ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES
COLLEGE OF NATURAL SCEINCES
DEPARTMENT OF MICROBIAL, CELLULAR AND MOLECULAR BIOLOGY

Actinomycetes from Unexplored Environmental Niches in Ethiopia and their Biotechnological Potentials for Antimicrobial Compound Production

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June, 2018

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A Thesis Presented to the School of Graduate Studies of the Addis Ababa University in Partial Fulfillment of the Requirements for the PhD Degree in Biology (Applied Microbiology)

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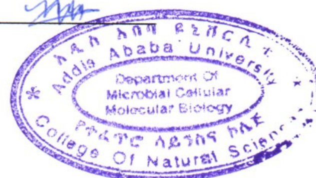

General Abstract

Actinomycetes have a widely recognized potential for the production of significant bioactive compounds. The major aim of this study was to isolate, screen and evaluate the biotechnological potential of selected actinomycete isolates particularily for antimicrobial compound production using standard bioassays methods, LC-MS, high resolution mass spectrometry (HR-MS) and NMR techniques. It consists of six distinct chapters. In the first chapter, general introduction, statement of the problem and major objectives are presented. The second chapter deals with the review of related literatures. The rest four chapters (3-6) are the experimental sections of the work. Hence, the third chapter concentrated on the isolation, screening, bioactivity detection and phylogenetic analysis of promising actinomycetes capable of producing bioactive secondary metabolites from various unexplored niche habitats in Ethiopia. Among the 416 isolates screened for bioactivities, 101 (24%) isolates were inhibiting the growth of *C. albicans*, and 88 (21%) isolates were inhibiting both *C. albicans* (ATCC 62376) and *C. neoformans* (clinical isolate). Ten isolates having considerable activities were chosen for further investigation and taxonomic identification studies. The polyphasic identification results of these isolates found to be in consistent with the genus *Streptomyces* described in Bregay's manual of systematic bacteriology. Identification of the isolates have been verified by the analysis of the 16s rRNA gene sequence. The phylogenetic relationships of the isolates to type strains and best matches based on BLAST search were inferred using the Maximum Likelihood algorithm in MEGA 7 software and confirmed that all the isolates belong to genus *Streptomyces.* The fourth chapter deals with the cultivation of five promising isolates namely Ac-029, Ac-125, Rv-355, Ac-464 and Go-475 for bioactive secondary metabolite production and subsequently evaluation of SSF process parameters on metabolite yield. Depending on the types of the isolates, variations were observed

in optimal fermentation process parameters on bioactive secondary metabolite production. It was demonstrated that wheat bran in the presence of supplementary nutrients, an initial moisture content of 65%, a pH value of 7.5, incubation temperature of 30 ^{o}C, an inoculum size of $3x10^7$ CFU/mL and incubation period of 12 days were the optimal SSF conditions for most of the isolates studied. The fifth chapter focused on antimicrobial potential of *Streptomyces* sp. Rv-355 cultivated in submerged culture. In its bioactivity profile, *Streptomyces* sp. Rv-355 produced antimicrobial compounds with wider spectrum of activities against yeasts, Gram positive and Gram negative bacterial pathogens. It was found that biomass production and bioactivity profiles of *Streptomyces* sp. Rv- 355 are positively correlated. Bioactivity guided analysis of the crude extract from *Streptomyces* sp. Rv-355 using TLC, column chromatography, HPLC, LC-MS showed the presence of potential compounds. The partially purified extract showed MIC values of 50µg/mL against *Candida albicans* and 100µg/mL against *Bacillus subtilis*. The result is found to be a prelude for further analysis of the crude extract from Rv-355 using HR-MS, and NMR methods. The sixth chapter was targeted on the bioactivity guided identification and structural elucidation of members of benz[a]anthraquinone antibiotics, 8-O-methyltetrangomycin and 8-O-methyltetrangulol from *Streptomyces* sp. Go-475 extracts using LC-MS, HR-MS/MS and ^{1}H NMR ^{13}C NMR methods. *Streptomyces* sp. Go-475 displayed potent activity against both yeasts and Gram-positive bacteria with MIC values of the crude extracts 100µg/mL and 50µg/mL against *Candida albicans* ATCC62376 and *Bacillus subtilis* ATCC6633 respectively. The analysis revealed that *Streptomyces* sp. Go-475 is able to produce at least three known secondary metabolites (4-Methoxy-1(3H)-isobenzofuranone, 3-Phenylpropionic acid or 1, 2-Benzenediol and Dehydrocineromycin B) that were not detected in the SmF extract. However, betaine was detected in both SSF and SmF extracts of this isolate. Two important anti-bacterial

compounds were purified from methanol extract of *Streptomyces* sp. Go-475 and their structures were elucidated by NMR and HR-MS/MS as 8-O-methyltetrangomycin and 8-O-methyltetrangulol. Besides, many potentially novel metabolites were detected, the majority of which were produced in SSF method. The findings enable us to conclude that *Streptomyces* sp. Go-475 and other isolates from Ethiopian soil have the capacity to produce potentially new antifungal secondary metabolites and warrant further investigations. The results also proved that SSF as promising economical and best option to produce potential bioactive secondary metabolites from *Streptomyces* spp. The genome sequence of *Streptomyces* sp. Go-475 was obtained using a hybrid assembly approach of high quality Illumina short read and low quality Oxford Nanopore long read data. The complete linear chromosome of 8,570,609 bp, featuring a G+C content of 71.96%, contains 7,571 predicted coding sequences, 83 t(m)RNA genes, and six *rrn* operons. Analysis of the genome for secondary metabolite biosynthesis gene clusters allowed us to connect certain clusters with experimentally confirmed molecules. The findings also verified great potential of *Streptomyces* sp. Go-475 for the production of chemically diverse secondary metabolites.

Key words: *Actinomycetes*, *antimicrobial bioassay, biosynthetic genes, Ethiopian soils, genome analyses, high resolution mass spectrometry, NMR-assisted structure elucidation, solid state fermentation*, *Streptomyces,*

Dedication

This Ph.D dissertation is dedicated to my late parents; my mother, Tadelech Gebeyehu and my father, Kibret Wondimagegn, both of whom gave me the foundation of learning they had never enjoyed, and set all the stages, so I have had access to education. I am indeed blessed to have them in my life. Let God keep your soul peacefully in heaven!!!

Acknowledgments

First and for most, I would like to offer my wholehearted thank to my almighty God who allowed me to live and enabled me to achieve this level. Next, I would like to give my heartfelt gratitude to my supervisor, Dr. Dawit Abate, for his guidance through out my journey, from the moment of admission all the way up to the final constructive editing of my dissertation. I am always inspired by his continuous critics and praiseworthy comments during the whole period of my study. I would also like to express my appreciation to Dr. Dawit once again for his successful effort to make available important instruments such as HPLC, freeze dryer, UV/ViS spectrophotometer and laminar air flow hood in the laboratory.

My deepest and special gratitude goes to my co-supervisors Prof. Dr. Sergey B. Zotchev and Prof. Dr. Judith M. Rollinger University of Vienna, Austria, who gave me an appealing opportunity to work in their world-class laboratory with their respected research team and for the invaluable supports given to me particularly on molecular analysis of my isolates using 16SrRNA sequencing, unlimited guidance in the most expensive activities such as LC-MS, HR-MS and NMR analysis of bioactive compounds and structural elucidation. I apperciate you, for introducing me to the most inspiring and high technology work.

I would also like to express my heartfelt thanks to the research team in University of Vienna specially Professor Ernst Urban for Nuclear Magnetic Resonance (NMR) measurement of my samples and spectra interpretation, Dr. Martin Zehl for high resolution mass (HRMS), LC-MS/MS analysis and interpretation of mass spectra, Jaime Felipe Guerrero-Garzón and Valerie-Katharina Wronski for their invaluable support on 16s rRNA sequencing and fractionation of the sample using preparative HPLC and mass spectrometry measurement.

I would also like to thanks to Zenebech Ayitenew, Gunter Seiringer and Olga Sekurova for their help in laboratory routines, and Samson Warkaye Lemma for his support in the preparation of the soil sample sites map.

I am really delighted to thank Dr. Gurja Belay, Dr. Fassil Assefa, Dr. Amare Gessesse, Dr. Tesfaye Alemu, Dr. Dereje Beyene, Dr. Kassahun Tesfaye, Dr. Tiliye Feyissa, Dr. Anteneh Tesfaye and Dr. Asnake Dessalegn, AAU who provided me with administrative support and various resources, facilitated my work, thought me PhD courses and gave me moral supports.

I want to use this special opportunity to express my deepest and heartfelt gratitude to my special friend Dr. Teshome Geremew, for his invaluable encouragement, unreserved and friendly values he gave me in my endeavor. I would also like to extend my special gratitude to my friends Muluye Teka, Alemayehu Godana, Mequanent Muneye, Yemiamrew Jorgi and all Ph.D. and M.Sc students in Microbiology Labs for continuous encouragement and support during my laboratory work. I would like to offer my special & wholehearted thanks to my lovely wife Almaz Zewdu, my father Kibret Wondimagegn, my brothers Atile Kibret and Telelle Kibret, my sisters Almaz Kibret, Albase Kibret, Demwoz Kibret & Meseret Mekonen for their encouragement and financial support.

I am grateful to Jigjiga University for sponsoring my PhD study and financial support. I owe due thanks to the Department of Microbial, Cellular and Molecular Biology, Addis Ababa University for giving me this special opportunity to pursue my PhD work and for all the assistance given to me during my study. University of Vienna, Austria and Bielefeld University, Germany are also highly akowledged for providing the invaluable and most expensive laboratory work. I am thankful to Swedish University of Agricultural Sciences for the PhD training given to me.

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List of Abbreviations and Acronyms

1D	One dimentional
2D	Two dimentional
3D	Three diemntional
AIDS	Acquired Immune Deficiency Syndrome
ATCC	American Type Culture Collection
BGCs	Biosynthetic gene clusters
CAD	Charged Aerosol Detector
CLSI	Clinical Laboratories Standard Institute
COSY	Correlation spectroscopy
DAD	Diode-array Detector
DAP	Diaminopimelic Acid
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DNP	Dictionary of Natural Products
DNTPs	Deoxyribosenucleotides triphosphates
DQF	Double quantum filtered
EASAC	European Academies of Science Advisory Council
EDTA	Ethylene Diamine Triacetic Acid
ELSD	Evaporative Light Scattering Detector

ESI	Electro Spray Ionization
ESI-Qq-TOF	Electro Spray Ionization, quadrupole-quadrupole-time-of-flight
EtOAc	Ethyl Acetate
eV	Electron Volt
H1N1	Hemagglutinin Type 1 and Neuraminidase Type 1 (swine flu)
H5N1	Hemagglutinin Type 5 and Neuraminidase Type 1 (Avian Influenza)
H7N9	Hemagglutinin Type 7 and Neuraminidase Type 9 (Avian Influenza)
HIV	Human Immunodeficiency Virus
HMBC	Hetro nuclei multiple bond correlation
HPLC	High performance Liquid Chromatography
HRESIMS	High resolution Electro Spray Ionization Mass Spectrometry
HRMS	High resolution Mass Spectrometry
HRMS/MS	High Resolution Tandem Mass Spectrometry
HSQC	Heteronuclear Single Quantum Coherence
ISG	Inorganic salts glucose
ISP	International Streptomyces Project
LC	Liquid Chromatography
LC-MS	Liquid Chromatography coupled with Mass Spectrometry
LC-MS/MS	Liqiud Chromatography coupled with tandem Mass Spectrometry
m/z	Mass to Charge ratio
mAU	milli-Absorbance Units

MeOH	Methanol
MIC	Minimum Inhibitory Concentration
MW	Molecular Weight
NMR	Nuclear Magnetic Resonance
PCR	Polymerase Chain Reaction
PDA	Photo Diode Array Detector
QDA	Quadruple mass detector
RAL	Reichsausschuß für Lieferbedingungen - German Institute for Quality Assurance and Labeling
rDNA	Ribosomal Deoxyribonucleic Acid
RDP	Ribosomal Database Project
Rf	Retention Factor
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute
Rt	Retention time
SARS	Severe acute Respiratory Syndrome
SrRNA	Small Ribosomal Ribonucleic Acid
TAE	Tris base, acetic acid and EDTA
TSB	Trypton soy brtoth
UPLC	Ultrahigh Performance Liquid Chromatography

Chapter 1: General Introduction

1.1. Background

Infectious diseases have been the main challenges to human development and survival (Morens, *et al*., 2004; Talbot *et al*., 2006) and are still continue to be among the most important causes of death throughout the world (Luzhetskyy *et al*., 2007) accounting for 15 million (26%) annual death globally (Morens *et al*., 2008). Nowadays, human beings have commonly encountered serious new diseases and re-emergent situations, including tuberculosis, cancer, AIDS, SARS, avian influenza (H5N1 and H7N9), swine flu (H1N1), Ebola and infections caused by multidrug resistant pathogens (French *et al*., 2002; Morens *et al*., 2008). Considering these backgrounds, new and potent therapeutic agents are essential as ever. More importantly, effective antifungal antibiotics are aindispensable for the treatment of opportunistic infections caused by *Candida albicans* and *Cryptococcus neoformans* which are serious threats for immunocompromised patients (Lia *et al*., 2012). In this regard the significant impacts of low-molecular weight compounds from natural sources remain very important in medicine (Grabley and Thiericke, 1999).

