SCREENING AND CHARACTERIZATION OF BIOACTIVE ACTINOMYCETES AND THEIR PRODUCTS FROM NEPAL AGAINST MULTIDRUG RESISTANT BACTERIA AND FUNGI

A THESIS SUBMITTED TO THE

CENTRAL DEPARTMENT OF MICROBIOLOGY

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TRIBHUVAN UNIVERSITY, NEPAL

FOR THE AWARD OF DOCTOR
OF PHILOSOPHY IN MICROBIOLOGY

BY

BINOD LEKHAK

MARCH, 2020

DECLARATION

Thesis entitled **"Screening and characterization of bioactive actinomycetes and their products from Nepal against multidrug resistant bacteria and fungi"** which is being submitted to the Central Department of Microbiology, Institute of Science and Technology (IOST), Tribhuvan University, Nepal for the award of degree of Doctor of Philosophy (Ph.D.) is a research work carried out by me under direct supervision of Prof. Dr. Dwij Raj Bhatta, Central Department of Microbiology, Tribhuvan University and co supervised by Prof. Dr. Anjana Singh, Central Department of Microbiology, Tribhuvan University.

This research is original and has not been submitted earlier in part or full in this or any other form to any university or institute, here or elsewhere, for the award of any degree.

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Binod Lekhak

RECOMMENDATION

This is to recommend that **Mr. Binod Lekhak** has carried out research entitled **"Screening and characterization of bioactive actinomycetes and their products from Nepal against multidrug resistant bacteria and fungi"** for the award of Doctor of Philosophy (Ph.D.) in **Microbiology** under our supervision. To our knowledge, this work has not been submitted for any other degree.

He has fulfilled all the requirements laid down by the Institute of Science and Technology (IOST), Tribhuvan University, Kirtipur for the submission of the thesis for the award of Ph.D. degree.

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LETTER OF APPROVAL

On the recommendation of Prof. Dr. **Dwij Raj Bhatta** and Prof. Dr. Anjana Singh, this PhD thesis submitted by Mr. **Binod Lekhak,** entitled **"Screening and characterization of bioactive actinomycetes and their products from Nepal against multidrug resistant bacteria and fungi"** is forwarded by Central Department Research Committee (CDRC) to the Dean, IOST, Tribhuvan University.

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ABSTRACT

Actinomycetes are versatile producers of diverse secondary metabolites including antibiotics. New therapeutics of microbial origin is essential to combat multidrug resistant human pathogens. This descriptive study was conducted to isolate, screen and characterize bioactive actinomycetes and their anti-microbial product along with optimization of cultural conditions for better metabolite production. A total of 240 soil and 48 water samples were collected from different parts of Nepal and actinomycetes were selectively isolated, primarily screened by perpendicular streak method and secondary screening was performed by agar well diffusion technique against selected bacteria and fungi. The minimum inhibitory concentration of ethyl acetate extract was carried out by tube dilution method. The screened actinomycetes strains were characterized by cultural, morphological, biochemical and 16SrRNA sequencing. Optimization of fermentation was carried out by cultivating the screened strains separately at different nutritional and cultural conditions. Characterization of ethyl acetate extract was performed by FT IR and LC-MS method.

Out of 320 actinomycetes isolates, 120 (37.5%) were found bioactive against one or more test microbes. Among them only 4(3.3%) were inhibitory against all test organisms. In secondary screening, the most potent strain A_3 showed highest activity against *C. albicans* with zone of inhibition of 41.33± 1.5mm and lowest minimum inhibitory concentration of 2.5mg/ml against *C. albicans* and *E. coli*. Phenotypic characteristics predicted that strains A_3, D_2, P_4 and J_1 were *Nocardiosis prasina*, *Streptomyces violarus, S. krainskii* and *S. tsusimaensis* respectively and the most potent strain A_3 was genotypically characterized as *N. prasina*. Optimization study revealed that starch, casein and potassium nitrate supported maximum bio mass and metabolite production in A_3, P_4 and J_1 strains. Likewise, incubation temperature of $30^0$with pH 7-8 for 7-8days incubation time were found optimum for all screened strains. FT-IR and LC-MS analysis revealed that the ethyl acetate extracts of all the strains contained diverse functional groups and compounds having molecular mass (m/z) ranging from 106 to 986.

Key words: Antibiotics, Multidrug Resistant, FT-IR, LC-MS, Actinomycetes.

LIST OF ACRONYMS AND ABBREVIATIONS

%	Percentage
µg	Microgram
µl	Microliter
16SrRNA	16 Svedberg unit ribosomal Ribonucleic Acid
CFU	Colony Forming Unit
DAP	Diamino Pimelic Acid
DNA	Deoxyribonucleic Acid
G+C	Guanine + Cytosine
GC-MS	Gas Chromatography Mass Spectroscopy
HPLC	High Performance Liquid Chromatography
LC-MS	Liquid Chromatography Mass Spectroscopy
MDR	Multidrug Resistant
MDRSA	Multidrug Resistant *Staphylococcus aureus*
mg	Milligram
MHA	Mueller Hinton Agar
MIC	Minimum Inhibitory Concentration
mm	Millimeter
MRSA	Methicilin Resistant *Staphylococcus aureus*
NA	Nutrient Agar
^{o}C	Degree Celsius

PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PKS	Polyketide Synthase
RAPD	Random Amplified Polymorphic DNA
Rpm	Revolution per minute
SCA	Starch Casein Agar
SIM	Sulphide Indole Motility
spp.	Species
SPSS	Statistical Package for Social Science
TLC	Thin Layer Chromatography
VRE	Vancomycin Resistant Enterococci
ZOI	Zone of Inhibition

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CHAPTER 1
INTRODUCTION

1.1 Introduction

Actinomycetes are diverse group of Gram positive and spore former aerobic organisms with mycelial growth and thread like filaments (Bhatti *et al.* 2017). Actinomycetes are filamentous bacteria possessing high G + C content (>55mol%) in their nucleic acid. They are ubiquitous organisms with wide physiological and morphological diversity and have been isolated from all kinds of terrestrial and aquatic habitats where they can exist as free-living bacteria as well as pathogens or in symbiotic association with plants and insects or as endophytes (Singh *et al.*2019, Srinivasan *et al.* 1991). Actinobacterial population has been recognized as one of the dominant groups of soil microbes that can participate in the cycling of the soil components especially in the degradation of organic components. Precisely, they are involved in the decomposition and mineralization cycles with the production of extra cellular enzymes, such as cellulase, chitinase, lignin peroxidase, etc. Since they can decompose complex mixture of polymers in dead plants, animals and fungal material, they have important role in soil biodegradation by recycling of nutrients associated with recalcitrant polymers (McCarthy and Williams, 1992). Actinomycetes are the most remarkable prokaryotes in medical and biotechnology industries due to their ability to synthesize a vast number of bioactive molecules like antibiotics, vitamins and enzymes (Singh *et al.* 2006, Thakur *et al.* 2007 & Dhananjayan *et al.* 2010).

These saprophytic microorganisms found broadly scattered in the soil, colonizing the plants and present in water. At the beginning the actinomycetes were recognized primarily on the basis of morphological criteria, but now a days molecular methods are frequently applied to make their precise characterization. In addition, the phylogenetic and molecular approaches when applied have been given a huge impact to facilitate their classification methods (Babalola *et al.* 2009,Hozzein and Goodfellow, 2011). On the other hand, a number of organisms that are accidentally placed in different groups are recently classified in the correct way due to the application of molecular techniques (Zhi *et al.* 2009). However, recently, the phylogenetic classification of the species is

broadly relied upon 16S rRNA sequencing and the use of polymerase chain reaction (Wood *et al.* 2007, Zhi *et al.*2009).

Remarkably, the actinobacteria are noteworthy as antibiotic producers, making three quarters of all known antibiotics. They are responsible for the production of about half of the discovered bioactive secondary metabolites, notably antibiotics, antitumor agents, immunosuppressive agents and enzymes. Among the actinobacteria, *Streptomyces*, a representative genus of actinobacteria of terrestrial soil origin, has accounted for the production of 80% of the total antibiotic products, followed by *Micromonospora* and *Actinopolyspora* as rare actinobacteria are the runner-up with less than one-tenth of *Streptomyces* (Hopwood *et al.* 2000). The role of rare actinobacteria as the source of bioactive molecules became apparent as these organisms provide about 25% of the antibiotics of actinobacterial origin reported during 1975- 1980 (Nisbet, 1982). Rare actinobacteria have usually been regarded as isolates of actinobacteria whose isolation frequency by conventional methods is much lower than that of *Streptomycetes.* Consequently, basic knowledge of the habitat ecology, physiology and productivity of molecules of rare actinobacteria gradually increased, which expands the screening source into virgin and unexplored environments.

Hundreds of antimicrobials agents for human and animals have been discovered following the discovery of penicillin during 1929. The discovery of streptomycin in 1943 caught the attention of researchers toward actinomycetes (Riedlinger *et al.* 2004). Streptomycin was first bioactive secondary metabolites derived from the genus *Streptomyces* and it has been established as the most reliable microbial antibiotic producers (Berdy, 2005). *Micromonospora* and *Streptomyces* strains have a uniquely proven capacity to produce novel antibiotics (Watve *et al.* 2001). Today 80% of the antibiotics are derived from the genus *Streptomyces,* the most important among all the actinomycetes (Procópio *et al.* 2012).

The widespread and often unwarranted use of antibiotics causes the emergence and spread of bacterial resistance to commonly practised antibiotics. Antibiotic resistance is a naturally occurring biological phenomenon, especially in antibiotic producers

where self- resistance mechanisms usually pre-exist coupled to antibiotic biosynthetic pathways that favors the spread of resistance mechanisms throughout and across bacterial populations (Hopwood, 2007). Inadequate use and widespread non therapeutic applications of antibiotics (as growth promotors or for prophylactic purpose) in animal husbandry also considerably contributed on the pace of resistance development (Hopwood, 2007). About 100,000 tons of antibiotics are produced annually for their use in agriculture, food industry and therapeutic purpose (Nikaido, 2009). Nowadays, antibiotic resistance of microorganisms is one of the biggest threats to global health, food security and development. The World Health Organization (WHO) Global Report on the surveillance of antimicrobial resistance has established that bacterial resistance to commonly used drugs in infection treatment has reached alarming levels in many parts of the world (WHO, 2014). In 2017, WHO released its first list of most concerning "priority pathogens" for human health – a catalogue of twelve families of bacteria for which new antibiotics are urgently needed (WHO, 2017). According to O'Neill's independent review about 700 000 people around the world die annually due to infections caused by multidrug-resistant microorganisms. If current trends continue, such infections could lead the death of 10 million people a year by 2050 (O'Neill, 2016). In this context, the discovery of new bioactive compounds, particularly those with new modes of action, is not only needed for modern medicine but absolutely required to avoid future pandemics.

Despite the alarming resistance development, the list of novel antibiotics with new modes of actions is limited. Since 2006, only one new antibacterial agent (doripenem) has been approved for therapeutic use in USA and the number of potential antibiotics in clinical trials is very limited (Boucher *et al.* 2009). Therefore, the discovery of new and potent antibiotics is a continuous challenge to tackle the rapidly spreading antibiotic- resistant pathogens.

Chemical synthesis and engineered biosynthesis of antimicrobial compounds have considerable contribution but nature still dominates as the richest and the most versatile source for new antibiotics (Koehn *et al.* 2005). Newmen *et al.* (2003) reported that 60% and 75% of new drugs, respectively, for cancer and infectious diseases originated from

natural sources between 1981 and 2002. Chemically synthesized compounds have limited structural diversity, often due to lack of chiral centers, aromatic rings, oxygen containing substituents and structural rigidity (Fehler and Schmidt, 2003).

Natural products play a predominant role in the development of new therapeutic agents (Newman and Cragg, 2016). Actinomycetes represent the most prominent group of microorganisms, which synthesize bioactive compounds. They claimed nearly two-thirds of all naturally derived antibiotics currently applied in medicine, veterinary and agricultural practice. Majority of these molecules derived from diverse species of *Streptomyces* (Barka *et al.* 2016, Chater, 2016). The diversity of natural products can provide physio-chemical properties such as specific interactions with multiple biological targets that are hardly found in molecules derived from combinatorial synthesis (Nussbaum *et al.* 2006). Natural product-derived antibiotics have been genuine advantage of having evolved to be active in target cells *in vivo*, abandoning a major obstacle of target -based approaches- the conversion of *in vitro* hits into whole-cell active leads (Baltz, 2008).

The actinomycetes have ability to produce diverse bioactive compounds including anthracyclines, glycopeptides, aminoglycosides, macrolides, polyenes, β-lactams, peptides, nucleosides, terpenes, polyethers and alkaloids which display broad variety of biological activities (Raja and Prabhakarana, 2011, Suthindhiran and Kannabiran, 2009).

Actinomycetes have been resourced for numerous important therapeutic drugs including antibacterial, antifungals, antitumor, antiparasitic, antivirals and immunosuppressants and represent undoubtedly the most prolific antibiotic producers. Actinomycetes are ubiquitous organisms with wide physiological and morphological diversity and have been isolated from all kinds of terrestrial and aquatic habitats where they can exist as free living bacteria as well as pathogens (Schaal and Lee, 1992) or in symbolic associations with plants (Benson and Silvester, 1993) and insects (Curie *et al.* 1999) or as endophytes (Bascom-Slack *et al.* 2009). It is also interesting to observe that despite extensive screening of terrestrial isolates, soil samples have still only been taken

from a minute fraction of the surface of the entire globe (Baltz, 2008). Consequently, the taxonomic diversity even within the well-studied group of terrestrial actinomycetes is likely to be far from exhausted (Watve *et al.* 2001).

However, the ability of actinomycetes strains to produce bioactive products is not a fixed property but can be greatly increased or completely lost under different conditions of nutrition and cultivation (Waksman, 1961). Therefore, designing an appropriate fermentation medium is of critical importance in the production of secondary metabolites (Gao *et al.* 2009). Changes in the nature and type of carbon and nitrogen sources have been reported to affect antibiotic biosynthesis in *Streptomyces* (Barratt and Oliver, 1994, Reddy *et al.* 2011). Also, several cultivation parameters like pH, incubation period, temperature and mineral salts play a major role in the production of bioactive metabolites (Usha Kiranmayi *et al.* 2011).

Biosynthesis of secondary metabolites depends on the growth conditions of each strain. For years researchers have been applying different modifications in nutrients and physicochemical factors during fermentation processes to optimize the production of bioactive compounds (Rajnisz *et al.* 2016). Currently, modeling and analysis of fermentation processes is performed by the statistical optimization approach, e.g. response surface methodology, which enables enhanced production of antibiotics, enzymes and probiotics (Latha *et al.* 2017). Identification and structure elucidation are an important and integral part of drug discovery. Various chemical and biochemical methods applied for this purpose are FT IR, HP LC, LC MS, GC MS, H-NMR, C-NMR and so many.

1.2 Rationale

The development of new antimicrobial agent, preferably naturally occurring ones with novel mechanism of action is an urgent medical need. Soil in particular is an intensively exploited ecological niche, the inhabitants of which produce many biologically active natural products, including clinically important antibiotics. However, emergence of multidrug resistant pathogens necessitates a continuing search for new antimicrobial compounds with anti-pathogenic activity. Searching for previously unknown microbial strain is an effective approach for obtaining new biologically active substances. There is an urgent need to find new drugs, especially antibiotics, to control the spread of

antibiotic resistant pathogens and to treat life threatening diseases such as cancer. The need for less toxic more potent antibiotics from non-infective organisms, which overcome the resistance exhibited against the existing antibiotic, is felt actually. During the past decades, the actinomycetes have provided many important bioactive metabolites of high commercial value. Consequently, they are continuing to be routinely screened for new bioactive substances. These searches have been remarkably successful and approximately two-third of naturally occurring antibiotics have been isolated from actinomycetes. Actinomycetes which are genuine producers of antibiotics and important suppliers to the pharmaceutical industry, can synthesize a wide array of secondary metabolites. Actinomycetes are widely distributed in nature and are typically useful in the pharmaceutical industry for their seemingly unlimited capacity to produce secondary metabolites with diverse chemical structures and biological activities. Although thousands of bioactive metabolites have been isolated from *Streptomyces* and other actinomycetes, they represent only a small fraction of the repertoire of bioactive compounds produced.

Nepal is rich in biodiversity and it has diverse geographical locations including Terai, Mountains and Himalayas. Therefore, isolation of new actinomycetes strains from natural resources such as soil and water of Nepal and characterization of secondary metabolites is a valuable endeavor. Very few studies regarding isolation and screening of actinomycetes from Nepalese soil have been conducted. In this regard isolation of novel strain with potent antimicrobial activity would open the door for further research regarding their application at industrial level to formulate new drug.

1.3 Objectives

1.3.1 General objective

Screening and Characterization of antimicrobial (antifungal and antibacterial) compound producing actinomycetes from soil and water bodies from different geographical regions of Nepal.

1.3.2 Specific objectives

1.3.2.1 To isolate bioactive actinomycetes from soil and water of different geographical locations.

1.3.2.2 To screen and characterize antibacterial compound producing actinomycetes specially against Multi Drug Resistant Bacteria.

1.3.2.3 To screen and characterize antifungal compound producing actinomycetes.

1.3.2.4 To optimize the fermentation conditions for potent antimicrobial producers.

1.3.2.5 To characterize the extracted bioactive compound.

CHAPTER 2
LITERATURE REVIEW

2.1 Actinomycetes

One of the major dominant groups of soil population has been identified as actinomycetes together with bacteria and fungi. Actinomycetes are aerobic Gram-positive prokaryotic organisms. They are free living saprophytic bacteria and are widely distributed in soil and water and colonizing plants (Pandey *et al.* 2004). They possess a large genome with a large number of transcription factor that allow them to produce a wide variety of metabolites (Ohmishi *et al.* 2008). It represents one of the largest taxonomic units among the 18 major lineages currently recognized within the domain bacteria. They possess a very high G+C content in their DNA, greater than 55% generally in the range of 65-75%, with the G+C content about 70-74% in *Streptomyces* (Ventura *et al.* 2007). Several filamentous organisms classified as actinomycetes, such as *Streptomyces, Micromonospora, Amycolatopsis, Actinoplanes* and *Streptoverticillium* bear a liner genome, sometimes accompanied by liner plasmids (Ventura *et al.* 2007). The actinomycetes are known to be the potential producers of antibiotics and of other therapeutically useful compounds. The bioactive secondary metabolites produced by actinomycetes are known to possess antibacterial, antioxidant, anti-cancer, anti-algal, anti-helminthic, anti- malarial and anti-inflammatory properties (Jayaprakashvel, 2012).

Medically important members of actinobacteria mainly lie in subclass actinobacteridae, among which actinomycetes lie in the order actinomycetales. The order Actinomycetales is composed of approximately 80 genera, nearly all from terrestrial soils, where they primarily live as saprophytes. The chemical composition of their cell wall is similar to that of Gram-positive bacteria but because of their well-developed morphological and cultural characteristics, actinomycetes have been considered as a well separates group from other common bacteria (Das *et al.* 2008). Actinomycetes are of universal occurrence in nature and have proved their ability to produce variety of bioactive secondary metabolites.

2.2 Habitat

Actinomycetes are the most abundant organisms in the soil. They from thread like filaments and grow as hyphae like fungi with characteristic earthy smell (Sprusansky *et al.* 2005). Actinomycetes are the ubiquitous group of microbes widely distributed in natural ecosystem worldwide and exist in various habitats in nature. Although they are major group of bacteria in soil, their distribution is affected by the type of soil (George *et al.* 2012). Besides soil, they have also been isolated from marine habitat. Actinomycetes from such sources are being explored as potential sources of novel antibiotics (Subramani and Aalbersberg, 2012).

They are found in large numbers in fresh water sources such as lakes and river bottoms, manure, compost, and dust, as well as on plant residues and food product (Bizuye *et al.* 2013). It has been reported that they are present in extreme environments, at cryophilic region for example soil from Antarctica and even from desert soil (Diraviyam *et al.* 2011). However, the diversity and distribution of actinomycets that produce secondary metabolites can be determined by physical, chemical and geographical factors (Torsvik and Ovreas, 2002).

2.3 Structure

Actinomycetes are a group of branching unicellular filamentous bacteria, that reproduce either by fission or by means of spores or conidia and are characterized by the formation of normally branching threads or rods. The hyphae are generally non-septate, sporulating mycelium may be branching or non-branching, straight or spiral shaped, spores may be oval, spherical or cylindrical (Waksman, 1940). The cell wall is rigid and consists of various complex compounds including peptidoglycan, teichoic and teichuronic acid and polysaccharides. Polysaccharides consist of glycan chains of alternating N-acetyl-D-glucosamine (NAG) and N-acetyl-D-muramic acid (NAM) that is unique in prokaryotic cell wall. Teichoic and teichuronic acid are chemically bonded to peptidoglycan (Davenport *et al.*2000). Actinomycetes form mycelia of branching filaments and historically called ray fungi and thought to be related to true fungi. In general, actinomycetes do not have membrane bound cell organelles. They are characterized by a filamentous or rod and coccus structure and the presence of lateral protuberance (Barka *et al*. 2016).

2.4 Classification of actinomycetes

Different schemes of prokaryotic classification have been proposed over the years:

i. The conventional method that uses microbial traits such as morphological features, growth and physical requirements and biochemical characteristics (Mohanraj and Sekar, 2013).

ii. Numerical taxonomy that explains the relationship between different organisms based on the analysis of huge volumes of Phenotypic data using computer programs (Garrity,2006).

iii. Chemotaxonomic method based on the chemical variation in actinobacteria and used chemical characteristics in classification and identification, and it deals with the discontinuous distribution of specific chemicals especially amino acids, lipids, sugars proteins and other substances in whole cells, part of cells of fermentation products, and with enzymes (Varalakshmi *et al.* 2014).

iv. Genotypic classification studied the genetic relatedness, inferred mainly from DNA-DNA hybridization and comparative sequence analysis of homologous macromolecules, especially rRNA. In recent years, more and more genotypic approaches were applied on the classifications of actinobacteria, such as multilocus sequence analysis (MLSA), average nucleotide identity (ANI) and whole genome analysis (Nouioui *et al.* 2018). The most widely accepted system in recent time is the polyphasic approach. This approach combines as many different data as possible, for instance, phenotypic, chemotaxonomic, genotypic and phylogenetic information (Singh *et al.* 2007). But the characterization of a strain is a key element in actinobacteria systematic in any period and prokaryotic morphologies are consistent with their phylogenetic re-constructions (Li *et al.* 2016).

Actinomycetes based on morphology, were described as an independent group of microorganisms, with close associations with bacteria, but with a fungus like growth form. The morphology, spore structure and mode of growth will be sufficient to indicate their taxonomic position (Waksman, 1940).

Actinomycetes are traditionally classified as part of the bacteria. In the Bergey's Manual of Determinative Bacteriology, actinomycetes are included in several sections of volume IV. All actinomycetes are included under the order actinomycetales (Garrity, 2006).

Actinomycetes were earlier studied as fungi, due to the similarly in morphology, mainly the presence of long branching mycelial structure and production of spores (Barka *et al.* 2016). However, later due to lack of a membrane bound cell organelles, presence of a peptidoglycan cell wall, presence of 70s ribosomes instead of the 80s type and other features, they were classified as prokaryotes. Thus, actinomycetes are bacteria with a fungal morphology (Nakeeb and Lechevalier, 1963).

The conventional method of actinomycete classification contains two schemes-one based on morphology and the other on chemical composition. Different groups of actinomycetes from a wide variety of structures that can be used as the basis of classification, unlike true bacteria (Lechevalier and Lechevalier, 1970). These include different types of conidia on the substrate or the aerial mycelium, sporangia containing either motile or nonmotile spores, and structures that can be equated to the sclerotia, coremia, or the pycnidia of fungi. Actinomycetes have a cell wall composition similar to that of Gram-positive bacteria and also indicated that the chemical composition of the cell wall might furnish practical methods of differentiating between various types of actinomycetes. Barka *et al.* (2016) suggested the use of color of the mycelium, branching nature of the mycelium and color of the diffusible pigments, in accurate identification of actinomycetes. Several media for the International Streptomyces Project (ISP) were suggest by Shirling and Gottlieb (1966) to differentiate and characterize actinomycetes at different levels. They also suggest the combination of morphological features including spore arrangement, mycelial characters, and production of melanin and diffusible pigments with the use of different types of sugars for the identification of *Streptomyces* species (Shirling and Gottlieb, 1966).

A comparative study used in characterization of actinomycetes by an international subcommittee concluded that the morphology of the sporophores, the shape of the spores are significant and constant characteristics, which along with the melanin production and color of aerial and vegetative mycelia provided valuable insight into the taxonomy of actinomycetes (Kuster, 1961). On the basis of chemical composition, Lechevalier and Lechevalier (1970) proposed a straightforward technique for differentiating between atypical *Nocardia*- like *Streptomyces* and atypical

Streptomyces- like *Nocardia* using paper chromatography of whole-cell hydrolysates and this method can be used for the classification of actinomycetes.

Currently, actinomycetes have been classified as follows: Super kingdom Bacteria, Phylum: Firmicutes, Class: Actinobacteria, Subclass: Actinobacteridae and Order: Actinomycetales, with 14 suborders, 49 families and over 40 genera (Adegboye and Babalola, 2012).

2.5 Isolation of Actinomycetes from Soil

Soil is diluted by serial dilution technique and plated on starch casein agar (Collins *et al.* 2004). Characteristic slower growth rate comparatively to that of other soil bacteria and fungi complicates isolation of actinobacteria (Hirsch and Christensen, 1983). This development of selective isolation procedures chiefly comprises the following approaches:

Pretreatment of soil is done chemically or mechanically, viz. $CaCO_3$ (CavaIla and Eberlin, 1994, EI-Nakeeb and Lechevalier, 1963, Qin *et al.* 2009, Tsao *et al.*1960 , Yi Ng and Amsaveni,2012), sodium propionate (Crook *et al.* 1950) and phenol treatments (Flayakawa *et al.*2004 , Lawrence, 1956) or heat treatments (Nonomura and Ohora,1969,Williams and Cross,1971), centrifugation (Jensen *et al.* 2005, Rehacek, 1959 , Yamamura *et al.* 2003) . Soil samples treated with CaCO3 gives higher total and relative plate counts than other methods (EI-Nakeeb and Lichevalier, 1962, Qin *et al.* 2009, Yi Ng and Amsaveni, 2012).

Nutritionally selective media are preferentially used by actinobacteria (Kuster and Williams, 1964, Lingappa and Lockwood, 1962, Osman *et al.* 2011, Porter *et al.* 1960). Starch-casein media (SCA) (Kuster and William, 1964, Okazaki and Okami, 1972, Osman *et al.* 2011 & Williams and Davies, 1965) is commonly used for isolation of actinobacteria. SCA plates containing cycloheximide (25 µg/ml) and nalidixic acid (25µg/ml) have been used to inhibit fungi and bacteria except actinobacteria (Magarvey *et al.* 2004). Rapidly assimilated carbon sources such as glucose are good for growth, but it represses the enzymes formation involved in the biosynthesis of secondary metabolites. Non repressing carbon sources such as polyalcohols (e.g. glycerol), polysaccharides (e.g. starch), oligosachharides (e.g. lactose) and oils (e.g.

soybean, methyloleate) are often preferred as the carbon source which is slowly hydrolyzed and metabolized (Demain and Fang, 2000, Trilli, 1990). Organic nitrogen sources are often used, as these compounds can be broken down into smaller units that are transported into bacterial cells, e.g. amino acids (e.g. casein) and ammonia (NH_3). Ammonia is the preferred inorganic nitrogen source for actinobacteria, where it is added as ammonium sulfate $(NH_4)_2SO_4$ or ammonium chloride (NH_4CI) (Dunn, 1985, Osman *et al.* 2011). Excessively assimilable nitrogen source exerts a repressive effect causing a decrease in the levels of secondary metabolites, mainly caused by ammonium salts and amino acids (Omura and Tanaka, 1984, Osman *et al.* 2011, Sanchez and Demain, 2002). Phosphate helps in primary metabolism, a shift down in primary metabolism de represses secondary metabolism (Drew and Demain, 1977, Osman *et al.* 2011, Sanchez and Demain, 2002). Phosphate shifts carbohydrate catabolic pathways, stops synthesis of the inducer of the secondary metabolite (SM) pathway and prevents the formation of SM precursors (Martin, 1977, Osman *et al.* 2011). Phosphate prevents or represses phosphatases which is essential for SM biosynthesis and also suppresses SM production by divesting the cell of an essential metal (Martin *et al.* 1989, Sanchez and Demain, 2002). SM biosynthesis is repressed or inhibited by $PO4^{3-}$ concentrations above 1 mM in liquid media whereas higher concentrations of 10 to 25 mM are required in solid media (Martin, 1989, Sanchez and Demain, 2002). Inorganic potassium cation is a cofactor of some SM biosynthetic enzymes involving in many transport processes. It is usually added as an inorganic K salt, e.g. K_2HPO_4 or KH_2P04. Magnesium is a catalyst of SM synthases and enzyme cofactor. Addition of magnesium ($MgSO_4.7H_20$) has been shown to improve the production of the macrolide antibiotic miocamycin. Iron is a catalyst for SM synthesis and is involved in redox processes. Presence of NaC1 helps in liberation of antibiotics from the mycelium into the medium (Egorov, 1985).

Actinobacteria producing secondary metabolite are mesophilic, with growth and secondary metabolism production being optimal at 25°C- 28°C (Iwai and Omura, 1982, Wang *et al.* 2010). Actinobacteria have a wide pH optimum for growth (usually between 6 and 8) while secondary metabolism can only tolerate a narrow range within 0.2 pH units (Chen *et al.* 1999, James *et al.*1991, Song *et al.* 2012). Secondary metabolite producing actinobacteria are obligate aerobes, thus providing cells with

adequate supplies of oxygen is critical for respiration and biosynthesis of metabolites (Liefke *et al.* 1990). The aeration varies from 0.5 to I v/v per minute while agitation is between 200 to 400 rpm is often required (Gurung, 2008).

2.6 Secondary Metabolite Production

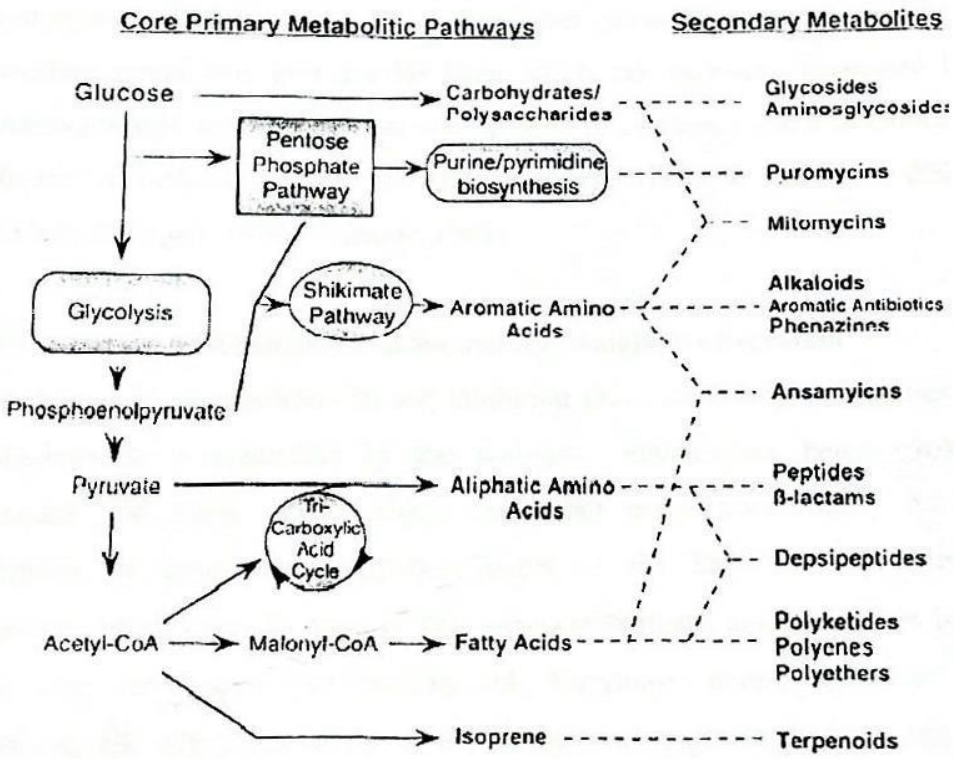


Figure 2.1: Primary metabolic pathways leading to the formation of secondary metabolites (August *et al.* 1999).

Microbially synthesized secondary metabolites are the metabolic intermediates or end products originated from complex and often lengthy biosynthetic pathways. Secondary metabolites (SM) may be found in various species in diverse genera or families and an array of metabolites can be expressed from a single species under different environmental conditions. Secondary metabolites are derived from the precursors and energy is generated through primary metabolic pathways (Figure 1). The SM groups commonly dealt out in nature are the polyketides, terpenes, steroids, shikimic acid and alkaloids (Herbert, 1989).

The biosynthesis of secondary metabolites in actinobacteria involves the following sequence of events; 1) Uptake of nutrients into the cell and conversion into intermediates of primary metabolism, 2) Accumulation of primary metabolites and signaling molecules causes secondary metabolite production, 3) Primary metabolites diverging into the pathway particular for a specific secondary metabolite. Several primary metabolic pathways have been identified as sources of precursors for synthesis of secondary metabolites. These are: fatty acid metabolism (acetate and propionate for e.g. polyketide biosynthesis), carbohydrate metabolism (hexose, pyruvate). The production of these secondary metabolites is regulated by pathway specific genes that determine the start of secondary metabolite production (Hodgson, 2000).

2.6.1 Functions of Secondary Metabolites

The production of secondary metabolites serves a number of useful functions; they act as chemical agents in inhibiting other microorganisms and in boosting the fitness and the survival of the producing organism in the natural environment. Certain secondary metabolites support in metal transport providing metal ions in a soluble form which can be easily harnessed by microorganisms, act as sex hormones, agent of symbiosis and also embrace effectors of differentiation in sporulating bacteria (Demain and Fang, 2000, McCann and Pogell, 1979, Neilands, 1995).

2.6.2 Resistance Mechanisms and Secondary Metabolite Secretion

The degree of susceptibility to self-inhibition from secondary metabolites in actinobacteria is controlled by the resistance mechanisms being evoked (Mendez and Salas, 2001) which can either occur concurrently during idiophase or sparked by sublethal levels of the SM. SM producing actinobacteria possess an array of defensive mechanisms against suicide from their own metabolites that includes; (a) Enzymatic detoxification of the antibiotic, (b) Alteration of the antibiotics normal target in the cell, and (c) Modification of the permeability to permit antibiotic to be pumped out of the cell and confine its re-entry. (d) Seclusion of secondary metabolite by cytoplasmic proteins, (e) Inhibition of antibiotic synthases during rapid growth (Cundliffe, 1989, Sheldon *et al.* 1999, Vining, 1990, Wilson and Cundliffe, 1999).

2.6.3 Microbial Screening for Secondary Metabolites

In the search for novel microbial metabolites, a number of lucid screens have provided an effective means in detecting secondary metabolites. Targeted screens based upon mechanisms of action have detected metabolites with the desired bioactivity which are either a known compound or have undiscovered new structural classes (Table 4) (Franco and Coutinho, 1991, Higashide, 1995, Osada, 1995, Silver and Bostian, 1990). The classical approach for testing new microbial isolates for antibiotic production is the cross- streak method (Madigan, 2000).

Table 2.1: Screening assays used for assessing bioactive metabolites (White *et al.* 1986)

Screen Type	Method
Antibacterial	Agar Diffusion Agar plate Assay organism
Anticoccidial	Primary chick kidney cell culture oocyst
Anticancer	Biochemical inducing assay (BIA)
Antiviral	Antibacteriophage assay
Enzyme inhibitors	Ligand-receptor competition assay

2.7 Importance of actinomycetes

Actinomycetes are the main group of soil microorganism that play a major role in recycling of organic matter in environment by production of hydrolytic enzymes (Remya, 2008). Actinomycetes are important bio-degraders in nature. They are industrially important sources of different secondary metabolites, including pharmaceutically important compounds like antibiotics. Actinomycetes are the important antibiotic producers, producing about 75% of all used antibiotics; the

Streptomyces are especially prolific and can produce a major antibiotics and other class of biologically active secondary metabolites (Pandey *et al.* 2004).

Actinomycetes are important sources for novel antibiotics and hence having a high pharmacological and commercial interest including control of infectious diseases. Actinomycetes and their bioactive compounds show antimicrobial activity against various pathogens and multidrug resistant bacteria such as Vancomycin resistant Enterococci (VRE), Methicillin resistant *Staphylococcus aureus* (MRSA), *Klebsiella spp.,* and *Pseudomonas aeruginosa* etc. (Singh *et al.* 2012,Tiwari and Gupta, 2012). The need for new, safe and effective antimicrobial agent is the major challenge to the pharmaceutical industry now a days, mainly with the obvious increase in opportunistic infections in the immune compromised host and multiple drug resistant strains. However, new approaches for the isolation of soil actinomycetes have revealed that other genera are also significant, and many news species have been isolated, most of them are also able to produce novel secondary metabolites. Although the first antibiotic from an actinomycete has been reported more than 50 years ago, and since then more than 4000 new bioactive compounds have been obtained, the search for new actinomycetes of interest to biotechnology is still important (Semedo *et al.* 2001).

The ability of actinomycetes of produce various bioactive substance has been utilized in a series of researches in numerous institutional and industrial laboratories. This promoted the isolation of certain agents which have found application in combating a variety of human infections. That is why more than 7 of naturally occurring antibiotics have been isolated from different genus of actinomycetes which have different biological activities such as antibacterial, antifungal, antiparasitic, antitumor, anticancer and immunosuppressive actions (Bizuye *et al.* 2013).

Actinomycetes have been a good source of a wide range of nutritional materials, cosmetics and vitamins (Remya, 2008) and have been associated with soil organic matter production, owing to their black pigments called melanin, which are related to soil humic acid in some respects. A special attention has been given to them in biotechnological applications because of their great metabolic diversity (Semedo *et al.* 2001).

2.8 Antibiotics

The word "antibiotic" is derived from Greek term antibiosis, which literally means "against life". In the 1940s, antibiotic was originally defined as a substance produced by one microorganism that inhibited the growth of other microorganisms in low concentration. It can be purified from microbial fermentation (β-lactams, Streptomycin, tetracycline, etc.) and modified chemically (Cephalosporin, Penicillin, etc.) or enzymatically (Chloramphenicol, Nalidixic acid, etc.) for either chemical use of for fundamental studies. The antibiotics are widely distributed in the nature, where they play an important role in regulating the microbial population of soil, water, sewage and compost. Out of the several hundred naturally produced antibiotics that have been purified, only a few have been sufficiently non-toxic to be of use in medical practice. Those that are currently of greatest use have been derived from a relatively small group of microorganisms belonging to the genera *Penicillium, Streptomyes, Cephalosporium, Micromonospora and Bacillus.* About 60% of the antibiotics, in use today are contributed by the genus *Streptomyces* (Subramani and Aalbersberg, 2012). Many antibiotics are produced by microorganism as secondary metabolites. The isolation of antibiotic from microorganism is relatively easy as compared to chemical synthesis of antimicrobial agents.

2.8.1 Types of antibiotics

Different antibiotics have different modes of action, owing to the nature of their structure, degree of affinity to certain target sites within bacterial cells, the mode of action, etc. Antibiotics are classified into different classes under the following groups:

i. β-lactam antibiotics: Penicillin, Cephalosporin

ii. Tetracycline antibiotics: Doxycycline, Minocycline

iii. Macrolides: Erythromycin, Azithromycin

iv. Sulphonamides, Trimethoprim and related drugs: Co-trimoxazole

v. Quinolones: Ciprofloxacin, Nalidixic acid

vi. Aminoglycosides: Vancomycin, Teicoplanin

vii. Miscellaneous antibacterial antibiotics: Clindamycin, Nitrofurantoin (Hugo and Russel, 2011).

2.8.2 Antibiotics from actinomycetes

Until the end of 1940s, fungi and to a lesser extent, bacteria furnished the greatest number of antibiotics discovered. Between 1955 and 1962, however, about 80% of antibiotics found to be originated from actinomycetes. Streptomycin was the first antibiotic to be isolated from actinomycetes, *Streptomyces griseus* (Mahajan and Balachandran, 2012). Since the discovery of Streptomycin from cultures of *Streptomyces* and *Streptoverticillium,* a large number of antibiotics, including major therapeutic agents such as aminoglycosides, chloramphenicol, tetracycline, macrolides and more recently β-lactam cephamycin group, have been isolated. As greater number of new antibiotics were discovered, the chances of finding novel antimicrobial compounds among conventional actinomycetes reduced. Thus, the focus of industrial screening has moved to less exploited genera of rare actinomycetes such as *Actinomadura, Actinoplanes, Amycolatopsis, Dactylosporangium, Microbiospora, Micromonospora, Planobispora, Streptosporangium* and *Planomonospora* (Grandgirard *et al.* 2002).

