of the isolates

S.No	Sample	Colour	Morphology	Remarks
1	S3 White	Purple	Beaded rod	Gram Positive
2	S4 white	Purple	Branched rod	Gram Positive
	S4 Pink	Purple	Beaded rod	Gram Positive
3	S8 White	Purple	Beaded rod	Gram Positive
	S8 yellow	Purple	Beaded rod	Gram Positive
4	S10 White small	Purple	Filamentous	Gram Positive
	S10 White	Purple	Beaded rod	Gram Positive
5	S12 White	Purple	Beaded rod	Gram Positive
	S12 Yellow	Purple	Beaded rod	Gram Positive
	S12 Blue	Purple	Beaded rod	Gram Positive
6	S19 White	Purple	Beaded rod	Gram Positive
	S19 Yellow	Purple	Branched	Gram Positive
	S19 Pink	Purple	Beaded rod	Gram Positive
7	S20 Pink	Purple	Beaded rod	Gram Positive
	S20 White	Purple	Beaded rod	Gram Positive
8	S24 White	Purple	Filamentous rod	Gram Positive
	S25 White	Purple	Filamentous rod	Gram Positive
9	S25 Pink	Purple	Filamentous rod	Gram Positive
	S25 Yellow	Purple	Filamentous rod	Gram Positive
10	S26 White	Purple	Filamentous rod	Gram Positive

4.2.3 Morphological characteristics

A series of biochemical tests were performed on the four antibiotic-producing isolates (Table 5) to characterize their metabolic capabilities. All four active isolates were confirmed Gram-positive, with no spore formation observed under the test conditions common for non-sporulating actinomycetes or those requiring specific cues to sporulate.

4.2.3.1 Biochemical tests

All four isolates tested positive for catalase gas bubble production with 3 % H_2O_2 and for oxidase (no color change on tetramethyl-p-phenylenediamine). This is in favor with many aerobic Actinomycetes such as *Streptomyces* which are typically catalase-positive (Mondal & Thomas, 2022). The negative catalase result in one isolate could indicate it belong to catalase-negative genera or reflect a slow enzyme reaction not detected in the assay. Some *Micromonospora* or rare actinomycetes might lack catalase, but further investigation is needed. Oxidase negativity is common in Actinomycetes because they do not use cytochrome c oxidase pathways. All four isolates were Indole-negative, out of four isolates three isolates were motile and one isolate was non motile. All four isolates gave a positive Methyl Red (MR) test indicated by red color in acidic broth because they produce stable acidic metabolites from glucose fermentation. All four isolates gave a negative Voges-Proskauer (VP) test indicated by no change in color because they were unable to produce acetoin from glucose. All four isolates could utilize citrate as a sole carbon source as Simmon's citrate agar. Citrate positivity is a trait found in many soil actinomycetes (Mondal & Thomas, 2022). Three of four isolates were urease-positive but one isolate (S25 Yellow) was urease negative. Urease production is fairly common in Actinomycetes, including some *Streptomyces* and relates to nitrogen metabolism in soil (Mondal & Thomas, 2022). In, sulfide indole motility (SIM) medium one isolate S25 Yellow produced H_2S indicated by blackening of the medium whereas the others did not. H_2S production by Actinomycetes is not usually a prominent trait, but this result suggests S25 Yellow might have the ability to reduce sulfur compounds (perhaps cysteine desulfurase activity). The majority showing H_2S-negative is consistent with typical actinomycetes (Mondal & Thomas, 2022). All isolates produced some acid in triple sugar iron (TSI) agar (yellow butt), but as expected for non-fermentative aerobes, the slants were not strongly acidified because they remained red or slightly yellow with limited glucose fermentation, and some gas was observed in one case.

In summary, the biochemical profile of bioactive isolates suggests they are non-fermentative or weakly fermentative aerobes, with an oxidative metabolism that does not produce indole or large amounts of neutral acetoin. The citrate positive, urease positive pattern in most isolates and indole negative, oxidase negative pattern aligns well with characteristics of known soil Actinomycetes (Mondal & Thomas, 2022). One published actinomycete strain, *Beijerinckia fluminensis* (marine-derived but an Actinobacteria), exhibited a very similar biochemical pattern: positive for starch, casein, gelatin hydrolysis, citrate, urease, and negative for MR, VP, indole (Mondal & Thomas, 2022). The isolates match this description closely, underscoring their actinomycete identity.

Table 5 Biochemical Tests For Actinomycetes Isolates

S. No.	Sample	Catalase	Oxidase	Indole	Methyl Red	VP	Citrate	Urease	TSIA	Motility	H_2S
1	S12 Blue	+	-	-	+	-	+	+	Alk/A G^+	Motile	-
2	S24 White	+	-	-	+	-	+	+	Alk/A	Motile	-
3	S25 Yellow	+	-	-	+	-	+	-	Alk/A	Motile	+
4	S25 White	+	-	-	+	-	+	+	Alk/A	Non motile	-

+ positive, - negative, A/A- acid/acid, Alk/A- alkali/acid, A/Alk- acid/alkali

4.2.3.2 Substrate hydrolysis tests

All isolates were tested for their ability to hydrolyze important polymeric substrates, and results were uniformly positive (Table 6). Every isolate formed a clear zone on starch agar upon iodine flooding, confirming they secrete amylolytic enzymes. This is a well-known trait of actinomycetes; they contribute to starch breakdown in soil, and most *Streptomyces* are amylase-positive (Mondal & Thomas, 2022). Likewise, all isolates liquefied gelatin agar each isolate formed a clear zone on gelatin agar upon mercuric chloride flooding indicating production of proteases (gelatinases). Actinomycetes are prolific producers of extracellular proteases and play a role in protein decomposition (Mondal & Thomas, 2022). All isolates hydrolyze Casein which was seen as the growth of colonies on starch casein agar which suggests they can utilize casein. In similar studies, a majority of isolates hydrolyze casein and other polymers (Mondal & Thomas, 2022).

Table 6 Substrate Hydrolysis Test

S. No	Starch Hydrolysis	Gelatin Hydrolysis	Casein Hydrolysis
1	+	+	+
2	+	+	+
3	+	+	+
4	+	+	+

+ positive, - negative

4.4 Screening of actinomycetes for antimicrobial activity

4.4.1 Primary screening of actinomycetes

Primary screening of antimicrobial activity of the isolates was conducted against test bacteria such as *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Morganella*, *Proteus*, *Acinetobacter*, *Pseudomonas aeruginosa* and *Serratia marcescens* (Table 7). Out of 21 isolates, four isolates exhibited measurable antibacterial activity, as mentioned earlier. The isolate S25 (Yellow colony) sample collected from Bhimdatta-6 (pH 7.1, moist soil) was the most potent and broad-spectrum. It produced clear inhibition zones against six of the nine test organisms. Notably, it inhibited *Staphylococcus aureus* (7 mm diameter zone) and *Enterococcus* (8 mm) very strongly. It also showed activity against *Escherichia coli* (3 mm) and *Klebsiella pneumoniae* (4 mm), indicating some Gram-negative coverage. *Morganella* was moderately inhibited (8 mm), and *Pseudomonas aeruginosa* showed a small zone (4 mm), which is significant since *Pseudomonas* is often highly resistant. However, this isolate had no effect on *Proteus*, *Acinetobacter,* or *Serratia* (0 mm zones). The broad activity suggests S25 Yellow produces one or more antibiotics with a fairly wide spectrum (covering both Gram-positive and certain Gram-negative bacteria). Given the strong zones, especially against *S. aureus* and *Enterococcus*, the antibiotic might be quite potent. This isolate is a prime candidate for further study (secondary metabolite extraction and characterization).

The Isolate S24 (White colony) Sample collected from Bhimdatta-6, (pH 8.1, dry soil) displayed moderate, multi-target activity. It inhibited five out of nine pathogens tested, chiefly Gram-positives and a couple of Gram-negatives. *Staphylococcus aureus* was suppressed with a 3 mm zone, and *E. faecalis* with 4 mm. Among Gram-negatives, it showed small zones against *E. coli* (2 mm) and *Klebsiella* (1 mm) and also inhibited *Morganella* (4 mm). No activity was seen against *Proteus*, *Acinetobacter*, *Pseudomonas*, or *Serratia* (all 0 mm). The spectrum of S24 White is thus primarily against Gram-positive bacteria (with marginal effect on a couple of Gram-negatives). The zones were relatively small, suggesting weaker antibiotic or lower production levels compared to S25 Yellow. Nonetheless, being active against *S. aureus* and *Enterococcus* is valuable (these are common Gram-positive pathogens).

The isolate S12 (Blue colony) sample collected from Krishnapur (pH 7.7, dry soil had a narrower spectrum, inhibiting three out of nine test microbes. It formed a 2 mm zone against *S. aureus*, 2 mm against *Klebsiella*, and 3 mm against *Enterococcus*. It also showed very small 1 mm inhibition against *E. coli* (borderline). Importantly, it did not inhibit most Gram-negatives (0 mm for *Morganella, Proteus, Acinetobacter, Pseudomonas, Serratia*). The activity profile here is mostly anti-Gram-positive (consistent with many known actinomycete antibiotics, like streptomycin, which target Gram-positive bacteria preferentially). The small zone sizes indicate the antibiotic yield or diffusion was low; it's possible S12 Blue produces a low concentration of an antibiotic that is only weakly active or diffuses poorly in agar.

The isolate S25 (White colony) sample collected from Bhimdatta-6 (pH 7.1, moist soil) was a second distinct colony from that sample. This isolate showed low but detectable activity against two or three pathogens. It gave a 2 mm zone against *S. aureus*, 2 mm against *Enterococcus*, and 1 mm against *E. coli*. Other test bacteria were unaffected (0 mm). The spectrum again leans toward Gram-positive targets. Given that this S25 White came from the same soil as S25 Yellow (which was much stronger), it is interesting that same soil contained two antibiotic producers, though one clearly outperformed the other. It is possible they produce different compounds, or one produces much less of the active metabolite.

To visualize the overall pattern all four active isolates were able to inhibit *Staphylococcus aureus* to some extent (2–7 mm zones), making *S. aureus* the most universally susceptible target in this panel. In contrast, none of the four inhibited *Serratia marcescens*, and only one (S25 Yellow) could inhibit *Pseudomonas aeruginosa*. *Proteus* and *Acinetobacter* were also completely resistant to all isolates. This susceptibility trend Gram-positive bacteria being more easily inhibited by actinomycetes metabolites than Gram-negative is well documented. Actinomycetes antibiotics (like β-lactams, aminoglycosides, etc.) often have more difficulty penetrating Gram-negative outer membranes or may be pumped out by efflux mechanisms. Our findings concur with those of Budhathoki & Shrestha (2020), who noted actinomycete isolates were more active against Gram-positive *S. aureus* than against Gram-negative organisms, likely due to differences in cell envelope structure and target accessibility (Budhathoki & Shrestha 2020). Indeed, they reported *S. aureus* was the most susceptible test organism, whereas Gram-negatives like *Salmonella* and *P. aeruginosa* were least susceptible (Budhathoki & Shrestha 2020). This is consistent with the idea that Gram-negative bacteria's outer membrane and efflux systems render them less sensitive to many actinomycete antibiotics (Budhathoki & Shrestha 2020). Nevertheless, the fact that our S25 Yellow isolate inhibited *Pseudomonas* is notable that relatively few soil Actinomycetes produce compounds that can breach Gram-negative defenses, so this isolate might produce a potent or broad-spectrum agent worth investigating.