The anti-infective areas still dependent on natural products and their structures (Newman and Cragg, 2016). Over 60% of approved products are either natural products or related to them and represent over $40 billion in sales (Demain, 2009). For instance, 70 out of the 90 antibiotics marketed in the years 1982-2002 originated from natural products (Newman and Cragg, 2003). Mainly, microbial products have been the sources of most of those therapeutic antibiotics

currently in clinical use throughout the world (Demain, 2009; Berdy, 2012). This might be because microorganisms are endowed with many vital pathways for the synthesis of metabolites and have been consistently screened for novel bioactive compounds (Berdy, 2005).

Of all the known microbes, actinomycetes represent the top producers of antibiotics, biosynthesizing secondary metabolites that have considerable importance in pharmaceutical industry (Kavitha and Vijayalakshmi, 2007; Berdy, 2012). They are among the most inexhaustible producers of microbial metabolites having remarkable biological actions and are used in medicine, agriculture and industry (Berdy, 2005; Hopwood, 2007). Particularily, actinomycetes of the genus *Streptomyces* are the most versatile producers of potential secondary metabolites and sometimes called antibiotic manufacturing factories (Berdy, 2005; Baltz, 2007; Demain, 2009). They synthesize antifungal antibiotics, antitumor agents, immunosuppressive agents, pesticides, herbicides and anti parasite agents (Iznaga *et al*., 2005; Li *et al*., 2008; El-Tarabily *et al*., 2009; Liu *et al*., 2009; Nakouti *et al*., 2012). Tetracycline, Neomycin and Gentamicin are some examples of antibiotics produced by *Streptomcyes aureofaciens, Streptomyces fradiae,* and *Streptomcyes lincolenensis*, respectively (Butay, 2000; Berdy, 2005).

However, drug development studies from natural products are now suffering from declining interest as it needs much effort, longer time and huge capital to isolate the active components and to elucidate the molecular structures (Demain, 2009). According to Grabley and Thiericke (1999), pharmaceutical companies invest $350 million to develop a single new drug indicating that drug development research is one of the biggest capital intensive works.

Furthermore, Berdy, (2012) illustrated that, it needs an average of 12 years for an experimental drug to go through the laboratory upto the medicine cabinet (market) and costs (800Million–2Billion, USD) per drug. Thus, in spite of an increasing frequency and severity of antimicrobial resistance, the future discovery of new anti-infective agents is in danger, because of the termination of many big pharmaceutical companies to invest on this crucial area (Wenzel, 2004; Norrby *et al*., 2005). Nevertheless, Newman and Cragg, (2016), recommended that, this area of natural product research must be extended extensively.

1.2. Statement of the problem

The growing of resistance in infectious agents has become a serious global phenomenon particularly in the developing world which increase morbidity and mortality (Demain, 2009; Laxminarayan and Heymann, 2012; Hamers *et al*., 2012). Hence, various reports certainly argued that there is a burning demand for novel drug development against these pathogens (Talbot *et al*., 2006). In this regard, nature have unlimited and diverse supply of substances mainly from plant and microbial origin to fight those infectious agents (Grabley and Thiericke, 1999; Demain, 2002; Berdy 2005; Demain, 2009). The reports by Berdy (2012) and Tiwari and Gupta (2014), pointed out that bioprospecting untouched habitats for the endless sources of natural product is indispensable for novel drug development.

Reports by Donadio *et al*. (2002), Berdy (2005), Goodfellow and Fiedler (2010) emphasized that microorganisms belonging to the most inexhaustible groups of antibiotic producers, actinomycetes, notably *Streptomyces*, remain to be the best choice to find out novel metabolites.

These microbes continue to attract attention as a source of invaluable medicinal compounds that have great socio-economic relevance and with many potential therapeutic applications (Hwang *et al*., 2014). Hence, it appears beneficial to explore untouched environmental niches, to discover new actinomycetes capable of producing potential bioactive compounds (Sanglier *et al*., 1996; Bull and Stach, 2007; Berdy, 2012; Matsui *et al*., 2012; Zotchev, 2012).

There is no comprehensive work on soil actinomycetes in various regions in Ethiopia showing their potential bioactivities in the drug development efforts. Based on this background information and the identified research gaps, it has been hypothesized that Ethiopian soils from various climatic, agro-ecological zones and with remarkable biodiversity might harbor new actinomycete strains. Those actinomycetes obtained from previously unstudied areas might be important sources of effective biomolecules (secondary metabolites with diverse biological activities). Thus, bioactive compounds from actinomycetes in untouched environments could be potential as drug lead compounds; or could meet the endless need for new antibiotics in the battle against the rising of drug-resistant infectious agents. Based on these hypotheses the current study has the following objectives.

1.3. Research objectives

1.3.1. General objectives

The major aim of the current study was isolation and characterization of bioactive compound producing actinomycetes from various unique environments and specific biotops in Ethiopia. And to evaluate the biotechnological potential of some selected isolates as source of antimicrobial compounds.

1.3.2. Specific objectives

- To isolate, screen and characterize bioactive secondary metabolite producing actinomycetes
- To optimize and produce bioactive compounds under different culture conditions.
- To analyze, characterize and elucidate the molecular structures of bioactive compounds from selected isolates

Chapter 2: Literature Review

2.1. Some important opportunistic infections and chemotherapy

Opportunistic pathogens mainly *Candida* and *Cryptococcus* spp. are medically important, usually avirulent in strong people, but could be disseminated to deep tissue and cause fatal systemic disease in immunocompromised individuals (Caston-Osorio *et al*., 2008). The morbidity and mortality rates caused by these opportunistic pathogenic yeasts are relatively higher, representing at least 1.5 million deaths rate annually worldwide (Pianalto and Alspaugh, 2016), but the impact of these diseases on human health is not well appreciated (Brown *et al*., 2012). The diseases they cause can be classified (in increasing severity) as superficial, subcutaneous and systemic infections (Chakrabarti, 2005).

These opportunistic yeasts differ in characteristics from that of conventional infectious pathogens. They are mainly low or non virulent; but they could evolve to develop infections mainly on the basis of 'host-parasite' interaction in particular circumstances (Casadevall and Pirofski, 2002). Recently, with an increase in the number of immuno-compromised patients, particularly the current pandemics of HIV/AIDS, opportunistic yeasts have great significance (Banerjee, 2005). In order to find out treatment measures, important description of the general characteristics of vital opportunistic pathogens and their infection processes are presented briefly. Besides, vital information on the already existing therapeutic agents, their origin and modes of actions and key natural sources of these therapeutic agents are reviewed in this particular paper.

2.1.1. *Candida albicans* and candidiasis

2.1.1.1. *Candida albicans*

Candida albicans is an oval, budding yeast cell that forms pseudohyphae in culture, tissues and exudates (Thompson, *et al*., 2011). It is an asexual yeasts and genetically diploid with the presence of eight chromosomes. It can be characterized by phenotypic switching, variant colony morphology, dimorphism and transition from budding yeast to pseudohyphal and hyphal growth forms (Fidel *et al*., 1999; Pfaller and Diekema, 2002). At least 70% of *Candida* strains isolated from sites of infections have been identified as *C. albicans* and it has been reported as pathogenic in all forms of candidiasis (Calderone, 2002).

Candida albicans is a member of the normal micro flora of the mucous membranes in the gastrointestinal, upper respiratory and female genital tracts (Nobel, 1980; Brown *et al*., 2012). It can be found in about 70% of the healthy population. However, *C. albicans* is an opportunistic pathogen, given the right situation, it has the ability to adjust itself to the varying backgrounds and cause local oral thrush, mucosal infections of the genitals, gastrointestinal and even, sever systemic infections (Ruhnke and Maschmeyer, 2002; Mayer *et al*., 2013). Risk groups are injured persons or patients with compromised immune systems including infants, pregnant women, diabetes, cancer, and AIDS-patients etc. http://www.cdc.gov/ncidod/dbmd/diseaseinfo.

C. albicans has several characteristics that facilitate it to quickly adapt to changing environmental cues and these aids it in take over of the host. One of these is the ability of *Candida* to switch between growing as unicellular yeast and growing as multicellular filaments (the yeast-hyphal transition) (Sudbery *et al*., 2004). In addition, it shows various forms of

"phenotypic switching" in which cells change their morphology and physiology by reversible switching between optional modification phenotypes (Soll, 2002; Soll *et al*., 2014). Both the yeast-hyphal transition and phenotypic switching have an effect on the virulence of *C. albicans* in systemic diseases. *C. albicans* has several genes that have been acquainted with a variety of environmental cues, including pH, temperature and the presence of serum (Smiths *et al*., 2005). These make *C. albicans* quickly adapt to diverse host microenvironments with both heritable and non heritable systems and these can help promote tissue invasion and evading of the host immune system (Smiths *et al*., 2005; Mayer *et al*., 2013). Furthermore, as *C. albicans* colonizes a niche, it most likely to modify that niche, for example by metabolizing available nutrients, probably altering the ambient pH via its metabolic activity and destroyed host tissues (Calderone, 2002).

2.1.1.2. Candidiasis

Candidiasis refers to the diseases caused by yeast of the genus *Candida* (Benett, 2001). Candidal infections are serious challenges in immunocompromised people. Interestingly, *C. albicans* differs from other medically important fungi such as *Histoplasma capsulatum*, *A. fumigatus*, and *C. neoformans* etc. in rarely being isolated from soil. Therefore, infections caused by it are grouped as endogenous and not exogenous as with others (Richardson, 2005). In normal situation, it continue living with other normal microbial flora of the host organs; but in patients with weakened immunity, who have undergone chemotherapy, bone-marrow transplantations etc., it act like an opportunistic pathogen and produces external to systemic infection (Odds *et al*., 2006). *Candia* is the fourth most important cause of nosocomial bloodstream infections and the third cause of bloodstream infections in the intensive care unit, with a mortality rate of

almost 50% (Park, *et al*., 2009; Lockhart, 2014; Kullberg and Arendrup, 2016). Over 95% of all fungal infections have been due to *Candida albicans, Aspergillus fumigates,* and *Cryptococcus neoformans* (Richardson, 2005).

Candidiasis includes minor or opportunistic infections ranging from acute, sub-acute and persistent to life-threatening systemic mycoses. Infections could be confined to a small area to mouth, throat, skin, vagina, fingers, bronchi, lungs and gastrointestinal tract, or sometimes become systemic as candidemia, endocarditis and meningitis etc. (Hayens and Westerneng, 1996; Brown *et al*., 2012). About 70% of women encounter vaginal candidiasis once in a life, and 20% undergo from relapse (Fidel *et al*., 1999). The occurrence of systemic candidiasis in patients with other serious illnesses has raised dramatically Pfaller (1996) with a mortality rate from 50 to 80%, although an adequate treatment (Fraser *et al*., 1992). Thus, control measures which mainly focus on such opportunistic pathogens that can cause candidiasis, is a vital area of research in relation to the current spread of HIV/AIDS and additional immune compromising diseases.

2.1.2. *Cryptococcus neoformans* and cryptococcosis

2.1.2.1. *Cryptococcus neoformans*

Cryptococcus neoformans is an oval encapsulated yeast, 4-6μm in diameter in clinical specimens and having a capsule ranging in size from 1 to > 30μm. In specimens isolated from nature, the cells tend to be smaller and weakly encapsulated (Neilson *et al.*, 1977). *Cryptococcus neoformans* is omnipresent in the environment. At the very beginning it was isolated in nature

from peach juice in 1894 by Francisco Sanefelice and was first isolated from soil by Emmons in 1951 (Levitz, 1991). *Cryptococcus neoformans* was also isolated from pigeon droppings in 1955, and has since been isolated from several geographic sites contaminated by pigeon or other bird excrement worldwide (Staib and Heissenkuber, 1989). Although *C. neoformans* commonly isolated from pigeon excreta and soil, it has been isolated less often from additional sources, including fruits and vegetables, dairy products and droppings from a large variety of bird species (Ellis and Pfeiffer, 1990).

When grown on agar media such as blood agar or Sabouraud's dextrose agar, *Cryptococcus neoformans* revealed white to cream colored, smooth, mucoid colonies. The amount of mucoidness of the colonies is related to the thickness of the capsule. Growths of Cryptococcus generally take places in 36 to 72 hours and are specifically slower than that of *Candida* and under the same situation (Min and Kwon- Chung, 1986). *Cryptococcus neoformans* grows at 37°C, whereas nonpathogenic species of *Cryptococcus* do not. A characteristic feature of *C. neoformans* is the capacity to synthesize melanin. On differential media supplemented with Niger seed (birdseed agar), smooth brown colonies are formed after quite a few days of incubation (Min and Kwon- Chung, 1986).

2.1.2.2. Cryptococcosis

Cryptococcosis is a systemic mycosis caused by the encapsulated yeast of the genus *Cryptococcus*. It affects both immunocompromised and strong individuals. It is mainly life-threatening, deep-seated fungal infection in AIDS patients, representing an emerging challenge

in the management of patients with AIDS complex-related diseases (Kwon-Chung *et al*., 2014). Infection is acquired from the environment (Richardson and Lass-Florl, 2008). Mostly, it attacks the lungs or central nervous system and less frequently the blood, skin, skeletal system and prostate with approximately a total rate of one million cases annually and over 600,000 deaths (Park *et al*., 2009; Perfect, *et al*., 2010). Since the occurrence of cryptococcusis is significantly greater than before in immunocompromised patients, particularly in patients with AIDS and organ transplant recipients, it is regarded as an importat opportunistic fungal disease (Rodrigues *et al*., 1999). The main route of entry for *Cryptococcus* is via the lungs, where the fungus may begin a primary infection. If the early pulmonary infection is not managed, it can spread to additional organs and the central nervous system, resulting in fatal cryptococcal meningoencephalitis (Aguirre *et al*., 2004).