Great screening programs started after 1945, which utilized simple methods for the isolation of a large number of strains from soil samples, resulted in the production of hundreds of antibiotics of actinomyces origin. Around 75% of total antibiotic product is covered by *Streptomyces,* Along other genera like *Micromonospora* with less than one- tenth as many as *Streptomyces* (Pandey *et al.* 2004). In recent years, new antibiotics have been isolated in great majority from cultures of microbes that are usually classified with the genus *Streptomyces.* They are being announced at an unending pace. It is the actinomyces which furnished the greatest part of antibiotics in commercial use (Sharma *et al.* 2014).

2.9 Commercial antibiotics produced by actinomycetes

2.9.1 Streptomycin

Streptomycin is the major antibiotic secreted by the actinomycetes, *Streptomyces griseus* that has been proven to be effective against *Mycobacterium tuberculosis* infections (Smith and Waksman, 1947) and is still used as second line drug for that

purpose, in spite of having few other applications. It consists of N- methyl-L-glucosamine, Streptose and Streptidine, linked glycosidically. Streptomycin inhibits protein synthesis similar to all aminoglycoside antibiotics. It irreversibly combines with 30s subunit of the 70s ribosomes found in bacteria and also binds to the S12 protein involved in the initiation of protein synthesis. Therefore, it prevents the initiation of protein synthesis by blocking the binding of initiator N-formyl methionine of the t-RNA to the ribosome. The disruption of the cell membrane of susceptible bacteria is also caused by Streptomycin (Mahajan and Balachandran, 2012).

2.9.2 Tetracycline

They were the first among broad- spectrum antibiotics discovered in 1945, chlortetracycline, secreted by *Streptomyces aureofaciens,* is the first in this group of antibiotics (Mahajan and Balachandran., 2012). They can be used against Gram positive and Gram-negative bacteria, Trichomonas, amoeba, mycoplasma. It inhibits the formation of the necessary complex of ribosome, mRNA, and amino- acyl tRNA (Hugo and Russel,2013). Since they inhibit the protein synthesis and not directly kill bacterial cells, they are classified as bacteriostatic agents (Mahajan and Balachandran, 2012).

2.9.3 Chloramphenicol

Chloramphenicol is a protein synthesis inhibitor displayed a broad spectrum of activity. It was the first actinomycetes antibiotic to be synthetically manufactured on a large scale. Chloramphenicol production is reported in *Streptomyces venezuelae.* Chloramphenicol inhibits protein synthesis by binding to the 50s ribosomal subunit (Mahajan and Balachandran,2012). It inhibits bacterial enzyme peptidyl transferase, thereby preventing the growth of the polypeptide chain during protein synthesis but to a lesser extent in eukaryotic cells (Mahajan and Balachandran, 2012).

2.9.4 Neomycin

Neomycin was first reported in 1949 as an aminoglycoside antibiotic produced by *Streptomyces* species and a *Micromonospora* species. They work by affecting various bacterial enzymes (Waksman *et al.* 1949).

2.9.5 Erythromycin

Erythromycin, a macrolide antibiotic, was first reported from *Streptomyces erythreus.* Similar to all macrolides, they inhibit protein synthesis by binding to the 50s ribosome. Erythromycin is rapidly absorbed and diffuses into most tissues and phagocytes ensuring their easy passage to the site of infection for release (Mahajan and Balachandran, 2012)

2.9.6 Vancomycin

Vancomycin is a glycopeptide antibiotic, produced by *Amycolatopsis orientalis,* discovered in 1953. This antibiotic was discovered when the staphylococcal menace of drug resistance to the existing therapies was looming large. When the penicillin and cephalosporins fail, it is adopted as a last line of treatment against Gram positive infections; prevents of cell wall synthesis. The incorporation of N- acetyl- glucosamine and N- acetyl- muramic acid in the peptidoglycan of the bacteria cell wall is prevented due the action of vancomycin. In recent times, due to emergence of Vancomycin Resistant Enterococci strains (VRE), the leading cause of endocarditis in hospital settings, its use is limited. Moreover, very recently there are report of emergence of Vancomycin Resistant *Staphylococcus aureus* (VRSA) (Mahajan and Balachandran, 2012).

2.9.7 Kanamycin

Kanamycin was first reported in 1957 from the soil actinomycete *Streptomyces kanamyceticus.* It is a water- soluble, broad- spectrum antibiotic and a mixture of at least 3 components, A, B and C with component A predominating over the others. This aminoglycoside antibiotic inhibits protein synthesis by interacting with the 30s ribosome and inhibits translocation of the ribosome. They are useful in the treatment of serious infections caused by Gram- negative bacteria (Barka *et al.* 2016).

2.9.8 Rifamycin

Rifamycin are a group of compounds (A, B, C, D, E, S and SV), discovered in the late fifties that are biosynthesized by *Amycolatopsis mediterranei.* They have a broad antibacterial spectrum, including activity against several forms of *Mycobacterium.* In the susceptible organisms, they inhibit DNA dependent RNA polymerase activity by

forming a stable complex with the enzyme, thus suppressing the initiation of RNA synthesis (Chaudhary *et al.* 2013).

2.9.9 Gentamicin

Among many antibiotics developed by *Micromonospora*, gentamicin has received the maximum attention. It is an aminoglycoside complex of various components which has been reported to be secreted by *Micromonospora echinospora* and *Micromonospora purpurea* (Mahajan and Balachandran., 2012).

2.9.10 Daptomycin

Daptomycin is a new lipopeptide antibiotic, derived from *Streptomyces roseosporus.* It has the bactericidal effect against several Gram-positive organisms, especially MRSA, VRSA and VRE. The structure consists of a 13-member amino acid peptide linked to 10-carbon lipophilic tail which is responsible for its unique mechanism of action, i.e. it involves calcium dependent binding to the bacterial plasma membrane and disrupting membrane function. Thus, a depolarization of cellular membrane affecting macromolecular synthesis and disruption of the cell membrane occurs (Mahajan and Balachandran., 2012).

Among the families and genera belonging to the order actinomycetales, in term of number of strains producing antibiotics, the very promising family is streptomycetaceae with genus *Streptomyces* (Waksman, 1946), followed by micromonosporaceae (mainly *Micromonospora* and *Actinoplane*), the family pseudonoocardiaceae (mainly *Amycolatopsis*, *Sachharopolyspora* and *Sachharothrix)* and the family thermomonosporaceae (mainly *Actinomadura*), the family nocardiaceae (*Nocardia* and related genera), the family Sreptosporangiaceae (mainly *Streptosporanguim*). Other microbial producers are found in the genus *Nocardioides* (Lazzarini *et al.* 2000).

2.10 Factors affecting antibiotic production

Microorganisms require specific temperature, pH and salinity for optimal growth. It is necessary to maintain the optimum temperature, pH and salinity for the proper growth, development and production of bioactive secondary metabolites (Akond *et al.* 2016).

Temperature is very important in the biosynthesis of antibiotics and growth of actinomycetes. The optimum temperature is in the range of 25^0 – 30^0C, probably closer to 28^0C. If the temperature rises over 30^0C, the synthesis of antibiotics is particularly discontinued (Roshan *et al.* 2013). Soil actinomycetes mostly show their optimum growth at neutral and slightly alkaline conditions, in the range from pH 7-8. The existence of large diversity of acidophilic actinomycetes differed morphologically and physiologically from neutrophilic species (Vijayakumar, 2016). Acidophilic isolates grow in the pH range 3.5-6.5, with the neutrophilic strains grow at the pH range 5.0-9.0. Alkalophilic actinomycetes are also known to occur in soil. Many of these isolates, initially assigned to the genus *Streptomyces,* were later reclassified in the genus *Nocardiopsis* (Thumar and Singh, 2007).

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 0.5-1 v/v per minute and agitation in between 200-400 rpm promotes antibiotic production (Nanjwade *et al.* 2010). Halotolerant actinomycetes grow in salt concentration above 0.5 molar NaCI (Ismet *et al.* 2016). They have been mostly isolated from marine habitats. A halophilic strain in soil isolates has only been observed in certain genera. Historically the most commonly isolated actinomycete genera have been *Streptomyces* and *Micromonospora.* As a result, the majority of metabolites identified in screening programs searching for new antibiotics were derived from a relatively limited pool of organism (Basilo *et al.* 2003).

It has been generally known for many years that most soil actinomycetes cease to grow at about pH 5.0 and therefore comprise a very small component of the microbial populations of acid soils (Akond *et al.* 2016). However, the early work of Kim *et al.* (2004) demonstrated that some soil actinomycetes, termed *Actinomyces acidophilus,* required acidity for growth.

2.11 Optimization of bioactive metabolite from actinomycetes

Enhancing the production of any bioactive compound from actinomycetes strains is normally achieved by a variety of physicochemical, molecular, nutritional, immobilization and mutational regulated processes. Regulation and operating of

physicochemical and nutritional variations for the maximum production is a fundamental and classical technique to augment the production. In addition, genetic engineering, mutations, and immobilization techniques are effective tools to improve the yield. Modern advances prepared in software and bioprocess tools results a number of highly integrated software-based techniques to attain the maximum production of the end product in any bioprocess or fermentation. Response Surface Methodology (RSM) with an appropriate statistical plan is one of the modern and important techniques to achieve this goal (Chang *et al.* 2002 & Aghaei Kohazani *et al.* 2012).

A large number of actinomycetes strains especially belonging to genera *Streptomycetes* have the ability to grow on nutrient agar, Muller-Hinton agar and trypticase soy agar with calcium chloride for the production of desired bioactive compounds (Busti and Yushi, 2006), as well as each of the carbon and nitrogen sources. Furthermore, oxygen, temperature pH, ions and some other precursors play pivotal roles and can affect the production of bioactive compounds from isolated actinomycetes species (Rafieenia, 2013).

Media composition has the most important impact on the production of antibiotics and other bioactive compounds, especially relating with both glucose and phosphate, known as suppressors for the production of some metabolites process. However, strategies to realize novel biologically active secondary metabolites are extremely reliant on the culture conditions. In order to determine how these bioactivities of a broad collection of actinomycetes changed according to growth conditions, and to investigate chemical or growth supplements ability to trigger antimicrobial production, the productivity of the collected species grown under disparate conditions and with the addition of various additives was assessed; starch (1% w/v) ,Peptone (0.8% w/v) and also with pH 10 in particular were found to be the most efficient conditions for activating the production of antibiotic among the 40 assessed conditions. All the conditions are without difficulty available at a low cost, with minimum batch to batch variation (Sanchez *et al.* 2010, Van Wezel and McDowall, 2011).

The enhanced production related to the improvement in yield amount or value of yield

greater than the former obtained by some strategies is followed. Commonly fermented yield can be enhanced by a suitable fermented design, optimization of process parameters, media optimization and recombination in microorganisms (Ren *et al.* 2013). Furthermore, improvement of the strain used in the study was done by different strategies like immobilization and mutation (Haq and Ali, 2006).

2.12 Importance and Diversity of Secondary Metabolites:

Novel bioactive compounds especially antibiotics, are of great value to counter the spread of antibiotic resistant microorganisms (Payne *et al.* 2006) and to combat life threating diseases such as cancer (Olano *et al.* 2009). Although several antimicrobials of chemical synthesis origin and engineered biosynthetic are manufactured, nature still remains the most potential and versatile source for new antibiotics (Koehn and Carter, 2005). Actinomycetes, which are reliable producers of antibiotics and important suppliers to the pharmaceutical industry, can produce a wide array of secondary metabolites (Baltz, 2005). Traditionally actinomycetes have been isolated from terrestrial sources but sediments of marine environment is also appeared as good ecological environment for them (Weyland, 1969) Marine actinomycetes are the best sources of secondary metabolites and the vast majority of these compounds are primarily derived from genus *Streptomyces*, whose species are distributed widely in the marine and terrestrial habitats (Pathom-Aree *et al.* 2006) and are of industrial interest because of their ability to produce new bioactive secondary metabolites.

Actinomycetes are known to have the ability to produce a wide variety of secondary metabolites. Indeed, each strain of actinomycetes is likely to have the genetic potential for the production of 10-20 secondary metabolites (Bentley *et al.* 2002 and Lam, 2006). About 23000 antibiotics have been discovered from microorganisms and 10000 of the have been isolated from actinomycetes. Various genus and strains of actinomycetes have enormous biosynthetic potential to produce pharmaceutically valuable products ranging from antibacterial, antifungal, anticancer, antiviral and so on.

2.12.1 Antibacterial metabolites

Antibiotics and antibacterial agents are regularly searched to overcome the problem of decreased efficacy and resistance of pathogens to available therapeutic agents (Ravi

Kumar *et al.* 2010b). Several actinomycetes are extensively studied for antibacterial activity. Abyssomicin C is a novel polycyclic polyketide antibiotic produced by *Verrucosispora* strain that can block para-aminobenzoic acid biosynthesis there by inhibiting folic acid biosynthesis as like sulfa drugs (Riedlinger *et al.* 2004). It showed good antibacterial activity against various Gram positive including multidrug resistant and vancomycin resistant *Staphylococcus*. So that it can be developed as commercial antibacterial agent (Rath *et al.* 2005). Bonactin, an antibiotic isolated from *Streptomyces* SPP BD 21-2, showed broad antimicrobial activity against Gram positive and Gram-negative bacteria along with many fungi (Schumacher *et al.* 2003). Diazepinomycin is a unique farnesylated dibenzodiazepione produced by *Micromonospora* strain exhibiting antibacterial, antitumor and anti-inflammatory activity (Charan *et al.* 2004). Similarly, Frigocyclinone is a novel angucyclinone antibiotic isolated from *Streptomyces griseus* strain NTK-97, consisting of a tetragonomycin moiety attached through a C-glycosidic linkage with the amino-deoxy sugar glucosamine. It showed antibacterial activities against Gram positive bacteria (Brunter *et al.* 2005).

Essramycin is a triazolopyrimidine antibiotic isolated from *Streptomyces* SPP which showed antibacterial activity against many Gram positive and Gram negative bacteria with MIC values of 2-8 µg/ml. (EI-Gendy *et al.* 2008).Novel Chlorinated bisindole pyrrole compounds named Lynamycins that have been isolated from *Marinispora* Spp. Lynamicins significantly showed broad antibacterial activity against both Gram positive and Gram negative bacteria. Likewise, this compound was highly active against MRSA and vancomycin resistant *Enterococcus faecium* (Mc Arthur *et al.* 2008). Caboxamycin is a new benzoxazole antibiotic detected by HPLC-diode array screening in extracts of *Streptomyces* spp. NTK 937. It showed inhibitory activity against Gram positive bacteria, Phosphodiesterase enzyme, tumor cell lines of gastric adenocarcinoma, hepatocellular Carcinoma cells (HEPG2) and breast carcinoma cells (MCF7) (Hohmann *et al.* 2009a). Himalomycins A and B are anthracycline antibiotics obtained from *Streptomyces* spp. 6921 showed strong antibacterial activity (Maskey *et al.* 2003a). Glyciapyrroles A, B and C are pyrrolo sesquiterpenes antibiotics obtained from *Streptomyces* spp. NPSOO 8187. These exhibited antibacterial activity (Macherla *et al.* 2005). Triandamycinc, a dienoyl terramic acid, isolated from *Streptomyces* spp. 307-9 displayed inhibitory activity against vancomycin resistant

Enterococcus facecalis (Carlson *et al.* 2009). Bisanthraquinone, an antibiotic isolated from *Streptomyces* spp. showed potent cidal activity against vancomycin resistant *E. faecium* (VRE), methicillin succeptible, methicillin resistant and tetracycline resistant *Staphylococcus aureus* (Socha *et al*. 2006).

Among various spirotetronate-class polyketides, maklamycin is a polycylic compound extracted from culture broth of an endophytic *Micromonospora* sp. GMKU326 isolated from Thailand. This compound displayed broad antimicrobial activity against *Micrococcus luteus, Bacillus Subtilis, Bacillus Cereus, Staphylococcus aureus and Enterococcus faecalis* with a MIC values of 0.2, 1.7,6.5, 13 and 13 µg/ml respectively. Comparatively it showed lower activity against *Candida albicans* with MIC value 50 µg/ml. In addition to this, it showed moderate cancer cell cytotoxicity. (Igarashi *et al.* 2011). Nomimicin is another spirotetronate antibiotic of polyketide origin biosynthesized by *Actinomadura* sp. TP-A0878 exhibited antimicrobial activities against *M. luteus*, *C. Olbicans* and *Kluyveromyces fragilis* with MIC values 6.3, 12.5 and 12.5 µg/ml respectively (Igarashi *et al.* 2012). Spirotetronate group includes Lobophorin F extracted from *Streptomyces* SCSIO 01127 showed MIC values against *Bacillus thuringinesis, S. aureus and E. faecalis* as 2, 8, 8 µg/ml respectively (Niu *et al.* 2011). Likewise, lobophorin A, B and G produced *Streptomyces* sp. MSI00061 isolated from South China Sea showed significant activity against *Mycobacterium bovis,* moderate activity against *M. tuberculosis* and diverse toward *B. subtilis* (Chen *et al.* 2013). Similarly, lobophorin H isolated from South Shina Sea derived *Streptomyces* sp. 12A35 exhibited antibacterial activity against *M. tuberculosis, B, subtilis* and *S. aureus*. This compound also displayed toxicity toward human CEM-TART cell line (Pan *et al.* 2013 and Lin *et al.* 2014). *Streptomyces* sp. C34 isolated from hyper-arid soil of Atacama, Chile was able to synthesize polyketides designated as chaxamycins A-D. Among these compounds, Chaxamycin D exhibited potent antibacterial activity against *S. aureus* ATCC 25923, *E coli* ATCC 25922 and a panel of clinical isolate of MRSA with a MIC value of 0.05 µg/ml, 1.21 µg/ml and 0.06-0.25 µg/ml respectively (Rateb *et al*, 2011a). In another research three polyketide antibiotics belonging to β-diketone group were purified from culture broth of *S. asenjonii* KNN42.f isolated from extreme hyper-aid desert soil of Chile. These three compounds were asenjonamide A, B and C of which asenjonamide C displayed highest antibacterial activity against MSSA (MIC=1.8

µg/ml) followed by *E. faecium* (MIC=3.9 µg/ml) and *E. coil* with a MIC 5.4 µg/ml (Abdelkader *et al.* 2018). Another glycoside polyketide, gilvocarcin HE, obtained from ethyl acetate extract of *Streptomyces* sp. QD 01-2 isolated from soil of Heniquin square of China proved promising antimicrobial activities against *S. aureus, B. subtilis, E. coli* and *C. albicans* with variable MICs ranging from 0.25-2.5 µg/ml. This compound was found cytotoxic to various cell lines such as MCF-7, K562 and P388. Vinyl side Chain was solely responsible for antimicrobial and cytotoxic activities of gilvocarcin type glycosides (Hou *et al.* 2012).

In the search of new metabolites of microbial origin, Lu *et al.* (2017) purified two new chloranthrabenzoxocinone antibiotics named zunyimycins B and C from culture broth of *Streptomyces* sp. FJS31-2 isolated form soil of Finking Mountain China. The compound Zunyimycin C displayed higher antimicrobial activity against *S. aureus* with a MIC 0.94 µg/ml and five clinical MRSA isolates with MIC from 3.75-8.14 µg/ml. Many new polyketide antibiotics named as formicamycins A-L were purified from culture broth of *Streptomyces formicae* KY5 isolated from a Kenyan ant *Tetraponera penzigi.* Among these types, formicamycin L was found most active against clinical isolates of MRSA and vancomycin resistant *Enterococcus faecium* with a MIC value 0.41 µg/ml and 0.82 µg/ml respectively (Qin *et al.* 2017). Among many new compounds synthesized by *Streptomyces* sp. Chaxalactins A-C are rare macro lactone polyketide antibiotics. These molecules showed comparatively higher inhibitory activity against Gram positive bacteria such as *S. aureus, L. monocytogens and B. subtilis* with MIC value ranging from 0.2 to 6.3 µg/ml. Chaxalactins A and C had MIC value of 12.5 µg/ml against *Vibrio parahemolyticus* and Chaxalactin C had MIC 20 µg/ml. (Rateb *et al.* 2011b). A new *Streptomyces* sp. strain CNH 365 isolated from marine sediment sample of Santa Barbara, California, USA is able to synthesize novel compound named as anthracimycin. This compound is a polyketide antibiotic having 14-membered lactone ring structurally similar to that of macrolide chlorotonil. The antibiotic displayed highest inhibitory activity against *Bacillus anthracis* with a MIC value of 0.03 µg/ml It showed extended antibacterial activity against a panel of pathogens *E. faecalis, S. Pneumoniae, H. influenzae, Moraxella catarrhalis,* MSSA, MRSA and Vancomycin resistant *S. aureus* with MIC ranging from 0.03 to 4 µg/ml. (Hensler *et al.* 2014).

Marine actinomycetes strain isolated from sediment from Chuuk, Federal States of Micronesia, was able to synthesize two macrolactins $A_1$and B_1, as well as lauramide diethanolamine. These compounds were inhibitory to Gram positive (*B. subtilis, S. aureus)* and Gram negative *(E. coil, P. aeruginosa)* bacteria with MICs ranging between 0.015 and 0.125 µg/ml. In addition to this, *S. cerevisiae* was also inhabited with a MIC value of 0.125 µg/ml. (Mondoll and Shin, 2014).

Ability of new actinomycetes *Actinoallomurus* sp. IDI 45698to synthesize hyperchlorinated anguacyclinones was explored. These compounds were designated as allocyclinones. These molecules are characterized by an unusual lactone ring and present up to four halogens per molecule, with one congener representing the first natural product containing a trichloromethlyl substitution in an aromatic system. The antibacterial activity of four isolated allocyclinones was determined to increase with the number of chlorine substituents on the methyl group. Allocyclinone A has three additional chlorine atoms at carbon C-13. This compound showed the highest antibacterial activity. The MICs were in the range of 0.25-0.5 µg/ml against *S. aureus, S. pyogenes and E. faecalis,* but for *E. faecium* it was 4µg/ml (Cruz *et al.* 2017). Soil derived actinomycetes *Streptomyces* RAB12 isolated from Hyderabad, India synthesized bicyclic chromopeptide lactones RSP01 and RSP 02 belonging to actinomycin group. Both RSP 01 and RSP02 displayed a chemical structure similar to actinomycin D. However, RSP01 has a ketocabonyl group at the fourth carbon of the proline moiety, which is absent in actinomycin D. Result of bioactivity assay explored that RSP 01 has a higher antimicrobial potential than actinomycin D. The MIC values for RSP 01 ranged from 0.007 to 0.06 µg/ml against *S. aureus, P. aeruginosa, S. typhi & B. subtilis*. (Rathod *et al.* 2018). Extensive exploration of antimicrobial potential of actinomycetes by Lu *et al.* (2015) resulted into isolation of two 15-membered macrolides (tylosin analogues) from a wbIA disruption mutant of *Streptomyces ansochromogenes.* Both of these products showed satisfactory activity against bacterial pathogens such as *Streptococcus pyogenes, S. pneumoniae, B. subtilis, B. cereus and S. aureus* with MIC values in the range of 3.53-58.5 µg/ml. It is noteworthy that tylsoin derivatives showed stronger bactericidal activity toward *S. Pneumoniae* than tylosin itself. Sawa *et al.* (2018) obtained a novel 48-memberd polyol macrolide compound quadoctomycin from culture broth of *Streptomyces* sp. MM168-141F8. This compound exhibited antibacterial activity against only Gram-

positive bacteria. The MIC values obtained were in between 1-2 µg/ml against three MSSA, five MRSA and six *E. faecalis.* A soil actinomycetes *Amycolatopsis* sp. MST-108494 isolated from southern Australia was found to synthesize three glycosylated polyketide macrolides named amycolatopsins A, B and C. Amycolatopsin A and C showed antibacterial activity toward *M. tuberculosis* and *M. bovis.* All of these compounds showed cytotoxic activity against mammalian NCIH-460 and SW620 cells (Khalil *et al.* 2017). Two novel polycyclic tetrameric acid macrolactams named isoikarugamycin and 28-N-methylikarugamycin were purified from culture broth of a marine *Streptomyces zhaozhouensis* CA-185989. These metabolites verified strong antibacterial and antifungal activities against *S. aureus, C. albicans* and *A. fumigatus* with MIC value between 1 and 8 µg/ml. (Lacret *et al.* 2014). Heterogenous expression of specific gene clusters corresponding to two eDNA- derived KSBS sequence tags in *Streptiomyces albus* resulted into the production of polyketide quinone antibiotics known as arenimycins C and D. These antibiotics showed strong antibacterial activity against MRSA USA 300 with MIC value 0.0988 and 0.19µg/ml respectively and *B. Subtilis* RM125 with MIC value 0.0015 & 0.39 µg/ml respectively (Kang and Brady, 2014). Marine actinomycetes *Pseudonocardia* sp. SCSIO 01299 isolated from deep sea sediment of south china sea synthesized pseudonocardians A-C, diazanthraquinone analogs during controlled fermentation. Pseudonocardians A and B were inhibitory against *S. aureus, E. faecalis,* and *B. thuringiensis* with MIC value ranging from 2-4 µg/ml. These compounds also displayed activity against tumor cell lines (Li *et al.* 2011).

In the search of novel metabolites Jiang *et al.* (2015) have purified Xiakemycin A, a pyranonaphthoquinone, from fermented broth of a soil isolate *Streptomyces* sp. CC8-201from China. It demonstrated antibacterial activity against several Gram-positive bacteria such as *S. aureus, S. epidermidis, E. faecalis* and *E. faecium* with MICs ranging from 2-16µg/ml. Similarly, in another research from the same country, *Streptomyces niveus* SCSIO 3406 isolated from marine sediment have produced four novel sesquiterpenoid napthoquinones named as marfuraquinocins A-D. Among these compounds marfuraquinocins A, C and D were found to be inhibitory against *S. aureus* ATCC 29213 with equal MIC 8 µg/ml. Furthermore, marfuraquinocins C and D displayed antibacterial activity against methicillin resistant *S epidermidis* clinical isolate Shhs-EL with equal MIC value of 8 µg/ml. (Song *et al.* 2013).In the series of

new metabolites, two new capomycin type quinone antibiotics fradimycins A and B were extracted and purified from culture broth of a marine *Streptomyces fradiae* PTZ 0025. Both of these compounds verified activity against *S. aureus* with MIC 6 and 2 µg/ml respectively. In addition, these compounds exhibited cytotoxic activity toward human cancer cells. (Xin *et al.* 2012). A novel chlorinated quinolone compound named as ageloline A was extracted from culture broth of *Streptomyces* sp. SBT 345 associated with marine sponge *Agelas oroides.* This compound was found inhibitory to *Chlamydia trachomatis* inclusion with IC_{50} value of 2.1 µg/ml. In addition to this, it also reduces nucleic acid damage induced by 4-nitroquinoline-1-oxide. (Cheng *et al.* 2016). In a study Shin *et al.* (2016) purified a broad-spectrum secondary metabolite actinomadurol from culture of *Actinomadura* KC 191 strain. This compound consists of a 19-niditerpenoid-carbon backbone and exhibited antibacterial activity against *B. subtilis* ATCC 6633, *S., aureus* ATCC 6538p, *K. rhizophila* NBRC 12708, *P. hauseri* NBRC 3851 and *S. enterica* ATCC 14208 with MICs ranging from 0.39-3.12 µg/ml. Novel cyclic peptides designated as pargamicins B, C and D were purified from culture broth of a soil actinomacetes *Amycolatopsis* sp. ML1 hF4 but paragamicin A was described structurally in 2008. All pargamicin consist of N-methyl-3hydroxy valine, 4-hydroxy piperazic acid (4-OH-pip), sarcocine, Phenylalanine, N-hydroxy isoleucine (NOH-Ile) and Piperazic acid (pip). All pargamicins are structurally same but only differ in the pip (NOH-Ile) moiety. These molecules verified antibacterial activities against MSSA, MRSA and enterococci. The MIC values of pargamicin C against MSSA and MRSA were in between 2 & 4 µg/ml. While it was 0.5 to 1 µg/ml toward *E. faecium* and *F. faecalis* respectively. On the other hand, paragamicin B and D showed MIC values of 8-16 and 32-64 µg/ml toward MSSA and MRSA respectively. Likewise, pargamicins B and D displayed an equal MIC value 8 µg/ml against *F. faecalis* and *E. faecium.* (Hashizume *et al.* 2017).

In a research conducted by Hassan *et al.* (2015) a new bicyclic depsipeptide antibiotic named as salinamide F was purified from marine *Streptomyces* sp. CNB-091. The salinamide F displayed antibacterial activity against *E. coil* D21f2tolC, *Haemophilus influenzae, E. faecalis and Neisseria gonorrhoeae* with MIC values of 0.20, 12.5, 12.5 and 25µg/ml respectively. In addition to this, inhibition of Gram positive and Gram-negative bacterial RNA polymerase with IC_{50} 4 µM for *S. aureus* RNAP and 2 µMfor *E. coil* RNAP. (Hassan *et al.* 2015). In a long research of 60 years conducted at Merck

Research Laboratories, Merk & co., a novel DNA gyrase inhibiting compound named as Kibdomycin was isolated. This hexacyclic polyketide-peptide hybrid metabolite containing Dichloropyrrole moiety was synthesized by *Kibdelo- sporangium* sp. MA7385. This compound selectively inhibits the activity of two subunits of topoisomerase IV and DNA gyrase. The Kibdelomycin inhabited a panel of bacterial pathogens with variable MICs. The MIC values were 0.5, 1, 2 and 2 µg/ml against MRSA, *S. pneumoniae E. faecalis* and *H. influenzae* respectively. (Phillips *et al.* 2011). In the extended study performed by Singh *et al.* (2015), Kibdelonycin was found significantly potent against *Acinetobacter baumanii* with MIC value of 0.125 µg/ml. Furthermore, there was no cross resistance observed with other gyrase inhibitors in assays with novobiocin and ciprofloxacin resistant *S. aureus.*

A novel lipopeptide, the armomycin A6, was isolated from *Streptomyces parvus* HCC B10043. With the use of Ultra performance liquid chromatography coupled with tandem quadrupole and time of flight high-resolution mass spectroscopy (UPLC-Q-TOF-HRMS). This compound was found inhibitory to *S. epidermidis* HCCB 20256 with the MIC of 1µg/ml (Rao *et al.* 2013). In another study carried out in Korea Um *et al.* (2013) extracted two new cyclic depsipeptide antibiotics named as ohmyungamycins A and B from fermented broth of *Streptomyces* sp. isolated from soil sample of a volcanic island. These compounds contain unusual amino acid units (N-methyl-4-methoxytryptophan, β-hydroxyphenylalanine and N, N-di-methyl-valine). These molecules displayed inhibitory activity against *B. subtilis, Kocuria rhizophila* and *Proteus hauseri* with MIC values ranging from 1.56-49.5 µg/ml. Ohmyungsamycin A was comparatively more cytotoxic and antibacterial than ohmyungamycin B. In an extended study Moon *et al.* (2014) discovered buanmycin, a new pentacyclic xanthone, from a culture broth of marine *Streptomyces* strain. The buanmycin showed broad antibacterial activities against several Gram-positive bacteria such as *S. aureus, B. subtilis, Kocuria rhizophila* and Gram-negative bacteria including *S. enterica* and *P. hauseri* with MIC values ranging *S enterica* and *P. hauseri* with MIC values ranging from 0.42-12.5 µg/ml. In addition to this, this compound also inhibited the *S. aureus* sortase A enzyme responsible for adhesion and host invasion with IC_{50} value of 43.2 µM. Furthermore, it also displayed potent cytotoxicity with sub-micromolar IC_{50} values as well as moderate antifungal activity toward *C. albicans* with MIC value 12.5 µg/ml. Novel xanthones designated as

citreamicin QA, citreamicin QB, citreaglycon A and dehydrociteraglycon were purified from fermented broth of a marine *Streptomyces Caelestis* strain. All of these compounds showed potent activity against *S. haemolyticus, S. aureus* and *B. subtilis* with MICs 0.25-16 µg/ml. Among them citreamicin QA and citeramicin QB were found more inhibitory probably due to the five-member nitrogen heterocycle in their structure. Besides antibacterial, all of the compounds displayed cytotoxicity against HeLa cells (Liu *et al.* 2012). The combined approach including phylogenetic and chemical analysis of marine Streptomyces strain revealed several secondary metabolites including two new bioactive novobiocin analogs. The compounds desmehylnovobiocin and 5-hydroxy novobiocin were found inhibitory to MRSA ATCC 33591 with MIC value of 16 and 8 µg/ml respectively. Structure activity relationship (SAR) studies demonstrated that analogues bearing different substituents at 3 – carbomoyl and 4 –Ome Noviose moieties or a 5-H hydroxybenzoate ring showed a dramatic decrease or complete elimination of inhibitory activity against MRSA (Dalisay *et al.* 2013).

Three novel meroterpenoids-rahinomycins named as 4-dehydro-4a-dechloronapyradiomycin A1, 4-dechloro-3-bromonapyradiomycin A1 and 3-chloro-6, 8-dihydroxy-8-a-lapachone along with other previously known metabolites were extracted from culture broth of *Streptomyces* sp. strain SCSIO10428 isolated from marine samples of China. Among these compounds, 3-dechloro-3-bromonapyradiomycin showed activity against *S. aureus, B. subtilis and B. thuringiensis* with MIC values in the range of 0.5-1 µg/ml. Moreover, the compound also exhibited cytotoxicity against four human cancer cell lines (Wu *et al.* 2013). A new thiazozyl peptide antibiotic kocurin (PM181104) was isolated from *Kocuria palustris* F.27,345. This antibiotic was found significantly inhibitory against *B. subtis* and antibiotic resistant isolate of *S. aureus, E. faecium* and *E. faecium.* The MIC values of the compound was very low in the range of 0.008-0.512 µg/ml in comparison to commercial standard antibiotic linezolid. The molecule successfully protected the mice from organ specific infections and systemic infections in an In-vivo study (Mahajan *et al.* 2013).

2.12.2 Antifungal metabolites

Various antibiotics have been isolated from a variety of microorganisms; however, studies are still being conducted to identify novel antifungal antibiotic (Atlas and Bartha, 1986). In general, *Streptomyces* spp. are soil inhabitant saprophytic organism play an important role in turnover of complex biopolymers and antibiotic production (Wanner, 2009).

Marine isolates of actinobacteria have tremendous potential of producing antifungal substances (Okami and Hotta, 1988). Chandrananimycin A obtained from *Actinomadura* spp. exhibited potent antifungal activity against *Mucor miehei* and antialgal activity against *Chlorella vulgaris* and *C. Sorokiniana.* In addition, this compound showed antibacterial activity against *S. aureus* and *B. subtilis* along with anticancer activity (Maskey *et al.* 2003b). N-(2-hydroxyphenyl)-2-Phenazinamine (NHP) is a novel compound isolated from *Nocardia dassonvillei.* This compound showed significant antifungal activity against *Candida albicans* with a MIC value of 64 µg/ml and anticancer activity against HePG2, A-549, HCT-116 and COC1 cells (Gao *et al.* 2012).

The macrolide polyene antibiotic Amphotericin B was earlier isolated from *Streptomyces nodosus* which showed broad antifungal activity but with limited clinical applications due to many side effects (Terjo and Bennett, 1963). Polyoxins obtained from *S. cacaoi var. asoensis* exhibited strong antifungal activity against *Alternaria and Piricularia oryzae* (Kimura and Bugg, 2003). Nikkomycins (nikkomycin Z) showed better antifungal activity than polyoxins against *Candida albicans.* Various nikkomycins are synthesized by *S. tendae* and *S. ansochromogens.* Plant pathogens *Rhizopus carcinans* and *Botrytis Cinerea* were susceptible to nikkomycins produced by *S. tendae.* (Kimura and Bugg, 2003). Nikkomycins X and Z are peptidyl nucleoside antibiotics biosynthesized by *S. ansochromogens* are inhibitors of chitin synthetase enzyme in fungi and insects. The later type is more potent than nikkomycin X (Liao *et al.* 2009). Oligomycins are macrolide antibiotics produced by *S. diastaticus, S. diastochromogens, S. avermitis* and *S. libani.* Among them, oligomycin A and C displayed promising antifungal activity against *Alternaria alternate, Botrytis cinerea and Phytophora capsici* (Yang *et al.* 2010). Recently oligomycins A and E produced by *Streptomyces* sp. strain HG29 were tested against a

large panel of fungi and bacteria. These compounds exhibited strong antifungal activity against *Aspergillus carbonarious* M333, *A. niger* OT 304, *Fusarium equiseti* and *F. monilioforme.* Against tested bacteria moderate activity (10-20 mm) was shown against *Bacillus subtilis* ATCC 6633 and MIC value was found 2-10 µg/ml for majority of fungi and >100 µg/ml against *Bacillus subtilis,* staphylococci, *Candida albicans* and other (Khebizi *et al.* 2018).

Streptomyces aureofaciens isolated from the root tissues of *Zingiber officianale* produced 5,7-dimethoxy-4-phenylcaumarian and 5,7-dimethoxy-4-p-methoxyl-phenylcaumarin which displayed antifungal activities (Taechowisan *et al.* 2005). Potent antifungal activity of a novel compound 210-A was proved against *Fusarium oxysporum* f. sp. *cubense* race four. This compound was extracted from *S. noursei* Dao7210 (Wu *et al.* 2009). Among the several bioactive metabolites extracted from broth of *Streptomyces* TK-VL 333 and 1H-indole-3- carboxylic acid (T1) verified antifungal activity against *Candida albicans, Epidermophyton floccosum, Aspergillus niger and Fusarium oxysporum* (Kavitha *et al.* 2010). Soil actinomycetes *S. lavenduligriseus* produces three novel compounds belonging to polyene macrolide group. Of these, 15-glycidylfilipin III showed strong activity against *Candida albicans* with MIC value of 6.25 µg/ml compared to MIC 3.13 µg/ml for nystatin used as positive control (Yang *et al.*2016).Similarly in another study, six new cyclic depsipetides and enduspeptide A-F, extracted from a *Sterptomyces* spp. isolated from soil sample of coal mine of china. These molecules proved strong antifungal activities toward *Candida glabrata* ATCC90030. The IC_{50} values of enduspeptide A, B and C were 5.33, 1.72 and 8.13 µg/ml respectively (Chen *et al.* 2017). In the series of research to explore novel bioactive metabolites, mohangamides A and B were extracted from *Streptomyces* sp. SNM55 isolated from soil of Korea. These compounds were found to inhibit isocitrate lyase (ICL) of *Candida albicans* with an IC_{50} value of 4.4 and 20.5 µM respectively. (Bae *et al.* 2015). A marine actinomycetes *Actinoalloteichus* sp. NPS 702 isolated from Japan synthesized various neomaclafungin A-I. These Compounds displayed a MIC value of 1-3µg/ml against dermatophyte *Trichophyton metagrophytes* ATCC 9533 (Sato *et al.* 2012)

2.12.3 Antiviral Metabolites:

Several antiviral secondary metabolites have been extracted and successfully assayed against various viruses. Ahmpactinin Bu is a novel linear peptide and pyrrolidine derivative containing unusual amino acid, 4-amino-3-hydroxy-5-(4-methoxy phenyl) pentanoic acid. It was produced by cultivating *Streptomyces* sp. CPCC 202950 on sterile soaked rice. Ahmpatinin Bu displayed potent inhibitory activity against HIV-1 protease resulting in IC_{50}=1.79 nM (Chen *et al.* 2018) In order to control spread of Zika virus in North and South American region, several previously used drugs were researched against Zika Virus. Daptomycin and nanchangmycin were found effective against Zika virus with IC_{50} values of 1µM and 0.1 µM respectively. Both of these drugs are bioactive secondary metabolites of *Streptomyces* sp. for which antiviral activity was not reported before. Daptomycin is a lipopeptide antibiotic used in the treatment of Gram-positive bacteria produced by *S. roseosporus.* While nanchangmycin is produced by *S. nanchangensis* with insecticidal activity against silkworm and antibacterial activity in vitro. (Barrows *et al.* 2016; Pascolino *et al.* 2016 and Rausch *et al.* 2017). Promising Chemical structure with antiviral properties are displayed by Xiamycins C-E produced by *Streptomyces* sp. HK18 isolated from soil sample of Korea. These compounds were chemically elucidated as carbazole-bearing indodosesquiterpenoids. Among them Xiamycin D showed strongest effect on porcine epidemic diarrhea virus (PEDV) with replication value Of EC_{50} equaling 0.93 µM (cytotoxicity=56.03µM and selective index = 60.31). Additionally, the inhibitory activity of xiamycin D was confirmed by quantitative real-time PCR after amplification of fragments of the genes encoding essential proteins (GP6 nucleocapsid, GP2 spike and GP5 membrane) for PEDV replication and by Western blotting of PEDV GP2 Spike and GP6 nucleocapsid proteins (Kim *et al.* 2016). In a study Ravesh *et al.* (2013) purified a novel metabolite antimycin A1a from marine *Streptomyces kaviengensis* of Ireland Coast. This molecule displayed promising activity against Western equine Encephalitis virus with IC_{50} value less than 4nM and selectivity index of greater than 550. Analysis of its mechanism of action revealed disruption of mitochondrial electron transport and pyrimidine biosynthesis. In addition, previously known antimycin A exhibited a broad antiviral activity against a wide Range of RNA viruses belonging to togaviridae, flaviviridae, bunyaviridae, picornaviridae and paramyxoviridae families. HIV-1 Protease is essential in the life cycle of HIV and has been used as a promising target for AIDS therapy. A novel

inhibitor of HIV-1 protease, 4862F was isolated from the culture broth of Streptomyces *albosporus* IO3A-04862. It was elucidated as N, N, N-(trimethylated)-Tyr-h-Leu-L-Val-L-Leu-(dehydrated)- His and determined to displayed inhibitory activity against HIV-1 protease with an IC_{50} value of 15.26 nM (Liu, Gan *et al.* 2012).

