SCREENING OF ANTIBIOTIC PRODUCING ACTINOMYCETES FOR ANTIBIOSIS FROM SOIL OF SUNSARI, NEPAL

A Dissertation Submitted to the **Department of Microbiology, Central Campus of Technology**, Tribhuvan University, Dharan, Nepal, in Partial Fulfillment of the Requirements for the Award of Degree of Master of Science in Microbiology **(Environment and Public Health)**.

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ABSTRACT

Actinomycetes, slow growing gram positive bacteria, are known as an organism that is useful in the search for bioactive compounds. The aim of this study was to screen antibiotic producing Actinomycetes for antibiosis from the soil of Sunsari, Nepal. Primary Screening and Secondary screening were performed by perpendicular streak method and agar well assay method respectively. Microbiological characterization were performed for identification of presumptive genera. Characterization of the antibacterial substances produced by this isolate was done by Thin-layer chromatography (TLC) using Chloroform:Methanol (10:90) as the solvent system and iodine vapor as the visualizing agent. Altogether twenty four actinomycetes were isolated from soil samples but only one (4.2%) of the isolate showed antibacterial activity against both gram positive (*Staphylococcus aureus* and *Bacillus* spp.) and gram negative (*Escherichia coli*, *Salmonella* Typhi and *Pseudomonas* spp.) test-bacteria in primary screening. This isolate (S_{11}) was selected for secondary screening because of its potent antibacterial activity. Minimum inhibitory concentration (MIC) of crude antibacterial substances extracted from broth culture of the isolate (S_{11}) was found to be 1.2 mg/ml against test organisms. The chromatogram in TLC showed only one spot with R_f value 0.87 by the isolate suggesting that the isolate produced only one compound which was completely different from the spot with R_f value 0.94 produced by gentamycin. According to identification by Microscopy (1000X) and overall biochemical, and physiological characteristics, the isolate was considered as *Streptomyces spp*, a distinct taxonomic group.

Key words: Actinomycetes, Antibacterial activity, Minimum inhibitory concentration, Thin layer chromatography

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LIST OF ABBREVIATIONS

16 S rRNA	:	16 Svedberg unit ribosomal Ribonucleic acid
ATCC	:	American Type Culture Collection
ATP	:	Adenosine Tri phosphate
BLAST	:	Basic local Alignment Search Tool
DABA	:	Diaminobutyric Acid
DAP	:	Diaminopimelic Acid
DNA	:	Deoxyribonucleic Acid
G + C	:	Guanine + Cytosine
HPLC	:	High Pressure liquid Chromotography
MHA	:	Mueller Hinton Agar
MIC	:	Minimum Inhibitory Concentration
MRSA	:	Methicillin Resistant *Staphylococcus aureus*
NA	:	Nutrient Agar
R_f	:	Retentation Factor
SCA	:	Starch Casein Agar
SIM	:	Sulphide Indole Motility
TLC	:	Thin Layer Chromatography
UV	:	Ultra violet
Viz	:	Namely

CHAPTER I

INTRODUCTION

1.1 Background

An antibiotic was originally defined as a substance, produced by one micro-organism, which inhibits the growth of other microorganism. The advent of synthetic now refers to a substances produced by one microorganism, or to a similar substances (produced wholly or partially by chemical synthesis), which in low concentrations inhibits the growth of other microorganism (Russell, 2005). Actinomycetes are the most widely distributed group of microorganism in nature which primarily inhabit the soil (Oskay et al 2004).Actinomycetes are facultative anaerobic (except *A. meyeri and A. israelii* both obligate anaerobe), they grow best under anaerobic conditions. Actinomyces species may form endospores and while individual bacteria are rod shaped Actinomycetes colony form fungus-like branched networks of hyphae (Holt JG et al 1994).

Actinomycetes are a diverse group of Gram positive, free living, saprophytic, filamentous bacteria that produce major source of antibiotic (Valli et al 2012). Microbial diversity is a vast frontier and potential goldmine for the biotechnology industry because it offers countless new genes and biochemical pathways to probe for enzymes ,antibiotics and other useful molecules(Singh and Agrawal 2002).The diversity of terrestrial Actinomycetes are of extraordinary significance in several areas of Science and medicine, particularly in antibiotic production (Magarvey et al 2004).

The Actinomycetes compromise an extensive and diverse group of Gram-positive mycelial bacteria having high G+C (>50%) content in their DNA. They are numerous and widely distributed microorganism in nature. They are the best common source of novel antibiotics (Okami and Hotta, 1988).

Actinomycetes belong to the order Actinomycetales (Superkingdom: Bacteria, Phylum:Firmicutes,class:Actinobacteria,subclass:Actinobacteridae).They have high G+C content (>55%) in their DNA. They provide approximately two-

third of naturally occuring antibiotics and are the best common source of antibiotics (Okami and Hotta, 1988).

Actinomycetes species synthesize a numerous natural metabolites with diverse biological activity such as antibiotics. Antibiotics of Actinomycetes origin evidence a wide variety of chemical structure, including aminoglycosides, Tetracyclines, beta lactams, nucleosides, peptides, polyenes, actinomycins and tetracycline (Barrios-Gonzalez et al 2005).Antibiotics have been used in many fields including agriculture ,veterinary and pharmaceutical industry (Kavitha, Vijayalakshmi, 2007). Actinomycetes have diverse clinical effects and are active against many pathogenic organisms. Actinomycetes and their bioactive compounds show antibacterial and antimicrobial against various pathogens and multi drug resistent pathogens e.g. Vancomycin-Resistant Enterococci , Methicillin-Resistant *Staphylococcus aureus, Shigella dysenteriae, Klebsiella sp* and *Pseudomonas aeruginosa* etc (Bhatnagar and Kim, 2011; Saadoun et al 1999; Selvameenal et al 2009 and Servin et al 2008).

Actinomycetes also synthesis and excrete dark pigments, melanin or malanoid (Zenova 1965; Arai and Mikami 1972; Amal et al 2011).These melanin compounds are irregular, dark brown polymers having radio protective and antioxidant properties that can effectively protect the living organisms from ultraviolet radiation (Romero-Martinez et al 2012).Melanins are frequently used in medicine, pharmacology and cosmetic preparation (Quadri and Asgar, 2012).

After the remarkable discovery of streptomycin from *Streptomyces griseus* in 1942 by Walksman, there has been a worldwide hunt of for antibiotics from various terrestrial substrates and geographical regions (Hockenhull,1963).

The *Streptomyces* spp is particularly fruitful and can produce great many antibiotic and other biologically active secondary metabolites. They cover around 80% of total antibiotic product with other genera trailling numerically; *Micromonospora* is the runner up with approximately one -tenth as many as *Streptomyces*. If we include secondary metabolites with biologically activities other than antimicrobial, actinomycetes are still out in front, over 60%; *Streptomyces* spp. accounting for 80% of these (Hopwood et al 2000).

Although the number of antibiotics substances discovered to date is great, the majority of those are venomous to man and animals, and thus have not curative use (Egorov, 1985). About 160 antibiotics are presently used in clinic for treatment of human and animal diseases. Besides that, effective antibiotics have been found to be ineffective due to the resistance developed by the bacteria towards them. Large number of emerging and re-emerging bacterial , fungal and viral pathogens continue to increase, and the threats of bioterrorism looms large worldwide (Spellberg et al 2004).Resistance of bacteria to the effects of antibiotic has become a major problem in the treatment of disease .Infectious disease are still the second leading cause of death worldwide (Luzhetsky et al 2007).

The rare Actinomycetes constitute a potentially important source of novel antibiotics. For that many laboratories endeavored to diversity the source of actinomycetes by the use of samples coming from the most extreme habitations (Lee and Hwang, 2002; Lemriss and Laurant, 2003).Therefore, Sunsari district is a district of Province no.1 of Nepal. The district is located in the Eastern Terai and covers an area of 1257 sq km. It is situated at 71-1430 m height from the sea level. As a result of the elevation difference, the district has two difference types of climate tropical and subtropical climate .The annual rainfall is about 114.3 mm and temperatures vary from 10^{o} C to 30^{o} C. Its altitude, Sun radiation and seasonal variation create extreme habitation that may harbour unusual type of microorganisms. Although much more researchers have been carried out from mountain and Himalayan region, no research has been done from Eastern Terai region of Nepal. So, this present study has been undertaken for isolation and screening of actinomycetes for antibiotics production from soil samples of Sunsari district.

1.2 Objectives

1.2.1 General Objective

- To screen actinomycetes showing antibacterial activity from the soil sample of Sunsari, Nepal and characterize the antibacterial substance.

1.2.2 Specific Objectives

- To isolate actinomycetes from soil samples of Sunsari, Nepal.
- To screen actinomycetes isolates for antibacterial property.
- To identify the isolated antibiotic producing actinomycetes
- To characterize the antibacterial isolates.
- To extract secondary metabolites from screened antibiotic producing actinomycetes.

CHAPTER II

LITERATURE REVIEW

2.1 Actinomycetes

The name "Actinomycetes" derived from the Greek aktis (a ray beam) and mykes (fungus) and was given to these organisms from initial observation of their morphology. Their taxonomic position is not well established within the kingdom of prokaryote. The properties relating them to bacteria are the absence of nuclear membrane, small hyphal diameter (one tenth to one fifth as that of eukaryotes, chemical nature of cell wall, structure of flagella when produced, and sensitive to antibacterial agents and Lysozyme. Many actinomycetes can form resting structure called spores which will facilitated them in rapid dispersal in aquatic habitats, dispersal in air, survival in soil and viability over many years.

2.2 Taxonomy

Actinomycetes are large group of Gram positive to Gram variable bacteria that usually grow by filament formation.This belong to the order Actinomycetes (Superkingdom; Bacteria). Phylum: Firmicutes, class: Actinobacter, Subclass: Actinobacter, Subclass: Actinobacteriaceae. According to Bergey's Manual of Determinative Bacteriology (2000), Actinomycetes are divided into eight families, viz. Actinomycetes, Mycobacteriaceae, Actinoplanaceae, Frankiaceae, Dermatophilaceae, Nocardiaceae, Streptomycetaceae, Micromonosporaceae (Holt 1989) and comprise of 63 genera (Nisbet and Fox 1991).

Actinomycetes were originally classified according to their morphology. Subsequent analysis of chemotaxonomic markers has assisted in the detection of genera and differentiation of species in some groups. On the basis of cell wall analysis actinomycetes family has been divided into eight types (type I-VIII) on the basis of presence of cell wall chemotypes-L-DAP, Meso-DAP,DABA, Aspartic acid, Glycine, Lysine, Ornithine, Arabinase and Galactose.

Actinomycetes form the type II –IV can be further distinguished by their whole organism sugar patter like Arabinose, Fructose, Galactose, Madurose and Xylose.

A whole array of taxonomic tools has been used to define genera and suprageneric groups of actinomycetes (Good fellow and O 'Donell 1989), but partial sequence analysis of 16s rRNA is the most significant. Based on 16s sRNA classification system they have recently been grouped in ten suborders. Actinomycineae, Corynebacterineae, Frankineae, Glymcineae, Micrococcineae,Micromonosporineae,Proionibaterineae,Pseudonocardinea e, Streptomycinea and Strektosporangineae and large of members of Actinomycetes are still remained to be grouped.

2.3. Taxonomic classification of the order actinomycetes

2.3.1.Micromonosporinea: The family of this suborder is Micromonosporoceae. The genus of this family are *Micromonospora, Actinoplanes,Catellospora, Couchioplanes, Pilimelia, Dactylosporangium*.