In the majority of cases, infection with *C. neoformans* is thought to be caused by inhalation of the organism, either in yeast form or perhaps as basidiospores, from environmental source such as bird droppings or soil (Duperval *et al*., 1977). Cryptococcosis can happen in many individuals without a known immunologic deficiency, but most patients have predisposing factors (Pappas *et al.*, 2001). Predisposing circumstances include AIDS, systemic corticosteroids, organ transplantation and diabetes mellitus (Dismukes, 1993; Pappas *et al*., 2001).

2.2. Anti-infective agents

There has been a constant battle between humans and a number of microorganisms that cause infection and disease. Around the middle of the 20th century, major advances in antimicrobial

drug development turn the tide in favor of humans. With respect to bacterial infections, the situation dramatically improved when penicillin became available for use in the early 1940s. However, the excitement over the potential conquest of infectious diseases was short lived (Spellberg *et al*., 2008). Almost as soon as antimicrobial drugs were deployed, pathogens responded by manifesting various forms of resistance (Hopwood, 2007). As antimicrobial usage increased, so did the level and complexity of the resistance mechanisms exhibited by pathogens. Nevertheless, the struggle to gain the upper hand against infections using antibiotics continues to this day (Spellberg *et al*., 2008).

2.2.1. Antibiotics, an overview

The word "antibiotic" was originated in 1889 by a French biologist, P. Vuillemin, but used very commonly to explain the inhibition of one organism, not necessarily a microbe, by another (Hopwood, 2007). Barrios-Gonzalez *et al*. (2004) define antibiotics as low-molecular-weight organic natural products produced by microorganisms, which are active against other microorganisms at low concentrations. Whereas, Demain defined antibiotics as low molecular weight organic natural products, produced by living organisms, which in low concentrations are able to inhibit growth of other organisms (Demain, 1998). Indicating that, there is no consistency in the use of the word antibiotic, however, most scientists agree to accept Waksman's definition that "an antibiotic is a substance produced by one micro-organism which is capable of interfering with the growth of others."

The introduction of antibiotics for human therapy is one of the most victorious achievements in medicine. Besides, it is likely that it is the most important human involvement in the micro biosphere and one of the few processes of evolution that can be focused on in real time (Martinez and Baquero, 2009). The selective action exerted by microbial secondary metabolites on pathogenic bacteria and fungi steered the antibiotic era. Thus, the increase in life expectancy seen during the twentieth century in many parts of the world is by now too familiar to require lengthy discussion. This remarkable jump has been attributed to advent of drugs for the treatment of infectious disease (Marinach *et al*., 2009). Due to their complicated and diversified structure, antibiotics often exhibit activity selectively against biological systems and become antimicrobial tools (Okami, 1982). Antibiotics eliminate or prevent growth of pathogens (bacterial and fungal) and can therefore cure infectious diseases caused by these microbes (Fernando, 2006).

Historically the origin of antibiotics is commonly depicted to 1929 when Alexander Fleming descovered the antibacterial activity of a substance secreted by *Penicillium notatum* on a contaminated culture plate. However, until the early 1940s, the consequent development of penicillin as a therapeutic agent was not realized; hence the antibiotic era was successfully launched after a consortium of academic and pharmaceutical scientists from England and the United States of America, developed sufficiently advanced fermentation technology to produce high-purity penicillin in large enough quantities for medical supplies (Bush, 2010). The discovery of penicillin and its use in the clinic in the 1940s was followed by the discovery of a huge number of antibiotics from microbes, in particular from members of the actinomycetes and fungi during the golden age of antibiotics. The discovery of streptomycin from soil *Steptomyces griseus* by Selman Waksman and his student Albert Schatz in 1943 was the breakthrough in the

era of antibiotics (Hopwood, 2007). Consequently, many antibiotics were discovered effectively until the early 1970s and reached the market (Pelaez, 2006).

2.2.2. Important antifungal antibiotics in clinical use

Until the 1950s, relatively few drugs were available for the treatment of superficial or invasive mycoses. Later, intravenous formulations of polyenes such as Nystatin B and Amphotericin B were developed as the first generation of antifungal agents (Caillot *et al.*, 1992). The discovery of the polyene antifungals nystatin and amphotericin B in 1955, was the beginning of the era of effective antifungal chemotherapy for systemic infection, followed by the discovery of the first topical azole antifungal agent, chlormidazole, in 1958 (Lewis and Fothergill, 2015). Until recently, the only systemic antifungal agents available for use were amphotericin B, ketoconazole, itraconazole, fluconazole, and 5-flucytosine (5FC) (Liao and Dunne, 2003).

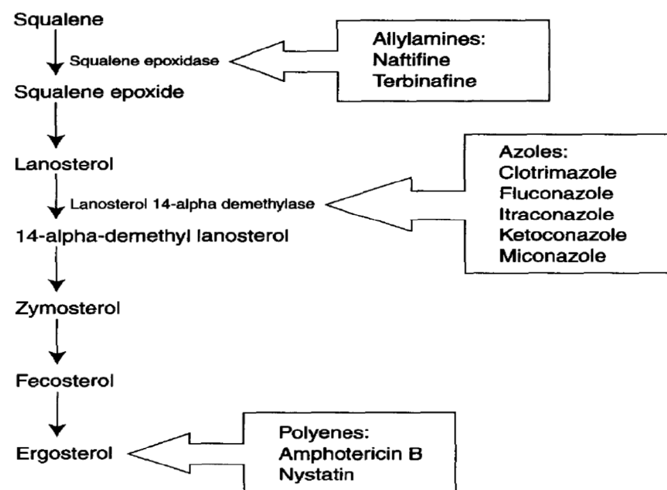


Figure 2.1. Fungal ergosterol biosynthesis and targets for various types of antifungal agents (Stone *et al.*, 2002)

In the next section some important systemic antifungal agents and their targets are presented; for instance, amphotericin B act on membrane integrity (Liao and Dunne, 2003; Lewis and Fothergill, 2015). Important illustrations are presented for the vital antifungal antibiotics targetes in Figure 2.2.

2.2.2.1. Polyenes

The polyene antibiotics are produced almost exclusively by *Streptomyces* spp. These groups of antifungal antibiotics are characterized by the large macrocyclic lactone ring containing a series of conjugated double bonds and one or more sugar residues (see Figure 2.3 and Figure 2.4), used extensively to treat systemic and non-systemic mycoses (Hammond, 1977; Aparicio *et al*., 2004). Nearly one hundred polyene antibiotics have been described but some of these may not be unique or even pure as a result of the scarce evidence provided by many authors (Bolard, 1986).

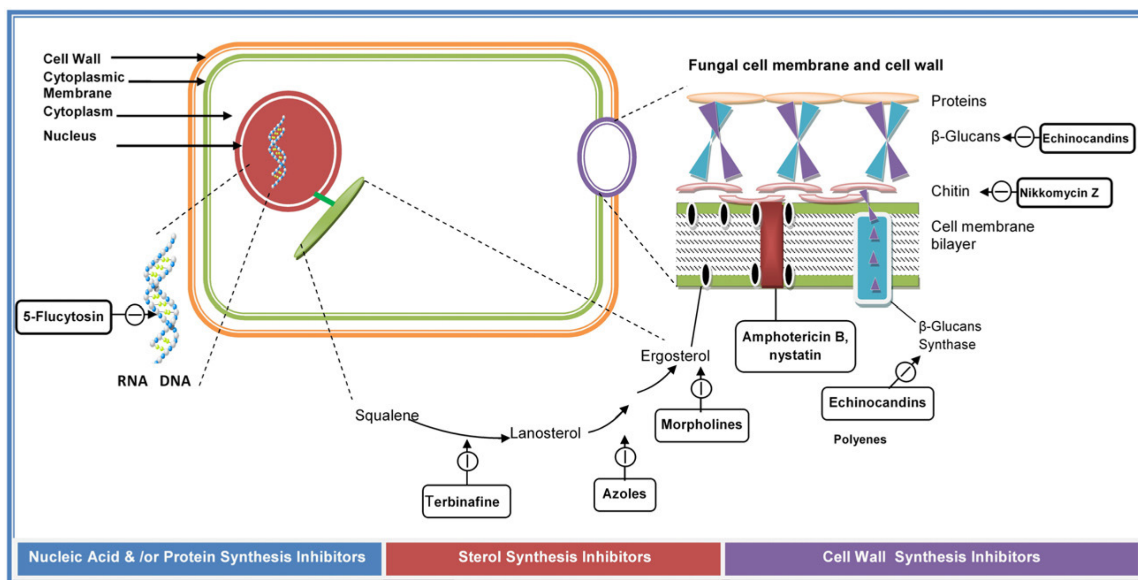


Figure 2. 2. Important targets for antifungal therapy (Kathiravan *et al*., 2012)

Table 2.1. Groups of antifungal antibiotics, mode of action and mechanism of resistance; adapted from Stone *et al*. (2002)

Class	Fungal Target	Action	Mechanism of Resistance
Polyene	Ergosterol	Binding to ergosterol	Altered or decreased amounts of ergosterol in cell membrane; defects in sterol biosynthetic pathways
Flucytosine	Nucleic acid synthesis	Inhibition of nucleic acid synthesis	Mutations in cytosine deaminase; decrease in uracil phosphoribosyl transferase activity
Azoles	Ergosterol	Inhibition of ergosterol biosynthesis	Mutations in **ERG11, CDR1, CDR2, MDR1**; overexpression of efflux pumps; overexpression/mutations of target enzyme
Echinocandins	β (1, 3) glucan synthetase	Inhibition of glucan biosynthesis	No data

Amphotericin B: Amphotericin B was produced by *Streptomyces nodosum* discovered by Gold and his colleagues in 1956 cited in Trejo and Bennett (1962). The mechanism of action of amphotericin B is not by inhibiting an enzyme; rather it binds to ergosterol, thereby perturbing membrane function to the point of causing leakage of cellular contents. As a result, intracellular univalent and divalent cations seep out, i.e. it leads to the leakage of sodium, potassium and hydrogen ions, followed by cell death (Odds *et al*., 2003). This agent is preferentially more toxic to the fungal cell membrane than to the mammalian cell membrane because of its relative selectivity for ergosterol (in fungi) over cholesterol (in mammalian cells) (Wingard and Leather, 2004). Structurally, the fungal sterol, ergosterol, exhibits a more cylindrical three-dimensional structure than the mammalian sterol, cholesterol, which largely explains the greater affinity of amphotericin B binding to ergosterol (Lewis and Fothergill, 2015).

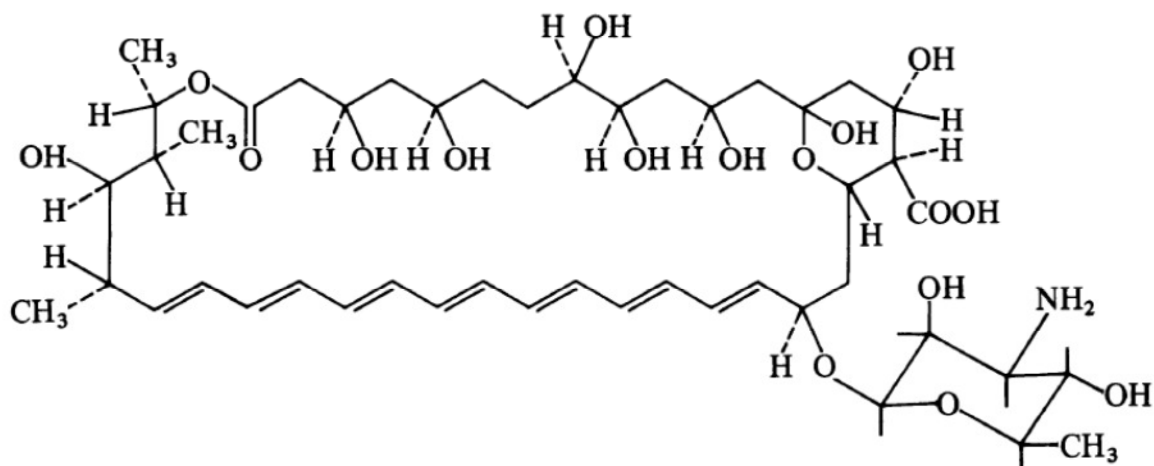


Figure 2.3. Molecular structure of Amphotericin B (Mechlinski et al., 1970)

As described in Wingard and Leather (2004), Amphotericin B has been used as the only antifungal treatment option that can be administered systemically to treat a visceral infection for many years. Its spectrum of activity against fungi is relatively wider. It is active against most fungal pathogens in humans including *Candida* and *Aspergillus* species. However, nephrotoxicity and hepatoxicity are the major limitation of the clinical usefulness of amphotericin B. Anemia, electrolyte wasting by renal tubules (potassium, magnesium and bicarbonate), hepatic dysfunction, are some of the other problems. It is poorly absorbed from the gastrointestinal tract and must be administered by injection to achieve adequate fungicidal concentrations (Wiebe and Karriker, 2005).