Marine actinobacteria *Streptomyces nitrosporeus* was found to synthesize a novel compound benzastatin, a 3-chlorotetrahydroquinolone alkaloid, showing antiviral activity in a dose dependent manner with EC_{50} values 1.92, 0.53 and 1.99 µg/ml against herpes simplex virus type 1 (HSV-1), herpes simplex virus type-2 (HSV-2) and vesicular stomatitis virus (VSV) respectively (Lee *et al.* 2007). In a study Takagi *et al.* (2010) have detected a compound JBIR-68 with a unique skeleton (5-o-Geranyl-5, 6-dihydrouridine) from *Streptomyces* sp. RI18. This compound revealed antiviral activity against influenza virus. A novel compound pimprinethine was extracted from culture broth of *Streptomyces* sp. displayed inhibitory activity against EV71 and ADV-7. It showed moderate activity against CVB3, HSV-1 and H1 N1 types (Wei *et al.* 2014).

2.12.4 Anticancer and antitumor metabolites

Cancer still remains one of the most serious human health problems and breast cancer is the second most universal cause of cancer death in woman. Several antitumor compounds have been derived from marine actinobacteria are used for the production of anticancer drugs (Ravikumar *et al.* 2012a). Actinomycin isolated from *Streptomyces antibioticus* was the first drug used in tumor treatment. Similarly, many anticancer drugs such as daunomycin, doxorubicin, anthracyclines were produced by *S. peuceticus*. Epirubicin is an anthracycline group compound approved by FDA in 1999 and has a better therapeutic profile than doxorubicin due to less adverse effects. It is used in breast cancer, ovarian cancer, lung cancer and leukemia (Soleka *et al.* 2012).Furthermore, bleomycin and streptozotocin produced by *S. verticillus* and *S. achromogens* were approved by FDA as antitumor drugs(Soleka *et al.*2012).While studying bioactive metabolites of actinomycetes Zhang *et al.*(2010) extracted a geldanamycin analogue 11-methoxy-17-formyl-17-demethoxy-18-O-dihydrogeldanamycin from culture broth of *S. hydroscopicus* Ao70101. This compound displayed promising toxicity against MCF-7 (breast cancer cell line), COR-L23 (lung cancer cell line) and SK-Mell (skin melanoma cell line). In a recent

study, *Streptmyces bingchenggensis* ULS14 isolated from sediment sample synthesized anticancer compounds ULD F_4 and ULD F_5 structurally similar to staurasporine and kigamicin. These two compounds displayed anticancer activity against HeLa cell line with IC_{50} value of 0.034 µg/ml and 0.075 µg/ml respectively. Furthermore, other eight actinomycetes strains also exhibited anticancer activity against Hela, AGS, MCF-7 and HL-6 cell lines with an IC_{50} values ranging from 0.030 µg/ml to 4.4 µg/ml (Olabisi *et al.* 2019). Actinobacterial isolates from mangrove soil samples were found to synthesize heterocyclic compounds such as phenolics, pyrazines and pyrrolopyrazines by three active strains. Among the isolates *Microbacterium flava* MUSC 78T and *M. mangrovi* HUSC115T exhibited anticancer effect on ca ski (human cervical carcinoma cell lines). Furthermore, the isolates also exhibited antibacterial activities against a panel of test bacteria including both Gram-positive and Gram negative (Azman *et al.* 2017). A depsipeptide antibiotic valgamyin containing the extremely rare amino acid cleonine and valgamycin compounds A, T and V were isolated from *Amycolatopsis* SP. ML1-hF4. The compound T showed mild toxicity toward cancer Cell lines such as HGC27, NB 16, ME 180 and others (Hashizume *et al.* 2018). *Amycolatopsis* sp. ICBB8242 isolated from black water ecosystem of Indonesia synthesized two new apoptolidins, 2-0-succinyl- apoptolidin A and 3-O-succinyl-apoptolidin A. metabolites were found to suppress the proliferation and viability of human H292 and HeLa Cells (Sheng *et al.* 2015).

An investigation of secondary metabolites from *Nonomuraea* species isolated from soil afforded a new S-bridged pyronaphthoquinone dimer, hypogeamicin A along with its monomeric precursors hypogeamicin B-D. The prior compound (A) had significant cytotoxic activity against colon cancer derived cell line TCT-1 with IC_{50}=6.4-12.8 µM, but it did not exhibit antibacterial activity, On the other hand the monomeric substances did not display cytotoxic activity but inhibited *B. subtilis* with MIC value 7-28 µg/ml. (Derewacz *et al.* 2014). Two novel compounds amethysione and amethyamide along with other derivatives were extracted from culture broth of *Streptosporangium amethystonenes* BCG27081 isolated from soil. Amethysione exhibited weak cytotoxicity toward KB and NCL-H187 cell lines with IC_{50} values 16.94 µg/ml and 36.99µg/ml respectively (Boonlarpradab *et al.* 2016). A new benzylanthraquinone compound named as PM07747 was isolated from a sponge associated marine actinomycetes *Saccharopolyspora taberi* PEM-06-F23-019B. This

compound showed cytotoxicity activities against MDA-MB 231 cells, HT-29 cells and A-459 cells with LC_{50} values 1.72, 72.73 and 6.82 µM respectively (Perez *et al.* 2009).

2.13 Techniques used for structure elucidation

2.13.1 IR Spectroscopy

IR Spectroscopy refers to the analysis of the interaction of a molecule with infrared light.; a light having a lower frequency than visible light. The application of infrared spectroscopy is in the identification of the functional groups in organic and inorganic molecules. In IR spectroscopy molecules absorb particular frequencies of light that are characteristic of the corresponding functional groups in the molecule. Therefore, IR spectroscopy detects frequencies of infrared light absorbed by a molecule and a graph is plotted with the infrared light absorbed on the Y-axis and frequency or wavelength on the X-axis. IR frequencies vibrate the bonds with more amplitude with the change in the vibrational energy and dipole moment in the molecule. The intensity of the absorption depends on the polarity of the bond. Symmetrical non-polar bonds in N≡N and O=O do not absorb radiation. Most of the bands corresponding to different functional groups present in the region from 4000 cm^{-1} to 1300 cm^{-1}. The band position identifies the functional group in the unknown compound. The region from 1500 to 500 cm^{-1} in the IR spectrum is called the finger print region. In this region peaks correspond to variety of bending and stretching within the molecule. A particular compound has a unique set of peaks in the fingerprint region that is exploited for the identification of the molecule. (Silverstein *et al.* 1991).

2.13.2 Liquid Chromatography-Mass Spectrometry (LC-MS)

Liquid Chromatography-Mass Spectrometry (LC-MS) or High-Pressure Liquid Chromatography-Mass Spectrometry (HPLC-MS) is an analytical technique that coupled high resolution chromatographic separation with sensitive and specific mass spectrum detection. This includes High Performance Liquid Chromatography (HPLC)-MS, Capillary Electrophoresis (CE)-MS and Capillary Electrochromatography (CEC)-MS. The combination of Gas Chromatography and Mass Spectrometry (MS) was first reported in 1958 and made available commercially in 1967. Combination of LC with MS is an important development in the history of

chromatography. Mass spectrometry in LC-MS helps to determine the elemental composition and structural elucidation of a sample.

Typical LC-MS system is combination of HPLC with MS using interface (ionization source). The sample is separated by LC, and the separated sample species are sprayed into atmospheric pressure ion source, where they are converted into ions in the gas phase. The mass analyzer is then used to sort ions according to their mass to charge ratio and detector counts the ions emerging from the mass analyzer and may also amplify the signal generated from each ion. As a result, mass spectrum (a plot of the ion signal as a function of the mass-to-charge ratio) is created, which is used to determine the elemental or isotopic nature of a sample, the masses of particles and of molecules, and to elucidate the chemical structures of molecules. LC- MS can be applied for various purposes such as molecular weight determination, Structural determination/elucidation, Pharmaceutical application*s:* It's used to determine the pharmacokinetic profile of the pharmaceuticals like drug, drug metabolites/degradation product, impurities and chiral impurities. It Is widely applied to identify microbial metabolites like antibiotics, aflatoxins, vitamins synthesized by microbes or present as contaminants.

2.13.3 Other Methods

Other techniques widely used for complete structure elucidation include GC-MS, H-NMR, C-NMR, MALDI-TOF, and many more. These methods rely upon different principles exploring complete structure of a chemical compound.

CHAPTER 3
MATERIALS AND METHODS

3.1 Study Type and duration

This laboratory based descriptive study was carried out during a period of 5 years from 2012 to 2016.

3.2 Sample Types

Soil and water samples were used for the isolation of actinomycetes.

3.3 Sample Collection sites

Soil samples were collected from 60 different districts of Nepal including Terai, Mountain and High Mountain. Similarly, water samples were collected from some rivers, lakes and hot water springs.

3.4 Sample Size

Four soil samples were collected from each district of Nepal constituting total 240 samples. Similarly, four water samples from each river, lake and pond were collected accounting a total of 40 samples from 10 sites. Altogether 288 soil and water samples were collected from all sampling sites.

3.5 Collection of samples

3.5.1. Collection of soil samples

Soil samples were collected from the top 4 cm of the soil profile. A soil sampling kit consisted of zip lock plastic bags each containing 1g of calcium carbonate ($CaCO_3$), a permanent marker and a soil digging instrument. Nearly 100 gram of soil sample was collected in plastic bags, mixed with $CaCO_3$, zip locked, labelled and transported to the laboratory. The soil samples were finely grounded in powdered form. Bacterial population was reduced by heating the samples at 45-50 ^{0}C for 3-4 hours and stored in a refrigerator at 4^{0}C (Tsao *et al*. 1960, Labeda and Shearer, 1990).

3.5.2 Collection of water samples

Approximately 50 ml of water sample was collected from each sampling site in a sterile autoclavable plastic bottle leaving an ampoule of air space. Then the bottles were tightly screw capped, labelled and transported to the laboratory by maintaining cold chain (4^0C) in an ice box.

3.6 Isolation of actinomycetes

Actinomycetes were isolated from soil and water samples by spread plate technique on Starch Casein Agar (SCA) medium following serial dilution technique. Ten ml of each water and 10 gram of soil sample was suspended separately in 90ml of sterile water and serially diluted up to 10^{-6} dilution with vortexing at each step to get properly homogenized suspension. An aliquot of 0.1ml of each dilution was evenly spread with the help of L- shaped sterile glass rod over the surface of SCA supplemented with cycloheximide and nalidixic acid (each at concentration of 50µg/ml). Then the plates were left undisturbed for 30 minutes and incubated at 30^0C for a week (Hayakawa *et al*. 2004, Magarvey *et al*. 2004).

3.7 Isolation of pure culture of actinomycetes

Typical actinomycetes colonies were identified based on dry, powdered, lichenoid and /or tough, leathery or butyrous colony morphology on SCA (Goodfellow *et al*., 2011). The colonies were picked up from SCA plate with the help of inoculating loop or straight wire and streaked on SCA by quadrant streaking technique (Collins et *al*. 2004). The inoculated plates were incubated for one week at 30^0C to get pure culture of actinomycetes (Ndejouong *et al*. 2010). The pure culture of actinomycetes were maintained in sterile soil and in SC broth added with 15% glycerol.

3.8 Primary screening of actinomycetes for antimicrobial activity

Primary screening of actinomycetes was done by perpendicular streak method on Muller Hinton agar (MHA) as described by (Marbrouk and Saleh, 2014). Actinomycetes were streaked along the diameter of MHA plate and incubated for 1 week for 30^0C. The test microbes *Bacillus subtilis,* Methicillin Resistant *Staphylococcus aureus* (MRSA) *Escherichia coli* ATCC25922, *Acinetobacter baumannii* (MDR), *Salmonella typhi* (MDR)and *Candida albicans* were each

inoculated in 5 ml of nutrient broth and incubated at 37^0C for 2-4 hours so as to obtain a turbidity comparable to 0.5 McFarland nephelometer standard. The broth culture of test microbes was streaked perpendicularly on either side of fully grown actinomycetes growth line while keeping the least distance (1-2 mm)between the test organism's streak line and actinomycetes. The plates were incubated at 37◦C for 24 hours and growth inhibition of test organism was recorded.

3.9 Inoculum preparation, Fermentation and extraction of bioactive metabolite

3.9.1 Inoculum preparation

The seed culture to be used as fermentation inoculum was prepared by transferring pure culture of actinomycetes strain from agar plates to starch casein broth in a 250 ml Erlenmeyer flask. Seed culture were grown in a water bath shaker at 30ºC with constant shaking 150rpm for 3-5 days. Then culture broth was centrifuged at 10000 rpm for 10 minutes. The pellet was washed twice with sterile distilled water and suspended in 20 ml sterile distilled water and used as inoculum (Ismet *et al*. 2004).

3.9.2 Fermentation

Two hundred ml of starch casein broth was taken in a 500 ml Erlenmeyer flask and aseptically inoculated with respective inoculum as mention above. The content of the flask was incubated at 30^0C in a water bath shaker for 7 days with 150 rpm. The visible pellets, clumps or aggregates and turbidity in the broth confirmed the growth of organism in the flask (Gebreyohannes *et al*. 2013).

3.9.3 Extraction of metabolite

After fermentation content of the flask were filtered through Whatman No.1 filter paper. The filtrate was then mixed with an equal volume (1:1) of ethyl acetate and shaken vigorously for 24 hours in a water bath shaker maintained at 30^0C. The organic phase (ethyl acetate fraction) and aqueous phase was separately collected in sterile beaker using separating funnel. The organic phase was dried at 50^0C in a desiccator and remaining content was dissolved in phosphate buffer saline (pH 7.4), labelled as partially purified metabolite and stored at 4^0C in a refrigerator. (Gebreyohannes et *al*. 2013).

3.10 Secondary screening

The crude extract preserved at 4^0C was subjected to centrifugation at 10,000 rpm for 10 minutes and supernatant was collected in separate sterile test tubes. Then both the supernatant and partially purified metabolite suspension was subjected to antimicrobial activity against test bacteria and fungi by agar well diffusion method (Boyanova *et al*. 2005) on Mueller Hinton Agar (MHA). MHA plates were uniformly swabbed with test microbial broth cultures with 0.5 Mc Farland Nephelometer standard. With the help of 6 mm cork borer wells were made on MHA plate. 50µl of partially purified metabolites were carefully transferred in separate well, kept undisturbed until complete diffusion in agar medium. The plates were incubated 37^0 C for 24 hours. If the zone of inhibition around the well was seen, it was measured and recorded.

3.11 Determination of Minimum Inhibitory Concentration

A set of ten tubes with sterile Mueller Hinton broth (one containing 10 ml and remaining containing five ml) were prepared. The solution of the extract in PBS was used to prepare 2-fold serial dilutions. Inoculum of the test organisms were prepared to turbidity of 0.5 McFarland standards. Equal volume of the prepared inoculum was dispensed in each tube. All the tubes were incubated at 37ºC for 24 hours. A tube with Mueller Hinton broth but without the antimicrobial agent and an uninoculated tube of medium were also incubated as growth control and sterility control respectively. After 24 hours, visual turbidity in each tube was noted (Wiegand *et al*.2008).

3.12 Characterization of actinomycetes

Those actinomycetes showing both antibacterial and antifungal activity in secondary screening were characterized phenotypically and sequenced.

3.12.1 Phenotypic Characterization

The pure culture of screened actinomycetes on SCA were examined for the color of aerial mycelium, substrate mycelium, diffusible pigments and other colony characteristics such as size, consistency and margin of colony (Waksman and Henrici, 1943, Kampfer, 2006).

3.12.1.1 Microscopic Characterization

Microscopic characterization was done by coverslip culture method (Kawato and Shinobu, 1959). The SCA plate was divided into 4 sectors. In each sector a sterilized coverslip was inserted at an angle of 45^0. Then potent isolates A_3, D_2, P_4 and J_1 were inoculated along the line where medium upper surface touched the coverslip. The plate was incubated for 10 days at 30^0C. After incubation the coverslip was carefully removed and placed on a clean slide with its inoculated side facing upward. Methanol was added on the surface of the coverslip and left for 15 minutes for fixation. Methanol was drained off and the coverslip was washed with distilled water. Crystal violet solution was added and left for five minutes and drained off. The coverslip was again washed with distilled water, air dried and observed under oil immersion objective of light microscope for the mycelium structure, configuration of sporophore, shapes and arrangement of spores. On the basis of morphology, the isolates were identified (Bergey and Holt, 2000).

3.12.1.2 Biochemical Characterization

Physiological, biochemical, temperature tolerance, salt tolerance and substrate utilization tests were studied for the identification of potent screened actinomycetes (Holt *et al.* 1994, Hopwood and Wright, 1973, Williams *et al.* 1989).

Catalase Test: This test was done by picking up an isolated colony with a sterile glass rod and mixing it with a drop of 3% H_2O_2 solution on a clean glass slide. Positive test was indicated by the appearance of gas bubbles.

Oxidase Test: This test was performed by picking up an isolated colony of actinomycetes with a sterile glass rod and rubbing it on the paper strip impregnated with oxidase reagent (1% tetramethyl-paraphenylene diamine dihydrochloride). Positive test was indicated by the development of intense deep purple color on the paper strip within a minute.

Carbohydrate Utilization Test: For this test, the basal medium containing peptone sodium chloride and phenol red was incorporated with carbohydrate at the

concentration of 1% (w/v). Carbohydrates used were fructose, Galactose, glucose, inulin, maltose, mannose, raffinose, salicin, sucrose and xylose. Each carbohydrate stock solution was heated for 15-20 minutes at 60^0C before adding appropriate volume to the already autoclaved basal medium. Then the carbohydrate containing basal medium was inoculated with the pure culture of actinomycetes colonies and incubated at 30^0C for 2 weeks. Positive test was indicated by the change in color of the medium from red to yellow.

Citrate Utilization Test: This test was done by streaking the slant of Simmon's citrate agar tubes with the pure culture of actinomycetes colonies and incubating the tubes at 30°C for 2 weeks. Citrate utilization was detected by change of color of the medium from dark green to Prussian blue.

Indole and Hydrogen Sulphide (H_2S) Production Tests: These tests were performed by stabbing sulphide indole motility (SIM) agar tube with the pure culture of actinomycetes and incubating the tubes at 30°C for 2 weeks. Indole production was detected by the development of cherry Red color at the interface upon the addition of Kovac's reagent. H_2S production was detected by blackening of the medium.

Nitrate Reduction Test: Nitrate broth was inoculated with the pure culture of actinomycetes from SCA plate and incubated at 30°C for 2 weeks. Nitrate reduction was detected by adding a few drops of sulphanilic acid reagent and alpha-napthylamine reagent into the culture broth. Development of red or pink color indicated positive test (Presence of Nitrite) in the absence of a positive reaction, 4-5 mg of Zinc dust was added to the tube previously tested for nitrite. The presence of nitrate (negative reaction) was demonstrated by the development of red color.

Urea Hydrolysis Test: In this test urea agar slants were streaked with the pure culture of actinomycetes colony and incubated at 30°C for 2 weeks. Positive test was indicated by the change of the color of the slant from orange to pink.

Starch Hydrolysis Test: Starch agar plates were inoculated with the pure culture of actinomycetes colony and incubated at 30ºC for 2 weeks. Starch hydrolysis was detected by flooding the plates with iodine solution. Positive test was indicated by a clear zone of hydrolysis around the colonies.

Gelatin Hydrolysis Test: Gelatin agar plates were inoculated with the isolated pure culture of actinomycetes and incubated at 30ºC for 2 weeks. Gelatin hydrolysis was detected by flooding the plates with mercuric chloride solution. Positive test was indicated by a clear zone of hydrolysis around the colonies.

3.12.1.3 Physiological Characterization

Temperature Tolerance Test: Nutrient agar plates were inoculated with the pure culture of actinomycetes and incubated at 15ºC, 37ºC and 45ºC for 2 weeks. Positive test was indicated by growth of the isolates.

Sodium chloride (NaCl) Tolerance Test: Nutrient agar plates with 3%, 5% and 7% NaCl were inoculated with pure culture of actinomycetes and incubated at 30^0C for 2 weeks. Positive test was indicated by the growth of the isolates.

Motility Test: This test was performed by stabbing sulphide indole motility (SIM) agar tube with the pure culture of actinomycetes colony and incubating the tubes at 30ºC for 2 weeks. Motility was detected by hazy growth of actinomycetes away from the stab line in the medium.

3.12.2 Genotypic Characterization

Genotypic characterization was carried out by 16SrRNA sequencing. The method of sequencing is given as:

3.12.2.1 Cultivation of Actinomycetes:

Pure culture of each actinomycetes strains were separately inoculated into conical flasks containing 50ml of SCA. The flasks were incubated at 30 ºC for 3 days. Then the broth was centrifuged to collect mycelia as pellet. The pellet was washed with sterile phosphate buffer for 3 times and subjected to DNA extraction.

3.12.2.2 DNA Extraction:

It was carried out using SpinStarTM Extraction Kit manufactured by ADT Biotech, Malaysia. The detailed procedure of DNA extraction is given as:

- The pellets containing approximately $5x10^6$ cells was separately taken in microfuge tubes and labelled properly.
- 20µl Proteinase K and 2µl Lysis Enhancer was added to the sample and mixed thoroughly by pulse-vortex and the tubes were briefly centrifuged.
- 200µl Buffer CB was added and mixed thoroughly by pulse-vortex.
- The tubes were incubated at 65°C for 10 min.
- The microcentrifuge tubes were briefly centrifuged. 20µl RNase A was added in each tube followed by mixing and incubation at 37ºC for 10min.
- 200µl absolute ethanol was added to the sample and was mixed immediately by pulse-vortex.
- All mixture (approx. 620ul) was transferred to a SpinStarTM column without wetting the rim. The cap was closed and the mixture was centrifuged at 6200 x g (8000 rpm) for 1 min. The SpinStarTM column was placed in a clean collection tube and tube containing the filtrate was discarded.
- The SpinStarTM column was carefully opened and 500µl Wash Buffer 1 was added without wetting the rim. The cap was closed and the mixture was centrifuged at 6200 x g (8000 rpm) for 1 min. The SpinStarTM column was placed in a clean collection tube and the tube containing the filtrate was discarded.
- The SpinStarTM column was carefully opened and 500µl Wash Buffer 2 was added without wetting the rim. The cap was closed and the mixture was centrifuged at 6200 x g (8000 rpm) for 1 min and the filtrate was discarded.
- The microcentrifuge tube was centrifuged at 17,000 x g (13,300 rpm) for 10 min.
- The SpinStarTM column was transferred to a clean 1.5 ml microcentrifuge tube and the collection tube containing trace Wash Buffer 2 was discarded.
- The SpinStarTM column was carefully opened and 60-100µl pre-heated Elution Buffer was added to the center of the membrane. It was incubated at room temperature (15–25°C) for 2 min, and then centrifuged at 9600 x g (10,000 rpm) for 1 min.
- The extracted DNA was stored at -20ºC if not used immediately.

3.12.2.3 16SrRNA sequencing:

The extracted pure DNA was maintained at -20ºC and sent to Macrogen, Inc., South Korea where 16SrRNA gene was amplified using universal eubacterial primer set, 27F 5' (AGA GTT TGA TCM TGG CTC AG) 3 and 1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3' for PCR and 785F 5' (GGA TTA GAT ACC CTG GTA) 3' and 907R 5' (CCG TCA ATT CMT TTR AGT TT) 3' for sequencing. The sequence obtained from Macrogen, South Korea was subjected to BLAST analysis and phylogenic tree was constructed by neighbor-joining method (Saitou and Nei, 1987).

3.13 Optimization of Fermentation

3.13.1 Inoculum preparation

The seed culture to be used as fermentation inoculum were prepared by transferring pure culture of screen actinomycetes strain from agar plates to basal medium consisted of g/l glucose 10, peptone 10, NaCl 10, $CaCO_3$ 2 in a 250 ml Erlenmeyer flask. Seed culture were grown in a water bath shaker at 30ºC with constant shaking 150rpm for 3-5 days. Then culture broth was centrifuged at 10000 rpm for 10 minutes. The pellet was washed twice with sterile distilled water and suspended in 20ml sterile distilled water and used as inoculum (Ismet *et al*. 2004)

3.13.2 Effect of carbon source on biomass and bioactive metabolite production

The effect of different carbon sources on growth and bioactive metabolite production was studied by replacing glucose in the basal medium with other carbon sources glucose, fructose, Galactose, maltose, Mannitol, sucrose, starch, and xylose. Triplicate flasks were setup for each carbon source used. Each flask was inoculated with 10% (V/V) on inoculum and incubated at 30ºC with 150 rpm for 7 days. After incubation, mycelia were separated by centrifugation and dried at 70ºC in a desiccator until constant weight was obtained which was expressed as mg/100ml (Bordodina *et al*. 2008). The content of flask was filtered and filtrate was mixed with equal volume of ethyl acetate. The mixture was vigorously shaken for 24 hours and solvent phase was dried at 50ºC in a desiccator and residue was dissolved in phosphate buffer saline (pH 7.4) and tested against test organisms by agar well method to determine effect of carbon source on bioactive metabolite production the diameter of zone of inhibition was recorded (Saadoum and Muhana, 2008).

3.13.3 Effects of nitrogen source on biomass and bioactive metabolite production

The effect of different nitrogen sources on growth and bioactive metabolite production was studied by replacing soybean meal in the basal medium with other nitrogen sources beef extract, casein, peptone, and tryptone. Triplicate flasks were setup for each nitrogen source used. Each flask was inoculated with 10% (V/V) on inoculum and incubated at 30^{o}C with 150 rpm for 7 days. After incubation, mycelia were separated by centrifugation and dried at 70^{o}C in a desiccator until constant weight was obtained which was expressed as mg/100ml (Bordodina *et al*. 2008). The content of flask was filtered and filtrate was mixed with equal volume of ethyl acetate. The mixture was vigorously shaken for 24 hours and solvent phase was dried at 50^{o}C in a desiccator and residue was dissolved in phosphate buffer saline (pH 7.4) and tested against test organisms by agar well method to determine effect of nitrogen source on bioactive metabolite production the diameter of zone of inhibition was recorded (Saadoum and Muhana, 2008).

3.13.4 Effect of initial pH on biomass and bioactive metaboliteproduction

The effect of pH on biomass and bioactive metabolite production was studied by adjusting initial pH of basal medium with superior carbon and nitrogen source at 4, 5, 6, 7, 8, 9, and 10. Flasks were inoculated with 10% inoculum and incubated at 30^{o}C with 150rpm for 7 days. After incubation biomass estimation and bioactive metabolite production was determined (Oskay, 2011).

3.13.5 Effect of temperature on biomass and bioactive metabolite production

The basal medium with superior carbon and nitrogen source was inoculated with 10% inoculum and incubated at temperatures 20, 25, 30, 35 and 40ºC for 7 days with 150rpm. After incubation biomass and bioactive metabolite production was determined (Oskay, 2011).

3.13.6 Effect of incubation period on bioactive metabolite production

Basal medium containing superior carbon and nitrogen source was inoculated with 10% inoculum in separate conical flasks and incubated at 30^0C up to 14 days with 150rpm. After each 2 days an aliquot of 10ml broth was withdrawn and subjected to concentration and extraction with ethyl acetate. The extract was dissolved in phosphate buffer saline and subjected to antimicrobial test by agar well method (Reddy *et al*. 2011).

3.13.7 Effect of minerals on biomass and bioactive metabolite production

The optimized medium with superior carbon and nitrogen source was supplied individually with different minerals $CuSO_4$, $MgCl_2$, $MgSO_4$, $FeCl_3$ and KNO_3 each at concentration 0.05% (w/v) and inoculated with 10% inoculum. Then all flasks were incubated at 30^0C with 150rpm for 7 days. After incubation biomass and bioactive metabolite production was estimated (Kiranmayi et *al*. 2011).

3.14 Identification of Bioactive metabolites

3.14.1 Fourier Transform Infrared Spectroscopy (FT IR)

The Fourier-transform infrared spectrum of ethyl acetate extract was analyzed to identify the functional groups present in the active compounds. Electromagnetic radiation ranging between 2500 nm and 20,000 nm was passed through the sample and was absorbed by the bands of the molecules in the sample causing them to stretching or bending. The wavelength of the radiation absorbed is characteristic of the bond absorbing it. IR spectrum was recorded on a PerkinElmer Spectrum version 10.53 FT-IR instrument equipped with ATR Golden gate accessories. The spectrum was scanned in the 400–4000/ cm range. The spectrum was plotted as percentage transmittance

versus wave number. The spectra obtained through those samples were compared and interpreted for the shifting of functional peaks (Parashuraman *et al*. 2014)

3.14.2 Liquid chromatography - Mass Spectroscopy (LC- MS)

Determination of the bioactive compound by LC/MS analyses was performed on Shimadzu LCMS-2020 system (ESI-Single Quad) equipped with a positive ionization source scan mode. The column oven temperature was adjusted at 30 °C using a Zorbas column (250 x 4.6 mm; C18 5micron particle size). Samples were injected using High Performance autosampler with a 10 µL injection volume at the flow rate 0.3 ml/min throughout the run. Separations were carried out by using mobile phases- mobile phase A 0.1% formic acid in water and mobile phase B acetonitrile. The column DL temperature was 250 ^{0} C and heat block temperature was 350 ^{0} C with drying gas flow rate 15L/min. Full mass scan spectra were recorded over a range of 100–1000 m/z (Silversteim *et al*. 1991)

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Results

4.1.1 Distribution of Actinomycetes in Samples

Among 288 samples processed, 240 were soil samples and rest 48 were water samples. A total of 320 actinomycetes strains were isolated. Of them 250 from soil samples, 50 from waters of ponds, lakes and rivers and rest 20 from hot spring water (Tatopani) as given in Table 4.1. Table 4.1 also depicted that 120 (37.5%) strains exhibited antimicrobial activity in primary screening of which 100 (40%) were from soil and 20 (40%) from different water samples. None of the isolate from hot water spring samples was found bioactive.

Table 4.1: Distribution of Actinomycetes

S.N.	Sample Types	Number of Actinomycetes Isolates	Number of Bioactive isolates {n (%)}
1.	Soil	250	100 (40%)
2.	Water from River, Ponds, Lakes	50	20 (40%)
3.	Water from Hot Spring (Tatopani)	20	0 (0%)
	Total	**320**	**120(7.5%)**

4.1.2 Types of Actinomycetes on the basis of pigmentation

Altogether 11 different pigment producing actinomycetes, dirty white and white isolates were most predominant each constituting 16.7% and least predominant were green and brown each 5% as presented in figure 4.1.

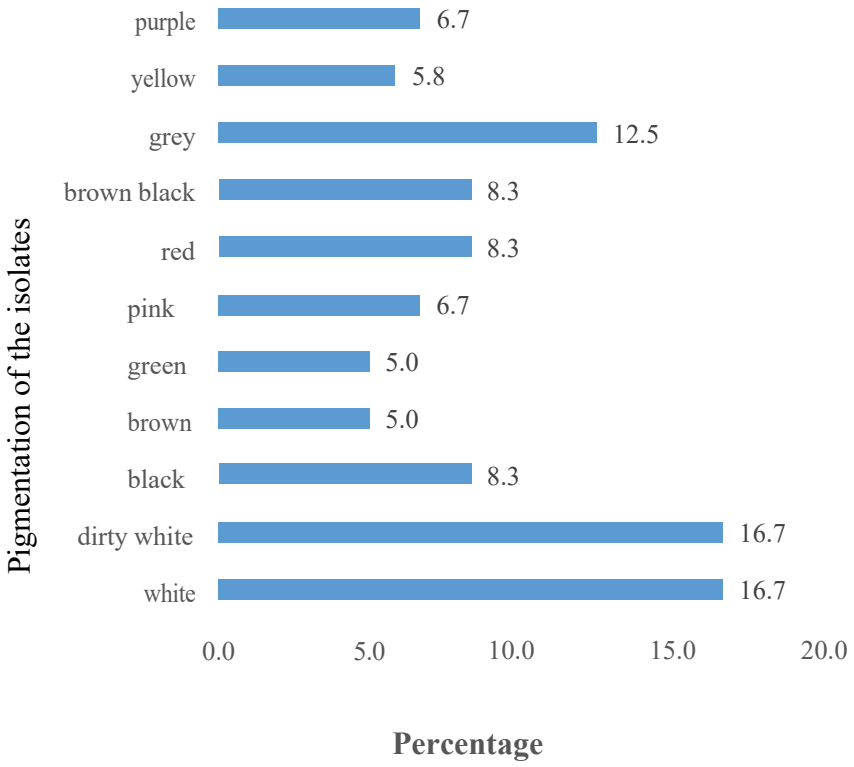


Figure 4.1: Types of Actinomycetes on the basis of pigmentation

4.1.3 Primary Screening of Actinomycetes

Among the isolated actinomycetes, 68(56.7%) strains were found to be inhibitory against only Gram positive, 42(35%) against only Gram negative, 6(5%) against both Gram positive and Gram-negative bacteria. Only 4(3.3%) strains were found to be active against tested fungi and all bacteria (Table 4.2). The antibacterial and antifungal activity of four screened strains during primary screening is shown in Photo plates 1 A_3 , 1 D_2 , 1 P_4 and 1 J_1.

Table 4.2: Activity of bioactive actinomycetes (N=120) against bacteria and fungi.

S.N.	Colony color	Active against			
		GP only {n (%)}	GN only {n (%)}	Both GP and GN {n (%)}	Fungi and Bacteria {n (%)}
1	White	12 (17.6)	4 (9.5)	2 (33.3)	2 (50.0)
2	Dirty White	8 (11.8)	8 (19.0)	3 (50.0)	1 (25.0)
3	Black	3 (4.4)	7 (16.7)	0 (0.0)	0 (0.0)
4	Brown	1 (1.5)	5 (11.9)	0 (0.0)	0 (0.0)
5	Green	4 (5.9)	2 (4.8)	0 (0.0)	0 (0.0)
6	Pink	6 (8.8)	2 (4.8)	0 (0.0)	0 (0.0)
7	Red	4 (5.9)	6 (14.3)	0 (0.0)	0 (0.0)
8	Brown Black	7 (10.3)	3 (7.1)	0 (0.0)	0 (0.0)
9	Grey	10 (14.7)	3 (7.1)	1 (16.7)	1 (25.0)
10	Yellow	7 (10.3)	0 (0.0)	0 (0.0)	0 (0.0)
11	Purple	6 (8.8)	2 (4.8)	0 (0.0)	0 (0.0)
	Total	**68 (56.7)**	**42 (35.0)**	**6 (5.0)**	**4 (3.3)**

GP: Gram positive bacteria; GN: Gram negative bacteria

4.1.4 Secondary Screening of Actinomycetes

Antimicrobial activity of bioactive compound extracted in ethyl acetate is shown in Table 4.3 and Photo plate 2. Out of 4 potent isolates A_3 showed highest antimicrobial activity against *Candida albicans* (41.33±1.15 mm), *Salmonella typhi* (24.33±2.08 mm), *Acinetobacter baumannii* (31.33±3.05 mm) and *Bacillus subtilis* (24.67±2.08 mm). Highest zone of inhibition against *E. coli* (26 ±0.00 mm) and MRSA (31.33±3.21 mm) was given by P_4. Strain.

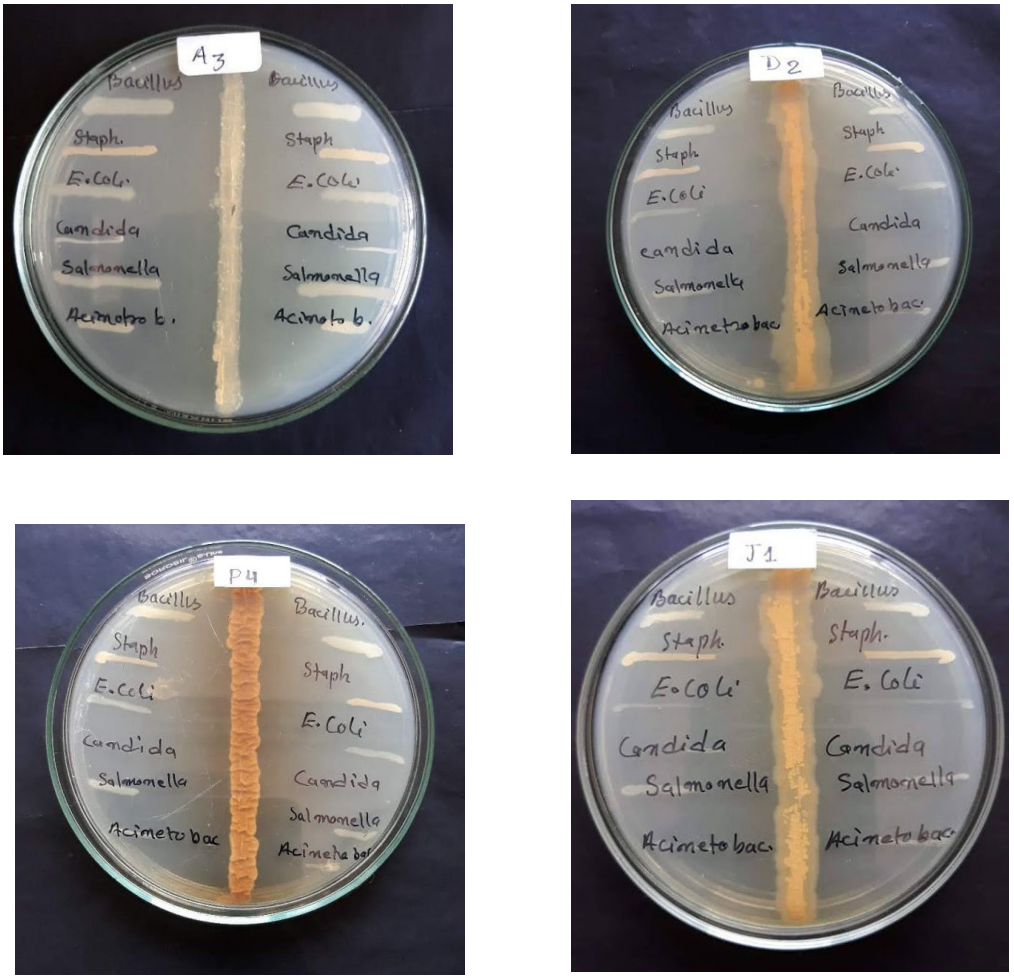


Photo plate 1: Primary screening of actinomycetes isolates (A_3, D_2, P_4 and J_1 against test microorganisms)

Table 4.3: Antimicrobial activity of ethyl acetate extract against test microorganisms

Strain	Zone of inhibition (Mean±S.D.) in mm					
	Bacillus subtilis	**MRSA**	***E. coli* ATCC 25922**	***Acinetobacter baumanii***	***Salmonella typhi***	***Candida albicans***
A_3	24.67±2.08	30.67±7.02	23.33±4.93	31.33±3.05	24.33±2.08	41.33±1.15
D_2	17.33±1.15	19.33±1.52	15.00±1.00	18.33±1.15	14.00±1.00	24.67±1.52
P_4	24.33±0.57	31.33±3.21	26.00±0.00	28.00±2.00	19.00±1.00	29.33±0.57
J_1	14.33±2.08	23.33±2.08	24.67±0.57	21.67±1.52	17.33±0.57	28.67±1.52

4.1.5 Minimum Inhibitory Concentrations (MIC) of Bioactive Metabolites

In MIC evaluation bioactive compound produced by A_3 strain showed lowest values against all test organisms 0.125mg/ml for *C. albicans* and *E. coli*, 2.5mg/ml for *S. typhi*, *A. baumannii* and MRSA. For *Bacillus subtilis* extract of all isolates gave same value 0.625mg/ ml. MIC values of bioactive compounds was found in a range 0.125- 5 mg /ml (Table 4.4).