Comparing our antimicrobial results with other studies: the proportion of active isolates (4 out of 21, 19%) is on the lower side of the range but not atypical for a primary screening using unoptimized conditions. Other studies in Nepal found 33–48% of isolates active in primary screening (Budhathoki & Shrestha 2020). For example, Sapkota et al, 2020 reported 43.3% of their isolates showed antimicrobial activity mostly against Gram-positive bacteria. Budhathoki & Shrestha, 2020 found 47.9% active, with many inhibiting only one test species. It is possible that if we tested our isolates against a different panel (e.g. including *Bacillus* or *fungi*), we might find a few more actives, as some actinomycetes target fungi or specific bacteria not in our panel. It's also likely that secondary screening (cultivating isolates in production media and extracting metabolites) would yield stronger activity. In our primary agar-based screen, zone sizes were relatively small, implying that higher antibiotic concentrations (via fermentation and extraction) could enhance detectable activity. For instance, Budhathoki & Shrestha, 2020 performed a secondary screening via agar well diffusion of ethyl acetate extracts and continued to see *S. aureus* as the most inhibited with larger zones up to 20 mm by some isolates (Budhathoki & Shrestha, 2020). A similar approach with our top producers (especially S25 Yellow) could reveal greater potency and possibly activity against those Gram-negatives that showed no zones in primary screening.

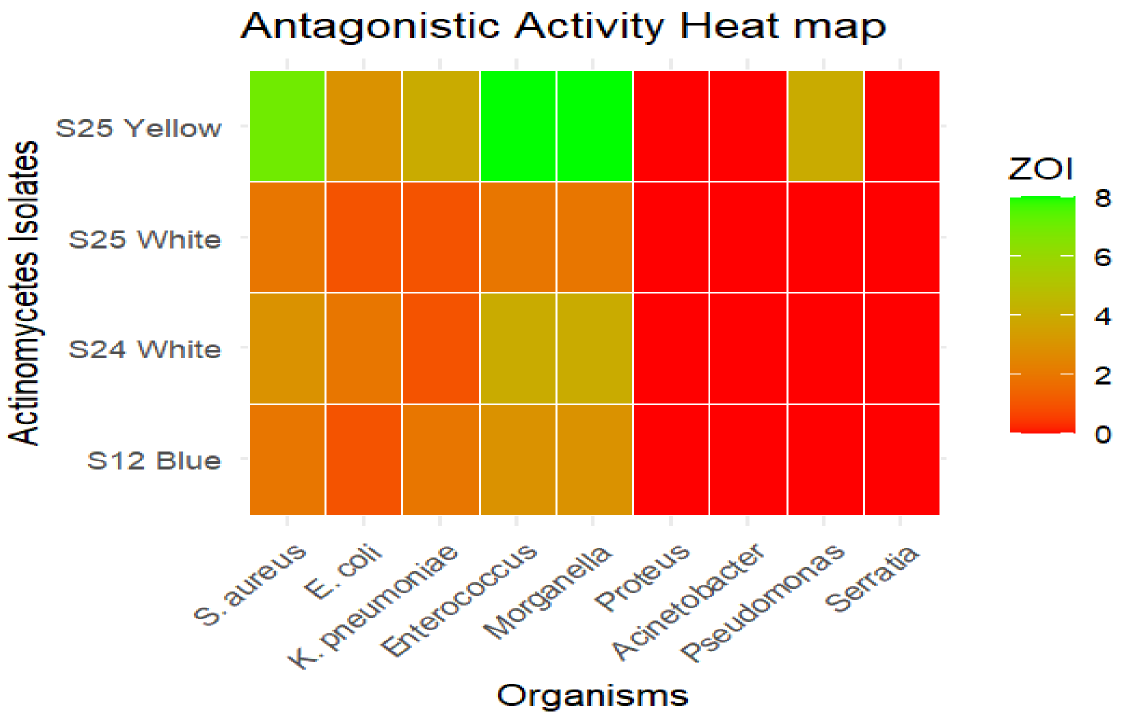
In summary, our antimicrobial screening establishes that a subset of actinomycetes from Kanchanpur soils produce antibiotic substances. These substances are particularly effective against Gram-positive bacteria (all active isolates hit *S. aureus*, some hit *Enterococcus* and *Bacillus* genus represented by *Morganella/B. subtilis* in analogous studies (Budhathoki & Shrestha, 2020). Gram-negative pathogens were generally resistant, with only the most potent isolate affecting *E. coli, Klebsiella,* and *Pseudomonas* to a limited extent. This pattern is typical for soil-derived antibiotics (Budhathoki & Shrestha, 2020). The isolate S25 (Yellow) stands out as a promising broad-spectrum strain, and S24 (White) also has multi-target activity worth further exploration. The other actives, while narrower in effect, could still be sources of specific anti-staphylococcal or anti-enterococcal agents.

Importantly, these findings emphasize the bioprospecting potential of even a small collection of actinomycetes from unexplored habitats. We have essentially scratched the surface with 30 samples; a richer sampling or dedicated screening program in the Terai region could uncover many more antibiotic producers. All four active isolates in this study came from neutral pH soils, reinforcing that such environments are fruitful hunting grounds for antibiotic-producing actinomycetes (Budhathoki & Shrestha, 2020). Our data contribute to the growing evidence that Nepal's diverse soils harbor actinomycetes capable of yielding novel antimicrobials (Sapkota et al., 2020; Budhathoki & Shrestha, 2020).

Table 7 Primary Screening results of the isolates

Sample	**Zone of Inhibition (mm)**								
	Staphylococcus aureus	***Escherichia coli***	***Klebsiella pneumoniae***	***Enterococcus***	***Morganella***	***Proteus***	***Acinetobacter***	***Pseudomonas***	***serratia***
S12 Blue	2	1	2	3	3	0	0	0	0
S24 White	3	2	1	4	4	0	0	0	0
S25 Yellow	7	3	4	8	8	0	0	4	0
S25 White	2	1	1	2	2	0	0	0	0

Figure 4 Primary Screening results of the isolates Heat map



4.5 Discussion

This study examined actinomycete communities in soil from Kanchanpur, Nepal, revealing insights into their diversity, characteristics, and bioactivities, and allows comparison with regional and global research. We found Actinomycetes in one-third of the samples, with a clear preference for neutral to alkaline soils. This agrees with the consensus that most Actinomycetes thrive at pH ~7–8 (Budhathoki & Shrestha, 2020). Our observation that no isolates were obtained below pH 6 reflects the inhibitory effect of acidity on actinomycete growth; acidic forest or peat soils often support fewer Actinomycetes (Budhathoki & Shrestha, 2020). Budhathoki & Shrestha (2020) similarly reported that effective antibiotic-producing isolates were predominantly from soils of pH 7.4–8.4, with very few from acidic ranges. This pH dependence is commonly attributed to Actinomycetes' physiology neutral pH favors their enzyme function and sporulation, whereas acidic conditions favor fungi and acid-tolerant bacteria, outcompeting Actinomycetes. There are exceptions acidophilic Actinomycetes exist, as noted in a Finnish study but such organisms (e.g. *Streptomyces acidiscabies*) are less common (Budhathoki & Shrestha, 2020). Our results support the idea that soil pH is a key factor shaping actinomycete distribution, and adjusting pH (e.g. liming acidic soils) can enhance actinomycete recovery for bioprospecting.

In terms of soil type and habitat, our isolates came from both agricultural (terai flatland) soils and forest soils at the foothills. Notably, samples from black or dark soils (likely to be richer in organic matter) yielded multiple Actinomycetes, whereas lighter brown/red soils did not yield any. A study by Budhathoki & Shrestha (2020) found black soils harbored the most antibiotic-producing Actinomycetes, more than red soils. Guo *et al.* (2015) reported red soils harbor diverse acidophilic Actinomycetes, but in Nepal's context black soils (neutral pH, high humus) were more productive (Budhathoki & Shrestha, 2020). This aligns with our findings. The moisture content at collection sites varied while moisture itself didn't show a strong effect in our limited sample, extremely high moisture (waterlogged conditions) can suppress Actinomycetes due to low oxygen. In Budhathoki & Shrestha's (2020) work, moderate moisture soils had plenty of Actinomycetes, but very high moisture (riverbank soils ~40–50% water) had Actinomycetes whose activity might be hampered by anaerobic pockets (Budhathoki & Shrestha, 2020). All our samples were from well-drained surface soils (dry or just moist), so aeration was likely sufficient in all cases.

In this study only 21 isolates were isolated from 30 samples which is lower than some studies, which could be due to environmental differences or the specificity of our media. In the Kathmandu valley, Budhathoki & Shrestha isolated 121 Actinomycetes from 15 samples possibly because those samples were richer in Actinomycetes populations (e.g. compost-amended garden soils, etc.) or because they used multiple isolation media and pretreatments (Budhathoki & Shrestha, 2020). Similarly, Sapkota *et al.* (2020) isolated 41 Actinomycetes from 11 high-altitude soil, employing enrichment techniques. Our approach (single medium, no pretreatment except dry heat for one hour) might have limited recovery. It's known that using selective pretreatments (e.g. $CaCO_3$ treatment, dry heating to kill non-spore-formers, or antibacterial antibiotics in media) can significantly increase actinomycete yields (Budhathoki & Shrestha, 2020). We might have missed some Actinomycetes in samples that also contained fast-growing fungi or bacteria. Future studies could employ such techniques to get a more exhaustive isolate collection from Kanchanpur soils.

Nevertheless, our isolates showed considerable morphological diversity in colony appearance and pigment production, indicating a heterogeneous actinomycete community. Sapkota *et al.* 2020 observed 11 pigment types among 41 isolates (with yellow most common). We observed 4 pigment types among 21 isolates (with white most common). This difference could arise from the specific species present. White-pigmented colonies often correspond to

Streptomyces that produce white/gray aerial spores; yellow and other colors might indicate species of *Streptomyces* or other genera that produce diffusible pigments (e.g. *Nocardia* can be orange, *Streptomyces cyaneus* is blue). The presence of a blue-producing isolate in our set is intriguing; relatively few Actinomycetes produce blue pigment (perhaps an actinorhodin-like compound). Pigment diversity may not directly correlate with antimicrobial production, as Sapkota *et al.* 2020 found no significant link between pigment color and antibiotic activity. Still, pigments themselves can be bioactive, so unusual colors are worth noting (Sapkota *et al.* 2020).

Upon Gram staining and microscopic findings confirmed all isolates as Gram-positive Actinomycetes with filamentous forms. This was expected, since the isolation medium is quite selective for such bacteria. We observed branching filaments breaking into rod-like units. The literature describes Actinomycetes as "Gram-positive, free-living saprophytes with filamentous branching morphology and spore formation, often called ray fungi" (Sapkota *et al.* 2020). Our data fits perfectly with this description. We did not perform 16S rRNA sequencing, but the ubiquity of Gram-positive filamentous rods strongly suggests the majority are *Streptomyces* the most abundant genus in soils. Supporting this, 70% of isolates in a similar Nepali study were *Streptomyces*, and globally *Streptomyces* tends to dominate soil actinomycete isolates (Sapkota *et al.* 2020). The remainder could be *Nocardia, Micromonospora,* or other genera some of our colonies were fastidious and small, possibly *Micromonospora* which often has small soft colonies. Identification to genus/species would require molecular tools and is recommended for follow-up.

Biochemical characteristics of the isolates highlight both commonalities and differences compared to known strains. The consistent citrate utilization and urease activity in most isolates indicate versatile metabolism of nitrogen and carbon sources a trait seen in many Actinomycetes including certain *Streptomyces* (Mondal & Thomas, 2022). The lack of typical fermentative pathways (indole, MR, etc.) is expected since Actinomycetes rely on oxidative metabolism and are not enteric bacteria. One curious result was the catalase-negative reactions. Most aerobic Actinomycetes (e.g. *Streptomyces, Nocardia*) have catalase to detoxify hydrogen peroxide; in fact, catalase tests are often positive for these genera (Malviya et al., 2013). Catalase-negative Actinomycetes do exist, however for instance, members of the genus *Actinomyces* which are anaerobic or microaerophilic, found in oral flora are catalase-negative. It's unlikely we isolated *Actinomyces* from soil, but perhaps some isolates lost catalase activity, or it wasn't expressed under our test conditions. Alternatively, the test might have been done on a tiny colony mass leading to a false negative. Regardless, this anomaly should be rechecked, as catalase is a key differentiator in actinomycete taxonomy. On the other hand, oxidase-negative is entirely normal for Actinomycetes; they typically do not have cytochrome c oxidase in their electron transport chain (Malviya et al., 2013).