2.3.2.Frankineae : Frankiaceae, Sporichthkyaceae, Geodermatophilaceae, Microsphaeraceae, Acidothermaceae are its family. The genus of this sub order are *Frankia, Sporichthya, Geothermophillus, Blastococcus, Microsphera* and *Acidothermus*.

2.3.3.Pseudonocardineae : It belongs to the family Pseudonocardiaceae. The genus of this family are *Pseudonocardi,Actinopolyspora, Kibelosporium,Kutzneria,Lentzea,Saccharomonospora,Saccharopolyspora , Saccharothrisx, Streptoalloteichus* and *Thermocrispum.*

2.3.4 Streptomycieae : The family of this suborder is Streptomycetaceae and the genus is *Streptomyces*.

2.3.5 Corynebacterium : The family of this suborder are Nocardiaceae, Gordoniaceae, Mycobacteriaceae, Dietziaceae, Tsukamurella and Corybactericeae. The genus are *Nocardia, Rhodococcus, Gordonia, Mycobacterium, Dietzia, Tsukamurella, Corynebacterium and Turicella.*

2.3.6 Actinomyineae : The family is Actinomycetaceae and its genus are *Actinomyces, Mobiluncus and Acanobacterium.*

2.3.7 Propionibacterianeae: The family of this suborder is Propioniabacteriaceae. The genus are *Propioniobacterium, Luteococcus, Microrolunatus and Propionoferans.*

2.3.8 Streptosporangineae: Streptosporangiaceae, Thermomonosporaceae and Nocardiopsaceae are its family. The genus are *Streptosporangium, Herbidospora, Microbiospora, Microteraspora, Planobispora, Planomonospora, Themomonospora, Actinomadura, Spirillospora* and *Nocardiopsis.*

2.3.9 Glycomycineae: Its family is *Glycoimycetaceae*. The genus of this sub order is *Glycomyces*.

Source : www.ncbi.nlm.nib.

2.4 Occurrence:

Actinomycetes are found almost entirely in every natural substances, such as soils, decayed mixture of plants ,fresh water basins, oceans sediment, foodstuffs and the atmosphere. These organisms live and multiply in large amounts in different depth of compost and soil in tropical and in temperate regions. Alkaline and neutral soils are more favourable habitats than acidic soils and neutral peats are more favourable than acidic peats.

Actinomycetes have been fingered as one of the very important groups of the soil organisms (Kuster, 1968) and their occurrence is greatly affected by humidity, P^H, temperature and vegetation. Conn (1916) reported that Actinomycetes make up as much as 40 percent of the microbial population in soil rich in plants roots .Waksman and Curtis (1916) also reported that soil contained a large a number of actinomycetes. Acidophilic isolated grow in the ph range 3.5-6.5,with optimum growth between p^H 4.5 and 5.5,while neutrophilic strains grow in the P^H range 5.09 (Khans and Willium 1975). Alkalophilic Actinomycetes are also known to occur in soils (Mikami et al 1982).Jiang et al (1993) isolated alkalophilic Actinomycetes from alkaline soil

samples(P^H 10) collected at Yunan. Halo tolerant actinomycetes grow in salt concentration above 0.2M NaCl (Kushner 1985). Therefore they have been mostly isolated from marine habitat. Halophilic among soil isolates has only been observed in certain genera (Embley,1992).Hirsch et al,2004 isolated three Cryptoendolithic Actinomycetes from Antartic Sand stone rock samples were characterized Penotypically and by molecular taxonomic methods. Actinomycetes were also isolated from three Brazalian tropical soils, using differential centrifugation procedure (Semedo et al 2001).Singh (1999) isolated various Actinomycetes from the hospital disposal site and the rhizosphere of different plants and from the compost soil.

2.4.1 Isolation of Actinomycetes from soil

There is presence of mixed micro flora in nature and it is complicated to isolate them because of their characteristic slow growth in comparison of other soil bacteria and fungi (Hirsch and Christensen, 1983). Due to this, it resulted in the development of selective isolation procedure primarily based on the following approaches.

1. Pretreatment of soil using different chemicals and methods such as calcium carbonates (Tsao et al 1960). In this method, air dried soil is mixed with calcium carbonate in a mortar and the mixture is incubated for 10 days at 28°C in a closed inverted sterile petridish. This creates high relative humidity maintained by water saturate filter discs. This method gives not only the highest total count of Actinomycetes , also the lowest relative number of bacteria and fungi (El-Nakeeb and Lichevalier,1962).Other chemicals and methods are sodium propionate (Crook et al 1950), Phenol (Lawrence,1969), centrifugation (Rehacek, 1959) etc.
2. Nutritional selection, the commonly used media are medium (kuster and William,1964; Williams and Davies, 1965; Okazaki and okami, 1972), chitin medium (Lingappa and lockwood, 1961), modified Benidicts medium (porter et al1960), Soybean meal –glucose medium (Tsao et al1960),Gauze's agar medium (Rehacek,1959), Czapek's agar

medium, glucose asparagines medium (Nomura and Ohara, 1969), glycerol asparagines apar II (Waksman, 1961).

Actinomycetes can produce exoenzymes and degrade chitin, cellulose and lignin which are polymeric substances. So, the appropriate medium is the main step in the selective isolation of actinomycetes. Generally, the best carbon sources are glycerol, starch and chitin, with casein, asparagines and arginine as organic nitrogen sources. Many media also contain an inorganic as organic nitrogen, mainly nitrate and phosphate.

3. Selective inhibition, in this process antibiotic are incorporated into a media to inhibit microorganism other than actinomycetes selectively (Williams and Davies, 1965).A large number of method have been advocated to facilitate the separation of actinomycetes from their relatives. It is not difficult to isolate them from an intimate mixture with fungi, since the physiological properties of these two group of microorganisms are different. For example, strictly antifungal antibiotic which donot affects the growth of actinomycetes can be used successfully. Cycloheximide has been used by Dulency et al (1955).Porter et al (1960) used Nystatin and Pimaricin.Williams and Davis (1965) found that a combination of Nystain and cycloheximide (each 50µg/ml of medium) inhibited most soil fungi, while having no deleterious effect on actinomycetes , use of cycloheximide alone was not so effective .Use of antifungal antibiotics is therefore an essential precaution in the isolation of soil actinomycetes. However , it is more difficult to separate them from true bacterial. Use of anti-bacterial antibiotic presents more problems as Actinomycetes are potentially sensitive to them (Williams and Willington,1980).The addition of Penicillin (1µg/ml) and polymixin (5µg/ml)to media can reduce bacterial development (Williams and Davis,1965).

a. The soil dilution plate technique

The soil dilution plate technique is one of the frequently used method for isolating and counting soil Actinomycetes and other soil microorganism. This basic procedure is used frequently and is described

in detail by Johnson et al (1959) .Generally , the diluents used is sterile water with most soil, suitable plates can be obtained by using dilution of the soil in water between 10^{-1} and 10^{-6}.The efficiency of this technique may be influenced by composition of nutrient medium ,and the procedure used by incorporate the diluted soil suspensions into apar medium.

b. Other technique

Although, the vast majority of workers have used the dilution plate technique to isolate soil actinomycetes, there are other method that can be applied, particularly in ecological studies. Soil is incorporated in culture medium in small quantities to make soil plates for isolation of soil fungi. Nonomura and Ohora (1960) found this method to be particularly useful for isolating strains of *Micromonospora.*

Hirsh and Christensen (1983) described a method in which a membrane filter is used. A nutrient agar medium is overlaid with a 0.2-0.45µm pore cellulose ester membrane, and the surface of the filter is inoculated with samples (soil, water, various vegetables matters etc). During inoculation , the branched mycelium of the actinomycetes penetrate the filter pores to underlying agar medium where as growth of non actinomycetes bacteria is restricted to the filter surface .

2.4.2 Screening of Actinomycetes for antimicrobial activity

New antibiotics were discovered in the past by screening. In the screening approach, a large number of isolated possible antibiotics producing micro organism is obtained from nature. Then the pure cultures were prepared in order to test these isolates for antagonistic activity. The test is done by exposing selected bacterial strain to the environs of the cultural isolates and looking for the zone of inhibition. The best bacteria used are selected from a variety of bacterial types. However, they are chosen to be representative of or related to bacterial pathogens. The classical procedure for testing new microbial isolates for antibiotic production is the cross streak method (Madigan, 2000). Alternatively, the organisms may be grown in liquid

medium and the filtered brew placed in cups cut into a seeded agar plate giving zone of inhibition confirms the antibiotic production.

Above 8000 substances are known, and several hundred antibiotics are discovered yearly. Most of the microorganism which belongs to the genera like *Streptomyces*, *Penicillum* and *Bacillus* have the ability to produce antibiotic. So, there are more antibiotic waiting to be discovered. Many antibiotic researchers believe that a number of new antibiotic will be discovered, if other group of microorganism were studied (Brock, 1997).

Waksman (1937) made a detailed survey of actinomycetes possessing antagonistic effect upon the activity of other microorganisms.

Agre and Orleanskly (1962) isolated 289 strains of thermophilic Actinomycetes from India pamer soil and it is found that 33% inhibited *S.aureus* and a lot of 48% had some sort of antibiotic activity .It was found that *Micromonospora vulgaris* was the most prevalent organism. Kudrim and Maksimova (1963) isolated 1100 cultures under thermophilic condition from 385 chinese soil samples and found that 17% had a broad spectrum antibiotic..Pandey (2003) found that 36 out of 106 isolates of actinomycetes having antimicrobial activity from Lobuche area (5000-5300 m in height) and Lukla area (2660 m) in Khumbu region of Nepal.

Muthu et al (2013) found 5 isolates of total 10 screened samples having antimicrobial activity from Cauvery river soil sample. Five Isolates actinomycetes were identified by 16s rRNA sequencing and were confirmed by using bioinformatics tool as BLAST (Isoptericola variabilis).

Tyagi et al (2014) reported that 11 Actinomycetes were isolated from 21 soil samples at two different temperatures 28^{o}C or 37^{o}C. These actinomycetes were screened with regard to potential against Gram-positive and Gram negative bacteria. They were evaluated for their inhibitory activities on test organisms like *Streptococcus aureous, E coli, Staphylococcus aureus* and *Bacillus strains.*These 11 actinomycetes isolates were highly active with an inhibition zone more than 16 mm in

diameter. These 11 actinomycetes isolates were isolated mostly from alkaline soil .

Rotich et al (2017), A total of 107 actinomycetes were isolated and only 39 (36.4%) showed antimicrobial activity against five of the six test isolates that included reference strains *Staphylococcus aureus* (ATCC 25923*),Escherichia coli* (ATCC 25922) and *Candida albicans* (ATCC 90028) and three clinical strains *Trichophyton mentagrophyte, Microsporum gypseum* and Methicillin Resistant *Staphylococcus aureus*. Two of the isolates showed activity against MRSA and four isolates showed a higher potency than the standard drug chloramphenicol (30µg) against *S. aureus*. DNA from the isolates was extracted and the 16S rRNA gene was amplified using primers specific for actinomycetes. The amplified gene was sequenced and phylogency analysis was done. The 16S rRNA gene was able to be amplified in only 15 of these isolates. Sequencing showed that 93.3% were of the genus *Streptomyces* while 6.7% were of the genus *Rhodococcus*.

2.4.3 Fermentation and Antibiotic production

Microbial biodiversity is a rich source for the biotechnology industry as it offers numerous genes and biochemical pathways to examine for enzymes antibiotics and other useful molecules (Agrawal, 2002).Since the first virgin effort of Flory and chain in 1939, the importance of antibiotic to medicine has lead to much research into discovering and producing them. The process of production usually includes screening of micro organism, testing and modification. Antibiotic production is carried out by using fermentation, it is anaerobic condition where there is no oxidation phosphorylation to maintain the production of Adinosine Triphosphate (ATP) by glycolysis.