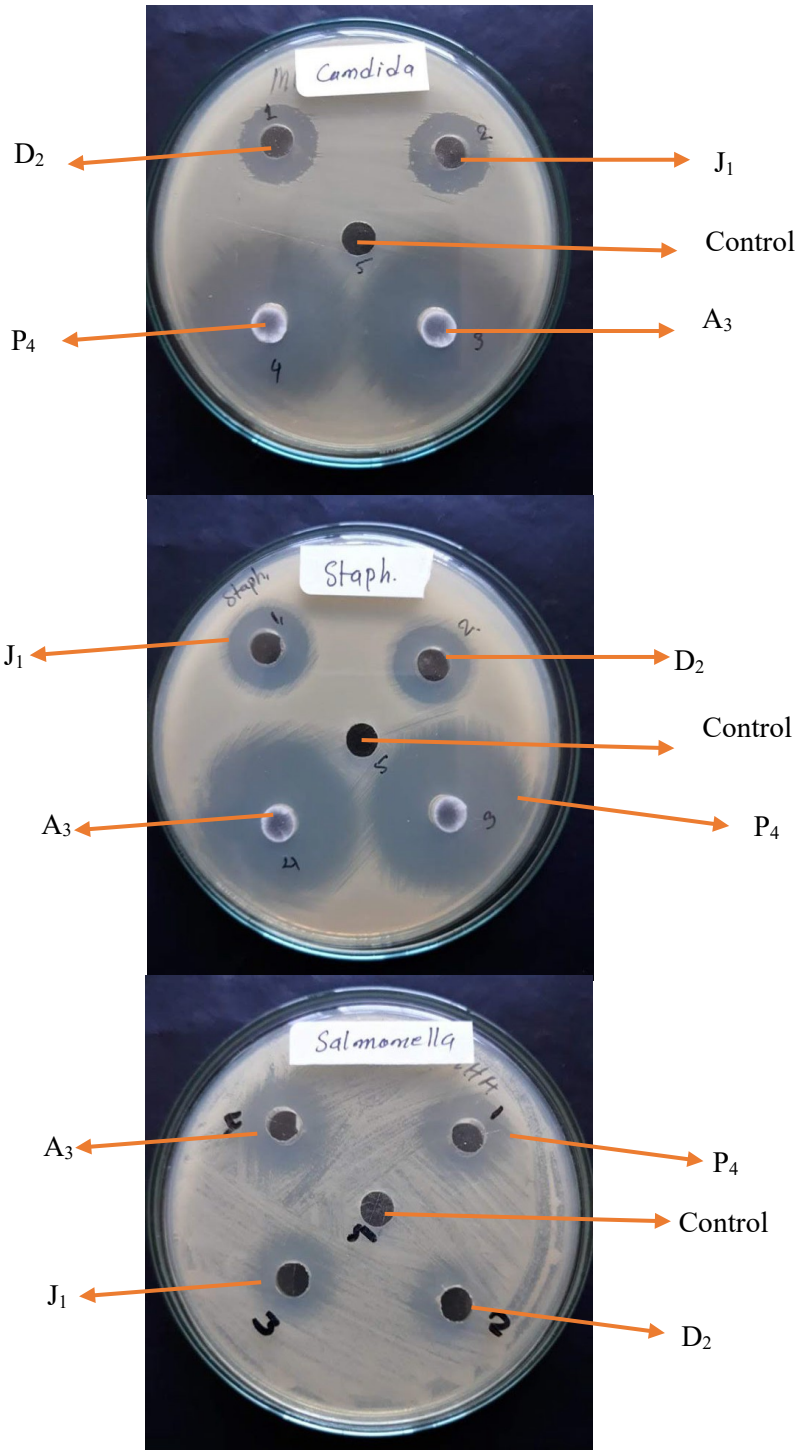
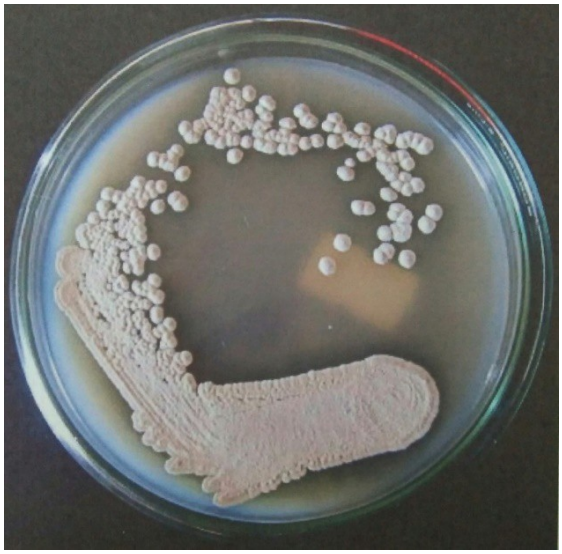


Photo plate 2: Antimicrobial activity of ethyl acetate extract against test microorganisms (Top to bottom: *C. albicans, S. aureus, S. typhi*)



A_3

D_2

P_4

J_1

Photo plate 3: Pure culture of actinomycetes on starch-casein agar (SCA)

Table 4.4: MIC Values of bioactive compounds against test microbes

Strains	MIC against(mg/ml)					
	Bacillus subtilis	**MRSA**	***E. coli*** **ATCC 25922**	***Acinetobacter baumannii*** **(MDR)**	***Salmonella typhi*** **(MDR)**	***Candida albicans***
A_3	0.625	2.5	0.125	2.5	2.5	0.125
D_2	0.625	5	2.5	5	5	2.5
P_4	0.625	5	2.5	2.5	2.5	2.5
J_1	0.625	5	5	5	2.5	2.5

4.1.6 Characterization of Screened Isolates

4.1.6.1 Cultural (Colonial) Characteristics

The screened isolates produced substrate mycelium with different colors. Out of four isolates, two produced yellowish and rest two produced light gray colored substrate mycelium. Isolates A_3 and P_4 produced white-floccose and creamy-floccose colony respectively. D_2 and J_1 produced powdery colony with dirty white and whitish-grey color respectively. Isolates A_3, D_2 and J_1 produced irregular, umbonate colonies. Strain P_4 had opaque, butyrous, umbonate colony with regular margin. Size of the colony varied from 2- 6mm. (Table 4.4 and Photo plate 3)

Table 4.5: Colonial characteristics of screened isolates on SCA

S. N.	Isolate code	Macroscopic Characteristics		
		Color of Substrate Mycelium	Color and Texture of aerial mycelium	Other Colony Characteristics
1.	A_3	Dark Yellow	White, Floccose	Irregular, some are punctiform, erose, concentric, opaque, butyrous, umbonate, 6mm sized colony.
2.	D_2	Light Gray	Dirty white, Powdery	Irregular, Some are punctiform, erose, concentric, opaque, dry, umbonate, 2mm sized colony.
3.	P_4	Yellow	Creamy, Floccose	Regular margin, erose, concentric, opaque, butyrous, umbonate, 2mm sized colony.

4.1.6.2 Microscopic Characteristics

Microscopic characterization was performed by cover-slip method. The cellular morphology revealed that all the isolates had recti flexible sporophore except D_2 that had short retinaculum sporophore. Mycelium of all the strains were not fragmented except A_3 with all having long chain of spores except D_2 that had spiral form of spores. (Table 4.6)

Table 4.6: Microscopic characteristics of screened isolates

S.N.	Isolate Code	Microscopic Characteristics
1.	A_3	Recti flexible sporophore, mycelium fragmented with branching, long chain of spores.
2.	D_2	Short retinaculum sporophore, mycelium not fragmented, spiral form of spore chains.
3.	P_4	Recti flexible sporophore, mycelium not fragmented with branching, chain of spores.
4.	J_1	Recti flexible sporophore, mycelium not fragmented with branching, long chain of spores.

Microscopy of screened isolates is shown in Photo plate 4.

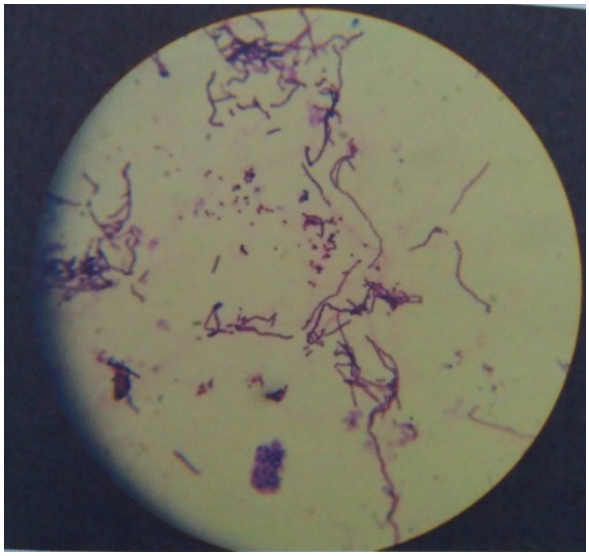
4.1.6.3 Carbohydrate Utilization Test

Glucose, Mannitol, salicin and sucrose were utilized by all strains but none of the strain utilized inulin. Galactose was utilized only by A_3, xylose by D_2 and P_4 only. Strains D_2 and J_1 were unable to utilize maltose which is presented in Table 4.7.

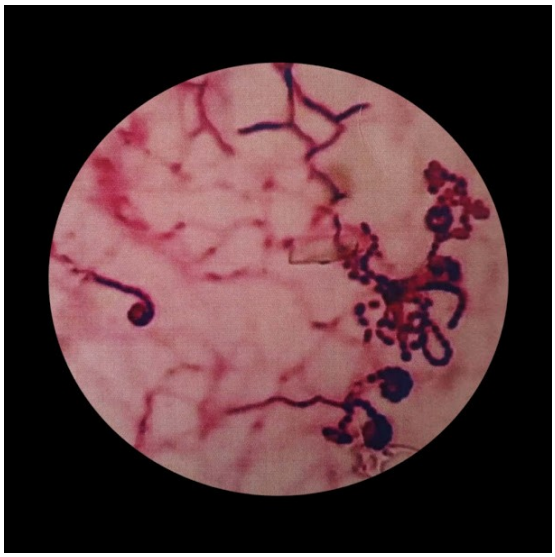
Table 4.7: Carbohydrate utilization test of potent isolates

Strains	Carbohydrate utilization tests									
	Fru	Gal	Glu	Inu	Mal	Man	Raf	Sal	Suc	Xyl
A_3	+	+	+	-	+	+	-	+	+	-
D_2	+	-	+	-	-	+	+	+	+	+
P_4	+	-	+	-	+	+	-	+	+	+
J_1	+	-	+	-	-	+	-	+	+	-

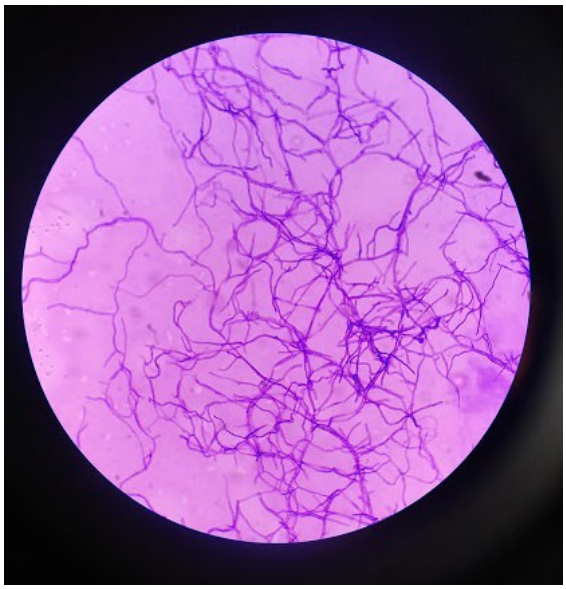
Fru: Fructose; Gal: Galactose; Glu: Glucose; Inu: Inulin; Mal: Maltose; Man: Mannose Raf: Raffinose; Sal: Salicin; Suc: Sucrose; Xyl: Xylose.



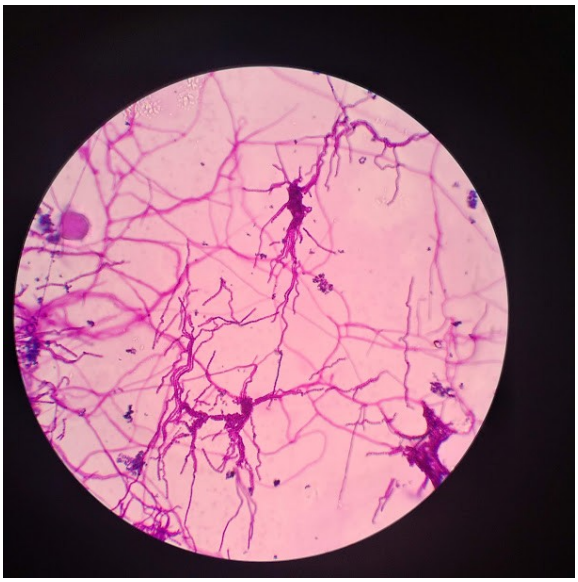
A_3



D_2



P_4



J_1

Photo plate 4: Microscopy of actinomycetes isolates.

4.1.6.4 Substrate hydrolysis test of potent isolates

All the isolates hydrolyzed gelatin starch and urea except D_2 that was unable to hydrolyze urea as shown in Table 4.8.

Table 4.8: Substrate hydrolysis test of screened isolates

Strains	Hydrolysis of		
	Gelatin	Starch	Urea
A_3	+	+	+
D_2	+	+	-
P_4	+	+	+
J_1	+	+	+

4.1.6.5 Morphological and biochemical tests

A four strains were Gram positive and catalase positive. All the isolates were H_2S production positive, Oxidase positive, Indole negative and non-motile. Citrate utilization and nitrate reduction was shown positive only by A_3 and P_4 which is given in Table 4.9.

Table 4.9: Morphological and other biochemical tests

Strains	Tests							
	Gram straining	Catalase	Oxidase	Citrate Utilization	H_2S	Nitrate reduction	Indole	Motility
A_3	+	+	-	+	-	+	-	-
D_2	+	+	-	-	-	-	-	-
P_4	+	+	-	+	-	+	-	-
J_1	+	+	-	-	-	-	-	-

4.1.6.6 Temperature and Salt Tolerance Test of Screened Isolates

All four potent isolates were able to grow in salt concentrations at 3, 5 and 7 %. Similarly, all strains were able to grow at 15, 37 and 45°C except A_3 which was unable to grow at 45°C as shown in table 4.10. On the basis of morphological, biochemical, substrate hydrolysis, temperature tolerance and NaCl tolerance, strain A_3 was probably identified as *Nocardiopsis prasina* whereas D_2, P_4 and J_1 were *Streptomyces violarus, S. krainskii* and *S. tsusimaensis respectively*.

Table 4.10: Temperature tolerance and salt tolerance test

Strains	Physiological tests					
	Temperature tolerance			NaCl tolerance		
	15˚C	37˚C	45˚C	3 %	5 %	7 %
A_3	+	+	-	+	+	+
D_2	+	+	+	+	+	+
P_4	+	+	+	+	+	+
J_1	+	+	+	+	+	+

4.1.7 Genotypic Characterization

The results of 16SrRNA obtained from Macrogen, Korea were compared with nucleic acid sequence of other actinomycetes retrieved from the NCBI gene bank database and phylogenetic position of the strains was determined by neighbor-joining method. On the basis of 99% similarity shown the most potent strain A_3 was confirmed as *Nocardiopsis prasina*.as shown in figure 4.2.

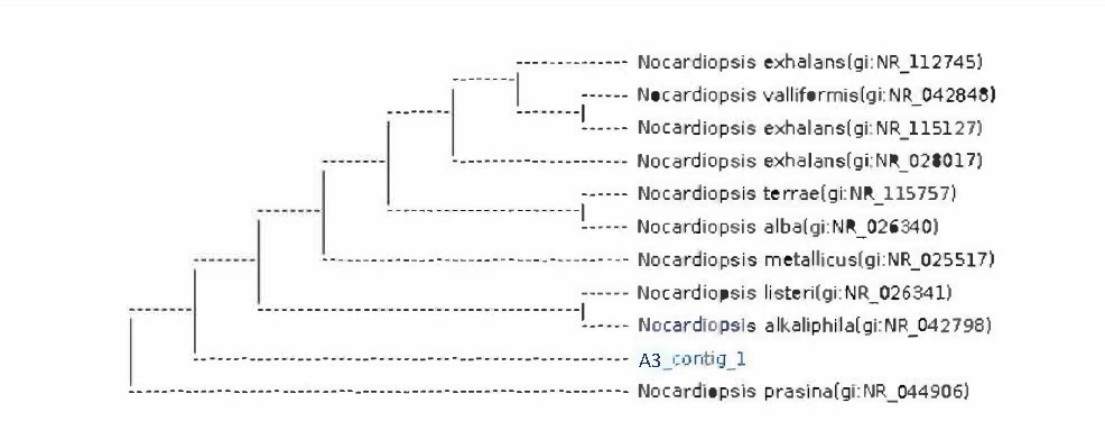


Figure 4.2: Phylogenetic tree of A_3 (*N. prasina*)

4.1.8 Optimization of Fermentation Process

4.1.8.1 Effect of Carbon Sources on Biomass and bioactive metabolite Production

Biomass production (mg/100 ml) of strain A_3 was high is all sugars except in Xylose and highest in glucose (311.67±10.48) but for D_2 strain, it was high presence of Mannitol (247.33±3.055) whereas for P_4 strain, biomass production was maximum in maltose (246.67±2.887). In case of J_1, maximum biomass production was in sucrose (203.33±5.774) as shown in Figure 4.2. All the strains showed maximum antimicrobial activity (mm) against test organisms when starch was used as carbon source. Maximum antimicrobial activity was shown by A_3 strain against all organism, 39.33±0.57 mm against *C. albicans* which is depicted in Figure 4.3. Statistically significant association between carbon source with biomass and metabolite production was found (p value= 0.000).

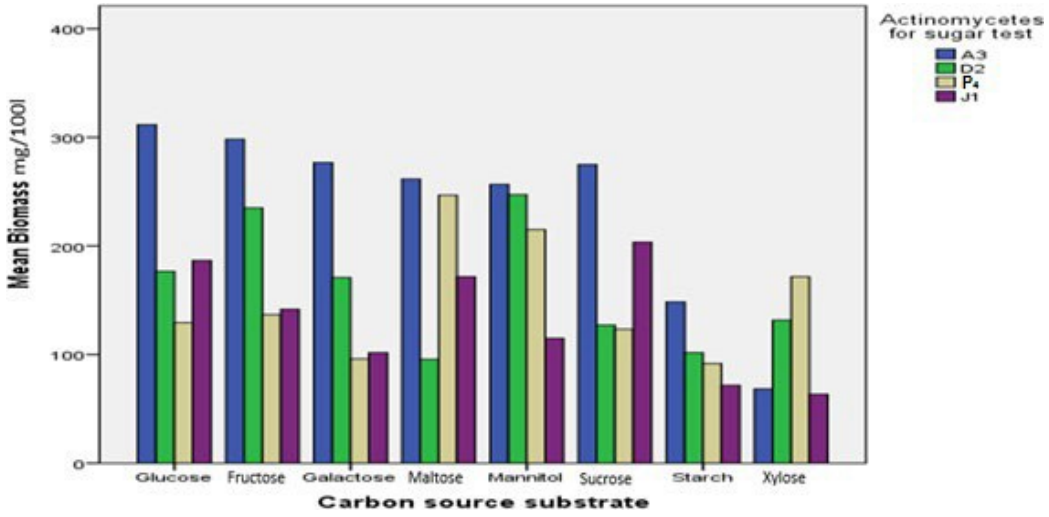


Figure 4.3: Mean biomass production of different actinomycetes based on carbon source (p value=0.000)

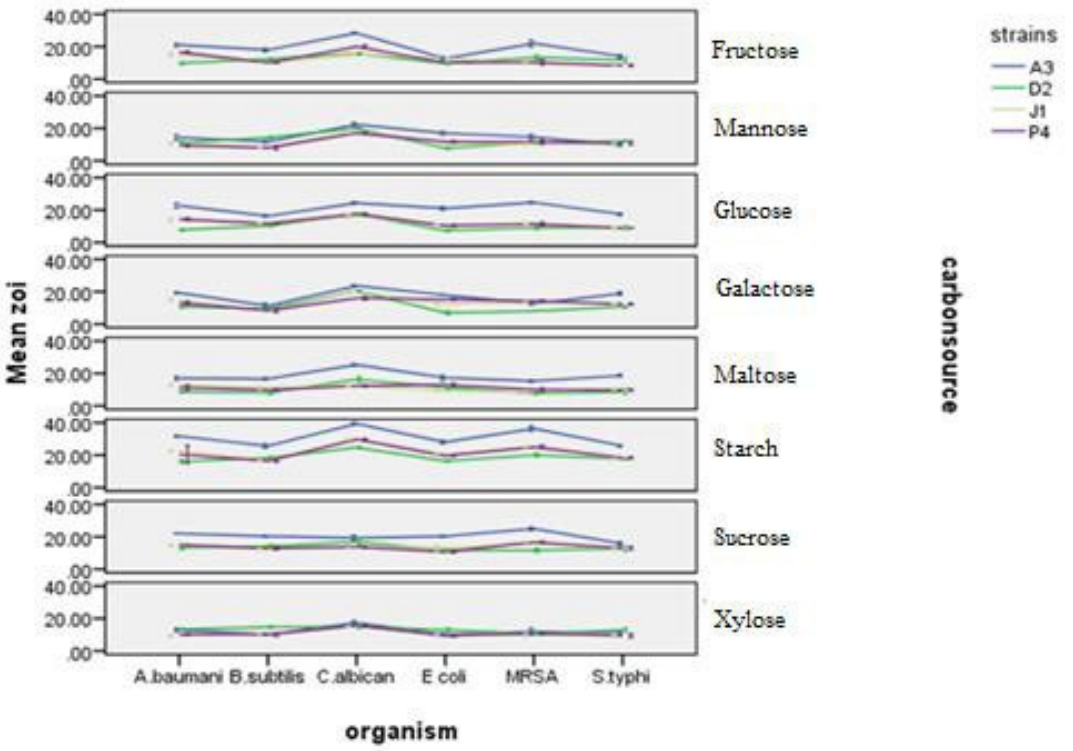


Figure 4.4: Effect of carbon source on antimicrobial activity/ bioactive metabolite production (p=0.000)

4.1.8.2 Effect of Nitrogen Sources on Biomass and Bioactive Metabolite Production

All the strains, except P_4 produced maximum biomass (mg/100ml) when casein was used as nitrogen source whereas for P_4 strain tryptone was found an appropriate nitrogen source. Highest biomass was produced by A_3 strain (298.333±7.637 mg/ 100 ml) and lowest biomass was produced by P_4 strain (105.00±8.66). Similarly, bioactive metabolite production by all strains except D_2 was found to be maximum when Casein was used as Nitrogen source. Maximum antimicrobial activity was exhibited by strain A_3 against *C. albicans* (34.667±0.577 mm) as shown in Figure 4.4 and 4.5. There was significant association between nitrogen sources with biomass and metabolite production (p value= 0.000).

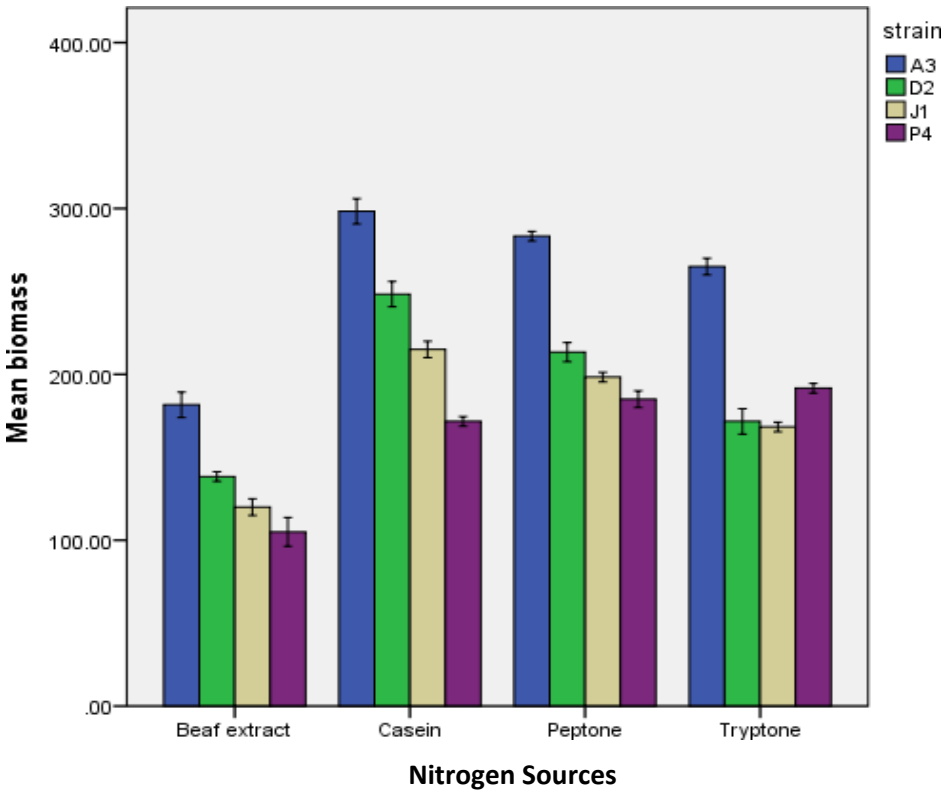


Figure 4.5: Effect of nitrogen source on biomass production (p=0.000)

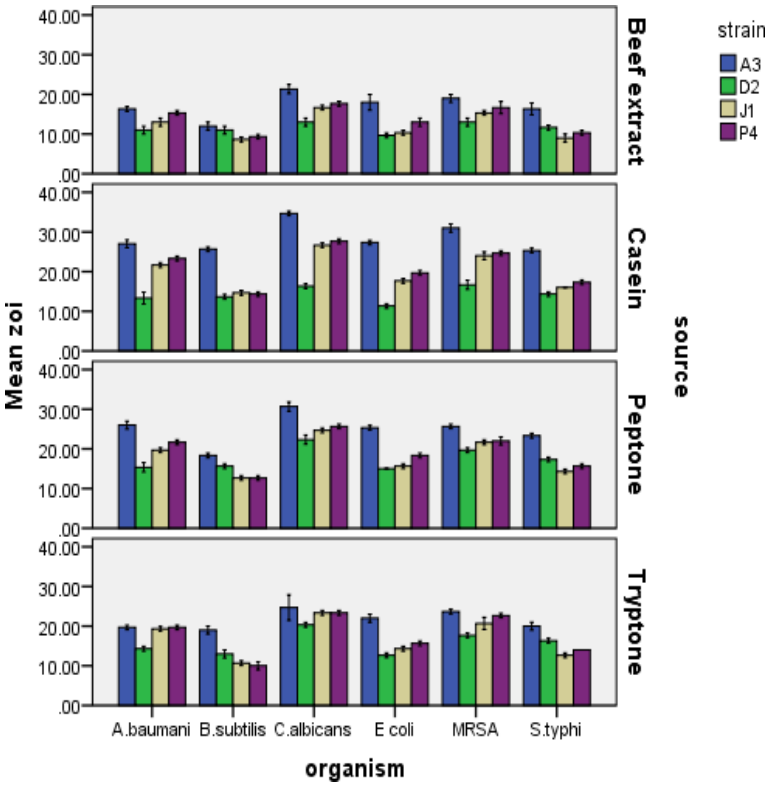


Figure 4.6: Effect of nitrogen source on bioactive metabolite production (p=0.000)

4.1.8.3 Effect of Temperature on Biomass and Bioactive Metabolite Production

As shown in Figure 4.6, biomass production (mg/100 ml) of strains A_3, D_2, and P_4 were found to be maximum at 30ºC but for strain J_1, 35ºC was found to be optimum. Maximum biomass was produced by A_3 (301.67±2.88) at 30ºC and minimum biomass was produced by D_2 (78.33±2.88) at 20ºC. Similarly, Figure 4.7 illustrates that all strains showed maximum antimicrobial activity at 30ºC and maximum inhibitory activitywas observed in case of strain A_3 which showed highest zone of inhibition against *C. albicans* (37.00±1.00 mm). There was a significant association between temperature with biomass and metabolite production (p value < 0.05).

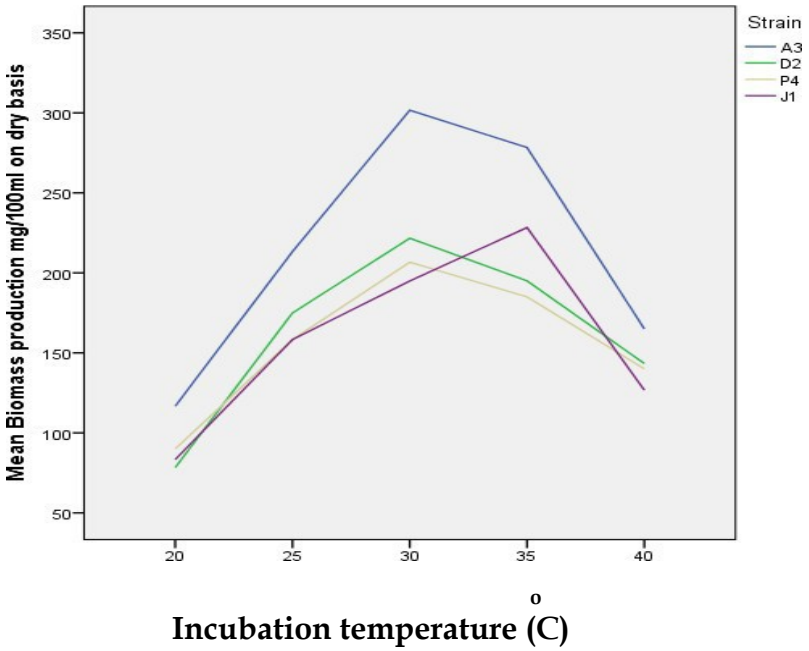


Figure 4.7: Effect of Temperature on Biomass Production (p=0.014)

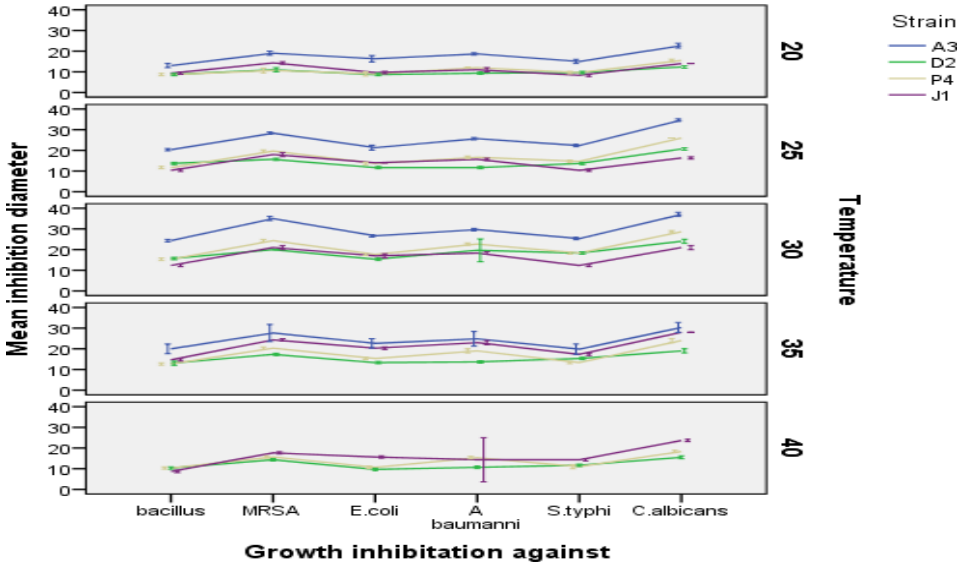


Figure 4.8: Effect of incubation temperature on bioactive metabolite production (p=0.000)

4.1.8.4 Effect of pH on Biomass and Bioactive Metabolite Production

As shown in Figure 4.8 and 4.9, strains A_3, D_2 and J_1 produced maximum biomass (mg/100ml) at pH 8 whereas in case of strain P_4, it was maximum at pH 7. Maximum biomass production was observed in A_3 strain at pH 8(318.33±2.88) and minimum biomass was found for J_1 at pH 4(58.33±7.63). Antimicrobial activity of strains A_3, D_2 and J_1 were found to be maximum against all test organisms at pH 8 whereas strain P_4 showed maximum activity at pH 7. Maximum zone of inhibition was displayed by strain A_3 against *C. albicans* (38.67±0.57mm) at pH 8. Minimum activity of all strains was observed at pH 4. The association between pH and biomass production was not significant (p value=0.074). However, significant association between pH and metabolite production was observed. (p value=0.000).

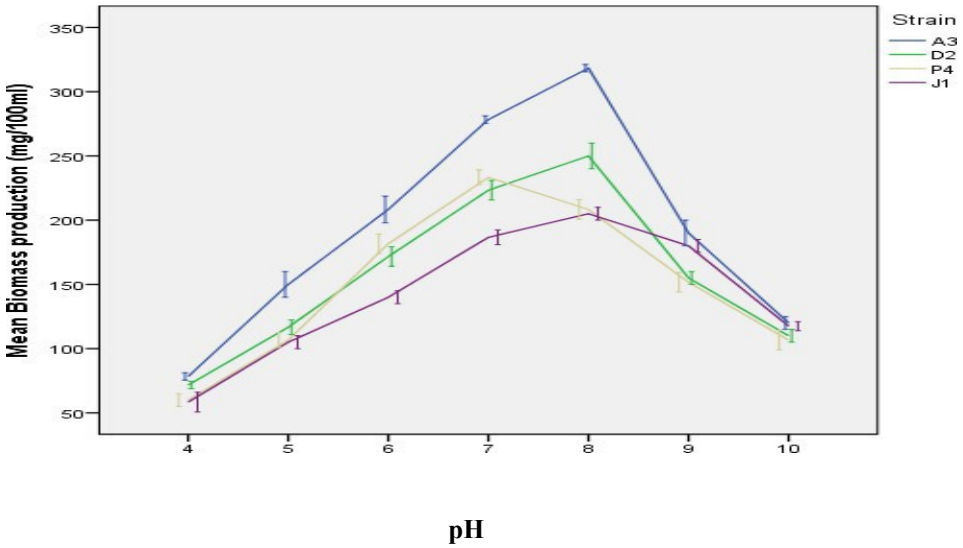


Figure 4.9: Effect of pH on Biomass Production (p=0.074)

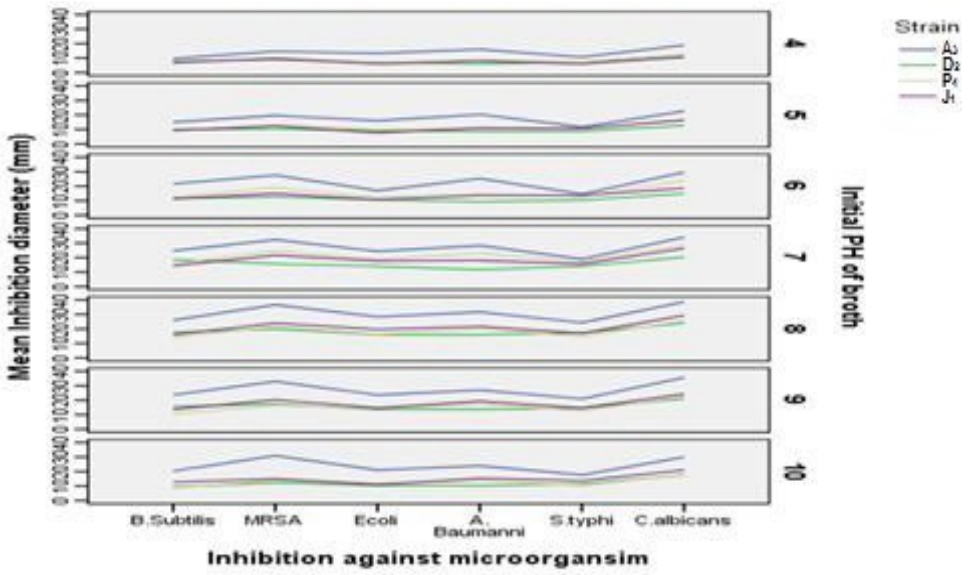


Figure 4.10: Effect of pH on bioactive metabolites production (p=0.000)

4.1.8.5 Effect of Incubation Period on Bioactive Metabolite Production

All the strains showed maximum antimicrobial activity on 8th day of incubation except P_4 which showed maximum antimicrobial activity on the 6th day. None of the strains produced bioactive metabolites on 2nd day. Strain A_3 exhibited highest antimicrobial activity against all test organisms than others. It showed maximum zone of inhibition (38.67±0.57mm) against *C. albicans* as presented in Figure 4.10. The metabolite production by actinomycetes varied significantly with incubation duration (p value=0.000).

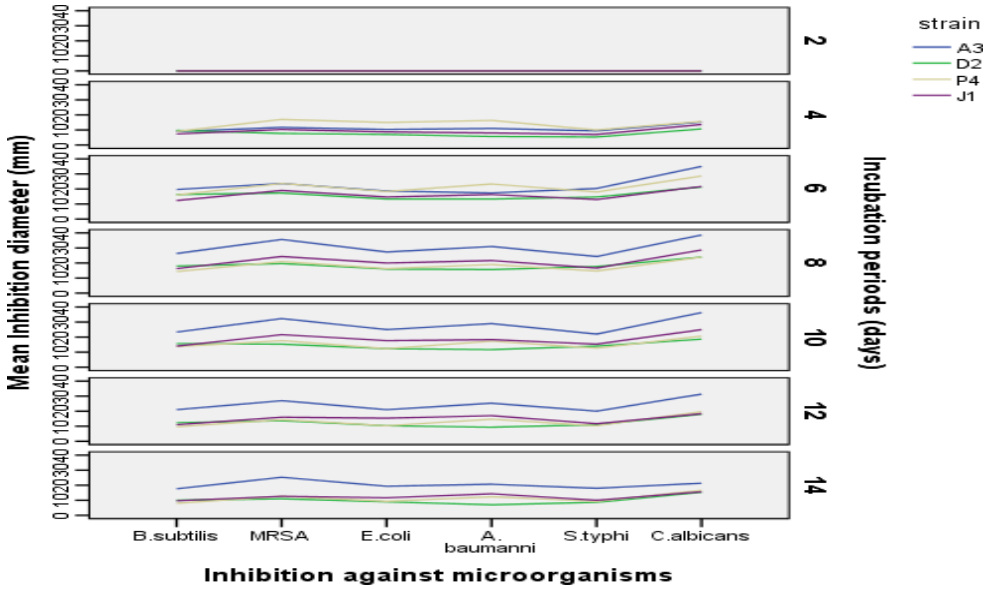


Figure 4.11: Effect of Incubation Period on Bioactive metabolite production (p=0.000)

4.1.8.6 Effect of Minerals on Biomass and Bioactive Metabolite Production

Figure 4.11 shows that all the strains produced maximum biomass (mg/100ml) in presence of $MgCl_2$ except strain D_2 for which KNO_3 was found to be most appropriate. Biomass production of A_3 strain was maximum than other strains in all minerals. Maximum biomass production of A_3 strain was found in $MgCl_2$ (315.00±5.00) and minimum biomass was produced by J_1 strain in $FeCl_3$ (76.67±5.77). Similarly, as depicted in Figure 4.12, strains A_3, P_4 and J_1 showed maximum antimicrobial activity in presence of KNO_3 in fermentation broth whereas D_2 showed highest antimicrobial activity against majority of test organisms in presence of $MgCl_2$. Maximum antimicrobial activity was shown by A_3 against *C. albicans* in presence of KNO_3 (35.33±1.15mm) and minimum antimicrobial activity was shown by J_1 against *B. subtilis* in presence of $CuSO_4$ (6.67±0.57mm). Highly significant association between minerals with biomass and metabolite production was observed (p value=0.000).