Among the isolates starch, casein and protein hydrolysis ability is consistent with Actinomycetes known enzymatic ability (Mondal & Thomas, 2022). Many *Streptomyces* secrete amylases, cellulases, xylanases, lipases, and proteases that are exploited in biotechnology (e.g. in laundry detergents or waste treatment). The universal positive result for starch/gelatin in our screening underscores that Actinomycetes from the soil of Kanchanpur could be sources of industrial enzymes. In fact, similar Nepali isolates have been examined for enzyme production; for example, a recent metabolite profiling study found soil Streptomyces with enzymes and bioactive compounds useful in agriculture and medicine (Bhattarai et al., 2022).

When comparing antimicrobial activity, our findings align with broader patterns in actinomycete research but also have some distinctive points. Consistent with virtually all studies, we saw a higher efficacy against Gram-positive bacteria than Gram-negative (Budhathoki & Shrestha, 2020). Actinomycetes historically have yielded many antibiotics effective on Gram-positives (penicillins, cephalosporins, vancomycin, rifamycin), whereas only a subset (aminoglycosides, tetracyclines) have broad Gram-negative activity. The cell wall differences are a fundamental reason Gram-negative possess an outer membrane that many large antibiotic molecules cannot penetrate (Budhathoki & Shrestha, 2020). Our active isolates, especially S25 Yellow, produced substances that at least partly overcame this barrier for certain Gram-negatives. For instance, it inhibited *Klebsiella* and *E. coli* (Enterobacteriaceae) and *Pseudomonas* to some degree, which is noteworthy. Many *Streptomyces* produce aminoglycosides (like streptomycin, gentamicin) that do target Gram-negatives. It is tempting to speculate that S25 Yellow might be a *Streptomyces* producing an aminoglycoside or a polyketide with broad-spectrum action. The relatively small zones against Gram-negatives could also mean the compound is produced in low concentration optimizing fermentation might increase potency.

Comparatively, in Budhathoki & Shrestha's large screening, 58 primary hits were narrowed to 22 for secondary screening, of which 14 had activity broad spectrum against 3 test organisms (Budhathoki & Shrestha, 2020). They found *S. aureus* was inhibited by 12 isolates, *Klebsiella* by 10, etc., while *Salmonella* Paratyphi was least inhibited by 5 isolates (Budhathoki & Shrestha, 2020). The isolates of this study overlaps this trend: inhibiting *S. aureus* strongly and *Klebsiella* moderately but not touching *Serratia*. This suggests the antibiotics produced by the isolates of this study are likely similar in class to those in other studies effective against a range of Gram-positives and some Gram-negatives, but not all. It is also possible that with a different panel (like including fungal pathogens or *Bacillus* species), we might discover antifungal or anti-*Bacillus* activity. Many Actinomycetes produce antifungals (e.g. nystatin from *Streptomyces noursei*). We did not test fungi in this study, which is a potential extension.

One interesting point for discussion is the percentage of active isolates we had 19% active isolates while Sapkota et al., 2020 found 43% active in primary screening Budhathoki & Shrestha found 48% active (Sapkota et al., 2020; Budhathoki & Shrestha, 2020). Our lower percentage might be due to the species composition in Kanchanpur soils. Perhaps a lot of isolates were benign *Streptomyces* that specialize in decomposition rather than antibiotic production. Environmental conditions also play a role; Actinomycetes from harsher or more competitive environments (e.g. high-altitude, or rhizosphere) might produce antibiotics more readily to compete with other microbes (Sapkota et al., 2020). Kanchanpur's lowland soils might have slightly less competitive pressure, or our isolation method may have picked up some fast-growing but weakly bioactive strains. Another factor is the screening method we used a straightforward agar plug/spot test on lab strains of pathogens, which might not be very sensitive. Using more sensitive assays or different indicator organisms might reveal activity in isolates we scored as "inactive." In any case, it's clear that some fraction (20–50%) of soil Actinomycetes have antibiotic biosynthesis capacity, which justifies continued screening efforts for novel drugs.

Finally, it's worth placing our findings in the global context of actinomycete bioprospecting. Actinomycetes remain the source of the majority of antibiotics in use (Budhathoki & Shrestha, 2020). The discovery of new strains is still relevant as resistance rises. Our study targeted a region (far-western Nepal) that is relatively unexplored for microbiological resources. Most prior Nepali studies focused on central Nepal Kathmandu valley (Budhathoki

& Shrestha, 2020). By documenting Actinomycetes in Kanchanpur, we contribute to mapping the geographic distribution of these organisms. Climate and soil in Terai (hot, subtropical plains) differ from alpine or temperate regions; interestingly, we still isolated typical *Streptomyces*-like organisms, indicating their adaptability. Some might expect more thermotolerant species due to the warmer climate; perhaps our isolates include thermotolerant Actinomycetes (future tests of temperature tolerance could verify this).

Our data also hint at untapped chemical diversity for instance, the S25 Yellow isolate's broad activity suggests it could produce multiple compounds or a particularly potent one. Secondary metabolite profiling (e.g. via HPLC, mass spectrometry) would be a logical next step. In a comparable case, Sapkota et al., 2020 identified one isolate (*Micromonospora* sp. C7) with broad-spectrum activity and suggested it as a candidate for further drug development (Sapkota et al., 2020). Similarly, our S25 Yellow might be a candidate for purification of its antibiotic. It could potentially yield a known antibiotic (like streptomycin) or something new. Given that *Pseudomonas* was inhibited, there's a chance it produces an antibiotic that targets Gram-negatives possibly a membrane-active compound or an enzyme inhibitor.

Our discussion highlights that the Actinomycetes from Kanchanpur soils share many characteristics with those from other regions. They prefer neutral pH, exhibit Gram-positive filamentous morphology, produce enzymes, and a subset synthesize antibiotics (mainly effective on Gram-positives). Minor differences, such as pigment distribution are noted and may indicate unique species or genera present. These findings underscore the value of exploring diverse habitats for Actinomycetes, as each locale can yield strains with potentially novel properties.

Chapter 5 Summary, Conclusion and Recommendations

5.1 Summary

Actinomycetes were characterized by a fairly diverse group of isolates from Kanchanpur soils, showing filamentous Gram-positive forms with varied colony pigmentation and remarkable biochemical profiles. It was observed that Actinomycetes were widely distributed in neutral to weakly alkaline soils well aerated but not in acidic soils. Only 4 out of the 21 isolates displayed remarkable activity against certain Gram-positive bacteria, including *Staphylococcus aureus* and *Enterococcus*, with one isolate being active against certain Gram-negative bacteria.

The active isolates thus confirmed that Actinomycetes from Kanchanpur are a potential source of antibacterial compounds, hence reemphasizing similar findings from different areas where a significant number of soil Actinomycetes produce antibiotics. Although most isolates did not display activity under the testing conditions, they maintain interesting features, such as enzyme production, which may be of interest from an industrial point of view or even show bioactivity under different conditions or against different targets.

This research has been designed to answer the following questions:

1. It describes the distribution of fairly good Actinomycetes diversity in Kanchanpur soils, although the pH is more or less neutral and the soils are darker.

2. While diverse in colony form and color, the isolates were all Gram-positive and filamentous, typical of an actinomycete profile.

2. They show citrate utilization, urease production, and strong extracellular enzyme activity (starch/protein hydrolysis) in accordance with their ecological role in the soil.

4. In vitro, quite a few of these isolates have inhibitory actions regarding pathogenic bacteria, especially the one with the strong broad-spectrum effect, which strongly suggests the existence of strains within these soils that are capable of synthesizing biologically active secondary metabolites.

5.2 Conclusion

The isolated actinomycetes isolates S25 (yellow) and other isolates were presumed to be *Streptomyces* genera. This study has added to our knowledge base concerning Actinomycetes present in the soils of the far-western parts of Nepal as a reservoir. Isolates showed diverse characters, right from the decomposition of lignocellulosic substrate materials to synthesize important bioactive secondary metabolites such as antibiotics. This emphasized again the importance of Actinomycetes in global ecology and their roles in biotechnological functions. Because of the very high potential in the discovery of newer bioactive molecules from these Actinomycetes and their further beneficial application in pharmaceutical and agricultural requirements, detailed identification and compound characterization of the former is important. It is only through exploration of under-exploited areas, like Kanchanpur, that we can hope to counter antibiotic resistance as a global menace, to find new Actinomycetes as the resource for next-generation antibiotics, and beneficial microbial products. The results further support the implication that the soil health, especially maintaining an adequate pH and organic carbon content, has to be conserved for the habitat of these natural antibiotic producers. The Actinomycetes' true potential is most promisingly going to be unveiled by the micro, chemical, and genomic integrated future research carried out in this field.

5.3 Recommendations

Based on the findings of this research work following recommendations are put forward:

1. Incorporation of cycloheximide along with nalidixic acid in media can be practiced for minimizing fungal and bacterial contamination.
2. Conventional methods including morphological, biochemical and cultural are still gold standard for the characterization of actinomycetes.
3. Optimization of nutritional parameters such as carbon, nitrogen and mineral sources is essential while conducting fermentation.
4. Parameters such as temperature, pH and duration of incubation period should be studied and optimized for individual strain for better metabolite production.
5. Molecular techniques such as 16 s rRNA can be used for identification of Actinomycetes and polymerase chain reaction (PCR) can be used for the detection of the biosynthetic gene clusters.
6. The techniques such as HPLC, GCMS, H-NMR and C-NMR can be used for complete structure elucidation of active compounds.

References

Anderson, A.S. & Wellington E.M.H. (2001). The taxonomy of streptomyces and related genera. *Int J Syst Evol Microbiol*, 3,797-814.

Arifuzzaman, M., khatun, M.R., and Rahman, H. (2010), "Isolation and screening of actinomycetes from Sundarbans soil for antibacterial activity." *Afr journal Biotechnol.*, **9,** 4615-4619.

Babalola, O. O., Kirby, B. M., Le Roes-Hill, M.; Cook, A. E., Cary, S. C., Burton, S. G. and Cowan, D. A. (2009). Phylogenetic analysis of actinobacterial populations associated with Antarctic dry valley mineral soils. *Environ Microbiol.*, 11:566-576.

Bailoori, S. N. (2020). "Antibacterial Screening of Actinomycetes isolated along the coast Bay of Bengal, Visakhapatnam," **9**, 12.

Barka, E. A., Vatsa, P., Sanchez, L., Gaveau-Vaillant, N., Jacquard, C., Meier-Kolthoff, J. P., Klenk, H. P., Clément, C., Ouhdouch, Y., & van Wezel, G. P. (2015). Taxonomy, Physiology, and Natural Products of Actinobacteria. *Microbiology and molecular biology reviews : MMBR*, *80*(1), 1–43. https://doi.org/10.1128/MMBR.00019-15

Barrios-Gonzalez, F., Fernandez, J., Tomassini, A., and Megia, A. (2005). "Secondary Metabolites Production by solid state Fermentation," *Malaysian Journal Microbiol.*, **1**, 1-6.

Baucheron S, Monchaux I,Le HS,Weill FX,and Cloeckaert A (2014).Lack of serovars Typhi efflux mediated quinolone resistance in *Salmonella enterica* and *Paratyphi* A. *Front.Microbial.* 5:12.doi:10. 3389 / fmicb. 2014. 00012.

Berdy, J. (2005). Bioactive microbial metabolites. The Journal of Antibiotics, 58(1), 1–26.

Bhattarai K, Tiwari KB and Agrawal VP (2007). Enhanced antibacterial activity of sodium azide treated mutant *Streptomyces* strain 44. Journal of Nepal Association for medical laboratory Sciences, **8** (1) : 67- 8.

