IMMUNOLOGICAL SCREENING OF ACTINOMYCETES OF KHUMBU REGION OF NEPAL

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This is to certify that Ms. SUMAN THAPA has completed this dissertation work entitled "IMMUNOLOGICAL SCREENING OF ACTINOMYCETES OF KHUMBU REGION OF NEPAL" as partial fulfillment of M.Sc. Degree in Microbiology under our supervision. To our knowledge, this work is original and has not been submitted to any other degree.

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ABSTRACT

The preserved isolates of actinomycetes from Lobuche area (5000-5300 meter in height) and Lukla area (2660 meter in height) of Khumbu region of Nepal were taken for the study. A total of eighty isolates of actinomycetes were revived on starch casein agar (SCA). Out of them, isolate Lobuche 137.2 was selected for further study. Isolate Lobuche 137.2 has already been identified as a new species of actinomycetes in the project work of Nepal Academy of Science and Technology (NAST) named "Biodiversity of Khumbu region: Population study of Actinomycetes"in year 2002.

The isolates of Lobuche 137.2 were streaked on starch casein agar (SCA). Isolates of Lobuche 137.2 are observed to be creamy in colour on starch casein agar (SCA) plate. Macroscopically, Lobuche 137.2 has creamy aerial mycelium with creamy substrate. Microscopically, Lobuche 137.2 has been identified by coverslip method. On 1000x of microscope, it has long non-septate hyphae with the branching filaments and its spores are present at the end of the filament. Shapes of the spores of Lobuche 137.2 are oval and they are present in a chain form.

To detect the immunological sensitivity of the spore 137.2 as Indirect Enzyme Linked Immunosorbent Assay (IND-ELISA) is considered more appropriate. Therefore, Indirect Enzyme Linked Immunosorbent Assay (IND-ELISA) for spore of Lobuche 137.2 was performed. Three control and a test was set on the ELISA plate. Intense colour was observed on the well having 1:20 dilution of test. Spore density of 0.6×10^6 spores/200µl was detected on 1:20 dilution of test sample.

Key words: -actinomycetes, hyphae, Indirect Enzyme Linked Immunosorbent Assay.

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LIST OF ABBREVIATIONS

AP	Alkaline Phosphatase
CFA	Complete Freund's Adjuvant
CUM	Carbohydrate Utilizing Media
DNA	Deoxyribose Nucleic Acid
ELISA	Enzyme Linked Immunosorbent Assay
HRP	Horse Radish Peroxidase
IFA	Incomplete Freund's Adjuvant
IND-ELISA	Indirect Enzyme Linked Immunosorbent Assay
OPD	Ortho-phenylenediamine Dihydrochloride
PBS	Phosphate Buffer Saline
rRNA	Ribosomal Ribose Nucleic Acid
SCA	Starch Casein Agar
SIM	Sulphate Indole Motility Agar
TSI	Triple Sugar Iron Agar
VP	Voges Proskauer
YEME	Yeast Extract Malt Extract

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CHAPTER – I

1. INTRODUCTION

Actinomycetes are gram-positive organisms that grow in the form of mycelia. They were considered to be intermediate group between bacteria and fungi. The name "Actinomycetes" derived from the greek word "Aktis" (a ray) and "mycus" (fungus). Actinomycetes are aerobic or facultative anaerobic organism with high G: C content (i.e. > 55%).

Actinomycetes have been identified as one of the major group of the soil microbial population (Kuster, 1967). They are also present in a variety of habitats including compost, river, mud and lake bottoms (Alexander, 1978). In some dry and alkaline soil samples actinomycetes were found at a concentration of 1/4 pound per cubic foot. They comprised 5% of soil's total bacteria population. They were even isolated from extreme environments like paleoglacial cores of the Central Antarctica. In their normal habitats the actinomycetes may occur as conidia or as the vegetative hyphae and both forms can give rise to colonies on the agar media (Singh, 1999).

Actinomycetes belong to the order: actinomycetales, superkingdom : bacteria, phylum**:** firmicutes, class**:** actinobacteria, subclass**:** actinobacteridae. Bergey's Manual of Determinative Bacteriology (2000) divides actinomycetes into eight diverse families. They are actinomycetaceae, mycobacteriaceae, actinoplanaceae, frankiaceae, dermatophilaceae, nocardiaceae, streptromycetaceae, micromonosporaceae (Holt, 1989). The families compromise of 63 genera (Nisbet and Fox, 1991). Based on 16S rRNA classification system, they have recently been grouped in ten suborders: actinomycineae,corynebacterineae, frankineae, glycomycineae, micrococineae,

micromonosporineae, propionibacterineae, pseudonocardineae, streptomycineae, streptosporangineae. The large number of actinomycetes still remains to be grouped (Khatri, 2002). The morphology and arrangement of spores, cell wall chemistry, physiology and the types of sugars present in the cell extracts are particularly important in actinomycetes taxonomy.

Various techniques have been used to study the phenotypic variation of microorganisms. They include: serotyping, monoclonal antibody typing, biotyping, bacteriophage typing, fimbrination typing, resistotyping, cell electrophoresis, and chemical profiling or fingerprinting. Practically, it has been found that IND-ELISA is far more sensitive than any other technique. IND-ELISA is used for membrane bound insoluble and soluble antigens. It involves the coating of the polystyrene wells of an ELISA plate with the antigen to be tested, followed by the binding of the antibody from the sample to the antigen in the coated well and then detection of the binding by an enzyme - linked secondary antibody and a specific substrate reaction. The result is a direct quantitative analysis of the amount of antibody bound to the coated antigen (Cheesbrough, 1994).

Natural products continue to be a useful source of new leads for the pharmaceutical industry. Actinomycetes are prolific producers of natural products and one strategy to increase the possibility of discovering novel chemical entities is to screen actinomycetes considered “rare” in the environment and previously under-represented in natural product screening collections.

Due to the large geographic variation, Nepal has varied microbial biodiversity. So, it is quite likely that the distribution of actinomycetes is also variable (Pandey, 2004). This

study is carried out to screen novel species of actinomycetes from higher altitude, Khumbu region, of Nepal which is situated at the lower part of Mount Everest base camp. Here, the immunological detection of actinomycetes was done by the IND-ELISA against spore of novel actinomycetes. This study had been undertaken to isolate novel actinomycetes from preserved isolates of actinomycetes in RLABB.

CHAPTER – II

2. OBJECTIVES OF THE STUDY

GENERAL OBJECTIVE

To screen actinomycetes isolated from soil sample using immunological method.

SPECIFIC OBJECTIVE

1) Isolation, identification and characterization of actinomycetes.

2) Detection of novel actinomycetes.

3) Production of antibodies against spore of novel actinomycetes.

4) Identification of novel actinomycetes using IND-ELISA.

CHAPTER III

3. LITERATURE REVIEW

3.1 OVERVIEW

The actinomycetes are a large group of filamentous gram-positive bacteria that form branching filaments. Basedon their G+C content, gram-positive bacteria include two major branches, the low G+C organisms (*Bacillus, Clostridia, Lactobacillus, Staphylococci, Streptococci, Mycoplasmas*) and high G+C organisms (*Actinomycetes, Mycobacteria, Micrococcus*). Actinomycetes resemble fungi in appearance, but their cells are prokaryotic. The properties relating them to bacteria are –absence of nuclear membrane, small hyphal diameter, sensitivity to lysozyme, chemical nature of the cell, sensitivity to antibacterial agents and presence of prokaryotic flagella when produced. In their normal habitats, the actinomycetes may occur as conidia or as the vegetative hyphae and both forms can give rise to colonies on agar media. The name of the group actinomycetes is derived from the first described anaerobic species *Actinomyces bovis* which causes actinomycosis, the "ray fungus disease" of cattle.

The actinomycetes are mostly aerobic or facultative anaerobic. They are gram positive, non-acid fast, non-motile and are related to coryneform bacteria and mycobacteria by an almost continuous sequence of intermediate forms. The rods or filaments are less than 1 mm in diameter, and no aerial hyphae are produced. They ferment carbohydrates to acids with no gas. They produce numerous secondary metabolites that include chromogenic substances,antibiotics and enzymes e.g., protease, amylase, lignase, chitinase etc.

Most actinomycetes are mesophiles that prefer to grow at temperature range of 25 to 30^{o} C and generally facultative thermophiles when growing at 55 to 65^{o} C as well as at 30^{o} C (Alexander, 1977). Many actinomycetes grow on common bacteriological media, such as nutrient agar and trypticase soy agar. Outgrowths from a spore or fragments of mycelium that is considered as colony forming units (CFU) develop into hyphae that penetrate the agar (substrate mycelium). Then the hyphae branch repeatedly, becoming cemented together on the surface of the agar forms a tough leathery or chalky type colony. The density and consistency of the colony depends on the composition of the medium.

3.2 OCCURRENCE

The actinomycetes are widely distributed in nature. Although actinomycetes are free living, saprophytic bacteria found in soil, water and colonizing plants, their natural occurrence is mostly restricted to soils. They are even isolated from extreme environments like Paleo-glacial cores of the central Antartica. They are present in the range of soil layers like surface soil, lower horizons and considerable depths. The population density will vary markedly in similar soil of any locality by cultural practices. In their normal habitats, the actinomycetes may occur as conidia or as the vegetative hyphae and both forms can give rise to colonies on agar media. The actinomycetes are affected directly by the presence of available carbon and their number is especially great on land rich in organic matter (Alexander, 1977).

Actinomycetes are important decomposers that participate in the turnover of the soil components, especially in the transformation of organic compounds (Kononova 1966, Kuster 1967, Kuzner 1968, Huntzens 1972). 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 materials (McCarthy 1987, Crawford 1988, Wong et al 1991), they have important role in soil biodegradation by recycling of nutrients associated with recalcitrant polymers (McCarthy and Williams, 1992). This replenishes the supply of nutrients in the soil and is an important part of humus formation. Actinomycetes also inhabit plants and animals, including a few pathogens, such as *Mycobacterium*. Actinomycetes are also responsible for the musty or earthy odor of a freshly plowed field and are helpful in improving soil crumb structure.