Besides its use in human medicine, amphotericin B is the most commonly used drug in veterinary medicine for systemic fungal infections in domestic animals. For instance, in avian medicine, amphotericin B is widely used to treat *Aspergillus* infections. The similarity between fungal ergosterol and human cholesterol is thought to account for the toxicity of amphotericin B

in humans. Liposomal and lipid formulations of the drug are recent attempts to overcome some of the toxic effects of the drug (Caillot *et al*., 1992).

Nystatin: Nystatin, produced by *Streptomyces noursei*, was the first polyene macrolide antifungal antibiotic discovered in 1950 from the fermentation broth of *Streptomyces noursei* (Hazen and Brown, 1950). However, the complete strereostructure elucidation of nystatin was conducted in 1989 using proton NMR (Lancelin and Beau, 1989). Nystatin is still used as a topical antifungal agent for topical treatment of *Candida* infections (Chamers and Sande, 1995b). It is non absorbable after oral administration but is effective topically in the treatment of oropharyngeal candidiasis. The world market for nystatin is estimated to have a yearly turnover in the range of 250–300 million US dollars (Jonsbu *et al*., 2002).

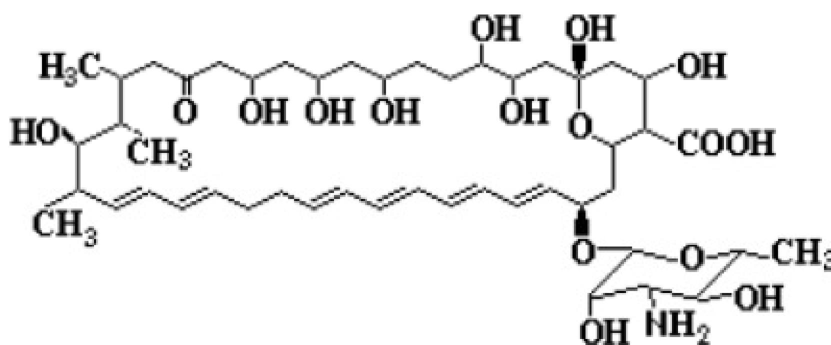


Figure 2.4. Molecular structure of Nystatin, (Lancelin and Beau, 1989)

Despite the severe toxicity, antifungal activity of nystatin is wider as compared to ampotericin B and could therefore, be more efficient when given by the intravenous method (Schaffner, 2002; Arikan *et al*., 2002). Its mode of action is by the formation of pores on the plasma membrane of the target cell (Bolard, 1986). These pores have size-selective characteristics, leak only to solutes not larger than glucose (Holz and Finkelstein, 1970). The disruption of the cellular

electrochemical components, caused by the enhanced plasma-membrane permeability to ions and small molecules, eventually results in cell lysis and death (Bolard, 1986).

2.2.2.2. Pyrimidine analogs (5-Fluorocytosine)

Fluoropyrimidine is a synthetic pyrimidine nucleoside analog, that is taken up by fungal cells and converted to 5-fluorouracil which is phosphorylated and incorporated in to fungal RNA, interferes with fungal nucleic acid synthesis causing miscoding (Waldorf, *et al*., 1983; Wingard and Leather, 2004). It was originally discovered as a potential antitumor agent Pauw (2000). It reaches to the target by transporting via cytosine permease and then delaminated to the active form (5-fluorouracil) (Wingard and Leather, 2004).

The incorporation of Fluorouracil into RNA, can cause premature chain termination and inhibits DNA synthesis through effects on thymidylate synthase and works as an antifungal agent after it has been converted to 5-fluorouracil within the target cells (Waldorf *et al*., 1983). For this mechanism of action, the target cells must possess cytosine permease to internalise the flucytosine molecule, cytosine deaminase to convert it to 5-fluorouracil and uracil phosphoribosyl transferase to convert 5-fluorouracil into a substrate for nucleic acid synthesis (Waldorf, *et al*., 1983; Vermes, *et al*., 2000; Hope *et al*., 2004).

The spectrum of flucytosine is restricted to pathogenic yeasts (*Candida* species and *C. neoformans*) because most filamentous fungi lack these enzymes. Flucytosine is used as adjunctive, rather than primary therapy, in the clinic, because primary and secondary resistance

resulting from defects in the permease, deaminase and/or phosphoribosyl transferase enzymes were thought to occur at a high frequency (Odds *et al* ., 2003).

2.2.2.3 Azoles

One of the land marks in the development of active antifungal antibiotics was the discovery of the first azole antifungal, benzimidazole, by Wooley in 1944 (Wooley, 1944; Sheehan *et al.*, 1999). Ketoconazole was introduced in 1981, and then a series of similar agents has followed. Azole classes of antifungals act on key enzymes in the biosynthesis of ergosterol which are fungal cytochrome P450 14-α-sterol demethylase and of 24-methylene dihydrolanosterol demethylation. As a result of inhibition of the conversion of lanosterol to ergosterol, accumulation of lanosterol occurs, and there is a reduction of ergosterol in the fungal cell membrane, leading to inhibition of fungal growth (Wingard and Leather, 2004). Inhibition of cytochrome P450-dependent lanosterol 14-alpha-demethylase by azoles, cause the accumulation of methylated sterols and toxicity of cells, depletion of ergosterol and inhibition of cell growth (Koltin and Hitchcock, 1997).

In general, cytochrome P450–Erg11p, is the principal molecular target of azole antifungals. It catalyses the oxidative removal of the 14α-methyl group of lanosterol in fungi by a typical P450 mono-oxygenase activity. This protein contains an iron protoporphyrin moiety located at the active site and the antifungal azoles bind to the iron atom via a nitrogen atom in the imidazole or triazole ring (Odds *et al.*, 2003). Azoles are typically fungistatic against yeasts and fungicidal against molds. Except for fluconazole, azoles are commonly used to treat systemic fungal infections (Verweij *et al.*, 2007).

Triazoles: Triazoles are distinguished from the azoles by the presence of a third nitrogen atom in the core structure. The mechanism of action is similar to that of azoles by preventing the synthesis of ergostero (Maertens, 2004). The structural differences might seem small, but they dictate antifungal potency and spectrum, bioavailability and drug interaction and toxic potential which are very important considerations for compounds that bind to haem groups in P450s (Odds *et al.*, 2003).

2.2.2.4. Echinocandins

Echinocandins are the most recently developed class of antifungal drugs. They are fungal secondary metabolites comprising a cyclic hexapeptide core with a lipid side chain responsible for antifungal activity (Odds *et al*., 2003). The mechanism of action of the echinocandins is inhibition of β (1, 3) - glucan synthase (complex of proteins responsible for synthesis of cell wall β-1, 3- D- glucan polysaccharides), which leads to interference in the synthesis of glucan, a major constituent of the fungal cell wall and a unique fungal target.

These groups namely caspofungin, anidulafungin and micafungin, offer the long-desired option of moving away from membrane sterols to the cell wall as the target of selective action because they are inhibitors of glucan synthesis and they have similar three-dimensional molecular configuration (Tkacz and DiDomenico, 2001; Odds *et al*., 2003). Echinocandins are fungicidal against most yeast species and fungistatic against molds. Echinocandins have few toxicities, since the target enzyme, 1, 3-β-D-glucan synthase, is absent in mammalian cells (Diekema *et al*., 2003). Echinocandins are often effective for the treatment of fungal infections caused by azole-resistant isolates. Nevertheless, the increasing emergence of strains resistant to echinocandins

can be considered as a clear sign for the urgent need to search for the next-generation of antifungals (Berenguer *et al*., 1997).

2.2.3. The challenges in drug resistance and shortage of new antibiotics

Antibiotic resistance has increased worldwide leading to treatment failures in infectious diseases that become a major concern in the anti-infective therapy (Ashkenazi *et al*., 2003). Pathogenic organisms are able to adapt rapidly to new environmental conditions (in the presence of antimicrobial molecules) and as a consequence, resistance may increases with increasing exposure to antimicrobials. Serious concerns about antibiotic resistance from nosocomial, community-acquired and food-borne pathogens have been growing for a number of years, and have been raised at both national and international levels (Okeke *et al*., 2005). To this end, Diamond (1991) described microbial infection as major threats to public health particularly in developing countries due to the relative unavailability of medicine and growing of drug resistance. Thus, resistance of pathogens to antibiotics has been a major problem in the treatment of diseases. As a result, infectious diseases are still the second leading causes of death worldwide (Luzhetskyy *et al*., 2007).

On the other hand, the pipelines of drug development in the area of anti-infective became vacant; this is mainly because new technologies such as combinatorial chemistry and high throughput screening methods have failed to provide novel drug candidates even though a lots of novel targets provided by the sequenced genomes of pathogens (Donadio *et al*., 2005a; Payne *et al*., 2007). The general failure of the involved antibiotic producing stakeholders (big

pharmaceuticals, small biotechnology enterprises, academic groups and governmental agencies) in delivering new antibiotics is reflected by the poor state of the antibiotic pipeline. If stakeholders and governments do not invest a lot in discovering and developing novel antibiotic classes, increasing antibiotic resistance danger, together with the current neglected antibiotic-discovery research, might return the society to the pre-antibiotic era (Berdy, 2012; Meer *et al*., 2014).

2.2.4. The need for new antibiotics

There have been no new broad-spectrum antibiotics developed in the last 40 years and the drugs we have currently are quickly becoming ineffective (Gill *et al*., 2015). Because of the prevalence of drug resistance, the effectiveness of all antibiotic agents in current use became compromised (Fischbach &Walsh, 2009). Hence, the need to search for new and efficient antibiotic keeps rising due to the emergening of multidrug resistant pathogens (Baltz, 2006; Berdy, 2012). Infections caused by drug-resistant pathogens are associated with increased morbidity, mortality and health care costs.

The European Academies Science Advisory Council (EASAC) has a long-standing attention on the opportunities and challenges in combating the infectious diseases. The EASAC organized a meeting in Hannover, Germany (in March, 2014) to bring together academic professionals on antibiotic research and development, so as to explore new ways in antibiotic discovery. The council noted that the "urgent action to tackle antimicrobial resistance must take account of all the scientific opportunities available, find new resources to support academia and emphasize the

importance of innovation to policy-makers and to the general public" (Meer *et al*., 2014). A number of investigators have also argued that this problem is aggravated by the current shortage of achievement in developing novel antibiotics. Thus, development of new antimicrobial agent, if possible from the naturally available ones with novel mode of action, is an imperative call for medicine (Ilic, 2005; Kumar *et al*., 2014).

In the search for new antibiotics, choice of natural materials such as soils by is based on the assumption that samples from widely diverse and unstudied locations are more likely to yield novel microorganisms that can produce novel metabolites as a result of the geographical variation (Moncheva *et al*., 2002). In addition, the important approaches helpful in discovering new microbial species or novel bioactive substances include isolation and characterization of microorganisms from the most extreme habitations (Goodfellow and Fiedler, 2010).

2.2.5. Microbial natural products as sources of antibiotics

Despite the decline in interest by large pharmaceutical companies, natural products represent the main source of approved drugs and still play an important role in providing chemical diversity in the future (Monciardini *et al*., 2014). In this regard, the exploration of micro-organisms as sources of therapeutically useful compounds has a much shorter and less well-known history than the use of plants and plant extracts in human medicine (Pettit, 2011). These micro-organisms may have evolved the ability to produce bioactive secondary metabolites because of the selection advantages conferred upon them as a result of the interactions of the compounds with specific receptors in other organisms (Demain, 1983). Hence, in spite of the high rate of

rediscovery, microbial natural products still appear to be an inexhaustible source of pharmaceutical lead structures discovery for new antimicrobials, antiviral, antitumor drugs, and agricultural and pharmacological agents (Vicente *et al*., 2003; Pettit, 2011).

Successful discovery of new antibiotics from microbial natural products, needed to solve the potential bottlenecks in the process. It requires a given microorganism grown in conditions appropriate to induce the production of the desired metabolite, which is then extracted and in a screened to detect the active substance. The compounds have to be isolated from the original mixture and identified (Pelaez, 2006). Besides, it is extremely time-consuming and expensive to isolate and elucidate the chemical structures of microbial products (Rishton, 2008).

It is well known that bioactive natural products are obtained from bacteria, within them the most prolific producers of novel structures, are the gram-positive filamentous bacteria called actinomycetes (Hopwood, 2007; Berdy, 2012). Among actinomycetes, the largest genus namely *Streptomyces,* is responsible for the production of over two thirds of all clinically useful antibiotics. The considerable role of *Streptomyces* to pharmaceutical industry is still a stimulus to examine secondary metabolites isolated from these strains as potential bioactive compounds in the future (Demain, 2009; Berdy, 2012; Taj and Sorensen, 2015).

2.2.6. Actinomycete as prolific sources of antibiotics

The name “actinomycete” goes back to 1877, when it was applied to a microbe responsible for a disease of cattle called “lumpy jaw.” From Greek ‘aktis’ (a ray) and ‘mykes’ (fungus) and given

to these organisms from initial observation of their morphology. It originated from the generic name *Actinomyces*, described for the causative agent of actinomycosis, the thread bacterium *Actinomyces bovis* (Harz, 1877 cited in Goldman and Lorrence, 2009). Actinomycetes were originally considered to be an intermediate group between bacteria and fungi. They were grouped in prokaryotes by Buchnanin in 1917 and now are recognized as prokaryotic organisms (Buchanan, 1917 cited in Lechevalier and Lechevalier, 1967).