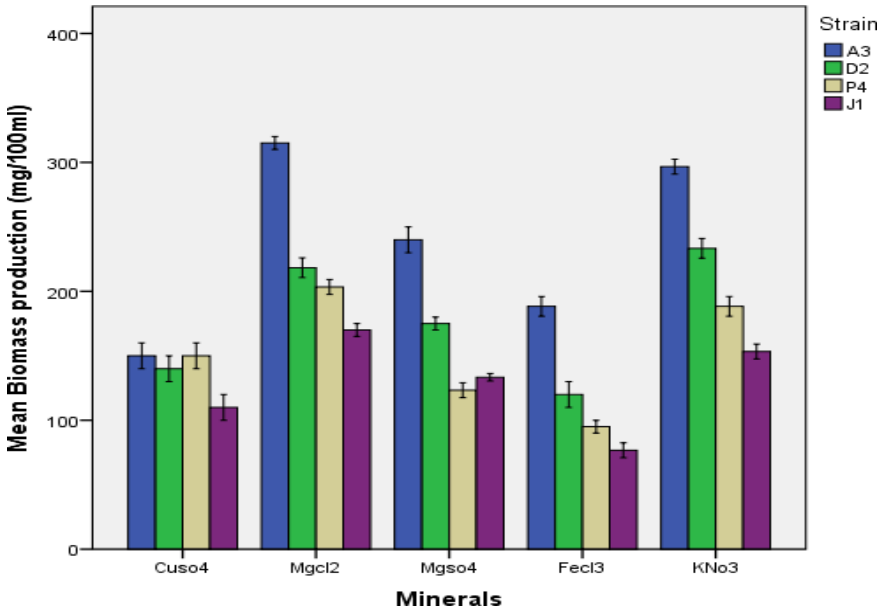


Figure 4.12: Effect of Minerals on Biomass Production (p=0.000)

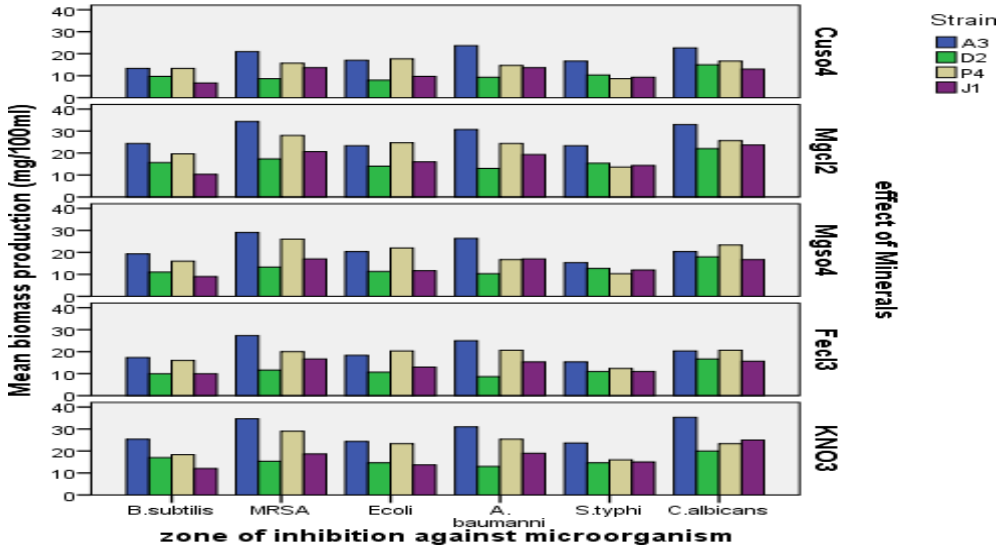


Figure 4.13: Effect of minerals on bioactive metabolites production (p=0.000)

4.1.9 Characterization of Ethyl Acetate Extract

4.1.9.1 FT IR analysis of the extract

The FT IR spectra of the compounds produced by all strains showed more or less similar pattern of functional groups. Ethyl acetate extract of compounds produced by A_3 strain

displayed different peaks at 3500, 2981, 1644, 1510 and 1030cm^{-1}. These spectra indicated OH, CH, C=C, Aromatic and C-O group respectively. Similarly, the compounds produced by D_2 strain showed different peaks at 3500, 2970, 1697, 1550 and 1042 cm^{-1} corresponding to OH, CH, C=O, Aromatic and C-O groups respectively. In case of compounds produced by P_4, the spectra exhibited bands at 3491, 2971, 1720, 1511 and 1048cm^{-1} that suggested the presence of OH, CH, C=O, Aromatic and C-O groups accordingly. The IR spectrum of compounds produced by J_1 showed various peaks at 3259, 2980, 1644, 1519 and 1064cm^{-1} demonstrated the functional groups OH, CH, C=C, Aromatic and C-O respectively as shown in Table 4.11. IR spectra of ethyl acetate extracts of different actinomycetes are shown in Figure 4.14, 4.15, 4.16 and 4.17.

Table 4.11: Results of FT IR Analysis

Strains	Band Absorbed and Functional Groups of Ethyl Acetate Extracts				
A_3	3500cm^{-1} ν(OH)	2981cm^{-1} ν(CH)	1644cm^{-1} ν(C=C)	1510cm^{-1} ν(Aromatic)	1030cm^{-1} ν(C-O)
D_2	3500cm^{-1} ν(OH)	2970cm^{-1} ν(CH)	1697cm^{-1} ν(C=O)	1550cm^{-1} ν(Aromatic)	1042cm^{-1} ν(C-O)
P_4	3491cm^{-1} ν(OH)	2971cm^{-1} ν(CH)	1720cm^{-1} ν(C=O)	1511cm^{-1} ν(Aromatic)	1048cm^{-1} ν(C-O)
J_1	3259cm^{-1} ν(OH)	2980cm^{-1} ν(CH)	1644cm^{-1} ν(C=C)	1519cm^{-1} ν(Aromatic)	1064cm^{-1} ν(C-O)

4.1.9.2 Results of LC-MS analysis

4.1.9.2.1 LC-MS profiling of ethyl acetate extracts of *N. prasina* (A_3)

Ethyl acetate extract of *N. prasina* revealed four major compounds eluted at 4.195, 20.254, 21.868 and 24.171 minutes (Table 4.12 and chromatogram). The molecular ion mass of prominent compounds eluted at a retention time 4.195 min. are of m/z 322, 160, 450, 478 and 672, 929 and 964. But only one major molecule having m/z 531 was

eluted at retention time 20.254 minutes. Similar pattern was observed in the retention time 21.868 min. and 24.171 min. at which only one major molecule having m/z 274 and 412 were observed respectively.

Table 4.12: LC-MS profiling of ethyl acetate extracts of *N. prasina* (A_3)

Retention Time(minutes)	Mass(m/z) of Ethyl Acetate Extracts of A_3
4.193-4.196	142, 160, 205, 322(base peak), 450, 478, 672, 949, 964
20.253-20.256	159, 224, 245, 489, 531(base peak)
21.867-21.869	106, 230, 274(base peak), 318, 362, 556, 559
24.171-24.173	320, 412(base peak)

4.1.9.2.2 LC-MS profiling of ethyl acetate extracts of *S. violarus* (D_2)

Six major compounds detected in ethyl acetate extract of *S. violarus* eluted atretention time 4.137, 18.780, 21.842, 23.263, 24.230 and 27.684 minutes. At retention time 4.137 it has displayed molecular ion mass(m/z)163, 293, 455, 615 and 673 with retention time 18.780 min. the molecules of m/z 453, 602, 679 and 702 appeared as major compounds. Seven different compounds of m/z 230, 274, 318, 362, 511, 556 and 599 were eluted at retention time 21.842 minutes. In the retention time 23.263 minutes various compounds eluted having molecule ion mass m/z were 115, 172, 219, 259, 302, 346, 401, 422, 489, 549, 577, 641, 673, 706, 736, 814, 849, 870, 913 and 977. Only one prominent compounds of m/z 412 was eluted at retention time 24.230 min. and two compounds of m/z 705 and 769 were eluted at retention time 27.684. (Table 4.13 and chromatogram).

Table 4.13: LC- MS profiling of ethyl acetate extracts of *S. violarus* (D_2)

Retention Time(minutes)	Mass(m/z) of Ethyl Acetate Extracts of D_2
4.136-4.139	163, 239, 293, 455, 615(base peak), 673, 806, 844, 943, 981
18.779-18.781	287, 387, 453, 508, 569, 602, 679(base peak), 702, 771, 805, 863, 903, 931, 969
21.840-21.843	106, 230, 274(base peak), 318, 362, 490, 511, 556, 599, 695, 810, 907, 932, 986
23.261-23.264	115, 172, 219, 259, 302(base peak), 346, 401, 422, 489, 549, 562, 577, 641, 673, 706, 736, 814, 849, 870, 931, 977
24.229-24.232	180, 320, 354, 412(base peak), 480, 535, 575, 632, 696, 761, 817, 848
27.683-27.685	174, 294, 412, 533, 597, 638, 705(base peak), 769, 839, 904, 964

4.1.9.2.3 LC-MS profiling of ethyl acetate extracts of *S. krainskii* (P_4)

The LCMS chromatogram of ethyl acetate extract of *S. krainskii* exhibited four major compounds eluted at different retention time 17.693, 20.293, 21.875 and 23.192 minutes. At retention time17.693-minute compounds of m/z 116,158,434,591 and 750 were eluted. Similarly, compounds of m/z 224, 489 and 531 were eluted at retention time 20.293 minute. Other compounds eluted at retention time 21.875 minutes exhibited m/z 230, 274, 318, 555 and 750. Various compounds of m/z eluted at retention time 23.192 minute were 258, 302, 346, 612 and 751. (Table 4.14 and chromatogram)

Table 4.14: LC-MS profiling of ethyl acetate extracts of *S. krainskii* (P_4)

Retention Time(minutes)	Mass(m/z) of Ethyl Acetate Extracts of P_4
17.69-17.695	116, 158, 434, 591, 750(base peak)
20.292-20.295	159, 224, 489, 531(base peak)
21.873-21.876	230, 274(base peak), 555, 750
23.191-23.193	258, 302(base peak), 612, 751

4.1.9.2.4 LC-MS profiling of ethyl acetate extracts of *S. tsusimaensis* (J_1)

Crude fermented product in ethyl acetate extract of *S. tsusimaensis* exhibited five major compounds eluted at different retention time 16.789, 18.892, 20.697, 21.825 and 24.193 minutes. At retention time 16.789 min. the prominent compound of m/z were 184, 202, 241, 302, 483, 544, 594, 603 and 763. The compounds of m/z 680 and 701 were eluted at 18.892 minute. With an increase in retention time 20.697min. different compounds eluted had m/z 133,165,206,275 and 433.Various major compounds eluted at retention time 21.825 min. displayed m/z 230, 274, 318, 362, 512, 556 and 600. In the retention time 24.193 min. two compounds having m/ z 320 and 412 were eluted. (Table 4.15 and chromatogram).

Table 4.15: LC-MS profiling of ethyl acetate extracts of *S. tsusimaensis* (J_1)

Retention Time(minutes)	Mass(m/z) of Ethyl Acetate Extracts of J_1
16.787-16.789	184, 202, 241, 302, 361, 406, 433, 483, 594, 603(base peak), 662, 763, 862
18.891-18.893	213, 390, 596, 680(base peak), 701, 771, 821, 846, 907, 950, 966
20.696-20.699	133, 165(base peak), 206, 275, 288, 363, 385, 433, 500, 574, 616, 663, 730, 760, 798, 830, 904, 946, 983
21.824-21.827	230, 274(base peak), 318, 362, 512, 556, 600, 664, 710, 859, 883, 952, 983
24.192-24.195	225, 275, 320, 412(base peak), 492, 602, 710, 804, 937, 971

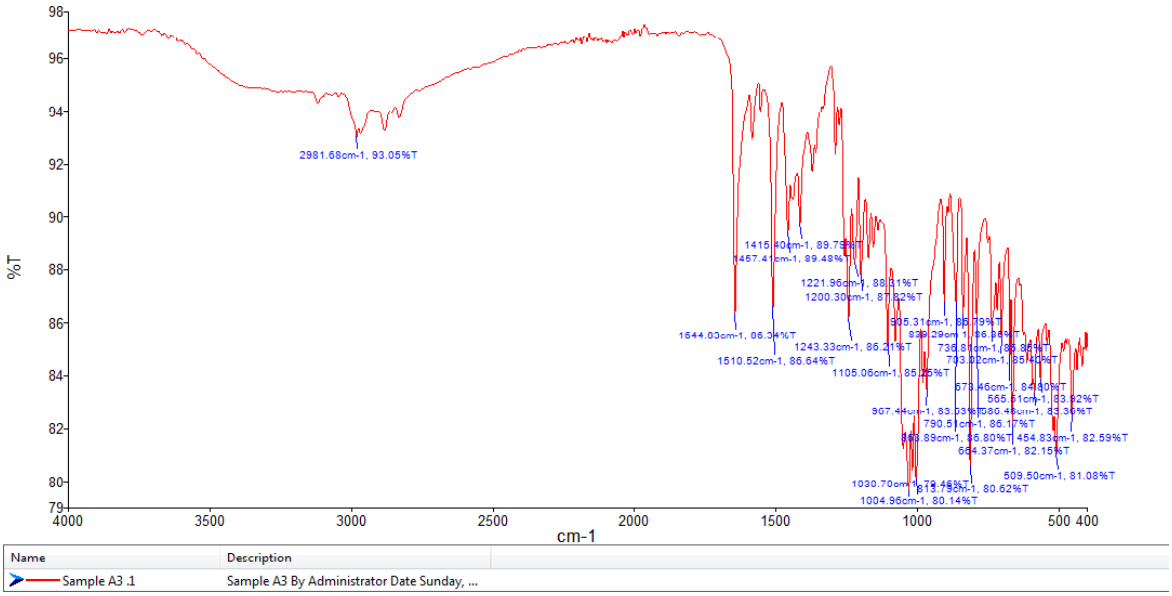


Figure 4.14: IR spectra of ethyl acetate extract of A_3 (*N. prasina*)

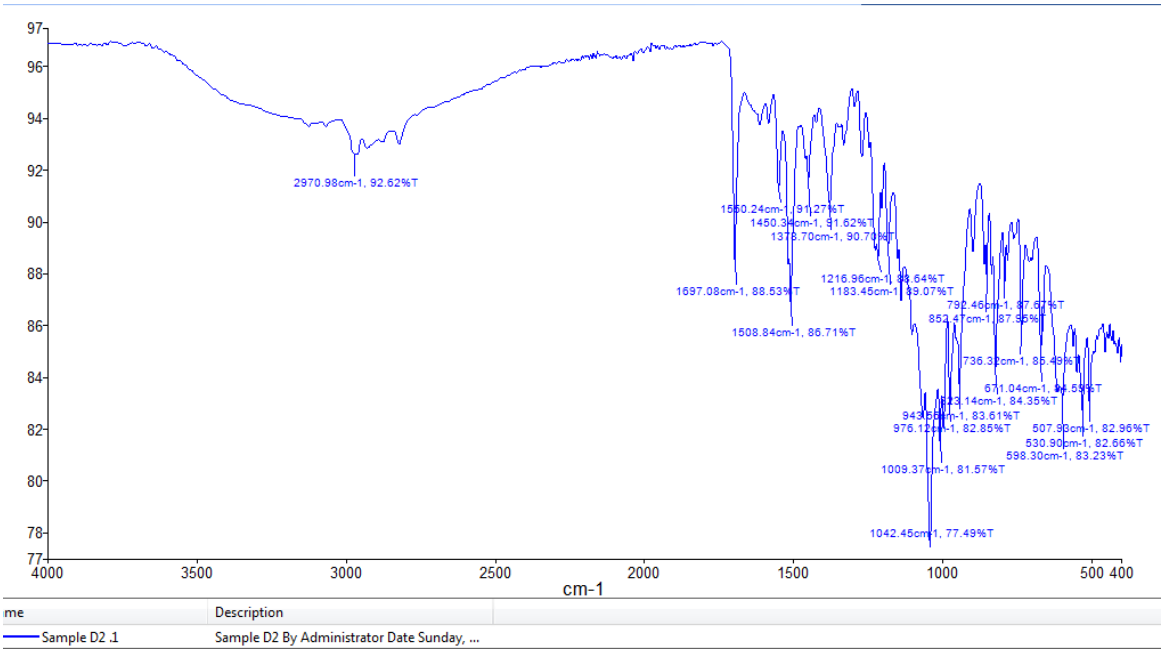


Figure 4.15: IR spectra of ethyl acetate extract of D_2 (*S. violarus*)

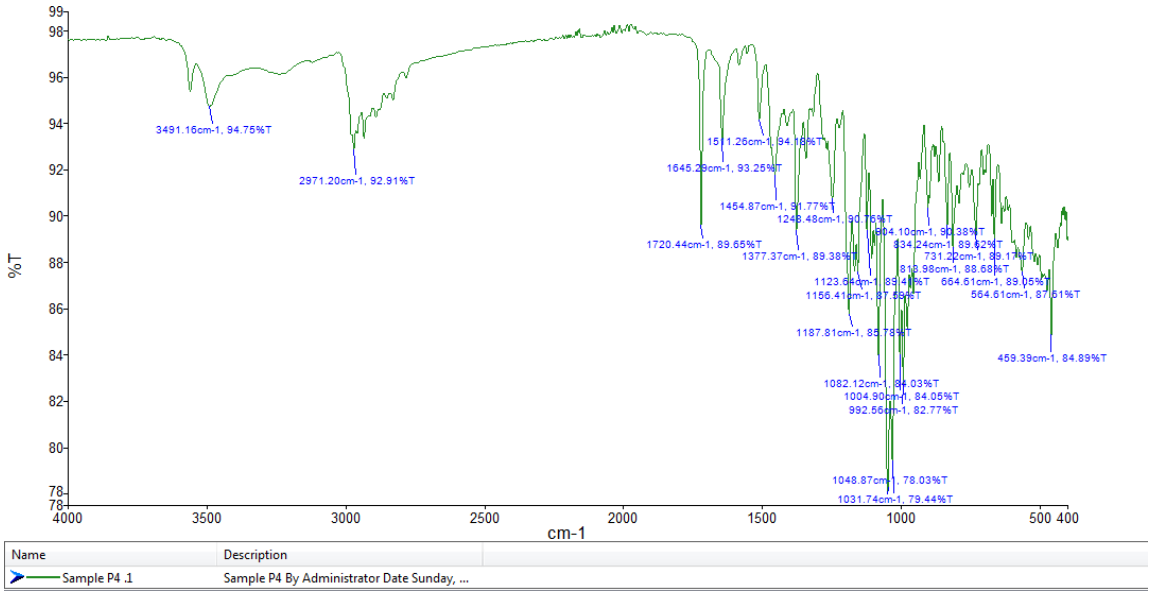


Figure 4.16: IR spectra of ethyl acetate extract of P_4 (*S. krainskii*)

Figure 4.17: IR spectra of ethyl acetate extract of J_1 (*S. tsusimaensis*)

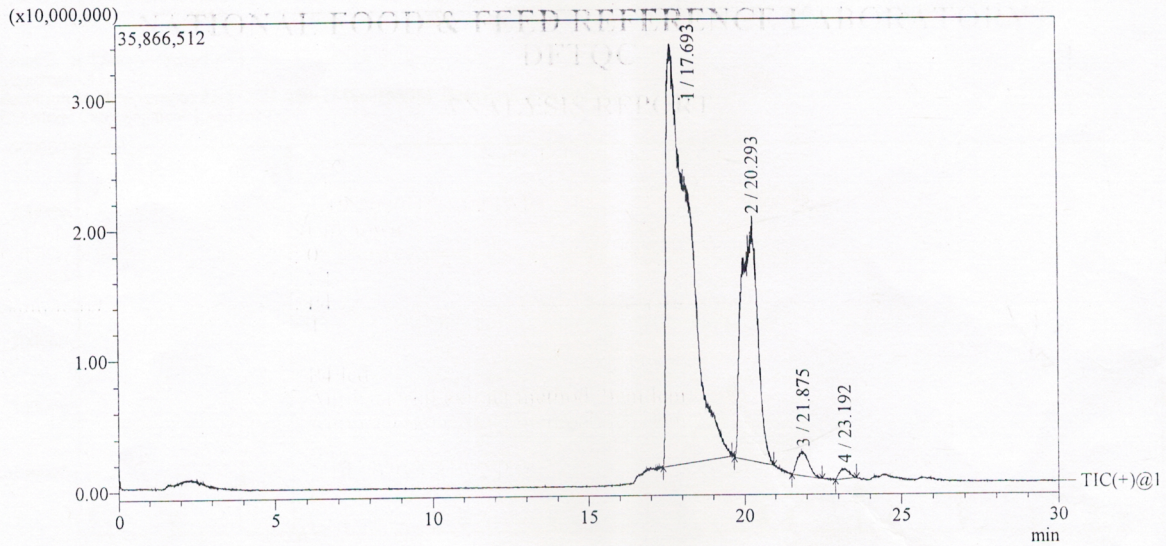
NATIONAL FOOD & FEED REFERENCE LABORATORY
DFTQC

ANALYSIS REPORT

Sample Information

Acquired by	: suraz
Date Acquired	: 2/19/2020 4:21:02 PM
Sample Type	: Unknown
Level#	: 0
Sample Name	: P4
Sample ID	: P4
Vial#	: 4
Injection Volume	: 5
Data File	: P4.lcd
Method File	: Antibacterial extract method 21cm.lcm
Original Method File	: Antibacterial extract method 21cm.lcm
Processed by	: suraz
Date Processed	: 2/19/2020 4:51:12 PM

MS Chromatogram



MS Spectrum

Line#:1 R.Time:----(Scan#:----)
MassPeaks:478
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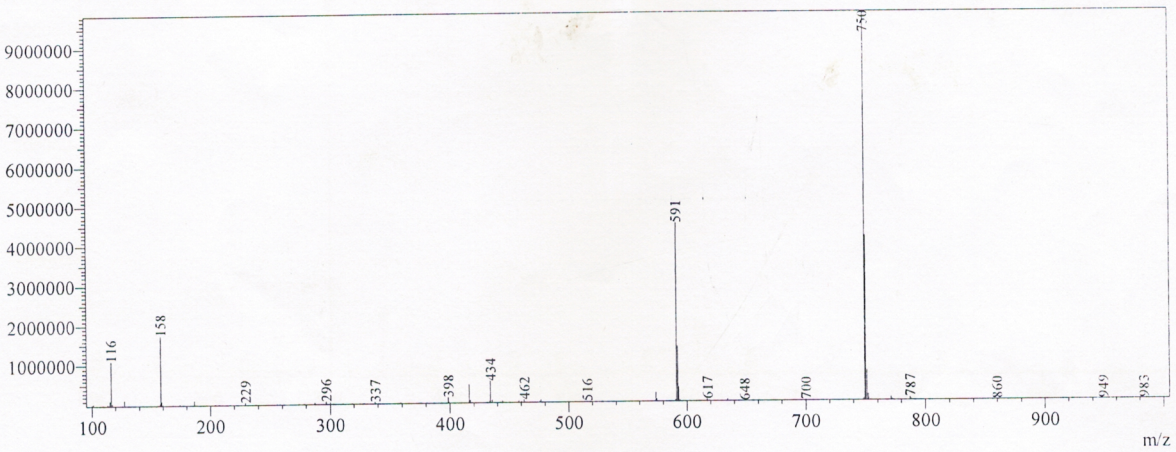
BG Mode:Calc Segment 1 - Event 1



Line#:2 R.Time:----(Scan#:----)
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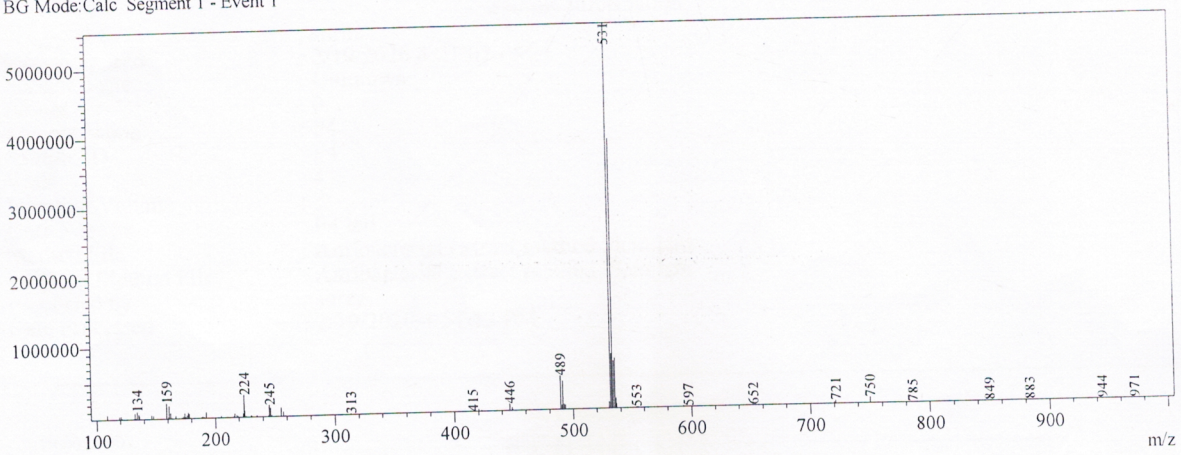

Line#:3 R.Time:----(Scan#:----)
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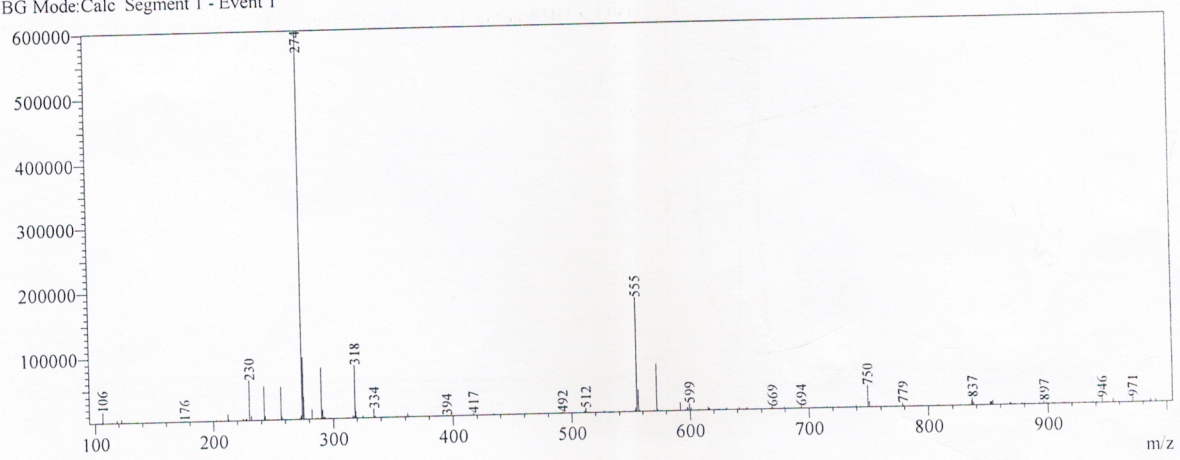
BG Mode:Calc Segment 1 - Event 1



Line#:4 R.Time:----(Scan#:----)
MassPeaks:442
Spectrum Mode:Averaged 23.191-23.193(17394-17396) Base Peak:302(334750)
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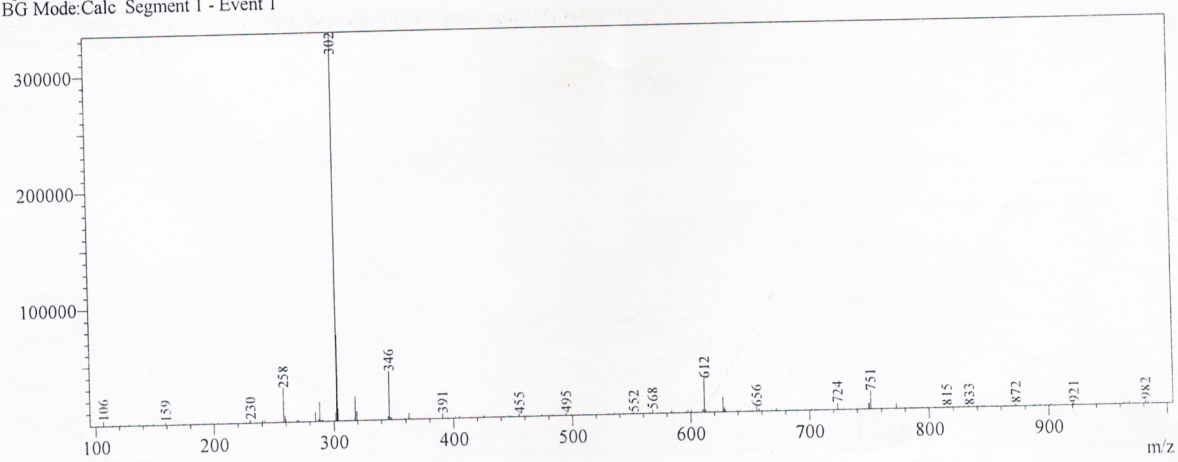


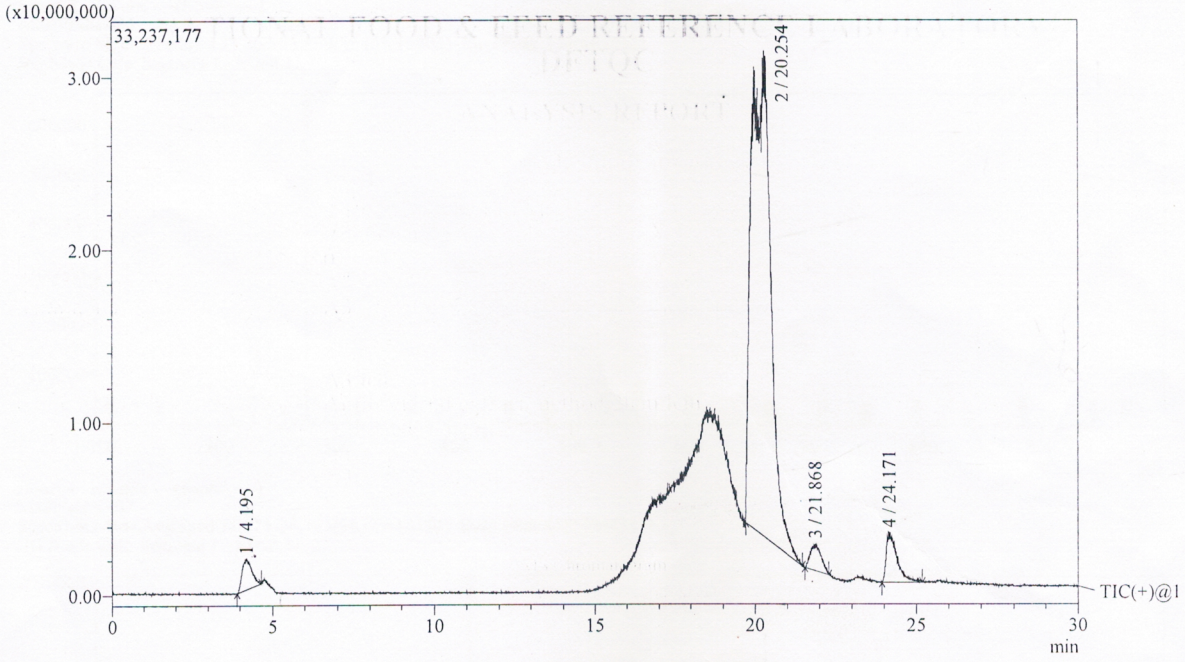
NATIONAL FOOD & FEED REFERENCE LABORATORY
DFTQC

ANALYSIS REPORT

Sample Information

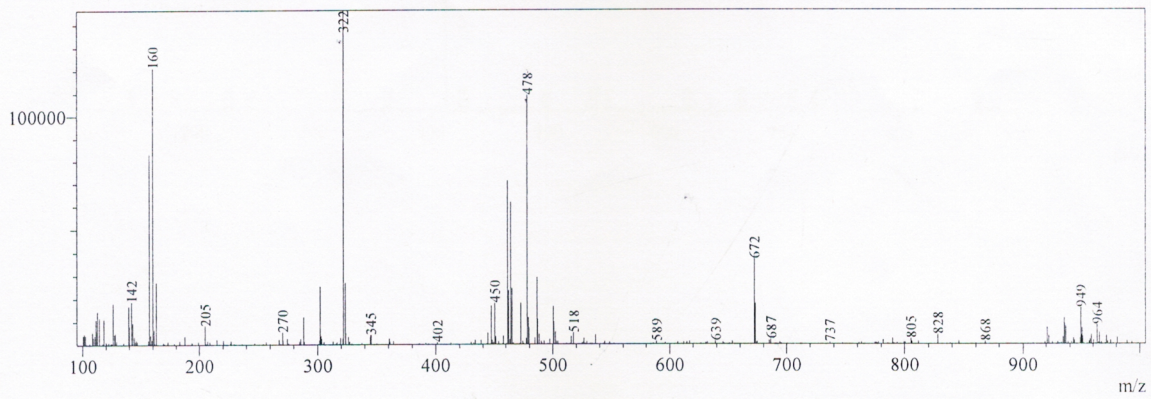
Acquired by	: suraz
Date Acquired	: 2/19/2020 2:48:14 PM
Sample Type	: Unknown
Level#	: 0
Sample Name	: A3
Sample ID	: A3
Vial#	: 1
Injection Volume	: 5
Data File	: A3.lcd
Method File	: Antibacterial extract method 2lcm.lcm
Original Method File	: Antibacterial extract method 2lcm.lcm
Processed by	: suraz
Date Processed	: 2/19/2020 5:05:58 PM

MS Chromatogram

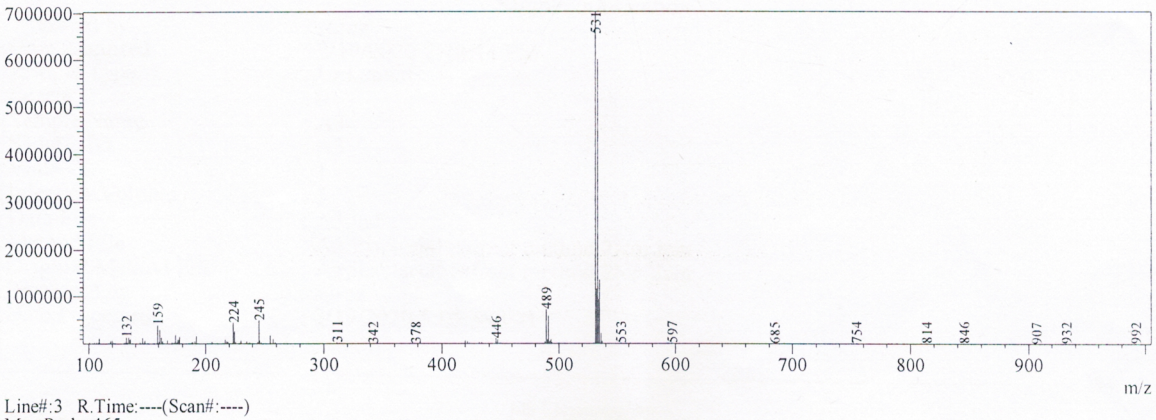


MS Spectrum

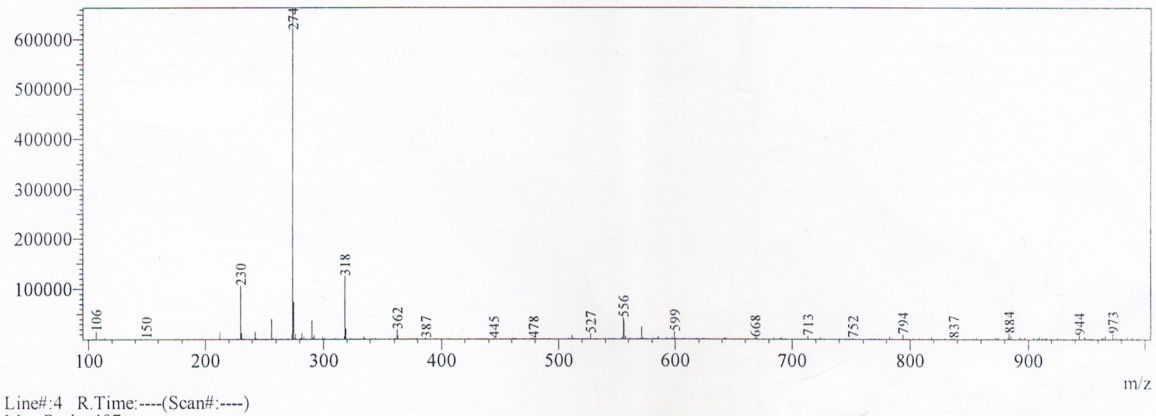
Line#:1 R.Time:----(Scan#:----)
MassPeaks:568
Spectrum Mode:Averaged 4.193-4.196(3146-3148) Base Peak:322(146339)
BG Mode:Calc Segment 1 - Event 1



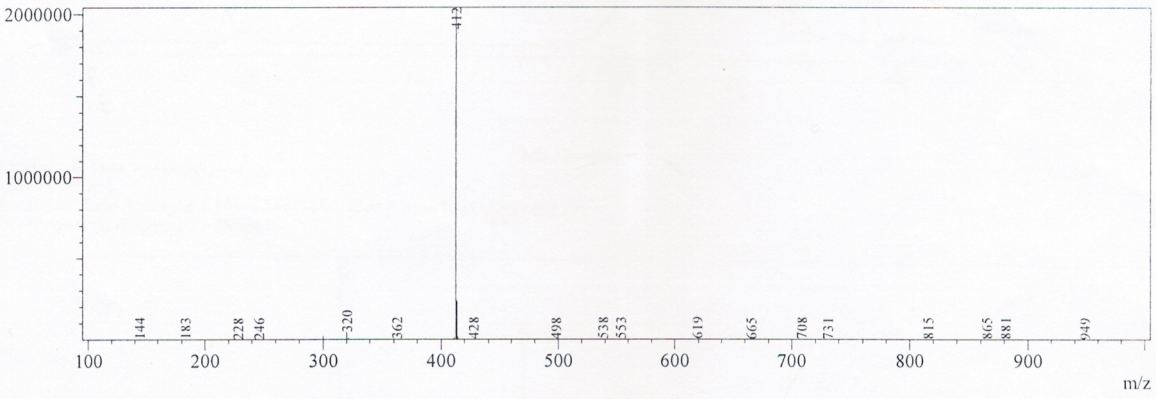
Line#:2 R.Time:----(Scan#:----)
MassPeaks:452
Spectrum Mode:Averaged 20.253-20.256(15191-15193) Base Peak:531(7020005)
BG Mode:Calc Segment 1 - Event 1



Line#:3 R.Time:----(Scan#:----)
MassPeaks:465
Spectrum Mode:Averaged 21.867-21.869(16401-16403) Base Peak:274(663517)
BG Mode:Calc Segment 1 - Event 1



Line#:4 R.Time:----(Scan#:----)
MassPeaks:407
Spectrum Mode:Averaged 24.171-24.173(18129-18131) Base Peak:412(2044551)
BG Mode:Calc Segment 1 - Event 1



NATIONAL FOOD & FEED REFERENCE LABORATORY
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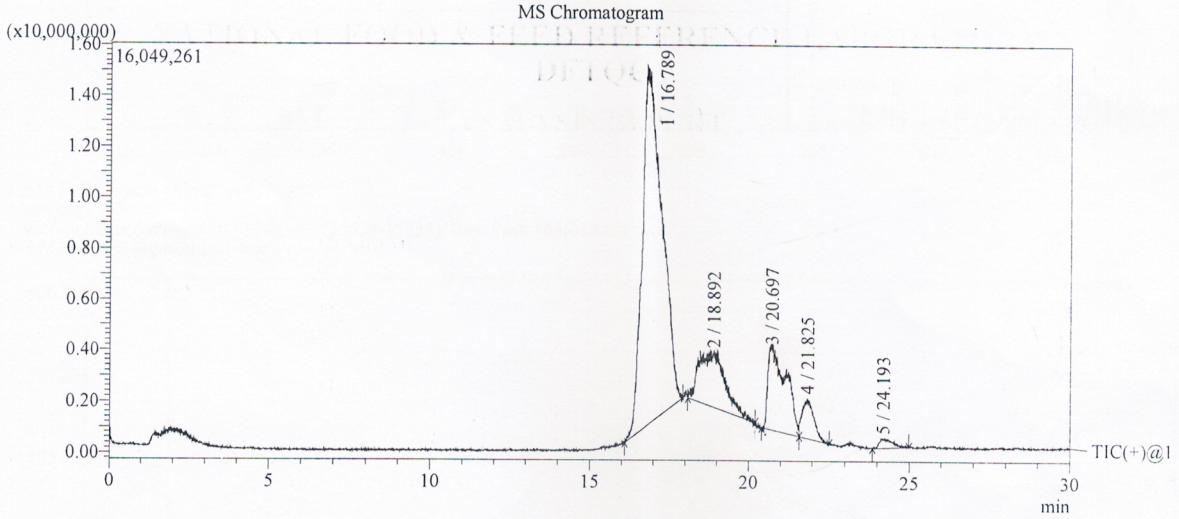
ANALYSIS REPORT

