CHAPTER-I

1. INTRODUCTION

After the discovery of penicillin in 1929 by Alexander Fleming and its isolation in 1940 by Florey and Chain, the science of antibiotics developed rapidly which led to intense search for more antibiotics substances produced by various microorganisms. They are bacteria, fungi and actinomycetes (Hussar and Holley, 1954).

The persistent search was crowned by the discovery of many new antibiotic producing microorganisms. Within few decades this branch of science developed so rapidly that more than 6500 antibiotics have been characterized so far. However, only about 100 of them are used therapeutically. Most antibiotics are not used in medicine because of their toxicity and instability in human body (Egorov, 1985).

The primary source of these antibiotics is bacteria, *Streptomyces* spp., *Nocardia* spp. and fungi with several other classes of microorganisms contributing to the lesser extent (Casida, 1968). No doubt soil is the natural habitat of most of the microorganisms where vast array of bacteria, actinomycetes, fungi and other organisms exist and provided with suitable growth condition and ability to proliferate. Thus most actinomycetes contributing to antibiotic production are screened from soil (Williams and Khan, 1974).

Actinomycetes were originally considered to be an intermediate group between bacteria and fungi but are now recognized as prokaryotic. The gram-positive bacteria include two major branches: the low G+C organisms, containing genera such as *Bacillus*, *Clostridium, Staphylococcus* and *Streptococcus*; and the high G+C (> 55%) organisms referred to as the actinomycetes. The name "Actinomycete" derives from the Greek *aktis* (a ray beam) and *mykes* (fungus) and was given to these organisms from initial observations of their morphology.

The majority of the actinomycetes are free living, saprophytic bacteria found widely distributed in soil, water and colonizing plants. Actinomycetes population has been identified as one of the major group of soil population (Kuster, 1968), which may vary

with the soil type. Actinomycetes can compose 10-33% of the bacteria in the soil (Waksman, 1961). The genera *Streptomyces* and *Nocardia* are the most abundant. *Micromonospora*, *Actinomyces* and many other actinomycetes are indigenous to soils but generally are present in low numbers. These organisms participate in the turn over of the soil components, especially in the transformation of organic compounds (Huntzens, 1972; Konova, 1966; Kuster, 1967; Kuzner, 1968). In soil they are involved in the decomposition and mineralization cycles with the production of extracellular enzymes, such as cellulases, chitinases, and lignin peroxidases. Since they can decompose complex mixtures of polymers in dead plant, animal and fungal material (Mc Carthy, 1987; Crawford, 1988), they have important role in soil biodegradation by recycling of nutrients associated with recalcitrant polymers (McCarthy and Williams, 1992).

The actinomycetes are noteworthy as antibiotic producers, making three quarters of all known products; the *Streptomyces* are especially prolific and can produce a great many antibiotics and other class of biologically active secondary metabolites and cover around 80% of the total antibiotic product, with other genera trailing numerically: *Micromonospora* is the runner-up with fewer than one-tenth as many as *Streptomyces* (Hopwood *et al*., 2000).

Currently, microbes can be recognized at the DNA or RNA level without the cultivation of pure cultures on selective media. Relying on these techniques, one can also determine the composition of a natural microbial population and interactions among members of this population in time and space. Nevertheless, to microbiologists, the isolation and characterization of pure cultures of some microbial species are as important as understanding their objective existence in natural ecosystem. The isolation of diverse and novel pure cultures of actinomycetes also provides a theoretical guide for the exploitation and utilization of actinomycetes resources. One of the aims of biodiversity studies of actinomycetes is to use effective isolation procedure to study the distribution of actinomycetes in various climatic and ecological environments (Hayakawa *et al*., 1988).

Choice of natural materials such as soils may be based on the assumption that samples from widely different locations are more likely to yield novel isolates and therefore

hopefully, novel metabolites as a result of the geographical variation (Sen *et al*., 1993). In Nepal, Actinomycetes were first isolated from the soils of Kathmandu valley by Tamrakar (1998) and Singh (1999) isolated Actinomycetes from the soil of 'Khumbu' region. Similarly, Pandey (2004) also reported the antibacterial Actinomycetes from 'Khumbu' region.

Considering the prevalence of antimicrobial Actinomycetes isolated from 'Khumbu' region, this study was also carried out to reveal more antibiotic producing Actinomycetes from the soil sample of that region and also to test their abilities to control their common plant pathogenic fungi and some bacterial species.

CHAPTER II

2. OBJECTIVES

2.1 General objective

The General objective of this study is to study the diversity and bioactivity of soil inhabiting actinomycetes of higher altitude of Nepal collected from 'Khumbu' region, base camp of Mount Everest.

2.2 Specific objectives

- To study the antibacterial activity of the identified isolates.
- To study the antifungal activity of the identified isolates against some common fungal plant pathogens.
- To carry out the fermentation of the priority isolates for extraction of secondary metabolite out of the culture.
- To screen and evaluate the antibacterial activity of the extract.
- To screen and evaluate the antifungal activity of the extract against the common fungal plant pathogens of Nepal.
- To analyse the extract by thin layer chromatography.

CHAPTER III

3. LITERATURE REVIEW

3.1 Antibiotics

Antibiotics (Greek anti, “against”, bios, “life”) are chemical substances elaborated by various species of microorganisms, such as fungi, actinomycetes and bacteria; they suppress the growth of other microorganisms and may ultimately destroy them in low concentrations (Satoskar *et al.*, 1999). The most important concept underlying anti-microbial therapy is selective toxicity, i.e. selective inhibition of the growth of the microorganism without damage to the host. Selective toxicity is achieved by exploiting the differences between the metabolism and structure of the microorganism and the corresponding features of human cells. For example, penicillins and cephalosporins are effective antibacterial agent because they prevent the synthesis of peptidoglycan, thereby inhibiting the growth of bacterial but not human cells (Levinson, 1998).

3.1.1 Classification of antibiotics

The Classification of antibiotics according to both theoretical and practical point of view is highly important. Antibiotics can be classified in several ways. The most common method classifies them according to their chemical structure as antibiotics sharing the same or similar chemical structure will generally show similar patterns of antibacterial activity, effectiveness, toxicity and allergic potential.

Table 1: Classification of Antibiotics according to their chemical structure and mechanism of action.

Class (chemical structure)	Mechanism of action	Examples
B-lactam antibiotics ⁾ Penicillins ⁾ Cephalosporins ⁾ Carbapenems	Inhibit bacterial cell wall synthesis	Penicillins ⁾ Penicillin G ⁾ Amoxicillin ⁾ Flucloxacillin Cephalosporins ⁾ Cefoxitin ⁾ Cefotaxime ⁾ Ceftriaxone Carbapenem ⁾ Imipenem
Macrolides	Inhibit bacterial protein synthesis	⁾ Erythromycin ⁾ Azithromycin ⁾ Clarithromycin
Tetracyclines	Inhibit bacterial protein synthesis	⁾ Tetracycline ⁾ Minocycline ⁾ Doxycycline ⁾ Lymecycline
Fluoroquinolones	Inhibit bacterial DNA synthesis	⁾ Norfloxacin ⁾ Ciprofloxacin ⁾ Enoxacin ⁾ Ofloxacin
Sulphonamides	Blocks bacterial cell metabolism by inhibiting enzymes	⁾ Co-trimoxazole ⁾ Trimethoprim
Aminoglycosides	Inhibit bacterial protein synthesis	⁾ Gentamicin ⁾ Amikacin
Imidazoles	Inhibit bacterial DNA synthesis	⁾ Metronidazole
Peptides	Inhibit bacterial cell wall synthesis	⁾ Bacitracin
Lincosamides	Inhibit bacterial protein synthesis	⁾ Clindamycin ⁾ Lincomycin

(Source: Ngan, 2008)

3.1.2 Sources of Antibiotics

The main source of antibiotics is found to be microorganisms although few synthetic antibiotics are known to be produced. Besides, semi-synthetic antibiotics are also widely used, in which, part of the molecules is produced by a fermentation process using appropriate microorganisms and the product is then modified by chemical process (Hugo and Russel, 1983). Antibiotics from biological sources are also of two types: antibiotics from microorganisms and antibiotics from higher form of life.

Programmes aimed at the discovery of antibiotics and other bioactive metabolites from microbial sources have yielded an impressive number of compounds over the past 50 years, many of which have found applications in human medicine and agriculture (Busti *et al.*, 2006). However, it soon became apparent during screening programmes that some microbial metabolites were discovered more frequently than others. As the number of described microbial metabolites increased, so did the probability of rediscovering known compounds. At the present time, with several thousands of described microbial metabolites, strategies must be introduced into screening programmes to increase the chances of discovering novel compounds (Bull *et al.*, 2000; Lancini *et al.*, 1995).

Currently about 10,000 antibiotics have been discovered from microorganisms. Two-thirds of these are produced by actinomycetes (mainly those belonging to the genus *Streptomyces*). Considering the practically useful compounds, today about 130 to140 microbial products and a similar number of derivatives (including semi-synthetic antibiotics) are applied in human medicine, mostly in chemotherapy and veterinary medicine. Furthermore, some of 15 to 20 compounds are used in agriculture mainly as pesticides, plant protecting agents and food additives. The majority of these compounds except fungal penicillins, cephalosporins and several bacterial peptides and few others, are also produced by actinomycetes. The high percentage of new compounds derived from new target oriented screening method is also of actinomycetal origin (Moncheva *et al*., 2002).

Bacteria such as *Bacillus* species and *Pseudomonas* species are also known to produce antibiotics. The antibiotics such as colistin, polymyxins B and M, gramicidin, gramicidin S, tyrothricin, bacitracin and atterimin are produced by *Bacillus* species; Pyocyanin and pyrrolnitrin from *Pseudomonas* species; and finally nisin from other eubacterials.

Large group of fungi are responsible for the production of antibiotics and it was found that nearly 1150 of them are produced (Egorov, 1985). Primarily *Penicillium* and *Aspergillus*, belonging to the Aspergillaceae family and some fungi imperfecti species are of greater practical value with respect to antibiotic production. The antibiotics of fungal origin that have been commercialized are penicillin G, penicillin O, cephalosporin, griseofulvin, fumagillin, fusidic acid etc. A wide variety of fungi are able to produce the same individual antibiotic type. The occurrence of cephemycins, helvolic acid, as well as that of penicillin among metabolites of streptomycetales, emphasize that fact even more.

A number of antibiotics and antitumor agents were found from higher form of life. Antibacterial agents of plant origin have long been well known (phytoncides); however, they never gained practical introduction except for the antitumor Vinca alkaloids. On the other hand, there is great development in the field of cytotoxic substances of plant origin, primarily in research on different terpene derivatives. Higher plants can produce a great diversity of chemicals that have antimicrobial activity *in vitro*. The group of plant formed antibiotics that has probably received the greatest amount of attention is the phytoalexins, a term originally coined by K.O. Muller for those plant antibiotics that are synthesized de novo after the plant tissue is exposed to microbial infection (Muller and Borger, 1940). By definition phytoalexin are low molecular weight, antimicrobial compounds that are both synthesized by and accumulated in plants after exposure to microorganisms (Paxton, 1980; 1981).

3.2 Actinomycetes

The actinomycetes are a large group of filamentous, Gram-positive Bacteria like organism that form branching filaments. As a result of successful growth and branching, a ramifying network of filaments is formed, called mycelium. Although it is of bacterial dimensions, the mycelium is in some ways analogous to the mycelium formed by the filamentous fungi. Most actinomycetes form spores; the manner of spore formation varies and is used in separating subgroups (Madigan *et al.*, 2000). The actinomycetes comprise a ubiquitous order of bacteria, which exhibit wide physiological and morphological diversity. The majority of species are aerobic, saprophytic (some species are pathogenic to man, animals and plants), mesophilic forms, whose natural habitat is soil although it has been isolated from a wide-variety of terrestrial and aquatic habitats (Porter, 1971).

Since the discovery of actinomycin in Selman Waksmans laboratory at Rutgers University in 1940, followed in 1943 by streptomycin, the first really effective drug to treat tuberculosis, the actinomycetes have been famous as producers of antibiotics and other 'secondary metabolites' with biological activity (Challis and Hopwood, 2003).

In the last decades actinomycetes became the most fruitful source for antibiotics. In the 60s and 70s of the 20^{th} century 75 to 80% of all discovered antibiotics derived from the order Actinomycetales, mainly from Streptomyces species. In 70s and 80s the ratio and significance of the other non-streptomycete Actinomycetes (so called rare actinomycetes) increased up to 20% of all microbial antibiotics and 30-35% of actinomycetes species (Moncheva *et al*., 2002).

Moncheva *et al*. (2002) isolated 47 actinomycetes strain from Antarctica soils, nineteen of which showed antagonistic activity against Gram-positive and Gram-negative bacteria. Their study indicates that six of the strains belonging to genera *Streptomyces*, *Actinomadura* and *Kitasatosporia* possessed a broad spectrum of antibacterial activity. When antibacterial activity was confirmed in batch culture they found that three strains

were not only active against clinical isolates from the species *Staphylococcus aureus* and *Streptococcus pneumoniae* but also showed antibacterial activity against the

phytopathogenic bacteria *Xanthomonas axonopodis* pv glycines, *X. vesicatoria, X. axonopodis* pv phaseoli, *Pseudomonas syringae* pv tomato and *Clavibacter michiganeasis* for which no biological means for control, had been developed yet. According to them antibacterial compounds produced by those strains probably possessed non-polar structure and consisted of several active compounds.

3.2.1 Taxonomy of Actinomycetes

Actinomycetes belong to the order Actinomycetales (Superkingdom: *Bacteria,* Phylum: *Firmicutes*, Class: *Actinobacteria*, Subclass: *Actinobacteridae*). According to Bergey's Manual (Vol. IV), actinomycetes are divided into eight diverse families: *Actinomycetaceae, Mycobacteriaceae, Actinoplanaceae, Frankiaceae, Dermatophilaceae, Nocardiaceae, Streptomycetaceae, Micromonosporaceae* and comprise 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 (Table 2). Actinomycetes from the types II-IV can be further distinguished by their whole organism sugar pattern (Table 3). A whole array of taxonomic tools has been used to define genera and supergeneric groups of actinomycetes (Goodfellow and Williams, 1983), but partial sequence analysis of 16 s rRNA is the most significant. Based on 16s rRNA classification system they have recently been grouped in ten suborders: *Actinomycineae, Corynebacterineae, Frankineae, Glycomycineae, Micrococineae, Micromonosporineae, Propionibacterineae, Pseudonocardineae, Streptomycineae* and *Streptosporangineae* (Table 4) and a large members of actinomycetes are still remained to be grouped.

3.2.2 Ecology of Actinomycetes

Actinomycetes are cosmopolitan in nature and found in most environments. Soil is colonized by actinomycetes hyphae, which can form aerial mycelia bearing hydrophobic spores dispersed by air, water, and microarthropods. Some groups such as the actinoplanes are adapted for water-mediated dispersion and produce motile zoospores within desiccation-resistant sporangia.

Table 2: Cell wall Chemotypes with illustrating Families

Chemotype	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**
L-DAP	+							
Meso-DAP		+	+	+				
DABA							+	
Aspartic Acid						V[a]		
Glycine	+	+					+	
Lysine					+		V	
Ornithine					+			+
Arabinose				+				
Galactose				+		V		
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**

Note: **1** Streptomycetaceae, Nocardiaceae

2 Micromonosporaceae

3 Dermatophilaceae, Mycobacteriaceae, Thermomonosporaceae, Streptosporangiaceae, Frankiaceae

4 Nocardiaceae, Mycobacteriaceae, Corynebacteriaceae, Pseudonocardiaceae

5 Actinomycetacea

6 Cellulomonadaceae, Micrococcaceae, Microbacteriaceae, Actinomycetaceae

7 Microbacteriaceae

8 Cellulomodaceae, Microbacteriaceae

DAP: Diaminopimelic acid, **DABA**: Diaminobutyric acid, **[a]V:** Variable amount.