3.3 DEVELOPMENT PATTERNS IN MYCELIAL ACTINOMYCETES

The life cycle of actinomycetes includes two distinct phases: a stage of vegetative mycelial growth, and a stage of spore formation. Sporulation is initiated by starvation for essential nutrients, it was observed that the portion of mycelium in the center of a colony may become deprived of nutrients even while the edge is still actively growing, resulting in concurrent appearance of these two stages. A mature colony may contain within it a complete developmental sequence: an actively growing mycelium at the periphery of the colony and different stages in a sporulation toward the center of the colony. This center often doesn't have any more repetitive mycelim as it completely lysed.

If an actinomycete mycelium is entirely within the substratum, it is termed substrate mycelium. This is the most common situation among the actinomycetes that do not form spores. Many actinomycetes form a structurally distinct aerial mycelium, which typically extends away from the substratum. In such organisms, spores are usually formed at the tips of the aerial hyphae.

The substrate mycelium is typically septate although the multinucleate cells are normally quite long (e.g., 20 µm or more). The hyphae are of constant diameter because, as in most other bacteria cell growth is accomplished by elongation with no increase in cell diameter. However, a few actinomycetes grow in width as well as length, and deposit cross walls longitudinally as well as across the mycelium. The result is a mass of cells forming tissue like thallus or sclerotium.

3.4 TAXONOMY

Actinomycetes comprise of 63 genera (Nisbet and Fox, 1991). The morphology and arrangement of spores, cell wall chemistry, physiology and the types of sugar present in the cell extracts are particularly important in actinomycetes taxonomy. A whole array of taxonomic tools have been used to define genera and supergeneric groups of actinomycetes (Goodfellow and O'Donnell, 1989), but partial sequence analysis of 16s rRNA is the most significant. Cell wall analysis was introduced by Cummins and Harris in 1956 and divided the family into eight types as shown in the Table 1. Actinomycetes from the types II-IV can be further distinguished by their whole organism sugar pattern (Table 2 and 3) and characteristics of actinomycetes are shown in Table 4. According to the Bergey's Manual of Determinative Bacteriology, the order actinomycetales is divided into eight families. They are as follows:

1. Actinomycetaceae

This family consists of organism that is predominantly diphtheriod in shape with a tendancy to form branched filaments. Due to frequent fragmentation, diptheriod or coccoid forms are made. Aerial mycelium and spores are not formed.

Genus: *Actinomyces, Arachnia, Bifidobacterium, Bacterionema, Rothia*.

2. Mycobacteriaceae

Filamentous or mycelium like growth may occur but slight disturbance usually becomes fragmented into rods or coccoid elements. These acid fast organisms are widely distributed in nature.

Genus: *Mycobaterium.*

3. Frankiaceae

It includes symbiotic, filamentous, mycelium forming bacteria which induce and live in root nodules of certain non-leguminous plants.

Genus: *Frankia.*

4. Actinoplanaceae

These organisms form distinct mycelium. Sporangia are spherical, subspherical, lobucheed, cylindrical, club shaped to irregular. They are borne at the tip of aerial hyphe. Each sporangium may contain several to many hundreds of spores. They are obligately aerobic and occur in humus rich soils.

Genus: *Actinoplanes, Spirillospora, Streptosporangium, Amorphosporangium, Ampullariella, Pimelia, Planomonospora, Planobispora, Dactylosporangium*

5. Dermatophilaceae

The mycelial filament from these groups of organisms divides transversely in at least two longitudinal planes forming masses of coccoid or cuboid cells. Aerial mycelium ordinarily absent.

Genus: *Dermatophilus, Geodermatophilus.*

6. Nocardiaceae

These actinomycetes form scant or well developed mycelium which eventually fragments with the production of cocci, bacilli or branched filamentous forms. They are aerobic.

Genus: *Nocardia, Pseudonocardia.*

7. Streptomycetaceae

Vegetative hyphae produce a well developed branched mycelium that does not fragment readily. They are primarily soil forms.

Genus: *Streptomyces, Streptoverticillium, Sporichthya.*

8. Micromonosporaceae

Aerial mycelium is present, except in *Micromonospora*. Spores are formed singly or in pairs on either aerial and substrate mycelium. They are generally mesophilic and some are thermophilic. They are primarily soil inhabitants.

Genus: *Micromonospora, Thermoactinnomyces, Actinobifida, Thermomonospora, Microbispora, Micropolyspora.*

Based on 16s rRNA classification system, actinomycetes have been grouped in 10 suborders. The use of ribosomal RNA sequence in defining the components of mixed natural microbial populations with highly conserved structures and nucleotide sequences and is used for investigating phylogenetic relationships. Abridged phylogenetic tree of actinomycetes based on almost full 16S rRNA sequences was constructed by using the neighbour – joining methods with *Bacillus subtilis* as the outgroup by Goodfellow in 1989. It is shown in figure 1.

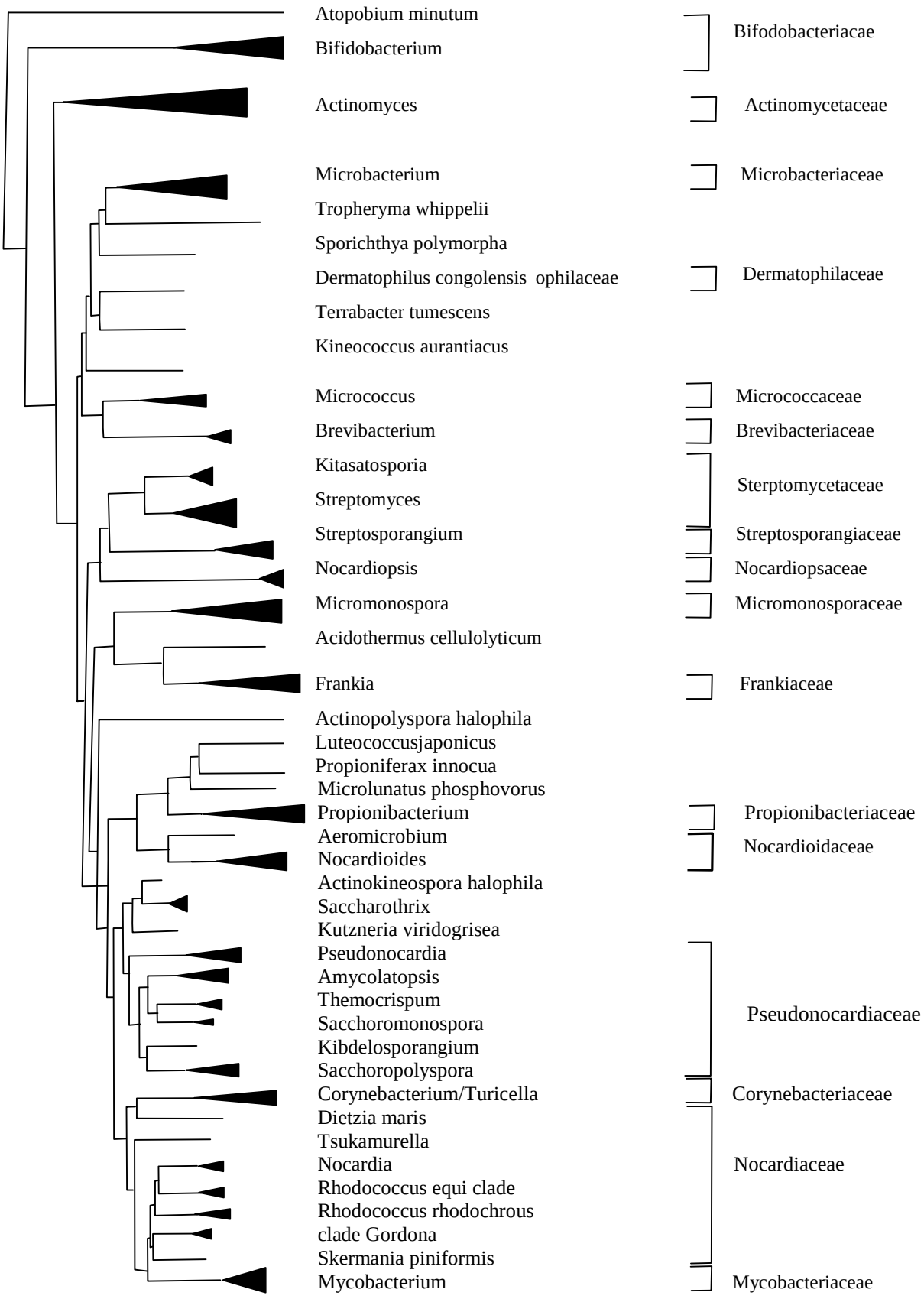

Fig. 1. Abridged phylogenetic tree of actinomycetes based on almost full 16S rRNA sequences.The tree was constructed by using the neighbour – joining methods with *Bacillus subtilis* as the outgroup. There are now over 100 validly described actinomycetes genera, but not all have been assigned to families. About half of the genera omitted from this abridged tree belong to one of the recognized families. (Goodfellow , 1989)

Table 1: Cell wall chemotypes with illustrating families (Cummins and Harris, 1958)

Chemotype	L - D A P	Meso-D A P	DABA	Aspartic A c i d	Glycine	Lysine	Ornithine	Arabinose	Galactose	Representative Genera
I	+	–	–	–	+	–	–	–	–	*Streptomycetaceae Nocardiaceae*
II	–	+	–	–	+	–	–	–	–	*Micromonosporaceae*
III	+	–	–	–	–	–	–	–	–	*Dermatophilaceae, Mycobacteriaceae, Thermomonosporaceae, Frankiaceae, Streptosporangiaceae*
IV	–	+	–	–	–	–	–	+	+	*Nocardiaceae, Mycobacteriaceae, Corynebacteriaceae, Pseudonocardiaceae*
V	–	–	–	–	–	+	+	–	–	*Actinomycetacea*
VI	–	–	–	V	–	–	–	–	V	*Cellulomonadaceae, Micrococcaceae, Microbacteriaceae, Actinomycetaceae*
VII	–	–	+	–	+	V	–	–	–	*Microbacteriaceae*
VIII	–	–	–	–	–	–	+	–	–	*Cellulomonadaceae, Microbacteriaceae*

Note :- (+) = Present; (-) = Absent; V = Variable

Table 2: Whole organism sugar pattern of actinomycetes from cell wall chemotypes II-IV (Cummins and Harris, 1958)

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

Note :- (+) = Present; (-) = Absent

Table 3: Distribution of cell wall type and whole cell sugar pattern in genera of actinomycetes (Cummins and Harris, 1958)