Bizuye, A., Moges, F., and Andualem, B. (2013). Isolation and screening of antibiotic-producing Actinomycetes from soils in Gondar town, Northwest Ethiopia. *Asian Pac J Trop Dis*, 3(5), 375-381.

Black, R. E.; Brown, K. H.; Becker, S. and Yunusm M. (1982). Longitudinal Studies of Infectious Diseases and Physical Growth of Children in Rural Bangladesh. I. Patterns of Morbidity. *Ameri J Epidemiol.* 115, 305–314.

Budhathoki, S., & Shrestha, A. (2020). Screening of Actinomycetes from Soil for Antibacterial Activity. *Nepal J Biotechnol.,* December 8 (3): 102–110.

Casidia (1959). Basis and development of industrial fermentation process, in industrial microbiology, 6th edition, Wiley Eastern Limited Publication

Ceylan, O.; Okmen, G. and Ugur, A. (2008). Isolation of soil *Streptomyces* as source antibiotics active against antibiotic-resistant bacteria. *Eur Asian J Bio Sci.* 2(1), 73-82.

Chamikara, P. (2016). "Advanced Study on selected taxonomic groups of Bacteria and Archaea Actinomycetes," *Microbial diversity in hotspots*, **9**, 1-9.

Chaudhary, H.S., Yadav, J., Shrivastava, A.R., Singh, S., Singh, A.K., Gopalan, N. (2013). "Antibacterial activity of actinomycetes isolated from different soil samples of Sheopur (A city of central India)." J Adv Pharm Technol Res., 2, 118-23.

Chavan D.V., Mulaje, S.S., Mohalkar, R.Y. (2013). "A Review on Actinomycetes and Their Biotechnological Applications." *International Journal of Pharmaceutical Sciences and Research*., **5**, 1730 -1742.

Crook P, Carpenter CC and Klens PF (1950). The use of sodium propionate in isolating actinomycetes from soils Science 112L656.

Deng Z, Shan Y, Pan Q, Gao X, and Yan A (2013). Anaerobic expression of the gadE-mdtEF multidrug effluxoperon is primarily regulated by the two component system Arc BA through antagonizing the H-NS mediated repression. *Front. Microbiol.* 4:194.doi:10.3389/ fmicb. 2013. 00194

Denyer, P.S., Hodges, N., Gorman, P.S ssss. (2011). "*Hugo and Russell's Pharmaceutical Microbiology*." Eighth Edition. Willey-Blackwell Publication.

Dhananjay, V., Selvan, N., and Dhanapal, K. (2013). "Isolation, characterization, screening, and Antibiotic sensitivity of Actinomycetes from locally (Near MCAS) collected soil samples*," J Boil Sci*.,**6**, 514.

Dulency EL, Larsen AH Stapley EO (1955). A note on the isolation of microorganism from natural Sources. *Mycologia* **47**:420-422

Egorov NS (1985). Antibiotics, A Scientific Approach. Mir publishers, Moscow.

Farnet, C.M., & Zazopoulos, E. (2005). Improving drug discovery from microorganisms. In L. Zhang and A. L. Demain (Eds.), Natural products: Drug discovery and Therapeutic medicine *Humana Press Inc.* pp.; 95.

Goodfellow, M. & Williams, S.T. (1983). Ecology of Actinomycetes. *Annual Review of Microbiol*. 37, 189–216.

Goodfellow, M. (1989). Suprageneric Classification of Actinomycetes. In: Williams, S.T., Sharpe, M.E. and Holt, J.G., Eds., *Bergey's Manual of Systematic Bacteriology*, Vol. 4, Williams & Wilkins Company, Baltimore, pp. 2333–2339.

Guo, H., Callaway, J. B., & Ting, J. P. (2015). Inflammasomes: mechanism of action, role in disease, and therapeutics. *Nature medicine*, *21*(7), 677–687. https://doi.org/10.1038/nm.3893

Gurung, K., & Rai, M. K. (2021). A Comparison of Antibacterial Potential of Different Soil Actinomycetes from Pokhara. *Prithvi Journal of Research and Innovation*, *3*(1), 1–8. https://doi.org/10.3126/pjri.v3i1.41625

Gurung, T.D., Sherpa, C., Agrawal, V.P., and Lekhak, B. (2009). Isolation and characterization of antibacterial Actinomycetes soil samples of Kalapatthar, Mount Everest region. *Nepal Journal of Science and Technology*, 10, 173-182.

Hacene, H., and Lefebvre, G. (1996). "HP17, a new pigment-like antibiotic produced by a new strain of *spirillospora*," *J. Applied Bact*., **89,** 565-569.

Harry W. Lewis and christopher J. Moody (1989). Experimental organic chemistry. Principle and Practice (illustrated ed). Wiley Blackwell. pp. 159-173. ISBN 978-0-632-02017-1.

Haseena, A., Nishad, V.M., Balasundaran, M. A. (2016). "Consortium of Thermophilic Microorganisms for Aerobic Composting," *IOSR Journal of Environmental Science, Toxicology and Food Technology*., **1**, 49-56.

Hassouni, E.M., Karkouri, E.A., Assou, S.A. (2019). "Isolation and screening of actinomycetes producing antimicrobial substances from an extreme Moroccan biotope," *Pan Afr Med J*, **33**, 329.

Hayakawa, M. (2008). "Studies on the Isolation and Distribution of Rare Actinomycetes in Soil*," Actinomycetologica*., **22**, 12-19.

Hayakawa, M., Sadakata, T., Kajiura, T., and Nonomura, H. (1991). "New methods for the highly selective isolation of *Micromonospora* and *Microbispora* from soil," *Journal of Fermentation and Bioengineering*., **72**, 320– 326.

Hirsh CF and Christensen DL (1983). Novel method for selective Isolation of actinomycetes. Applied and Environmental Microbiology, pp. 925 - 929.

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Stanley, J. T. & Williams, S. T. (1994). Bergey's Manual of Determinative Bacteriology, 9th ed. *Williams & Wilkins, Co.,* Baltimore.

Holt, J.G. (1989). "*Bergey`s Manual of Systematic Bacteriology*, "Williams and Williams, Baltimore, MD.

Hopwood, D.A., Buttner, M.J., Bibb, M.J., Kieser, T. and Charter, K.F. (2000). Antibiotic production by Streptomyces. *Pract Streptomyces Genet*, 1, 1–42.

Hozzein, W. N. and Goodfellow, M. (2011). Actinopolyspora egyptensis sp. nov., a new halophilic actinomycete. *Afr J Microbi. Res.* 5:100-105.

Ikekawa, N. (1963). Gas Chromatography of Amino Acids I. Analysis of the Mixture of Methyl-N-trifluoroacetyl Derivatives. *The Journal of Biochemistry*, *54*(3), 279-282.

Jeffrey, L.S. (2008). "Isolation, characterization, and identification of actinomycetes form actinomycetes from agriculture soils at semongok. Sarawak." *Afr. J. biotechol.*, **9**, 4615-4619.

Jiang CL, Xu LH, Yang YR, Wang LF, Shi ZW and Guo GY (1993). A study of Alkalophilic Actinomycetes in Yunn, *Actinomylogica* **7**:58-64.

Karki, R., & Magar, B. A. (2024). ISOLATION AND SCREENING FOR ANTIBACTERIAL ACTIVITY OF ACTINOMYCETES COLLECTED FROM THE SOIL OF KATHMANDU VALLEY. *Xavier International College Journal (XICJ)*, 6(1), 1-9.F

Kumagai, T. (1993). Estimates of transition densities for Brownian motion on nested fractals. *Probability theory and related fields*, *96*(2), 205-224.

Kumar, N., Ravi, K.S., Mishra, S.K., Singh, R.K., and Pachouri, U.C. (2010). "Isolation and screening of soil actinomycetes as source of antibiotics active against bacteria." *International Journal of Microbiology Research.*, **2**, 12–16.

Kumar, P.S., Raj, J.P., Duraipandiyan, V., Ignacimuthu, S. (2012). "Antibacterial activity of some actinomycetes from Tamil Nadu, India," *Asian Pac J Trop Biomed.*, **12**, 936-43.

Kushner DJ (1985). The Halobactericeae. InWoese CR and Wolfe RS(eds) The Bacteria. Academic Press ,London,UK,.**8**:171-214.

Kuster, E and Williams ST (1964). Selection of Media for the Isolation of Streptomycetes, *Nature*, 202.

Kuster, E., and Williams, S.T. (1964). "Production of hydrogen sulphide by *Streptomyces*." *Microbial Espanola*., **16**, 193-202.

Lechevalier, M. P., & Lechevalier, H. A. (1975). Actinoplanete with Cylindrical Sporangia, Actinoplanes rectilineatus sp. nov. *International Journal of Systematic Bacteriology*, *25*(4), 371-376.

Lekhak, B., Singh, A. and Bhatta, D.R., (2018). Antibacterial and Antifungal Property of Actinomycetes Isolates from Soil and Water of Nepal. *J Nepal Health Res Counc* Apr-Jun,16(39)136-9.

Lingappa Y and Lockwood JL (1961). A Chitin Medium for Isolation ,Growth and Maintenance of Actinomycetes. Nature **189**:158-159.

Liu CM, Westley JW, Herman TE, Prasser BLT, Palleroni N, Evans RH and Miller PA (1986). Novel polyether antibiotics, X- 14873 A, G and H produced by *Streptomyces*. Taxonomy of the producing culture, fermentation, biological and ionophores properties of the antibiotics. *Journal of Antibiotics*, 39 (12):1712-1718.

Malviya, M. K., Pandey, A., Sharma, A., & Tiwari, S. C. (2013). Characterization and identification of Actinomycetes isolated from 'fired plots' under shifting cultivation in northeast Himalaya, India. *Annals of Microbiology*, *63*(2), 561–569. https://doi.org/10.1007/s13213-012-0504-x

McCarthy, A.J. & Williams, S.T. (1992). Actinomycetes as agents of biodegradation in the environment: a review. *Gene*, pp. 115, 189–192.

Meklat, A., Sabaou, N., Zitouni, A., Mathieu, F., Lebrihi, A. (2011). "Isolation, taxonomy, and antagonistic properties of halophilic *Actinomycetes* in Saharan soils of Algeria," *Appl Environ Microbiol*., **77**, 6710–6714.

Mohseni, M., Norouzi, H., Hamedi, J., Roohi, A. (2013). "Screening of Antibacterial Producing Actinomycetes from Sediments of the Caspian Sea," *Int J Mol Cell Med,* **2**, 64-71.

Mondal, H., & Thomas, J. (2022). Isolation and Characterization of a Novel *Actinomycete* Isolated from Marine Sediments and Its Antibacterial Activity against Fish Pathogens. *Antibiotics (Basel, Switzerland)*, *11*(11), 1546. https://doi.org/10.3390/antibiotics11111546

Muthu, M., Rajesh & Ramasamy., Subbaiya & Balasubramanian., Murugesan &Ponnusamy, P., and Masilamani, S. (2013). "Isolation and Identification of Actinomycetes Isoptericola variabilis From Cauvery River Soil Sample." *International journal of current Microbiology and Applied sciences*., **2**, 236-245.

Nabi SA, Khan MA, Khowaja SN and Almuddin (2006). Thin Layer Chromatographic Seperation of Penicillins on Stannic arsenate-Cellulose layers. *Acta Chromatographica,* No.16

Narayana, K.J.P *et al.* (2007). "L-asparaginase production by Streptomyces albidoflavus*,"* *Indian J. Microbiol*., **3**, 331-336.

Nonomura H and Ohora Y (1969) Journal of Fermentation Technology, **47**: 463-469

Okami Y. K. Hotta. (1988). Search and discovery of new antibiotics. In: Actinomycetes in biotechnology (Eds. M. Good Fellow, S.T. Williams and M. Mordarski). *Academic Press, London,* pp.; 37-67.