Table 3: Whole organism sugar pattern of Actinomycetes from cell wall Chemotypes II-IV

Pattern	Arabinose	Fructose	Galactose	Madurose	Xylose
A	+		+		
B				+	
C					
D	+				+
E		+			

Table 4: Taxonomic Classification of Order Actinomycetales

Suborder	Family	Genus
Micromonosporineae	Micromonosporaceae	*Micromonospora, Actinoplanes, Catellatospora, Couchioplanes, Catenuloplanes, Pilimelia Dactylosporangium*
Frankineae	Frankiaceae Sporichthyaceae Geodermatophilaceae Microsphaeraceae Acidothermaceae	*Frankia* *Sporichthya* *Geothermatophills, Blastococcus* *Microsphera* *Acidohermus*
Pseudonocardineae	Pseudonocardiaceae	*Pseudonocardia, Actinopolyspora, Actinosynnema, Amycolatopsis, Kibdelosporium, Kutzneria, Lentzea, Saccharomonospora, Saccharopolyspora, Saccarothrix, Streptoalloteichus, Thermocrispum.*

Contd…

Streptomycineae	Streptomycetaceae	*Streptomyces*
Corynebacteriuam	Nocardiaceae	*Nocardia, Rhodococcus.*
	Gordoniaceae	*Gordonia*
	Mycobacteriaceae	*Mycobacterium*
	Dietziaceae	*Dietzia*
	Tsukamurellaceae	*Tsukamurella*
	Corynebacteriaceae	*Corynebacterium, Turicella*
Micrococcineae	Micrococcaceae	*Micrococcus, Arthrobacter, Kocuria, Nesterenkonia, Rorhia, Renibacterium, Stomatococcus*
	Brevibacteriaceae	*Brevibacterium*
	Cellulomondaceae	*Cellulomonas, Oeskovia,*
	Dermabacteraceae	*Rarobacter*
	Intrasporangiaceae	*Dermatobacter, Brachybacterium Intrasporangium, Sanguibacter,*
	Jonesiaceae	*Terrabacter*
	Microbacteriaceae	*Jonesia Microbacterium, Agrococcus, Agromyces, Aureobacterium, Clavibacter, Curtobacterium,*
	Promicromonosporaceae	*Rathaybacter Promicromonospora*
Actinomyineae	Actinomycetaceae	*Actinomyces, Mobiluncus, Arcanobacterium*
Propionibacterianeae	Propionibacteraceae	*Propionibacterium, Luteococcus, Microlunatus, Propioniferax*

Contd…

Streptosporangineae	Streptosporangiaceae	*Streptosporangium, Herbidospora, Microbispora, Microtetraspora, Planobispora, Planomonospora*
	Thermomonosporaceae Nocardiopsaceae	*Thermomonospora, Actinomadura, Spirillospora* *Nocardiopsis*
Glycomycineae	Glycomycetaceae	*Glycomyces*

(Source: Bergey's Manual of Systematic bacteriology, Vol-IV, 1989)

3.2.3 Antibiotic producing Actinomycetes

Antibiotics have been used in many fields including agriculture, veterinary and pharmaceutical industry. Actinomycetes have the capability to synthesize many different biologically active secondary metabolites such as antibiotics, herbicides, pesticides, anti-parasitic, and enzymes like cellulase and xylanase used in waste treatment. Of these compounds, antibiotics predominate in therapeutic and commercial importance (Lacey, 1973; McCarthy and Williams, 1990; Ouhdouch *et al*., 2001; Saadoun and Gharaibeh, 2003; Waksman, 1961). The antibiotics from actinomycetes are diverse in chemical structure in contrast to antibiotics from the unicellular bacteria, which are more commonly peptides or modified peptides (Vining, 1990).

Among the different genera and families belonging to the order *Actinomycetales*, the very prolific genus *Streptomyces* is followed – in terms of number of strains producing antiinfectives – by the family *Micromonosporaceae* (mainly *Micromonospora* and *Actinoplanes*), the family *Pseudonocardiaceae* (mainly *Amycolatopsis*, *Saccharopolyspora* and *Saccharothrix*) and the family *Thermomonosporaceae* (mainly *Actinomadura*), the family *Nocardiaceae* (*Nocardia* and related genera), the family *Streptosporangiaceae* (mainly *Streptosporangium*) (Lazzarani *et al*.,2001).

Some genera of Actinomycetes known to produce antibiotics are:

Actinoplanes

Actinnoplanes are aerobic, mesophilic and world wide distributed in many different habitats. Soils of neutral pH are good sources. They produce a fine non -fragmenting, branching mycelium. Aerial mycelium scanty or absent but some species produce aerial mycelium that imparts to the colonies a powdery appearance (Lechevalier and Corke, 1975). Colonies are usually bright colored. The common is orange, but some have red, yellow, rusty brown, blue, purple, green or black mycelia. Growth takes place usually from 10⌈-50⌈C.

Antibiotic producing species with corresponding antibiotics are:

A. philippinensis – macrocyclic lactone antibiotics; *A. utahensis* – cyclic peptide antibiotics; *A. ianthinogenes* – naphthoquinone antibiotics; *A. deccanensis* – lipiarmycin; *A. garbadinensis, A. liguriae* – gradimycin; *A. teichomyceticus* – teichomycin; *A. brasiliensis* – acidic antibiotics; *A. caeruleus* – heptaene antifungal antibiotic etc.

More than 120 antibiotics have been reported from Actinoplanes strains. Amino acid derivatives, such as peptides and depsipeptides are prevalent and among these compounds some are of clinical relevance such as teicoplanin from *Actinoplane teichomyceticus* ATCC31121 (Bardone *et al*., 1978) and ramoplanin from *Actinoplane sp* ATCC33076 (Ciabatti and Cavalleri, 1989).

Micromonospora

As name designated, spores are borne singly on sporophores. Aerial mycelium is absent or in some cultures appearing irregularly as a restricted white or grayish bloom. Colonies are initially pale yellow or light orange becoming orange, red or brown. Growth occurs normally between 20⌈- 40⌈C but not above 50⌈C. *Micromonospora* is considered to encompass the second largest group of culturable actinomycetes in soil (Lazzarini *et al*., 2001). The isolation of *Micromonospora* has been mainly based on the use antibiotics such as gentamicin and novobiocin as selective agents (Williams and Wellington, 1982) or deleterious agents such as phenol and chlophexidine gluconate solutions (Hayakawa *et al.*, 1991).

Some antibiotic producing species are:

M. purpurea, M. echinospora – gentamicin antibiotics complex; *M. carbonacea* – everninomicin antibiotic complex; *M. halophytica* – halomicin antibiotic complex; *M. olivasterospora* – fortimicin; *M. inositola* – XK – 41 antibiotic complex.

Important antibiotics produced by *Micromonospora* strains include the aminocyclitols, such as gentamicins; sisomicin; fortimicin; macrolides such as the mycinamicins and rosamicins and polysaccharide antibiotics such as eyerninomycin (Lancini and Lorenzetti, 1993)

Actinomadura

Actinomadura possess extensively branching vegetative hyphae forming a dense non-fragmenting substrate mycelium. Aerial mycelium moderately developed or absent, which at maturity form short or occasionally long chains of arthrospores. Colonies exhibit a cartilaginous or leathery appearance. Aerial mycelium at maturity is of white, cream, pale yellow, pink, blue or gray color. Temperature range is 20⌈-45⌈C although some species grow at thermophilic range (up to 55⌈C). Most of the species are originated from soil (Nonomura and Ohara, 1971), while *A. madurae* and *A. pelletieri* are known to cause human actinomycetoma.

Antibiotic producing species are:

A. roseoviolacea – antibacterial compound similar to daunomycin (Nonomura and Ohara, 1971); *A. carminata* – carminomycin; *A. pusilla* – sulfur containing peptide antibiotic named actinotiocin; *A. cremea* – rifamycin; *A. lauzonensis* – antitumor antibiotic complex; *A. azurea* – cationomycin; *A. oligospora* – polyether antibiotics; *A. kijaniata* – complex of novel acid enol antibiotics, the major component of which was designated kijanimicin.

Out of more than 250 antibiotics produced by *Actinomadura* strains, the most frequently found are the ionophoric polyethers, which are used as animal feed additives (Westly, 1977). The antifungal compounds benanomicin and pradimicin were also recently discovered from strains of this genus (Debono and Gordee, 1994).

Planomonospora

Substrate mycelium was found to grow profusely into the medium forming a compact layer on the surface of agar. Sporangia formed on aerial mycelia only. Colonies on agar media are flat or elevated with smooth surfaces, occasionally wrinkled. Optimum temperature for growth is 28⌠-37⌠C. Members of this genus are world wide distributed in the soil. Of the antibiotic producing strains, *P. parontospora* sub sp. *antibiotica* is known to produce sporangiomycin.

Streptosporangium

Sporangiospores are formed by septation of a coiled, unbranched hypha within the sporangium. Aerial mass color is pink, white, pale greenish, gray or dusty green. Most species grow best within the range 28⌠-30⌠C. Slightly acid, humus rich soils are favorable habitat.

Antibiotics producing species are:

S. violaceochromogenes – victomycin; *S. albidum* – sporoviridin like antibiotics; *S. viridogriseum* sub sp. kofuense – chloramphenicol; *S. viridogriseum* sub sp. viridogriseum – sporoviridin etc.

Since the first sporaviridin complex was reported in Japan in 1966, about thirty different bioactive molecules have been described in the last thirty years. Many of them are produced as antibiotic complex ranging from two to ten structurally related components. These molecules belong to a broad spectrum of diverse chemical classes making the metabolic potential expressed by members of this genus very attractive to industrial screening programmes. However, streptosporangia are difficult to isolate by traditional culturing methods due to their very slow growth on isolation plates and hence their inability to compete with faster growing actinomycetes. It is apparent from the recent literature that combinations of different isolation methods can be used to isolate Streptosporangiaceae. Some of the selective procedures exploit the ability of Sporangiospores to withstand physical treatments such as dry heating or microwave

irradiation or resistance to treatment with toxic chemical agents such as benzethonium chloride and chloramines-T or to specific antibiotics such as leucomycin and tobramycin (Hayakawa *et al*., 1991)

Saccharomonospora

They produce predominantly single spores on aerial hyphae. They are isolated from soil, lake sediments and peat but are common in manures and compost and over heated fodders (Nonomura and Ohara, 1971). Three antibiotics produced by *Saccharomonospora* strains have been described. The first, thermoviridin, was produced by the original isolate on which the genus was found. The remaining two are produced by strains considered as belonging to this genus - an antitumor agent from *Micropolyspora coerulea* and the antibacterial antibiotic primycin from *Thermomonospora galeriensis.*

Saccharopolyspora

They produce well-developed substrate mycelium fragmenting into rod- shaped elements. Aerial mycelium is straight or in spirals, characteristically segmented into bead like chains of spores. Colonies are thin, raised or convex, slightly wrinkled, mucoid or gelatinous in appearance. They are mostly isolated from soil. *S. hirsuta* sub sp. kobensis was found to produce sporaricin. Similarly, *S. erythraea* isolates produce erythromycin.

Nocardia

Rudimentary to extensively branched vegetative hyphae, growing on the surface of and penetrating agar media, often fragmenting into bacteroids. Colonies may have a superficially smooth appearance or more commonly a matte, chalky or velvety. Growth temperature range is 15⌈-37⌈C, although many will grow at higher or lower temperature. They are widely distributed and are abundant in soil. Some strains are pathogenic opportunists for man and animals.

Some of the antibiotics producing strains are:

N. Orientalis – vancomycin; *N. mediterranei* rifamycins; *N. sulphurea* – chelocardin; *N. kuroishii* – neonocardin.

A new family of ℘lactam antibiotics was reported in 1976 from the actinomycete *Nocardia uniformis* sub sp. Tsuyamanensis ATCC 21806 (Aoki *et al*., 1976, Hashimoto *et al*., 1776). Nocardicin A is the major product as well as several other structurally related metabolites in smaller amounts nocardicins B to G (Hashimoto, *et al*., 1976, Hosoda *et al*., 1977). The nocardicins were the first naturally occurring monocyclic ß-lactams that display any significant antibacterial activity to be isolated, and they bear structural and stereo-chemical similarities to the naturally occurring penicillin N (Kelly and Townsend, 2005).

Streptoverticillium

Substrate mycelium is branching. The aerial mycelium consist of long straight filaments bearing at more or less regular interval branches arranged in whorls colonies are somewhat similar to that of *Streptomyces* except for cottony consistency of the aerial growth of *Streptoverticillium.* Optimum temperature for growth is between 26⌈-32⌈C. They are mostly saprophytes in soil.

Some of antibiotics produced by *Streptoverticillium* species are:

Distamycins, fervenulin, actinospectacin, takacidin, raisomycin, netropsin, pentaene antibiotic, narangomycin, citromycin, olivomycins, trichomycin, soedomycin, carbomycin, enteromycin, eurocidin, protomycin, tertiomycin, etc.

Streptomyces

This group of actinomycetes is important both numerically and as producers of useful metabolites. Most actinomycetes isolated from any habitat are likely to be *Streptomyces* spp. They are widely distributed and abundant in soil, including composts. A few species are pathogenic for man and animals. Optimum temperature for growth is 25⌈-30⌈C. They produce aerial mycelium, which at maturity forms chains of 3 to many spores. Colonies

are discrete and lichenoid, leathery or butyrous. Initially colonies are relatively smooth surface but later they develop a weft of aerial mycelium that may appear floccose, granular, powdery or velvety. Many strain produce one or many antibiotics. Some of the commonly used are: streptomycin, tetracycline, puromycin, perimycin, neomycins, oxytetracycline, polyoxin, proactinomycins, narbomycin, actinomycin, chloramphenicol, collinomycin, rubromycin, folimycin, levomycin, pyridomycin, hygromycin, glebomycin, chlorotetracycline, amphotericin, camphomycin, carbomycin, spinamycin, mutomycin, pimarcin, xantjomycin, viomycin, novobiocin, etc.

Since the discovery of streptomycin, a large number of antibiotics including major therapeutic agents such as aminoglycosides, chloramphenicol, tetraclines, macrolides and more recently, lactam cephamycin group have been isolated from cultures of *Streptomyces* and *Streptoverticillium* (Miyadoh *et al*., 1997).

Out of more than eight thousand antimicrobial products described in the Antibiotic Literature Database (ABL), 45.6% are produced by Streptomycetes (Lazzarini, *et al.*, 2001)

It is interesting that *Streptomyces* strains continue to provide a larger number and wider variety of new antibiotics than any other actinomycete genus, suggesting that substantial numbers of *Streptomyces* species or strains with novel antibiotic productivity exist in nature. In search for bioactive antibiotics, *Streptomyces* strains have been isolated from various types of soils, including rice paddy, lake mud and water, deciduous forest, tropical forest, waste land and cave soils (Jiang and Xu, 1996, Kim *et al*., 1995, Suzuki *et al*., 1994 and Xu *et al.*, 1996).

Thermoactinomycetes

Thermoactinomycetes comprises only one genus, consisting mostly of thermophilic organisms growing at temperature between 45⌈-60⌈C. They are characterized by the formation of single spores on both the aerial and substrate mycelium. Colonies are flat or

ridged with entire or filamentous margins. Habitats are soil, molding and decaying plant materials and composts. Cultures of *T. thalpophilus* have been reported to produce thermorubin which is more inhibitory to gram positive than gram-negative bacteria but it is also highly toxic to mammals.

3.2.4 Isolation of Actinomycetes

Actinomycetes are the most widely distributed groups of microorganisms in nature. They are attractive bodacious and charming filamentous gram positive bacteria. In comparison with their main competitors, bacteria and fungi, they have certain deficiencies. The rate of radial growth on culture media is lower than that of fungi, and their rate of cell production is generally lower than that of bacteria. Therefore methods for their isolation must be designed to compensate, at least partially, for their generally poor competitive ability under laboratory conditions though they make up in many cases especially under dry alkaline conditions, a large part of the microbial population of the soil (Athalye *et al.*, 1981; Goodfellow and Williams, 1983; Lacey, 1973; Lacey 1997; Waksman, 1961).