Cell wall type	Sugar pattern	Genera
I	NC (No characteristic sugar)	Streptomyces, Streptoverticillium, MicrocelLobucheiospora, Sporichthya
II	D	Micromonospora, Actinoplanes, Ampulleriella, Amorphosporangium, Ductylospoangium
III	B	Microbiospora, Streptosporangium, Spirillospore, Planomonospora, Dermatophilus, Nocardia-madure type (Actinomadura)
IV	C	Actinobifida, Thermoactinomyces, Geodermaphilus
V	A	Mycobacterium, Nocardia, Pseudonocardia, Thermomonospora, Micropolyspora

Table 4: General characteristics of actinomycetes and similar genera of nonmotile, gram positive (Cummins and Harris, 1958)

Genera	Morphology	Filaments			Spores	Oxygen Requirement	Cell wall Chemotype	Mol% G+C	Mycolic Acid (No. of carbon)	Catalase
		Substrate	Aerial	Fragmentation						
Corynebacterium	Pleomophic rods, club shaped cells	–	–		–	A, F	IV	51-59	+(22-38)	+
Actinomyces	Rods, filaments, some branching	+	–	+	–	A, F, An	V,VI	57-69	_	_
Arachnia	Rods, filaments, some branching	+	–	+	–	A	I	63-65	_	_
Bifidobacterium	Irregular rods with branching	–	–		–	An	VIII	55-67	_	_
Mycobacterium	Rods, some branched filaments	V	–	+	–	A	IV	62-70	+(60-90)	+
Dermatophilus	Large, irregular branched filaments and cocci	+	–	+	+[c]	A	III	57-59	–	+
Rhodococcus	Rods, fragmentation into cocci	V	+	+	–	A	IV	59-72	+(34-64)	+

Note :- (+) = Present; (-) = Absent; V = Variable; A= Aerobic; F=Fermentative; An= Anaerobic.

Continued…..

Genera	Morphology	Filaments			Spores	Oxygen Requirement	Cell wall Chemotype	Mol% G+C	Mycolic Acid (No. of carbon)	Catalase
		Substrate	Aerial	Fragmentation						
Propionibacterium	Pleomorphic rods, branched forms, cocci	_	_		_	F	I	53-68	_	+
Actinomadura	Rods, filaments, cocci	+	V	_	+	A	III	65-77	_	+
Streptomyces	Branched filaments	+	+	_	+	A	I	69-78	_	+
Frankia	Branched filaments	+	+	V	+	A	IV	66-68	_	+
Thermoactinomyces	Branched filaments	+	+	_	+	A	III	53-55	_	+

Note :- (+) = Present; (-) = Absent; V = Variable; A= Aerobic; F=Fermentative; An= Anaerobic.

Table 5: Taxonomic classification of order actinomycetales (Agarwal, 2002)

Suborder	Family	Genus
Micromonosporineae	Micromonosporaceae	*Microsmonospora, Actinoplanes, Catellatospora, Couchioplanes, Catenuloplanes, Pilimelia, Dactylosporangium*
Frankineae	Frankiaceae Sporichthyaceae Geodermatopilaceae Microsphaeraceae Acidothermaceae	*Frankia* *Sporichthya* *Geothermopills, Blastococcus* *Microsphera* *Acidohermus*
Pseudonocardineae	Pseudonocardiaceae	*Pseudonocardia, Actinopolyspora, Actinosynnema, Amycolatopsis, Kibdelosporium, Kutzneria, Lentzea, Saccharomonospora, Saccharopolyspora, Saccarothrix, Streptoalloteichus, Thermocrispum*
Streptomycineae	Streptomycetaceae	*Streptomyces*
Corynebacterium	Nocardiaceae Gordoniaceae Mycobacteriaceae Dietziaceae Tsukamurellaceae Corynebacteriaceae	*Nocardia, Rhodococcus* *Gordonia* *Mycobacterium* *Dietzia* *Tsukamurella* *Corynebacterium, Turicella*
Micrococcineae	Micrococcaceae Brevibacteriaceae Cellulomondaceae Dermabacteraceae Intrasporangiaceae Jonesiaceae Microbacteriaceae Promicromonosporaceae	*Micrococcus, Arthrobacter, Kocuria, Nesterenkonia, Rorhia, Renibacterium, Stomatococcus, Brevibacterium* *Cellulomonas, Oeskovia, Rarobacter* *Dermatobacter, Brachybacterium* *Intrasporangium, Sanguibacter, Terrabacter* *Jonesia* *Microbacterium, Agrococcus, Agromyces* *Aureobacterium, Clavibacter, Curtobacterium, Rathaybacter* *Promicromonospora*
Actinomyineae	Actinomycetaceae	*Actinomyces, Mobiluncus, Arcanobacterium*
Propionibacterianeae	Propionibacteraceae	*Propionibacterium, Luteococcus, Microlunatus, Propioniferax*
Streptosporangineae	Streptosporangiaceae Thermomonosporaceae Nocardiopsaceae	*Streptosporangium, Herbidospora, Microbispora, Microtetraspora, Planobispora, Planomonospora* *Thermomonospora, Actinomadura, Spirillospora* *Nocardiopsis*
Glycomycineae	Glycomycetaceae	*Glycomyces*

For many years, sequencing of 16S rRNA gene has served as an important tool for determining phylogenetic relationship between microorganisms. Development of recombinant DNA technology in *Streptomyces* spp has enabled the investigator to clone individual genes, thus providing direct genetic evidence for their close relationship (Honnouchi and Beppu, 1992).

The production of antibiotics by soil-borne microorganisms, the actinomycetes, has considerable economic importance. The manipulation of antibiotic producers has become a prime target for the application of recombinant DNA technology. Certain technical requirements have had to be met for gene cloning to be successful in the actinomycetes. These requirements, including the development of cloning vectors and transformation procedures, have been satisfied, in part, for some members of the Streptomyces genus. Some problems including sequence rearrangement and stability of plasmid maintenance are now being recognized. A number of genes have been cloned in Streptomyces and some preliminary results characterizing the gene for a Streptomyces-derived beta-galactosidase-like activity were described.

3.5 ACTINOMYCETES AS ANTAGONISTS

Actinomycetes are widely distributed in nature. They are the primary decomposers of tough plant materials like bark, newspapers and woody stems. They are especially effective at attacking tough raw plant tissues (cellulose, chitin and ligmin), softening them for their less enterprising relatives.

Actinomycetes are successful in biocontrol of *Fusarium* spp because of their ability to possess ectoenzyme system to breakdown chitinous walls present in fungi. Workers have obtained some control of *Fusarium solani, Fusariu solani f.* spp.*Phaseoli, Fusarium oxysporium f.* spp. *Cubense and Fusarium oxysporium f.* spp. *Conglutinam.*

In addition to their ability to colonize bulk soil, they successfully colonize rhizosphere (Watson and Williams, 1974). This ability may be partly due to the antagonistic characteristics in competition with other rhizospheric bacteria such as *Pseudomonas* and *Bacilli.* An added advantage actinomycetes have over gram-positive soil bacteria is their ability to spread through relatively dry soil via hyphal growth. In water, motile bacteria such as *Pseudomonas fluorescens* show more expensive colonization of rhizosphere than actinomycetes (Elliott-Juhnke et al, 1987, Karagouni et al, 1993, Milus and Rothrock, 1993).

3.6 ACTINOMYCETES AND THEIR ANTIBIOTICS

Antibiotics are best-known products of actinomycetes metabolism. Other metabolites such as extracellular enzymes, pigments and growth promoting factors (probioties) are also produced. Many previously fatal diseases are now easily controlled by using antibiotics produced by *Streptomycetes* and other actinomycetes. Actinomycetes are noteworthy as antibiotic producers, making three quarters of all known products. The *Streptomyces* species are especially prolific producers of many antibiotics and other class of biologically active secondary metabolites. The reports in literature indicate that around 80% of the total antibiotic products are of *Streptomyces* origin. *Micromonospora* is the runner-up with fewer than one-tenth as many as *Streptomyces.*Even if we include

secondary metabolites with biological activities other than antimicrobial, actinomycetes are still out in front, with over 60% of actinomycetes accounting for 80% of ; *Streptomyces* spp. (Hopwood *et al*., 2000).

Relatively few actinomycetes that produce antibiotics are non-streptomycetes. Non-streptomycetes are not frequently encountered on isolation plates and they often possess more complex nutritional requiremenmts and grow more slowly than *Streptomyces.* In 1940 Selmon Waksman discovered that the soil bacteria made actinomycin. This discovery granted him a Nobel prize. Since then hundreds of naturally occurring antibiotics have been discovered from these terrestrial microorganisms, especially from the genus *Streptomyces.*Approximate numbers of secondary metabolites produced by different groups of organisms as in 1994 are shown in Table 6.

Table 6 Approximate numbers of secondary metabolites produced by different groups of organisms as in **1994 (Agarwal, 2002)**

Source	**Bioactive metabolites**			**"inactive" metabolites**
	Antibiotics	Others	Total	
Non-actinomycete bacteria	1400 (12%)	240 (9%)	1640 (11%)	2000-5000
Actinomycetes	7900[1] (66%)	1220[1] (40%)	9120[1] (61%)	8000-10,000
Fungi	2600 (22%)	1540 (51%)	4140 (28%)	15,000-25,000
Total mocroorganisms	11,900	3000	14,900	25,000- 40,000
Lichens	150	200-500		1000
Algae	700	800-900		1000-2000
Higher plants	5000	25,000-35,000		500,000
Terrestrial animals	500	10,000-15,000		800,000
Marine animals	1200	1500-2000		200,000
				300,000
				2000-3000
Total higher Organisms	7500	35,000-50,000		> 1,000,000

[1] In each category, nearly 80% were found in *Streptomyces* and 20% in other actinomycetes.

3.7 PATHOLOGICAL SIGNIFICANCE

Many species of actinomycetes that occur in soil are harmless to animals and higher plants while others are important pathogens. Actinomycetes, which mostly cause infections, are members of genuses *Mycobacterium, Actinomadura, Nocardia* and *Actinomyces.* Infections caused by actinomycetes are actinomycosis, nocardiosis and actinomycetoma. Disease caused by aerobic actinomycetes are worldwide and affect tissue and the immune state of the patient.