Okazaki T and Okami Y (1972).Search on Marine Microorganism II. Actinomycetes in Sagami Bay and their Antibiotic Substances. *Journal of Antibiot.* 25, 461-466.

Oppong, E. K., Edwards, R. L., Maitland, D. J., & Hanson, R. (2010). Metabolites from static cultures of the fungus Xylaria badia. *Int J Appl Chem*, *6*(3), 387-394.

Oskay, M., Tamer, A.U., and Azeri, C. (2004). 'Antibacterial activity of some actinomycetes isolated from farming soils of Turkey," *African J Biotechnology*., **9,** 441-446.

Pandey, B., Ghimire, P., and Agrawal, V. P. (2004). Studies on the antibacterial activity of the Actinomycetes isolated from the Khumbu region of Nepal. *Journal of Applied Microbiology*, 12, 421-423.

Porter JN, Wilhelm JJ, and Tresner HD (1960). Method for the Preferential Isolation of Actinomycetes from Soils.Appl. Microbiol. **8**:174-178.50

Radhakrishnan, M., Suganya, S., Balagurunathan, R., Kumar,V. (2010). "Preliminary screening for antibacterial and antimycobacterial activity of actinomycetes from less explored ecosystems" World *J Microbiol Biotechnol*., **26**, 561–566.

Rahman, M. A.; Islam, M. Z. and Islam, M. A. (2011). Antibacterial activities of actinomycete isolates collected from Rajshahi, *Bangladesh Biotech Res Int soils.* 6,18-24.

Rai, K., Khadka, S., & Shrestha, B. (2018). Actinomycetes: Isolation, characterization, and screening for antimicrobial activity from different sites of Chitwan, Nepal. *International Journal of Microbiology and Biotechnology*, 3(1), 25-30.

Rai, M., Bhattarai, N., Dhungel, N., and Mandal, PK. (2016). Isolation of antibiotic-producing Actinomycetes from the soil of Kathmandu Valley and assessment of their antimicrobial activities. *International Journal of Microbiology and Allied Sciences*, 2(4), 22-26.

Raja, A., Prabakaran, P., Gajalakshmi, P. and Rahman, A. H. (2010). A Population Study of Psychrophilic Actinomycetes Isolated from Rothang Hill-Manali Soil Sample. J Pure *Applied Microbiol*. 4,847-451.

Ranjani, A., Dharumadurai, D., and Manogaran, G.P. (2016). "An introduction to Actinobacteria: Chapter 1,".

Rehacek Z (1959).Isolation of Actinomycetes and Determination of the Number of their spores in Soil Microbiology USSR (English Transl) **28**:220-225.

Rotich M.C, Magiri E,Bii C,Maina N (2017). Bio-Prospecting for Broad Spectrum Antibiotic Producing Actinomycetes Isolated from Virgin Soils in Kericho country, Kenya. Department of Biochemistry, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya, 7, 56-70.

Sah, S. N., & Lekhak, B. (2017). Screening of antibiotic-producing Actinomycetes for antibiosis from the soil of Siraha, Nepal. *Himalayan Journal of Science and Technology*, 1(1), 20-25.

Saha S, Roy P and Samanta S (2012). Actinomycetes from hospital dump soil produce highly active antibiotic. *International Journal of Microbiology research,* pp 258 - 262.

Schneegurt, M.A., and Caton, I.R. (2012). "Culture-independent analysis of the soil bacterial assemblage at the Great Salt Plains of Oklahoma," *J. Basic Microbiol.*, **52**, 16-26.

Schulz, D.; Nachtigall, J.; Riedlinger, J.; Schneider, K.; Poralla, K.; Imhoff, J. F.; Beil, W.; Nicolson, G.; Fiedler, H. P. and Sussmuth R. D. (2009). Piceamycin and its N-acetylcysteine adduct are produced by Streptomyces sp. GB 4-2. *J Antibi.* 62, 513-518.

Selman, A., Waksman. (1959). "The Actinomycetes-Nature, Occurrence and Activities. Baltimore," The Williams & Wilkins Company.

Shi K, Caldwell SJ, Fong DH and Berghuis AM (2013).Prospects for circumventing aminoglycoside kinase mediated antibiotic 51 resistance. *Front. Cell. Infect. Microbiol.* 3:22.doi:103389/fcimb2013.00022

Shirling, E.B., and Gottlieb, D. (1966). "Methods, classification, identification and description of genera and species." William and Wilkin company, Baltimore, MD, USA. 61-292.

Singh D and Agrawal VP (2002). Microbial Biodiversity of Mount Sagarmatha Region, In Proceedings of International Seminar on Mountains, March 6-8, Kathmandu. Nepal Academy of Science and Technology, Khumaltar, Lalitpur, Nepal: 357-360.

SpellBerg, B., Powers, J.H., Brass, E.P., Miller, l.G., and Edwards, J.E. (2004). "Trends in antimicrobial drug development; implications for the future," *Clinical Infectious Diseases.,* **38,**1279-1286.

Srinivasan, M.C., Laxman, R.S., Deshpande, M.V. (1991). "Physiology and nutrition aspects of actinomycetes an overview." *World J Microbial Biotechnol*, **7**, 171-184.

Stach, J.E. & Bull, A.T. (2005). Estimating and comparing the diversity of marine actinobacteria. *Antonie van Leeuwenhoek,* 87, 3-9.

Suthindhiran, K. & Kannabiran, K. (2009). Cytotoxic and Antimicrobial Potential of Actinomycete Species Saccharopolyspora salina VITSDK4 isolated from the Bay of Bengal Coast of *India. Ameri J Infect Dis.* 5(2),90–8.

Thakur, D.; Yadav, A.; Gogoi, B. K. and Bora, T. C. (2007). Isolation and screening of Streptomyces in the soil of protected forest areas from the state of Assam and Tripura, India, for antimicrobial metabolites. *J mycol and med.* 17, 242-249.

Tsao PH, Leven C, and Keitt GW (1960) An Enrichmentmethod for isolating Actinomycetes that produce Diffusible Antifungal Antibiotics. *Phytopathohlogy* **50**:88-89.

Tyagi, J., Bhatnagar, T., and Pandey, F.K. (2014). "Isolation and characterization of actinomycetes from soil and screening their antibacterial activities against different microbial isolates," *International journal of life science research.*, **2**, 101-105.

Usui M, Nagai H, Hiki M, Tamura Y and Asai T (2013) . Effect of antimicrobiol exposure on AcrAB Expression in *Salmonella enterica* subspecies enterica serovar choleraesuis. *Front Microbiol.* **4**:53. doi:10. 3389/ fmicb.2013.00053.

Ventura, M., Canchaya, C., Tauch. A., Chandra. G., Fitzgerald, GF., Chater. KF. & Van, Sinderen D. (2007) Genomics of Actinobacteria: Tracing the Evolutionary History of an Ancient Phylum. *Microbiol Mol Biol Rev* 71. https://doi.org/10.1128/mmbr.00005-07

Vogel AI, Tatchell A.R; Furnis B.S, Hannaford A.J and Smith PWG (1989). Vogel's Textbook of Practical organic chemistry (5th edition).ISBN 0-13-141601-7.

Wahab, A., Shumaila, S., Subhan, S. A., Ali, S. T. and Mujahid, T.Y. (2015). Isolation and identification of Actinomycetes isolated from Karachi soil and screening of antimicrobial compounds. *International Journal of Current Research,* 7(2), 12760-12765.

Waksman SA (1961). The Actinomycetes Vol 2. Classification, Identification, and Description of Genera and Species. The Williams and Wilkens Co, Baltimore.

Waksman SA (1968). Actinomycetes ; Nature ,Formation and Activities, Applied Microbiology,**5**:235-293.

Waksman, S. A., Schatz, A., & Reynolds, D. M. (2010). Production of antibiotic substances by actinomycetes. *Annals of the New York Academy of Sciences*, *1213*, 112–124. https://doi.org/10.1111/j.1749-6632.2010.05861.x

Watkins, R. R., Papp-Wallace, K. M., Drawz, S. M., & Bonomo, R. A. (2013). Novel β-lactamase inhibitors: a therapeutic hope against the scourge of multidrug resistance. *Frontiers in microbiology*, *4*, 392.

Wilkins, K. (1996).Volatile metabolites from Actinomycetes. *Chemosphere*, 32, 1427– 1434.

Williams, S.T. & Davies, F.L. (1965). Use of antibiotics for selective isolation and enumeration of Actinomycetes in soil. *Journal of General Microbiology*, pp. 38, 251–261.

Williams, S.T., & Cross, T. (1971). Actinomycetes, Methods Microbiology *Academic Press, Inc. (London) LTD,* Volume 4, pp.; 295-334

Wood, SA; Kirby, B.M.; Goodwin, C.M.; Le Roes, M. and Meyers, P.R. (2007). PCR screening reveals unexpected antibiotic biosynthetic potential in *Amycolatopsis sp*. strain UM16. *J. Appl. Microbiol.,* 102:245-253.

World Health Organization. (2020, September 22). "Surveillance standards for antimicrobial resistance."

Yadav, J., Shrestha, U.P., Tiwari, K.B., Sahukal, G.P. and Agrawal, VP. (2008). Streptomycin-like Antibiotic from Streptomyces spp. Isolated from Mount Everest base camp. *Nepal Journal of Science and Technology*, 9, 73-77.

Zhang, L., Xi, L., Ruan, J., and Huang, Y. (2012). "Micro bacterium marinum sp. nov., isolated from deep-sea water," *Syst. Appl. Microbiol.*, **35**, 81–85

Zhi, X.Y.; Li, W.J. and Stackebrand, E. (2009). An update of the structure and 16S rRNA gene sequence-based definition of higher ranks of the class Actinobacteria, with the proposal of two new suborders and four new families and emended descriptions of the existing higher taxa. *Int. J. Syst. Evol. Microbiol.* 59:589-608.

APPENDICES

Appendix 1

Materials used

Equipment's

1. Autoclave
2. Incubator
3. Incubator
4. Hot air oven
5. Binocular microscope
6. Refrigerator
7. Electronic weighing balance
8. Bunsen burner
9. Centrifuge
10. Water bath shaker

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. Sulphide indole motility media (SIM)

6. Urea agar base
7. Simmons's citrate agar
8. Hugh and Leifson's media
9. MR/VP medium
10. Triple sugar iron agar
11. Gelatin 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