3.2.4.1 Isolation from soil

Soil is indeed almost a perfect laboratory for the creation of natural medicines. Soil contains a wide array of tiny microhabitats that creates an enormous variation in the appearance and survival strategies of soil microbes. This diverse group of microbes, of which there are billions in an average teaspoon of soil, must then compete with one another for every available nourishing piece of organic matter. The most prolific species found in soils are bacteria. The second key soil species are the actinomycetes which resembles both bacteria and fungi. Actinomycetes give soil its earthy smell; it comes from compound, geosmin, which is released as actinomycetes breakdown organic matter. Though they play an important role in soil quality, actinomycetes are more commonly known as the source of antibiotics such as actinomycin, tetracycline and neomycin. Actinomycetes are also nitrogen fixers; they convert atmospheric nitrogen into a form that can be used by plants (Brady and Weil, 2004).

Many workers isolate actinomycetes as a group from soil, but unless specialized techniques are used: these isolates consist of a very high percentage of Streptomyces strains, which are the most numerous group in soil. The relatively low occurance of non-streptomycetes species is due to difficult techniques required for the isolation of these strains from the environment (Berdy *et al*., 1982).

The selective isolation of *Actinomadura* and *Microtetraspora* strains is achieved by air-drying and heating soil and using isolation media supplemented with antibiotics such as streptomycin and rifamycin (Li, 1989).

Singh and Agrawal (2002) isolated 68 isolates of actinomycetes from 120 soil samples collected from Lobuche. They identified 42 isolates belonging to genera: *Catellospora, Intrasporangium, Kibdelosporium, Kitasatospora, Micromonospora, Nocardia, Saccharopolyspora, Streptomyces and Streptoverticilium.*

Oskay *et al.,* (2004) isolated actinomycetes from farming soil of Turkey using Glycerol-yeast extract Agar (Glycerol 1gm, Yeast extract 0.4gm, K_2HPO_4 0.02gm, Peptone 5.0gm, Agar 3gm, Distilled water 200ml) complemented with nystatin (50⇑g/ml) at 27⌈C (Waksman, 1961).

Moncheva *et al*., (2002) used selective media such as AV agar and starch- casein- nitrate agar (Atlas, 1997) for the isolation, cultivation and maintainance of actinomycetes strains from soil of Antarctica.

Xu *et al*., (1996) isolated temperate actinomycetes from soil of Yunnan, China by spread plate method using glycerol- asparagines agar, starch casein agar, modified AV agar (Jiang *et al*., 1991) and HV agar with some modifications. For modified HV medium, humic acid was dissolved in 1M NaOH. After boiling, 1ml of HCl was added. Precipitates were discarded after cooling and centrifugation. Potassium dichromate (25⇑g/ml) was used as an inhibitor. While for thermophilic actinomycetes air dried soil samples were pretreated at 120⌈C for 1 hour and diluted soil samples were spread on Yeast extract- malt extract agar and glycerol- asparagines agar.

3.2.5 Screening of the antibiotic producing actinomycetes

Need of screening for Antifungal and Antibacterial characteristics

- Resistance development by pathogens
- New emerging pathogens and their medical and economic value importance

Target cultures:

Plant pathogenic fungi:

a) *Aterneria* spp.: *Alterneria solani* attacks members of the family Solanaceae as well as other hosts. It causes a disease called early blight in potato and tomato. It also causes leaf spot in lady's finger, chilli and sunflower. Fruit rot in tomato is also caused by *A. solani* (Rangaswami).
b) *Fusariu oxysporum*: *Fusarium oxysporum* causes an important wilt disease of sweet potato (*Ipomoea batetas*) in USA. The fungus also infects the roots of many plants without causing any external symptoms viz. cabbage, cotton, lowpea, maize, okra, soyabean, tobacco and water melon (Booth, 1971).
c) *Sclerotinia* spp.: *Sclerotinia sclerotiorum* causes lettuce drop and other vegetable diseases as well. Some species of Sclerotinia causes brown rot of fruit apple, apricot, plum etc. It causes stem rot of mustard (Rangaswami, 1996).

Bacterial pathogen:

a) *Bacillus* spp.: It is often associated with food poisoning.
b) *Enterobacter* spp.: Enterobacter aerogenes and E. cloacae are opportunistic pathogens involved in infections of wounds and of the urinary and respiratory tracts and are occasional causes of septicemia and meningitis.
c) *E. coli*: Although *E. coli* is considered as commensal flora, it is able to cause frequent opportunistic infections, as for example, appendix abscesses, peritonitis, cholecystitis septic wound (Collee *et al*, 1996).

Nonetheless, the actinomycetes that produce antibiotics are abundant in soils. According to some estimates, the top 10cm of global soil contains 10^{25}-10^{26} actinomycetes, but only about 10^{7} have been screened for antibiotic production in the past 50 years, leaving plenty of room for further screening (Baltz, 2007).

Several Decades ago, researchers at Rutgers University in New Brunswick, N.J., and Boyd wood ruff and his collaborators at Merck in Rah way, N.J., found streptothricin in about 10% of randomly collected soil actinomycetes, mainly *Streptomyces* species. Streptomycin is found in about 1% of random soil actinomycetes, whereas tetracycline and actinomycin are present at about 0.1%. It is no wonder that these were among the first antibiotics discovered. The erythromycin and vancomycin biosynthetic pathways are much less abundant present at frequencies of about 5×10^{-6} and 1.5×10^{-6} respectively. Even so, they are sufficiently abundant that they could be isolated by low- throughput methods in the 1950s. Many other antibiotics, perhaps as many as 2000, are isolated at frequencies of $1\text{-}2 \times 10^{-7}$, including daptomycin, which was recently approved for clinical use (Baltz, 2007).

The screening of active strains of actinomycetes is an important process. Especially, Streptomyces have received attention as the tool for this purpose (Haque *et al*., 1995). The main way in which new antibiotics were discovered in the past was by screening. In the screening approach, a large number of isolates of possible antibiotic producing microorganisms are obtained from nature in pure culture and these isolates are then tested for antibiotic production by seeing weather they produce any diffusible materials that are inhibitory to the growth of test bacteria. The classical procedure for testing new microbial isolates for antibiotic production is the cross-streak method, first used by Fleming in his pioneering studies on penicillin (Madigan *et al.*, 2000).

Some new screening programs have been already developed for discovering of new species or unknown bioactive substances. One of the modern approaches is isolation and screening of microorganisms from relatively unknown or unstudied areas (Franzmann *et al*., 1997,). In the recent years using molecular genetics methods it was found that the majority of the isolated microorganisms are new species.

The development and massive application of genus-oriented selective isolation methods, mainly applied by industrial researchers, has given a significant impetus to the discovery of new microbial products of medical importance. Furthermore, this approach also helps to answer the question: are these less exploited actinomycetes less abundant in the environment or are they just more difficult to isolate and cultivate (Lazzarani *et al.*, 2001)

Ellaiah *et al*., (2002) investigated sediment samples from Krishna River, at Nagayalanka of Andra pradesh, India as a source of actinomycetes to screen for the product of novel bioactive compounds. During their investigation on fresh water actinomycetes from 5 different river sediment samples, a total of 80 actinomycetes were isolated. Out of those 80 isolates, 30 isolates, which showed distinct morphological characteristics, were selected. The antimicrobial activity was studied for all the 30 isolates. The preliminary study for antimicrobial activity by cross streak method indicated that 16 isolates (53.3%) have excellent antagonistic properties. When all the 16 isolates were subjected to detailed submerged fermentation studies, 12 isolates (40%) exhibited antibacterial activity and 9 isolates (30%) showed antifungal activity while 5 isolates (16.6%) showed both antibacterial and antifungal activities.

Soares *et al*., (2006) observed significant interaction between the actinomycete isolates and the phytopathogenic fungi for spore germination and mycelium growth inhibition. *Streptomyces soecies* codified as AC 26 was more efficient in inhibiting spore germination of *Curvularia eragrostides,* while *S. thermotolerans* and *Streptomyces* spp. N0035 were more efficient for *C. gloeosporioides. Streptomyces* spp. (AC26) was the most efficient in inhibiting mycelium growth of both fungi and the inhibitory effect was positively correlated to metabolite concentration.

Oskay *et al*., (2004) recovered 50 different actinomycetes strains from farming soil samples collected from Manisa Province and its surroundings which were then assessed for their antibacterial activity against four phytopathogenic and six pathogenic bacteria. Results indicated that 34% of all isolates are active against at, least one, of the test organisms; which includes *Agrobacterium tumefaciens, Erwinia amlovora, Pseudomonas viridiflova, Clavibacter michiganensis*, *Bacillus subtillis* ATCC 6633, *Klebsiella*

pneumoniae ATCC 10031, *Enterococcus faecalis* ATCC 10541, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC29998 and *Sarcina lutea* ATCC 9341.

Additional actinomycete- produced antibiotics likely could be discovered by subjecting soils and marine sediments to innovate enrichments and whole- cell screening methods (Baltz, 2007).

3.2.6 Physico-chemical Parameters for antibiotic production

Temperature is very important in the biosynthesis of antibiotics and growth of actinomycetes. The optimum temperature is in the range of 25⌈-30⌈C, probably closer to 28⌈C (Casida, 1968). If the temperature rises over 30⌈C, the synthesis of antibiotics is particularly discontinued (Egorov, 1985).

The optimum pH of the medium for cultivation of actinomycetes is 7 and the antibiotic is formed in the range from pH 7 to 8 (Casida, 1968).

Being highly aerobic organism, the actinomycetes consume considerable quantities of oxygen. Thus antibiotic yield responds strongly to high aeration and agitation. Generally the aeration varies from 0.5v/v per minute to 1v/v per minute while agitation is between 200 to 400 rpm.

The growth of the actinomycetes producing antibiotics and the physiological and biochemical changes occurring in the medium and the microorganism has a pronounced two stages character. During first stage, mycelial growth takes place rapidly, glucose consumption is rapid, pH gradually increases, oxygen consumption is maximum and there is maximum soluble nitrogen and mineral phosphate with slow antibiotic production. The second phase is characterized by gradual autolysis of mycelium, less consumption of glucose, maximum attainment of pH, decrease in oxygen consumption to minimum, increase in concentration of soluble nitrogen, release of mineral phosphate into medium and maximum rate of antibiotic production.

Casida in1968 divided growth into three stages; the first one characterized by vigorous growth of mycelium followed by rapid diminish of growth rate in the second phase where production of the antibiotic accelerate rapidly. However the third phase; which approximately being after 7 days incubation brings the cessation in the production of antibiotics. Harvest of antibiotics from culture medium is done during the idiophase, when maximum titre of antibiotic is attained.

Liu *et al*., (1980) carried out fermentation for 5 days for polyether antibiotics by *S. malachitofuscus.*

CHAPTER IV

4. MATERIALS AND METHODS

A list of materials, chemicals, equipments, media and reagents required for the study is presented in Appendix 1.

4.1 METHODS

4.1.1 Sample Collection

Sites choosen for sample collection were, 'Chaurikharkha' (altitude 2900m), 'Chaurikharkha' (altitude 2840m), 'Lobuche' (altitude 5050m) and 'Feriche' (altitude 4800m) of 'khumbu' region, of Nepal, which is situated at the lower part of Mount Everest base camp. Soil samples were collected in fresh, sterile polyethene bags. The surface soil was removed and approximately 20-50 grams of soil along with the piece of root, from depth of 3-4 inches were collected and kept in fresh polyetene bag and well tightly closed with a rubber grider.

The soil samples were transported to Natural Product Research Laboratory at NAST, 'Khumaltar' where it was air dried and finely ground in a sterile mortar with a sterile pestle.

4.1.2 Isolation

Isolation of actinomycetes from soil was carried out by spread plate technique (Collins and Lyne, 1989). One gram of air-dried and grounded soil sample was suspended in a test tube containing 10ml of sterilized distilled water. The cotton plug was inserted and homogeneous suspension was made by rotating the tubes between the palms of the hand. 0.2 ml of each soil suspension was applied to the center of the surface of solidified Glycerol- Asparagine agar (Shriling and Gottlieb, 1996), in petri plate, with the help of sterilized pipette. Then it was spread over the medium by pushing the sterile bent glass rod backward and forward while rotating the plate. The glass rod was sterilized by dipping it in alcohol and then burning off the residual alcohol. The plates were left for 30 minutes to dry before inverting and incubated at 27⌈C for one week. Actinomycetes colonies were further isolated in pure form on the solidified Glycerol Asparagine agar by streak plate method and incubated at 27⌈C for one week. Thus obtained colonies were subjected to screening process for the determination of antimicrobial activity.

4.1.3 Maintenance of Actinomycetes culture

Each pure culture of isolate was given a code and stored in sterile distilled water and Glycerol Asparagine agar slant at 4⌈C.

4.1.3.1 Storage in sterile distilled water

For storage purpose, 3ml of distilled water was pipetted and dispensed in a number of capped autoclavable plastic vials and made sterile by autoclaving at 15 lbs pressure at 121⌈C for 15 minutes. The water in the vial, after sterilization, was allowed to cool. The surface of the Actinomycetes colony on the Glycerol Asparagine agar was scrapped with sterile inoculating loop and transferred to the sterile distilled water in the vial aseptically so as to prepare suspension of broken mycelium and spores in water. The vial was capped tightly and sealed with a parafilm. The vials were labeled with the isolate code and date and stored at 4⌈C from which cultures could be revived by pipetting 1-2 ml of suspension

on Glycerol Asparagine agar and incubating at 27⌈C for 1-2 weeks as per the requirement.

4.1.3.2 Storage in Glycerol Asparagine agar slants

Actinomycetes culture from a typical sporulating colony was transferred aseptically with the sterile inoculating loop onto the Glycerol Asparagine agar slant and incubated at 27⌈C for 1-2 weeks. The slant was observed for good colony growth. Finally the screw cap was sealed with parafilm, the tubes were labeled with isolate code and date and stored at 4⌈C.

4.1.4 Characterization of actinomycetes

Each of the soil actinomycetes isolated from soil samples were characterized by morphological and physiological methods for identification of the isolates.

4.1.4.1 Morphological methods

4.1.4.1.1 Macroscopic methods

The actinomycetes were grown in Glycerol Aspsragine agar for 7 days at 27⌈C and color, texture and morphology of the colony were noted.

4.1.4.1.2 Microscopic methods

The microscopic characterization was done by cover slip culture method (Kawato Sinobu, 1979).

The sterilized cover slip was inserted in nearly solidified Glycerol Asparagine agar in petri dish at an angle of about 45 until half of the cover slip was buried in the medium. The medium was allowed to solidify properly. Then the isolates were inoculated along the line where the medium meets the upper surface of the cover slip. It was incubated at 27⌈C for 7-10 days. Then the cover slip was removed carefully and placed on a slide upward. It was then examined under oil immersion (1000X) for the mycelium structure, color and arrangement of conidiospore and arthrospore on the mycelium. The observed

structure was compared with *Bergey's manual* of Determinative Bacteriology, Ninth edition (1994) and the organism was identified.

4.1.4.2 Biochemical characterization

Various biochemical tests performed for the identification of the potent isolates are as follows:

4.1.4.2.1 Casein hydrolysis

It was carried out in the Skim milk agar (Appendix II). Solidified skim milk agar plates were divided into 6 sectors with each being streaked with the isolate. Observations were made after 7 days of incubation at 30⌈C, for the clear zone around the colony. To confirm, the plates were flooded with Mercuric chloride solution (Appendix III).

4.1.4.2.2 Gelatin Hydrolysis

Each of the solidified gelatin agar plates (Appendix II) was divided into 6 sectors where isolates were streaked. The plates were incubated at 30⌈C for 7 days. Hydrolysis was confirmed by flooding the plates with Mercuric chloride solution (Appendix III).