In 1888, Nocard first recognized the pathogenic potential of aerobic actinomycetes. Since then, several aerobic actinomycetes have been a major source of interest for the commercial drug industry and have proved to be extremely useful for producing novel antimicrobial agents. They have also been well known as potential veterinary pathogens affecting many different animal species. The medically important aerobic actinomycetes may cause significant morbidity and mortality, in particular in highly susceptible severely immunocompromised patients, including transplant recipients and patients infected with human immunodeficiency virus. Very few plant diseases are caused by actinomycetes. The best known is potato-scab (Lechevalier, 1968).

Of the specific type of actinomycetes, *Actinomyces israelii* causes skin and mouth lesion at wound sites in human being, *Actinomyces viscous* and *Actinomyces naeslundii* causes dental caries or dental decay in human being. *Nocardia otitidiscariarum* causes an ear disease in guinea pigs, and *Dermatophilus congolensis* causes dermatophilosis, a severe dermatitis of cattle, sheep, horses, and occasionally humans. Several species of

Streptomyces cause the disease actinomycosis in humans and cattle. Taxonomy of the common pathogenic aerobic actinomycetes is shown in Table 7.

Table 7: Taxanomy of the common pathogenic aerobic actinomycetes (Khatri, 2002)

Aggregate group	Description	Organism	Disease	Habitat	Appearance in host tissue	
					Exudates	Stained Sections
Nocardioform	Diphtheroid to filamentous, true branching, mycelial elements, commonly fragmented to give coccoid to bacillary spores. Some species are partially acid- fast and produce aerial hyphae.	*Nocrdia asteroids*	Pulmonary transient to chronic suppurative abscesses. Generalized systemic disease often with central nervous system involvement. Mycetoma rare.	Ubiquitous in soil, animals, and humans.	Granules are small (25-150 μm), soft, white to yellowish, Lobuchelated, sometimes clubbed, occurring only rarely, generally in Mycetoma; usually occurs as clumped or scattered, acid- fast, branched filaments, undergoing fragmentation.	Granules are irregularly oval, staining lightly with hematoxylin, often with a distinct eosinophilic periphery fringed, and sometimes clubbed. Gram stain gives similar patterns; more commonly, colonies are either loose mycelium or scattered, fragmenting filaments.

Aggregate group	Description	Organism	Disease	Habitat	Appearance in host tissue	
					Exudates	**Stained sections**
		Nocardia brasiliensis	Frequent agent of mycetoma and isolated sporotrichoid lesions. Pulmonary and generalized disease rare.	Ubiquitous in soil: predominantly North and South America, Central Plateau of Brazil and India.	As above, but granule are common in mycetomata; branched filaments in cutaneous abscesses.	As above, but granules are common in mycetoma.
		Norcardis otitidiscaviarum	Generalized systemic disease mycetoma.	Ubiquitous in soil: predominantly Tunisia, Japan, India, Mexico, and United states.	As observed for *N. brasiliensis*	As observed for *N.* brasiliensis
		Rhodococcus spp.	Generalized granulomatous disease, central nervous system involvement, eczematous and granulomatous dermatitis septicemia, and pericarditis.	Ubiquitous in soil and plant material.	Small, white to yellowish soft granules. May appear as loose colonies of coccobacilli. Some species are acid-fast.	Granules are similar to those of botryomycosis masses of gram positive coccobacilli surrounded by suppuration. Also form granulomata with epithelial and giant cells.

Aggregate group	Description	Organism	Disease	Habitat	Appearance in host tissue	
					Exudates	**Stained Sections**
Maduromycetes	Filamentous with mycelial elements fragmenting to cocobacillary forms. Spores formed within a sheath on aerial or substrate mycelium.	*Actinomadura madurae*	Mycetoma	Ubiquitous in soil, plants, thorns, and decaying vegetation.	Large (1-5 mm) soft, white to yellowish or raddish granules, irregularly oval, serpiginous, or Lobucheted.	H & E[a]: center of granule is hollow or tenous, surrounded by denser network staining dark purple, wide, dense, pink border with long fringes and usually clubs.
		Actinomadura pelletieri	Mycetoma	Soil, thorns, plants, and decaying vegetation. Found in West Africa, Trans Africa Belt and India.	Granules are soft, small (300-500 µm), and deep red, smooth-edged or finely denticulate, irregularly spherical, sometimes with large Lobuchees.	H & E: granules round, shrply delimited, characteristically fracturing into large segments; stain basophilic with a lighter purple peripheral band.
		Nocardiopsis dassonvillei	Mycetoma	Ubiquitous in soil.	Insufficient information; granules not reported.	Insufficient information.

3.8 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

ELISA is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample. The ELISA has been used as a diagnostic tool in medicine and plant pathology, as well as a quality control check in various industries.

ELISA makes use of the antibodies to which enzymes have been covalently bound such that the enzyme's catalytic properties and the antibody's specificity are unaltered. It is highly specific, sensitive and requires only microlitre quantity of test reagent. It is widely used in detection of a variety of antibody and antigens such as hormones, toxins and viruses.

Enzymes are molecules which function as catalysts for certain biochemical reactions. A small amount of enzyme can react with much larger amounts of substrate to produce detectable levels of breakdown products in a given time. Enzymes which bring about chromogenic or fluorescent changes in their substrates are selected for enzyme immunoassay. Typically linked enzyme includes peroxidase, alkaline phosphatase, and galactosidase, all of which catalyze reactions whose products are colored and can be detected in very low amounts.

When some of the reactants are adsorbed on a solid phase it is called heterogeneous enzyme linked or Enzyme Linked Immunosorbent Assay (ELISA). Antibodies are conjugated or labelled with an enzyme by addition of glutaraldehyde in such a way that resulting conjugate retain both immunological and enzyme activity. They can be assayed by their ability to break down a suitable substrate. The most commonly used enzyme is Alkaline Phosphatase (AP) and Horse Radish Peroxidase (HRP) with their respective

substrates of P- nitrophenyl phosphate and O-phenylenediamine dihydrochloride (OPD). The color changes in the substrate due to enzyme activity are assayed visually or in a simple spectrophotometer.

Two basic ELISA methodologies have been developed, one for detecting antigen (direct or sandwich ELISA) and the other for detecting antibodies (indirect ELISA). In indirect ELISA technique, known antigen is attached to the inside surface of the well and the antiserum produced against antigen is added. After incubation and washing, enzyme labelled second antibody is reacted with the antibody that has attached to the antigen. The uncombined labelled enzyme is washed from the well and a substrate is then added. The presence and concentration of antibody that has reacted with the antigen is shown by a change in color of the substrate. The more intense the color, the higher the concentration of antibody in the serum.

The ELISA has to be optimized by means of chequerboard titration. In this antigen is titrated by reciprocal dilution in one direction on the microtitre plate and reciprocal dilution of test sample are titrated in the opposite direction. The antigen and serum dilution which give high optical densities and good signal to ratios when compared to the negative control serum are chosen.

ELISA tests are generally highly sensitivity and specific and compare favorably with radioimmune assay (RIA) tests. They have the added advantages of not needing radioisotopes or a radiation-counting apparatus. Several researches have conducted through IND-ELISA for identification of disease and taxonomical study.

CHAPTER-IV

4. MATERIALS AND METHODS

4.1 Revival of Isolates

Isolates of Actinomycetes preserved in Glycerol based media were revived. They were revived by streaking on Starch Casein agar and incubated at 28^0C for 7 days (Pandey, 2004).

4.2 Characterization

4.2.1. a. Macroscopic Characterization

Macroscopic study was done by observing the color and texture of the colony from both side of the Petri dish.

4.2.1. b. Microscopic Observation

Cover Slip Method

The sterilized cover slips were carefully inserted at an angle of about 45^0 into solidified Starch casein agar medium in Petri dish, until about half of the cover slip was buried in the medium. The isolates were inoculated along the line where the medium meets the upper surface of the cover slip. It was incubated at 28^0C for 7-10 days. Then the cover slip was carefully removed and was placed upward on the slide (Williams and Cross, 1971). The growth on the cover slip was fixed with a few drops of absolute methanol for 15 minutes. It was washed with tap water and blot dried. Then it was stained with crystal violet reagent for 1 minute followed by washing and drying and examined under microscopic field (1000X).

During microscopic examination substrate mycelium and mycelium were observed according to William et al, (1976). Colour arrangement and texture of conidiospore and

arthrospore on the mycelium were observed and compared with those described in Bergey's Manual of Determinative Bacteriology, 9th Edition (2000).

4.2.2 Physiological and Biochemical Test

Sugar utilization Test

Carbohydrate utilizing media (CUM) was prepared and filter sterilized sugar was added in it. After this, isolates of sample 137.2 were added in prepared media. It was then incubated at 28° C for 7 days. Changes in color of media were noted after incubation. The sugars used for this test are Adonitol, Arabinose, Dextrose, Galactose, Mannitol, Raffinose, Salicin, andxylose.

Hydrolysis Test

Gelatin Hydrolysis

Solidified gelatin agar plates were divided into 4-sectors and isolates were streaked on it. The plates were incubated at 28° C for 7 days. Hydrolysis was confirmed by flooding the plates with mercuric chloride solution.

Casein Hydrolysis

Solidified Skim milk agar plates were divided into 6 sectors. Each sector was streaked with the isolate Lobuche 137.2. It was incubated at 30° C for 7 days. After incubation, plates were flooded with mercuric chloride solution and observed.

Starch Hydrolysis

Solidified starch agar plates were inoculated with the isolates and incubated for 7 days at 30° C. Then iodine solution was flooded onto the plates to see for the clear zone of hydrolysis around the colony.

Tween – 20 Hydrolysis

The isolates were streaked on the solidified Tween - 20 agar plates and incubated at 28^{o} C for 7 days. Observations were made for clear zone around the colony.

Hypoxanthine hydrolysis

Hypoxanthine (0.5 gm suspended in 10ml of distilled water) was autoclaved. It was then mixed into 100 ml of sterile nutrient agar cooled at 45^{o} C and was poured into sterile petriplates. Isolates of Lobuche 137.2 were streaked on it and was incubated and observed.

Xanthine Hydrolysis

To 100 ml of melted nutrient agar, 4 gm of xanthine was added and was steamed for 30 mins. It was mixed well to suspended aminoacid and was poured into plates. Isolates were streaked. It was incubated and observed.

Arginine Hydrolysis

The isolate was incubated in arginine broth for 24 to 28 hrs and a few drop of Nessler reagent was added. A brown color indicates hydrolysis.

Urea Hydrolysis

Each test tube dispensed with urea agar was inoculated with the isolates and incubated at 28^{o} C for 7 days. Observations were made daily for positive test. Positive test was confirmed by the change of color of the agar to pinkish red.