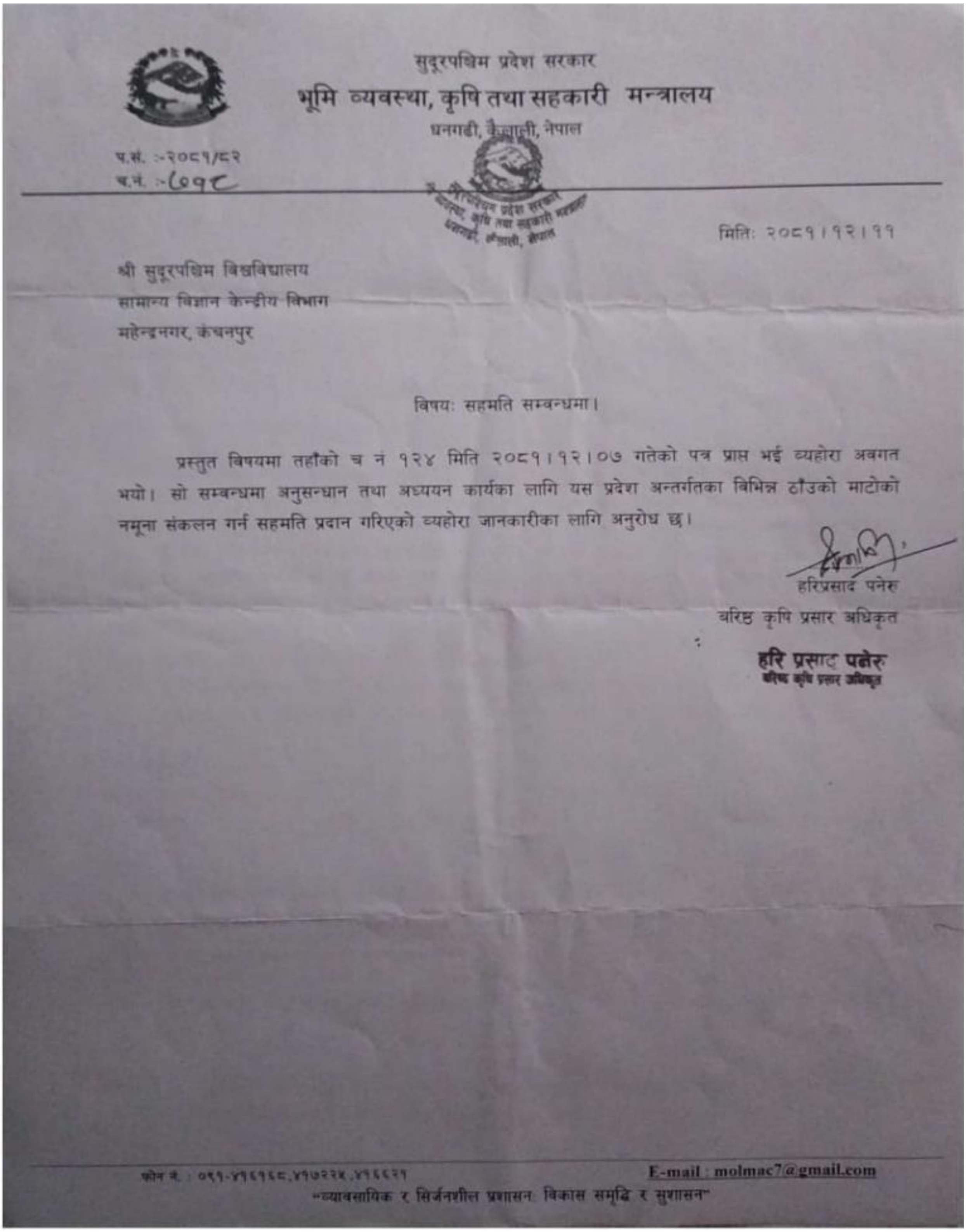
Test organism

1. *Proteus mirabilis*
2. *Escherichia coli*

3. *Staphylococcus aureus*
4. *Klebsiella pneumoniae*
5. *Acinetobacter baumannii*
6. *Pseudomonas aeruginosa*
7. *Enterococcus faecalis*
8. *Serratia* spp.
9. *Morganella* spp.

Appendix 2

Permission for collection of Sample from Ministry of Land Management, Agriculture and Cooperatives, Sudurpaschim Government, Dhanghadi Kailali



सुदूरपश्चिम प्रदेश सरकार

भूमि व्यवस्था, कृषि तथा सहकारी मन्त्रालय

धनगढी, कैलाली, नेपाल

प.सं. :-२०८१/८२

च.नं. :-६९८

मिति: २०८१।१२।११

श्री सुदूरपश्चिम विश्वविद्यालय

सामान्य विज्ञान केन्द्रीय विभाग

महेन्द्रनगर, कंचनपुर

विषय: सहमति सम्वन्धमा।

प्रस्तुत विषयमा तहाँको च नं १२४ मिति २०८१।१२।०७ गतेको पत्र प्राप्त भई व्यहोरा अवगत भयो। सो सम्वन्धमा अनुसन्धान तथा अध्ययन कार्यका लागि यस प्रदेश अन्तर्गतका बिभिन्न ठाँउको माटोको नमूना संकलन गर्न सहमति प्रदान गरिएको व्यहोरा जानकारीका लागि अनुरोध छ।

हरिप्रसाद पनेरु

वरिष्ठ कृषि प्रसार अधिकृत

हरि प्रसाद पनेरु

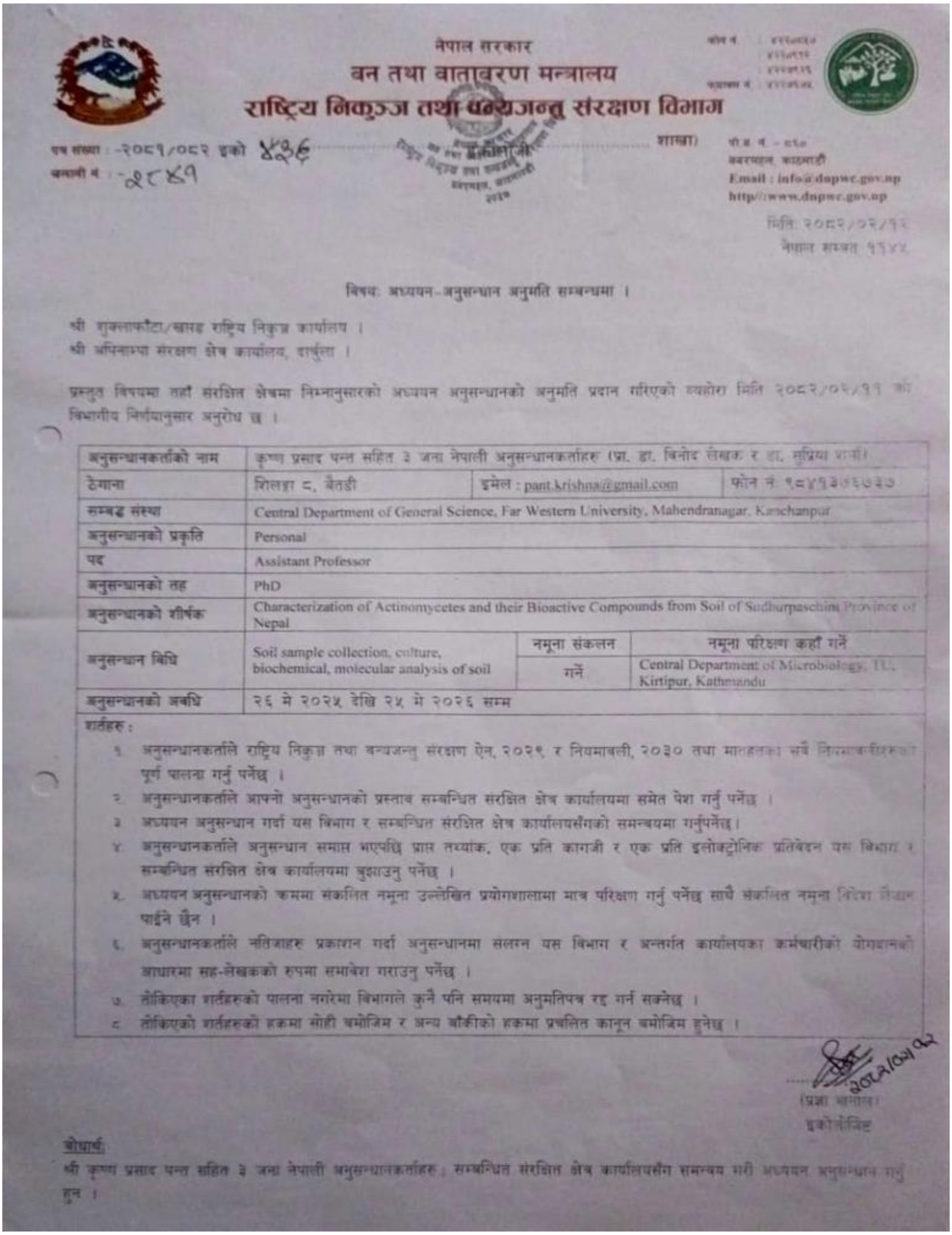
वरिष्ठ कृषि प्रसार अधिकृत

फोन नं. : ०९१-४१६१६८,४१७२२४,४१६६२१ E-mail : molmac7@gmail.com

"व्यावसायिक र सिर्जनशील प्रशासन: विकास समृद्धि र सुशासन"

Appendix 3

Permission for collection of Sample Department of National Parks and Conservation, Babarmahal, Kathmandu



नेपाल सरकार
वन तथा वातावरण मन्त्रालय
राष्ट्रिय निकुञ्ज तथा वन्यजन्तु संरक्षण विभाग

पत्र संख्या : २०८१/०८२ इको ४३६
चलानी नं : २८४१

शाखा

पो.ब.नं. - ८६०
बबरमहल, काठमाडौं
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मिति: २०८२/०२/१२
नेपाल सम्वत ११४५

विषय: अध्ययन-अनुसन्धान अनुमति सम्बन्धमा ।

श्री शुक्लाफाँटा/खप्तड राष्ट्रिय निकुञ्ज कार्यालय ।
श्री अपिनाम्पा संरक्षण क्षेत्र कार्यालय, दार्चुला ।

प्रस्तुत विषयमा तहाँ संरक्षित क्षेत्रमा निम्नानुसारको अध्ययन अनुसन्धानको अनुमति प्रदान गरिएको व्यहोरा मिति २०८२/०२/११ को विभागीय निर्णयानुसार अनुरोध छ ।

अनुसन्धानकर्ताको नाम	कृष्ण प्रसाद पन्त सहित ३ जना नेपाली अनुसन्धानकर्ताहरु (प्रा. डा. विनोद लेखक र डा. सुप्रिया शर्मा)		
ठेगाना	शिलङ्गा ८, बैतडी	इमेल : pant.krishna@gmail.com	फोन नं: ९८४१३३६७३७
सम्बद्ध संस्था	Central Department of General Science, Far Western University, Mahendranagar, Kanchanpur		
अनुसन्धानको प्रकृति	Personal		
पद	Assistant Professor		
अनुसन्धानको तह	PhD		
अनुसन्धानको शीर्षक	Characterization of Actinomycetes and their Bioactive Compounds from Soil of Sudhurpaschim Province of Nepal		
अनुसन्धान विधि	Soil sample collection, culture, biochemical, molecular analysis of soil	नमूना संकलन	नमूना परिक्षण कहाँ गर्ने
		गर्ने	Central Department of Microbiology, TU, Kirtipur, Kathmandu
अनुसन्धानको अवधि	२६ मे २०२५ देखि २५ मे २०२६ सम्म		

शर्तहरु :

१. अनुसन्धानकर्ताले राष्ट्रिय निकुञ्ज तथा वन्यजन्तु संरक्षण ऐन, २०२९ र नियमावली, २०३० तथा मातहतका सबै निर्देशिकाहरुको पूर्ण पालना गर्नु पर्नेछ ।
२. अनुसन्धानकर्ताले आफ्नो अनुसन्धानको प्रस्ताव सम्बन्धित संरक्षित क्षेत्र कार्यालयमा समेत पेश गर्नु पर्नेछ ।
३. अध्ययन अनुसन्धान गर्दा यस विभाग र सम्बन्धित संरक्षित क्षेत्र कार्यालयसँगको समन्वयमा गर्नुपर्नेछ।
४. अनुसन्धानकर्ताले अनुसन्धान समाप्त भएपछि प्राप्त तथ्यांक, एक प्रति कागजी र एक प्रति इलेक्ट्रोनिक प्रतिवेदन यस विभाग र सम्बन्धित संरक्षित क्षेत्र कार्यालयमा बुझाउनु पर्नेछ ।
५. अध्ययन अनुसन्धानको क्रममा संकलित नमूना उल्लेखित प्रयोगशालामा मात्र परिक्षण गर्नु पर्नेछ साथै संकलित नमूना विदेश लैजान पाईने छैन ।
६. अनुसन्धानकर्ताले नतिजाहरु प्रकाशन गर्दा अनुसन्धानमा संलग्न यस विभाग र अन्तर्गत कार्यालयका कर्मचारीको योगदानको आधारमा सह-लेखकको रुपमा समावेश गराउनु पर्नेछ ।
७. तोकिएका शर्तहरुको पालना नगरेमा विभागले कुनै पनि समयमा अनुमतिपत्र रद्द गर्न सक्नेछ ।
८. तोकिएको शर्तहरुको हकमा सोही बमोजिम र अन्य बाँकीको हकमा प्रचलित कानून बमोजिम हुनेछ ।