4.1.4.2.3 Starch hydrolysis

Solidified starch agar plates (Appendix II) were inoculated with the isolates and incubated at 30⌈C for 7 days. Hydrolysis was confirmed by flooding the plates with iodine solution (Appendix III) to see the clear zone of hydrolysis around the colony.

4.1.4.2.4 Tween 20 hydrolysis

The isolates were streaked on the solidified Tween 20 agar plates (Appendix II) and incubated at 30⌈C for 7 days. Observations were made for clear zone around the colony.

4.1.4.2.5 Urea hydrolysis

Each test tube dispensed with sterile urea broth (Appendix II) was inoculated with the isolate and incubated at 30⌈C for 7 days. Observations were made daily. Positive test was confirmed by the change in color of the broth to pinkish red.

4.1.4.2.6 Nitrate reduction

Each of the isolates was inoculated into test tubes dispensed with nitrate broth (Appendix II) and incubated at 30⌈C for 5-7 days. Nitrate reduction was detected by adding few drops of Sulphanilic acid reagent (Appendix III) and Alpha-napthylamine reagent (Appendix III) into the culture broth. A distinct red or pink color indicates the reduction of nitrate. Negative test was confirmed by addition of zinc dust into the negative tubes. Development of distinct red color after addition of zinc dust confirms the test negative. Absence of development of reddish color even after addition of zinc dust was considered positive.

4.1.4.2.7 Acid Production from sugar

For this test basal medium containing peptone, sodium chloride and phenol red (Appendix II) was incorporated with carbohydrate at the concentration of 1% (w/v). Carbohydrates used were Arabinose, Fructose, Galactose, Glucose, Lactose, Maltose, Mannose, Raffinose and Sucrose. Each carbohydrate was added to the separate broth that had been autoclaved. Then the mixture broth was dispensed into the test tubes, which were inoculated with the isolates and incubated at 30⌈C for 7-14 days. Positive result was determined by the change in color of the broth from red to yellow.

4.1.4.2.8 Sodium chloride resistance

For this basal medium containing Glucose, Yeast extract, Malt extract and Agar (Appendix II) was prepared in three batches and supplemented with 5%, 7% and 10% sodium chloride. The medium was autoclaved and solidified in petriplates. Agar plates

were then incubated at 30 ⌈C for 7 days and observations were made to record highest concentration of salt that allow the growth.

4.1.4.2.9 Temperature tolerance

The isolates were streaked in Nutrient agar plates and incubated at 15 ⌈C, 37 ⌈C and 50 ⌈C for 7 days and growth was observed.

4.1.5 Antimicrobial screening of Actinomycetes

All the identified isolates were subjected to the screening process. As antimicrobial activity of the isolated actinomycetes was detected against two different groups of organism i.e, bacteria and fungi, two different procedures were followed.

4.1.5.1 Antibacterial activity

The antibacterial activity of the isolated actinomycetes was determined by perpendicular streak method (Egorov, 1985) on Nutrient agar (NA). The test organisms used were: *Bacillus subtilis, Staphylococcus aureus, Enterobacter* spp*, Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, Pseudomonas aeruginosa, Citrobacter* spp*, Salmonella paratyphi* A *and Shigella* spp. The actinomycetes and test bacteria were grown on the same nutrient agar medium. Each of the isolate was streaked as a single cross streak through the diameter of the plate and incubated at 27 ⌈C for 5 days for sufficient growth of organisms. Then test organisms were streaked on the either sides of the fully grown isolates without touching it, making a least distance between the actinomycetes streak and perpendicular streak. The plates were then incubated at 37 ⌈C for 24 hours to allow the growth of the test organisms. Presence of the antibacterial activity of the actinomycetes was presumed by noting a clear zone between the test organisms and the cross streak at the center. The zone of inhibition was measured.

4.1.5.2 Antifungal activity

The antifungal activity of the isolated actinomycetes was determined by dual culture method used by Carruthers *et al*., 1994 (Shrestha, 2002)

Petridish containing solidified sterile PDA was inoculated 2 cm from its edge with a fungal agar disc (5mm diameter) cut out from actively growing plant pathogenic fungus (7 day old culture) grown on PDA.

Similarly, a colony of actinomycetes growing on Glycerol Asparagine agar was also inoculated at the center of the plate and spread making a round inoculum of about 3mm in diameter with the sterile inoculating loop. The plates were then incubated at 27 ⎡C for 1-2 weeks and observed for the antagonistic effect of the Actinomycetes on the plant pathogenic fungal colony. The isolates were noted as highly inhibitory (+++), moderately inhibitory (++), low inhibitory (+) and not inhibitory (-) on the basis of extent of inhibition shown on the plant pathogenic fungal colony. The fresh cultures of *Alternaria* spp., *Fusarium oxysporum, Fusarium moniliforme, Geotricum* spp., *Gloeosporium sporioides, Helminthisporium* spp. and *Sclerotinia* spp. were used for studying antagonistic or inhibitory properties.

4.1.6 Fermentation process

Fermentation was carried out in Erlenmeyer flask (1 lit.) following the procedure as described by Liu *et al*., (1980).

Selected strains of actinomycetes were grown separately on Glycerol Asparagine agar plates. The actively growing 7-day culture was used for inoculation. Some colonies of actinomycetes were transferred aseptically to the 25ml sterilized starch casein broth in

100ml Erlenmeyer flask and incubated at 27 ⌈C at for 3 days. It was then transferred aseptically to 75ml sterilized cool starch casein broth in 200ml Erlenmeyer flask and incubated at 27 ⌈C for 4 days. Thus prepared inoculum was poured aseptically in sterile 1 lit. capacity Erlenmeyer flask containing 400ml sterile starch casein broth and further incubated at 27⌈C for 14 days. The above mentioned fermentation process was carried out in duplicate flask for each isolate. For control similar procedure was carried except inoculation of organism, i.e. for control, the fermentation process was carried without inoculation of actinomycetes colony.

4.1.7 Extraction of secondary metabolites:

After 3 weeks, the culture media was filtered through four folds of cheesecloth to remove solids. The filtrate was mixed with equal volume of chloroform and extracted two times in a 2L-separating funnel. The chloroform fraction was combined, dried over anhydrous sodium sulphate and evaporated in a rotary vacuum evaporator under reduced pressure (Shrestha, 2002). The process was carried out separately for different flasks of culture media. The control was also processed in the similar manner.

4.1.8 Determination of dry weight of chloroform extract

After evaporation of the extract in rotary evaporator, the round bottom flask in which evaporation was carried (whose weight was already taken before the filtrate was transferred to it) was left such for complete drying. When it was completely dried, the weight of the round bottom flask with extract was taken and dry weight of the extract obtained from each of 500ml of media was calculated as:

Dry weight of extract = Total weight of R.B. flask and extract – Weight of empty R.B. flask.

The arithmetic mean was calculated for the yield from two flasks and was recorded as the average dry weight of chloroform fraction of the actinomycetes extract. The dry weight of extract of control media, if obtained any, is found out in the similar way.

The extract was then dissolved in small volume of chloroform and transferred to a small vial taking care that no extract was left over. The vial was left as such overnight to allow chloroform to evaporate. When the extract was completely dried, the vial was capped tightly and preserved until used.

4.1.9 Bioassay

4.1.9.1 Preparation of inoculum of bacteria:

The test bacteria were inoculated in 5ml nutrient broth each and incubated at 37⌈C for 4hours. Then the turbidity of the broth was compared with the 0.5 nephlometer standard. If the turbidity matched with the standard, it was used for swabbing.

While swabbing, a sterile cotton swab dipped in the broth completely and it was pressed against the wall of the tube without touching the broth. Then it was swabbed on the antibiotic assay medium plate at an angle of 60⌈ with three rotations. The plates were let for about 30 minutes and well were made by a sterile cork borer.

4.1.9.2. Preparation of inoculum of fungi:

The test fungi were inoculated in sterile Potato Dextrose Agar plates each and incubated at 27⌈C for 7 days. Well-grown fungal colony with spores was used for swabbing. While swabbing, a sterile cotton swab was used.

4.1.9.3 Determination of the antimicrobial activity of the actinomycetes extract extracted with chloroform

To the extract obtained, 300⇑l of DMSO was added and dissolved well. The extract was screened for its antibacterial and antifungal activity against six different (three bacteria and three fungi) organisms.

4.1.9.3.1 Determination of antibacterial activity

The antibacterial activity was determined by Dingley's method or agar cup assay method (Sen *et al*., 1993). Three wells of 6mm diameter were made towards the edge in solidified Muller Hinton Agar (MHA) plates by removing agar disc from the media using a sterile cork borer. The test bacteria (0.5 McFarland turbidity standard) was swabbed over the plate with the help of sterilized cotton swab and 50⇑l of the actinomycetes extract was put carefully with the help of a micropipette. Into the second of the three wells, 50⇑l of the extract of the control media was put carefully and into the third well, 50⇑l of the solvent (DMSO) was added to test if the solvent itself exhibited the antibacterial activity. The plates were then left for half an hour with lid closed so that the extract diffused into the media. The plates were then incubated at 37 ⌈C for 24hrs and examined. The diameter of the zones of complete inhibition was measured to the nearest whole millimeter.

4.1.9.3.2 Determination of antifungal activity

The antifungal activity was determined by Agar well diffusion method. As in the determination of antibacterial activity three wells of 6mm diameter were made towards the edge in the solidified PDA plates by removing agar disc from the media using a sterile cork borer. The test fungus was inoculated on the plate and 50⇑l each of actinomycetes extract, extract of control media and solvent (DMSO) were put carefully with the help of micropipette in each well as in case of determination of antibacterial activity. The plates were then left for half an hour with lid closed so that the extract diffused into the media. After diffusion, the inoculated plates were incubated at 28 ⌈C for 4 days. Growth inhibition was observed after 4days. The zones of inhibition were measured using scale to the nearest whole millimeter and recorded.

4.1.10 TLC analysis of the actinomycetes extract

The chloroform fraction of the actinomycetes extract was developed on a precoated silica plate (20x5cm, 0.25mm thickness) along with extract of control medium in chloroform: methanol (40:1v/v) as the solvent system.

The TLC tank was well equilibrated with the lower layer of solvent at the bottom and the filter paper was equilibrated for at least 16 hours prior to the development. 5⇑l of each of actinomycetes extract and extract of control media each of which dissolved in about 40⇑l of acetone was loaded on to TLC plate, making at least 1.5cm from the edge, 2cm from the bottom and 1cm between each spot with the help of a 5⇑l capillary. Blow drier was used to prevent the unwanted diffusion. Then, the plate was kept in a tank with the solvent system and developed until the solvent front reached 2/3 rd of the length of the plate. The solvent front was marked as soon as the plate was taken out of the solvent system. The plate was then dried and the spots were first visualized under UV light with the wavelength of 254nm. Then, the plate was sprayed with vanillin/sulphuric acid (1%w/v) reagent followed by heating (Cardellina, 1991). The plate was also observed 24 hours after the reagent was sprayed. R_f values were calculated for each spot obtained.

The movement of the analyte was expressed by its retardation factor, R_f:

R_f = Distance moved by analyte from origin/Distance moved by solvent from origin.

CHAPTER- V

5 RESULTS

5.1 Isolation and Identification of soil Actinomycetes

Four soil samples were collected from four different regions viz. ‘Chaurikharka’ (2900m), ‘Chaurikharka’ (2840m), ‘Feriche’ (4800m) and ‘Lobuche’ (5050m) of ‘Solukhumbu’ district.

After the collection and the transportation of soil to the laboratory, the isolation and identification of Actinomycetes was carried out following the standard methodology.

5.1.1 Isolation of Actinomycetes from soil

A total of 22 isolates of actinomycetes were isolated as given in the table 1. Each isolate was given an identification code and forwarded for the identification on the basis of the morphological and physiological characteristics.

5.1.2 Identification

A total of 22 isolates were identified on the basis of their morphological and physiological characteristics. Morphological method includes macroscopic and microscopic identification. Physiological method consists of various biochemical tests.

5.1.2.1 Morphological characteristics

Table 5 : Morphological characteristics of the isolates observed in the study

Isolate Number	Colony characteristics	Microscopy	Remarks
Ch1	Cream colored colonies with white aerial mycellium	Branched mycelium with spores in chains.	*Streptomyces* spp.
Ch 2	Cream colored substrate mycelium and gray colored aerial mycellium	Long branched mycelium with few chains of spores	*Streptomyces* spp.
Ch 3	Brown colonies with aerial mycelium developed at the center as white bloom.	Thin filaments with few single spores.	*Micromonospora* spp.
Ch 4	Cream substrate and off white aerial mycelium with grayish tinge.	Long straight on curve spore chains seen.	*Streptomyces* spp.
Ch 5	White colored colony which turns gray on maturity	Branching mycelium with several chains of spores.	*Streptomyces* spp.
Ch 6	Creamish pale colonies, which at later stage developed to white cottony colony	The aerial mycelium consists of long filaments bearing branches arranged in whorls.	*Streptoverticillium* spp.
Ch 7	Light brown colony, which turned to dark brown in	Thick filaments fragmenting at some	*Nocardia* spp.

	color at later stage.	places.	

Contd...

Ch 8	Pale cream-colored substrate mycelium with creamish white aerial mycelium.	Aerial mycelium was branched flexuously with few chains of spores.	*Streptomyces* spp.
Ch 9	White colored substrate mycelium with gray aerial mycelium.	Spore chains hooked i.e. open loops and irregular spirals	*Actinomadura* spp.
Ch 10	Creamish substrate with white aerial mycelium, which becomes gray when matured.	Mycelium was found to be fragmented and some bacteroid shaped fragmens seen.	*Nocardia* spp.
Ch 11	Pale cream substrate and white aerial mycelium.	Straight branching of filaments with spore chains.	*Streptomyces* spp.
X1	Cream colored colony which developed white colored aerial mycelium.	Branched mycelium with long spore chains.	*Streptomyces* spp.
X2	Forms dark gray substrate mycelium with white aerial;	Branched mycelium with chains of spiral spores.	*Streptomyces* spp.
Lo1	Pale orange colonies which are circular, convex, smooth	Spores scattered and few pieces of filaments seen.	*Micromonospora* spp.
Lo2	Pale off white circular colonies.	Flexuous mycelium with spore chains of varying length.	*Streptomyces* spp.

Contd...

Lo3	Pale off white aerial appearing chalky with pale substrate mycelium.	Branched mycelium with long spore chains.	*Streptomyces* spp.
G1	White colored smaller colonies.	Few numbers of longer mycelium observed	Not identified
Q1	Light yellow colored colony	Only long, branched, tuff of mycelium observed.	Not identified
I2	Cream colored small sized colony.	Very thin and few mycelium observed	Not identified
I9	Yellow colored colony	Only few short mycelium were found to be scattered.	Not identified
C2	Cream colored substrate and white aerial mycelium	Spores scattered and few pieces of filaments seen.	*Micromonospora* spp.
C4	Cream colored substrate mycelium with gray aerial mycelium	Branched filaments with short spore chains.	*Streptomyces* spp.

As given in above table out of 22 isolates, highest frequency of isolates i.e 11 were identified as *Streptomyces* spp., 3 were identified as *Micromonospora* spp., 2 were identified as *Nocardia* spp. and only one of each were identified as *Streptoverticillium* spp. and *Actinomadura* spp. whereas 4 isolates could not be identified due to limited information and examining facility.