Nitrate Reduction

Each of the isolates was inoculated into test tubes dispensed with nitrate broth and incubated at 30^{o} C for 5-7 days. Nitrate reduction was detected by adding few drops of Sulphanilic acid reagent and Alpha-napthylamine reagent into the culture broth. A distinct red or a pink color indicated the reduction of nitrate.

Temperature Tolerance

The isolated were grown at 30^{o} C and 50^{o} C and growth was recorded after 7 days of incubation.

Resistance Towards Sodium Chloride

Basal medium consisting of 5%, 7%, and 10% sodium chloride was prepared in three batches. The medium were autoclaved and solidified in petriplates. Agar plates were then divided into sectors. Each sector was streaked with isolates. The plates were incubated at 30^{o} C for 7 days. Observations were made daily. The observations were made to record highest concentration of salt that allow the growth.

Motility Test

Motility test of the organism was carried out in the SIM medium where the organism was stabbed and incubated at 28^{o} C for 18 to 72 hrs. The motility was confirmed by the turbidity of the medium.

H_2S Production Test

This test was done to determine the production of hydrogen sulphide (H_2S) gas from sulphur containing amino acids. By the reduction of organic sulphur source such as thiosulphate (- S_2O_3) or the dilution of organic sulphur supplied by the R_1 – SH functional group of the amino acid. Cysteine , which is present in peptone produces H_2S gas. Lead

acetate, the salt of the metal, is an indicator of H_2S production. H_2S is a colorless gas, which upon contact with lead acetate produces the sulphide of a black precipitate, indicated by a visible black color reaction.

$$Pb(C_2H_3O_2)_2\ 3H_2O + H_2S \rightarrow PbS + H_2O + CH_3COO$$

The test organism was stabbed in TSI and SIM media and incubated at 28^oC for 18 to 72 hours. The blackening of the medium along the line of inoculation or through out entire butt indicated the positive test for H_2S production.

Triple Sugar Iron (TSI) Agar Test

This test was done to determine the ability of an organism to utilize a carbohydrate incorporated in a basal medium, with or without the production of gas, along with the determination of hydrogen sulphide (H_2S) gas production.TSI medium contains three carbohydrates: lactose and sucrose at 1% concentration and glucose at 0.1% concentration. Some organisms have ability to ferment all the three carbohydrate, whereas some organisms ferment only glucose. But some are unable to ferment any kind of sugar present in the TSI agar. TSI agar also contains sodium thiosulphate. Those organisms which can reduce sulfur produces H_2S gas. This gas reacts with ferrous sulphate present in the medium into ferrous sulphide, which is black in color. Carbohydrate fermentation can be interpreted as follows:

Table 8 Carbohydrate fermentation in TSI agar test.

Serial Nos.	REACTION (slant/butt)	SUGAR USED
01.	Alkaline/Acid (Red/Yellow)	Glucose only utilized
02.	Acid/Acid (Yellow/Yellow)	Glucose and lactose utilized or glucose and sucrose utilized or glucose, lactose and sucrose utilized
03.	Alkaline/Alkaline(Red/Red)	Neither glucose nor lactose attacked, peptone utilized
04.	Alkaline/No change (no change in slant and butt	Neither glucose nor lactose attacked, peptone utilized

The test organism was inoculated in the TSI agar slant and butt with the help of straight wire. The medium was incubated at 28^{o} C for 18 to 72 hours and the result was interpreted.

Indole Test

Indole test was used to determine the ability of an organism to decompose aminoacid tryptophan present in peptone into indole. These organisms contained tryptophanase enzyme, which catalyzes the degradation of tryptophan. The enzyme tryptophanase reduces tryptophan from its side chain, leaving aromatic ring in the form of indole. Indole production was detected by Kovac's reagent which contains 4(p)-dimethyleaminobenzaldehyde. This reacts with indole to produce a red colored compound. For this test medium was inoculated and incubated at 28^{o} C for 18 to 72 hrs. After incubation a few drops of Kovac's reagent was added in it and a red ring at the surface of the medium was observed.

Methyl Red Test

This test is performed to detect the ability of an organism to produce sufficient acid from the fermentation of glucose to give a red color with the indicator methyl red. This results in decrease of pH of medium up to 4.4. The methyl red test medium was inoculated and

incubated at 28^{o} C for 18 to 72 hrs. Methyl red indicator was added in it to see the change in color. The positive test was confirmed by red color, produced in the medium.

Voges-Proskauer (Acetoin Production) Test

Some micro-organisms produce acetyl methyl carbinol or its reduction product 2, 3 butylene glycol from pyruvic acid in the media. It can be detected by colorimetric method. The diacetyl formed during the test by oxidation of acetyl methyl carbinol or 2, 3 butylene glycol reacts with the peptone and produces red color. The medium was inoculated and incubated at 28^{o} C for 18 to 72 hrs. After incubation, VP reagent was added in it. The positive reaction was indicated by the development of the pink color in the medium.

Citrate Utilization Test

This test was performed to determine the ability of an organism to utilize citrate as sole source of carbon and energy for growth and an ammonium salts as sole source of nitrogen. Ammonium salts broken down to ammonia resulting in salt alkalinity that causes change in color of indicator used in the medium. The test organism was streaked on the slant of Simmon's citrate agar medium and incubated at 28^{o} C for 18 to 72 hours. Positive test was confirmed from the growth of an intense blue color on the slant.

Catalase Test

This test is used to differentiate those bacteria that produce the enzyme catalase. Catalase acts as a catalyst in the breakdown of hydrogen peroxide to oxygen and water.

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

A speck of test organism was picked from nutrient plate with a clean sterile glass rod and placed on a clean glasslide having a drop of 3% H_2O_2. Positive test was indicated by the production of gas bubbles from the surface of the solid culture material.

Oxidase Test

This test was done to detect the presence of a cytochrome oxidase that catalyses the oxidation of reduced cytochrome by molecular oxygen, which in turn acts as an electron transfer system. The test organism was picked with a clean sterile glass rod and smeared over the Kovac's reagent coated strip (Whatman's No.1 filter paper impregnated with 1% tetramethyl-p-phenylene diamine dihydrochloride). A positive reaction was indicated by an intense deep purple blue.

4.3 ISOLATION OF SPORE

The test organism was grown on petriplate as confluent lawn. 10ml of sterile saline was added to 96 hrs old cultures. The spore contain in them were scrapped. This suspension was transferred into microfuge tube and was vortexed. Then it was filtered through sterilized cotton. About 1ml of this seed culture contain more than 10^7 spore/ml was inoculated on YEME medium aseptically. It was incubated on water bath shaker (150 rpm/min) at 30^o C. After proper growth of spores, spores were harvested by centrifuging 5000g /20 min at 4^o C and washed five times with PBS (pH 7.2). Supernatant was discarded and pellet was suspended on 1ml of PBS and was stored at - 20^o C.

4.4 IMMUNOLOGICAL WORK

4.4.1 Selection of Animal

For antibody production against spore, two New Zealand White rabbits (two for each) of 3-5 kg of 2-3 months were tested for parasites and were found free of parasites and diseases.

4.4.2 Collection of Control Serum

Rabbits were bled from lateral marginal vein of ears. About 5-10 ml of blood was collected from syringe with 26G needle. The serum was isolated and decomplemented by incubation at 56^{o} C for 30 min. This was stored in microfuge tube.

4.5 ANTISERUM PREPARATION AND IMMUNIZATION

4.5.1 Inactivation of Spore

Spores were inactivated by formalin and incubated at room temperature for 30 mins. Then it was incubated overnight at 4^{o} C. The cell suspension was centrifuged at 10,000g for 10 mins. at 4^{o} C and washed with PBS (pH 7.2) and resuspended in sterilized PBS (pH 7.2).

4.5.2 Spore Standardization

The antibody production requires the cell density greater than 1×10^{7} (A_{450}) spores/ml (Hopwood et al). Thus, spore suspension used for immunization was matched with 1^{st} tube of McFarland Nephalometer standard i.e. the approximate cell density was 3×10^{8} spore/ml. When equal proportion of adjuvant was added to the suspension the final cell number in emulsion was 7.5×10^{7} spores/ml.

4.5.3 Immunization

This spore suspension was taken as antigen which emulsified, immunized and sample bleed as shown in Figure 2.

Antigen Selection

(Spore Concentration ≥ 3×10^8 spores/ml)

↓

0.5 ml of spore + 0.5 ml of Complete Freund's Adjuvant (CFA)

(Homogenate with double hubble needle of 18G)

↓

Check for stable oil in emulsion

↓

1^{st} dose

Inject (7.5×10^8 spore/ml)

After 14 days interval

↓

2^{nd} dose

Inject (7.5×10^8 spore/ml) mixed with Incomplete Freund's Adjuvant (IFA)in opposite flank

After 14 days interval

↓

3^{rd} dose

Inject same as 2^{nd} dose

After one week

↓

Serum collection

↓

Store in aliquots at - 20^o C

Figure 2: Immunization Schedule for Spore (Khatri, 2002)

4.6 INDIRECT PROCEDURE OF ELISA FOR SPORES OF LOBUCHE 137.2 (Khatri, 2002)

4.6.1 Choice of Solid Phase

The solid phase used was cobalt irradiated polystyrene microplates (Nunc Immunoplates I, Nunc, Denmark).

4.6.2 IND-ELISA for Spores

1. Coating

Spore suspension, sensitized with formalin and matched with the 1st tube of the McFarland Nephalometer standard (3×10^8 cell density/ml) was taken for coating. The dilution made were 1:20, 1:50, 1:100, 1:200, 1:500 and 1:5000. 100µl of this coating solution were added in the wells. The plate was covered with parafilm and incubated at 30^{o} C for two hours.

2. Blocking

Wells were washed with 100µl of PBS/Tween-20 for 3 times and were dried by tapping on a clean and dry towel. 100µl of blocking buffer was added to each of the wells. It was covered and incubated at 30^{o} C for 1 hour.

3. Addition of 1st Antibody

Wells were washed with PBS/Tween-20 and dried by tapping on a clean and dry towel. 100µl of primary antibody (whole antiserum) diluted (1:100) in blocking buffer was added vertically in the microtitre plate. It was covered with parafilm and incubated at 30^{o} C for 2 hrs.