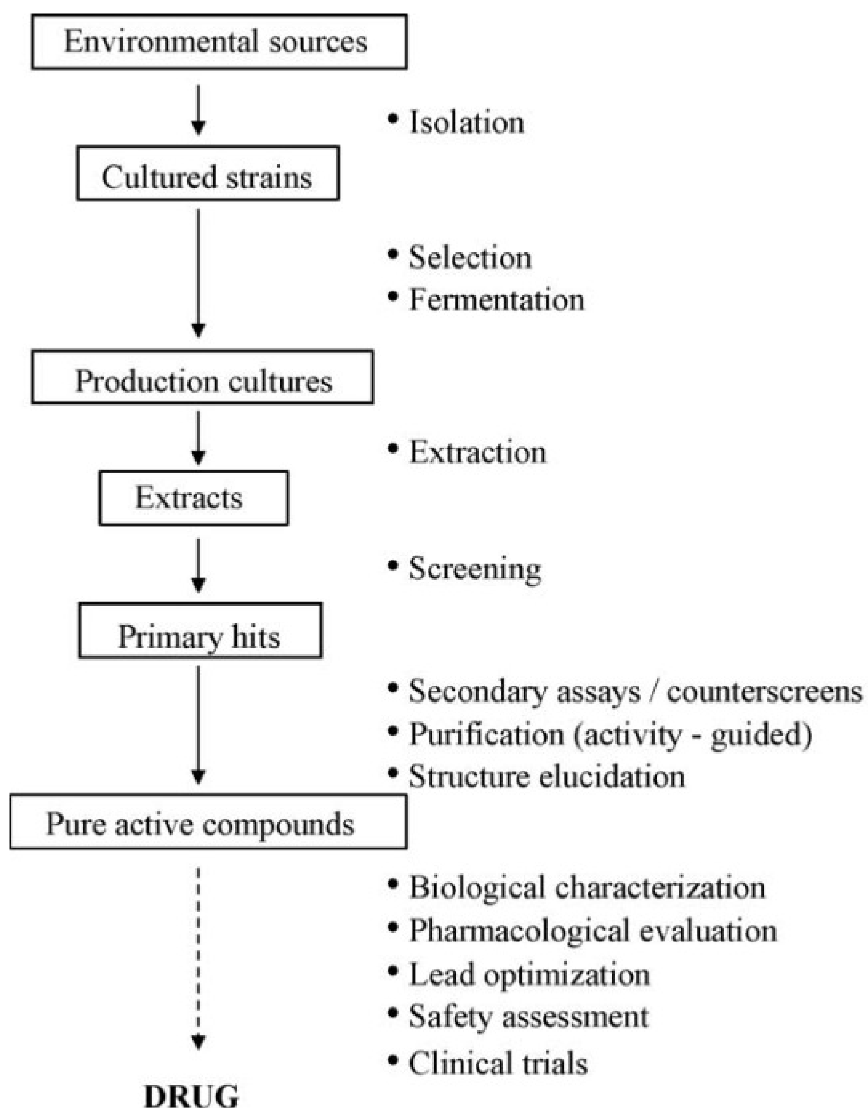


Figure 2.5. The process of antibiotic discovery from microbial natural products (Pelaez, 2006)

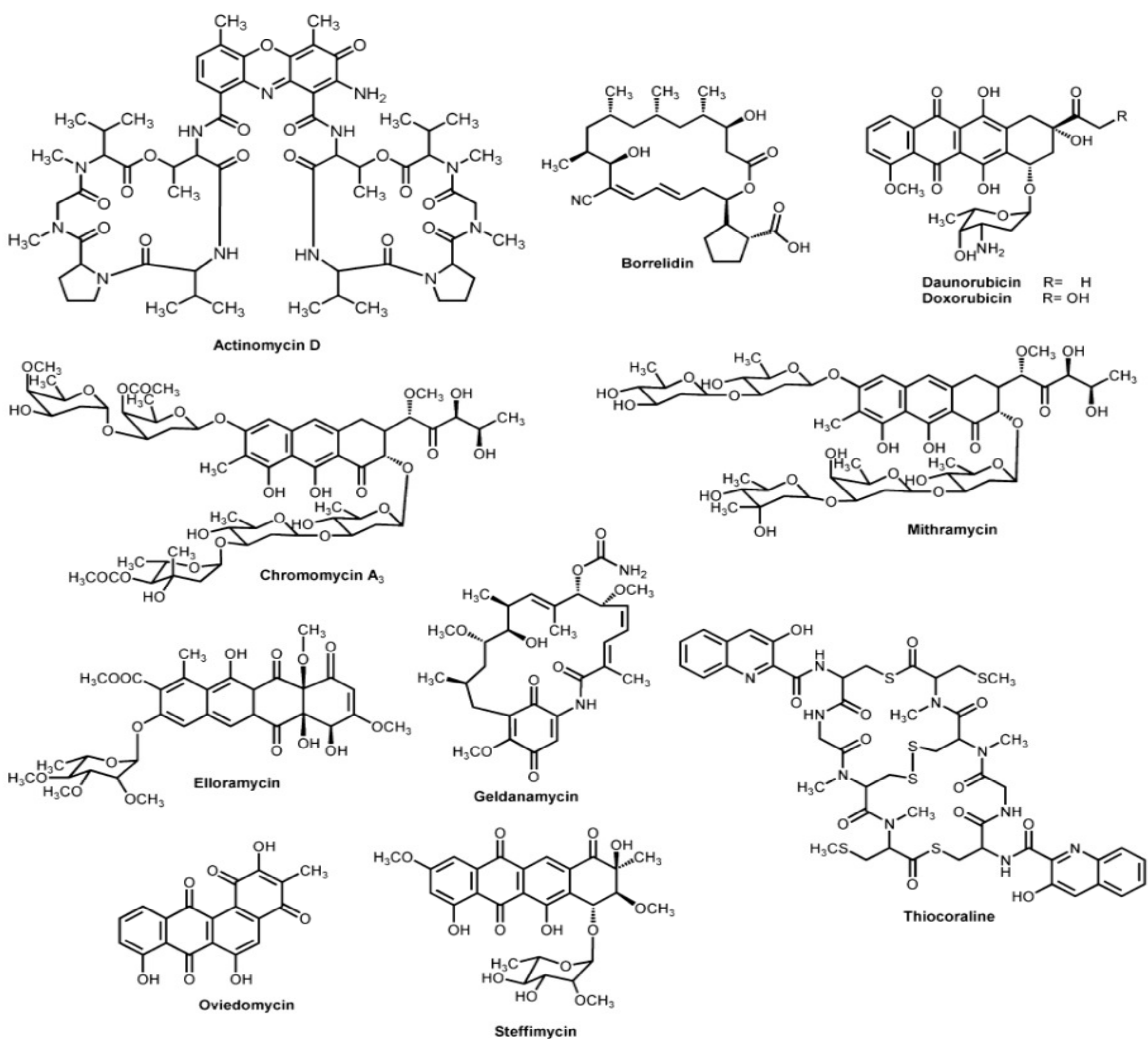


Figure 2.6. Structures of some polyketide and non-ribosomal peptide antitumour compounds from microbial sources (actinomycetes) (Olano *et al*., 2011)

It was due to Waksman's discovery that actinomycetes, known to be nature's inexhaustible producers of antibiotics. Streptomycin, the first effective antibiotic discovered from actinomycetes in 1943 was able to cure the most dangerous human disease, tuberculosis (TB). As a result, the actinomycetes were to become some of the most significant players in applied microbiology as the basis of a multibillion dollar antibiotics industry in the history of chemotherapy (Hopwood, 2007).

2.2.6.1. General characteristics of actinomycetes

Actinomycetes are filamentous bacteria typically revealing various levels of actual branching. The group comprise wide spread and diverse group of Gram-positive, mostly aerobic, heterotrophic and saprophytic, mycelial bacteria with a high guanine plus cytosine content in their DNA (Goodfellow and Williams, 1983). They make a range of spore types including the endospores in *Thermoactinomycetes*, zoospores in *Actinoplanes,* and arthrospores in *Streptomyces* (Goodfellow and Cross, 1974). These spores are specifically devoid of any thermal resistance; however, they do withstand desiccation quite satisfactorily, and thus exhibit considerable adaptive value (Kar, 2008).

Actinomycetes are well-differentiated microorganisms in their morphology. They encompasse genera covering a wide range of morphologies extending from the coccus such as *Micrococcus* and rod-coccus (e.g. *Arthrobacter*), through fragmenting hyphal forms (e.g. *Nocardia*), to genera with a permanent and highly differentiated branched mycelium (e.g. *Micromonospora, Streptomyces* and others) (Bhattacharya, 2007). Members of the group are often mesophilic and neutrophilic. Besides, psychrophilic and thermophilic actinobacteria are not uncommon, and when sought, acidophilic and alkalophilic are readily found. Different nutritional modes, anaerobic and fermentative (*Actinomyces, bifidobacteria, propionibacteria*), chemolithotrophic (*Acidimicrobium ferrooxidans*), nitrogen-fixing (*Frankia*), are found in the taxon (Goldman and Lorrence, 2009). Most actinomycetes in the order actinomycetales possess cell wall type I to IV with peptidoglycans containing L-diaminopimelic acid (DAP) and glycine (type I), meso-DAP

and glycine (type II), meso-DAP (type III), and meso-DAP and arabinose, or galactose (type IV) (Goodfellow, 1989).

2.2.6.2. Ecological distribution

Actinomycetes are mainly free living, saprophytic bacteria and some of them can exist in mutualistic or parasitic associations in the bodies of plants and animals (Goodfellow and Williams, 1983). They are commonly disseminated in terrestrial and aquatic ecosystems, mostly in soil, where they play significant roles in the degradation and recycling of complex mixtures of biopolymers in dead plant, animal and fungal materials (Stach and Bull, 2005). Culture-dependent and culture independent methods have showed that indigenous marine actinomycetes exist in the oceans and are widely distributed in different marine habitats (Lam, 2006). Recently, the isolation of actinomycetes from marine habitat, shallow costal to the deepest sediments, revealed that actinomycetes are omnipresent in marine ecosystems, but in fewer numbers than in soil (Fiedler *et al*., 2005; Ward and Bora, 2006).

A phylogenetic analysis on actinomycete diversity in dry valley soils by Babalola *et al*. (2008) cited in Donald *et al*. (2010), demonstrate that, very large pool of previously undescribed species and genera exist in such habitats. Generally, actinomycetes are flourishing group of bacteria that occur in diversified natural and man-made environments (Trujillo *et al*., 2006; Castillo *et al*., 2007). They exist in their natural habitats in either metabolically active or relatively inactive states. Hyphae and spore of most genera, for instance, can be equated with the active growth and

inactive dispersal phases, respectively (Williams and Wellington, 1980 cited in Goodfellow and Williams, 1983).

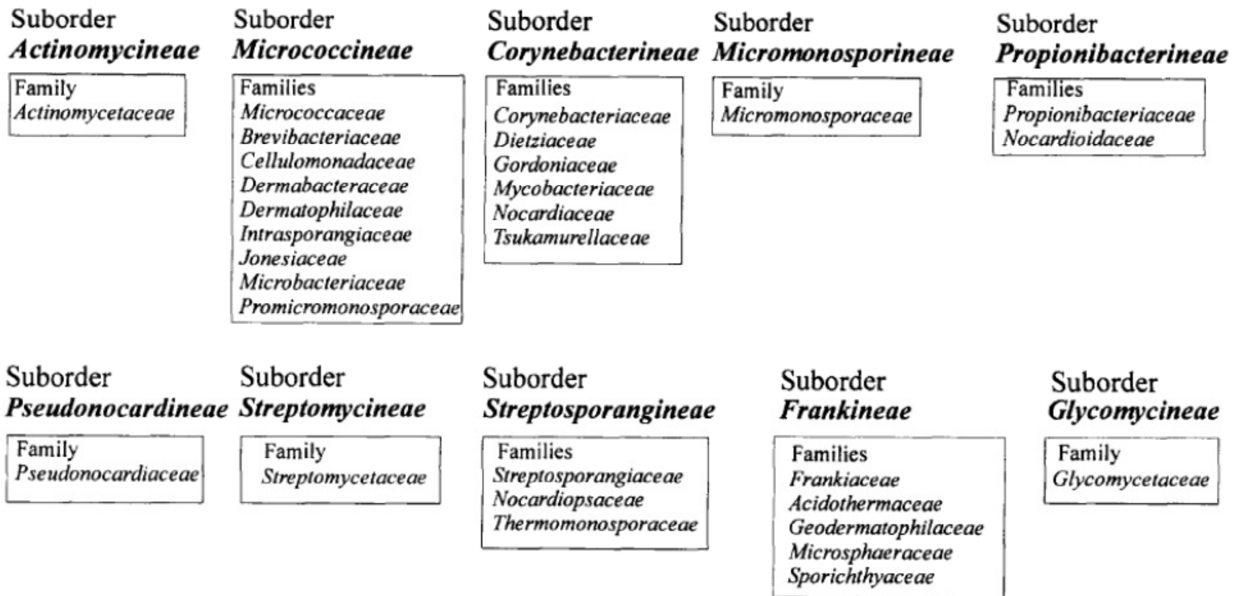
2.2.6.3. Taxonomy

Actinomycetes are highly diversified group of microorganisms and their taxonomy is in a state of flux. Several taxonomically significant characteristic features and useful techniques are employed for the taxonomic identification of these groups. The phylogenetic positions of actinomycetes are determined using morphological and cultural characteristics (Shirling & Gottlieb, 1966), micro-morphological features (Williams and Cross, 1971) chemotaxonomic properties, and molecular analysis (16srRNA sequencing) in polyphasic approach (Trujillo and Goodfellow, 1997; Basilio *et al*., 2003). Various classification schemes were proposed by different authorities to classify actinomycetes at different time. The hierarchic classification for *Actinobacteria* proposed by Stackebrandt *et al*. (1997), based on nucleotide sequences of the 16SrRNA genes was the most important one and there have been few changes in the higher phyla since 1997.