Antibiotic produced from actinomycetes are aminoglycodies ,anthrocycline, glycopeptide, β-lactams, macrolides, nucleocides, peptides, polymers, polypeptides, actinomycin, and tetracycline (okami and Hotta,1988).

Some of the actinomycetes strain of same species could generate different antibiotics, whereas strains belonging to the different species generated the same antibiotics (Lechevalier, 1975).

Antibiotics are produced as defense mechanism to prevent the multiplication of other organism when environmental stress that triggers a metabolic response. The co-culture of *Streptomyces* with a bacterium *T. pulmonis* cause the production of secondary metabolites which were not produced in a pure culture of *Streptomyces* (Garashi,2005).The other inducers of antibiotic production are mutagens like Sodium Azide (Bhattarai et al 2007),and intercalating agents like ethidium bromide and daunorabicin (Malikina and Dudnik,1995).

Microorganisms differ in the ways they respond to the fermentation condition and one condition are favourable they may produce a vast array of bioactive compounds. The mass production of antibiotic began during World War II with Streptomycin and Penicillin. Now most antibiotics are produced by staged fermentation in which strains of microorganism producing high yield are grown under optimum condition in nutrient media in fermentation tanks holding several thousand gallons. The microbial cell mass is stained out of the fermentation broth, and then the antibiotic is removed from the broth by filtration, precipitation and other separation methods.

During the idio-phase, Harvest of antibiotic from culture medium is done when maximum titer of antibiotic is obtained. Liu et al (1980) performed fermentation for 5 days for polyether antibiotics by *Streptomyces* malachite-fuscus where as Kumagai (1993) did it for 6 days for *Nocardia brasiliensis* .Casidia (1959) divided antibiotic production into three stages, the first one characterized by severe growth of mycelium followed by quick decrease of mycelium but production of antibiotic increases rapidly in the second phase. In the third phase, after 7 days of incubation it cause ending of the production of antibiotic.

2.4.4 Media Composition

For the production of antibiotic, it usually involves satisfactory media .It can be obtained by organized study of the suitability of large number of carbon and nitrogen sources. The medium for antibiotic production must provide all the basis constituents for growth formation that includes carbon, nitrogen, phosphates and trace elements.

Normally glucose is used in the fermentation as a recommended carbon source and catabolite repression is escape by feeding glucose during fermentation (Srinivivasan et al 1991). In order to do down this process, the addition of carbon source to the culture must be carefully controlled .In some cases, use of other carbohydrates such as lactose or starch have been found to be better carbon sources for antibiotic production (Srinivasan et al 1991).

Maximum production was obtained with ammonium nitrate among all inorganic and amino acid nitrogen sources, denoting that the level of antibiotic may be greatly influenced by the nature, type and concentration of the nitrogen source supplement in the culture medium. Waksman (1946) demonstrated that meat extract as the source of nitrogen source along with glucose as source of carbon, peptone and sodium chloride for streptomycin production. However, meat extract was replaced by yeast extract (Egorov, 1985).Yeast extract helps to sustain growth in fermentation medium and it is an effective nitrogen sources. Kobinata et al (1993) reported as a dispersal of mycelium in the fermentation broth of many Actinomycetes .

According to Huck et al (1991), for the effective and selective fermentation of bioactive substances from actinomycetes, a combination of carbon, starch, glycerol and glucose can be used with other complex nutrients, buffering agents and salts. Shomura et al (1983) used a complex medium containing soluble starch, glucose, peptone, yeast extract, Soybean meal and calcium carbonate for the production of dapiramicin by *Micromonospora Spp* SF-1917. Phosphate helps to regulate the biosynthesis of many different types of antibiotics and other secondary metabolites. Phospherous is very important for the growth of actinomycetes

and production of antibiotics (Egorov, 1985).At the same time ,a concentration beyond certain limit fails to produce a sensible effect on the growth of the mycelim and reduces the production of antibiotics Since excess phosphorous alters the biochemical composition of the protoplasm, changes the cycle of the actinomycetes development, and some physiological function of the cells . The optimum concentration of phosphorous in the medium is 0.04-0.07µg/ml according to Egorov (1985).Over the last four decade, an impressive number of antibiotic and secondary metabolite have been shown to be regulated negatively by phosphate. This includes Streptomycin C and thienamycin. Surprisingly the molecular mechanism of phosphate control has remain opaque (Martin et al 1994).

Iron, Zinc and Magnesium (trace elements) are very important for development of actinomycetes and for antibiotic production. The maximum bearing of Streptomycin is guaranteed with the concentration of $Feso_4.7H_2O$ of 0.0007-0.005%. High concentration of Ferrous sulphate inhibits the synthesis of the antibiotic, decrease the rate of consumption of carbohydrate and slow down the growth of actinomycetes (Egorov,1985).

2.4.5 Physiochemical parameters

Casida, 1959 reported that temperature is very important in the biosynthesis of antibiotic and growth of actinomycetes. The optimum temperature is in the range of 25^oC-30^oC, likely close to 28^oC. According to Egorov, 1985 if the temperature rises over 30^oC, the biosynthesis of antibiotic is particularly discontinued.

The P^H value within the growth allowances range of the strain has an effect on antibiotic production. The optimum P^H of the medium for cultivation of Actinomycetes is 7 and the antibiotic is formed in the range from P^H 7-8 (Casidia, 1959).

2.4.6 Extraction and characterization of antimicrobial Compounds

The most important duty after fermentation is the development of efficient purification method. Detailed methods of extraction and purification of the

antibiotic are necessary because of relatively small amount of antibiotic present in the fermentation broth. If the antibiotic is soluble in an organic solvent then it is immiscible in water, it may be relatively simple to purify the antibiotic by extracting it into a small volume of solvent, thus concentrating the antibiotic (Waksman, 1968). If the antibiotic is not soluble in organic solvent, then it must be removed from the fermentation. In all case, the aim is to obtain a crystalline product of high purity ,although some antibiotic do not crystalline readily and are difficult to purify.

Waksman (1968) used n-butanol to extract actinomycin from fermentation broth, obtained by submerged culture. Egorov (1985) described the method to concentrate streptomycin by adsorption on an active carbon. Liu et al (1986) determined the amount of antibiotic produced in the fermentation broth by ethyl acetate extraction and High pressure liquid chromatography (HPLC). Makoto (1995) extracted macrolide antibiotic of *Streptomyces violaceusniger* by acetone extraction of mycelium followed by butanol extraction.

Ikekawa et al (1963) studied 50 samples of known antibiotic using Thin layer chromatography (TLC). Nabi et al (2006) used TLC for the separation of penicillins. Kumagai et al (1993)used reverse phase high pressure liquid chromatography (HPLC) analysis to characterize macrolide antibiotic using methanol–water (80:20) as mobile phase and detected by ultraviolet (UV) rays absorption at 254nm. Saha et al (2012) performed TLC to understand the chemical nature of the Ethyl acetate extracted antimicrobiol compound present in the supernatant of isolated actinomycetes.

2.5 Antibiotics

An antibiotic is a substance produced by microorganism, or to a similar substances produced wholly or partially by chemical synthesis which in low concentration inhibits the growth of other microorganism (Russell, 2005). Antibiotic relate commonly to antibacterial. All antibiotic share the property of selective toxicity .They are more toxic to pathogen than they are able to animal or human host.Unlike compounds like organic acids and alcohol,

antibiotic substances are very effective even at low concentration .In addition; they are selective in their activity i.e. potent only against certain organism while hamless against others. Antibiotic are the secondary metabolite and have no physiological role in the growth phase of the organism, so they are produced as idiophase metabolites after the active phase is over.

2.5.1 History of Antibiotic Development

More than three hundred years ago (ancient time) people used Mold as antimicrobial agents. Warm soil was used in Russia by peasant to cure infected wounds in ancient period. In order to treat the infection, the ancient Chinese, Egyptians and Greeks used Mold and plants. As early as 350 A.D, the Sudanese-Nubian civilization used a type of tetracycline antibiotic. During the mid ages, crude plant extract and cheese curds were used to fight against infection.

The search for antibiotic starts in the late 1800s with the growing acceptance of the germ theory of disease. After that, Scientists starts to reserve time in searching drugs that would kill the disease causing bacteria. The main aim of such research was to find so called "magic bullet" that would destroy microbes without toxicity to the person taking the drugs (Wainwright, 1990).

2.5.2 Mechanism of antibiotic resistance

The multidrug efflux systems contribute significantly to the increased resistance to multiple antibiotics in bacteria. A major challenge in developing efficacious antibiotics against drug-resistant pathogens is to identify compounds that can counteract the efflux functions. The wealth of bacterial genomic information available suggests the presence of a variety of efflux systems in bacteria. Even a single bacterium may possess multiple efflux transporters of different families, with the overlapping substrate spectra. Accumulating evidence has indicated that the MexXY multidrug efflux system is a primary determinant of aminoglycoside resistance in *Psedomonas aeruginosa*. Morital et al (2012) provided a timely review on the *P. aeruginosa* MexXY pump and other aminoglycoside efflux pumps in a range of different bacteria. The expression of bacterial multidrug efflux

system is usually controlled by transcriptional regulators that either repress or activate the transcription of the multidrug efflux genes.

The articles by Usui et al (2013) and Deng et al (2013) further illustrated the complexity of regulation of multidrug efflux systems. However, the importance of multi drug efflux system may not be overstated for the specific antibiotic or organism, which is supported by the findings of Baucheron et al (2014).

Epidemiological work by chuma et al (2013) demonstrated a recent emergence of beta-lactamase-mediated cefotaxime resistance in *Salmonella enterica* Serovar Infantis. Watkins et al (2013) reviewed the novel beta-lactamase inhibitors that are close to being introduced in the clinical practice. Despite the successful development of beta-lactamase inhibitors for the combination therapy, the use of beta-lactamase inhibitors is still challenged by the variable affinity of inhibitors to different beta-lactamase and the vast quantity of beta-lactamase produced by the resistant strains.

A major mechanism of aminoglycoside resistance is the production of aminoglycoside-modifying enzymes. Shi et al (2013) provided a comprehensive overview of the structure of aminoglycoside kinase and reported on the recent progress in the discovery of aminoglycoside phospotransferase inhibitors using structure guided strategies.

2.5.3 Confirmation of antibiotic presence

Various methods like Biological, Serological, Molecular, Thin layer chromatography(TLC), Basic Local Alignment Search Tool (BLAST) analysis etc are used for the confirmation of antibiotic presence. But, Thin layer chromatography is the most familiar and efficient technique method used for the detection, analysis and separation of the bioactive compounds, so it is probably that 60% of the analyzed compounds are performed based on TLC over international. Thus, it is important to know the basic operation and performance of the TLC protocol (Maltland et al 2010).Thin - layer chromatography (TLC) is a chromatography technique used to separate non - volitile mixtures (Harry et al 1989). Thin layer chromatography is performed

on a sheet of glass, plastic, or almunium foil, which is coated with a thin layer of adsorbent material, usually sillica gel, aluminium oxide (alumina), or cellulose. This layer of adsorbent is known as the stationary phase. After the sample has been applied on the plate, a solvent or solvent mixture (known as mobile phase) is drawn up the plate via capillary action. Because different analytes the TLC plate at different rates, separation is achieved (Vogel et al).