5.1.2.2 Biochemical and Physiological characteristics

The isolates were subjected to different biochemical and physiological test and results are given below:

Table 6: Biochemical characteristics of the isolates

Isolate number	Tests performed														
							Acid from carbohydrate								
	Starch hydrolysis	Caesin hydrolysis	Gelatin hydrolysis	Tween 20 hydrolysis	Urease test	Nitrate reduction	Arabinose	Fructose	Galactose	Glucose	Lactose	Maltose	Mannose	Raffinose	Sucrose
Ch1	+	-	+	+	+	-	+	+	-	+	-	+	+	+	+
Ch2	+	-	+	+	-	-	+	+	+	+	+	+	+	+	+
Ch3	+	-	+	+	-	-	-	+	-	+	+	+	+	-	+
Ch4	+	+	+	+	-	+	-	+	-	+	-	-	-	-	-
Ch5	+	-	-	+	+	-	-	+	-	-	-	+	-	+	+
Ch6	+	-	-	+	-	+	+	+	+	+	-	+	+	-	-
Ch7	+	-	+	+	-	-	+	+	+	+	-	+	+	+	+
Ch8	-	+	+	+	-	+	+	-	-	-	-	+	+	-	+
Ch9	-	-	+	+	+	-	+	-	-	+	-	-	+	-	-
Ch10	+	+	-	-	+	+	-	+	-	+	+	+	-	-	+
Ch11	-	+	+	+	-	+	-	+	+	+	-	+	-	-	+
X1	+	-	+	+	-	-	-	+	+	+	+	+	+	+	+
X2	-	+	+	+	-	+	-	+	+	+	+	-	-	-	+
Lo1	+	-	+	-	+	-	+	+	+	+	+	+	+	-	+
Lo2	+	+	+	+	-	-	-	+	+	+	+	+	+	-	+
Lo3	+	+	-	+	-	-	-	+	+	+	+	+	+	+	+
G1	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+
Q1	-	-	+	+	+	+	+	+	+	+	-	+	+	-	+
I2	+	-	-	-	-	+	+	+	+	+	-	+	+	-	-
I9	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
C2	-	-	-	+	+	-	+	-	+	-	-	+	-	-	-
C4	-	-	+	+	-	+	-	+	+	+	-	-	-	+	+

+ = Positive reactions

- = Negative reactions

Table 7: Physiological tests of the isolates

Isolate number	Salt tolerance test			Growth at 50îC
	5%	7%	10%	
Ch1	-	-	-	-
Ch2	-	-	-	-
Ch3	-	-	-	-
Ch4	-	-	-	-
Ch5	-	-	-	-
Ch6	+	-	-	-
Ch7	-	-	-	-
Ch8	-	-	-	-
Ch9	-	-	-	-
Ch10	-	-	-	-
Ch11	-	-	-	-
X1	-	-	-	-
X2	-	-	-	-
Lo1	-	-	-	-
Lo2	+	+	+	-
Lo3	-	-	-	-
G1	-	-	-	-
Q1	-	-	-	-
I2	+	-	-	-
I9	-	-	-	-
C2	-	-	-	-
C4	-	-	-	-

+ = Positive reactions

- = Negative reactions

5.2 Screening of Actinomycetes possessing antimicrobial activity

After the identification of the isolates, those 13 isolates were subjected to the screening for their antimicrobial activities.

5.2.1 Screening for antibacterial activity

The 13 isolates were subjected to antibacterial screening by perpendicular streak method on NA. The fresh (24 hours) culture of bacteria viz. *Bacillus subtilis, Staphylococcus aureus, Enterobacter* spp., *Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, Pseudomonas aeruginosa, Citrobacter* spp., *Salmonella paratyphi* A *and Shigella* spp. were used for studying inhibitory properties of the Actinomycetes isolates. Most of the test organisms were obtained from the pathological laboratory of Tribhuban University Teaching Hospital (TUTH) while few were found to be preserved in the Labolatory of NAST. The inhibitory effect is indicated by distance between the perpendicular growth of test organisms and isolates in millimeters (mm.).

Table 8 Screening of the identified isolates of actinomycetes for their inhibitory activities against bacteria

Isolate number	Zone of inhibition (mm)									
	Bacillus subtilis	***Citrobacter* spp.**	***Enterobacter* spp.**	***Escherichia coli***	***Klebsiella pneumoniae***	***Proteus vulgaris***	***Pseudomonas aeruginosa***	***Salmonella paratyphi A***	***Shigella* spp.**	***Staphylococcus aureus***
Ch1	-	-	-	-	-	-	-	-	-	-
Ch2	11	-	-	-	-	-	-	-	-	-
Ch3	-	3	-	-	-	-	-	4	3	-
Ch4	-	-	-	-	-	4	-	-	-	-
Ch5	-	-	-	-	-	-	-	-	-	-
Ch6	10	-	-	-	-	-	-	-	-	-
Ch7	7	-	-	3	-	-	-	-	-	-
Ch8	-	3	-	-	-	3	-	-	-	-
Ch9	14	-	-	5	-	4	-	-	-	-
Ch10	-	-	-	-	-	3	-	-	-	-
Ch11	-	-	-	-	-	-	-	-	-	-
X1	16	-	-	-	-	-	-	-	-	-
X2	8	-	-	-	-	-	-	-	-	-
Lo1	-	-	-	-	-	-	-	-	-	-
Lo2	3	-	-	-	-	-	-	-	-	3
Lo3	-	-	3	-	-	4	-	-	-	4
C2	3	-	-	-	-	-	-	3	3	-
C4	12	5	6	-		-	-	-	-	-

Out of 18 isolates, isolate number Ch3, Ch9, C4, C2, Lo3 showed inhibitory activities against three different types of bacteria whereas isolate number Ch1, Ch5, Ch11 and Lo1 didn't showed inhibitory activities against all types of bacteria which were used during the test. Highest zone of inhibition was shown by isolate number X1 against *Bacillus subtilis.* Results are shown in above table.

5.2.3 Screening for antifungal activity

The 13 of the isolates were also subjected to the screening of their antifungal activities according to the dual culture method on PDA**.**

The fresh cultures of plant pathogenic fungi viz. *Alternaria* spp.*, Fusarium oxysporum, Fusarium proliferum, Geotricum* spp.*, Gloeosporium sporioides, Helminthisporium* spp. and *Scleretonia* spp. were used for studying antagonistic or inhibitory properties of the actinomycetes isolates. The isolates were noted as highly inhibitory (+++), moderately inhibitory (++), low inhibitory (+) and not inhibitory (-) to the plant pathogenic fungal colony.

Table 9 Screening of the identified isolates of actinomycetes for their inhibitory activities against plant pathogenic fungi

Isolate	**Test plant pathogenic fungal organism**

number	*Alternaria* spp.	*Fusarium oxysporum*	*Fusarium moniliforme*	*Geotricum* spp.	*Gloeosporium* spp.	*Helminthosporium* spp.	*Sclerotonia* spp.
Ch1	-	-	-	-	-	-	-
Ch2	+	-	-	-	-	+	-
Ch3	++	-	+	-	-	++	+
Ch4	++	+	++	+	-	+	-
Ch5	-	-	+	+	-	+	-
Ch6	-	+	+	+	-	-	+
Ch7	+	-	-	+	-	+	+
Ch8	+	+	-	++	-	++	-
Ch9	-	+	-	+	-	+	-
Ch10	++	+	-	+	-	+	-
Ch11	-	-	-	-	-	-	-
X1	++	+++	+++	++	+	+++	+++
X2	+	-	+	+	-	++	+++
Lo1	-	+	+	+	-	++	+
Lo2	+	+	+	-	+	+	+
Lo3	+	-	-	-	-	++	-
C2	+	+	+	-	-	+++	-
C4	+	++	+	-	-	+	-

Where+++ = Highly inhibitory

++ = Moderately inhibitory

+ = Low inhibitory

- = Not inhibitory

Among 18 isolates of actinomycetes, isolate number X1 showed highest inhibitory activities against all the fungi used during the test. Isolate number Ch1 didn't show any inhibitory activities against all speceies of fungi used during the test.

5.3 Extraction of secondary metabolites of a priority isolates of anctinomycetes

The isolate, X1, *Streptomyces* spp., Ch 9, *Actinomadura* spp., and C4, *Streptomyces* spp were selected for the purpose of fermentation and extraction of bioactive secondary metabolite because X1 showed largest zone of inhibition in case of both bacteria and fungi and showed broad spectrum of activity against fungi. C4 was chosen because of its broad spectrum of activity i.e. the organism showed good activity against gram-negative bacteria too, while screening for its antibacterial property. Ch 9 was chosen because it showed quite good activity against both bacteria and fungi and according to Leeuwenhoek, 2001 *Actinomadura* spp. is one of the rare actinomycetes and less work is done on its activity against fungi. According to Hegde *et al*., 1992, three novel antifungal antibiotics, Sch 38518, Sch 39185 and Sch 38516 were isolated from the fermentation broths of *Actinomadua* spp.

The fermentation of the isolates was carried out in 1liter Erlenmeyer flask containing 500 ml starch casein broth, including 100ml inoculum. It was fermented at 28⌈C for 7 days. After fermentation the broth was twice extracted by equal volume of chloroform. Each of the culture media in the flasks was processed separately and finally dry weight of the extract was determined and average dry weight was recorded. The control media was also processed in the similar manner**.**

Table 10 Extraction of secondary metabolite and yield per 500 ml of starch casein broth

Isolate number	Dry weight of extract (mg/500ml)	Average dry weight (mg/500ml)
C (Control)	18.30 14.15	14.90

	12.27	
X1: Flask 1	20.5	
Flask 2	46.5	33.5
Ch 9: Flask 1	36.7	
Flask 2	19.5	28.1
C4: Flask 1	13.1	
Flask 2	28.01	20.55

Out of 3 isolates of actinomycetes used for extraction of secondary metabolites, highest yield was given by the isolate number X1 i.e. 33.5 mg/500ml and least yield was given by the isolate number C4 i.e. only 20.55 mg/500ml of starch casein broth.

5.4 Study of antimicrobial activity of the actinomycetes extract extracted with chloroform

About 20.5 mg of the extract of X1, 19.5 mg of the extract of Ch9 and 13.1 mg of the extract of C4 was dissolved in 300⇑l of DMSO each separately so that the final concentration of each extract could be 20.5mg/300⇑l, 19.5mg/300⇑l and 13.1mg/300⇑l respectively. Similarly, 18.30 mg and 14.15 mg of the control media extract was also dissolved separately each in 300⇑l of DMSO so that the final concentration of the extract could be 18.30mg/300⇑l and 14.15mg/300⇑l respectively. During the study of antimicrobial activity of the extracts by Agar well diffusion method, 50⇑l of the extract dissolved in DMSO was loaded into a well on media plate. Similarly, 50⇑l of control media extract and solvent (DMSO) each were loaded separately into two wells on the same media plate inoculated with the test organism for assaying both the antibacterial and antifungal activity. The results confirmed that the extract of control media and solvent themselves were not inhibitory to the test organisms and the bioactivity have been solely due to the bioactive secondary metabolite present in the actinomycetes extract.

Table 11 Potency of the extracts for antibacterial activities

Code	Source of	Dry wt of	Vol. Of solvent in	Vol. Loaded	Diameter of zone of inhibition (mm)

	extract	extract used (mg)	dissolving extract	into the well	*Bacillus* spp.	*E.coli*	*Enterobacter* spp.
E (X1)	Culture broth	20.5	300⇑l of DMSO	50⇑l	-	12	20
C	Control media	18.30	300⇑l of DMSO	50⇑l			
S (solvent)				50⇑l			
E (Ch9)	Culture broth	19.5	300⇑l of DMSO	50⇑l	-	17	15
C	Control media	18.30	300⇑l of DMSO	50⇑l			
S (solvent)				50⇑l			
E (C4)	Culture broth	13.1	300⇑l of DMSO	50⇑l	17	17	-
C	Control media	18.30	300⇑l of DMSO	50⇑l			
S (solvent)				50⇑l			

Where - = No zone of inhibition

Diameter of the well = 6mm

Height of the well = 4mm

Among 3 extracts of three different isolates, highest zone of inhibition was given by the extract of X1 against the bacteria pathogen *Enterobacter* spp.

Table 12 Potency of the extracts for antifungal activities

Code	Source of extract	Dry wt of extract used (mg)	Vol. of solvent in dissolving extract	Vol. Loaded into the well	Diameter of zone of inhibition (mm)		
					Alternaria spp.	*Fusarium oxysporum*	*Sclerotinia* spp.
E (X1)	Culture broth	20.5	300⇑l of DMSO	50⇑l	19	37	30
C	Control media	14.15	300⇑l of DMSO	50⇑l			
S				50⇑l			

(solvent)							
E (Ch9)	Culture broth	19.5	300⇑l of DMSO	50⇑l	-	-	15
C	Control media	14.15	300⇑l of DMSO	50⇑l			
S (solvent)				50⇑l			
E (C4)	Culture broth	13.1	300⇑l of DMSO	50⇑l	12	-	15
C	Control media	14.15	300⇑l of DMSO	50⇑l			
S (solvent				50⇑l			

Where - = No zone of inhibition

Diameter of the well = 6mm

Height of the well = 4mm

Among three extract of three different isolates, highest zone of inhibition was given by the extract of X1 against the fungal plant pathogen *Fusarium oxysporum.*

5.5 TLC analysis of the actinomycetes extract

All three extracts and extract of control media were made to run on precoated silica plate (20x10 cm, 0.25mm thickness). 46.5 mg of the extract of X1, 36.7mg of the extract of Ch9 and 28.01 mg of the extract of C4 were made to dissolve in 40⇑l of acetone; and 12.27 mg of the extract of control media was also dissolved in 40⇑l of acetone, so that the concentration of the extract of X1, extract of Ch9, extract of C4 and extract of control media loaded would be 46.5mg/40⇑l, 36.7mg/40⇑l, 28.01mg/40⇑l and 12.27mg/40⇑l respectively. Small amount of each of them was loaded with the help of capillary tube on a precoated silica and run using chloroform: methanol (40:1) as the solvent system. The solvent front was marked at 18cm. The plate was then dried and the spots were visualized under UV light with the wavelength of 254nm. The control extract gave no spots; the extract of X1 gave two spots having R_f of 0.58 and 0.66; extract of Ch9 gave a single spot having R_f of 0.79; and extract of C4 also gave single spot having R_f of 0.66. The fluorescence color of the spots was blue. Then, the plate was sprayed with vanillin/sulphuric acid (1% w/v) reagent followed by heating.

Table 13 R_f values of the spots obtained on the TLC analysis

Analyte	Number of spots	Solvent front (cm)	Distance moved by the spots (cm)	R_f
X1crude extract	2	18	10.5, 12	0.58, 0.66
Ch9 crude extract	1	18	14.3	0.79
C4 crude extract	1	18	12	0.66
Control extract	-	18	-	-

Where R_f = distance moved by analyte from origin/distance moved by solvent from origin

\- = No spot obtained on TLC

CHAPTER-VI

6. DISCUSSION AND CONCLUSION

6.1 Discussion

Although the quantitative and qualitative population of actinomycetes is highest in the soil in comparison to other habitats, the soil amendments, its properties greatly reflect their number and type. Antibiotic producing actinomycetes being aerobic in nature are abundant in upper zone not below 5-6 inches. Most antibiotic producers are commonly associated with rhizosphere of plants (Whaley and Boyle, 1967). Quantitatively, actinomycetes number is higher in grassland soil and least in forest soils, with field soils intermediate. However, population of natural habitats can be altered by man's activities. Indiscriminate use of fertilizers and pesticides can bring about changes in proportion of microorganisms and reduction in number, respectively. Sometimes, introduction of alien substrate to natural habitats may inadvertently causes rarer microbes to predominate. Isolation of actinomycetes has been always encountered with difficulties since they have certain deficiencies in comparison to their competitors, bacteria and fungi (Williams and Cross, 1971).

In Nepal, few research works in isolation, identification and antimicrobial activities of Actinomycetes has been done in Central Department of Microbiology, Tribhuvan University. Tamrakar (1998) studied antimicrobial activities of Actinomycetes isolated from soils of Kathmandu valley then Singh (1999) did a study on Antimicrobial activity of Actinomycetes isolated from various geographical region of Nepal and characterization of their antibacterial agents. Similarly, Pandey (2004) studied antibacterial activity of Actinomycetes isolated from Khumbu region of Nepal.

The main objective of this work was the study of biodiversity and bioactivity of soil actinomycetes of the soil from unique environmental settling of 'Chaurikharka' (altitudes 840m. and 2900m), 'Feriche' (altitude 4800m) and 'Lobuche' (altitude 5050m) at 'Solukhumbu' district.