Sample Information

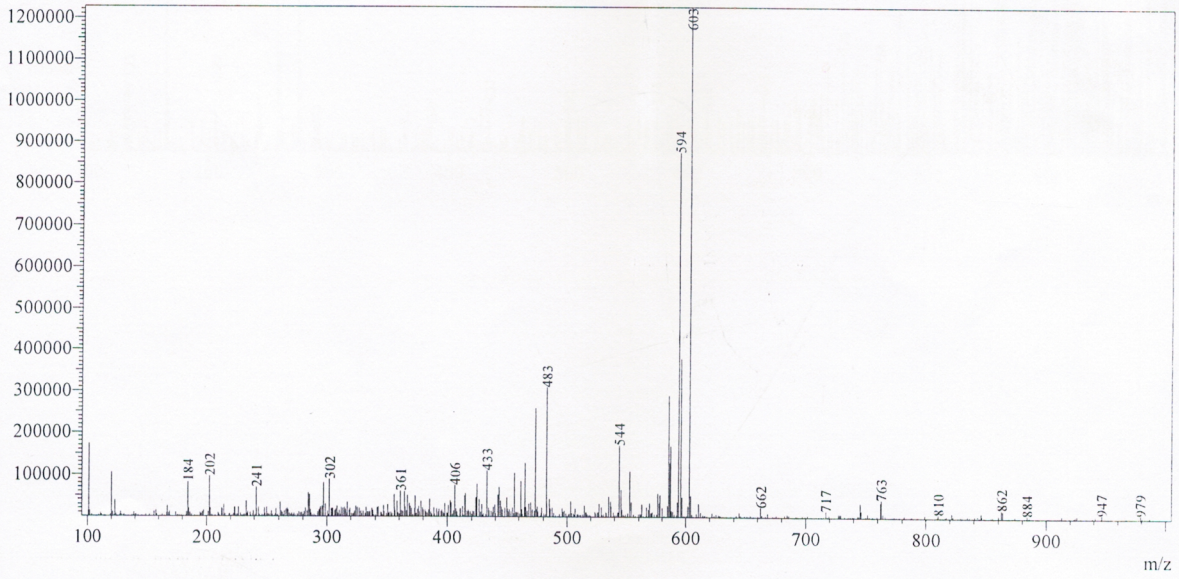
Acquired by	: suraz
Date Acquired	: 2/19/2020 3:50:10 PM
Sample Type	: Unknown
Level#	: 0
Sample Name	: J1
Sample ID	: J1
Vial#	: 3
Injection Volume	: 5
Data File	: J1.lcd
Method File	: Antibacterial extract method 2lcm.lcm
Original Method File	: Antibacterial extract method 2lcm.lcm
Processed by	: suraz
Date Processed	: 2/19/2020 5:11:23 PM

MS Chromatogram



MS Spectrum

Line#:1 R.Time:----(Scan#:----)
MassPeaks:716
Spectrum Mode:Averaged 16.787-16.789(12591-12593) Base Peak:603(1226145)
BG Mode:Calc Segment 1 - Event 1



Line#:2 R.Time:----(Scan#:----)
MassPeaks:482
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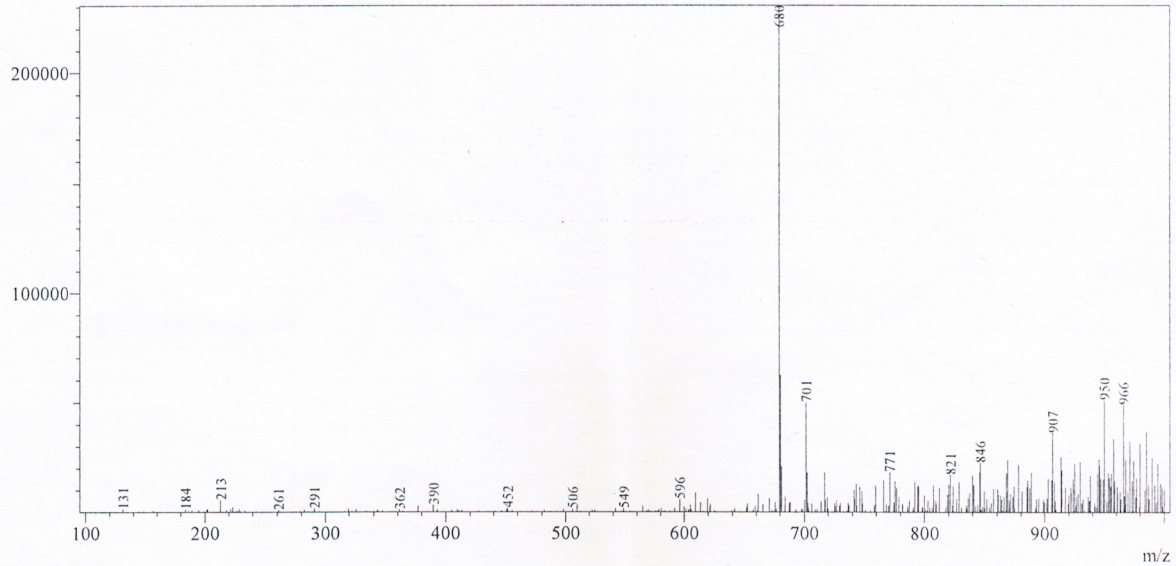
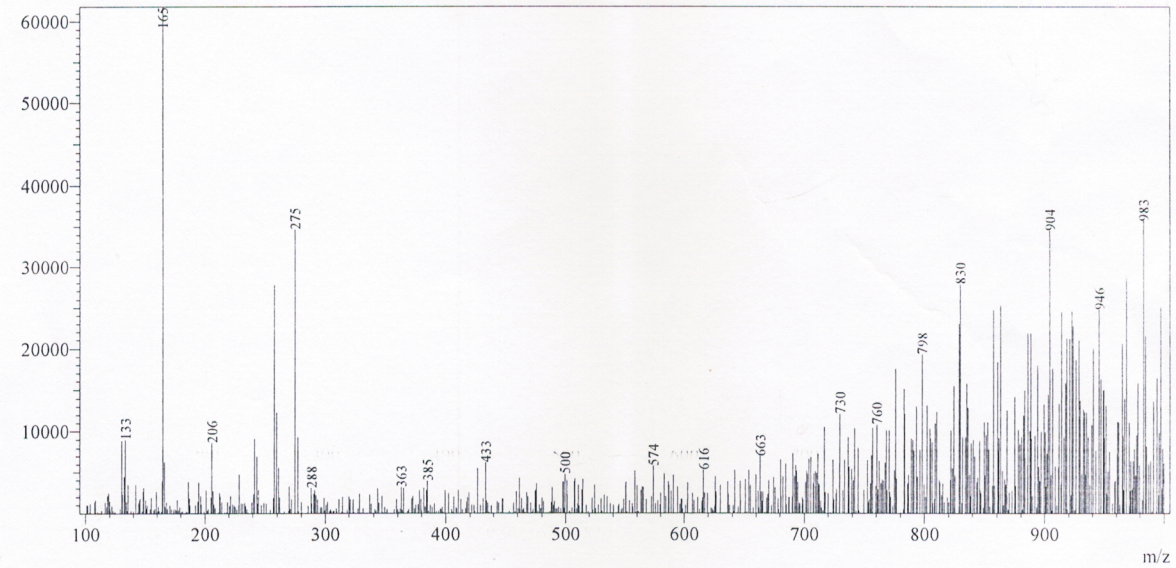
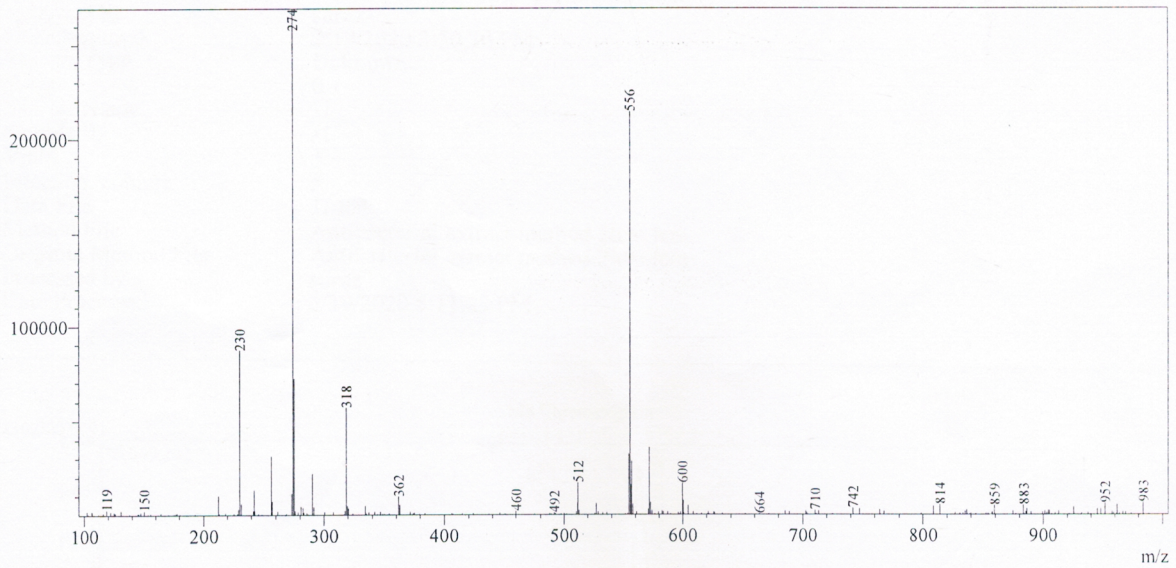
BG Mode:Calc Segment 1 - Event 1



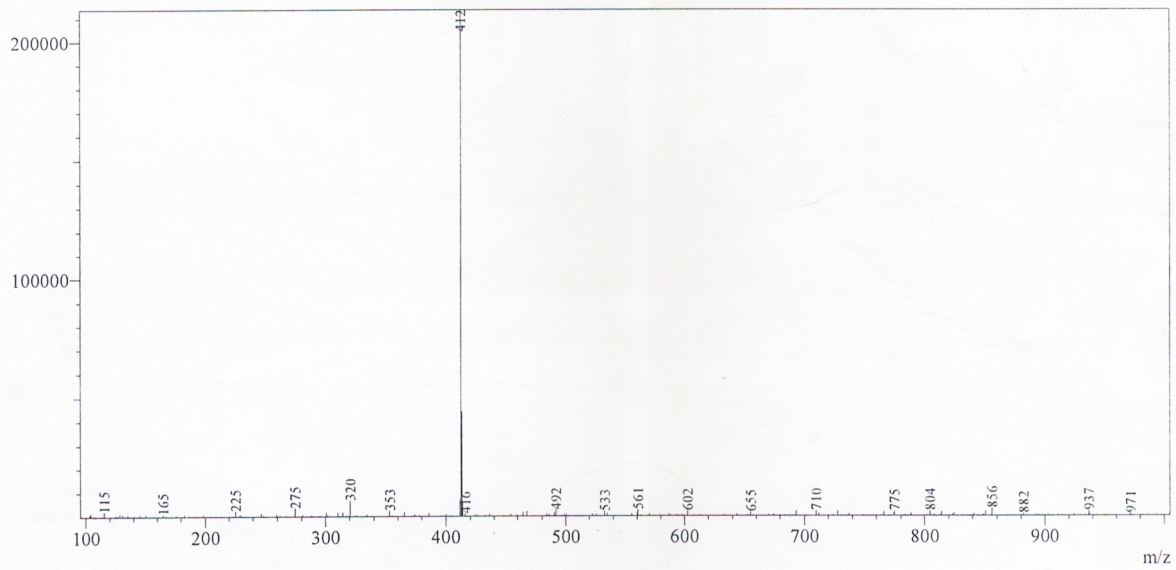
Line#:3 R.Time:----(Scan#:----)
MassPeaks:707
Spectrum Mode:Averaged 20.696-20.699(15523-15525) Base Peak:165(61820)
BG Mode:Calc Segment 1 - Event 1



Line#:4 R.Time:----(Scan#:----)
MassPeaks:483
Spectrum Mode:Averaged 21.824-21.827(16369-16371) Base Peak:274(269438)
BG Mode:Calc Segment 1 - Event 1



Line#:5 R.Time:----(Scan#:----)
MassPeaks:452
Spectrum Mode:Averaged 24.192-24.195(18145-18147) Base Peak:412(213552)
BG Mode:Calc Segment 1 - Event 1



NATIONAL FOOD & FEED REFERENCE LABORATORY
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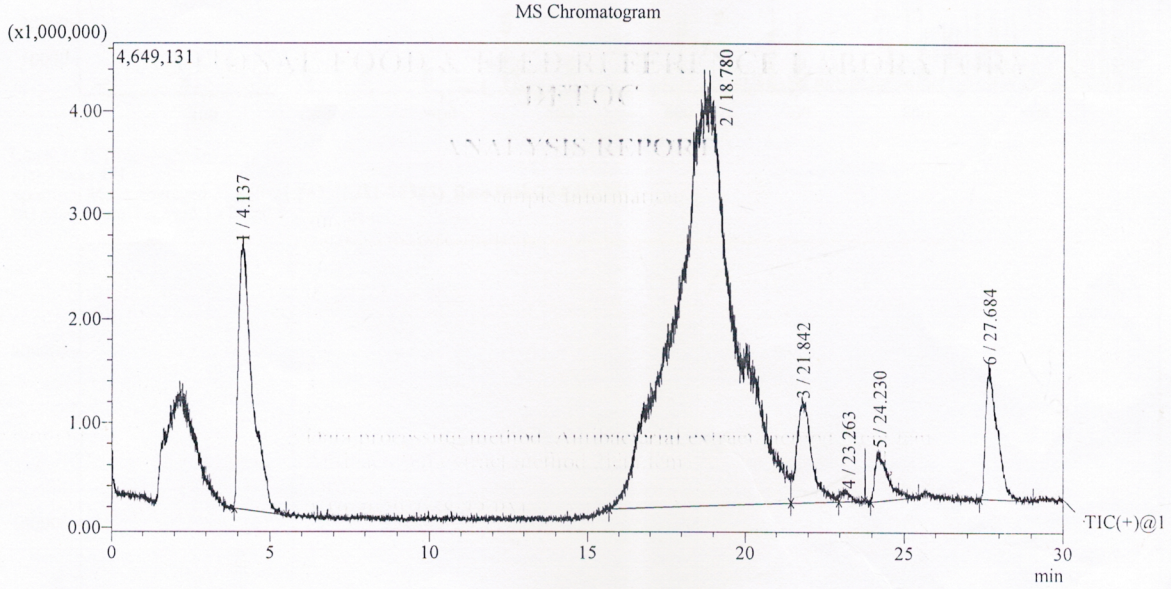
ANALYSIS REPORT

Sample Information

Acquired by	: suraz
Date Acquired	: 2/19/2020 3:19:06 PM
Sample Type	: Unknown
Level#	: 0
Sample Name	: D2
Sample ID	: D2
Vial#	: 2
Injection Volume	: 5
Data File	: D2.lcd
Method File	: Data processing method_Antibacterial extract method 2lcm.lcm
Original Method File	: Antibacterial extract method 2lcm.lcm
Processed by	: suraz
Date Processed	: 2/19/2020 4:58:32 PM

MS Chromatogram



MS Spectrum

Line#:1 R.Time:----(Scan#:----)
MassPeaks:671
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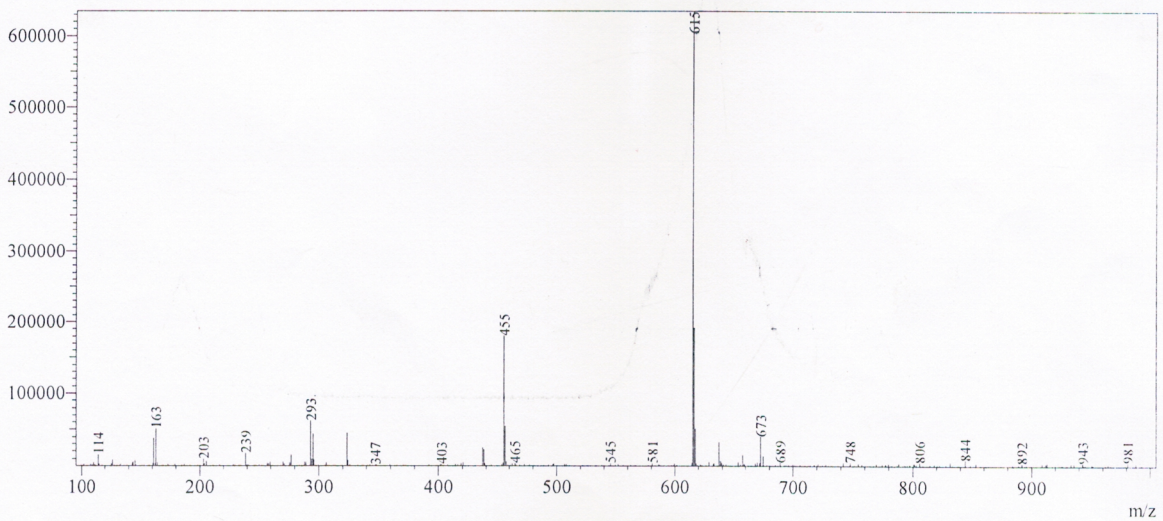
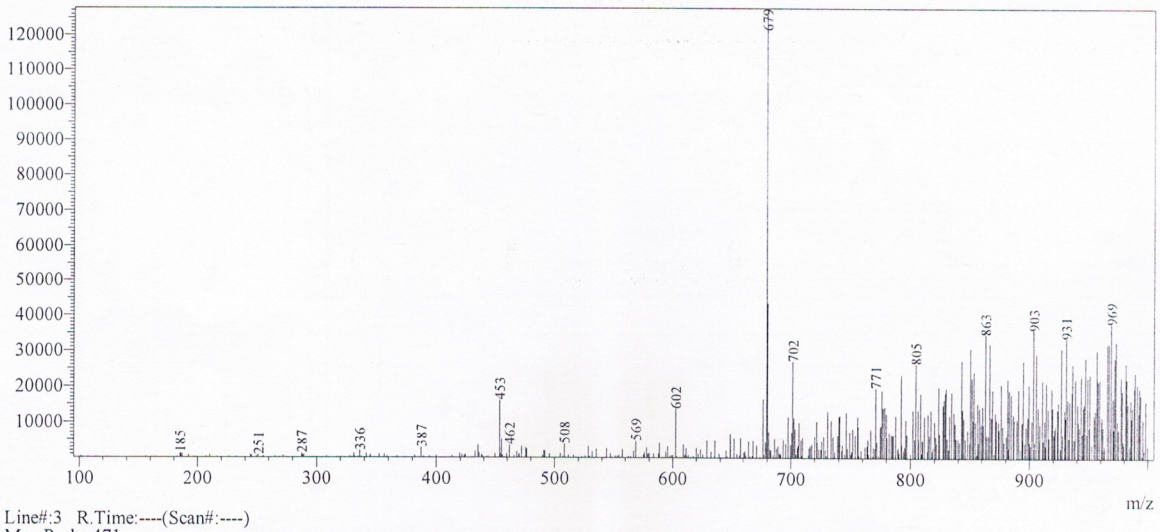
BG Mode:Calc Segment 1 - Event 1



Line#:2 R.Time:----(Scan#:----)
MassPeaks:582
Spectrum Mode:Averaged 18.779-18.781(14085-14087) Base Peak:679(127734)
BG Mode:Calc Segment 1 - Event 1



Line#:3 R.Time:----(Scan#:----)
MassPeaks:471
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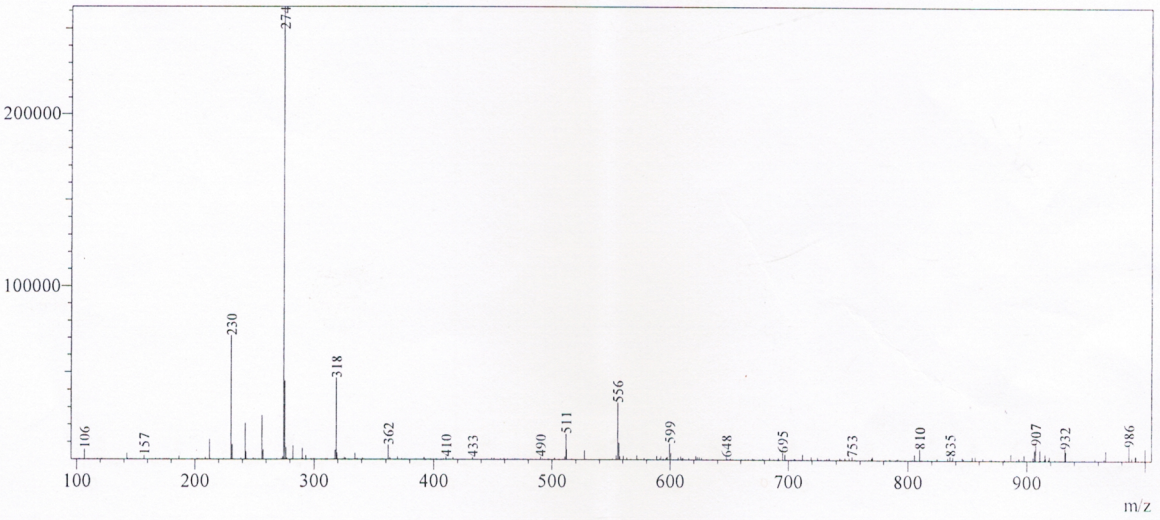
BG Mode:Calc Segment 1 - Event 1



Line#:4 R.Time:----(Scan#:----)
MassPeaks:409
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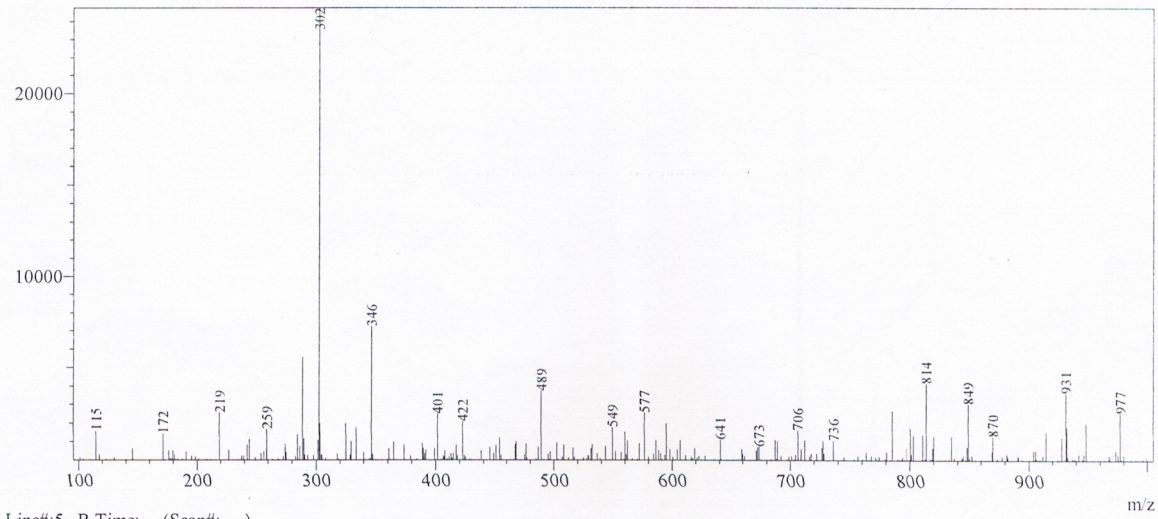
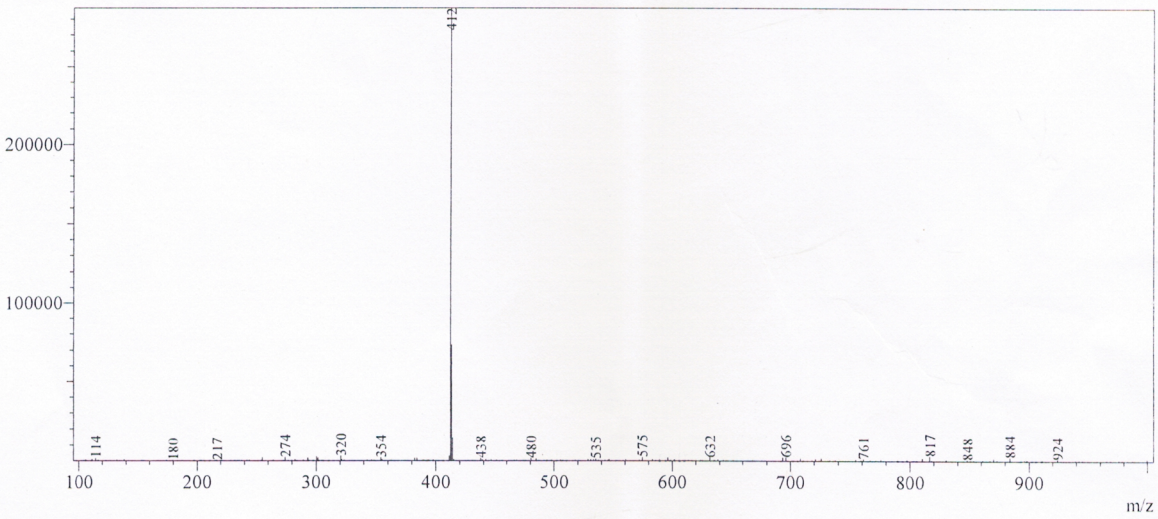
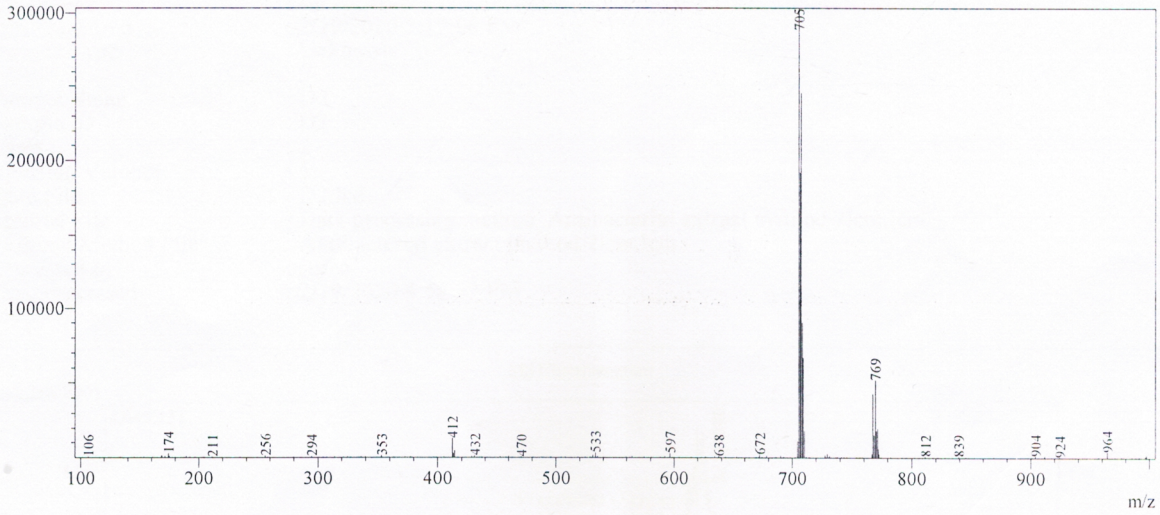
BG Mode:Calc Segment 1 - Event 1



Line#:5 R.Time:----(Scan#:----)
MassPeaks:411
Spectrum Mode:Averaged 24.229-24.232(18173-18175) Base Peak:412(287065)
BG Mode:Calc Segment 1 - Event 1



Line#:6 R.Time:----(Scan#:----)
MassPeaks:429
Spectrum Mode:Averaged 27.683-27.685(20763-20765) Base Peak:705(303386)
BG Mode:Calc Segment 1 - Event 1



4.2 Discussion

Microbial biodiversity is a huge area and potential discipline for the biotechnology industry because it provides plenty of new genes and biosynthetic pathways to produce enzymes, antibiotics, vitamins, amino acids and other useful molecules (Agrawal, 2002). The diversity of terrestrial actinomycetes has been of extraordinary significant in several areas of science and medicine, particularly in antibiotic production (Magarvey *et al*. 2004). The chemical diversity of naturally produced antibiotics by actinomycetes is unparallel to that observed in any other microorganism or by chemical synthesis. Approximately two-third of the known, naturally derived antibiotics, including many medications in current clinical use, were discovered as fermentation products by cultivating actinomycetes under optimum conditions (Berdy, 2005,Okami and Hotta, 1988).

The need for new antibiotics is greater than ever because of the emergence of multidrug resistance in common pathogens, the rapid emergence of new infections and the potential use of multidrug-resistant microbes in bio weapons (Spellberg *et al*. 2004). One of the most successful approaches to increase the probability of discovering novel antibiotics is the isolation and screening of actinomycetes from relatively unexplored areas (Moncheva *et al*. 2002). Accordingly, soil and water samples from different parts of Nepal were collected to isolate potent actinomycetes capable of producing antimicrobial bioactive metabolites in this study. The isolated strains were then screened for antibiotic production, and most promising producers were further subjected for characterization and optimization of fermentation process along with characterization of bioactive metabolite.

In this study fairly high number of actinomycetes (320) were isolated on starch casein agar medium (Table 4.1). Results of this study is in agreement with Ganesan *et al*. (2017) who isolated 120 strains from soil samples taken from five different places. Likewise, Muharram *et al*. (2013) reported 33 actinomycetes strains from only five soil samples indicating abundance of actinomycetes in terrestrial habitats. Such high isolation might be due to the pre-treatment of soil sample with calcium carbonate (Tsao *et al*. 1960). Treatment of soil sample with calcium carbonate has been found to be most effective for the isolation of actinomycete (El- Nakeeb and Lechevalier, 1963, Qin *et al*. 2009, Yi and Amsaveni, 2012).

Although the precise mechanism of the calcium carbonate effect is not clear, previous work has shown that mixing of powdered calcium carbonate makes the soil alkaline that favor the growth of actinomycetes spores (Tsao *et al*. 1960) and calcium ions have the ability to stimulate the formation of aerial mycelia (Natsume *et al* 1989). Besides, incorporation of nalidixic acid (25 µg/ml) and cycloheximide (25 µg/ml) also inhibited the bacterial and fungal contaminants respectively. Starch casein agar incorporated with nalidixic acid (25 µg/ml) and cycloheximide (25µg/ml) has been found to selectively make the environment suitable for the growth of actinomycetes (Hop *et al*. 2011, Magarvey *et al*. 2004 and Qin *et al*. 2009). Cycloheximide binds the ribosome and inhibits EF2- mediated translocation of fungi (Salkin and Hurd, 1972 & Schneider-Poetsch *et al*. 2010), thereby inhibiting them. Nalidixic acid is a specific inhibitor of bacterial DNA synthesis resulting into killing of bacteria (Pedrin *et al*. 1971).

The results of primary screening revealed that 37.5% (120) isolates exhibited antimicrobial activity out of which majority of isolates 40% (100) were from soil (Table 4.1). On the basis of color of colony white and dirty white were found most predominant each 16.7% followed by grey (8%) as shown in figure 4.1. Results of this study are comparable with Mabrouk and Saleh (2014) who found 35 soil isolates out of 75 showed antimicrobial activity. In this study white and gray colored strains were found in large proportion which is in accordance with Ramesh and Mathivanam (2009) who observed 115 grey and 79 white colored strains. They also recorded that 53% (111) strains were bioactive against majority of test microbes which is slightly higher than finding of this study. In another study Ganesan *et al*. (2017) explored that 41.5% of soil isolates displayed good antimicrobial activity against many pathogenic bacteria which is comparable with findings of this study. It has been observed that the environment of the soil such as humidity, pH and diversity of plant species grown on that particular soil influence the growth rate of microorganisms (Singh *et al*. 2009). Majority of strains in present study were recovered from soil that might be attributed to the presence of vegetation, enough organic and inorganic content with sufficient moisture. Furthermore, higher number of soil samples were processed than water samples might be another reason for low incidence of actinomycetes in fresh water habitats.

In primary screening higher proportion 68(56.7%) out of 120 strains of actinomycetes were found inhibitory to Gram positive bacteria than Gram negative and fungi reflecting inborn susceptibility of Gram positive towards antimicrobial compounds. This result is comparable with findings of Mabrouk and Saleh (2014) who reported 64.3% actinomycetes were found active against Gram positive bacteria. Results of this study is also supported by Singh *et al*. (2014) who isolated *Streptomyces sannanensis* SU118 active against only Gram-positive bacteria. The results (Table 4.2) revealed that only 4 (3.3%) isolates were found to be inhibitory to all test organisms which is comparable with the findings of Saravana Kumar *et al*. (2014) who recorded only 5% of *Streptomyces* exhibited good activity against test bacteria and fungi. Results of this study is also in accordance with Sengupta *et al*. (2015) in which only 9(4%) isolates with broad spectrum activity have been documented. Gram positive bacteria are more sensitive to metabolites of actinomycetes (Scherrer and Gerhardt, 1971). This can be attributed to the cell wall structure of Gram-negative bacteria that have an outer polysaccharide membrane carrying the structural lipopolysaccharide moiety that makes the cell wall impermeable to lipophilic solutes; while Gram positive bacteria have only an outer peptidoglycan layer which is not an effective permeability barrier (Willey *et al*. 2008).

Broad spectrum activity of all screened strains was confirmed in secondary screening. Among four strains selected ethyl acetate extract of A_3 showed highest antimicrobial activity against four test microbes and *Candida albicans* (41.33±1.15mm) was found to be most susceptible (Lekhak *et al.* 2018) as depicted in Table 4.3. Similar to findings of this study Sengupta *et al*. (2015) stated broad spectrum activity of three *Streptomyces* isolates against *Vibrio cholerae, S. aureus, S. typhi*, *Bacillus subtilis, Candida albicans* and others with highest activity against *V. cholerae* (34±2mm). Furthermore, results of this study are also in harmony with Muharram *et al*. (2013) who demonstrated broad spectrum activities of three *Streptomyces* species against *S. aureus, B. subtilis, E. coli, C. albicans* and *Aspergillus niger* with maximum zone of inhibition against *C. albicans* (32mm). Outcome of this study are comparable with Raghava Rao *et al*. (2017) who reported both antibacterial and antifungal activity of *Streptomyces coelicoflavus* BC01 with 19mm zone of inhibition against *C.albicans*. Broad spectrum activity of actinomycetes is due to mixture of diverse metabolites produced in medium. A new Thiopeptide TP-1161 compound produced

by Nocardiopsis was found highly active against many bacterial and fungal pathogens including vancomycin resistant enterococci and multidrug resistant *C. albicans* (Engelhardt *et al*. 2010). A mixture of antibiotics such as trimethoprim, nalidixic acid, fluconazole, ketoconazole and rifampicin was produced by *Streptomyces cyaneofuscatus* KY 287599 found to be active against many test organisms including *E. coli* and *C. albicans.* (Zothanouja *et al*. 2017). A diverse array of natural products is produced by some actinobacteria which make them a subject of research in finding new therapeutic drugs to combat the problem of multidrug resistance in microbes. Table 4.4 indicates that bioactive compound produced by A_3 strain had lowest Minimum inhibitory concentration (MIC) values against all test organisms which is 0.125mg/ml for *C. albicans* and *E. coli*, 2.5mg/ml for *S. typhi*, *A. baumannii* and MRSA. For *Bacillus subtilis* extract of all isolates gave same value 0.625mg/ml. MIC values of bioactive compounds was found in a range 0.125- 5 mg/ml (Lekhak *et al.* 2018). These observations are quite similar with Sengupta *et al*. (2015) who recorded MIC value of ethyl acetate extract of *Streptomyces* species in a range 0.5-5mg/ml against many bacteria and *Candida albicans*. Singh *et* al. (2016) found MIC value of metabolite as 1.25mg/ml for MRSA but it was 2.5mg/ml against VRE, *Shigella dysenteriae* and *Klebsiella pneumoniae* which is in accordance with this result. In contrast low MIC value of 12.5-75µg/ml against bacteria and 50-125µg/ml against fungi was evaluated by Ganesan *et al*. (2017). Unlike the result of this study Mangamuri *et al*. (2016) reported lowest MIC of purified metabolite as 4µg/ml and 8µg/ml against *Proteus vulgaris* and *C. albicans* respectively. In another study Satish and Kotaki (2017) reported low MIC value of 1mg/ml against MDRSA. MIC value is affected by many parameters including susceptibility of organisms, type of microorganism, concentration and type of bioactive metabolites, composition of cultural medium, incubation temperature and time (Satish and Kotaki, 2014). Higher MIC values displayed by extracts in this study could be due to use of partially purified product or differences in type of metabolites.

In this study the classical approaches (cultural, morphological, biochemical and physiological) for classifying the active isolates was used. Macroscopic characteristics of the isolates were chiefly used to differentiate the isolates (Table 4.5). All the isolates in this study showed good sporulation with compact,

powdery or floccose colonies of different colors ranging from creamy, yellow to brownish-black substrate mycelium. The color of aerial mycelium ranged from creamy, white to dirty white and grey in color. Other colony characteristics of the isolates include irregular shape, concentric appearance, butyrous consistency, umbonate elevation, erose margin and opaque colonies. The colony diameter of the isolates ranged from 2-6mm. All the characteristics are comparable to that of *Streptomyces* spp. All the isolates were found to be Gram positive with simple or branched sporophores and were recti flexible as they were straight with flexuous spore chain or spiral with many spores. Haque *et al*. (2015) reported flexuous to spiral spore chain in a *Streptomyces* isolates similar to that of strain D_2 of this study. Findings of macroscopic and macroscopic observations are comparable with the results of Mangamuri *et al*. (2016) who isolated *Streptomyces cheonanesis* that developed grey to white branched aerial mycelia with short chain of spores. The strain A_3 produced white colored colony with long chain of spores similar to *Nocardiopsis alba* isolated by Janardhan et *al*. (2014). Results of this study are quite contrasting than findings of Vimal *et al*. (2009) who observed unbranched aerial mycelium in *Nocardiopsis* spp. VITSVK(FJ973467). Actinomycetes exhibit diverse morphological and colonial characteristics with varied ability to utilize nutrients even within same genera (Kampfer, 2006, Waksman and Henrici, 1943). Color of substrate and aerial mycelium of actinomycetes is also governed by media used for their cultivation (Mabrouk and Saleh, 2014). Hence due to variable colonial and microscopic features of actinomycetes strains in the same genera it is difficult to pin point characterize them up to species level.

Results of carbohydrate utilization test showed variable ability of all strains as shown in Table 4,7. Glucose, Mannitol, salicin and sucrose were utilized by all but inulin was by none of the strain. Galactose along with majority of sugars were utilized by only A_3 strain showing its superior metabolic ability over others. Diverse ability of sugar utilization has been shown by three *Streptomyces* isolates that could utilize most of sugars such as glucose, galactose, mannitol, arabinose, sucrose but not meso-inositol (Muharram *et al*., 2013). In another study *Streptomyces rimosus* was found to utilize glucose, mannitol, xylose and inositol but not sucrose, lactose, arabinose, raffinose and sorbitol (Ganesan, 2017). Similarly, Mangamuri *et al*. (2016) have isolated *Streptomyces cheonanesis* VUK-A able to utilize different

sugars including glucose, lactose, Galactose, maltose but not xylose and mannitol. All of these studies indicated the massive physiological diversity among actinomycetes regarding utilization of sugars as carbon source and these variations do not allow to identify them solely on the basis of sugar utilization test.

All the isolates were catalase positive, oxidase negative, indole negative, H_2S production negative nonmotile. Similarly nitrate reduction and citrate test positive except D_2 and J_1 (Table 4.9). Likewise, all strains hydrolyzed starch, gelatin and urea except D_2 (Table 4.8). Results of tests exhibited by strains D_2 and J_1 are in accordance with Mangamuri *et al*. (2016) but contrasting for strains A_3 and P_4. Similar to findings of this study starch and gelatin hydrolysis test was found positive for *Nocardiopsis* (Janardhan *et al*. 2014). Results of this study are in agreement with Haque *et al*. (2015) who reported positive gelatin and starch hydrolysis in *Streptomyces* species. Substrate hydrolysis test can be used as an aid to identify actinomycetes but only this test cannot differentiate them.

All of the isolates were found to grow successfully at 15ºC, 37ºC and 45ºC except isolate A_3 which was failed to grow at 45ºC. Similarly, all the strains were found to grow efficiently in presence of 3%, 5% and 7% NaCl concentration as depicted in Table 4.10. Similar to the result displayed by A_3 strain in this study, *Nocardiopsis* sp. VITSVK 5 (FJ973467) isolated by Vimal *et al*. (2009) was found unable to grow at 45^0C but able to grow up to 7% NaCl concentration. Results of this studyare comparable with Mangamuri *et al*. (2016) who observed growth of *Streptomyces cheonanensis* VUK-A within a range 25-45^0C. Similarly, Sreevidya *et al*. (2016) isolated *Streptomyces* species grew well in a temperature range 20- 40^0C. Results of this research are also supported by Mangamuri *et al*. (2016) who observed a temperature range of 25-45^0C and salt tolerance up to 7% for *Streptomyces* spp. Several thermophiles and psychrophilic species are known, however the optimum temperature for most of the species is 25-35ºC. (Kampfer, 2006). The isolates were able to grow at three different temperatures. HSP18, a protein belonging to the small heat shock protein family has been found to be responsible for providing temperature tolerance to actinomycetes (Servant and Mazodier, 1996). Some *Streptomyces* contain 7kDa cold shock like protein which imparts psychrotrophic character (Av-Gay *et al*. 1992). Tolerance to high salt

concentration is attributed to P5CR and *mtlD* gene in *Streptomyces* (Mohamed *et al*. 2013). Wide ecological distribution of actinomycetes could be the reason make them tolerant to acidic, alkaline, saline and high or low temperatures conditions. On the basis of morphological, cultural and biochemical characteristics, the strain A_3 was identified as *Nocardiopsis prasina* whereas rest three belong to *Streptomyces* genera. Further identification by 16SrRNA typing, the most potent strain A_3 was found to be *Nocardiopsis prasina*.