To quantify the results, the distance travelled by the substance being considered is divided by the total distance travelled by the mobile phase. This ratio is called the retardation factor (R_f).

Thin layer chromatography can be used to monitor the progress of a reaction, identify compounds present in a given mixture, and determine the purity of the substance (Reich et al 2007).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials required

Various materials used in this study are listed in Appendix A.

3.2 Research methodology

3.2.1 Research design

This research design was basically descriptive type and qualitative in nature. The research work was mainly focused on antibiotic producing actinomycetes found in soil of Sunsari (an extreme environment) and their antibiotics for antibiosis against pathogenic bacteria.

3.2.2 Population and Sample

As actinomycetes are found in soil worldwide, soil of Sunsari was population for this study and collected soil representing different sites of Sunsari were the samples.

3.2.3 Sample size:

Altogether 32 soil samples from different places of Sunsari were collected. The places of soil sample sites are listed in Appendix D.

3.2.4 Laboratory Set up :

The study was completed at the laboratory of Department of Microbiology, Central Campus of Technology (CCT), Hattisar, Dharan-14.

3.2.5 Data collection procedure and data analysis plan

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

The collected data and information were processed, tabulated and analysed quantitatively by using different statistical methods such as Tables, Pie chart and Bar-diagram in final report.

3.3 Method

3.3.1 Collection of soil samples

Soil samples were collected from different sites from Sunsari district in June 2016. Four to five grams of preferably dry soil samples were collected from a depth of 4-5 cm and were placed in clean polyethylene bag and were mixed well with approximately 1 gm of $CaCO_3$ already added to the bag. Then the soil samples were further dried at room temperature for about 3 weeks.

3.3.2 Isolation of actinomycetes

Isolation of actinomycetes was done by spread plate technique following the serial dilution of soil samples, on starch casein agar (Williams & Davies,1965) plates containing nystatin and cycloheximide (each at concentration of 50 μg/ml of medium).One gram of soil was dissolved in 10 ml of sterile distilled water in a test tube. It was vortexed for a minute and serially diluted to 10^{-3} for each soil sample. Only 0.1 ml of the aliquot from 10^{-2} and 10^{-3} dilutions were plated on two different SCA plates by spread plate technique and then the plates were left a side undisturbed for 15 minutes after which the plates were incubated at 28^{o}C for 2-4 weeks.
Typical actinomycetes colonies characterized by their dry and tough wrinkled nature were picked up from SCA plates by a sterile inoculating loop or wire and streaked on SCA by quadrant streaking technique. The inoculated plates were then incubated for 2-4 days at 28^{o}C to isolate pure colonies of actinomycetes.

3.3.3 Screening of Actinomycetes for Antimicrobial activity

3.3.3.1 Primary Screening

Primary screening was done by perpendicular streak method on Nutrient Agar (NA). Actinomycetes was streaked along the centre of nutrient Agar plate and then was incubated for 2-4 weeks at 28^{o}C .The test bacteria (*Escherichia coli, Salmonella* Typhi, *Pseudomonas aeruginosa, Bacillus subtilis and Staphylococcus aureus)* were each inoculated in 5 ml of nutrient broth and

incubated at 37^{o}C for four hours in order to obtain visual turbidity comparable to 0.5 Mc Farland Nephalometer standard (AppendixE). The test bacterial broth cultures were streaked perpendicularly on either side of fully grown actinomycetes growth line while keeping the 1 mm between the bacterial streak line and the actinomycetes growth. The plates were incubated at 37^{o}C for 24 hrs. If the zone of inhibition of bacterial growth along the bacterial streak line was detected, it was measured and noted.

3.3.3.2 Secondary Screening

Secondary screening of actinomycetes isolates was done by agar well assay method on Mueller Hinton agar (MHA).The fresh and pure culture of each isolate selected from the primary screening was inoculated aseptically in 100 ml starch casein broth in 500 ml Erlenmeyer flask and incubated at 28^{o}C for 7 days in water bath shaker at 160 rpm. The visible pellets, clumps or aggregates and turbidity in the broth confirmed the growth of the organism in the flask. After the completion of incubation, the contents of the flasks were filtered through Whatman no 1 filter paper. MHA plates were swabbed with test bacterial broth cultures with 0.5 Mc Farland Nephalometer Standard and three to five cups (bores) were made on each of them. Hundred µl of filtrate was pipette in to the cups and kept undisturbed for few hours, allowing diffusion of Antibacterial substances on the agar medium. The plates were incubated at 37^{o}C for 24 hrs. If the zone of inhibition around the cups was detected, it was measured and noted.

3.3.4 Characterization of Actinomycetes

3.3.4.1 Macroscopic Characterization

The isolated colonies of actinomycetes on SCA were studied for the color of the aerial mycelium and diffusible pigments and other colony characteristics such as size, consistency, margin of colony etc.

3.3.4.2 Microscopic characterization

The microscopic characterization was done by cover slip culture method. They were then observed for their mycelial structure, configuration of sporophore (conidiospore and arthrospore) and arrangements and shape of spores on the mycelia under microscope (1000X).

The observed morphology of the isolate was compared with the Actinomycetes morphology described in Bergey's Manual of Determinative bacteriology, Ninth edition (2000) for the presumptive identification of the isolates.

3.3.5 Biochemical characterization

Various biochemical tests like Catalase, Oxidase, Carbohydrate utilization, Citrate utilization, Indole and Hydrogen Sulphide production, Nitrate Reduction, Urea hydrolysis, Tween 20 hydrolysis, Starch Hydrolysis, Esculin Hydrolysis, Temperature Tolerence, Nacl Tolerance, and motility tests were performed (Appendix C).

3.3.6 Fermentation

Fermentation was carried out by the submerged state culture in Erlenmeyer flask. The strains processing broad-spectrum antibacterial activity were selected for fermentation. They was inoculated in 25 ml starch casein broth in 100 ml Erlenmeyer flasks and incubated in water bath shaker at 28^{o}C at 160 rpm for 4 days .Thus prepared inoculums was poured in sterile 1 litre capacity Erlenmeyer flask containing 400 ml sterile starch casein broth and further incubated in water bath shaker at 160 rpm at 28^{o}C for 7 days. Potency of the culture broth was estimated by agar cup assay method using test organisms.

3.3.7 Extraction of antibacterial metabolites by using Ethyl acetate

After completion of fermentation, the broth (500ml) was filtered through Whatman no. 1 filter paper in sterile zone. The residue was discarded and the filtrate was taken for further analysis. The filtrate was subjected for solvent extraction method to recover antibacterial substances in pure form

to some extent, following the process described by Liu et al 1986. Ethyl acetate was added to the filtrate in the ratio of 1:1(v/v).From the 500 ml filtrate 100 ml was taken and to it 100 ml of ethyl acetate was added. It was shaken vigorously for 1 hour for complete extraction, poured in the separating funnel and left undisturbed for half an hour to allow separating from the aqueous phase through the separating funnel. Antibacterial activity was tested from both phases against test organisms (*E. coli and S. aureus)*. The phase having antimicrobial property was poured in porcelain basin for evaporation. It was evaporated to dryness in water bath at 40^{o}C and the residue obtained was weighed and dissolved in phosphate buffer for preservation. It was used to determine antimicrobial activity, minimum inhibitory concentration and to perform thin layer chromatography.

3.3.8 Determination of Antimicrobial Activity

The antimicrobial activity of the concentrated extract was determined by agar cup assay method against the test organisms. The residue obtained by the evaporation was weighed and dissolved in 2ml 0.1 M phosphate buffer at P^{H} 7. Then 100 ml of it was loaded into the cup (bore) on the MHA plate which was already swabbed with *S. aureus* (0.5 McFarland turbidity standards). The plate was incubated at 37^{o}C for 24 hours. The diameter of zone of complete inhibition was measured and noted.

3.3.9 Determination of Minimum Inhibitory Concentration

The minimum inhibitory concentration of the metabolites was determined by macro tube dilution method in nutrient broth against *E. coli.* A set of ten tubes were prepared with 5ml sterile nutrient broth in each. The solution of the extract in phosphate buffer was used to prepare 2 folds serial dilutions. Inoculums of the test organisms were prepared to the turbidity of 0.5 Nephalometer standards. 50 µl of the prepared inoculums was dispensed in each tube. All the tubes were incubated at 37^{o}C for 24 hours. A tube with nutrient broth but without the antimicrobial agent and an uninoculated tube of medium were also incubated. The former one served as a positive growth control and the later as negative growth control. After 24 hours, visual turbidity in each tube was noted and 0.01 ml

from each tube was sub-cultured on nutrient agar medium, followed by incubation at 37^{o}C overnight.

3.3.10 Chromatographic analysis

a. Thin Layer Chromatography

Silica gel plates, 20cm×20cm, 1mm thick, were prepared. They were activated at 80^{0}C for 2 hours. Ten microliters (µl) of the solution of antimicrobial extract in phosphate buffer and a reference antibiotic (Gentamycin) was applied on the plate and the chromatogram was developed using Chloroform: Methanol (10:90) as solvent system. The plates were run in duplicate and one set was used as the reference chromatogram. The obtained spots were visualized in the iodine vapor chamber.

Flow chart for the screening of actinomycetes for antibiosis

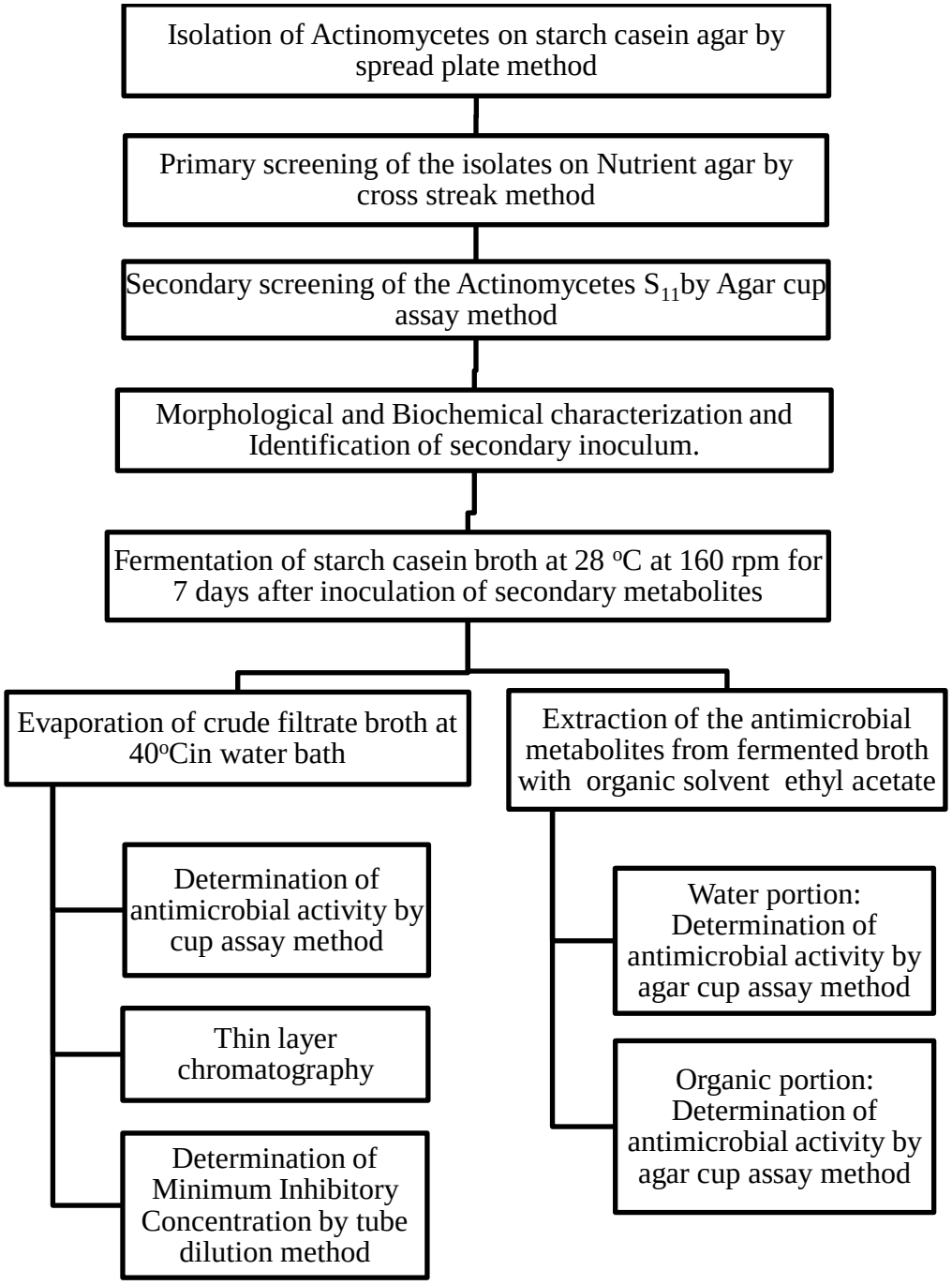


Figure 1: Flow chart for the screening of actinomycetes for antibiosis.