Soil samples from each site were collected following standard sampling procedure and transported to Natural Product Research Laboratory at NAST. Soil samples were then air dried and ground with the help of mortar and pestle as done by Tamrakar (1998).

In general, actinomycetes grows on ordinary laboratory media, but their growth is usually slower than that of ordinary bacteria. A division cycle in actinomycetes may take 2-3 hour as compared with 20min for *E.coli*. Choice of better medium would virtually encourage the growth of actinomycetes. Generally, the best carbon sources are glycerol, starch and chitin, with casein, asparagines and arginine as organic nitrogen (Williams and Cross, 1971).

Glycerol-asparagines agar medium was designed during isolation of actinomycetes by spread plate technique, preparing dilutions of soil in water. Xu *et al*., (1996) also used glycerol-asparagine agar for isolation of actinomycetes from soil of Yunnan, China employing spread plate technique.

Actinomycetes generally takes 7-14 days to develop mature aerial mycelium with spores thus the dilution plates were at least incubated for 7 days, up to which colony counts were made. For more pronounced growth the plates were incubated for 3 more days (that makes total of 10 days incubation). Lengthy incubation time can result in the evaporation of the medium, so thick agar plates were employed.

A total of 22 isolates, 11 isolates from soil of 'Chaurikharka' (altitude 2840m), 5 isolates from soil of 'Chaurikharka'(altitude 2900m), 4 isolates from soil of 'Lobuche' (altitude 5050m) and 2 isolates from soil of 'Feriche' (altitude 4800m) were isolated. Though the difference in isolate number was not large, comparatively, highest number was recovered from 'Chaurikharka' (altitude 2840m) might be because it was the fresh sample while others were brought to the laboratory more than a year before. Lowest numbers of isolates were recovered from soil of 'Feriche'.

Streptomyces spp., which has been the best recognized genera of actinomycetes for its wide distribution in nature, harbours a great number of the most important producers of antibiotics and other secondary metabolites from pharmaceutical and economical point of

view (Waksman and Henrici, 1943). *Bergey's manual* of determinative Bacteriology (Buchanan and Gibbons, 1974) listed 463 species of *Streptomyces*. *Streptomyces* are differentiated from closely related groups on the basis of spore chain morphology, pigmentation and ecological requirements (Jensen, 1943). They form long chains of arthrospores on aerial mycelium and occasionally on mycelium unlike some closely related genera such as *Streptoverticillium, Microellobosporia, Actinosporangium* which do not form spore chains on substrate mycelium (Gibbs and Shapton, 1968). *Micromonospora,* unlike *Streptomyces* are less abundant in soil and even slow grower. The characterstic features of the *Micromonospora* that differ from other genera are absence of aerial mycelium and only single spores are borne on substrate mycelium, which may branch.

Out of 22 isolates, 18 isolates could be identified up to genus level. Microscopic examination of the cover slip culture of the isolates revealed that most of the isolates belong to the genus *Streptomyces* (11) followed by *Micromonospora* (3), *Nocardia* (2), *Streptoverticillium* (1) and *Actinomadura* (1). They were distinguished from other genera on the basis of the characteristic spore chain formed on the aerial mycelium and few substrate mycelium. The cover slip culture technique was also carried out by Pandey(2004), to study the morphology of soil actinomycetes.

Biochemical tests (Table 6) showed that *Streptomyces* spp. hydrolyse tween 20. Most of them hydrolyse starch, casein and gelatin. Some of them are can reduce nitrate to nitrite and few ware urease positive. The pattern of acid production from carbohydrate was variable. Most of them contributed in production of acid from fructose, glucose maltose and sucrose while very few were capable of producing acid from arabinose. According to physiological tests (Table 7) only one isolate belonging to genera *Streptomyces* (Lo1) was able to grow in presence of 5%, 7% and also10% (w/v) sodium chloride in the medium. No growth has been found at 50⌈C, which indicates that thermophilic *Streptomyces* have not been isolated.

Micromonospora species were found to be variable in hydrolyzing starch, gelatin and tween 20 though two out of three were capable of hydrolyzing starch, gelatin and tween

20. No isolates were able to contribute in casein hydrolysation and nitrate reduction. They were unable to tolerate even 5% (w/v) sodium chloride.

Nocardia species were found to hydrolyse starch. Other characterstics were variable. They were also not able to tolerate even 5% (w/v) sodium chloride.

Actinomadura species was found to hydrolyse gelatin and tween 20. It was found to be urease positive. The isolate could produce acid from arabinose, glucose and mannose. It was not able to tolerate even 5% (w/v) sodium chloride.

Isolate belonging to *Streptoverticillium* species was able to hydrolyse starch and tween 20. It was found to reduce nitrate to nitrite. It could produce acid from arabinose, fructose, galactose, glucose, maltose and mannose. It could grow in presence of 5% (w/v) sodium chloride in medium.

Although various biochemical tests were performed, it was unable to identify the actinomycetes up to species level and few isolate even up to genera level due to lack of other tests. According to Kuster (1968) for proper identification of genera and species of actinomycetes, besides morphological and physiological properties, various other biochemical properties such as cell wall chemotype, whole-cell sugar pattern, peptidoglycan type, phospholipids type and G+C% of DNA should be determined.

Screening of actinomycetes for antibacterial property was carried out in Nutrient agar by perpendicular streak method also called cross streak method, while for antifungal property screening was carried out in PDA by dual culture method. The previously used crowded plate screening technique crowded plate screening technique allow screening directly from the dilution plate, since colony, around which inhibition zone was noted would be considered active and selected. The drawback of this technique is that the activity is given against unknown organisms. The use of cross streak method, an improved technique, allow numerous test organisms for antibacterial activity to screen on the same plate where actinomycetes to be screened has been grown. Cross streak method is useful for qualitative screening in determining the activity of isolate against specific test organisms (Haque *et al.*, 1995). In dual culture method colony interaction between

the test fungal plant pathogen and the actinomycetes giving zone of inhibition was considered as the indication of their antifungal activity. The use of dual culture method for antifungal activity provides isolated environment for isolate and test organism and provides sufficient incubation period for growth, minimizing the disturbance from fast growing organism. Dual culture method is easy and rapid method for screening or in vitro selection of antagonist for the purpose of biocontrol of plant pathogenic fungi.

Out of 18 actinomycetes subjected for screening process, only 14 showed antibacterial activity while 16 showed antifungal activity against test organisms. 0f the 14 isolates showing antibacterial activity, 5 were active against gram positive bacteria, 4 against gram negative bacteria and 5 against both gram positive and gram negative bacteria. Among them 9 isolates were active against *Bacillus subtilis,* 2 against *Enterobacter* spp., 3 against *Citrobacter* spp., 2 against *E.coli*, 5 against *Proteus vulgaris,* 2 against *Salmonella paratyphi* A, 2 against *Shigella* spp. and 2 against *Staphylococcus aureus.*

Of the 16 isolates showing antifungal activity, eleven isolates were found to be inhibitory towards the plant pathogenic *Alterneria* spp. but none of them showed potent inhibition against the fungi i.e. none of them were found to be highly inhibitory. Similarly, ten of the identified isolates were inhibitory towards plant pathogenic *Fusarium oxysporum* out of which X1 was found to be highly inhibitory. Similarly ten of the identified isolates were inhibitory towards plant pathogenic *Fusarium moniliforme* out of which X1 was found to be highly inhibitory. Similarly, ten of the identified isolates were inhibitory towards plant pathogenic *Geotricum* spp. but none of them possessed highly inhibitory activity. Similarly only two of the identified isolates were inhibitory towards plant pathogenic *Gloeosporium* spp. while none were able to show potent inhibition against the fungi. Similarly, fifteen of the identified isolates were inhibitory towards plant pathogenic *Helminthosporium* spp. out of which X1 and C2 were highly inhibitory. Seven of the identified isolates were inhibitory towards plant pathogenic *Sclerotinia* spp. out of which X1 and X2 were highly inhibitory.

The observation showed that actinomycetes possessed maximum inhibitory activities against *Helminthosporium* spp. The isolate that was able to show highly inhibitory activities against maximum number of plant pathogenic fungi was X1.

The result of the antibacterial screening reveals that most of the active isolates were active against gram positive bacteria (*Bacillus subtilis)* than gram negative bacteria. The reason for different sensitivity between gram positive and gram negative bacteria could be ascribed to the morphological differences between these microorganisms, gram negative bacteria having an outer polysaccharide membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to lipophilic solutes, while porins constitute a selective barrier to the hydrophilic solutes with an exclusion limit of about 600 Da (Nikaido and Vaara, 1985). The gram positive should be more susceptible having only an outer peptidoglycan layer which is not an affective permeability barrier (Scherrer and Gerhardt, 1971).

Maximum zone of inhibition (ZOI) in antibacterial screening was 16 mm for *Bacillus subtilis.* Singh, (1999) found maximum zone of inhibition of 44 mm for *E. coli*, and 70mm for *S. aureus* while screening the actinomycetes isolated from different parts of the country. Pandey, (2004) found maximum zone of inhibition of 7mm for *E. coli,* 11mm for *S. aureus* and 7 mm for *Proteus.*

In this study, soil actinomycetes, X1 (*Streptomyces sp.)* has also shown good amount of bioactivity against plant pathogenic *Fusarium oxysporum* and *Fusarium moniliforme*, which is believed to be due to the production of novel bioactive secondary metabolite.

As less amount of extract was recovered after fermentation and the number of test organisms was quite high in screening process in my study, only three test bacteria and three test plant pathogenic fungi were selected for antimicrobial test on the basis of the antimicrobial activity during screening process.

Three isolates, X1 (*Streptomyces sp.),* Ch9 *(Actinomadura sp.*) and C4 (*Streptomyces sp.*) were chosen for carrying out the fermentation process. The fermentation of the priority isolates was carried out in Starch-casein broth for three weeks at 28 ˚C. After three

weeks, the culture broth was filtered and extracted with the equal volume of chloroform two times. The fermentation was carried out in two separate flasks for each isolates and processed separately. Finally dry weight of the extract was determined which is mentioned in table 6. The average dry weight of the X1, Ch9 and C4 extract was 33.5, 28.1 and 20.55 respectively.

Then after, 20.5mg of X1 extract, 19.5mg of Ch9 extract and 13.1mg of C4 extract was dissolved in 500ml of DMSO each separately and the antimicrobial activity of the extract was studied qualitatively by Agar well diffusion method. The X1 extract gave ZOI of 12mm against *E. coli* and of 20mm against *Enterobacter* sp. but no ZOI against *Bacillus* sp. during antibacterial test, while on subjecting to antifungal activity X1 extract gave ZOI of 19mm against *Alternaria* sp., 37mm against *Fusarium oxysporum* and 30mm against *Scleretonia* sp. Similarly, Ch9 extract gave ZOI of 17mm against *E. coli* and 15mm against *Enterobacter* sp. but no ZOI against *Bacillus* sp. in antibacterial test, while during antifungal activity test Ch9 gave ZOI of 15mm against *Sclerotinia* sp. but no ZOI was given against *Alternaria* sp. and *Fusarium oxysporum.* Finally, C4 extract gave ZOI of 17mm against *Bacillus* sp. and *E. coli* and no ZOI against *Enterobacter* sp. in antibacterial test and gave ZOI of 12mm against *Alternaria* sp. and 15mm against *Sclerotinia* sp. but no ZOI was observed against *Fusarium oxysporum* during antifungal activity test.

The extracts were made to run on a pre-coated silica plate along with control extract using chloroform: methanol (40:1) as the solvent system in which extract of X1 gave two spots having R_f of 0.58 and 0.66, extract of Ch9 gave single hazy and larger spot having R_f of 0.79 and C4 also gave a single hazy and larger spot with R_f 0.66. The spots in the TLC plates were light colored, larger and not distinctly separated which might be due to the presence of very low concentration of antimicrobial secondary product.

As the inhibitory activity against bacteria as well as fungi in antimicrobial activity test signifies the presence of antimicrobial metabolite in the extract. The visualization of florescent spot under UV light even emphasizes the presence of such antimicrobial

metabolite in the extracts. Hence, it can be concluded that all the three extract from three different isolates possessed such antimicrobial metabolite.

7.2. CONCLUSION

Hence, a total of 22 isolates were isolated from four soil samples of 'Solukhumbu' district. Eighteen of the isolates were identified and were subjected to screening procedure for their antimicrobial and antifungal properties. 3 isolates were found to be potent in screening procedure were subjected to fermentation in Starch Caesin Broth. Then the fermented broth was filtered. The culture free supernatant was thendouble extracted with equal volume of chloroform. Then after, antimicrobial property of the chloroform extract of the identified isolates was observed. Extract of isolate X1 was found to be most potent against both bacterial pathogen and fungal plant pathogen. Three of the identified isolates showing better antimicrobial property were subjected to TLC using chloroform: methanol (40:1) as the solvent system. The visualization of florescent spot under UV light also confirms the presence of the bioactive compound in the chloroform extract of the isolates.

CHAPTER VII

7. SUMMARY AND RECOMMENDATION

7.1 Summary

1. **Collection of Soil sample:** Four soil samples from four different places of 'Solukhumbu' district were collected randomly, in fresh sterile polyethylene bag and transported to Natural Product Research Laboratory at NAST.

2. **Isolation:** After air-drying the soil samples, it was subjected to serial dilution in sterile distilled water. Isolation of actinomycetes from soil samples was carried out by spread plate technique in Glycerol Asparagine agar and a total of 22 isolates were obtained.

3. **Identification:** A total of 22 isolates were identified following standard morphological and biochemical methods. Only 18 of them could be identified, where majority of the isolates belonged to the following genera *Streptomyces* (11), *Micromonospora* (3), *Nocardia* (2), *Actinomadura* (1) and *Streptoverticillium* (1).

4. **Screening of actinomycetes possessing antimicrobial activity:** Antimicrobial activity of the identified actinomycetes was observed against both bacteria and fungi. Screening for antibacterial property was done on nutrient agar by perpendicular streak method. Out of 18 identified isolates only 14 showed the activity against the test bacteria. Of the 14 active isolates 5 were active against gram positive only, 4 against gram negative and 5 against both gram positive and gram negative bacteria. Antifungal property of actinomycetes, which has been observed for the first time in Nepal, was carried out on PDA plates by dual culture method. Among 18 identified isolates of actinomycetes 1 isolate showed highest inhibitory activities against all the fungi used during the test, where as 2 isolate didn't show any inhibitory activities against all the species of fungi used.

5. **Fermentation:** Three isolates, which were observed to be potent in screening procedure were selected for the extraction of bioactive secondary metabolite(s) and were subjected to the fermentation procedure. The fermentation was carried out in Starch-Casein broth and bioactive secondary metabolite(s) was extracted by solvent extraction method using equal volume of chloroform twice. The average dry weight of extract after the evaporation in rotary vacuum evaporator was recorded to be 33.5 mg/500ml from isolate X1 (*Streptomyces* spp.), 28.1 mg/500ml from isolate Ch9 (*Actinomadura* spp.) and 20.55 mg/ 500ml from isolate C4 (*Streptomyces* spp.).

6. **Antimicrobial activity of the actinomycetes extract:** The extract was forwarded for the study of its antimicrobial activity by Agar well diffusion method. In antibacterial

activity test largest ZOI was given by X1 against *Enterobacter* spp. In antifungal activity test largest ZOI was given by X1 against *Fusarium oxysporum.*

7. **TLC analysis:** All the three actinomycetes extracts and the extract of the control media were made to run on a precoated silica plate using chloroform: methanol (40:1) as the solvent system. Extract of X1 gave two spots with R_f 0.58 and 0.66, extract of C9 gave single spot having R_f 0.79 and C4 gave single spot having R_f value 0.66, however, control gave no spots. The fluorescence of the spots was blue.