4. Addition of 2nd Antibody

It was washed 3 times with PBS/Tween-20. About 100µl of second antibody (antirabbit-goat- IgG- conjugated with Horse Radish Peroxidase (HRP) was at the dilution of 1:10,000. It was covered and incubated at 30^{o} C for 2 hrs.

5. Addition of Substrate and Reading

It was washed with PBS/Tween-20 for 3 times. 100µl of fresh substrate (Ortho-phenylenediamine dihydrochloride (OPD) was added. It was incubated at room temperature for 10-15 mins. The reaction was stopped with addition of 50µl of 3M sulfuric acid. Plate was read for its absorbance at 490nm setting with the blank.

Dilute antigen in coating buffer

(1:20, 1:50, 1:100, 1:200, 1:500, 1:5000)

↓

Coat the plate with antigen

↓

Incubate 2 hours at 30^{o} c

↓

3 × wash

↓

Add blocking buffer

↓

Incubate at 30^{o} c for 1 hour

↓

3 × wash

↓

Add first antibody (ab_1) (1:100)

↓

3× wash

↓

Add conjugate (ab_2) (1:10,000)

↓

Incubate at 30^{o} c for 2 hour

↓

3× wash

↓

Add substrate

↓

Incubate at room temperature and determine optimum color

↓

Stop the reaction by adding stopping buffer

↓

Read absorbance at 490nm

Figure 3: Chequerboard titration (Modified method of Voller et al, 1976)

CHAPTER-V

5. RESULTS

5.1 REVIVAL AND COLONY CHARACTERIZATION

Preserved isolates of Actinomycetes species were revived on Starch casein agar and incubated at 28^{o} C for 7 days. The colonies of actinomycetes were light in color at the beginning and later become dry, stout and concentrated in the centre producing powdery spore at the top of the top of the colony but never spread like fungi. Different color of colonies observed were chalky white, grey, bluish white, Creamy, and greyish white. They left footmark when scrapped. Out of 80 isolates revived from the preserved soil samples of Khumbu. Lobuche 137.2 was selected for further study.

5.2 MORPHOLOGICAL CHARACTERISTICS

Macroscopic Characteristics

Macroscopic characteristics were observed from the both side of the Petri dish. Macroscopically, colonies have round margin, entire configuration, opaque, convex elevation and left footmark when scrapped. Colour of colony of few samples are shown below in Table 9.

Table 9: Colour of colony of actinomycetes isolated from different samples.

AREA SELECTED	Colour of colony	
	ISOLATES OBTAINED	NATURE
Lobuche (5000 – 5300 m)	137.2	Chalky white
	28.2	Yellow
	20.2	Creamy
	55	Yellowish white
	7.1	Creamy
Lukla (2660 m)	5	Dirty white
	14.1	Creamy
	8.8	Dirty creamy
	17.5	Creamy
	16.2	Yellowish

Photo1. Pure colonies of different samples of Lobuche viz., A = Lobuche 137.2 , B = Lobuche 28.2, C = Lobuche 137.2, D = Lobuche 20.2 in starch casein agar

Photo2. Mycelium of Lobuche 137.2 in 1000X

Microscopic Characteristics

Isolates of Lobuche 137.2 has been isolated for further study. So, only its microscopic study has been done. Coverslip method was used for the microscopic identification of isolates of Lobuche 137.2. Isolates of Lobuche 137.2 was inoculated and incubated on Starch casein media at at 28^{o} C for 7 days. After seven days of incubation, coverslips were stained and observed under microscope.

Under microscope, it was found that Lobuche 137.2 has long mycelium without fragments. Sporangia are spherical in shape. Sporangia are borne at the tip of aerial hyphae and present in chain form. Number of spore range from seven and above. On comparing the structure of Lobuche 137.2 with Bergey's manual of bacteriology, it has distinct structure than other known actinomycetes. So, it can be considered as rare actinomycetes.

5.3 PHYSIOLOGY, BIOCHEMISTRY AND IMMUNOLOGY OF Lobuche 137.2

For detail study of physiology, biochemistry, and immunology, isolates of Lobuche 137.2 was selected as it has shown distinct mycelia growth and spore arrangement. Results of physiological, biochemical and immunological tests have been graphically shown in figure 4, 5, 6 and 7. Further detailed work on this species help to find new species of actinomycetes.

5.3.1 PHYSIOLOGICAL CHARACTERISTICS

5.3.1 a. Carbohydrate utilization test

During observation, the conversion of the color of the phenol red on carbohydrate utilizing media was recorded through out seven days of incubation. It was found that arabinose, dextrose, galactose, mannitol, and salicin were utilized. But adonitol, raffinose, and xylose were not utilized.

Table 10: Different sugar utilized by Lobuche 137.2 on carbohydrate utilizing media

CARBOHYDRATE UTILIZATION MEDIA (CUM)	
SUGAR USED	**RESULT**
Control	-
Adonitol	-
Arabinose	+
Dextrose	+
Galactose	+
Mannitol	+
Raffinose	-
Salicin	+
Xylose	-

Note: (+) = Utilized, (-) = Not utilized

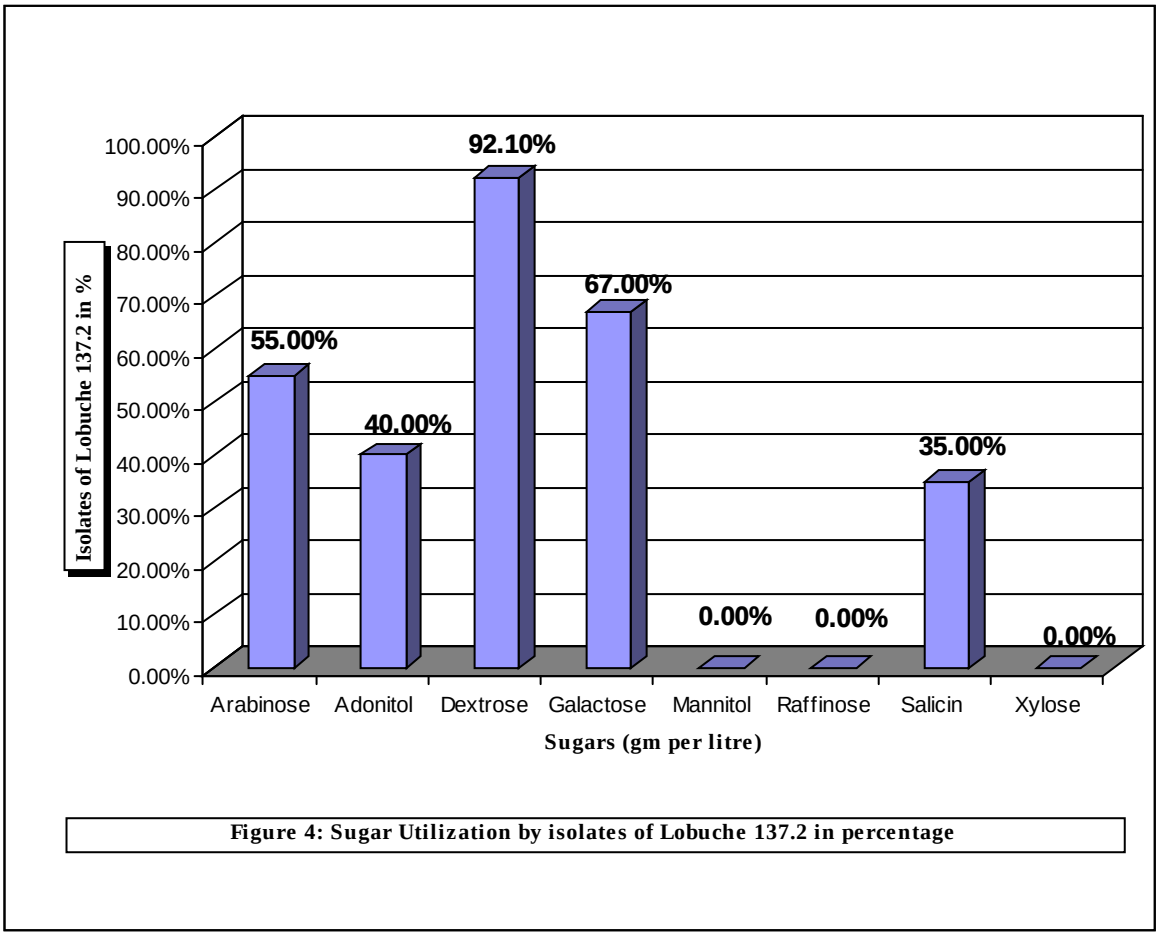

Figure 4: Sugar Utilization by isolates of Lobuche 137.2 in percentage

Photo3. Structure of novel actinomycetes isolated from sample Lobuche 137.2 in 1000X

Photo4. A mature sporangium of novel actinomycetes isolated from sample Lobuche 137.2 in 1000X

Table 11: Different biochemical tests used for the identification of Lobuche 137.2

TEST INVOLVED	SUBSTRATE INVOLVED	RESULT
Hydrolysis test	Hypoxanthine	+
	Xanthine	+
	Arginine	+
	Strach	+
	Gelatin	+
	Casein	+
	Tween-20	+
	Urea	+
Others	Gram stain	+
	Catalase	+
	Oxidase	+
	Nitrate reduction	+
	H_2S Indole Motility	+
	MR/VP	+/-
	Citrate	+
	TSI	Alkali/ Acid
	Oxidative/Fermentative	+/-
	Growth at 30^oC	+
	Growth at 50^oC	-
Antimicrobial test	Against Gram positive	-
	Against Gram negative	-
	Against Fungi	-