CHAPTER IV

RESULTS

4.1 Screening of Actinomycetes for Antimicrobial activity

4.1.1. Primary Screening of Actinomycetes

Altogether 32 soil samples were collected from Sunsari district and are listed in Appendix D. A total of 24 different actinomycetes were isolated in SCA from 32 soil samples of Sunsari .They were subjected to primary screening against two gram positive (*Bacillus subtilis* and *Staphylococcus aureus*) and the three gram negative bacteria (*Escherichia coli*, *Salmonella Typhi* and *Pseudomonas aeruginosa*) by perpendicular streak method in Nutrient agar.An antibiotic producing actinomycetes coded as S_{11}, S_{13}, S_{20}, S_{26} and S_{29}were isolated. Among them,most potent actinomycete was S_{11}.

Table 1: Number of Actinomycetes isolate from Soil samples.

Total soil Samples	Isolated Actinomycetes	Antibiotic Producing Actinomycetes	Potent Actinomycetes
32	24	5	1

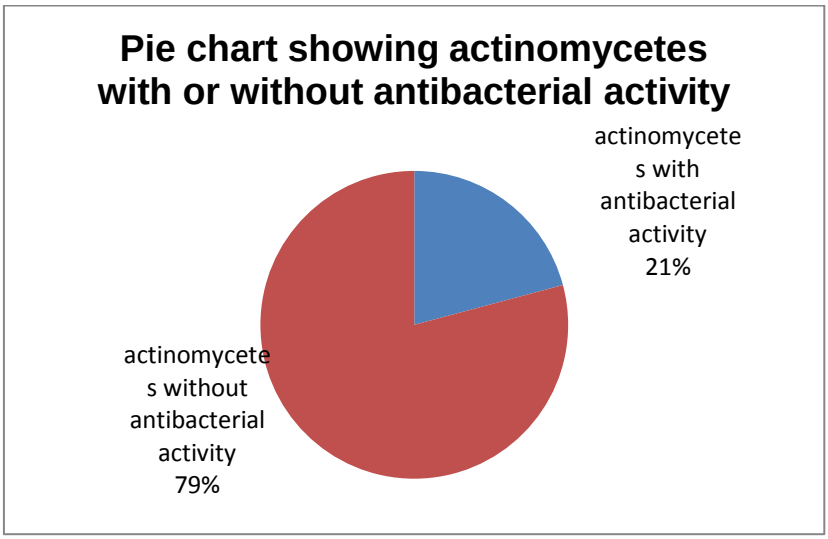
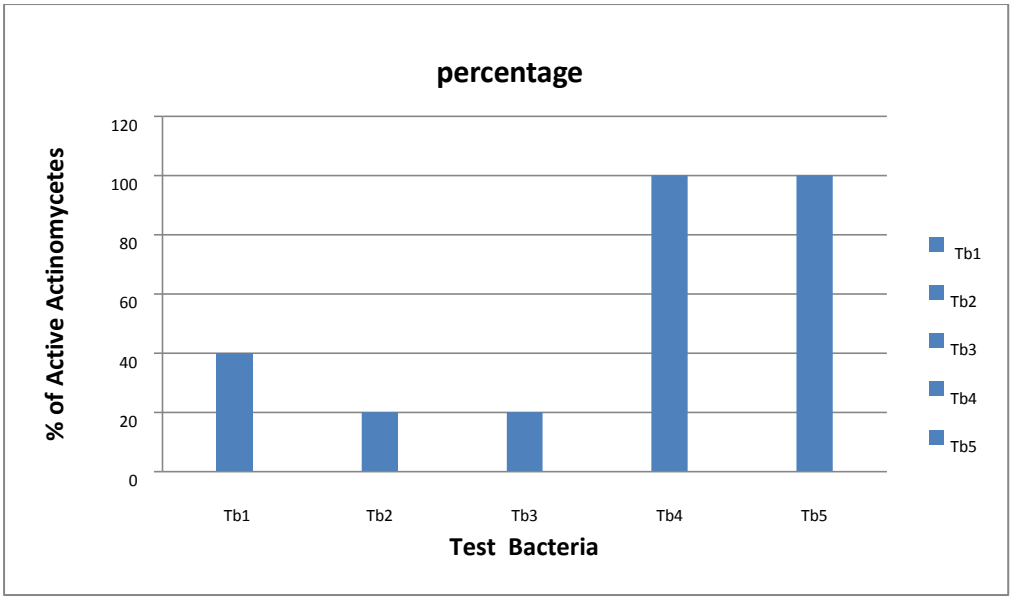


Fig 2 : Pie chart showing actinomycetes with or without antibacterial activity

Out of 24 actinomycetes isolates, only 5 (20.83%) showed antibacterial activity against test bacteria (Fig 2).



Tb1, *E.coli*; Tb2, *S.Typhi*;Tb3, *Pseudomonas* spp; Tb4, *S.aureus*;Tb5, *Bacillus subtilis.*

Fig 3 : Antibacterial activity shown by Actinomycetes isolates in primary Screening.

Out of the total active actinomycetes 2 (40%) showed activity *E. coli*, 1 (20%) showed activity against *Salmonella Typhi* , 1 (20%) showed activity against *Pseudomonas spp*, 3(60%) showed activity against *Staphylococcus aureus* and 5 (100%) showed activity against *Bacillus subtilis* (Fig 3).

Table 2: Zone of inhibition of active isolates in primary Screening

S.N.	Isolate code	Zone of inhibition (in mm) against test bacteria				
		Gram negative bacteria			Gram positive bacteria	
		Tb_1	Tb_2	Tb_3	Tb_4	Tb_5
1	S_{11}	9	30	14	26	26
2	S_{13}	0	0	0	16	17
3	S_{20}	0	0	0	18	16
4	S_{26}	5	0	0	7	9
5	S_{29}	0	0	0	7	6

Tb_1, *E.coli*; Tb2, S.*Typhi*;Tb_3, *Pseudomonas* spp;Tb_4, *S.aureus*;Tb_5, *B.subtilis*

Among the active isolates, 3(60%) showed activity against Gram positive bacteria and 2(40%) showed activity against both Gram positive and Gram negative bacteria (Table 2).

4.1.2. Secondary Screening of Actinomycetes

Among 5 active isolates, 1(S_{11}) was selected and subjected to submerged culture fermentation in Starch Casein broth. It showed inhibitory action against all test bacteria in Mueller Hinton agar. It was found to have antibacterial activity against both Gram positive and Gram negative bacteria (Table 3).

Table 3 : Zone of inhibition of active isolates in secondary screening

S.N.	Isolate code	Gram negative bacteria			Gram positive bacteria	
		Tb_1	**Tb_2**	**Tb_3**	**Tb_4**	**Tb_5**
1	S_{11}	6	18	9	16	16

Tb_1, *E. coli*; Tb_2, *S. Typhi*; Tb_3, *Pseudomonas spp*; Tb_4, *S. aureus*; Tb_5, *B. subtilis.*

4.2 Characteristics of the active isolates

4.2.1. Macroscopic Characteristics

The active actinomycetes isolates produced substrates mycelium in different shades of three colors i.e. Creamy, Brown and dark brown. Out of 5 isolates, 1(20%) produced brown substrate mycelium, 1(20%) produced dark brown colored substrate mycelium, 3(60%) produced creamy colored substrate mycelium.

The active actinomycetes isolates produced aerial mycelium in white color shade .Out of the 5 isolates, 5 (100%) produced white aerial mycelium.

Two types of textures i.e. powdery and smooth of the aerial mycelium of the Active actinomycetes were observed. The texture of the aerial mycelium powdery in 4(80%) and smooth 1(20%) of the total active isolates.

The colony diameter of the active isolates varied from 1-2 mm with most of them being 1mm in size. The colonies had entire round margin (table 4).

Table 4: Macroscopic characteristics of the active Actinomycetes isolates on SCA

S.N.	isolate code	Macroscopic characteristics		
		Color of substrate mycelium	color and texture of aerial mycelium	Other colony characteristis
1	S_{11}	Creamy	White ,powdery	Colony entire round ,1mm diameter
2	S_{13}	Brown	White ,powdery	Colony entire round ,1mm diameter
3	S_{20}	Dark brown	White ,powdery	Colony entire round ,1mm diameter
4	S_{26}	Creamy	White smooth	Colony entire round ,1mm diameter
5	S_{29}	Creamy	White powdery	Colony entire round ,2mm diameter

4.2.2 Microscopic Characteristicss

Microscopic characteristics was done by cover-slip method .The mycelium and cellular morphology revealed that all the active isolates were presumable identified as *Streptomyces spp.* Among them, 3(60%) had rectiflexible Sphorophore morphology and 2 (40%) had rectiflexible Sphorphore morphology mycelium unfragmented (Table 5).

Table 5 : Microscopic characteristics of the active Actinomycetes isolates (1000 X)

S.N.	isolate code	Microscopic characteristics	Presumptive genera
1	S_{11}	Mycelium unfragmented sporphore morphology rectiflexible	*Streptomyces*
2	S_{13}	Mycelium unfragmented sporphore morphology rectiflexible	*Streptomyces*
3	S_{20}	Sphorophore morphology:rectiflexible	*Streptomyces*
4	S_{26}	Sphorophore morphology:rectiflexible	*Streptomyces*
5	S_{29}	Sphorophore morphology:rectiflexible	*Streptomyces*

4.2.3 Biochemical and physiological characteristics

4.2.3.1Carbohydrate utilization tests

The active actinomycetes isolates were tested for their ability to utilize 6 different carbohydrates namely Mannose, Fructose, Maltose, Sucrose, Lactose and Mannitol were utilized by 1(20%), 4(80%), 0(0%), 1(20%) respectively (Table 6).