   In this study, though the chromatogenic analysis confirms the presence of bioactive secondary metabolite(s) in the actinomycetes extract, the exact chemical nature of the compounds of the extract could not be identified. As for the isolation and identification of the compounds of the extracts, it is necessary to obtain in pure form, which require series if purification procedures like Column Chromatography, High performance Liquid Chromatography, Preparative TLC and Identification processes like Co-chromatography, Melting point analysis, Infrared Spectroscopy, Nuclear magnetic Resonance Spectroscopy (NMR), Mass Spectrometry etc.

7.2 Recommendation

- Here, soil actinomycetes were identified only up to genus level and some were left unidentified. So, further identification up to the species level and also the identification of the unidentified ones are stressed, which may require standard morphological and molecular biological methods.
- The medium optimization to increase the yield of the extract is recommended.
- In this study, only chloroform was used as the solvent for the extraction. Other active compounds insoluble in chloroform could not be extracted with chloroform. So, other solvents may also be used.
- More varieties of target culture can be used for the study of antimicrobial activity of the extract.
- MIC determination of the bioactive secondary metabolite(s) is recommended.

- Purification, characterization and structure elucidation of the extract.
- Invivo test of the extract is also recommended.

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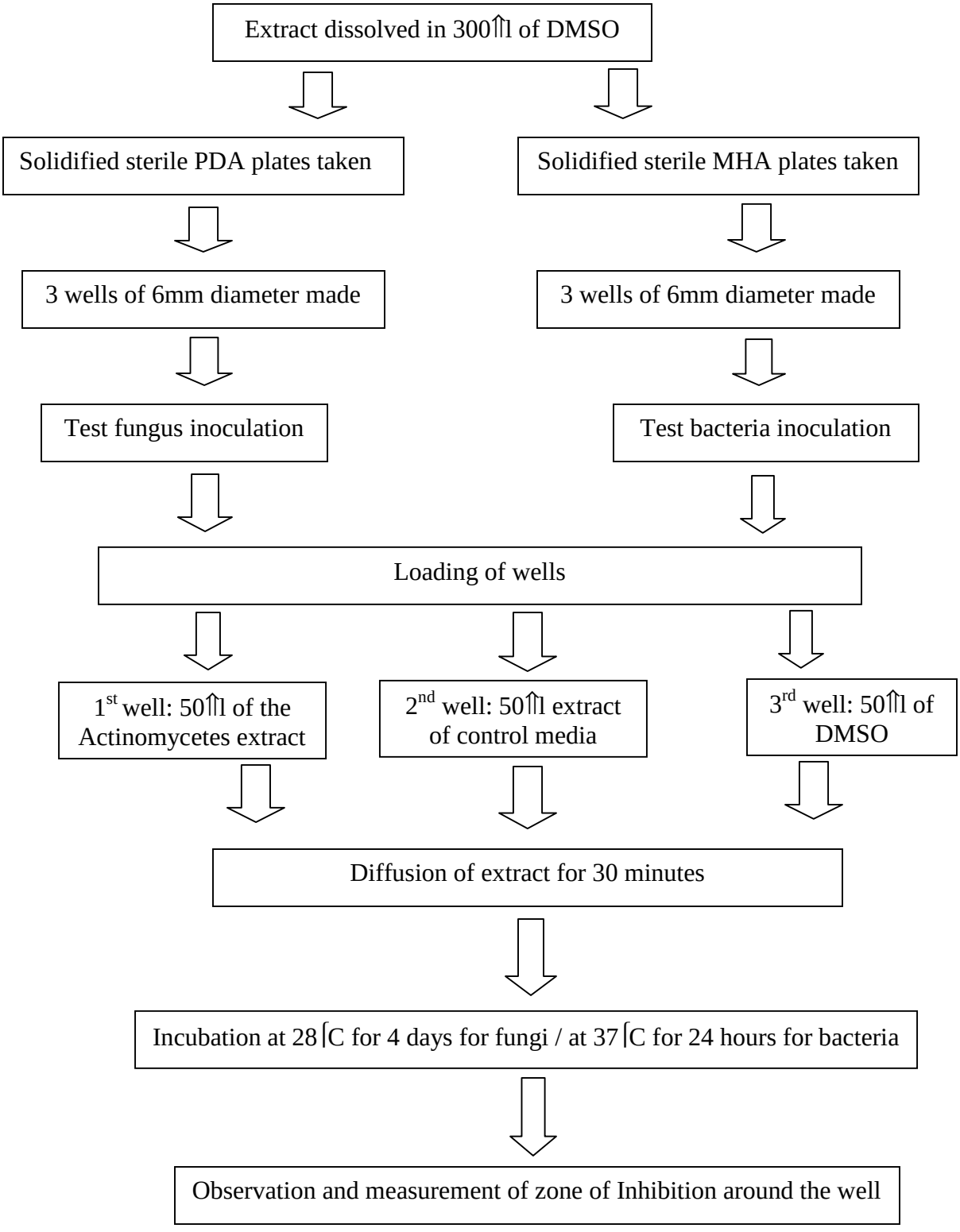
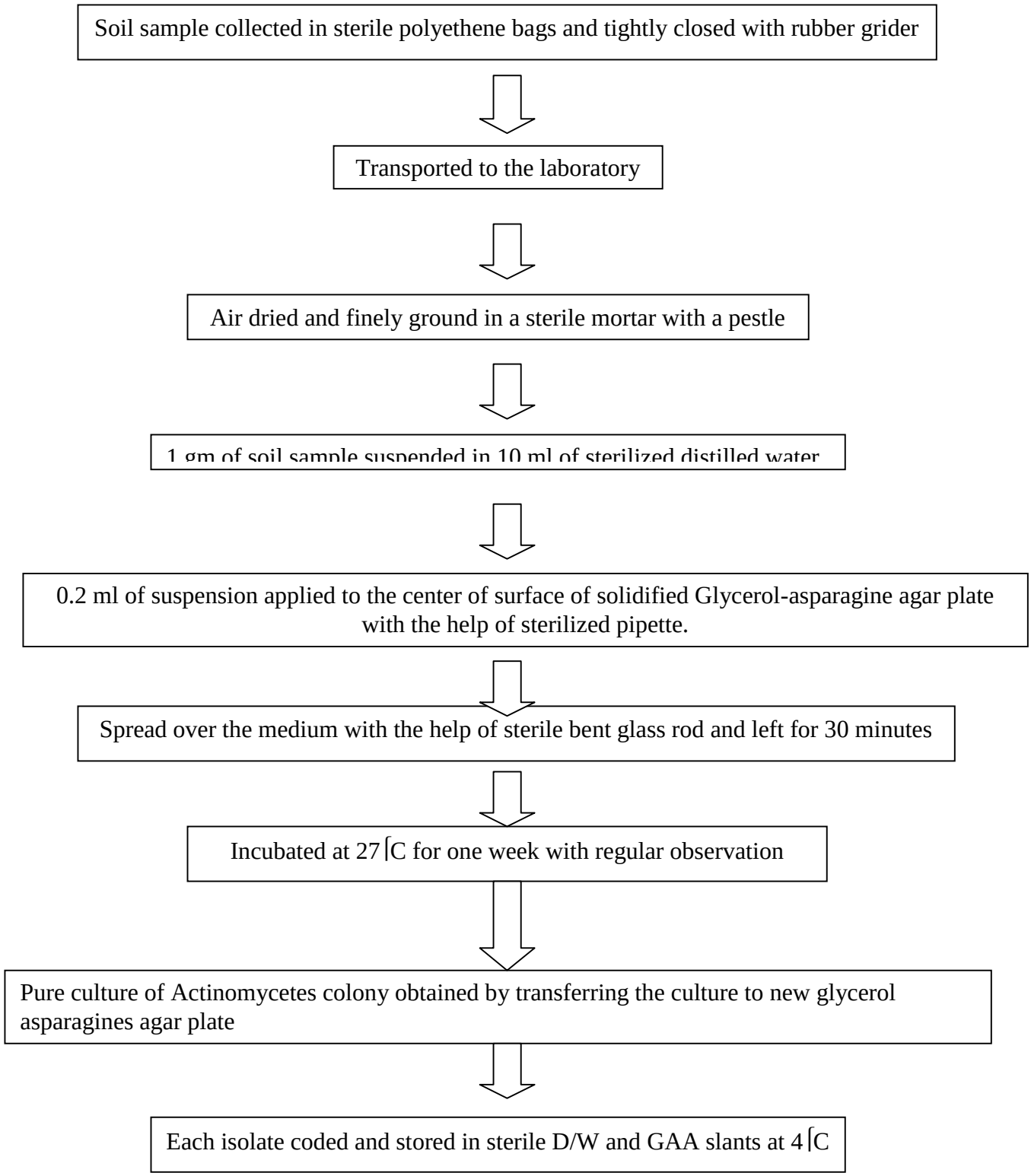


Figure 2 Schematic diagram for the study of antifungal and antibacterial activity of the extract of the Actinomycetes isolate.


Soil sample collected in sterile polyethene bags and tightly closed with rubber grider
Transported to the laboratory
Air dried and finely ground in a sterile mortar with a pestle
1 gm of soil sample suspended in 10 ml of sterilized distilled water
0.2 ml of suspension applied to the center of surface of solidified Glycerol-asparagine agar plate with the help of sterilized pipette.
Spread over the medium with the help of sterile bent glass rod and left for 30 minutes
Incubated at 27⌈C for one week with regular observation
Pure culture of Actinomycetes colony obtained by transferring the culture to new glycerol asparagines agar plate
Each isolate coded and stored in sterile D/W and GAA slants at 4⌈C

APPENDIX-I

LISTS OF MATERIALS

1.1 Equipments

1. Autoclave (Life steriware, India)
2. Electric Balance (Denver Instrument)
3. Grinder (Relsh GmbH, West Germany)
4. Hot air oven (Universal, India)
5. Incubators (Universal, India)
6. Laminar flow cabinet (Bassaire, 96 Co.Pvt. Ltd., USA. Module, England)
7. Microscope (Olympus, Japan)
8. pH meter (TOA pH meter HM-10P)
9. Refrigerator (Samsung)
10. Rotary vacuum evaporator (Aikakai, Japan)
11. Water distillation plant (Ogasweseiki)
12. Water bath shaker (Narang, India)

1.2 Microbiological Media

1. Agar Powder (Hi media, India)
2. D(-) Arabinose (Sigma Chemical Co. USA)
3. L- Asparagine (Sarabhai M. Chemicals)
4. Beef extract (Qualigens)
5. Casein (Merck, Germany)
6. Fructose (Sigma Chemical Co. USA)
7. D(+) Galactose (Loba-Chemie indoaustranal Co.)
8. Gelatin (Difco)
9. D-Glucose (Qualigens)
10. Glycerol (Qualigens)
11. Lactose (Hi media)
12. Maltose (Merck)

13. Malt extract
14. Mannose (Hi media)
15. Nutrient Agar (Hi media)
16. Nutrient Broth (Hi media)
17. Peptone (Qualigens)
18. Potato Dextrose Agar (Hi media)
19. Raffinose (Qualigens)
20. Starch (Hi Media)
21. Skim Milk powder (Nestle)
22. Sucrose (Qualigens)
23. Tween 20 (S.D Fine Chem.)
24. Urea Broth Base (Hi media)
25. Urea crystal (Merck)
26. Yeast extract

1.3 Chemicals and Reagents

1. Acetic acid (Qualigens)
2. Alpha-Napthalamine
3. Calcium Carbonate (Sd. Fine Chem., India)
4. Calcium Chloride (Sd Fine Chem., India)
5. Chloroform
6. Crystal violet
7. Dehydrated alcohol (Bengal Chemicals and Pharmaceutical Ltd.)
8. Dimethyl Sulfoxide (Qualigens)
9. Ferrous Sulfate (Sd. Fine Chem., India)
10. Hydrochloric Acid (Qualigens)
11. Iodine
12. Magnesium Sulfate (Loba Chemie.)
13. Mercuric Chloride (Merck)
14. Methanol (Qualigens)

15. Phenol red
16. Potassium iodide (Qualigens)
17. Potassium nitrate
18. Potassium Hydrogen Phosphate
19. Safranin
20. Silica Gel – for thin layer chromatography (Merck)
21. Sodium Hydroxide (S.D. Fine Chem., India)
22. Sodium Hydrogen Phosphate
23. Sodium Chloride (S.D. Fine Chem., India)
24. Sodium acetate (S.D. Fine Chem., India)
25. Sulphanilic Acid (S.D. Fine Chem., India)
26. Zinc dust

1.4 Glass wares

1. Beakers
2. Conical flasks
3. Cover slips
4. Funnel
5. Glass rods
6. Glass vials
7. Measuring cylinder
8. Microscopic slides
9. Micropippetes
10. Petri plates
11. Plastic vials
12. Round bottom flasks
13. Separating funnel
14. Screw- capped test tubes

1.5 Essential Requirements for TLC

1. Precoated silica plate (20x5 cm)
2. Solvent tank with lid
3. Solvent system- chloroform: Methanol (7:1)
4. Oven
5. Capillary
6. Blow drier
7. Sample spotting template
8. UV chamber
9. Chromogenic reagent: Vanillin/Sulphuric acid (1% w/v) reagent
10. Spray apparatus

1.6 Miscellaneous

1. Aluminum foil
2. Inoculating loop
3. Inoculating loop
4. Lens paper
5. Cotton roll
6. Measuring scale
7. Transport tray
8. Cork borer
9. Parafilm roll
10. Vaseline
11. Dropper
12. Sticker

1.7 Test organisms

Bacteria

1. *Bacillus* spp.
2. *Citro bacter* spp.
3. *Enterobacter* spp.
4. *Escherichia coli*
5. *Klebsiella pneumoniae*
6. *Proteus vulgaris*
7. *Pseudomonas aeruginosa*
8. *Salmonella paratyphi*
9. *Shigella dysenteriae*
10. *Staphylococcus aureus*

Fungi

1. *Alternaria* spp.
2. *Fusarium oxysporum*
3. *Fusarium moniliforme*
4. *Geotricum* spp.
5. *Gloeosporium sporioides*
6. *Helminthosporium* spp.
7. *Sclerotinia* spp.

APPENDIX-II

A.COMPOSITION AND PREPARATION OF MEDIA

1. Glycerol- Asparagine agar

Ingredients	Grams/liter
L- Asparagine	1.00
Dipotassium phosphate	1.00
Trace salt solution (ml)	1.00
Agar	20.00
pH	7.4 ± 0.2

Note: 23.00 gm of above mentioned mixture is suspended in 1000ml of distilled water containing 10 ml glycerol.

2. Starch- Casein agar

Ingredients	Grams/liter
Soluble starch	10.00
Casein	0.3
Potassium nitrate	2.00
Di-potassium hydrogen orthophosphate	2.00
Magnesium sulphate	0.05
Calcium carbonate	0.02
Ferrous sulphate	0.01
Sodium chloride	2.00
Agar agar	20.00
pH	7.2

3. Potato Dextrose Agar

Ingredients	Grams/liter
Potato peel	200.00
Dextrose	20.00
Agar	15.00
pH	5.6±0.2

4. Nutrient agar

Ingredients	Grams/liter
Peptone	5.00
Sodium chloride	5.00

Beef extract	1.50
Yeast extract	1.50
Agar	15.00
pH	5.8±0.2

5. Gelatin agar

Ingredients	**Grams/liter**
Nutrient agar	28.00
Gelatin	10.00
pH	7.2

6. Starch agar

Ingredients	**Grams/liter**
Nutrient agar	28.00
Starch	10.00
pH	7.2

7. Tween 20 agar

Ingredients	**Grams/liter**
Nutrient agar	28.00
Tween 20	10.00
Calcium chloride	50.00
pH	7.2

8. Skim milk agar

Ingredients	**Grams/liter**
Nutrient agar	28.00
Skim milk powder	28.00
pH	7.2

9. Nitrate broth

Ingredients	**Grams/liter**
Beef extract	3.00
Peptone	5.00
Potassium nitrate	1.00
Final pH	6.8±0.2

10. Urease broth

a. Urea broth base

Ingredients	Grams/liter
Monopotassium phosphate	9.1
Di-sodium phosphate	9.5
Yeast extract	0.1
Phenol red	0.01
pH	6.8±0.2

Urea	**40% (w/v)**

Note: 1.87 gm of mixture (a) was suspended in 95 ml of distilled water and sterilized by autoclaving. When cooled 5 ml of 40% urea (w/v) was mixed to it.