Biomass production of A3 strain was highest in all sugars except xylose. Maximum biomass production shown by A3 in presence of glucose (311.67±10.48 mg/100ml). Maximum antimicrobial activity was shown by A3 against all test organisms with highest toward *C. albicans* (39.33±0.57 mm) as shown in figure 4.3 and 4.6. Similar results have been reported by Bundale *et al* (2015). They reported mono and disaccharides for maximum biomass and starch for highest metabolite production. Maximum bioactive metabolite production in presense of starch was also reported by El-Naggar *et al.* (2003) and Narayan and Bijayalakshmi (2008). Soluble starch has been found as best carbon source for antibiotic production by many researchers. (Haque *et al.* 1995; Theobald *et al.* 2000).

All the strains, except P_4 produced maximum biomass when casein was used as nitrogen source. Similarly, bioactive metabolite production by all strains except D_2 was found to be maximum when casein was used as nitrogen source (Lekhak *et al.* 2018) as depicted in Figure 4.5 & 4.6. Alike result was observed by Satyapathy and Mohapatra (2017) who documented casein for maximum metabolite production in *Streptomyces* species. In Other studies Peptone followed by casein has been observed as best nitrogen source for bioactive metabolite production by *Streptomyces* strains (Bundale *et al*. 2015, Reddy *et al*. 2011). Excessively utilizable nitrogen source exerts a repressive effect causing a decrease in the levels of secondary metabolites, mainly caused by ammonium salts and amino acids (Omura and Tanaka, 1984., Osman *et al*. 2011, Sanchez and Demain, 2002).In this study both biomass and metabolite production of all strains except J_1 was found optimum at 30^0C as shown in Figure 4.7 &4.8.These results are in agreement with Bundale *et al*. (2015), who reported 30 ^{0}C temperature as an optimum for four different *Streptomyces* species studied. Findings of this study is also supported by Oskay (2011) who found 30ºC as optimum for growth and metabolite production of *Streptomyces* spp. KGG32. Similar result was

presented by Hassan *et al*. (2004) while studying effect of temperature on *Streptomyces violarus* andalso, by Ibtisam (2013) on *Streptomyces* spp. MS-266.

In this study strains A_3, D_2 and J_1 produced maximum biomass at pH 8 whereas in case of strain P_4, it was maximum at pH 7. Antimicrobial activity of strains A_3, D_2 and J_1 were found to be maximum against all test organisms at pH 8 whereas strain P_4 showed maximum activity at pH 7 (Figure 4.9 & 4.10). Result exhibited by P_4 strain is supported by Bundale *et al*. (2015) who found pH 7 as best for both growth and metabolite production by *Streptomyces purpurascens.* Study of El-Naggar *et al*. (2003) reported maximum antibiotic production by *Streptomyces violarus* at pH 7.5 which is more or less similar with the results displayed by strains A_3, D_2 and J_1. pH 7.5 was found to be best for antibiotic production by *Streptomyces* spp. 201 (Thakur *et al*. 2009). Satyapathy and Mohapatra (2017) observed highest antibiotic production by *Arthrobacter* spp. SAS 16 at pH 8 which is similar with A_3, D_2 and J_1 in this study. pH of the growth medium significantly influences cell morphology, cell membrane permeability and activity of enzymes catalyzing metabolic reactions thereby influencing bioactive metabolite production rate in actinomycetes (Guimaraes *et al*. 2004).

All the strains showed maximum antimicrobial activity on 8^{th} day of incubation except P_4 which showed maximum antimicrobial activity on the 6^{th} day. None of the strains produced bioactive metabolites on the 2^{nd} day (Lekhak *et al.* 2018) as shown in Figure 4.11. This result is in agreement with Satyapathy and Mohapatra (2017) who noticed maximum antimicrobial activity of *Arthrobacter* spp. SAS16 on the 8^{th} day. Similarly, Ibtisam *et al*. (2013) recorded maximum biomass production after 7^{th} day and antibiotic production before 9^{th} day by *Streptomyces* sp. MS-266 Dm4. Likewise, Bundale *et al*. (2015) found maximum antibiotic production by two species of *Streptomyces* on 8^{th} and 10^{th} day. In this study, P_4 strain showed maximum antibiotic production on 6^{th} day which is in agreement with Bundale *et al.* (2015) who observed maximum antibiotic production on 4^{th} and 6^{th} day by two species of *Streptomyces.* Actinomycetes massively increase biomass under excess availability of nutrients rather than the secondary metabolite production; but under nutrient deficient condition, cell cycle is shifted to stationary phase and signals the transition from primary metabolism to secondary metabolite production (Abd-Allah and El-Mehalawy,

2002). In this study biomass and metabolite production in majority of strains was found to be supported by $MgCl_2$ and KNO_3 respectively as depicted in Figures 4.12 & 4.13. This result is in accordance with Bundale *et al*. (2015) who reported positive impact of $MgCl_2$ and KNO_3 on both growth and bioactive metabolite production of *Streptomyces* sp. Similarly, El-Nagar (1991) stated that the salts of Magnesium and Potassium are considered as most appropriate for the growth and bioactive metabolite production of *Streptomyces*. For growth and synthesis of secondary metabolites, microelements (minerals and metal ions) are necessary, which are an important part of enzyme reactive center or could maintain the structure stability of biomolecules and balance of cell osmotic pressure (Wang *et al*. 2010).

The results of FTIR analysis revealed that ethyl acetate extract of all four potent actinomycetes strains contain -OH, CH, aromatic, C-O as common functional group but only the extract of strain *S. violarus*(D_2) contained C=O as shown in Table 4.11. In this study, IR spectra of ethyl acetate extract of strain of *S. tsusimaensis*(J_1) showed peak at 1519cm^{-1} which is in accordance with El Naggar *et al.* (2018). This peak is an indicative of C=C stretching of alkene which they confirmed by the detection of 1-henicosene and 1-octacosene through GCMS profiling. The n-henicosene has been previously reported as antibacterial component of *Streptomyces* spp. (Nandhini *et al.* 2015). The FTIR spectra exhibited by all the strains in this study is in agreement with Maleki and Mashinchian(2011) who also reported absorption peak at 3411, 2915, 2856 and 1649cm^{-1} denoting a double bond of polygenic compound. Similar pattern of absorption peaks at 3296, 1639 and 1031 cm^{-1} were noticed in ethyl acetate extract of *S. albidoflavus* PU23 producing non-polyene antifungal compound (Augustine *et al.* 2005). The findings of the study are in harmony with Maria *et al.* (2018) who observed alcohol (OH), alkene(C=C) and ether(C-O) in ethyl acetate extract of *Streptomyces* suggesting phenolic compound. Chemicals containing phenolic derivatives are known for their disinfectant and antiseptic properties. Various functional groups of the bioactive compounds commonly cause interaction with metabolite receptors in the cell of microbes resulting into specific biological response (Retnovati *et al.* 2018). The characteristic FT-IR spectra of ethyl acetate extract of all actinomycetes strains studied are in agreement with Arulappan *et al.* (2012) and Fatima *et al.* (2017) demonstrating alcohol (OH), alkane(C-H) and alkene(C=C) group in their study. The results of this study can be highly co-related with Bhosale *et al.* (2018) who reported OH, C=O, C-H

ether and ester functional groups in purified compound produced by *S. indiaensis.* This compound exhibited an ion peak m/z 664 in LCMS spectral analysis which is in accordance with IR spectra and LC MS ion peak m/z 664 of *S. tsusimaensis* (J_1) as shown in Table 4.15 and was identified as 2-amino-3-ethyl-benzofuran which is an established anti-fungal agent. Results of LCMS profiling of ethyl acetate extract of *N. prasina* (A_3) revealed a molecular ion peak m/z 245 in average retention time 20.253-20.256 as shown in Table 4.12. Dammak *et al.* (2017) also suggested the presence of cyclo(L-Phe-L-Pro), a dipeptide having m/z 245 in ethyl acetate extract produced by *Paludifilum halophilum*. The cyclic dipeptides are economically important and biologically active compounds among the most common peptide derivatives existing in nature. The cyclic nature of dipeptide makes them more stable than linear peptide. So, they are regarded as more promising candidates for making drugs. (Lucietto *et al.* 2006). Both natural and synthetic diketopiperazines, peptide derivatives, have broad biological activities including antibacterial (Kwon *et al.* 2000) and antifungal (Houston *et al.* 2004). *Streptomyces* species are potential producers of peptide antibiotics inhibiting wide range of microbes including Gram positive, Gram negative bacteria and *Candida albicans*. Such antibiotics contained OH and carboxylic functional group with amide bond (Nasri *et al.* 2017) Although ethyl acetate extract of *Nocardiopsis* sp GRG2(KT235641) screened by Rajivgandhi *et al.* (2018) exhibited similar FT-IR spectra to this study, the LCMS profiling of active compound showing inhibitory activity against ESBL producers (m/z 136) was found to be inconsistent with this study. This variation might be use of purified compound in their study. Also compounds may have similar pattern of functional groups but molecular ion mass may be different.

Actinomycetes are unlimited source of novel antibiotics used in medicine, agriculture, biotechnology and food industry. To overcome the problem of multidrug resistant microorganisms, search of new antibiotics with modified approach is essential. (Meij *et al.* 2017). Hence, this study was attempted to isolate, screen and identify potent actinomycetes strains from soil and water of Nepalese geography that could produce antifungal and antibacterial metabolites. Furthermore, this work also aimed to optimize cultural conditions for better antibiotic production along with minor attempts to characterize bioactive compounds. However, this study provides baseline information regarding better cultural conditions for antibiotic production by potent strains,

especially, *Nocardiopsis prasina.* The findings of the study may form the basis for undertaking further research in the fields of drug discovery and production using actinomycetes strains.

CHAPTER 5
CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The outcomes of the current study suggested that conventional morphological, biochemical and cultural methods are of gold standard to characterize actinomycetes. However, aid of molecular techniques such as 16SrRNA sequencing would be helpful to identify them upto species level. Growth and bioactive metabolite production by actinomycetes were found to be altered by many cultural parameters during this study. The incorporation of starch and casein can be practiced while conducting fermentation for optimum bioactive metabolite production. In addition to this, mesophilic temperature of 30^0C and slightly alkaline pH 8 was found optimum for 75% of the strains in this study. This result reflects importance of optimum temperature and pH for better growth and metabolite production. The result of optimization also suggests an incubation period up to 8 days along with incorporation of suitable minerals is essential for maximum metabolite production. Minor attempts regarding characterization of bioactive metabolites revealed that the mixed compounds in ethyl acetate extract exhibited diverse functional groups such as OH, C-H, C-O, C=C, C=O and aromatic groups in IR spectroscopy. The LC-MS profiling revealed that different actinomycetes strains can produce diverse antimicrobial components of variable molecular ion m/z value from 106 to 986. Actinomycetes of Nepal have tremendous capacity to produce antibacterial and antifungal compounds that may be utilized at industrial level after their complete study.

5.2 Recommendations:

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

1. Incorporation of cycloheximide along with nalidixic acid in media can be practiced to minimize fungal and bacterial contamination.
2. Conventional methods including morphological, biochemical and cultural are still gold standard for the characterization of actinomycetes.
3. Optimization of nutritional parameters such as carbon, nitrogen and mineral sources is essential while conducting fermentation.
4. Parameters such as temperature, pH and duration of incubation period should be studied and optimized for individual strain for better metabolite production.
5. The techniques such as HPLC, GCMS, H-NMR and C-NMR can be used for complete structure elucidation of active compounds.

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CHAPTER 6
SUMMARY

In this study a total of 288 soil and water samples were processed by standard selective microbiological technique to isolate actinomycetes. Altogether 320 strains were subjected to primary screening by perpendicular streak method to explore bioactive strains against some test bacteria and fungi (*Candida albicans*). Selected strains exhibiting broad spectrum activity against all test microbes were subjected to cultivation in starch casein broth and secondary screening was performed by agar well method. Minimum inhibitory concentration (MIC) of ethyl acetate was evaluated by tube dilution method. Phenotypic characterization of screened strains was carried out by cultural, microscopic and several bio chemical test. Optimization of cultural parameters was carried out by cultivating the strains at different nutritional and cultural conditions.

Out of 320 actinomycetes isolates 120 (37.5%) were found bioactive against one or more test microbes. Among 120 bioactive isolates 100 were recovered from soil sample. The most pre-dominant bioactive isolates produced dirty white (16.7%) and white (16.7%) colored pigments followed by grey (12.5%) and least were green (5%) and brown (5%). The results revealed that 68 (56.7%) bioactive isolates were inhibitory to Gram positive only followed by 42 (35%) against Gram negative, 6 (5%) against both Gram-positive and Gram-negative bacteria and only 4 (3.3%) against all test organisms during primary screening. In secondary screening greatest antimicrobial activity was shown by A_3 strain against *C. albicans* (41.33 ± 1.15mm). In addition, P_4 strain gave highest zone of inhibition (ZOI) against *E. coli* ATCC 25922 (26.00 ± 00mm) and MRSA (31.33 ± 3.21mm). In MIC evaluation of ethyl acetate extract A_3 strain showed lower values against all test microbes and lowest against *C. albicans* (0.125mg/ml). Phenotypic characteristics revealed that the strains D_2, P_4 and J_1 were most probably *S. violarus, S. krainskii* and *S. tsusimaensis* respectively whereas the most potent strain A_3 was genotypically characterized as *Nocardiopsis* prasina.

During optimization it was found that mono saccharides and disaccharides supported maximum biomass production by all strains. Starch was found to be most appropriate for maximum bioactive metabolite production by all strains. Biomass production of all strains except *S. krainskii* was found to be maximum in presence of casein as nitrogen

source. Similarly, bioactive metabolite production by all strains except *S. violarus* was observed in presence of casein. In case of temperature maximum biomass production by all strains except *S. tsusimaensis* was found maximum at 30^0C and same temperature supported bioactive metabolite production by all strains. Likewise, maximum biomass production by *N. prasina, S. violarus* and *S. tsusimaensis* was found at pH 8 whereas for *S. krainskii* it was at pH 7. Bioactive metabolite production by majority of strains was optimum at pH 8. Regarding duration of incubation maximum metabolite production by all strains except *S. krainskii* was recorded on 8^{th} day. Among the minerals tested in this study $MgCl_2$ supported for maximum biomass production but all strains except *S. violarus* for which KNO_3 was found most suitable. Maximum metabolite production by all strains except *S. violarus* was observed in presence of KNO_3.

The FT-IR analysis of ethyl acetate extract of all actinomycetes strains exhibited common functional groups, OH, CH, aromatic and C-O but the extracts from *N. prasina* and *S. tsusimaensis* showed C=C stretching whereas *S. violarus* and *S. krainskii* showed C=O stretching. The LC-MS analysis of the bioactive metabolites of *N. prasina,* revealed four major compounds; likewise, six major compounds were eluted at different retention time in case of *S. violarus* Similarly, *S. krainskii* and *S. tsusimaensis* displayed four and five major compounds respectively.

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APPENDIX I

A. Reagents/ Chemicals

Chemicals	Manufacturers
1. Acetic acid	Qualigens, India
2. Agar powder	Hi Media, India
3. Alpha-naphthylamine	Bengal chemicals, India
4. Barium chloride	Merck, India
5. Beef extract	Qualigens, India
6. Bromo phenol blue	Merck, India
7. Calcium carbonate	Merck, India
8. Casein	Merck, Germany
9. Chloramphenicol	Hi Media, India
10. Chloroform	Qualigens, India
11. Crystal Violet	Lobo chemicals, India
12. Cycloheximide	Hi Media, India
13. Dehydrated alcohol	Bengal chemicals, India
14. Dextrose	Qualigens, India
15. Dichloromethane	Qualigens, India
16. Dipotassium hydrogen phosphate	Qualigens, India
17. Ethyl acetate	Qualigens, India
18. Ferric citrate	Qualigens, India
19. Ferrous sulphate	S.D. fine-chem. Ltd. India
20. Fructose	Lobo chemicals, India
21. D (+) Galactose	Qualigens, India
22. Gelatin	Qualigens, India
23. Glacial acetic acid	Qualigens, India
24. D (+) Glucose	Qualigens, India

25. Glycerol	Qualigens, India
26. Hydrochloric acid	Qualigens, India
27. Hydrogen peroxide	Qualigent, India
28. Iodine	Qualigens, India
29. Isoamyl alcohol	Qualigens, India
30. Linezolid	Hi Media, Indi
31. Magnesium sulphate	Lobo chemicals, India
32. Magnesium chloride	S.D. fine-chem, Ltd. India
33. Maltose	Hi Media, India
34. Mannose	Hi Media, India
35. Mercuric chloride	Merck, India
36. Methanol	Qualigens, India
37. n-Butanol	Qualigens, India
38. Nalidixic acid	Hi Media, India
39. Nitrofurantoin	Hi Media, Indi
40. Peptone	Qualigens, India
41. Phenol	Qualigens, India
42. Phenol red	Qualigens, India
43. Potassium iodide	Qualigens, India
44. Potassium nitrate	Merck, India
45. Potassium dihydrogen phosphate	Qualigens, India
46. Proteinase K	Merck, India
47. Raffinose	Hi Media, I
48. Salicin	Merck, Indi
49. Sodium chloride	Hi Medium, India
50. Sodium hydroxide	Hi Media, India
51. Starch	Hi Media, India
52. Sucrose	Qualigens, India
53. Sulphanilic acid	S.D. fine-chem. Ltd. India
54. Sulphuric acid	Qualigens,
55. Tetracycline	Hi Media, India

56. Urea crystal	Merck, India
57. Xylose	Hi Media, India
58. Zinc powder	Merck, India

B. Glassware

1. Beaker
2. Conical Flask
3. Glass Rod
4. Measuring Cylinder
5. Petri dish
6. Pipette
7. Slides
8. Separating flask
9. Test-Tubes
10. Volumetric Flask

C. Equipments

Equipment	**Manufacturer**
1. Autoclave	Life, India
2. Cooling Centrifuge	Remi
3. Distillation unit	Mirage
4. Electric Weighing Machine	OHAUS
5. FT-IR instrument	PerkinElmer, USA
6. Hot water bath	Grant
7. Incubator	InDOEXIM
8. LCMS-2020 system (ESI-Single Quad)	Shimadzu, Japan
9. Microcentrifuge tubes	Eppendorf
10. Microcentrifuge	Eppendorf
11. Micropipette tips	Eppendorf

12. Microscope	Olympus
13. Microwave oven	LG
14. Oven	InDoEXIM
15. PCR tubes	Eppendorf
16. pH meter	Philip Harris
17. Refrigerator	Samsung
18. Thermocycle	GeneAmp
19. Water bath shaker	GRANT

D. Pipettes and Tubes

1. Adjustable pipettes for 10, 20, 200 and 1000µl
2. Eppendorf Tubes
3. Micropipette
4. Micropipette tips
5. PCR tubes

E. Miscellaneous

1. Bunsen Burner
2. Cotton
3. Forceps
4. Gloves
5. Inoculating loop
6. Labelling stickers
7. Soaps
8. Staining rack
9. Test-Tube holder
10. Tissue paper

APPENDIX II: Test Microorganisms

A. Referenced culture

1. *E. coli* ATCC 25922

B **Clinical isolates**

1. *Bacillus subtilis*
2. *Salmonella typhi*
3. *Acinetobacter baumannii* (MDR)
4. *Candida albicans,*
5. 5. *Staphylococcus aureus* (MDR*)*

APPENDIX III: Bacteriological Media

A. Composition and preparation of different media:

(Note: All compositions are given in grants per liter and at 25^0C temperature)

1. Basal Medium for carbohydrate utilization tests

Ingredients	**gm/l**
Peptone	10.0
Sodium chloride	5.0
Phenol red	0.018

Final pH at 25^0: 7.4

2. Gelatin Agar

Ingredients	**gm/l**
Nutrient agar	28.0
Gelatin	10.0

Final pH at 25^0C: 5.8

3. Muller Hinton Agar (MHA)

Ingredients	**gm/l**
Beef infusion	300.0
Starch	1.5
Casein	17.5
Agar	10.0

Final pH at 25^0C: 7.3 ± 0.2

4. Nitrate broth

Ingredients	**gm/l**
Beef extract	3.0

Peptone	5.0
Potassium nitrate	1.0

Final pH at 25^0C:6.8$\pm$0.2

5. Nutrient agar

Ingredients	**gm/l**
Peptone	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Agar	15.0

Final pH at 25^0:7.2

6. Nutrient Broth

Ingredients	**gm/l**
Peptone	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5

Final pH at 25^0C: 7.2

7. SIM (Sulphide indole motility) agar

Ingredients	**gm/l**
Peptone	30.0
Beef extract	3.0
Ferrous ammonium sulphate	0.2
Sodium thiosulphate	0.025
Agar	3.0

Final pH at 25^0C: 7.3

8. Simmon's citrate agar

Ingredients	**gm/l**
Ammonium dihydrogen phosphate	1.0
Dipotassium hydrogen phosphate	1.0
Sodium chloride	5.0
Sodium citrate	2.0
Magnesium sulphate	0.2
Bromothymol blue	0.08
Agar	15.0

Final pH at 25^0C: 6.9

9. Starch agar

Ingredients	**gm/l**
Nutrient agar	28.0
Starch	10.0

Final pH at 25^0C: 5.8

10. Starch casein agar

Ingredients	**gm/l**
Soluble starch	10.0
Casein	0.3
Potassium nitrate	2.0
Dipotassium hydrogen orthophosphate	2.0
Sodium chloride	2.0
Magnesium sulphate	0.05
Calcium carbonate	0.02
Ferrous sulphate	0.01
Agar-agar	20.0

Final pH at 25^0C: 7.2

11. Starch casein broth

Ingredients	**gm/l**
Soluble starch	10.0
Casein	0.3
Potassiumnitrate	2.0
Dipotassium hydrogen orthophosphate	2.0
Sodium chloride	2.0
Magnesium sulphate	0.05
Calcium carbonate	0.02
Ferrous sulphate	0.01

Final pH at 25^0C: 7.2

12. Urea agar base

Ingredients	**gm/l**
Peptone	1.0
Dextrose	1.0
NaCl	5.0
KH_2PO_4	2.0
Phenol red	0.012
Agar	12.0

Final pH at 25^0C: 6.8

Twenty-four gram of the medium was dissolved in 950 ml of distilled water and then boiled to dissolve completely and autoclaved at 121^0C for 15 minutes. After cooling to 50^0C, 50 ml of sterile 40% urea solution was poured into the medium and mixed with gentle rotation. Then 5 ml of the medium was dispensed in each tube and slant was prepared.

APPENDIX IV: Reagents/Chemicals

A. Composition and preparation of different staining reagents

a. Crystal Violet Solution

Crystal violet	20.0 gm
Ammonium oxalate	9.0 gm
Ethanol or Methanol	95.0 ml
Distilled water	1000 ml

Preparation: Twenty grams of crystal violet was weighed in a clean piece of paper and transferred to a clean brown bottle. Then 95.0 ml of ethanol was added and mixed until the dye was completely dissolved. To the mixture, 9.0 grams of ammonium oxalate dissolved in 200 ml of distilled water was added. Finally, the volume was made 1000 ml by addition of distilled water.

b. Lugol's Iodine

Potassium iodide	20 gm
Iodine	10 gm
Distilled water	1000 ml

Preparation: To 250 ml of distilled water, 20 gm oof potassium idide was dissolved and 10 gm of iodine was mixed to it until it was dissolved completely. Finally, the volume was made 1000 ml by addition of distilled water.

c. Mercuric chloride solution

Mercuric chloride	10.0 gm
Conc. Hydrochloric acid	20.0 ml
Distilled water	100.0 ml

B. Biochemical test reagents, preparation procedures and tests

a. Carbohydrate Utilization Test

For this test, the basal medium (Appendix II) containing peptone, sodium chloride and phenol red (Appendix II) was incorporated with carbohydrate at the concentration of 1% (w/v). Carbohydrates used were glucose, galactose, fructose, maltose, mannose, xylose, silicon, raffinose, inulin and sucrose. Each carbohydrate stock solution was heated for 30 minutes at 60^0 before adding appropriate volume to the already sterilized basal medium. Then, the carbohydrate containing basal medium was inoculated with the active actinomycetes colonies and incubated at 28^0C for 2 weeks. Positive test was indicated by the change in color of the medium from red to yellow.

b. Catalase Test

Catalase reagent (3% H_2O_2)

Hydrogen peroxide	1.0 ml
Distilled water	9.0 ml

Preparation: To the 9.0 ml of distilled water, 1 ml of hydrogen peroxide was added and mixed well so as to make 3% solution of hydrogen peroxide.

Picking up a few colonies with a sterile glass rod and mixing it with a drop of 3% h_2O_2 on a clean glass slide. Positive test was indicated by the appearance of gas bubbles.

c. Citrate Utilization Test

In this test, the slant of Simmon's Citrate Agar was streaked with the active actinomycetes colonies and incubated at 28^0C for 2 weeks. Change of dark green color to Prussian blue color of the medium implied utilization.

d. Nitrate reduction test

Sulphanilic acid	0.8 gm
a-naphthylamine	0.5 gm
Acetic acid	5.0 M

Preparation: Eight-hundred milligram of sulphanilic acid was dissolved in 100ml of 5.0 M acetic acid and 5.0 of a-naphthylamine was dissolved in 5.0 M acetic acid. Then, both are mixed in 1:1 v/v.

Nitrate broth (Appendix II) was inoculated with the active actinomycetes colonies incubated at 28^0C for 2 weeks. Nitrate reduction was detected by adding a few drops of sulphanilic acid reagent and a-naphthylamine reagent into the culture broth. Development of red or pink indicated the presence of nitrate reductase enzyme.

e. Oxidase Test

Oxidase strip soaked in oxidase reagent Tetramethylparaphenylenediaminedihydrochloride (TPD)	1 gm
Distilled water	100.0 ml

Preparation: One gram of TPD was dissolved in 100 ml of distilled water and strip of Whatman no. 1 paper was soaked and drained for about 30 seconds. Then the strip was freeze and stored in dark bottle tightly.

Picking up a few colonies with a sterile glass rod and rubbing it on the oxidase paper (Whatman No.1filter paper impregnated with 1%tetramethyl-p-phenylenediamine dihydrochloride). Positive test was indicated by the

development of Intense deep purple color on the oxidase paper within 10 seconds.

f. Sulphide Indole Motility test

Kovac's indole reagent

Para dimethyl amino benzaldehyde	2.0 gm
Isoamyl alcohol	30.0 ml
Concentrated hydrochloric acid	10.0 ml

Preparation: In 30 ml of isoamyl alcohol, 2 gm of para amino benzaldehyde was dissolved and transferred to clean brown bottle. Then to this solution, 10 ml of concentrated hydrochloric acid was added and mixed well.

Sulphide indole motility (SIM) medium was stabbed with the active actinomycetes colonies and incubated at 28^0C for 2 weeks.

H_2S production was detected by blacking of the medium. Indole production was decteted cherry red color at the interface upon addition of Kovac's reagent (Appendix II). Motility was indicated by hazy growth of the actinomycetes away from the stabled line on the medium.

g. Urea hydrolysis Test

Urea agar (Appendix II) slants were streaked with the active actinomycetes colonies and incubated at 28^0C for 2 weeks. Positive test was indicated by the change of the color of the medium from orange to red-violet.

C. Physiological tests

a. Gelatin Hydrolysis Test

Gelatin agar (Appendix II) plates were inoculated with the active isolates and incubated at 28^0C for 2 weeks. Appearance of zone of hydrolysis around

the colonies that produce proteases was detected upon flooding the plate with mercuric chloric solution.

b. Starch Hydrolysis Test

Starch agar (Appendix II) plates were inoculated with the isolates and incubated at 28^0C for 2 weeks. Starch hydrolysis was confirmed by flooding the plates with iodione solution to see for the clear zone of hydrolysis around the colonies that produce a-amylase.

c. Sodium Chloride Tolerance Test

NA plates with 3%, 5% and 7% NaCl were inoculated with the isolates with the isolates and incubated at 28^0C for 3 weeks. Positive test was indicated by growth of the isolates.

d. Temperature Tolerance Test

NA percent were inoculated with the active isolates and incubated at 15^0C, 37^0C and 45^0 for 2 weeks. Positive test was indicated by growth of the isolates.

D. Turbidity standard equivalent to McFarland 0.5

One percent v/v solution of sulphurica acid was prepared by adding 1 ml of concentrated sulphuric acid to 99 ml of distilled water. 1% w/v solution of Barium Chloride was prepared by dissolving 0.5 gm of dehydrate barium chloride in 50ml of distilled water. Then to the 99.5 ml of 1% sulphuric acid solution, 0.5 ml of barium chloride solution was mixed and stirred continuously. The solution was transferred into the clean screw capped tube and stored at dark place until used.

APPENDIX: V

A. Reagents, Solutions and Kits for DNA extraction

1. Absolute Ethanol
2. Buffer CB
3. Elution Buffer
4. Lysis Enhancer
5. Proteinase K
6. RNase A
7. SpinstarTM DNA Extraction Kit
8. Wash Buffer 1
9. Wash Buffer 2

B. Primers used in 16SrRNA Sequencing

1. PCR Primer Name Primer Sequences

   27F 5' (AGA GTT TGA TCM TGG CTC AG) 3

   1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'

2. Sequencing Primer Name Primer Sequences

   785F 5' (GGA TTA GAT ACC CTG GTA) 3'

   907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'

C. Sequence of *N. prasina* (A_3)

AAAGTCGCGTCTCCCATGCGGGCGCTTAATGCGTTAGCTACGGC
GCGGAAACCGTGGAAAGTCCCCACACCTAGCGCCCAACGTTTA
CGGCATGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCATGC
TTTCGCTCCTCAGCGTCAGGTAAGGCCCAGAAACCCGCCTTCGC
CACCGGTGTTCCTCCTGATATCTGCGCATTTCACCGCTACACCA
GGAATTCCAGTTTCCCCTACCTACCTCTAGCATGCCCGTATCCA
CTGCAGAACCGGAGTTAAGCCCCGGTCTTTCACAGCAGACGCG
ACATGCCGCCTACGAGCTCTTTACGCCCAATAATTCCGGACAAC
GCTCGGACCCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGC
CGGTCCTTATTCCCCACCTACCGTCAACCCCACGAGAACGTGGA
GCCTGCGTTGGTGGTAAAAGAGGTTTACAACCCGAAGGCCGTC
ATCCCCCACGCGGCGTCGCTGCATCAGGCTTTCGCCCATTGTGC
AATATTCCCCACTGCTGCCTCCCGCAGGAGTCTGGGCCGTGTCT
CAGTCCCAGTGTGGCCGGTCGCCCTCTCAGGCCGGCTACCCGTA
ATCGCCTTGGTAGGCCGTTACCCCACCAACAAGCTGATAGGCC
GCGAGCCCATCCCTGACCGATAAAACTTTCCACCCTCCACCATG
AGGTGGCGGGTCGTATCCGGTATTAGACGGCGTTTCCACCGCTT
ATCCCGGAGTCAGGGGCAGGTTGCTCACGTGTTACTCACCCGTT
CGCCGCTCGTGTACCCCGAAGGGCCTTACCGCTCGACTTGCATG
TGTTAAGCACGCCGCCAGCGTTCGTCCTGAGCGAGGGAAAAAA
ACAAAAAAAAAGAAAAAAACAAAGGGGGGGGGGGGGGTGTT
TTTTTTTTCCACCCCAAAAAAAAAAAAAAAAGGGTTGTATCCC
CCCCCCGGGCCGGGAAAGAAAAAAAAGGAATTTTTTTTTGGGG
GGGGGAGGGGGGGGGGGGGGGGCCCCCCCCCCCCCCCCGTTGG
GGAAGGGGGGGGGGTAAAAAAAAAACCCCAAAAACCCCCCTT
TTCTTTTTTTTCCTTCCTCCTTATGGGGGGGGGGGGGTGAAAAA
AAGGAGAGGTTCTCCCCCCAAAAGGGGGGGGAGGGGGGGAAA
AAAACCTTTTCTCTTTTTCCCTTTGGGGGGGGGGGGCCCAAAAA
AGGGGGAAAAGGGGGGGGGAAAGGGGGGGGGGAAAAAAA

D. 16SrRNA Sequencing of *N. prasina*(A_3)

Standard ID

16S rRNA Full Sequencing — Humanizing Genomics macrogen

16S rRNA service report

Order Number : 200217FN-079
Sample name : A3_contig_1

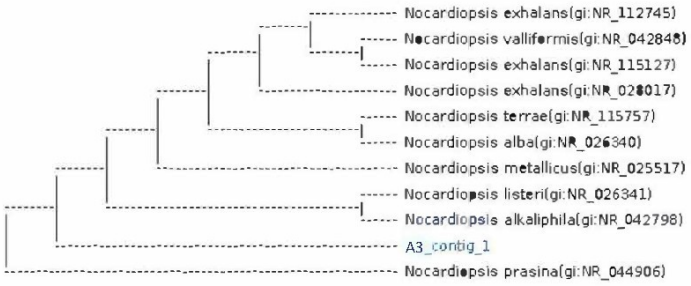
Information

Primer Information

Sequencing Primer Name Primer Sequences	PCR Primer Name Primer Sequences
785F 5' (GGA TTA GAT ACC CTG GTA) 3'	27F 5' (AGA GTT TGA TCM TGG CTC AG) 3
907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'	1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'

Subject						Score		Identities	
Accession	Description	Length	Start	End	Coverage	Bit	E-Value	Match/Total	Pct.(%)
NR_044906.1	Nocardiopsis prasina	1461	1	1458	99	2686	0.0	1457/1458	99

Kingdom	Family	Genus	Species
Bacteria	Nocardiopsaceae	Nocardiopsis	Nocardiopsis prasina



APPENDIX: VI

Paper Published

1. Lekhak, B., Singh, A. and Bhatta, D.R. (2018). Antibacterial and antifungal property of actinomycetes isolates from soil and water of Nepal. *J Nepal Health Res Counc*, 16(39), 136-139.

2. Lekhak, B., Singh, A. and Bhatta, D.R. (2018). Optimization of cultural parameters for bioactive metabolite production by potent actinomycetes isolated from soil of Nepal. *Physiol. Ecol. & Environ. Sci.,* 9(1&2), 15-27.

Original article

J Nepal Health Res Counc 2018 Apr-Jun;16(39): 136-9

Antibacterial and Antifungal Property of Actinomycetes Isolates from Soil and Water of Nepal

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[1]Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal.

ABSTRACT

Background: Human pathogens are rapidly acquiring resistance to antibiotics leading to treatment failure. We carried out this study to isolate and screen actinomycetes strains that have potential to kill bacterial and fungal pathogens.

Methods: In this descriptive study 288 soil and water samples were processed by standard microbiological techniques at Central Department of Microbiology,Tribhuvan University from 2013 to 2015. Screened actinomycetes were cultivated for bioactive metabolite production and minimum inhibitory concentration (MIC) of metabolites were determined against bacterial pathogens including multidrug resistant bacteria and fungi.

Results: One hundred twenty isolates having antimicrobial property were screened. Out of them, four most potent strains, *Nocardiopsis prasina, Streptomyces violarus, Streptomyces krainskii* and *Streptomyces tsusimaensis* were identified all having both antibacterial and anti-fungal property.Highest zone of inhibition (ZOI)was given by *N. prasina* against *Candida albicans*(41.33 ±1.15mm) and among bacteria, maximum ZOI was against *Acinetobacter baumannii*(31.33±3.05mm). MIC value of metabolite of *N.prasina* was 0.125mg/ml for *E.coli* and *C. albicans.* It was 2.5 mg/ml each for methicillin resistant *Staphylococcus aureus* (MRSA), *A. baumannii* and *Salmonella* Typhi and 0.625 mg/ml for *Bacillus Subtilis*.

Conclusions: Bioactive metabolite producing actinomycetes were recovered from soil and tested against human pathogenic bacteria and fungiand found to have antibacterial and antifungal property.

Keywords: Actinomycetes; bioactive metabolite; MIC; zone of inhibition.

INTRODUCTION

Antimicrobial resistance creates a great threat for effective prevention and control of several diseases caused by bacteria, fungi, viruses and other parasites. Multidrug resistant *Klebsiella pneumoniae*, fluoroquinolone resistant *E.coli*, third generation cephalosporin resistant *Neisseria gonorrhoae*, methicillin resistant *Staphylococcus aureus* (MRSA) and Colistin resistant enterobacteriaceae and many other microbes have been reported from different parts of the globe.[1] Human pathogens such as carbapenem resistant *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and enterobacteriaceae have been given top priority. Similarly, vancomycin resistant enterococci, MRSA, fluoroquinolone resistant *Salmonella*, *Campylobacter* and *Shigella* spp. along with many other multidrug resistant pathogens create problem so that new antibiotics should be developed to address resistance problem.[2] Antibiotics are bioactive secondary metabolites produced by bacteria, fungi and plants. Among diverse microbes actinomycetes are most capable candidates of producing antibiotics. Out of 22,500 biologically active compounds obtained from microbes, 45% are from actinomycetes, 38% from fungi and 17% from other bacteria.[3] Over 5000 antibiotics have been identified from the culture of Gram +ve, Gram -ve bacteria and fungi.[4] Among actinomycetes, various *Streptomyces* spp. account for more than 70% of total antibiotic production followed by other species.[5] Actinomycetes are Gram +ve, filamentous bacteria with high guanine+cytosine content of over 55% in their DNA.[6] Actinomycetes are natural inhabitant of soil, fresh water, marinewater, lakes and sediments and even found in extreme environment such as Himalayas and hot springs.[7] Hence, this study was conducted to isolate and screen potent antibiotic producing strains of actinomycetes against bacterial and fungal pathogens.

METHODS

This study was carried out at Central Department of Microbiology, Tribhuvan University. In this study, 240 soil and 48 water samples were collected from different geographical locations of Nepal and actinomycetes

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were isolated by spread plate technique on starch casein agar.[8] Isolates were primarily screened for antimicrobial property against bacterial and fungal pathogens by perpendicular streak method on Mueller Hinton agar.[9] The strains showing antimicrobial property were cultivated under optimum conditions in starch casein broth at 30°C for 7 days at 150 rpm and bioactive metabolites were extracted in ethylacetate. The extracts were subjected to secondary screening against *Bacillus subtilis*, MRSA, *E.coli*ATCC25922, *Acinetobacter baumannii*(MDR), *Salmonella* Typhi(MDR) and *Candida albicans* by agar well diffusion method.[10] Minimum inhibitory concentration(MIC) of extracts were determined by tube dilution method.[11] Strains exhibiting both antibacterial and antifungal activities were characterized phenotypically on the basis of gram straining, sugar utilization test (Glucose, Mannitol, Sucrose, Fructose, Xylose etc.) substrate utilization test such as starch casein and gelatin, tolerance to different temperatures and sodium chloride concentration.[12] Molecular characterization was carried out by extracting DNA running PCR using universal primer and sequencing.[13]All the data generated were entered in excel file and SPSS version 20 and mean, standard deviation, frequency and percentage were calculated.

RESULTS

A total of 288 soil and water samples from different geographical locations of Nepal were subjected to isolation of actinomycetes. Altogether 120 different actinomycetes showing antimicrobial properties were separated on the basis of pigmentation (Figure 1). Actinomycetes strains producing white and dirty white pigments were most predominant each 17%.

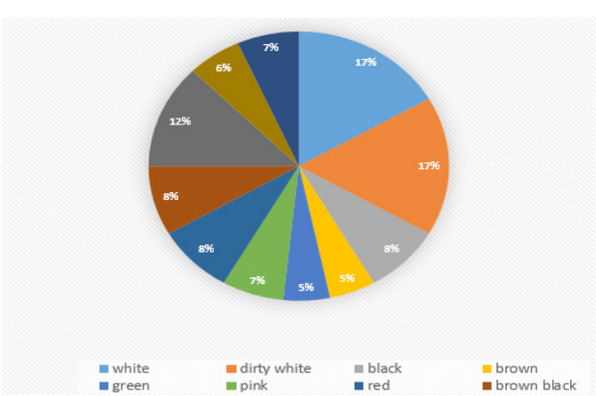

Figure 1: Types of actinomycetes on the basis of pigmentation

Among them 60 isolates were active against only Gram +ve bacteria, 44 isolates against only Gram -ve bacteria, 6 isolates showed activity against both Gram +ve and Gram -ve bacteria while 4 isolates exhibited both antifungal and antibacterial activity. Four most potent isolates were identified as *Nocardiopsis prasina*(A_3), *Streptomyces violarus* (D_2), *Streptomyces krainskii* (P_4) and *Streptomyces tsusimaensis* (J_1) (Table 1).

Table 1. Activity of bioactive actinomycetes against bacteria and fungi.

S.N.	Colony color	Active against			
		Gram +ve only	Gram -ve only	Both Gram +ve and Gram -ve	Fungi and Bacteria
1	White	8	8	2	2
2	Dirty White	8	8	3	1
3	Black	3	7	0	0
4	Brown	1	5	0	0
5	Green	4	2	0	0
6	Pink	6	2	0	0
7	Red	4	6	0	0
8	Brown Black	7	3	0	0
9	Grey	10	3	1	1
10	Yellow	7	0	0	0
11	Purple	2	0	0	0

Antimicrobial activity of bioactive compound extracted in ethyl acetate is shown in Table 2. Out of 4 potent isolates *N. prasina* showed highest antimicrobial activity against *Candida albicans* (41.33±1.15), *Salmonella* Typhi(24.33±2.08), *Acinetobacter baumannii* (31.33±3.05), MRSA (30.67±7.02) and *Bacillus subtilis*(24.67±2.08) while *S.krainskii* showed highest zone of inhibition against *E.coli* (26±0.00).