NOTE: + = utilized; - = not utilized

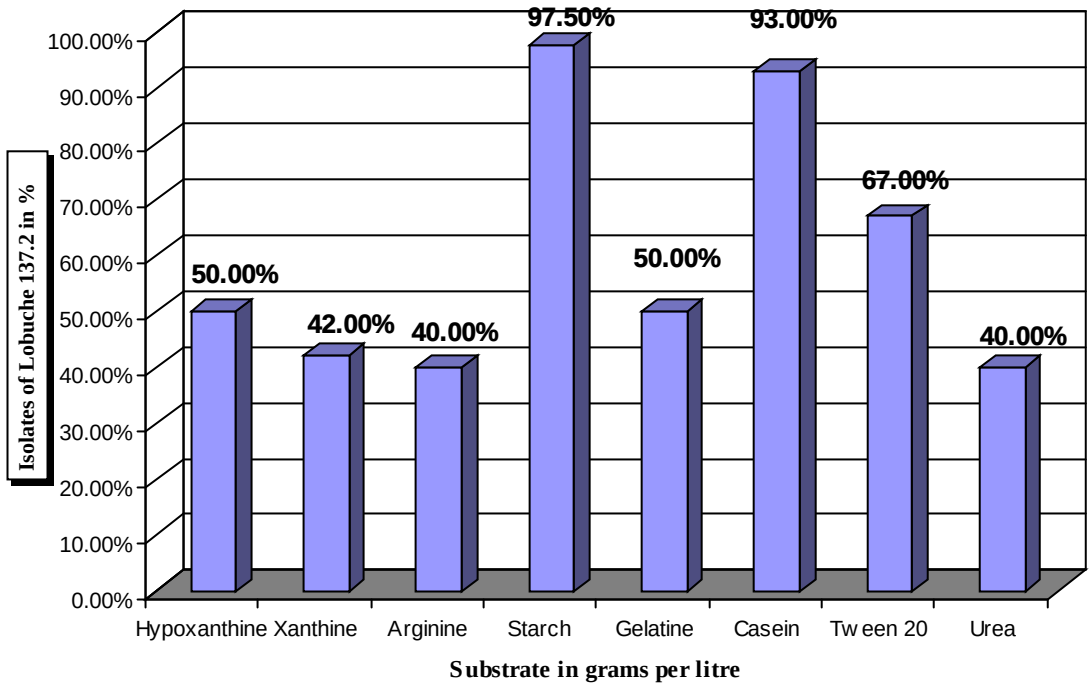

Figure 5: Substrate Utilization by isolates of Lobuche 137.2 in percentage

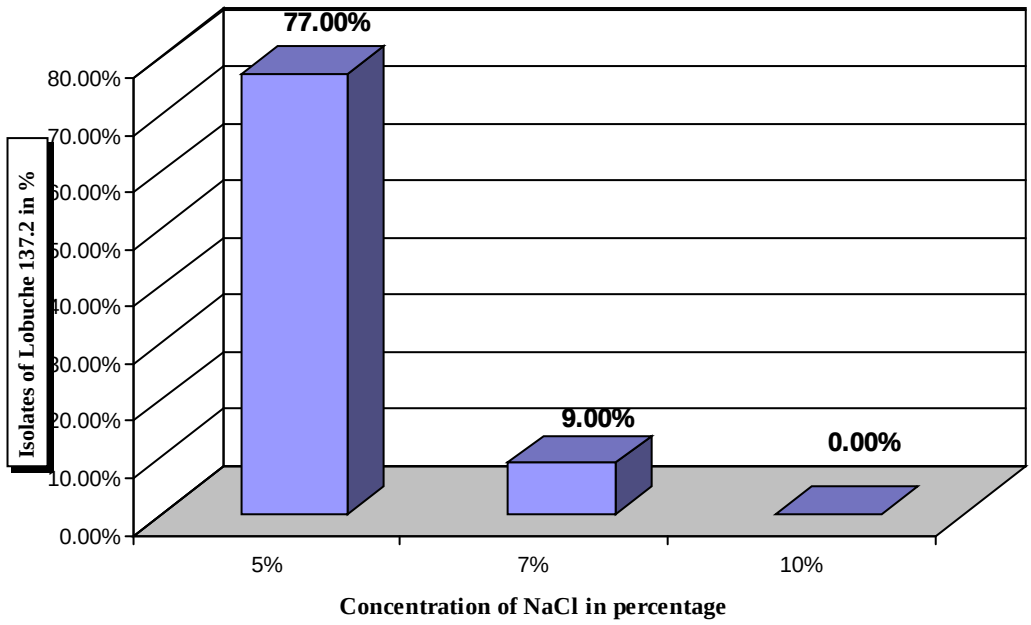

Figure 6: NaCl Resistance Test by isolates of Lobuche 137.2 in percentage

5.4 IND-ELISA FOR SPORE SUSPENSION OF LOBUCHE. 137.2

Spore suspension matched with the 1st tube of McFarland Nephalometer containing approximate cell density of 3×10^8 spore/ml was taken for coating by chequerboard titration. Serial dilutions of spore suspension of 1:20 to 1:5000 were reacted with Ab_1 of 1:100 and Ab_2 of 1:10,000. It was found that spore antigen of 1:20 dilution; primary antibody (Ab_1) of 1:100 and secondary antibody conjugated with HRP (Ab_2) of 1:10,000 were distinctly visible. Optimum density value of 1:20 spore dilution for Ab_1 (1:100) and Ab_2 (1:10,000) was 0.601.The probable number of spore was 0.6×10^6 spores/200 µl of coating buffer. Series of well is also used for detecting reactivity of secondary antibody with subrate. Change in colour in this row shows that reaction in test row is true.

Table 12: IND-ELISA FOR SPORES OF SAMPLE LOBUCHE 137.2

ANTIGEN →	**1:20**	**1:50**	**1:100**	**1:200**	**1:500**	**1:5000**
WELL → ↓	**1**	**2**	**3**	**4**	**5**	**6**
A (Control) (+Ag-Ab_1(1:100)-Ab_2(1:10,000))	0.000	0.002	0.001	0.004	0.007	0.069
B (Control) (+Ag-Ab_1(1:100)+Ab_2(1:10,000))	0.004	0.032	0.008	0.001	0.040	0.073
C (Control) (-Ag+Ab_1(1:100)+Ab_2(1:10,000))	0.037	0.025	0.045	0.011	0.023	0.076
D (Test) (+Ag+Ab_1(1:100)+Ab_2 (1:10,000))	0.601	0.600	0.584	0.500	0.500	0.165

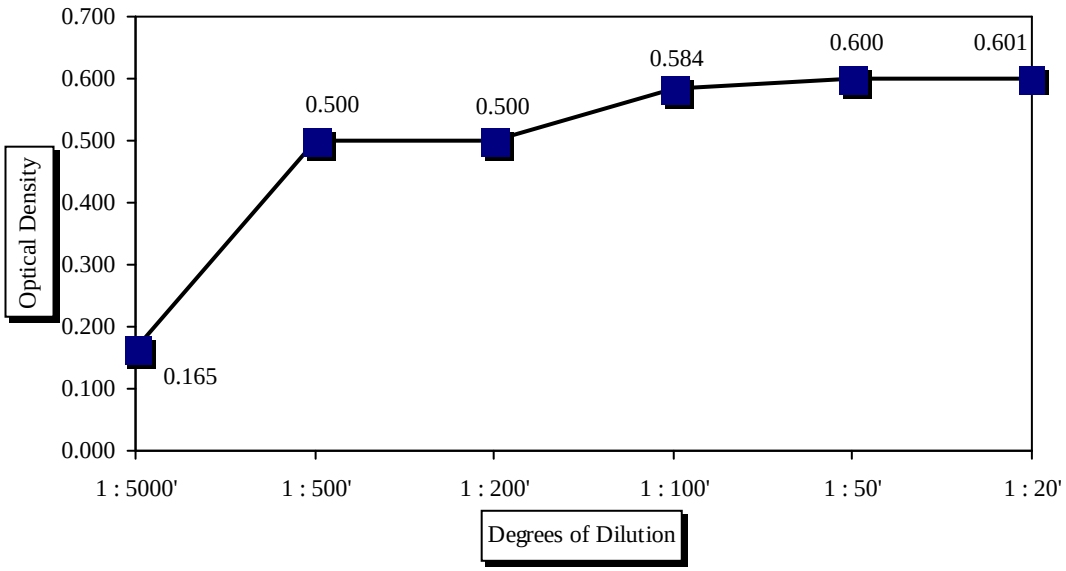

Figure 7: Determination of Optimal No. of Spore for coating IND-ELISA Plate

Photo5. ELISA plate for spore of Lobuche 137.2 (Row A = Control, B = Control, C = Control, D = Test, E = Ab_2 + OPD)

CHAPTER VI

6. DISCUSSION AND CONCLUSION

6.1 DISCUSSION

Actinomycetes are a large group of family, which are distinguished from one another by the nature of their mycelia and spores. In order Actinomycetales, there are eight families. But some actinomycetes have different microscopic characters. Such actinomycetes can be considered as new actinomycetes and must be studied further. The major emphasis is now on 16S rRNA homologies in addition to cell wall chemistry along fatty acid and lipid pattern (William et al, 1971). So, sequence analysis of 16s rRNA in such actinomycetes is essential.

For the new actinomycetes, proper detection and recovery method is an important prerequisite.It helps to know the fate of such actinomycetes released into the environment. Immunological technique has long been used for the detection and identification of the microorganism in microbiology laboratory and also in soil, water and other environmental samples. The immunological study of actinomycetes was done by performing IND-ELISA against spores of selected isolate.

Isolation, identification and characterization of actinomycetes were followed by immunological technique here IND-ELISA. Actinomycetes preserved in glycerol base media were revived on Starch casein agar. The test sample was streaked on SCA plate and was incubated at 28^{o} C for 7 days. The sample Lobuche 137.2 has an opaque white color colony with convex elevation, entire margin, round configuration and dry consistency.

Microscopically, Lobuche 137.2 has long mycelium without fragments. Sporangia are spherical in shape and present at the tip of the hyphae. Sporangia are present in chain. The number of spores range from seven and above.

Carbohydrate utilizing media has peptone and sodium chloride. So, it is very obvious that actinomycetes can utilize it as protein and could proliferate and multiply. While on addition of sugar of interest, utilization by particular organism differs. For carbohydrate utilization test, carbohydrate utilizing media (CUM) with phenol red 0.02% was used. In this test, Arabinose, Dextrose, Galactose, Mannitol and Salicin were utilized but Adonitol, Raffinose and xylose were not utilized. Utilization was determined by change of colour. In case of carbohydrate utilizing media, red colour change into yellow. In this research, for the determination of degree of conversion of phenol red, the incubated tube was interpreted according to the CMYK colour combination chat. Due to which, the rate of utilization of different sugar could be enumerated and could compare on taxonomical study.

In hydrolysis test, Hypoxanthine, Xanthine, Arginine, Strach, Gelatin, Casein, Tween-20 and Urea were hydrolysed. On Gram test, it was found that Lobuche. 137.2 is gram positive. Lobuche 137.2 did not show activity against test microbial organisms.