11. Medium for Sugar Fermentation test

Ingredients	Grams/liter
Peptone	10.00
Sodium chloride	5.00
Phenol red	0.018
Sugar	1%
Final pH	7.4±0.2

Note: The sugar used are: arabinose, fructose, galactose, glucose, lactose, mannose, maltose, raffinose and sucrose.

12. Basal medium for Sodium chloride tolerance test

Ingredients	Grams/liter
Glucose	4.00
Yeast extract	4.00
Malt extract	10.00
Agar	15.00
pH	7.4

Note: The basal medium prepared in 3 batches are supplemented with Nacl (gm/lit): 50, 70 and 100.

13. Starch- casein broth

Ingredients	Grams/liter
Soluble starch	10.00
Casein	0.30
Potassium nitrate	2.00
Di-potassium hydrogen Orthophosphate	2.00
Magnesium sulphate	0.05
Calcium carbonate	0.02
Ferrous sulphate	0.01

14. Nutrient broth

Ingredients	Grams/liter
Peptone	5.00
Sodium chloride	5.00
Beef extract	1.50
Yeast extract	1.50
pH	5.8±0.2

B.COMPOSITION AND PREPARATION OF REAGENTS

1. Gram's Iodine

Ingredients

Iodine crystals	1.0 gm
Potassium iodide	2.0 gm
Distilled water	300.0 ml

Preparation: To 250ml of distilled water, 20gm of potassium iodide was dissolved. Then 10gm of iodine was mixed to it until it was dissolved completely. Finally the volume was made 1 liter by adding D/W.

2. Mercuric chloride solution

Ingredients

Mercuric chloride	15.0 gm
Conc. Hydrochloric acid	20.0 ml
Distilled water	100 ml

3. Crystal violet

Crystal violet	20.0gm
Ammonium oxalate	9.0gm
Ethanol or Methanol	95ml
Distilled water (D/W)	1000ml

Preparation: In a clean piece of paper, 20gm of crystal violet was weighed and transferred to clean brown bottle. Then, 95ml of ethanol was added and mixed until the dye was completely dissolved. To the mixture 9gm of ammonium oxalate dissolved in 200ml of D/W was added. Finally the volume was made 1 liter by adding D/W.

4. Nitrate test Reagents

i) 5N acetic acid: 15 ml of glacial acetic acid was mixed with 85 ml distilled water to make 5N acetic acid.

ii) Sulphanilic acid: 0.8 gm of sulphanilic acid was dissolved in 100 ml 5N acetic acid.

iii) Alpha-napthylamine: 0.5 gm of alpha-napthylamine was dissolved in 100 ml of 5N acetic acid.

APPENDIX-III

3. PREPARATION OF THE McFARLAND NEPHLOMETER STANDARD

A chemically induced precipitation reaction can be used to approximate the turbidity of a bacterial suspension.

Ten clean test tubes of equal size were set up. 1% chemically pure Sulphuric acid (H_2SO_4) and 1.175% aqueous solution of Barium chloride ($BaCl_2.2H_2O$) were prepared in separate conical flasks. Slowly and with constant stirring, the designated amounts of two solutions were added to the tubes to make a total of 10ml/tube.

The suspended Barium Sulphate precipitate corresponds approximately to homogenous *E.coli* cell densities per milliliter through out the range of standard as (x108/ml). The tubes were sealed and stored in the dark at the room temperature.

The first tube which was 1 Nephlometer standard corresponds to 3x108 cells. 5ml of it was poured in another tube and mixed with 5ml distilled water. That was the 0.5 Nephlometer standard and corresponds 1.5x108 cells. It was used for swabbing the plates for antibiotic assay.

Table: Volume of Sulphuric acid and Barium chloride for Nephlometer standard

Tube number	Sulphuric acid (ml)	Barium chloride (ml)	Corresponding cell densities (x108)
1	9.9	0.1	3
2	9.8	0.2	6
3	9.7	0.3	9
4	9.6	0.4	12
5	9.5	0.5	15

6	9.4	0.6	18
7	9.3	0.7	
8	9.2	0.8	
9	9.1	0.9	
10	9.0	1.0	

APPENDIX – IV

A. Characteristics of Fungal Plant Pathogens used in this study

1. ***Fusarium oxysporum***

Class-Deuteromycetes
Order- Hyphomycetales
(Butler and Jones, 1986)

Fusarium oxysporum, apart from being the most economically important member of the genus *Fusarium*, is one of the most liable and variable species. The average growth rate of cultures is 4.5 cm. Cultures are pale, salmon, rosy buff, vinaceous, and violet to pale slate on media of pH 6.7 to 7. Mycelium is delicate, felted to floccose. Microconidia are always present that are unicellular or bicellular, ellipsoidal to allantoid and born on lateral phialides or on phialides produced from short lateral conidiophores. Macroconidia are falcate and are 3-5 septate when mature and are initially formed from branched lateral phialides but later often formed from sporodochia. Chalmydospores are intercalary or terminal on short lateral branches solitary or in chains, hyaline, smooth to rough walled.

The distribution is worldwide and occurs chiefly as a soil saprophyte and appears to survive in winter in mycelial or chlamydospore state.

Numerous strains of this species are wilt pathogens of many crop plants. *Fusarium oxysporum* causes an important wilt disease of sweet potato (*Ipomoea batetas*) in USA but is less important in tropcs. Intervienal yellowing of the leaves is followed by distortation and stunting, and the old leaves fall. There is extensive vascular necrosis that may appear purplish below the soil level; the cortex may rupture. Infected tubers may rot in storage. The fungus infects the roots of many plants without causing any

external symptoms viz. cabbage, cotton, cowpea, maize, okra, soyabean, tobacco, and 89watermelon (Booth, 1971).

2. ***Fusarium moniliforme***

Class-Deuteromycetes
Order-Moniliales
Family-Tuberculariaceae
(Alexopoulos and Mims, 1979)

Fusarium moniliforme is wide spread in both humid and sub-humid temperate zones and extending into sub-tropical zones through out the world. Growth is initially rather filmy, colorless and rapid (4.6 cm). Culture from below typically dark violet but occasionally paler, pink, lilac, vinaceous or even cream. Aerial mycelium is generally dense and delicately floccose to felted, often with a powdery appearance due to formation of microconidia. Microconidiophores are simple, lateral, subulate phialides formed on the aerial hyphae; rarely may they be formed on short lateral branches. They are 20-30 µ long by 2-3 µ at the base narrowing to the approximately 1.5µ at the apex.
Fusarium moniliforme is a major parasite of several Gramineae such as rice, sugarcane, maize and sorghum. It also occurs on a very wide range of other hosts. F. moniliforme causes seedling blight, foot rot, stunting and hypertrophy of shoots of rice (bakanae disease), also causes seedling blight, root rot and pink boll of cotton. It also attacks flowers and fruits of banana. It is also associated with storage rots of Freesia corms, pineapple and tomatoes. (Booth, 1971)

3**. *Sclerotinia*** spp.

Class-Ascomycetes
Order-Heliotales
Family-Sclerotiniaceae
(Alexopoulos and Mims, 1979)

This family comprises of economically important groups of fungi. Most fungi of this family live parasitically on plants. *Sclerotinia* spp. is an important plant pathogen. These species mostly live saprobically on the soil, on dead wood, on dung or on other organic matter from which they draw nourishment.

Apothecial initials arise from stromata or sclerotia. The apothecia are of medium size, generally brown and are most often borne on long stalks. The ascospores are generally hyaline, one-celled, oval or somewhat elongated (Alexopoulos and Mims, 1979).

Sclerotinia spp. causes a number of diseases in plants. *Sclerotinia sclerotiorum* cause lettuce drop and other vegetable diseases as well. *Sclerotinia frutigena* causes brown rot of fruit apple. *Sclerotinia frutigena*, *Sclerotinia cinerea* and *S. americana* causes brown rot of stone fruits e.g. apricot, plum etc. *Sclerotinia ricini* causes stem rot of castor. *Sclerotinia sclerotiorum* causes sclerotinia rot in wheat and other crops causing leaf spot and blight. It also causes stem blight of mustard (Rangaswami, 1996).

4. ***Helminthosporium*** spp.

Class-Deuteromycetes
Order-Moniliales
(Butler and Jones, 1986)

Helminthosporium is a widespread fungus whose mycelium consists of inter- and intra-cellular prostrate hyphae and more or less erect conidiophores. The mycelium develops as grayish brown to dark brown mat on host parts and on culture. On the host hyphae are short segmented and the conidiophores arise as lateral branches from these hyphae. The conidiophores are characteristically bent and possess knee joints, the points where conidia are attached. The conidia are sub hyaline to yellowish-brown, thin walled, straight, cylindrical to slightly tapering, having rounded ends, 1-7 septate and without constrictions at the septa. This genus causes a number of diseases in plants. *Helminthosporium gramineum* causes stripe disease of barley; Helminthosporium orizae causes brown leaf spot or Helminthosporium disease of rice, an important disease of rice that occurs in almost all rice growing areas of the world, especially under

semi dry conditions; Helminthosporium sativum causes spot blotch of barley and seedling blight and foot rot of wheat and barley; Helminthosporium teres causes net blotch of barley, etc (Singh, 1998).

5. ***Alternaria*** spp.

Class-Deuteromycetes
Order-Moniliales
Family-Dematiaceae
(Alexopoulos and Mims, 1979)

Alternaria is a large, universally occurring genus. Several form species are found as sprobes on dead and dying plant parts in soil from which the conidia are picked up by the wind.

Conidiophores dark, simple, rather short or elongate, typically bearing a simple or branched chain of conidia. Conidia dark, typically with both cross and longitudinal septa; variously shaped, obclavate to elliptical or ovoid, frequently borne acropetally in long chains; less often borne singly and having an apical simple or branched appendage.

A number of form species are also parasitic on plants. *Alternaria solani* attacks members of the family solanaceae as well as other hosts. It is particularly troublesome on potato, tomato where it causes the disease known as early blight. Blight is caused in linseed (*A.liniday*), castor (*A.ricini*), mustard (*A.brassicae*).In onion *Alternaria* causes blight, *Alternaria brassicae* and *Alternaria melongenae. solani* cause leaf spots with concentric rings in raddish and egg plant respectively. Leaf spots in lady's finger, chilli, sunflower is shown to be caused by *Alternaria nelianthis*. *Alternaria* also causes fruit rot in tomato (*A.solani*) (Rangaswami, 1996).

6. ***Gloeosporium sporioides***

Class-Deuteromycetes

Order-Melaconiales

(Butler and Jones, 1986)

Gloeosporium is a widespread fungus whose perfect stage is *Glomerella* found under family Hypocreaceae of order Sphaerials of class Pyrenomycetes under the subdivision Ascomycotina which has small, spherical perithica with dark parenchymatous walls and are formed single or in clusters mostly in host tissues. The ascospores are small, translucent, hyaline, and one-celled. They are mostly parasitic on higher plants causing anthracnose, leaf spots, fruit rots and stalk rot. The conidial stage is found under order Melanconiales of class deuteromycetes where conidia are produced in acervulus. The mycelium consists of sparsely septate hyphae, which at first are hyaline but later take on slightly dark color. The conidia are hyaline but in mass they look pinkish (Singh, 1998).

Besides these, the other test pathogenic fungal organism used in the study was *Geotrichum* spp.

B. General characteristics of bacteria used in this study and their pathogenicity

1. ***Bacillus subtilis***

a. General/identifying character: It is gram-positive, rod, spore former, spores are ellipsoidal, not bulging sporangium, spores are centrally located in the vegetative cell, very resistant to heat. *B. subtilis* is commonest saprophyte found as contaminants in food, clinical specimens and laboratory culture.
b. Pathogenicity: It is less commonly found opportunistic pathogen. It sometimes causes food poisoning (Collee et al., 1996).

2. ***Citrobacter*** spp.

a. General/identifying character: It is gram- negative, rod. Most strains of *Citrobacter* are motile, gas-producing and citrate-utilizing.

b. Pathogenicity: Previously considered as environmental contaminants species of *Citrobacter* have been isolated from human urine and faeces and a variety of serious infections, especially those of infants and immunocompromised patients. *Citrobacter freundii* and *C. koseri* are the species most frequently isolated from clinical material.

3. ***Enteorbacter*** spp.

a. General/identifying characters: It is gram-negative, rod. They are usually motile, ornithine positive and Voges-Proskauer positive.

b. Pathogenicity: *Enterobacter aerogenes* and *E. cloacae* are the species most commonly isolated from clinical material. They are opportunistic pathogens involved in infections of wounds and of the urinary and respiratory tracts and are occasional causes of septicemia and meningitis.

4. ***Escherichia coli***

a. General/ identifying character: This species lies in the family Enterobacteriaceae. They are gram-negative motile, rods. Their natural habitat is human and animal intestine. They cannot exist in outer environment for long time.

b. Pathogenecity: Although *E.coli* is considered as commensal flora, it is able to cause frequent opportunistic infections, as for example, appendix abscesses), peritonitis, cholecystitis, septic wound (Collee *et al.* 1996)

5. ***Klebsiella pneumoniae***

a. General/identifying characters: *Klebsiella* are non-motile members of the Enterobacteriaceae. They are usually capsulated and lactose fermentative. This organism is commonly isolated from water and human and animal faeces.
b. Pathogenicity: This is the most frequently encountered klebsiella in clinical specimens, especially from hospitalized patient, in whom it causes infections of surgical wounds and the urinary tract.

6. ***Proteus vulgaris***

a. General/identifying characters: They are gram-negative, motile, lactose-non-fermenting. They hydrolyse urea rapidly. They are found widely distributed in soil, on vegetables, in sewage and faeces and on rotting animal protein.
b. Pathogenicity: This organism is found in infected wounds and abscesses and in cases of otitis media, meningitis, septicaemia and osteomylitis.

7. ***Pseudomonas aeruginosa***

a. General/identifying characters: It is gram-negative motile, aerobic non-fermentative, non-spore forming, rod. It is distributed in nature. Unlike other *Pseudomonas, P. aeruginosa* will grow at 42⌈C. After overnight aerobic incubation in nutrient agar at 37⌈C six distinct colonial type of P. aeruginosa may be observed (Phillip, 1969).
b. Parhogenicity: It is a classical opportunist pathogen with innate resistance to many antibiotics and disinfectants. It may cause ear infection, corneal ulceration. This organism is major cause of malignant otitismedia and burning wound infection.

8. ***Salmonella paratyphi* A**

a. General/identifying characters: It is gram-negative motile, rod, 2-4x0.6⇑m, non-acid fast, non-capsulated and non spore former.

b. Pathogenicity: Strain of Salmonella is mostly responsible for enteric fever, which include typhoid fever. Other *Salmonella* spp.cause gastrointestinal tract infection.

9. ***Shigella dysenteriae***

a. General/identifying characters: It is a member of enterobacteriaceae hence it is gram-negative, rod fermentative, facultatively anaerobic, oxidase negative, catalase positive.

b. Pathogenicity: It is main etiological agent of bacillary dysentery. Although all *Shigella* species cause bacillary dysentery, the disease caused by *Shigella dysentriae* is most severe of all.

10. ***Staphylococcus aureus***

a. General/identifying characters: It is gram-negative, non-motile, cocci. In gram staining they are found in three-dimensional bunches of grapes like structure. Cell is approximately 1⇑m in diameter.

b. Pathogenicity: It causes localize infection when enters through breaks in skin. It causes pyogenic infections, which include impetigo, furuncles, carbuncles, breast abscess; post operative wound infection, osteomyelitis, septic arthritis (bone), lung abscess etc. It also causes boils, secondary infection, bacterimea, septicemia, pneumonia, meningitis, acute endocarditis and conjunctivitis in new burns and more commonly food poising.