According to Stackebrandt *et al*. (1997) classification scheme, the actinomycetes phyla are clustered into class Actinobacteria, with five subclasses, six orders, and ten suborders, with 31 families (Figure 2.7). On the other hand, as described in Garrity and Holt (2001), (http://www.bacterio.cict.fr), the phylum *Actinobacteria* comprises one class, five subclass, 6 orders, 39 families and more than 130 genera. However, as described in Goldman and Lorrence (2009), there are 195 validly described genera and 1936 validly described species as of mid-2007; many families contain one genus and many genera contain one species (Table 2.2).

Class ***Actinobacteria***

Subclass *Acidimicrobidae* Order *Acidimicrobiales* Family *Acidimicrobiaceae*
Subclass *Rubrobacteridae* Order *Rubrobacterales* Family *Rubrobacteraceae*
Subclass *Coriobacteridae* Order *Coriobacteriales* Family *Coriobacteriaceae*
Subclass *Sphaerobacteridae* Order *Sphaerobacterales* Family *Sphaerobacteraceae*
Subclass *Actinobacteridae* Order *Actinomycetales*



Order *Bifidobacteriales* Family *Bifidobacteriaceae*

Figure 2.7. Hierarchic classification of the actinomycetes based on the phylogenetic analyses of the 16S rDNA sequence data adapted from Stackebrandt et al. (1997)

Recently, Girard *et al*. (2013) published a novel taxonomic marker that discriminates between morphologically complex actinomycetes. The method utilizes the complete conserved SsgB amino acid sequence (related with the regulation of growth and cell division) between members of the same genus and its high divergence between even closely related genera as a high-quality marker for the classification of morphologically complex actinomycetes. In general actinomycetes belong to the order *Actinomycetales* (in the Super Kingdom: Bacteria, Phylum: *Firmicutes*, Class: *Actinobacteria*, Subclass: *Actinobacteridae*). However, the recent hierarchical

classification of the phylum actinobacteria organized these microbes in to the class actinobacteria with 5 subclasses, 9 orders, 55 families, 240 genera and around 3000 species (Goodfellow and Fiedler, 2010).

Table 2.2. Taxonomic groups of Actinobacteria; as described in Goldman and Lorrence (2009)

Actinobacterial Orders

Phylum	Class				
"Actinobacteria"	Actinobacteria	Number of Members in Each Taxonomic Rank			
Subclass	**Order**	**Suborder**	**Family**	**Genus**	**Species**
Acidimicrobidae	*Acidimicrobiales*	*"Acidimicrobineae"*	1	1	1
Rubrobacteridae	*Rubrobacterales*	*"Rubrobacterineae"*	5	5	8
Coriobacteridae	*Coriobacteriales*	*"Coriobacterineae"*	1	8	19
Actinobacteridae	*Bifidobacteriales*	—	1	5	37
	Actinomycetales	*Actinomycineae*	1	5	49
		Catenulisporineae	2	2	3
		Corynebacterineae	7	13	318
		Frankineae	7	12	24
		Glycomycineae	1	2	7
		Micrococcineae	15	72	311
		Micromonosporineae	1	15	73
		Propionibacterineae	2	18	96
		Pseudonocardineae	2	19	99
		Streptomycineae	1	3	749
		Streptosporangineae	3	17	142

In this particular study, it is worth to illustrate the taxonomic position of the most prolific bioactive secondary metabolite producers among actinomycetes, known as *Streptomyces*. Previously, the genus *Streptomyces* were grouped under the order *Actinomycetales,* suborder *Streptomycineae and* family *Streptomycetaceae.* However, in the recent volume five of Bergey's manual of systematic bacteriology (2012) the suborder *Streptomycineae* was elevated into a new order *Streptomycetales.* Hence their taxonomic position became under phylum *Actinobacteria*, class *Actinobacteria,* order *Streptomycetales* and family *Streptomycetaceae.* In the new

classification scheme, this family consists of two other genera (*Kitasatospora and Streptacidiphilus*) in addition to the genus *Streptomyces* (Zhi *et al*., 2009). Hence, in the recent volume of Bergey's manual of systematic bacteriology, more than 550 species of the genus *Streptomyces* have been described (Gontang *et al.,* 2007; Kampfer, 2012c).

2.2.6.4. Exploitable biology

Actinomycetes are the most significant microorganism in industrial application and in providing environmental services. They are the major sources of exploitable biology in modern biotechnology. The group is considered not only as an antibiotic manufacturers but also central microorganisms in the degradation of organic compounds and carbon recycling. It is evident that actinomycetes have the capability to synthesize many different biologically active metabolites such as antibiotics, herbicides, pesticides, anti-parasites and enzymes used for various purposes. Based on several studies among bacteria, the actinomycetes, particularly, *Streptomyces* are remarkable as antibiotic producers, making three quarters of all known microbial products (Solans & Vobis, 2003; Oskay *et al*., 2004; Berdy, 2005; Demain, 2009).

Most notably, the prolific genus *Streptomyces* are the sources of most of the commercially significant bioactive metabolites used as antibiotics, anticancer drugs, and immunosuppressant (Lam, 2006; Berdy, 2012). *Streptomyces* species synthesize a large number of metabolites that have various functions ranging from mineral chelating (siderophores for iron uptake), up to the vast arrays of enzymes (cellulases, amylase, pectinases, xylanase etc.) used in the degradation of biopolymers (Bentley and Chater, 2002; Flardh, 2003; Hopwood, 2007). These make

streptomycetes not only as antibiotic manufactures but also central microorganisms in the carbon recycling, which offer a green and clean remedy for the emerging challenges of waste management technology. Furthermore, *Streptomyces* sp. H668 is reported to produce of a new antimalarial polyether substance (Na *et al*., 2008). Other actinomycetes such as *Thermonospoa fusca* are the source for enzymes with a wide range of thermo stability, broad pH range, secreted cellulases and xylanases that have been cloned and expressed with a view to degrading agricultural waste to sugars and subsequently to ethanol (Bunch, 1998).

In agriculture, most interest has centered on the biocontrol potential of actinomycetes to protect plants from fungal pathogens (Xue *et al.,* 2013) in which many do readily produce of antifungal antibiotics in vitro (Iznaga *et al*., 2005). Actinomycetes have also showed potential towards the production of an applicable eco-friendly post harvest biocontrol products (Sadeghian *et al*., 2016). Besides, they are thought to be of most significance in the degradation of relatively complex, recalcitrant polymers and organochlorine pesticides (which is an important process for pesticide bioremediation) (Fuentes *et al*., 2010). To this end, potentials of actinobacteria in the bioremediation processes of pesticides and heavy metals clearly described in Alvarez *et al*. (2017). They have been shown to posses the potential to degrade many other polymers occurring in soils and litter including hemicelluloses, pectin, chitin and that of humus materials (Lechevalier & Lechevalier, 1967). Others significantly contribute in symbiotic nitrogen fixation (Gadelhak *et al*., 2005).

2.2.6.5. Methods of cultivation

A variety of standard media have been used for the cultivation of actinomycetes (Shirling & Gottlieb, 1966). Usually actinomycetes have been cultivated in liquid medium commonly known as submerged fermentation for the production of bioactive metabolites (antibiotics and enzymes etc.), either in laboratory or at industrial scale for commercial purposes. However, it has been noted that actinomycetes could also be cultivated using solid substrates in a process called solid state fermentation to produce valuable bioactive natural products. A number of authors Venkateshwarlu *et al*. (2000), Krishna *et al*. (2003), Sharmila *et al*. (2012) reported production of valuable antibiotics (rifamycin-B, rifamycin SV, cyclosporin- A respectively) from various actinomycetes using solid state fermentation method.

2.2.6.6. Solid state fermentation as alternative cultivation method

2.2.6.6.1. Definition and historical backgrounds

A number of authors have defined solid state fermentation in different ways. For instance, Durand and Blachere, (1988), defined it as a technique to grow and cultivate microorganism on or inside moistened solid substrates. According to these authors, the moisture content complexed with the solid substrate retained at the level equivalent to the water activity enabled for better growth and metabolism of the strains. On the otherhand, Raimbault (1998) described SSF as microbial transformation of solid substrates and defined it in terms of some important properties that use solid absorbent medium, allow high rates of biochemical processes and intends for the growth of specific cultures of microorganisms. SSF also refers to microbial growth and product formation using moist solid substrates particles in the absence of excess or free aqueous phase,

however, the substrate contains sufficient moisture to permit the microorganism growth and metabolism (Pandey, 2003).

Frequently the two terms i.e. solid-state fermentation and solid substrate fermentation have been used vaguely. However, the well known authority on the area Pandey *et al*. (2008) distinguish these two concepts, and stated that solid substrate fermentation is a processes in which the substrate itself act as carbon/energy source, occurring in the absence or near-absence of free water; whereas solid-state fermentation refers to the fermentation process taking place in the absence or near-absence of free water, that make use of a natural substrate or an inert substrates used as solid support. Unlike submerged fermentation, the water content in SSF is low and the microorganisms are almost in direct contact with gaseous oxygen in the air (Hesseltine, 1972). The required moisture in SSF is available in absorbed or complexed form within the solid matrix; the intraparticle space in the solid substrate mainly filled with a continuous gas phase, which is expected to be more helpful for growth and metabolite production due to the easy oxygen transfer in the process (Barrios-Gonzalez, 2012).

Historical developments showed that SSF originated from areas of food fermentation in Asia before the birth of Christ (Pandey *et al*., 2008). According to Pandey and his colleagues, Egyptian bread making using SSF processes in 2000BC and the use of soy sauce koji in China are some historical evidences showing the origin of solid state fermentation from the eastern world. According to Pandey (2003) solid state fermentation is the source of the other fermentation techniques since antiquity and its principles were the starting point for almost all the other fermentation processes practiced in the remote past. It has been well-known for

centuries that solid state fermentation was mainly used in making oriental foods (Barrios-Gonzalez and Mejia, 1996; Pandey *et al*., 2008). In the 1940s, which was considered as the golden era of fermentation industry, the golden drug penicillin was produced using both solid state and submerged fermentation methods (Pandey *et al*., 2008).

Even though it was known since centuries, SSF has got bright attention recently by researchers from different parts of the world Chen (2013), mainly from the eastern world like (Japan, China and India) and Latin America (Brazil and Mexico). This is because its development techniques are economical, used in solid waste management and in its role to produce valuable bioproduct (Pandey, 1992; Ellaiah *et al*., 2004; Holker *et al.,* 2004; Pandey *et al*., 2008).

2.2.6.6.2. Bioprocess development and control parameters

Solid-state fermentation (SSF) is a three-phase development, consisting of solid, liquid and gaseous phase, which provide advantatges in the microbial cultivation for bioprocesses and products development (Thomas *et al*., 2013). Due to its less energy demand, higher product yields and less wastewater production and less risk of bacterial contamination, recently SSF has gained considerable attention for the development of industrial bioprocesses (Barrios-GonzBlez and Mejia, 1996; Pandey *et al*., 2008). Besides, it is environmentally friendly, as it mostly makes use of solid agricultural and agro-industrial wastes (resides) as the substrate (Thomas *et al*., 2013). It has continued to build up trustworthiness in recent years in biotechnological industries because of its promising applications in the production of biologically active secondary

metabolites, apart from feed, fuel, food, industrial chemicals and pharmaceutical products (Pandey, 2003).

Recently, much potential have been observed in the development of quite a lot of bioprocesses and products (Pendey *et al*., 2008). A number of reports evidently showed great progression of bioprocess developments in solid state fermentation. To this end, biotechnological potential of SSF processes in biodegradation and removal of bioplastics (Emadian *et al*., 2017), degradation of feather waste (Mazotto *et al*., 2013), removal of waste grease (Kumari *et al*., 2017), bioremediation process for textile industry effluents (Asgher *et al*., 2014) and dye bioremediation (Waghmare *et al*., 2014), have been reported. Besides, different publications reported that bioethanol from cellulosic materials (Idris *et al*., 2017), production of biomass degrading enzymes for the bioenergy sector (Farinas, 2015), production of biopulp (Chen *et al*., 2002), production of biomass valorizing enzymes (Gopalan *et al*., 2016) and substitution of chemical dehairing by proteases in leather industry (Abraham *et al*., 2014) in SSF.