Table 6: Carbohydrate utilization tests

S.N	Isolate code	Presumptive general	Man	Fru	Mal	Sucro	Lac	Mannitol
1	S_{11}	*Streptomyces*	-	+	-	+	-	+
2	S_{13}	*Streptomyces*	-	+	-	-	-	-
3	S_{20}	*Streptomyces*	-	+	-	-	-	-
4	S_{26}	*Streptomyces*	+	-	-	-	-	-
5	S_{29}	*Streptomyces*	-	+	-	-	-	-

4.2.3.2 Substrate hydrolysis tests.

The active Actinomycetes isolates were tested for their ability to hydrolyse Urea, tween 20, Starch, Esculin, gelatin. Urea, tween 20, Starch, Esculin, gelatin were hydrolysed by 4(80%), 4(80%), 5(100%), 5(100%) and 5 (100%) of the active actinomycetes respectively.

The potent antibiotic producing isolate S_{11} hydrolysed all five substrates (Table 7).

Table 7: Substrate hydrolysis tests of active isolates

S.N	isolate code	presumptive genera	Hydrolysis tests of				
			Urea	Tween 20	Starch	Esculin	Gelatin
1	S_{11}	*Streptomyces*	+	+	+	+	+
2	S_{13}	*Streptomyces*	+	+	+	+	+
3	S_{20}	*Streptomyces*	+	+	+	+	+
4	S_{26}	*Streptomyces*	+	+	+	+	+
5	S_{29}	*Streptomyces*	-	-	+	+	+

4.2.3.3 Other Biochemical tests

The active isolates were also subjected to various other biochemical tests namely oxidase, catalase H_2S production, citrate utilization, nitrate reduction and indole production. All of the isolates were catalase positive and were oxidase negative, 1(20%) of the active isolate was able to produce H_2S on Sulphide indole motility (SIM) medium. Among 5 isolates, 1 (20%) of the active isolate was able to utilize citrate as the sole sources as carbon and 2 (40%) of the active isolates were able to reduce nitrate. None of the active isolates were able to produce indole (Table 8).

The potent antibiiotic producing isolate S_{11} was catalase positive, H_2S production positive and oxidase, citrate utilization, Nitrate Reduction test were negative.

Table 8: Other biochemical tests of the active isolates

S. N.	Isolate code	Presumptive genera	other biochemical tests				
			Catalase	Oxidase	H_2S production	Citrate utilization	Nitrate reduction
1	S_{11}	*Streptomyces* spp	+	–	+	–	–
2	S_{13}	*Streptomyces* spp	+	–	–	–	+
3	S_{20}	*Streptomyces* spp	+	–	–	+	–
4	S_{26}	*Streptomyces* spp	+	–	–	–	–
5	S_{29}	*Streptomyces* spp	+	–	–	–	+

4.2.3.4 Physiological Tests

Motility test, temperature tolerance test and NaCl tolerance test were performed on all active isolates. All the active isolates were found to be non motile when tested for their motility in SIM medium..Although all the active isolates were able to grow at 15^{o}C and 37^{o}C and non of the active isolates grew at 45^{o}C.

In respect to salt concentration, all the active isolates were able to grow well at 5%, 7% and 10% NaCl.

The potent antibiotic producing isolate S_{11} Showed growth at 15^{o}C, 37^{o}C but did not show growth at 45^{o}C. It was able to tolerate NaCl 5%, 7% and10% concentrations (Table 9).

Table 9 : Physiological tests of the active isolates

S.N	isolate code	Presumptive genera	Physiological tests					
			Temperature tolerance			Nacl tolerance		
			15°C	37°C	45°C	5%	7%	10%
1	S_{11}	*Streptomyces*	+	+	-	+	+	+
2	S_{13}	*Streptomyces*	+	+	-	+	+	+
3	S_{20}	*Streptomyces*	+	+	-	+	+	+
4	S_{26}	*Streptomyces*	+	+	-	+	+	+
5	S_{29}	*Streptomyces*	+	+	-	+	+	+

4.3 Fermentation

The isolate S_{11} was selected for fermentation on the basis of their activity against gram positive and negative and larger zone of inhibition. The antibacterial substances from broth extracted with organic solvent ethyl acetate. Ethyl acetate solvents could extract the potent metabolites in detectable level from the fermented broth.

4.4 Minimum Inhibitory Concentration of active compound

The filtrate of fermented broth was evaporated at 40^{o}C in porcelain basin to dryness. The residue obtained was light brownish white in color and had flaky and greasy consistency. The amount of the residue from the isolate S_{11} was 1 gm per 100 ml of the broth. The residue was dissolved in minimum amount of phosphate buffer and subjected for determination of minimum inhibitory concentrations (MIC) against *E. coli*, *Salmonella Typhi*, *Pseudomonas spp, Staphylococcus aureus* and *Bacillus spp*. The MIC was found to be 1.2 mg/ml for the isolate (Table 10).

Table 10: Minimum inhibitory concentration

Tube number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
concentration of antibiotic (mg/ml)	0.4	0.8	1.2	1.6	2.0	2.4	2.8	3.2	3.6	4.0
E. coli	+	+	+	—	—	—	—	—	—	—
Salmonella spp	+	+	+	—	—	—	—	—	—	—
Staphylococcus aureus	+	+	+	—	—	—	—	—	—	—
Pseudomonus spp	+	+	+	—	—	—	—	—	—	—
Bacillus spp	+	+	+	—	—	—	—	—	—	—

Note : Growth = (+) , No Growth =(—)

4.5 Characteristics of the Antibacterial Substances

The crude extract of the isolate S_{11} was dissolved in phosphate buffer to make concentration of 30 mg/ml.10 µl of each extract along with 10 µl of 1% of

Gentamycin prepared in distilled water, a standard were made to run thin layer chromatography using chloroform: methanol (10:90) as solvent system.

Only one spot was detected from the extract in iodine vapour near the solvent front. The retention factor (R_f) was 0.87 for S_{11} extract (Table 12).

Table 11: Concentration of antibacterial Substances

Isolate code	volume of crude broth (ml)	Temperature of evaporation (^oC)	Time of evaporation (hr)	Amount of residue (mg)	Color of residue	Consistency of residue
S_{11}	100	40	15	300	Light brownish white	Flaky/greasy

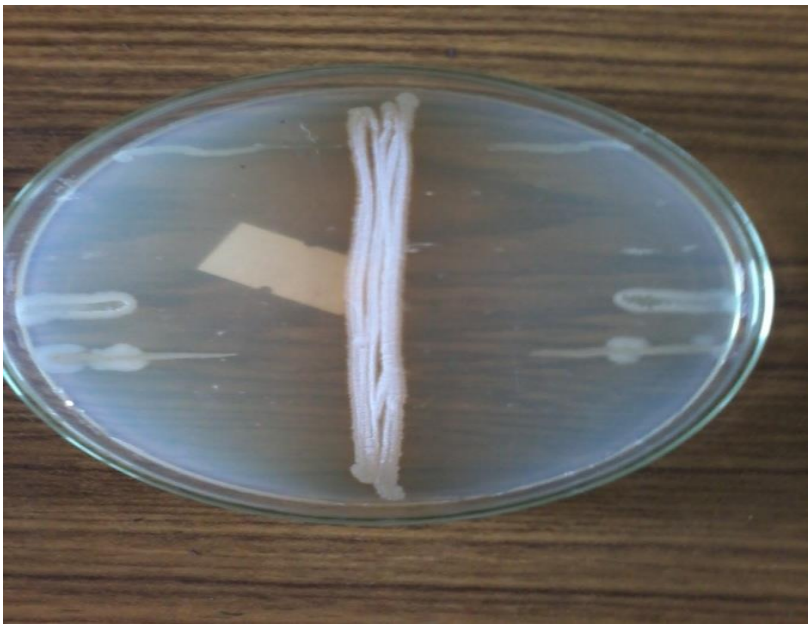
4.6 Thin layer chromotography

The active metabolites were analyzed by thin layer chromatography (TLC) on silica gel using chloroform: methanol (10:90) as solvent system and gentamycin as reference antibiotic. When chromatogram was visualized under iodine vapor, the crude extract produced only one spot, indicating the presence of only one compound

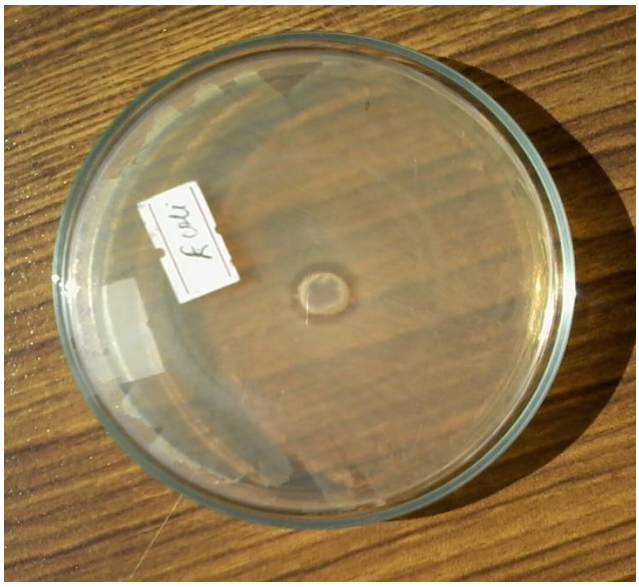
Table 12: Thin layer Chromatography of the antibacterial substances

Anti bacterial Substances (s)	Concentration (mg/ml)	Amount of load (µl)	Solvent System (M:C)	No of moved spot	Distance travelled by		R_f value
					Solvent front	Antibacterial substances	
S_{11}	30	10	90:10	1	9.4	8.2	8.7
Gentamycin	30	10	90:10	1	9.4	8.8	9.4

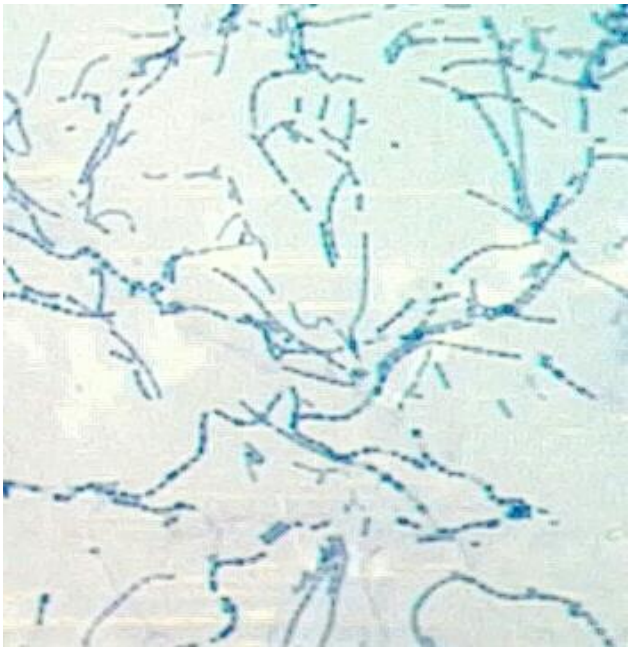
Photograph 1: A view of Sample site of Sunsari.



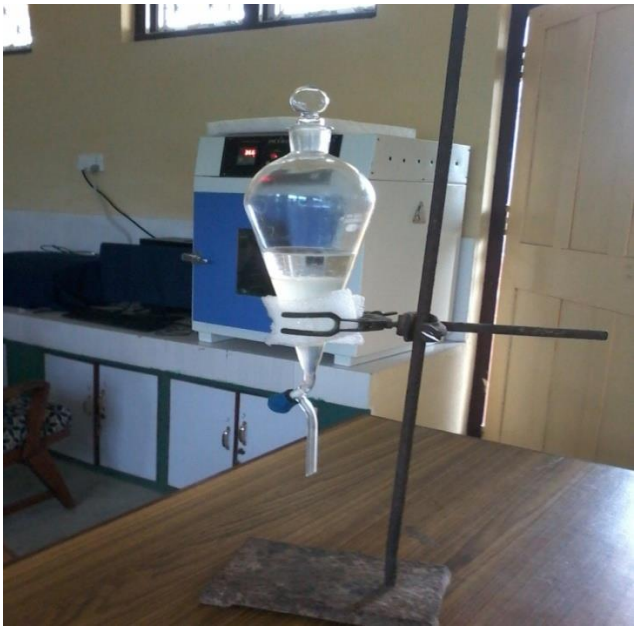
Photograph 2: Primary screening of isolate S_{11} against test bacteria by Perpendicular Streak method. Tb1, *Escherichia coli*; Tb2, *Salmonella* Typhi; Tb3, *Pseudomonas* spp; Tb4, *Staphylococcus aureus*; Tb5, *Bacillus* spp.