२०८२/०२/१२
(प्रज्ञा थमाल)
इकोलोजिष्ट

बोधार्थ:
श्री कृष्ण प्रसाद पन्त सहित ३ जना नेपाली अनुसन्धानकर्ताहरु : सम्बन्धित संरक्षित क्षेत्र कार्यालयसँग समन्वय गरी अध्ययन अनुसन्धान गर्नु हुन ।

Appendix 4

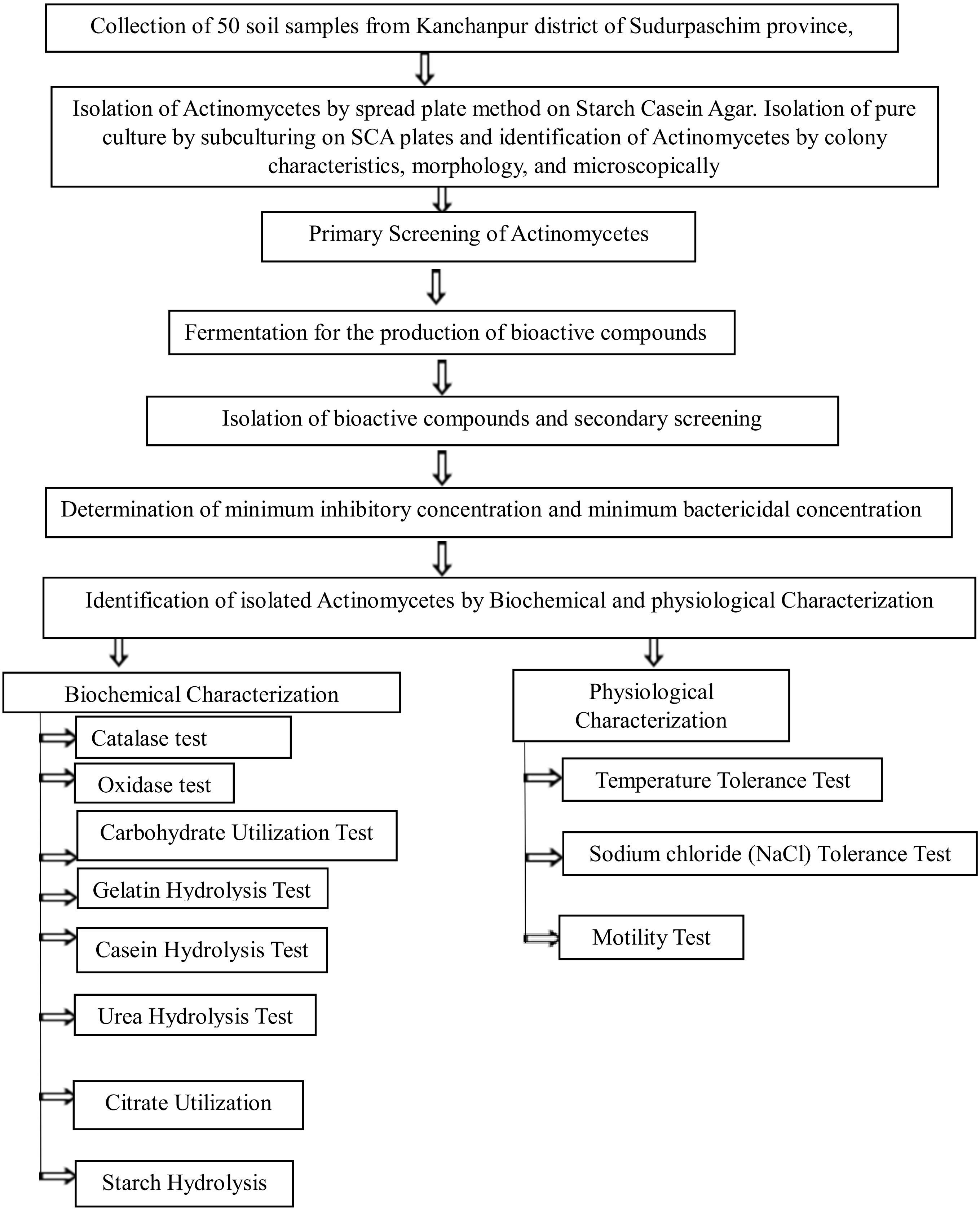
Places of Soil Sample Site

S No	Soil sample collection site	code	Land	Altitude (m)	Longitude	Lattitude	Temperature (ºC)	pH
1	Shuklaphanta National Park, Beldandi	S1	Barren	224	$28^0$52'40"N	$80^0$17'39"E	29.5	5.5
2	Beldadi 1	S2	Agricultural	204	$28^0$46'31"N	$80^0$16'22"E	30.7	6.5
3	Belauri-9	S3	Sugarcane Rhizosphere	205	$28^0$44'56"N	$80^0$18'46"E	28.1	6.4
4	Belauri-9	S4	Barren	205	$28^0$44'55"N	$80^0$18'46"E	31	7.8
5	Belauri-8, Kalkatta	S5	Wetland	147	$28^0$41'47"N	$80^0$19'9"E	31.9	7.9
6	Belauri-3, Laxmipur	S6	Agricultural	153	$28^0$41'1"N	$80^0$23'15"E	28.9	7.4
7	Belauri-4 shreepur	S7	Agricultural, low land	156	$28^0$41'2"N	$80^0$23'16"E	30.1	7.5
8	Belauri-4, Shreepur	S8	Agricultural	156	$28^0$41'3"N	$80^0$23'14"E	31.7	6.8
9	Punarvas-11, Dokebazar	S9	Barren	155	$28^0$41'30"N	$80^0$32'55"E	36.9	8
10	Krishnapur-6, Shantipur	S10	Barren	184	$28^0$46'40"N	$80^0$32'53"E	34.5	8.3
11	Krishnapur-Gularia	S11	Barren, forest	225	$28^0$50'24"N	$80^0$28'8"E	31.3	5.5
12	Krishnapur- , Bank	S12	Barren	225	$28^0$53'14"N	$80^0$23'54"E	33.2	7.7
13	Shuklaphanta , Kaluwapur	S13	Barren	208	$28^0$54'5"N	$80^0$22'19"E	31.8	7.1
14	Shuklaphanta National Park, Arjuni	S14	Barren	215	$28^0$55'24"N	$80^0$18'57"E	31.1	5.4
15	Bedkot-3, Chhure hill	S15	Barren	667	$29^0$1'40"N	$80^0$19'22"E	33.3	5.4
16	Bedkot-3, Chhure hill	S16	Barren, Termite	671	$29^0$1'41"N	$80^0$19'23"E	33.3	5.6
17	Bedkot-3, near lake	S17	Barren	507	$29^0$1'22"N	$80^0$19'3"E	31.1	5.1
18	Bedkot-3, Chella	S18	Barren	458	$28^0$59'27"N	$80^0$18'33"E	365.4	5.1
19	Bedkot-, Daiji	S19	Garden	206	$28^0$56'54"N	$80^0$15'59"E	29.6	7.8
20	Bhimdatta-4, Gobariya	S20	Barren	206	$28^0$58'2"N	$80^0$9'57"E	32.2	7.6
21	Bhimdatta-18, Katan	S21	Garden	199	$28^0$57'22"N	$80^0$10'48"E	30.3	8.4

22	Bhimdatta-18, Katan	S22	Barren	208	28^{0}57'18"N	80^{0}10'47"E	31.1	6.9
23	Bhimdatta-18, Katan	S23	Barren	208	28^{0}57'15"N	80^{0}10'40"E	31.4	6
24	Bhimdatta-6, Aithpur	S24	Barren	200	28^{0}58'26"N	80^{0}10'52"E	32.1	8.1
25	Bhimdatta-6, Aithpur	S25	Barren	200	28^{0}58'26"N	80^{0}10'52"E	27.8	7.1
26	Bhimdatta-6, Aithpur	S26	Agricultural	200	28^{0}58'26"N	80^{0}10'52"E	30.6	7.9
27	Bhimdatta-15, Nimbukheda	S27	Agricultural	208	28^{0}57'15"N	80^{0}10'40"E	28.9	7.4
28	Bhimdatta-15, Nimbukheda	S28	Agricultural	208	28^{0}57'15"N	80^{0}10'40"E	28.7	7.6
29	Bhimdatta-15, Nimbukheda	S29	Agricultural	208	28^{0}57'15"N	80^{0}10'40"E	28.8	7.4
30	Bhimdatta-15, Nimbukheda	S30	Agricultural	208	28^{0}57'15"N	80^{0}10'40"E	28.9	7.5

Appendix 5

Flow chart of the laboratory procedure



Appendix 6

Gantt Chart

SN	Activities	2024			2025			
		August	**Sept-Oct**	**Nov -Dec**	**Jan-April**	**May- Jun**	**July**	**August**
1	**Proposal writing and submission**	↔						
3	**Laboratory setting**		↔					
4	**Sample collection**			↔				
5	**Isolation and characterization**				↔			
6	**Preparation of research report draft**					↔		
7	**Preparation and submission of final report**						↔	
8	**Dissemination of finding and preparation of manuscript**							↔

Appendix 7

Budget Framework

Particulars	**Amount (NRS)**
Transportation for sample collection	20000
Remuneration for assistant	15000
Reagents/instruments/glass wares	25000
Stationary	5000
Typing, printing, and binding of reports	10000
Contingency/ other expenses	5000
Statistical analysis/software	-
Publication in indexed journal	-
Total	**80000**