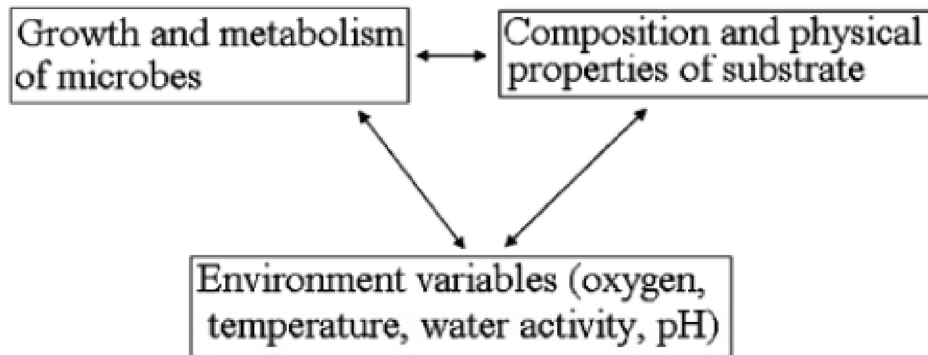


Figure 2.8. Relationship of microbial metabolism, physical properties of substrates and fermentation parameters (Chen 2013)

A number of process control parameters can affect the whole process design and product development in SSF. These are the substrates used in SSF, composition, mechanical properties, particle size (including inter-and intra-particle spaces), water holding capacity, moisture content, pH, temperature and microbial type etc. (Thomas *et al*., 2013). If SSF could benefit from a deeper understanding of microbial physiology in a solid environment, process parameter regulating factors should be investigated in detail (Barrios-Gonzalez and Mejia, 2008; Barrios-Gonzalez, 2012; Thomas *et al*., 2013). In SSF process, the selection of a suitable nutrient substrate is a critical factor which in turn is governed by the properties of the micro-organism (Nagavalli *et al*., 2015).

2.2.6.6.3. Microbial products developed by SSF

Microbial secondary metabolites are useful high value products that are normally produced by liquid culture; but could be advantageously produced by solid-state fermentation (SSF). A variety of secondary metabolites have even been shown to be produced solely during growth on solid substrates. For this reason, SSF could play an interesting role in the screening for new metabolites production (Holker *et al*., 2004). Recent research indicates that different kinds of secondary metabolites can be produced by SSF. Among the vital collection of bioactive compounds, which have been developed via solid state fermentation, antibiotics, immuno-suppressive drugs, alkaloids, mycotoxins, plant growth factors, etc. are some of the most important ones (Pandey *et al*., 2000).

2.2.6.6.4. Advantages and disadvantages of SSF

SSF has key benefits and weaknesses because of its physicochemical properties, namely, relatively low water activity and formation of significant products (Viniegra-Gonzalez, 1997). There are a number of advantages of SSF over the usual submerged fermentation (SmF) these include higher concentration of the products, high volumetric productivity and less effluent generation (environmental friendly). Besides, some enzymes and secondary metabolites can only be produced in SSF (Barrios-González, 2012). According to Barrios-Gonzalez, this is related to the higher transcription of biosynthetic genes and the expressing of specific genes in solid state environmental signals that could turn on cryptic secondary metabolite pathways.

Table 2.3. Some important comparison of SSF with SmF processes (Barrios-Gonzalez and Mejia, 1996)

Characteristics	**Solid state Fermentation**	**Submerged Fermentation**
Capital investment	Low	High
Human energy requirement	High	Low
Physical energy requirement	Low	High
Liquid waste produced	Negligible	Significant volume
Volume of fermentation mash	Smaller	Larger
Oxygen supply by	Diffusion	Aeration
Water usage	Limited	Unlimited
Microorganism, substrate	Static	Agitated

SSF also has its own limitations. In SSF only microbes that can survive in low moisture content can be used, exact regulation and scale-up of the fermentation conditions are difficult, growth rate of microbes on solid substratum is fairly slow, fermentation generally releases too much heat in the medium, difficulties in mixing, and the environmental conditions of the microbes are difficult to control (Chen, 2013; Papagianni, 2014).

Chapter 3: Actinomycetes Producing Antimicrobial Compounds: Screening, Bioactivity and Phylogeny

Abstract

The prime objective of this chapter was to isolate, screen and identify antimicrobial compound-producing actinomycetes from various unique environmental niches in Ethiopia. A total of 416 actinomycete cultures were isolated. During the primary screening, ten isolates having considerable activities against *Candida albicans* (ATCC 62376) and *Cryptococcus neoformans* (clinical isolate), were chosen for further investigation and taxonomic identification study. Some of these isolates showed substantial broad spectrum activities against standard Gram positive pathogens *Bacillus subtilis* (ATCC6633), *Staphylococcus aureus* (ATCC25923) and the others on Gram negative bacterial pathogens such as *E.coli* (ATCC 25922), and *Sallmonella typhomeriumn* (ATCC6539). Micro and macro-morphological, cultural, physiological and biochemical properties were interestingly fit with the genus *Streptomyces* described in Bregay's manual of systematic bacteriology. The identification of the isolates was confirmed by the analysis of the 16s rRNA gene sequences. Isolate Go-475 with ACC NO. MF474326 exhibited 99% similarity with *Streptomyces steffisburgensis* JCM4833 and that of Rv-355 ACC NO. MF474329 showed 99% similarity with *Streptomyces* sp. 13-3-10. Two isolates, namely Ac-006 with ACC NO. MF474330 and Ac-123 having ACC NO. MF474327 exhibited 99% similarity with *Streptomyces lividans* TCA20046 and *Streptomyces* sp. SAUAC2-1 respectively. Whereas, isolate Ac-146 and Ed-065 with ACC NOs. MF474325 and MF474328 respectively represented interestingly in the same clad with 99% similarity with *Streptomyces* sp. 13-2-25. In general isolates phylogenetic analysis confermed that all the isolates belong to the genus *Streptomyces*.

Key words: *Actinomycetes*, *antimicrobial bioassay*, *solid state fermentation*, *Streptomyces*

3.1. Introduction

Actinomycetes have been known for more than a hundred years mainly on morphological properties and well thought-out to be bacteria that can produce branching hyphae at some stage of their growth (Goodfellow and Williams, 1983; Sanglier *et al*., 1993). They belong to the order *Actinomycetales* (in the Super Kingdom: Bacteria, Phylum: *Firmicutes*, Class: *Actinobacteria*, Subclass: *Actinobacteridae*). The current hierarchical classification of the phylum actinobacteria grouped these microbes into the class actinobacteria with 5 subclasses, 9 orders, 55 families, 240 genera and around 3000 species (Goodfellow and Fiedler, 2010).

These filamentous, Gram positive bacteria have a widely recognized potential for the production of bioactive secondary metabolites and valuable enzymes (van der Meij *et al*., 2017). Actinomycetes are abundant in soils, marine sediments as well as in symbiotic association with various plants and animals (Goodfellow and Williams, 1983). They play vital roles in the processes of degradation and recycling of complex organic materials (Budihal *et al*., 2016), produce plant growth promoting hormones and biocontrol agents (Chen *et al*., 2005; Macagnan *et al*., 2008) and significantly contribute to symbiotic nitrogen fixation (Gadelhak *et al*., 2005). Most importantly, secondary metabolites of actinomycetes play prominent roles in the history of drug development, in particular as antibiotics and anticancer agents (Katz and Baltz, 2016). During the golden age of microbial natural product-based drug discovery (1950s through1970s), most of the antibiotics currently used to treat various infections were discovered from actinomycetes; e.g. the antifungals nystatin and amphotericin or the antibacterials erythromycin and vancomycin. Besides antibiotics, actinomycetes are known to produce antitumor agents (Li

et al. (2008), immunosuppressives, pesticides, herbicides and antihelmintics (El-Tarabily *et al*., 2009; Liu *et al*., 2009; Nakouti *et al*., 2012). Actinomycetes of the genus *Streptomyces* are the most prolific producers of bioactive secondary metabolites and they continue to be important sources of new antibiotics (Berdy, 2005; Baltz, 2007; Demain, 2009). *Streptomyces* usually possess 20-50 gene clusters dedicated to the biosynthesis of secondary metabolites in their genomes, underscoring their potential for the discovery of new bioactive compounds (Nett *et al*., 2009). Most of such clusters remain “silent” in laboratory conditions, but tools are being developed for activation of these genes, thus prompting production of previously undetected compounds (Kealey *et al*., 2017).

Despite the onset of the genomic era and some progress in awakening “silent” gene clusters in actinomycetes, screening for new natural products remains an important part in the drug discovery process. The major challenge in this approach is to avoid the re-discovery of already known bioactive compounds, even from phylogenetically distinct actinomycetes. Therefore, efficient de-replication (Carrano and Marinelli, 2015), novel screening techniques and investigating new sample sources from underexplored habitats (Nolan and Cross, 1988; Tan *et al*., 2009; Goodfellow and Fiedler, 2010) become more and more vital. In the search for actinomycetes capable of producing novel bioactive metabolites, it appears especially beneficial to look into extreme habitats and unique environmental niches (Sanglier *et al*., 1996; Bull & Stach, 2007; Berdy, 2012; Matsui *et al*., 2012; Zotchev, 2012). In this regard, Ethiopian soils from various climatic zones and possessing remarkable biodiversity may harbor new actinomycete strains that have the potential for new antibiotic discovery.

Hence, the current study was intended for the isolation of antibiotic-producing actinomycetes from soils collected at various unique natural environmental niches and specific biotopes in Ethiopia. Standard screening methods were employed for initial detection of antimicrobial activities targeting known microbial pathogens. This was followed by cultivation of bioactive secondary metabolites with solid state fermentation method, bioactivity profiling and taxonomic identification of selected isolates.

3.2. Objectives

3.2. 1. General objectives:

The general aim of this study was to isolate, screen and phylogenetically analyze promising actinomycetes capable of producing bioactive secondary metabolites from various unexplored habitats in Ethiopia.

3.2.2. Specific objectives: this particular study was intended to:

- Isolate and screen bioactive secondary metabolite producing actinomycetes using standard bioassay methods and determine the taxonomic position of potentially selected antimicrobial compound producing actinomycetes at genus level
- Cultivate bioactive secondary metabolite producing actinomycetes using SSF and evaluate the antimicrobial activities of the extracts
- Check the bioactivity spectrum of bioactive secondary metabolites from selected isolates

3.3. Materials and Methods

3.3.1. Sample sites and soil sampling

The sample collection sites encompassed 13 specific habitats in five geographical regions, with diverse natural environments in Ethiopia. Soil sampling sites were chosen based on the rational that diverse agro ecological, climatic and other environmental factors might be the source of promising actinomycetes for potential bioactive secondary metabolite production (Goodfellow and Fiedler, 2010). One of the peculiar soil sampling sites namely Gode is located in the eastern boarder geographical region in Ethiopia. The climate is tropical in Gode, mostly characterized by desert with high temperatures and low precipitation. The area is characterized by sandy soil and no vegetation cover. The maximum average temperature is 37.5 ^{o}C in March and the minimum average is 20.9 ^{o}C in January. Gode's maximum average rain fall is 102mm in April and the lowest average rain fall is 0mm in January, July and August https://en.climate-data.org/location/3660/. All specific soil sampling locations and their corresponding coordinate values are presented in (Table 3.1 and Figure 3.1) for details.

A total of 39 soil samples (about 500g each) were collected using sterile spatula by removing 2-3 inches of top soil and placing the material in sterile zipped polythene bags (Bharti *et al*., 2010). Sample bags were labeled on the basis of their source place and collection time. The collected sample was transported aseptically to Microbiology laboratory, Addis Ababa University, for isolation of actinomycetes.

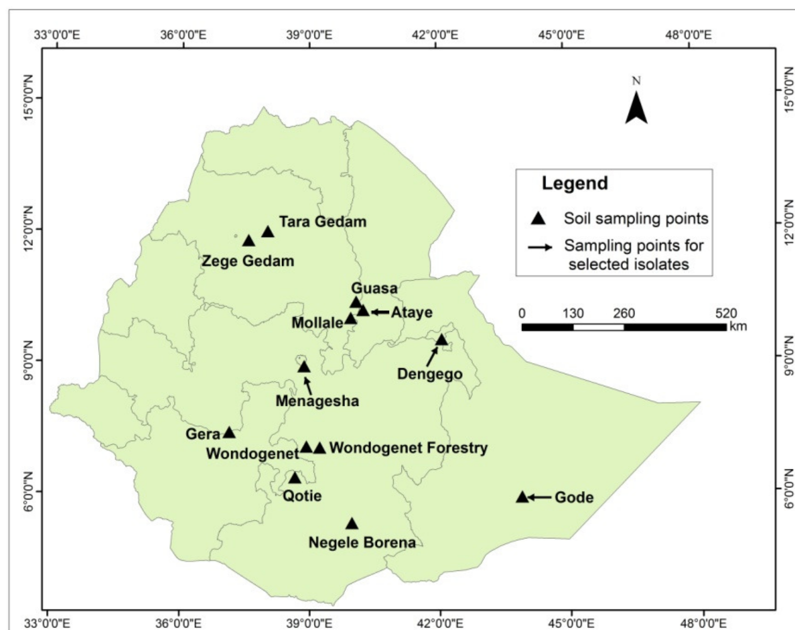


Figure 3.1. Map of Ethiopia, the black triangles showing 13 specific soil sample collection sites; the arrows signify the sampling points of the selected isolates for further investigation

Table 3.1. Coordinate values for the 13 soil sampling site (clustered in five geographical regions) in Ethiopia

SN	Geographical Regions	The specific soil sampling points	Longitude	Latitude
1	Central to south west	Menagesha Suba	38° 50'54''	8° 56'12''
		Gera	37° 7'23''	7° 25'12''
		Qotie	38° 38'20''	6° 24'32''
2	Rift valley area	Ataye	40° 13'12''	10° 14'11''
3	Northern	Guassa	40° 3'56''	10° 17'40''
		Mollale	39° 56'56''	10° 2'45''
		Tara Gedam	38° 1'48''	12° 3'21''
		ZegaeGedam	37° 35'3''	11° 50'36''
4	Southern Region	Wondogenet forestry	38° 55'33''	7° 5'54''
		Wondogenet	38° 12'48''	7°4'45''
		NegelleBorena	39° 57'27''	5°20'9''
5	Eastern Region	Gode	43° 52'23''	5° 55'41''
		Dengego	42° 2'55''	9° 34'27''

3.3.2. Isolation and maintenance of actinomycete cultures

Actinomycete cultures were isolated by soil serial dilution and plating technique on various media including starch casein agar, starch nitrate agar, yeast malt extract agar and actinomycete isolation agar medium supplemented with 50µg/mL cycloheximide and 25 µg/mL nystatin. The cultures were incubated at 30±2 °C for 7-12 days (Okazaki *et al*., 1983; Hayakawa and Nonomura, 1987; Seong *et al*., 2001). Morphologically identified colonies and those showing a characteristic inhibition (clear zone) in the crowded plate were picked up with sterile wire loop and streaked on starch casein agar medium plates; and incubated at 30 ^{o}C for 7-12 days. Repeated streak plating technique was conducted for further purification of the cultures. Pure actinomycete cultures were designated by alphabet and number coding system in which letters indicate the source place and the numbers show the isolation order; the pure isolates were stored at 4 ^{o}C on starch casein agar slants as stock cultures for further investigation.