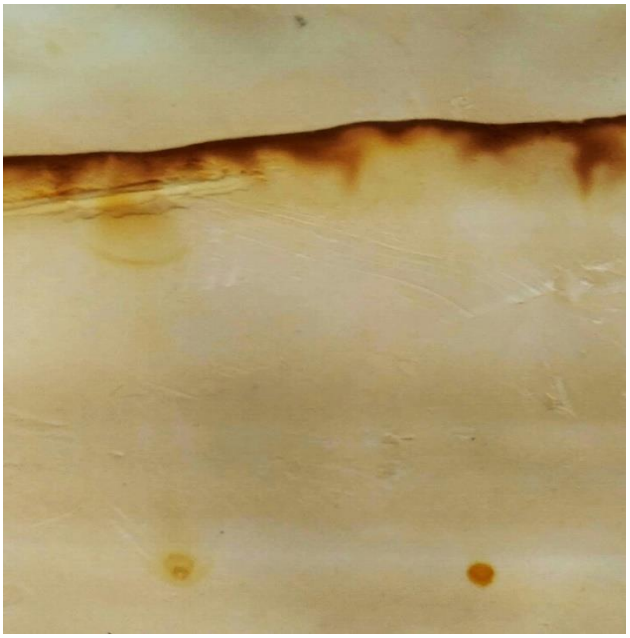
Photograph 3: Secondary screening of active isolate against *Escherichia coli* by agar well assay method



Phtograph 4: Micrograph of Potential actinomycetes isolate S_{11} (Presumptive *Streptomycetes* spp.) (X1000)



Photograph 5: Extraction of antimicrobial metabolites by using organic solvent (ethyl acetate) in a separating funnel



Photograph 6: Thin Layer Chromatography of extracted metabolites

CHAPTERV

DISCUSSION

The actinomycetes are remarkably antibiotic producer, making three quarters of all known products. The *Streptomyces* are especially prolific and can produce great many antibiotics and other class of biologically active secondary metabolites and cover around 80% of total antibiotic products with other genera trailing numerically. *Micromonospora* is the runner up with fewer than one-tenth as many as *Streptomyces*. If we include secondary metabolites with biological activities other than antimicrobial, actinomycetes are still out in front, over 60% *Streptomyces spp* accounting for 80% of these (Hopwood et al 2000).

The actinomycetes produce numerous substances essential for health such as antibiotics (Tamamura et al 1985). During the last few decades, they have become the most fruitful source of antibiotics. In the 60's and the 70's of the twentieth century (75-85%)of the discovered antibiotis were derived from order actinomyceteles, mainly from *Streptomyces spp* (Moncheva et al 2002).

During past 40 years, the isolation and subsequent screening of microbes form diverse habitats had lead to discovery of many novel and useful secondary metabolites (Williams, 1978) and this effort has not been exhausted till now since the investigators have been frequently rewarded with them, discovery of new antibiotics from these organism and even sometime new and rare species of actinomycetes. Due to large geographic variation, there is large variation in soil type and their contents in Nepal and hence it is quite likely that the distribution of antibiotic producing actinomycetes is also variable.

Nepal has a large geographical variation and hence it is quite likely that with its vast biodiversity an opportunity to investigate its diverse biota and utilize them for the benefit of mankind and ultimately for its own economic uplift exists. Soil is one of the natural habitats of most of the actinomycetes. It has large surface area that supports in their growth cycle of Spore-mycelium - spore. They are also adapted to the physical condition of this habitat, such as

moisture and aeration, which may undergo dramatic change with a short time according to weather and season. Therefore, like in many other studies on actinomycetes, this study employed soils as the source of potentially beneficial Actinomycetes. The soils of Sunsari districts were analysed as potential sources of prospective novel actinomycetes and hence novel antibiotics.

A good number of actinomycetes were isolated from the soil of Sunsari districts. The ability of actinomycetes to survive in this harsh and challenging habitat may be due to their adaptation to such environment and their abillity to produce resistant structures like spores. Though their spores are not as resistant towards unfavourable condition as bacterial endospores, they certainly contribute to their survival over long period of drought and anaerobic condition produced by water saturation .

Isolation of actinomycetes has always been faced with difficulties in comparison to their competitors namely other bateria and fungi (Williams and Cross1971). An effort was made to isolate increased ratio of Actinomycetes /microorganism in a soil sample by combining chemical ($CaCO_3$) pre-treatment in starch casein media.

Actinomycetes generally take one to two weeks to develop matured aerial mycelium with spores but the shape, size and the color of these actinomycetes colonies were different from that of other ordinary ones,in the sense that they were very small (0.4-0.6mm) in diameter, round shaped with hard texture and different color substrate mycelium with different aerial spores. The isolates which did not shows any antibacterial activity ,and hence they were not analysed further.

In screening cultures for antibiotic activity ,it is desirable to have methods that are rapid and sensitive. Both the primary and secondary steps were used to screen actinomycetes for antibiotic production. The first screening was used (i) to determine what cultures were active. (ii) to get information concerning the group of organisms against which the cultures were active. The secondary screening method was used mainly to select cultures for further studies and since various media were used, information was obtained concerning media requirements for antibiotic production.

Primary screening of the actinomycetes was carried out in nutrient agar by perpendicular streak/cross streak method (Egorov, 1985). The previously used crowded plate screening method allows primary screening diectly from the dilution plate, since the colony around which zone of inhibition was noted would be considered active and selected. The drawback of this technique is that the activity is against unknown organism. The use of perpendicular streak method, an improved technique, allows numerous test organisms to be screened on the sample plate where tested actinomycetes are grown. Cross streak method is useful for qualitative screning in determining the activity of isolate against specific test organisms (Haque et al 1992).The active isolates were subjected for secondary screening to test the capabilities of the primarily screened organisms in liquid medium. Secondary screening may be qualitative or quantitative in its approach. The qualitative approach is used to determine the range of the microorganisms that are sensitive to a potential newly discovered antibiotic. The quantitative approach provides the information about the yield of the antibiotic that can be expected when the organism is grown in different media (Casida,1959). Secondary screening should yield the type of information which is needed in order to evaluate the true potential of a micro organism.

The antibiotic producing actinomycetes from soil of Sunsari district was isolated and confirmed by primary screening and also confirmed by secondary screening. Secondary screening needs fermentation process for production of antibiotic. Fermentation and Identification of actinomycetes are going on in the laboratory of Central Campus of Technology, Dharan. According to result of microscopy given above, the antibiotic producing isolate might be *Streptomyces* sp.

Our result showed that Total twenty four different types of actinomycetes were isolated from the thirty two soil samples of Sunsari but out of them, only one showed active antimicrobial activities against both gram positive and gram negative bacteria.

The survival of the microorganisms in such harsh and challenging habitation might be due to their adaptation to the environment and ability to produce resistant structure like spores.

Isolation of actinomycetes has always been faced with difficulties in comparison to their competitors like other bacteria and fungi (Williams & Cross 1971). This may be due to their long incubation period. However, an effort was made to isolate increased ratio of actinomycetes isolation by pretreatment of the samples by calcium carbonate and subjecting them into air dry for three weeks. Use of starch casein agar (selective media) incorporation with antibiotics, nystatin (50µg/ml) and cycloheximide (50µg/ml) was good to prevent the growth of contaminating microorganisms like other bacteria and fungi. .

For antibacterial activity, both the primary and secondary screening methods were used to screen actinomycetes. The first screening was used to select the antibacterial isolates and determine the range of microorganisms that were sensitive to the antibiotic. The secondary screening method was crucial to select the isolates for further studies. The screening may be qualitative or quantitative in its approach. The qualitative approach is used to determine the range of the microorganisms that are sensitive to a potential antibiotic while the quantitative approach provides the information about the yield of antibiotic that can be expected when the organism is grown in different media (Gurung et al 2009).

The result of the screening revealed that the isolate were active against gram positive bacteria than Gram negative bacteria. This might be due to differences in their cell wall composition between those two types of microorganisms. gram negative bacteria have an outer lipopolysaccharide membrane; hence their cell wall is impermeable to lipophilic solutes, while porins constitute a selective barrier to the hydrophilic solutes (Nokaido and Vaara 1985). The Gram positive bacteria have only peptidoglycan layer which is not an effective permeability barrier for the antibacterial agents.

According to the result of primary and secondary screening, isolate S_{11} was found to be best strains as they showed big zone of inhibition against both gram positive and gram negative bacteria, it was chosen for fermentation to produce antibiotic.

The antimicrobial metabolites from fermented broth were tried to extract in organic solvent ethyl acetate by solvent extraction method. As, ethyl acetate

solvent could extract the potent metabolites in detectable level from the fermented broth. This might be because of the highest solubility of the metabolites in ethyl acetate solvent rather than others.

The minimum inhibitory concentration (MIC) for antibacterial metabolite extracted from S_{11} was 1.2mg/ml. Since this metabolite was obtained by the evaporation of ethyl acetate solvent, it is likely to have this high MIC value. The extract was analyzed by thin layer chromatography (TLC) on silica gel using chloroform: methanol (10:90) as solvent system and Gentamycin as reference antibiotic. When chromatogram was visualized under iodine vapor, the extract produced only one spot, indicating the presence of one compounds. The spot was at near solvent front with R_f value of 0.87 for the extract S_{11}. Similarly, Gentamycin produced one spot with R_f value 0.94. Similar findings of results were reported by Gurung et al (2009) where the $R_f$value was 0.88.

The microscopic observation of the actinomycetes was determined as according to Bergey's Manual of Determinative Bacteriology (1974).The active isolates was identified as *Streptomyces* species.

The soils of Sunsari were found to contain actinomycetes that produce antibacterial substances. Most potent actinomycetes isolate like *Streptomyces* sp (S_{11}) which showed presence antibacterial property against both gram positive and gram negative bacteria were isolated from soils of Sunsari.. Hence the present study clearly reveals the distribution of antibiotic producing actinomycetes in the Sunsari district of Nepal.

CHAPTER VI

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

The soils of Sunsari were found to contain *Streptomyces* genera that produce antibacterial substances. Hence, this study clearly reveals the distribution of antibiotic producing actinomycetes in soils of the Sunsari district of Nepal.

6.2 Recommendation

1. As Nepal has wide biodiversity, possibility of finding novel actinomycetes in different regions is great and should be exploited more.
2. Optimization of the culture media and growth conditions for the isolates should be done so as to minimize the incubation period of the isolates.
3. Optimization of parameters of fermentation should be done to enhance the production of antibiotic from the actinomycetes.
4. The broad spectrum antibiotics of actinomycetes isolated from such an extreme habitat may be novel and efficient to counterfeit the emergence of multidrug resistant pathogens. Hence isolation, purification, structure determination and identification of the antibiotic compounds should be done.
5. The whole research input can be applied to the commercial production of the antibiotics which can strengthen economy of the country.