In MIC evaluation of bioactive compound, *Nocardiopsis prasina* showed lowest values against all test organisms 0.125mg/ml for *C. albicans* and *E.coli*, 2.5mg/ml for S. Typhi, *A. baumannii* and MRSA. For *Bacillus subtilis* all isolates gave same value 0.625mg/ml (Table 3).

Table 2. Antimicrobial activity of extract against selected pathogens.

Strain	Zone of inhibition (mean±S.D.)mm					
	Bacillus subtilis	MRSA	*E.coli* ATCC 25922	*Acinetobacter baumanii*	*Salmonella Typhi*	*Candida albicans*
A3	24.67±2.08	30.67±7.02	23.33±4.93	31.33±3.05	24.33±2.08	41.33±1.15
D2	17.33±1.15	19.33±1.52	15.00±1.00	18.33±1.15	14.00±1.00	24.67±1.52
PY	24.33±0.57	31.33±3.21	26.00±0.00	28.00±2.00	19.00±1.00	29.33±0.57
J1	14.33±2.08	23.33±2.08	24.67±0.57	21.67±1.52	17.33±0.57	28.67±1.52

Table 3.MIC values of bioactive compounds from different strains.

Strains	MIC against(mg/ml)					
	B. Subtilis	MRSA	*E.coli* ATCC25922	*A.baumannii*(MDR)	*S.*Typhi(MDR)	*C. albicans*
A_3	0.625	2.5	0.125	2.5	2.5	0.125
D_2	0.625	5	2.5	5	5	2.5
P_4	0.625	5	2.5	2.5	2.5	2.5
$1(J_1)$	0.625	5	5	5	2.5	2.5

DISCUSSION

In this study, we have isolated and screened bioactive compound producing actinomycetes strains. White and gray colored actinomycetes were predominant and most of them were active against Gram +ve bacteria. This result is similar with Mabrouk and Saleh(2014) who reported dominance of white and gray actinomycetes with 64.3% active against Gram +ve bacteria.[9] Higher susceptibility of Gram +ve bacteria is due to lacking outer lipopolysaccharide which is impermeable to lipophilic compounds.[14] Results of our study is supported by Vengadesh et al., who found actinomycetes isolate A_5 was inhibitory to *Bacillus subtilis, E.coli, C.albicans and Aspergillus flavus*.[15] Our results are in agreement with Singh et al (2016) who observed high antibacterial activities of three actinomycetes strains against many test bacteria including MRSA, vancomycin resistant enterococci (VRE) and *Klebsiella pneumoniae*.[16] Results of this study showed that all four potent actinomycetes were active against bacteria and fungi with *N. prasina*as best candidate. MIC values of bioactive metabolite ranging from 0.125mg/ml to 5mg/ml. Similar to our findings, MIC value of metabolite of active actinomycetes as 1.25mg/ml for MRSA and other many bacteria. Similarly, MIC was 2.5mg/ml against VRE, *Shigella dysenteriae* and *Klebsella pneumoniae*.[17] In contrast, Satish and Kokati(2017) reported low MIC value of 1mg/ml for MDRSA.[18] MIC value is affected by many parameters including susceptibility of organisms, type of microorganism, concentration and type of bioactive metabolites, composition of cultural medium, incubation temperature and time.[15]

CONCLUSIONS

Our study showed that soil and water of Nepal contained diverse actinomycetes strain that can inhibit the growth of some bacteria and *Candida albicans*. Among screened isolates *N. prasina* was found to be the most effective against test bacteria and fungi. Further studies regarding characterization of bioactive compound is essential.

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I am grateful to my supervisor Prof Dr Dwij Raj Bhatta and co-supervisor Prof Dr Anjana Singh for their valuable suggestion to develop concept and designing research and also guiding me during my laboratory work.Equally. I would like to acknowledge Prof Dr Giorgio Palu, Director, Department of Molecular Medicine, Padova University, Italy for providing laboratory facility to study antimicrobial activities of actinomycetes and sequencing actinomycetes strains.

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OPTIMIZATION OF CULTURAL PARAMETERS FOR BIOACTIVE METABOLITE PRODUCTION BY POTENT ACTINOMYCETES ISOLATED FROM SOIL OF NEPAL

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ABSTRACT

Actinomycetes are versatile producers of diverse secondary metabolites like antibiotics. The purpose of this investigation was to optimize environmental and nutritional conditions of screened actinomycetes isolates from soil of Nepal. Laboratory based descriptive study, submerged fermentation has been done in basal media to study the effect of the environmental and nutritional parameters on growth and bioactive metabolite production. Effects of various sources (carbon, nitrogen, and minerals), temperature, pH and incubation duration on metabolite production was determined by agar well diffusion method. Among screened isolates, 4 isolates were found to produce bioactive metabolites inhibitory to all test organisms (Gram positive, Gram negative and fungi) and identified as *Nocardiopsis* spp. (A_3) and *Streptomyces* spp. (D, P_4 and J_1). Out of all potent actinomycetes, *Nocardiopsis* spp. were found to be most active. Isolate A_3 showed maximum bioactive metabolite production with starch, casein and KNO_3 as carbon, nitrogen and mineral sources respectively, on the 8th day at pH 8 and temperature of 30°C. The optimum conditions for production by isolate D_2 were observed to be starch, casein and $MgCl_2$ as the carbon, nitrogen and mineral sources, pH 7, temperature 30°C and incubation period of 8 days. Similarly, for isolate P_4, optimal production was observed on 6th day at pH 7 and temperature 30°C with starch, casein and KNO_3 as carbon, nitrogen and mineral sources. Likewise, for isolate J_1, high levels of bioactive metabolite production were observed when starch, casein and KNO_3 were used as carbon, nitrogen and mineral sources on 8th day at pH 8 with an incubation temperature 30°C. All the four strains responded differently requiring specific conditions for maximum growth and bioactive metabolite production. All of them have capacity to synthesize active metabolites and hence further investigation regarding molecular characterization along with structure elucidation of compounds is necessary for their further application at commercial level.

Key words: Nocardiopsis, Streptomyces, Bioactive metabolites, Antibiotic

INTRODUCTION

Actinomycetes are Gram positive, filamentous, spore forming bacteria with tremendous capacity to biosynthesize several secondary metabolites (Jorgensen *et al.* 2010, Kim *et al.* 2015). The ability of *Streptomyces* to synthesize several broad spectrum antibiotics have attracted research due to global emergence of multi-drug resistant bacterial and fungal pathogens (Kuti *et al.* 2002). Bioactive compounds are increasingly required for diverse biotechnological applications. One of the main targets is focused on the discovery of new drugs, such as antibiotics,

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to combat antibiotic resistant pathogens (Payne *et al.* 2007). It is crucial that new groups of actinomycetes and other microbes from unexplored habitats are pursued as source of novel antibiotics and other bioactive compounds (Goodfellow and Fiedler 2010).

However, the ability of actinomycetes strains to produce bioactive products is not a fixed property but can be greatly increased or completely lost under different conditions of nutrition and cultivation (Waksman 1961). Therefore, designing an appropriate fermentation medium is of critical importance in the production of secondary metabolites (Gao *et al.* 2009). Changes in the nature and type of carbon and nitrogen sources have been reported to affect antibiotic biosynthesis in *Streptomyces* (Barratt and Oliver 1994, Reddy *et al.* 2011). Also several cultivation parameters like pH, incubation period, temperature and mineral salts play a major role in the production of bioactive metabolites (Usha Kiranmayi *et al.* 2011).

Soil samples were collected from different areas of Nepal and processed to isolate and screen actinomycetes strains having both antibacterial and antifungal activities. The potent isolates were characterized on the basis of cultural, morphological and biochemical tests and identified probably as *Nocardiopsis* spp. (A_3) and three *Streptomyces* spp. (D_2, P_4 and J_1). This investigation evaluated the influence of different carbon and nitrogen sources, temperature, pH, incubation duration and mineral salts on growth and bioactive metabolite production of four screened actinomycetes strains and would be useful for designing fermentation process at large scale production of antibiotics.

MATERIALS AND METHODS

The seed culture to be used as fermentation inoculum was prepared by transferring pure culture of screened actinomycetes strain from agar plates to basal medium consisted of g/l glucose 10, peptone 10, NaCl 10, $CaCO_3$ 2 in a 250 ml Erlenmeyer flask. Seed culture were grown in a water bath shaker at 30°C with constant shaking 150 rpm for 3 - 5 days. Then culture broth was centrifuged at 10000 rpm for 10 minutes. The pellet was washed twice with sterile distilled water and suspended in 20 ml sterile distilled water and used as inoculum (Ismet *et al.* 2004).

Effects of environmental factors and nutrient sources on growth and bioactive metabolite production: (i) carbon source: The effect of different carbon sources on growth and bioactive metabolite production was studied by replacing glucose in the basal medium with other carbon sources glucose, fructose, galactose, maltose, mannitol, sucrose, starch, and xylose. Triplicate flasks were set for each carbon source used. Each flask was inoculated with 10% (v/v) on inoculum and incubated at 30°C with 150 rpm for 7 days. After incubation, mycelia were separated by centrifugation and dried at 70°C in a desiccator until constant weight was obtained which was expressed as mg/100 ml. The content of flask was filtered and filtrate was mixed with equal volume of ethyl acetate. The mixture was vigorously shaken for 2 hours and solvent phase was dried at 50°C in a desiccator and residue was dissolved in phosphate buffer saline (pH 7.4) and tested against test microorganisms by agar well method to determine the effect of carbon source on bioactive metabolite production by measuringdiameter of zone of inhibition (Saadoum and Muhana 2008).

(ii) Nitrogen source: The effect of different nitrogen sources on growth and bioactive metabolite production was investigated by replacing soybean meal in the basal medium with other nitrogen sources beef extract, casein, peptone and tryptone. Triplicate flasks were set for each nitrogen source used. Each flask was inoculated with 10% (v/v) on inoculum and incubated at 30°C with 150 rpm for 7 days. After incubation, mycelia were separated by centrifugation and dried at 70°C in a desiccator until constant weight was obtained which was expressed as mg/100 ml. The content of flask was filtered and filtrate was mixed with equal volume of ethyl acetate. The mixture was vigorously shaken for 2 hours and solvent phase was dried at 50°C in a desiccator and residue was dissolved in phosphate buffer saline (pH 7.4) and tested against test organisms by agar well method to determine effect of nitrogen source on bioactive metabolite production the diameter of zone of inhibition was recorded (Saadoum and Muhana 2008).

(iii) pH: The effect of pH on biomass and bioactive metabolite production was studied by adjusting initial pH of basal medium with superior carbon and nitrogen source at 4, 5, 6, 7, 8, 9, and 10. Triplicate flasks were inoculated with 10% inoculum and incubated at 30°C with 150 rpm for 7 days. After incubation growth and bioactive metabolite production was determined (Oskay 2011).

(iv) Temperature: The triplicate basal medium with superior carbon and nitrogen source was inoculated with 10% inoculum and incubated at temperatures 20, 25, 30, 35 and 40°C for 7 days with 150 rpm. After incubation biomass and bioactive metabolite production was determined (Oskay 2011).

(v) Incubation period: Triplicate basal medium containing superior carbon and nitrogen source was inoculated with 10% inoculum in separate conical flasks and incubated at 30°C up to 12 days with 150 rpm. After each 2 days an aliquot of 10 ml broth was withdrawn and subjected to concentration and extraction with ethyl acetate. The extract was dissolved in phosphate buffer saline and subjected to antimicrobial test by agar well method (Reddy *et al.* 2011).

(vi) Minerals: The triplicate optimized medium with superior carbon and nitrogen source was supplied individually with different minerals $CuSO_4$, $MgCl_2$, $MgSO_4$, $FeCl_3$ and KNO_3 each at concentration 0.05% (w/v) and inoculated with 10% inoculum. Then all flasks were incubated at 30°C with 150 rpm for 7 days. After incubation growth and bioactive metabolite production was estimated

The association between different nutrient sources and other parameters with biomass production and bioactive metabolite production was analyzed by SPSS version 20.

RESULTS AND DISCUSSION

(i) Carbon sources: Biomass production of strain A_3 was high in all sugars except in xylose and highest in glucose (311.67 ± 10.48) but for D_2 strain, it was high in case of mannitol (247.33 ± 3.06) whereas for P_4 strain, biomass production was maximum in maltose (246.67 ± 2.89). In case of J_1, maximum biomass production was in sucrose (203.33 ± 5.77) (Fig. 1). All the strains

showed maximum antimicrobial activity against test organisms when starch was used as carbon source. Maximum antimicrobial activity was shown by A_3 strain against all organisms, 39.33 ± 0.57 mm against *C. albicans* (Fig. 2). Biomass and metabolite production varied with different carbon sources. Similar results have been reported by Bundale *et al.* (2015). They reported mono- and disaccharides for maximum biomass and starch for highest metabolite production for 3 out of 4 *Streptomyces* spp. Maximum bioactive metabolite production in presence of starch was also reported by El-Naggar *et al.* (2003) and Narayan and Vijayalakshmi (2008). Soluble starch has been found as best carbon source for antibiotic production by many researchers (Haque *et al.* 1995, Theobald *et al.* 2000). Biosynthesis of secondary metabolites such as antibiotics have been found to be reduced in presence of monosaccharides (Huck *et al.* 1991). In presence of sufficient readily metabolizable carbon source, metabolism of actinomycetes is directed towards cell mass production with lower secondary metabolite biosynthesis (Bundale *et al.* 2015). An excess amount of glucose in culture medium causes catabolite repression thereby inhibiting the key enzymes responsible for secondary metabolite biosynthesis (Reddy *et al.* 2011 and Lounes *et al.* 1996). Biosynthesis and optimum production of secondary metabolites is usually enhanced by cultivating microbes in slowly metabolizable nutrient sources such as polysaccharides (Jonsbu *et al.* 2002).

(ii) Nitrogen sources: All the strains, except P_4 produced maximum biomass when casein was used as nitrogen source whereas for P_4 strain tryptone was found to be an appropriate nitrogen source. Highest biomass was produced by A_3 strain (298.33 ± 7.64 mg/100 ml) and lowest biomass was produced by P_4 strain (105.00 ± 8.66). Similarly, bioactive metabolite production by all strains except D_2 was found to be maximum when casein was used as nitrogen source. Maximum antimicrobial activity was exhibited by strain A_3 against *C. albicans* (34.68 ± 0.58 mm) (Figs 3 and 4). There was a close association between nitrogen sources with biomass and metabolite production. Satyapathy and Mohapatra (2017) documented casein for maximum metabolite production. Peptone followed by casein has been observed as best nitrogen source for bioactive metabolite production by *Streptomyces* strains (Bundale *et al.* 2015, Reddy *et al.* 2011).

(iii) Temperature: Growth of strains A_3, D_2 and P_4 were found to be maximum at 30°C but for strain J_1, 35°C was found to be optimum (Fig. 5). Maximum growth was found in A_3 (301.67 ± 2.88) at 30°C and minimum biomass was produced by D_2 (78.33 ± 2.88) at 20°C. All strains showed maximum antimicrobial activity at 30°C and maximum inhibitory activity was observed in case of strain A_3 which showed highest zone of inhibition against *C. albicans* (37.00 ± 1.00) (Fig. 6). There was a significant association between temperature with growth and metabolite production ($p < 0.05$). This finding is in agreement with Bundale *et al.* (2015), they showed the effect of temperature on four different *Streptomyces* spp. and is also supported by Oskay (2011) who found 30°C as optimum for growth and metabolite production of *Streptomyces* spp. KGG32. Similar result was presented by Hassan *et al.* (2004) while studying the effect of temperature on *Streptomyces violarus* and also by Ibtisam *et al.* (2013) on *Streptomyces* spp. MS-266.

(iv) pH: Strains A_3, D_2 and J_1 produced maximum biomass at pH 8 whereas in case of strain P_4, it was maximum at pH 7 (Fig.7 and 8). Maximum growth production was observed in A_3 strain

at pH 8 (318.33±2.88) and minimum biomass was found in J_1 at pH 4 (58.33 ± 7.63). Antimicrobial activity of strains A_3, D_2 and J_1 were found to be maximum against all test organisms at pH 8 whereas strain P_4 showed maximum activity at pH 7. Maximum zone of inhibition was given by strain A_3 against *C. albicans* (38.67 ± 0.57) at pH 8. Minimum activity of all strains was observed at pH 4. The association between pH and biomass production was not found significant. However, significant association between pH and metabolite production was observed. Result for P_4 strain is supported by Bundale *et al.* (2015); they found pH 7 as best for both growth and metabolite production by *Streptomyces purpurascens*. Study of El-Naggar *et al.* (2003) reported maximum antibiotic production by *Streptomyces violarus*at pH 7.5 which is more or less similar with present result for strains A_3, D_2 and J_1. pH 7.5 was found to be the best for antibiotic production by *Streptomyces* spp. 201 (Thakur *et al.* 2009). Satyapathy and Mohapatra (2017) observed highest antibiotic production by *Arthrobacter* spp. SAS 16 at pH 8 which is similar with A_3, D_2 and J_1 in this study. pH of the growth medium significantly influences cell morphology, cell membrane permeability and activity of enzymes catalyzing metabolic reactions (Guimaraes *et al.* 2004).

(v) Incubation period: All the strains showed maximum antimicrobial activity on 8th day of fermentation except P_4 which showed maximum antimicrobial activity on 6th day of fermentation. None of the strains produced bioactive metabolites on 2nd day of fermentation. Strain A_3 exhibited highest antimicrobial activity against all test organisms. It showed maximum zone of inhibition (38.67±0.57) against *C. albicansas* (Fig. 9). The metabolite production by actinomycetes differed significantly with incubation duration. This result is supported by Satyapathy and Mohapatra (2017). Ibtisam *et al.* (2013) recorded maximum biomass and antibiotic production after 7th day but before 9th day by *Streptomyces* sp. MS-266 Dm4. Bundale *et al.* (2015) found maximum antibiotic production by two species of *Streptomyces* on 8th and 10th day. In the present investigation P_4 strain showed maximum antibiotic production on 6th day which is in agreement with Bundale *et al.* (2015). Actinomycetes massively increase biomass under exccess availability of nutrients rather than the secondary metabolite production; but under nutrient deficient condition, cell cycle is shifted to stationary phase and signals the transition from primary metabolism to secondary metabolite production (Abd-Allah and El-Mehalawy 2002).

(vi) Minerals: It was found that all the strains produced maximum biomass in presence of $MgCl_2$ except strain D_2 for which KNO_3 was found to be most appropriate (Fig. 10). Biomass production of A_3 strain was highest in all minerals. Maximum biomass production of A_3 strain was found in $MgCl_2$ (315.00 ± 5.00) and minimum biomass was produced by J_1 strain in $FeCl_3$ (76.67 ± 5.77). Strains A_3, P_4 and J_1 showed maximum antimicrobial activity in presence of KNO_3 in fermentation broth whereas D_2 showed antimicrobial activity against majority of test organisms in presence of $MgCl_2$ (Fig. 11). Maximum antimicrobial activity was shown by A_3 against *C. albicans* in presence of KNO_3 (35.33 ± 1.15) and minimum antimicrobial activity was shown by J_1 against *B. subtilis* in presence of $CuSO_4$ (6.67 ± 0.57). Variation of minerals used in fermentation broth significantly affected biomass and metabolite production by actinomycetes. This result is in

agreement with Bundale *et al.* (2015) who reported positive impact of $MgCl_2$ and KNO_3 on both growth and bioactive metabolite production of *Streptomyces* sp. El-Nagar (1991) explained that the salts of magnesium and potassium are considered as most appropriate for the growth and bioactive metabolite production of *Streptomyces*.

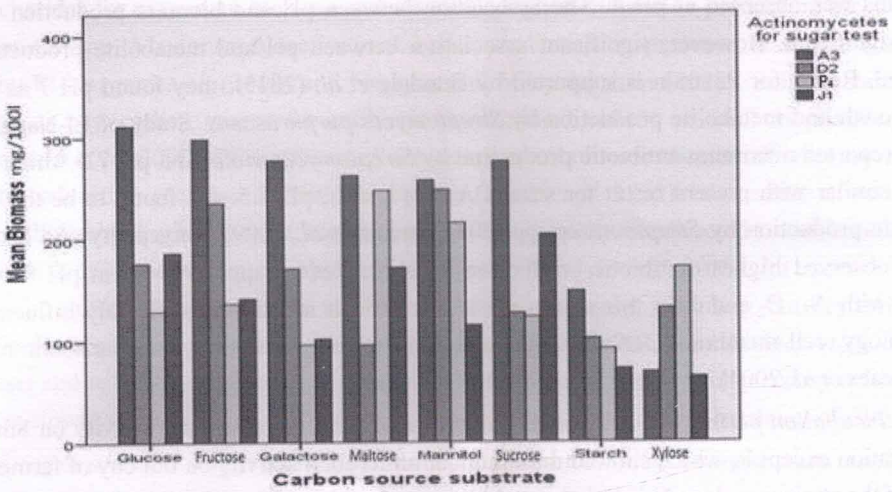

Fig. 1. Mean biomass production of different actinomycetes based on carbon source.

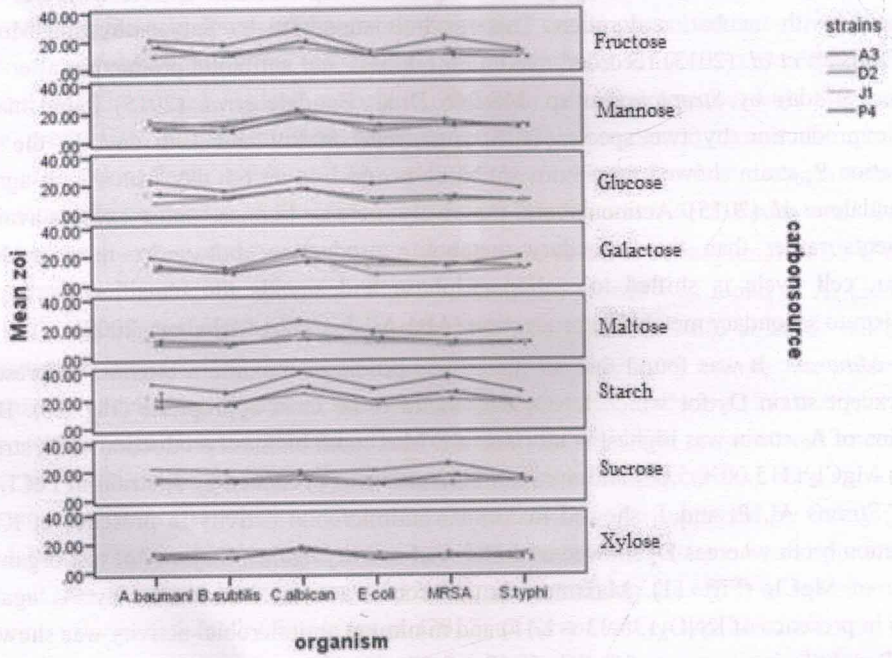

Fig. 2. Effects of carbon source on antimicrobial activity/ bioactive metabolite production.

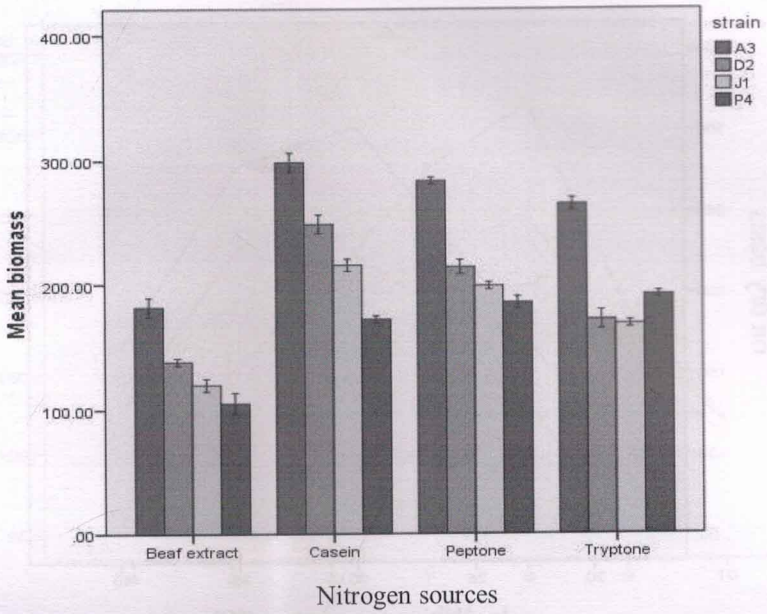

Fig. 3. Effects of nitrogen source on biomass production.

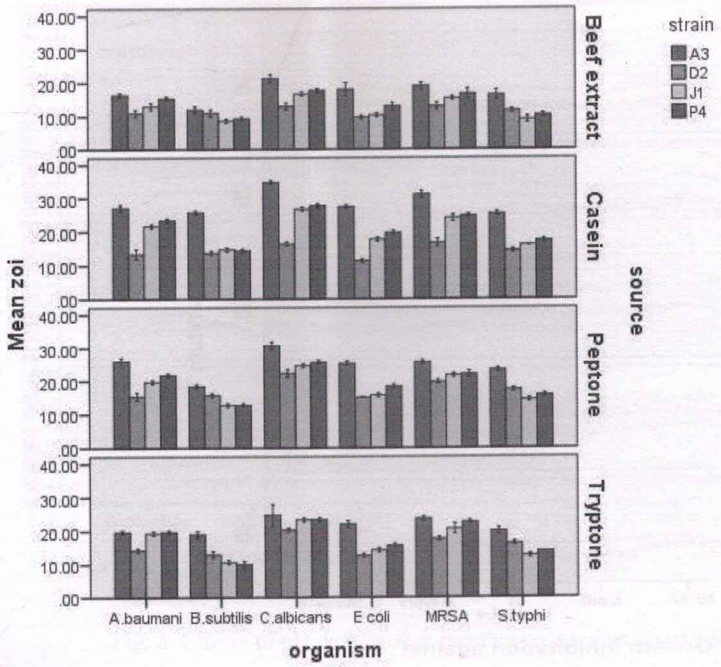

Fig. 4. Effects of nitrogen source on bioactive metabolite production.

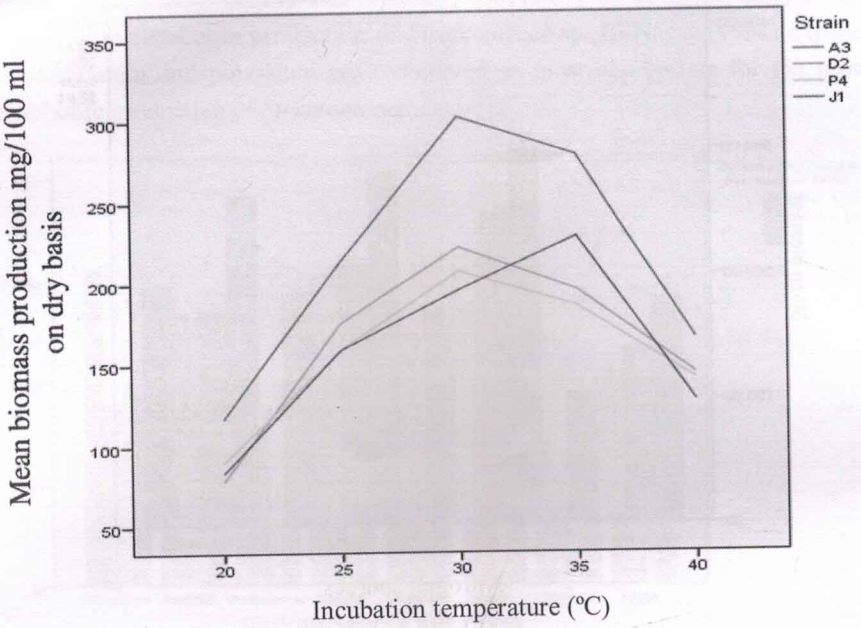

Fig. 5. Effects of temperature on biomass production.

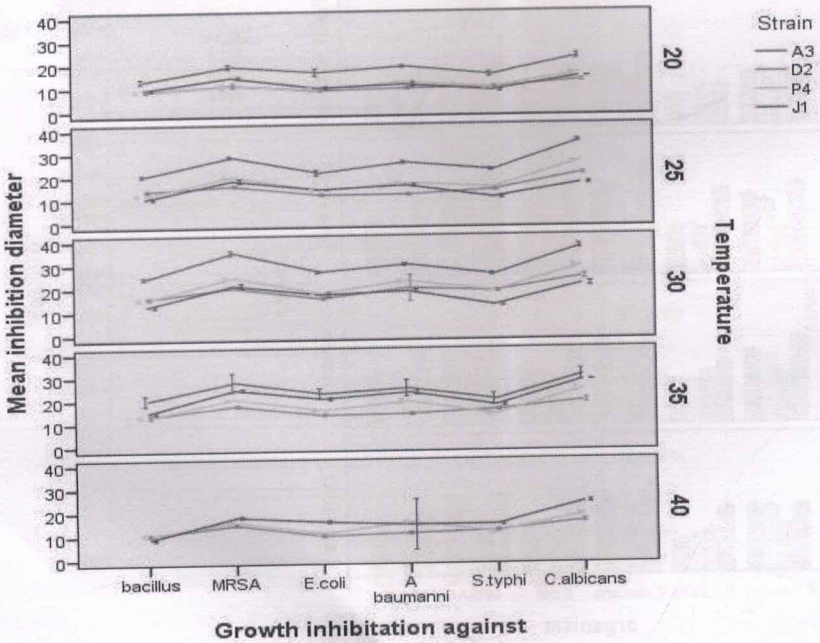

Fig. 6. Effects of incubation temperature on bioactive metabolite production ($p = 0.000$).

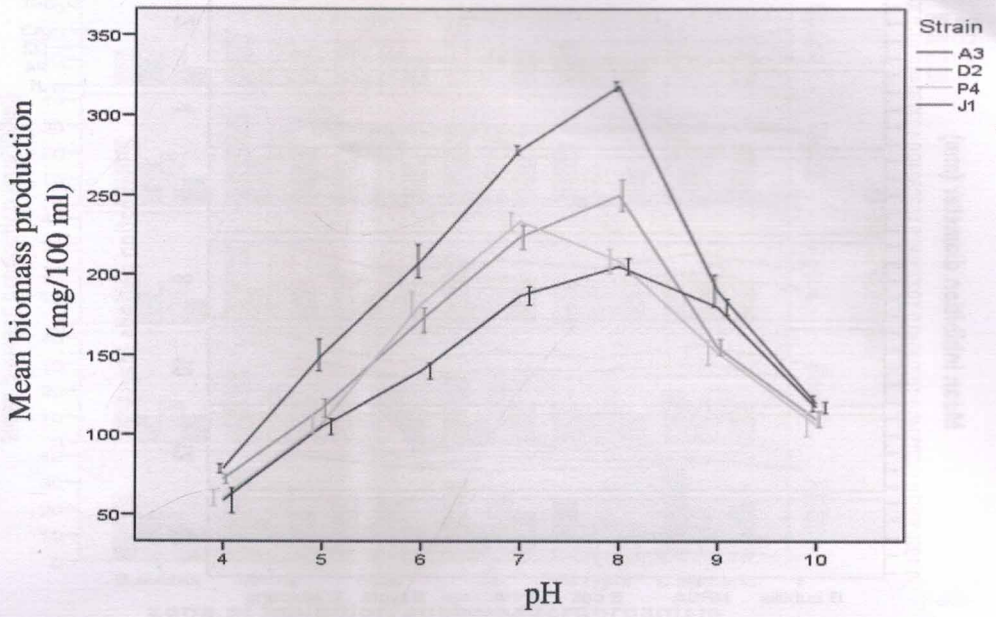

Fig. 7. Effects of pH on bio-mass production (p = 0.074).

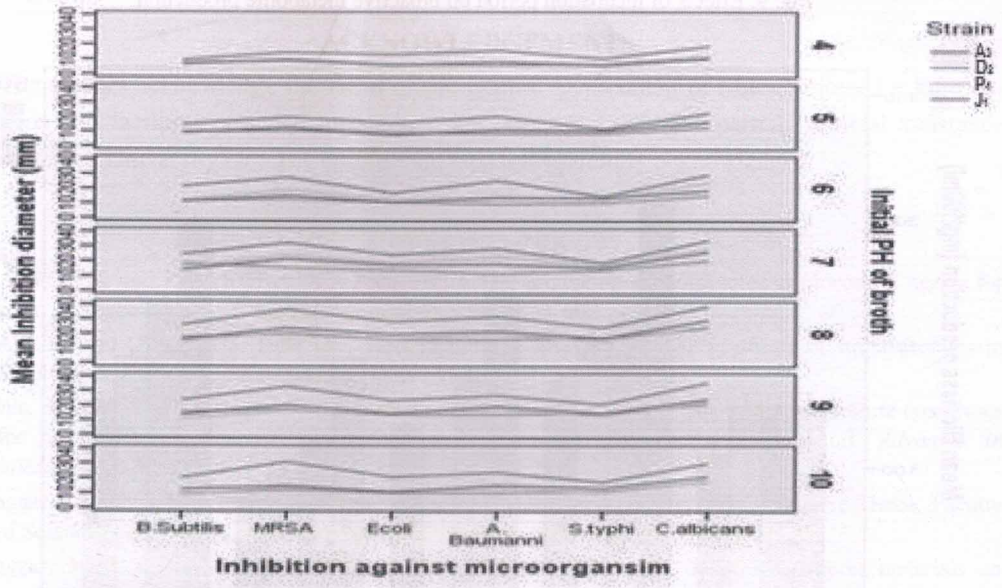

Fig. 8. Effects of pH on bioactive metabolites production (p = 0.000).

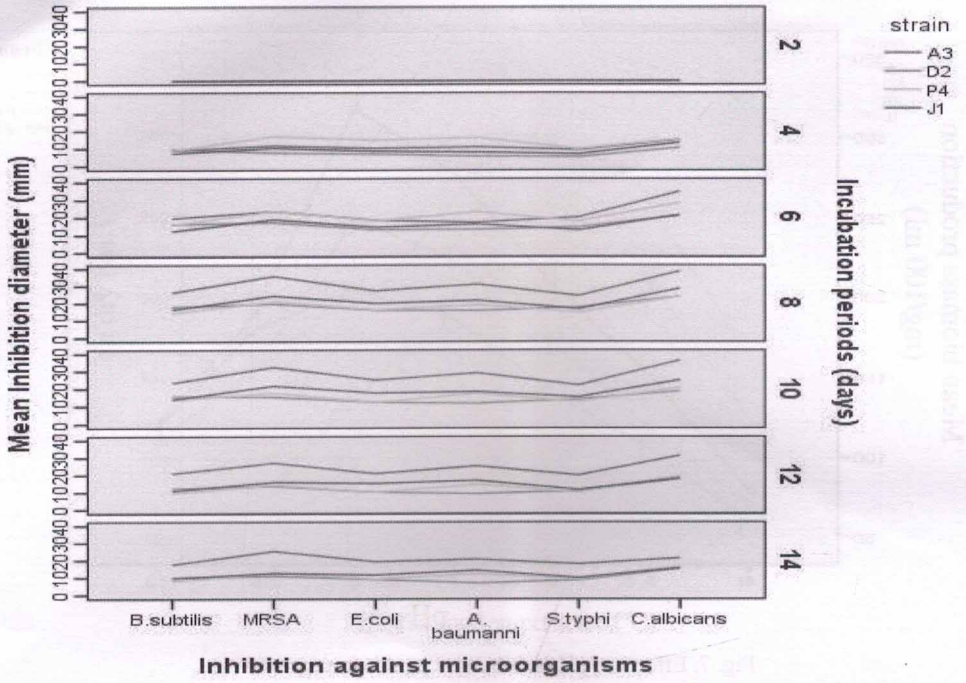

Fig. 9. Effects of incubation period on bioactive metabolite production.

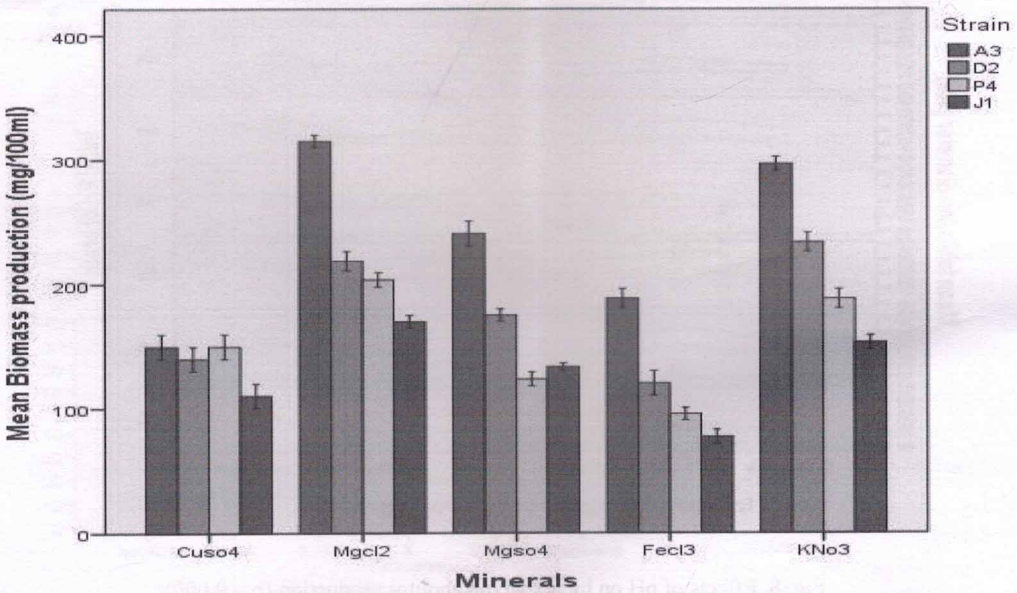

Fig. 10. Effects of minerals on biomass production.

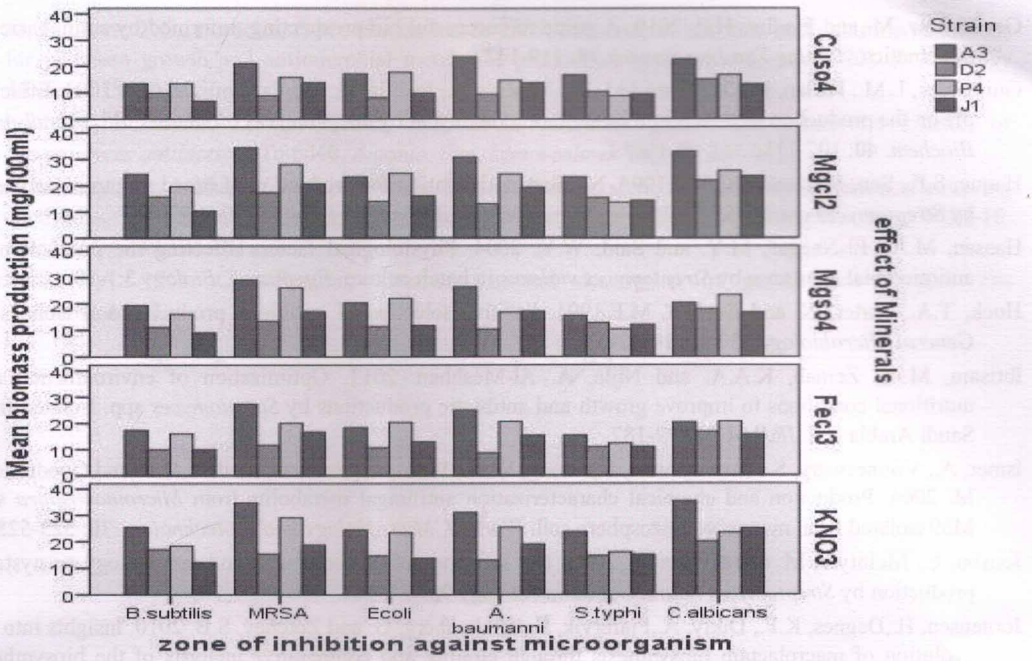

Fig. 11. Effects of minerals on bioactive metabolites production

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APPENDIX VII

Paper Presentation

"Promote Microbiology to Strengthen one Health concept in Nepal"

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Nepalese Society for Microbiology (NESOM) and American Society for Microbiology (ASM) jointly *awards this*

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for making Oral/Poster presentation on

Antibiotic Producing Actinomycetes of Nepal

at one day symposium on Microbiology and One Health Concept in Nepal" held in 3rd September 2016.

Prof. Dr. Rajani Malla
President, NESOM

Dr. Prakash Ghimire
ASM Ambassador to Nepal

Prof. Dr. Jibaraj Pokharel
Vice Chancellor
NAST
(Chief Guest)