Photographs

Photo1 Researcher Collecting Soil Samples in agricultural land

Photo 2 Researcher taking permission from landowner for collecting soil

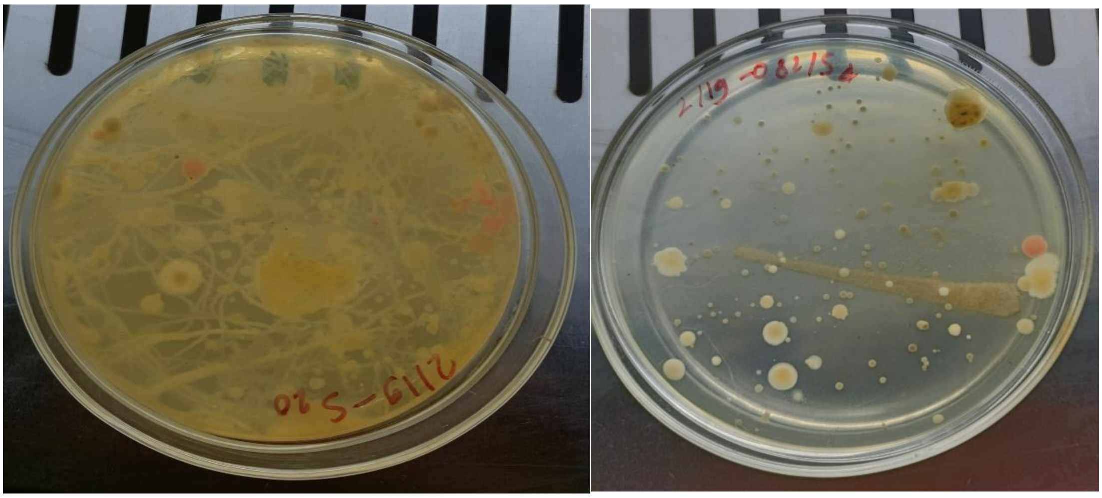


Photo 3 Colonies of Actinomycetes isolates on SCA plate

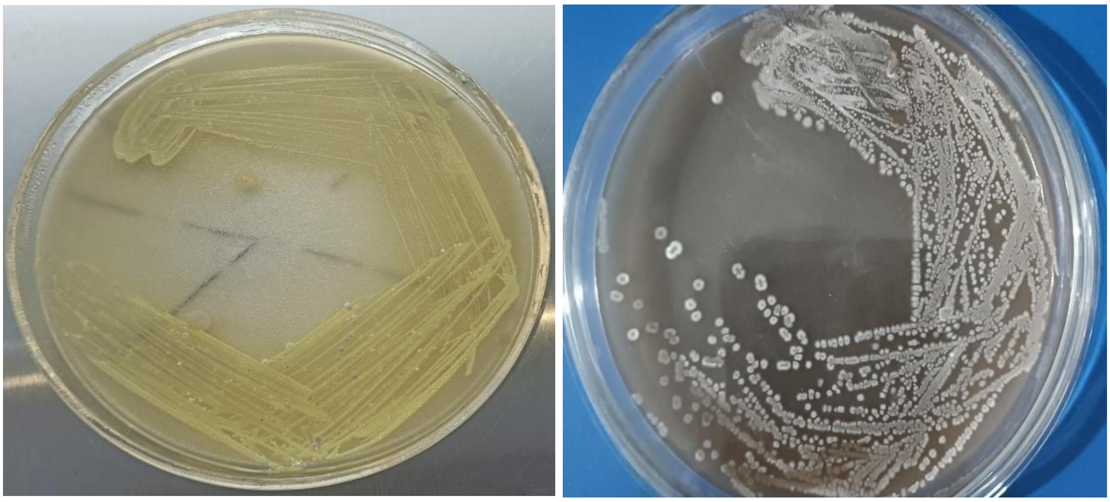


Figure 5 Pure culture of S25 yellow on SCA plate

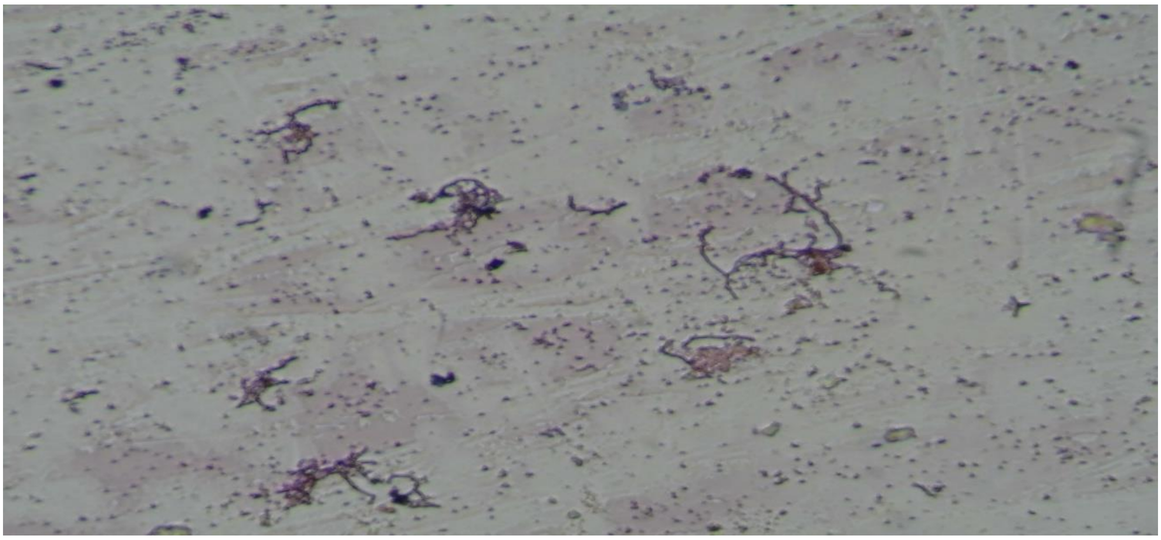


Photo 6 Gram positive filamentous Actinomycetes at 100X oil immersion

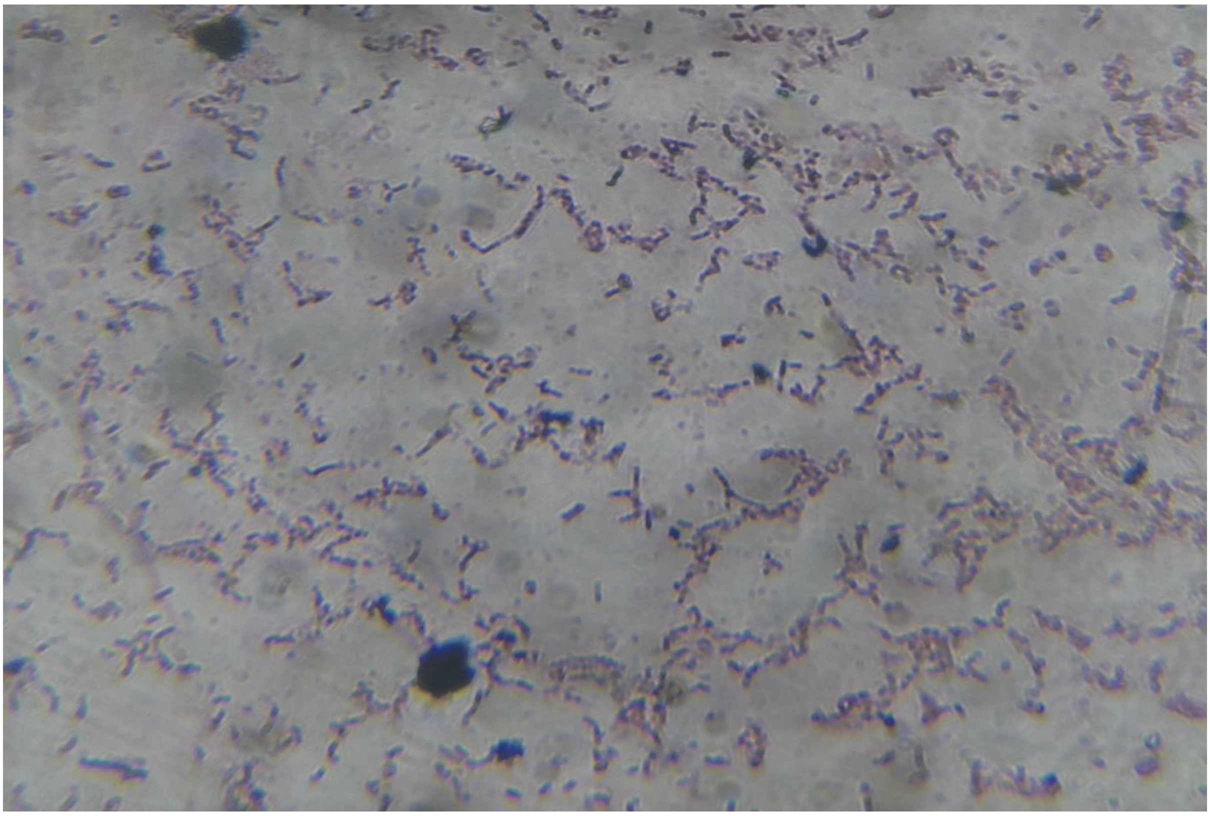


Photo 7 Gram Positive rod shaped Actinomycetes isolates at 40X

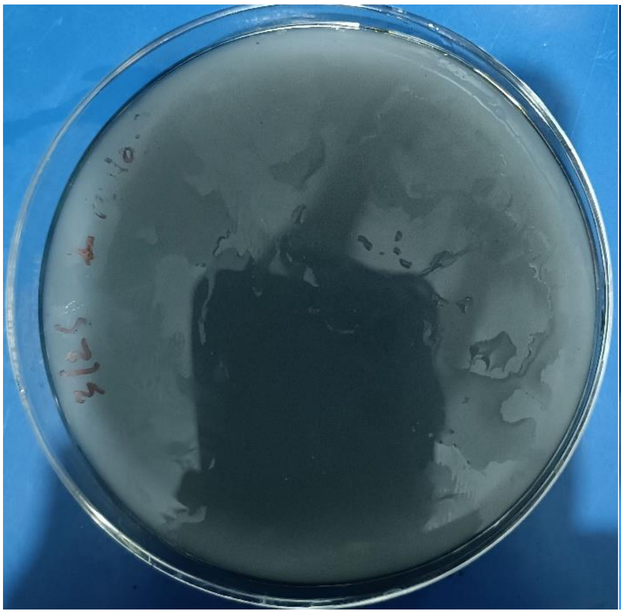


Photo 8 Starch hydrolysis test

(S25yellow)

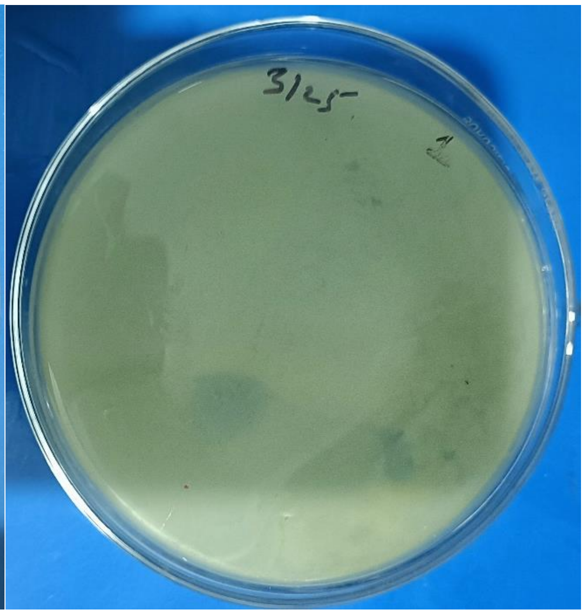


Photo 9 Gelatin Hydrolysis test

(S25 yellow)

Photo 10 Biochemical test for S25 yellow isolate from left indole -ve, MR +ve, VP -ve, Urease + ve, Citrate +ve and TSIA Alk/A

Photo 11 Biochemical test for S25 white isolate from left indole -ve, MR +ve, VP -ve, Urease + ve, Citrate +ve and TSIA Alk/A

Photo 12 Catalase test +ve (left) and Oxidase test -ve (right) for S25 isolate

Photo 13 Researcher in microbiology laboratory

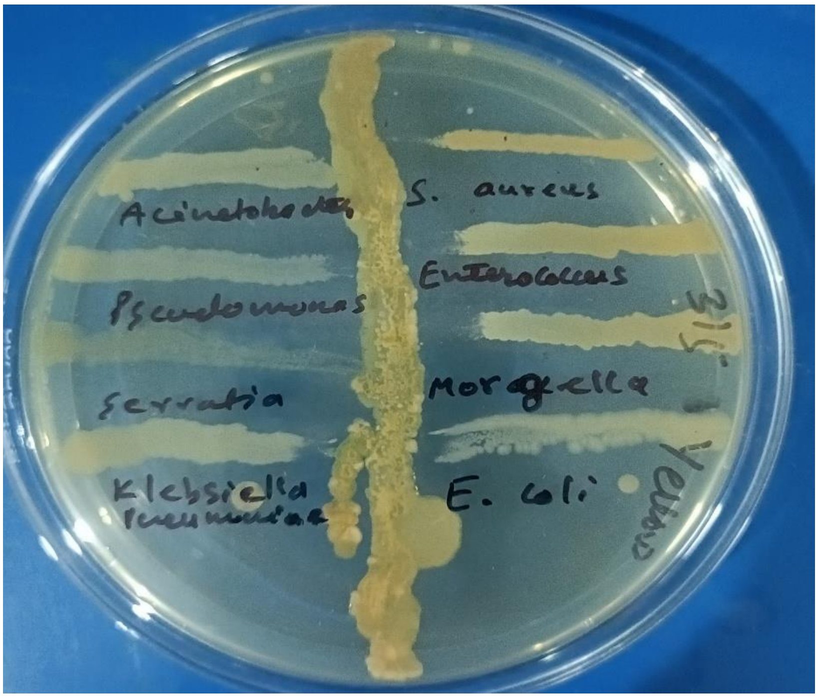


Photo 14 Primary Screening of S25 Yellow against test organism (*S aureus, Enterococcus, Morganella* were inhibited)