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APPENDIX A

Materials requires

Media and chemicals

Sulphite indole Motility media	Merch , Germany
Urea agar base	Merch , Germany
Simmon's citrate agar	Hi Media, India
Mueller Hinton agar	Hi Media, India

Chemicals

Agar powder	Hi Media, India
Alpha napthalamine	Bengal chemicals, India
Barium chloride	Merck , India
Beef extract	Qualigens, India
Calcium carbonate	S.D.fine –chem Limited, India
casein	Merck, Germany
chloroform	Qualigens, India
Crystal violet	Lobo chemicals, India
Dehydrated alcohol	Bengals chemical and Pharma.Ltd.
Dextrose	Qualigens, India
Dichloromethane	Qualigens, India
Dipotassium hydrogen phosphate	Qualigens India
Esculin	Qualigens India
Ferric citrate	Labo-chemie indoaustranal Co.
Ferrous sulphate	S.D.fine chem.Limited, India
Fructose	Labo-chemie indoaustranal Co.
Fructose	Qualigens, India
D(+)Galactose	Qualigens, India
Gelatin	Hi media, India
D(+) Glucose	Labo-chemie indoaustranal Co.

Glycerol	Qualigens, India
Glacial acetic acid	Qualigens, India
Hydrochloric acid	Qualigens, India
Hydrogen peroxide	Qualigens, India
Iodine	Qualigens, India
Lactose	Hi media, India
Magnesium sulphate	Labo-chemie indoaustranal Co.
Magnesium chloride	S.D.fine chem.limited. India
Maltose	Merck, Germany
Mannitol	Hi media, India
Mannose	Hi media , India
Mercuric chloride	Merck, Germany
Methanol	Qualigens, India
Peptone	Qualigens,India
Phenol Red	Qualigens, India
Potassium iodide	Qualigens, India
Potassium dihydrogen phoshate	Qualigens, India
Phenol	Qualigens, India
Salicin	Hi media, India
Sodium chloride	S.D.fine chemi,limited India
Sodium hydroxide	S.D.fine chemi,limited India
Silica	Hi media, India
Starch	Hi media, India
Sucrose	Qualigens, India
Sulphanilic acid	S.D.fine chem,limited, India
Sulphuric acid	Qualigens, India
Tween 20	S.D.fine chem,limited, India
Urea crystal	Merck, Germany
Xylose	Hi media, India

Zinc powder	S.D.fine chem,limited , India

Equipments

Autoclave	life,India
Cooling centrifuge	Remi
Deep freeze	Mirage
Electronic weighting machine	Mettler AJI00C3419 Model
Glasswares	Pyrex and Borax
Hot water bath	NSW, India
Incubator	NSW, India
Microfuge tubes	Genei, India
Microscope	Olympus
Micropipette	Genei, India
Micropipette tips	Vinay Trading Co.India
PH meter	Universal India
Refrigerator	Samsung
Water bath shaker	NSW, India

Test organisms

B.subtilis

E.coli. ATCC 25922

S.Typhi

Pseudomonas spp.

S.aureus

APPENDIX B

Composition of Media

Starch caesin agar

Ingredients		**gm/ml**
Soluble starch	:	10
Casein	:	0.3
Potassium nitrate	:	2
Di-potassium hydrogen orthophosphate	:	2
Magnesium sulphate	:	0.05
Calcium carbonate	:	0.05
Ferrous sulphate	:	0.01
Agar-agar	:	20
Final pH	:	7.2

Nutrients agar

ingredients		**gm/ml**
peptone	:	5
Sodium chloride	:	5
beef extract	:	1.5
Yeast extract	:	1.5
agar	:	15
Final p^H	:	7.2

Basal medium for carbohydrate utilization tests

Ingredients		**gm/l**
peptone	:	10
Sodium chloride	:	5
Phenol red	:	0.018
Final pH	:	7.4

Simmons citrate agar

Ingredients		**gm/l**
Ammonium dihydrogen phosphate	:	1
Dipotassium hydrogen phosphate	:	1
Sodium chloride	:	5
Sodium citrate	:	2
Magnesium sulphate	:	0.2
Bromothymol Blue	:	0.08
Agar	:	15
Final P^H	:	6.9

SIM(Sulphide Indole Motility)Agar

Ingredients		**gm/l**
Peptone	:	30
Beef extract	:	3
Ferrous Ammonium sulphate	:	0.2
Sodium thiosulphate	:	0.025
Agar	:	3
Final P^H	:	7.3

Urea Agar Base

Ingredients		**gm/ml**
peptone	:	1
Dextrose	:	1
Nacl	:	5
KH_2PO4	:	0.012
Phenol red	:	12
Agar	:	12
Final pH	:	6.8

Note:50 ml of sterilized 40% urea solution was added to one liter of autoclaved and cooled (45-55^0 C) urea agar base to prepare urea agar.

Tween 20 Agar

Ingredients		**gm/ml**
Nutrients agar	:	28
Tween 20	:	10
Calcium chloride	:	50mM
Final P^H	:	5.8

Esculin Agar

Ingredients		**gm/l**
Beef extract	:	3
Peptone	:	5
Esculin	:	1
Ferric citrate	:	0.5
Agar	:	15
Final pH	:	6.6±0.2

Starch Agar

Ingredients		**gm/l**
Nutrients Agar	:	28
Starch	:	10
Final P^H	:	5.8

Gelatin Agar

Ingredients		**gm/l**
Nutrient Agar	:	28
Gelatin	:	10
Final pH	:	5.8

Nitrate Broth

Ingredients		**gm/l**
Beef extracts	:	3
peptone	:	5
Potassium nitrate	:	1

Final P^H	:	(6.8 ±0.2)

Mueller Hinton Agar

Ingredients		gm/l
Beef infusion	:	300ml
Starch	:	1.5
Casein hydrolysate	:	17.5
Agar	:	10

Starch casein broth

Ingredients		gm/l
Soluble Starch	:	10
Casein	:	0.3
Potassium nitrate	:	2
Di potassium hydrogen Orthophosphate	:	2
Magnesium sulphate	:	0.05
Calcium carbonate	:	0.02
Ferrous Sulphate	:	0.01
Agar-Agar	:	20
Final pH	:	7.2

Nutrient broth

Ingredients		gm/l
Peptone	:	5
Sodium chloride	:	5
Beef extract	:	1.5
Yeast extract	:	1.5
Final pH	:	7.2

APPENDIX C

Methodology of biochemical test for the identification of Actinomycetes

A. Catalase test

This test was done by picking up a few colonies with a sterile glass rod and mixing it with a drop of 3% H_2O_2 on a clean glass slide. Positive test was indicated by the appearance of gas bubbles.

B. Oxidase test

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

C. Carbohydrate Utilization test

For this test, the basal medium containing peptone, sodium chloride and phenol red was incorporated with carbohydrate at the concentration of 1% (w/v). Carbohydrates used were glucose, fructose, maltose, mannitol, lactose, and sucrose. Each carbohydrate stock solution was heated for 30 minutes at 60^{o}C before adding appropriate volume to the already autoclaved basal medium. Then, the carbohydrate containing basal medium was inoculated with the actinomycetes colonies and incubated at 28^{o}C for 2 weeks. Positive test was indicated by the change in color of the medium from red to yellow.

D. Citrate utilization test

This test was done by streaking the slant of Simmon's Citrate Agar tubes with the actinomycetes colonies and incubating the tubes at 28^{o}C for 2 weeks. Citrate utilization was detected by change of color of medium from dark green to Prussian blue.

E. Indole and Hydrogen Sulphide Production Test

These two tests were done by stabbing Sulphide indole motility (SIM) medium tube with the actinomycetes colonies and incubating the tubes at 28^{o}C for 2-4 weeks.

Indole production was detected by the production of cherry red color at the interface upon the addition of Kovac's reagent. H_2S production was detected by blackening of the medium.

F. Nitrate Reduction Test

Nitrate broth (Appendix B) was inoculated with the actinomycetes colonies incubated at 28^{o}C for 2-4 weeks. Nitrate reduction was detected by adding a few drops of sulphanilic acid reagent and alpha-napthylamine reagent into the culture broth. Development of red or pink was proof of the presence of nitrate (positive reaction). In the absence of a positive reaction, 4-5mg of zinc dust was added to the tube previously tested for nitrite. The presence of nitrate (Negative reaction) was demonstrated by the development of a red color.

G. Urea Hydrolysis Test

Urea agar slants was streaked with the actinomycetes colonies and incubated at 28^{o}C for 2-4 weeks. Positive test was indicated by the change of the color of the slant from orange to Pink.

H. Tween 20 Hydrolysis Test

The isolates was streaked on solidified tween 20 agar plates and incubated at 28^{o}C for 2-4 weeks. Positive test was indicated by the appearance of clear zone around the colony.

I. Starch Hydrolysis Test

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

J. Esculin Hydrolysis Test

Esculin agar slants were inoculated with the isolates and incubated at 28^{o}C from 2-4 weeks. Hydrolysis was detected by appearance of zone of hydrolysis around the colonies upon flooding the plate with mercuric

K. Temperature Tolerance Test

Nutrient agar plates were inoculated with the isolates and incubated at 15^{o}C, 37^{o}C, and 45^{o}C for 2 weeks. Positive test was indicated by growth of the isolates.

L. NaCl Tolerance Test

Nutrient agar plates with 5%, 7% and 10% NaCl were inoculated with the isolates and incubated at 28^{o}C for 2 weeks. Positive test was indicated by growth of the isolates.

M. Motility Test

This test was done by stabbing sulphide indole motility (SIM) medium (Appendix B) tube with the actinomycetes colonies and incubating the tubes at 28^{o}C for 2-4 weeks. Motility was detected by growth of actinomycetes away from the stab line on the medium.

APPENDIX - D

Places of soil samples site

Sample code	Sample site	Isolated Actinomycetes	Antibiotic producing actinomycetes	Potent Actinomycetes
S_1	Itahari 3	—	—	—
S_2	Itahari 5	+	—	—
S_3	Itahari 8	+	—	—
S_4	Itahari 10	+	—	—
S_5	Itahari 15	—	—	—
S_6	Dharan15	+	—	—
S_7	Dharan 16	+	—	—
S_8	Dharan 17	+	—	—
S_9	Dharan 13	—	—	—
S_{10}	Dharan 11	—	—	—
S_{11}	Dharan 14	+	+	+
S_{12}	Inaruwa	+	—	—
S_{13}	Madhesa	+	+	—
S_{14}	Dumraha	+	—	—
S_{15}	Duhabi	+	—	—
S_{16}	Bhaluwa	—	—	—
S_{17}	Sonapur	+	—	—
S_{18}	Simariya	+	—	—
S_{19}	Tanbuna	+	—	—
S_{20}	Ramdhuni	+	+	—
S_{21}	Baklauri	+	—	—
S_{22}	Baraha kshhetra	+	—	—
S_{23}	Prakashpur	+	—	—
S_{24}	Madhalii	—	—	—
S_{25}	Auraboni	+	—	—
S_{26}	chimadi 2	+	+	—
S_{27}	Jhumka	+	—	—
S_{28}	Gadhi	—	—	—
S_{29}	Babhia	+	+	—
S_{30}	Chadbela	—	—	—
S_{31}	East-kusha	+	—	—
S_{32}	Madhuban	+	—	—

Note :(+) = Present, (—) = Absent.

APPENDIX - E

Procedure of 0.5 Mc Farland Nephalometer standard

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

2. A solution of 1% sulphuric acid (H_2SO_4) was also prepared.

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

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