IND-ELISA was performed to determine the immunological property of sample Lobuche 137.2. IND-ELISA was done against spore of sample Lobuche 137.2. For the validity and reproducibility of the results, three sets of control and a test were set. Control A (+Ag-Ab_1-Ab_2), control B (+Ag-Ab_1+Ab_2), control C (-Ag+Ab_{1+} Ab_2) and

test (+Ag+Ab_1+Ab_2) were set. The quantity of enzyme -labelled antibody bound to the antibody bacterial complex on the solid phase is measured as increase in absorbance per unit time, incubating with appropriate substrate. Here, the optical density of test sample was higher than the blanks. The main purpose of this study was to establish best easiest method for distinguishing the large population of actinomycetes through IND-ELISA.

6.2 CONCLUSION

Isolates were preserved on Glycerol based media and revived on Starch-Casein agar plate at 28°C for 7 days. The isolate Lobuche 137.2 (new species) were selected for physiological, biochemical and immunological study. It was identified as species of actinomycetes. Antiserum was produced against spores of isolate Lobuche 137.2 (new species). IND-ELISA was done for spore antigen. During this, coating was done by using 1:20 dilution (containing spore density of 0.6×10^6 spore/200µl), primary antibody of 1:100 and second antibody of dilution 1:10,000. It was distinct. Hence, IND-ELISA can detect 0.6×10^6 spores in 200µl of coating buffer.

CHAPTER – VII

7. SUMMARY AND RECOMMENDATION

7.1 SUMMARY

Isolates of Actinomycetes preserved on Glycerol based media were revived on Starch casein agar by streak plate method. Samples were chosen and streaked on plate having SCA media. They were incubated at 28^{o} C for 7 days. The isolates obtained are brown, chalky white, grey, white, creamy, brownish, blue, greenish, green, yellowish, yellow and when scrapped from the media the foot marks was retained.

The isolate Lobuche 137.2 were selected for further study. The colony of Lobuche 137.2 was creamy in colour. Physiological, biochemical and immunological study of Lobuche 137.2 were done. Different sugars were tested by using carbohydrate utilizing media (CUM) was done. On performing carbohydrate utilization test, it was found that Arabinose, Dextrose, Galactose, Mannitol and Salicin were utilized and Adonitol, Raffinose and Xylose were not utilized by Lobuche 137.2.

On biochemical test, it was found that isolates of Lobuche 137.2 could hydrolyse Hypoxanthine, Xanthine, Arginine, Starch Gelatin, Casein, Tween-20, and Urea. It was found that Lobuche 137.2 was gram-positive on gram staining. Lobuche 137.2 was catalase positive, oxidase positive, nitrate reductive, H_2S indole positive, Methyl red positive,citrate positive but Voges-Proskauer negative without motility. Lobuche 137.2 was oxidative and can grow at 30^{o} C. It did not grow at 50^{o} C. Lobuche 137.2 had not shown antimicrobial action against gram positive bacteria, gram negative bacteria and

fungi. Therefore by performing sugar utilization test and other biochemical test, the isolate Lobuche 137.2 was found to be related with actinomycetes.

On microscopic test, Lobuche 137.2 has long non-septate hyphae with the branching filaments, and spores are present at the end of filament in chain form. Spores are spherical in shape and present at the tip of hyphae in chain form. The number of spores range from seven to above. This makes isolate 137.2 structurally different from other actinomycetes. From this isolate 137.2 can be considered as rare species of group actinomycetes.

This test is further moved to serological test like IND-ELISA. For immunological work, antisera has raised against the spore isolate 137.2. Hyperimmunesera thus produced were checked. The IND-ELISA for spore was able to detect the spore density of 0.6×10^6 spores/200 µl of coating buffer. The OD (Optical Density) of the titer was sufficiently higher than the negative control and could be detected by naked eye.

7.3 RECOMMENDATIONS

- Nepal has varied microbial biodiversity. Due to this, there is high possibility of finding new species. So, screening of “rare” actinomycetes should be done.

- Actinomycetes are producers of natural products. So, detection of rare actinomycetes increases the possibility of finding new chemical entities.

- IND-ELISA against spore of other actinomycetes should be done.

CHAPTER-VIII

8. REFERENCES

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APPENDIX I

A. LIST OF EQUIPMENT, CHEMICALS, MEDIA/INGREDIENTS AND REAGENTS USED IN THE PRESENT STUDIES

EQUIMENTS USED

1. Microscope (Olympus, Japan)
2. Centrifuge (Sigma, 2KIs, Philip Harris)
3. Cryocentrifuge (Germany)
4. ELISA reader (Dyanatech)
5. Weighing balance (OHAUS, USA)
6. Water bath shaker (Grant)
7. Incubator
8. Shaker
9. Autoclave
10. pH mater
11. Oven
12. Microfuge tube
13. Micropipets
14. Parafilms

CHEMICAL USED

Calcium carbonate (Sd. Fine Chem., India), Crystal violet, Dehydrated Alcohol (Pharma Ltd.), Ethyl Acetate (Merck), Ferrous Sulphate (Sd. Fine Chem., India), Hydrochloric acid (Qualigens), Iodine, Magnesium Sulphate (Lobuchea Chem.), Mercuric Chloride (Merck), Methanol (Qualigens), Phenol red, Potassium Hydrogen Sulphate, Sodium Chloride (Sd. Fine Chem., India), Sodium Hydrogen Phosphate, Sodium Dodecyl Sulphate (Merck), beta-mercaptoethanol (Sigma), Second antibody conjugated with Horse Radish Peroxidase (HRP) (Genei), O- Phenylenediamine Dihydrochoride (OPD) (Sigma).

MEDIA AND INGREDIENTS

Agar (Hi-media, India), Casein (Merck), Nutrient agar (Hi-media), Nutrient broth (Hi-media), Urea Agar (Hi-media), Urea Crystal (Merck), Mueller Hilton Agar (Hi-media), D (-) Glucose (Loba : Chem. Indus. Co.), Sucrose (Qualigens), Mannitol (Hi-media), Raffinose (Sigma), Galactose (Hi-media), Fructose (Hi- Media), Starch (Hi-media), Gelatine, OF media, Sulphide Indole Motility (SIM) (Hi-media), Triple Sugar Iron (TSI) (Hi-media), Skim milked agar (Hi-media), Nessler reagent (Loba).

B. COMPOSITION AND PREPARATION OF DIFFERENT MEDIA

1. **Starch casein agar (SCA)**

Composition (gm/lit)	
Soluble starch	10.0 gm
Casein	0.30 gm
Potassium nitrate	2.00 gm
Di-potassium hydrogen Orthophosphate	2.00 gm
Magnesium Sulphate	0.05 gm
Calcium carbonate	0.02 gm
Ferrous sulphate	0.01 gm
Agar agar	20.0 gm
Final pH	7.20

All the ingredients are weighted and poured into conical flask. The volume was adjusted to liter by adding distilled water.

2. Carbohydrate utilizing media (CUM)

Composition (gm/lit)	
Bactopeptone	10.00 gm
Potassium dihydrogen phosphate	00.50 gm
Magnesium sulphate	0.50 gm
D/W	1000 ml
Phenol red	0.02%
Final pH	7.2

All the dry ingredients are weighted and poured into conical flask. The volume was adjusted to liter by adding distilled water.

3. YEME (Yeast extract malt extract)

Composition (gm/lit.)	
Difco yeast extract	3.00 gm
Difco bactopeptone	5.00 gm
Oxoid malt extract	3.00 gm
Glucose	10.00 gm
Surcose	340.00 gm
D/W	973.00 ml
Magnesium Chloride (2.5M)	2.00 ml

All the ingredients are weighted separately. Magnesium chloride was maintained 2.5M. Then sucrose was first dissolved into distilled water and volume was adjusted to liter. Then it was poured into a conical flask having rest of the ingredient.

C. REAGENTS

1. Crystal Violet Stain

Solution A: 2.0 gm of crystal violet was dissolved in 20 ml of 95% ethyl alcohol.

Solution B: 0.8 gm of ammonium oxoalate was dissolved in 80.0 ml of D/W.

Then the solutions A and B are mixed.

2. Gram Iodine

20 gm of potassium iodine was dissolved in 300ml of D/W. Then it was added with 100gms of iodine crystals. The volume was adjusted to 1000 ml by adding D/W.

3. Barritt's Reagent

Solution A: 5 gm of Alpha-Napthol in 100ml of Ethyl alcohol (absolute)

Solution B: 40 gm of Potassium hydroxide in 100 ml D/W.

4. Catalase reagent (2% H_2O_2)

Hydrogen peroxide	3 ml
D/W	97 ml

5. Kovac's reagent

Pure amyl or isoamyl alcohol	150 ml
p-dimethyl aminobenzaldehyde	10 gm
Conc. Hydrochoric acid	50 ml

6. Methyl red (indicate reagent)

Methyl red	0.1 gm
Ethyl alcohol	300 ml
Distilled water	200 ml

7. Oxidase reagent

Tetramethyl-p-phenylene

Diamine dihydrochoride	1 gm
D/W	100 ml

Once the above reagents are prepared, small filter paper strips were soaked with it, dried and then kept aseptic to test the oxidase activity.

8. Phosphate buffered saline (PBS, pH 7.2)

Sodium chloride	8 gm
Potassium dihydrogen Orthophosphate	0.2 gm
Disodium hydrogen Orthophosphate	1.15 gm
Potassium chloride	0.2 gm
Double distilled water	1000 ml

9. PBS/ T (Phosphate buffered saline/ Tween-20)

Add 0.5 ml of Tween-20 to a liter of PBS.

10. Blocking buffer

PBS/T and 0.5% BSA.

11. OPD solution

Dissolve a tablet in 0.05M phosphate citrate buffer, pH 5.0 to desired concentration (typically an OPD concentration of 0.4 mg/ml is used). Add 40µl of fresh 30% hydrogen peroxide per 100ml of substrate buffer solution in it, immediately prior to use.

APPENDIX II

1. Mcfarland Nephalometer tube

	0.5	1	2	3	4	5	6
Barium Chloride (ml)	**0.05**	**0.1**	**0.2**	**0.3**	**0.4**	**0.5**	**0.6**
Sulfuric acid (ml)	**9.95**	**9.9**	**9.8**	**9.7**	**9.6**	**9.5**	**9.4**
Approximate cell density ($\times 10^8$/ml)	**1.5**	**3**	**6**	**9**	**12**	**15**	**18**