3.3.3. Target pathogens

The current study targeted different pathogens namely *Candida albicans* (ATCC 62376) and *Cryptococcus neoformans* (clinical isolate); standard *C. albicans* was obtained from Ethiopian Food and Heath Research Institute. *Cryptococcus neoformans* was obtained from Addis Ababa University (Black lion referral hospital). Besides, Gram positive bacterial pathogens such as *Staphylococcus aureus* (ATCC25923), *Bacillus subtilis* (ATCC6633) and Gram negative bacterial pathogens, such as *E.coli* (ATCC 25922), *Sallmonella typhomerium*n (ATCC6539), *Shigella boydii (*clinical isolate), stored in Microbiology laboratory, were used as targetes to evaluate the bioactivity spectrum of antimicrobial compunds.

3.3.4. Primary screening (bioactivity detection)

All pure cultures of actinomycetes were screened for antifungal activities using agar plug assay method as described by Bharti *et al*. (2010). Briefly, pure culture of actinomycete strains were grown over the entire surface of yeast-malt extract agar (ISP2) medium plates composed of (g/L): malt extract 10, yeast extract 4, glucose 4 and agar 20, incubated at 30 ^{o}C for 7 days. At the end of incubation period, agar discs were cut out from the actively grown plate by sterile cork borer (6mm in diameter) and transferred to the surface of Sabouraud's dextrose agar plates seeded with *Candida albicans* and *Cryptococcus neoformans* cultures, with an inoculum size of 5×10^{6} CFU/mL. The culture plates were kept in an incubator at 37 °C to allow the growth of test organisms (24 hrs for *Candida albicans* and 48hrs for *Cryptococcus neoformans*). The bioactivity of the isolates against the test pathogen was confirmed by the formation of a dead (clear) zone around the agar plugs. Ten isolates were chosen on the basis of their bioactivity profile for further investigation.

3.3.5. Solid state fermentation (SSF) and recovery of the products

The inoculum for each of the isolates was prepared as described by Venkateswarlu *et al*. (2000), in which three loopfulls of the spores from selected actinomycete cultures were inoculated into 250mL Erlenmeyer flasks each containing 50mL of ISP2 liquid medium and incubated at 30 ^{o}C on a rotary shaker (200rpm) for five days. On the fifth day, the culture was transferred to sterile test tubes and centrifuged at 2000 rpm. The supernatant was discarded and the culture pellet was resuspended in10mL of 0.1M phosphate buffer (pH 7.0) and 0.01% of Tween 80. The suspension prepared in such a way was used as an inoculum for solid state fermentation (SSF) experiments.

For the SSF, 10 g of wheat bran was placed in 250mL Erlenmeyer flasks and supplemented with mineral salt solution containing 0.1% NaCl, 0.1% $MgSO_4$, 0.5% NH_4NO_3, 0.2% KH_2PO_4 and 1% each of the soluble starch and peptone. Trace elements solution (1mL) containing (g/L): $CuSO_4.5H_2O$, 3.3; $FeSO_4.7H_2O$, 10.0; $ZnSO_4.7H_2O$, 50.0; $MnSO_4.2H_2O$, 4.0; $CoCl_2$, 2.0; and $(NH_4)_2MoO_4$, 1.0, was added and sterilized and the initial moisture conten was adjusted to 65%. Three mL of the inoculum suspensions (containing 2.8 x 10^6 CFU/mL) was added, shaked well to distribute the inoculum uniformly and incubated at 30 ^oC for 12 days (Bussari *et al*., 2008).

At the end of fermentation process crude bioactive secondary metabolites were recovered from the fermented materials by solvent extraction system as described by Venkateswarlu *et al*. (2000). The fermented material and solvent mixtures (1:10) placed in 500mL flasks and shaked vigorously with glass beads overnight on a rotary shaker at 200rpm. In order to optimize the extraction of bioactive secondary metabolites from the fermented material different solvents ranging from more polar to less polar, such as ethyl acetate, methanol and hexane were used separately on the basis of best solubility and maximum antimicrobial yield. The material was filtered through Whatman No. 1 filter paper having a pore size of 11µm and the solvent was evaporated using BUCHI rotavapour, RE 121, Switzerland, under vacuum to achieve the concentrated dry product.

3.3.6. Antimicrobial bioassay and preparation of McFarland standard

3.3.6.1. Preparation of a 0.5 McFarland Standard

A 0.5 McFarland standard was prepared using the guideline given by NCCS (2004) as follows. In a 250mL Erlenmeyer flask, 0.5mL of 0.048 mol/L $BaCl_2$ (1.175% w/v $BaCl_2.2H_2O$) was combined with 99.5mL of 0.18 mol/L H_2SO_4 (1%v/v), the solution was mixed thoroughly. The turbidity standard (6mL) was placed in the same size screw-cap test tubes that were used for the preparation of yeast inoculum suspensions, sealed with Parafilm and stored in the dark at room temperature. The McFarland standard turbidity was validated by reading the optical density (OD) in a 1cm light path and matched cuvette containing 1mL of the standard at a wave length of 530nm using UV/ViS Spectrophotometer, model 6405, Frequency 50/60Hz, JENWAY, UK and verified by colony count method to be equivalent to 1-5×10^6 CFU/mL.

3.3.6.2. Antimicrobial bioassay

The bioactivity testing for potential crude extracts against *Candida albicans* and *Cryptococcus neoformans* were examined using disc diffusion assay method as described by Galvan *et al*. (2008). Inoculum for pathogenic yeasts was prepared by direct colony suspension method, in which 24hr old three to five pure colonies of each test pathogen (48hr for *Cryptococcus neoformans*) were picked up using sterile wire loop and suspended into test tubes containing 5mL of 0.85% sterile NaCl solution. The suspension was vortexed and the turbidity was adjusted to 0.5MacFarland spectrophotometrically with an optical density of 0.1 at a wave length of 530nm and confirmed by viable count to be equivalent to 2x10^6 CFU/mL. Each suspension was inoculated by spread plating over the entire surface of Sabouraud's dextrose agar plates uniformly using sterile cotton swab in a laminar air flow hood in 15minutes time. Sterile paper discs (6 mm in diameter) were impregnated with 50µL of the 1mg/mL of the crude extract from each isolate. The impregnated discs were dried inside laminar air flow hood and placed on the

surface of agar plates seeded with the target pathogens. Ketoconazole with the same concentration dissolved in DMSO was used as a positive control and discs impregnated with ethyl acetate was used as negative control. Inhibition zone diameters were measured in millimeter (mm) and the results were recorded.

Disc diffusion method usually known as Kirby-Bauer method described in CLSI (2012) was used for antibacterial susceptibility testing. Three to five 24hr old agar plate colonies of each of the test pathogens were picked up using a sterile inoculating wire loop and suspended in 5mL of sterile physiological saline, vortexed to create uniform suspension. The turbidity of the suspension was adjusted to a 0.5 McFarland standard either by adding more organisms or diluting with sterile saline depending on the optical density reading at 625nm wave length and the inoculum density was adjusted to a final concentration of 2 x10^8 CFU/mL. Each suspension was inoculated on the entire surface of Mueller-Hinton agar plates with sterile cotton swabs inside a laminar air flow cabinet. Paper discs (6mm in diameter and impregnated with 50µL of the 1mg/mL crude extract) were dried and placed on the surface of agar plates seeded with the target pathogens inside the laminar air flow hood. Chloramphenicol with the same concentration was used as positive control and discs impregnated with the solvent used as a negative control.

3.3.7. Taxonomic identification of the selected isolates

3.3.7.1. Morphological, cultural and microscopic examination

Morphological and cultural characteristics of the isolates were determined as per the International Streptomyces Project (ISP) guide lines. Standard culture media, such as ISP2 (yeast

extract-malt extract agar), ISP3 (oatmeal agar), ISP4 (inorganic salts-starch agar), and ISP5 (glycerol-asparagine agar) were used to study the cultural and morphological properties of the isolates (Shirling and Gottlieb, 1966). The color of aerial and substrate mycelium and that of various soluble pigments were determined by RAL code (Edition of 1990 - Germany Institute for Quality Assurance and Labeling) (Stackebrandt *et al*., 2001).

Micro-morphological structures of the isolates were studied as described in Williams and Cross (1971) and Williams *et al*. (1989). A sterile cover slip was inserted with around 45 degree inclination in the starch casein agar plates (inoculated with the isolates) and incubated at 30 ^{o}C for 5-7 days. At the end of incubation period, the cover slips were withdrawn from the plate using sterile forceps followed by wet mounting for microscopic examination. The structure of the hyphae, and spore chain morphology were investigated using light microscope (model BX-51; Olympus, Tokyo, Japan) and spore diameters were measured using image analysis software and the result was recorded. Observed macro and micro-characteristics were compared with the actinomycete already described in Bergey's manual of systematic bacteriology for identification.

3.3.7.2. Physiological characterization

3.3.7.2.1. Optimum growth temperature, pH and sodium chloride tolerance

Inorganic salts glucose medium (ISG) consisting of (g/L): glucose 10, K_2HPO_4 1.0, $MgSO_4.7H_2O$ 1.0, NaCl 1.0, NH_4NO_3 1.2, $CaCO_3$ 2.0, agar 18 and distilled water 1000mL was used to investigate growth of isolates at different temperatures. The cultures were incubated in temperature range of (15 °C - 40°C). The ability of the isolates to grow at different pH values

were evaluated using the same ISG medium adjusted with either NaOH or HCl to give pH values of 6.5, 7, 7.5, 8.0, 8.5, 9.0 and 9.5. Tolerance of isolates to NaCl was tested by incorporating various concentrations of NaCl (0, 2.5, 5, 7.5 and 10 % (w/v)) individually into ISG medium before autoclaving. The isolates were inoculated in triplicate plates and incubated at 30 ^{o}C; growth conditions were observed and the results were recorded (Shirling & Gottlieb, 1966).

3.3.7.2.2. Utilization of different carbon and nitrogen sources

The ability of the strains to utilize different carbon sources were examined with the basal medium composition (g/L): $(NH_4)_2SO_4$ 2.64, KH_2PO_4 2.38, K_2HPO_4 4.31, $MgSO_4.7H_2O$ 1.0, agar 20, and 1.0mL trace element solution containing (g/100mL) ($CuSO_4.5H_2O$ 0.64, $FeSO_4$.7H2O 0.11, $ZnSO_4.7H_2O$ 0.15, $MnCl_2.4H_2O$ 0.79). Carbon sources used were glucose, soluble starch, maltose, sucrose, fructose, and glycerol to a final concentration of 1% (Locci, 1989). Similarly, the ability of the strains to grow in the presence of different nitrogen sources were tested using a basal medium containing (g/L): 10g glucose, 1.0g K_2HPO_4, 0.5g $MgSO_4$, 1.0g NaCl and 20g agar. The nitrogen sources supplemented were (asparagine, casein, peptone, KNO_3, $(NH_4)_2SO_4$ and NH_4Cl) at a concentration of 1% each and the growth pH was adjusted at 7.5 in all the cases.

3.3.7.3. Biochemical characterization

3.3.7.3.1. Melanin production test

Melanin production was determined based on the guidelines given by the International *Streptomyces* Project on ISP7 (Tyrosine agar) media (Shirling and Gottlib, 1966). The

formations of melanin pigment were determined by comparing the inoculated tubes with that of uninoculated controls. Cultures that showed the formation of greenish brown to brown or darker brown diffusible pigment or a distinct brown pigments modified by other colors were considered as positive and absence of brown to black colors was considered as negative.

3.3.7.3.2. Catalase test

Cultures grown on the entire surface of starch casein agar medium for 7 days were flooded with 3-5 drops of 3% H_2O_2 solution. The formation of bubble indicates a positive reaction and no bubble formation were considered as a negative reaction for catalase test (Collins and Lyne, 1976).

3.3.7.3.3. Oxidation fermentation test

Oxidation and fermentation tests were carried out following the method described by Anejia (1993), with the medium composed of (g/L): peptone 2.0, NaCl 5, K_2HPO_4 0.2, Glucose 10, bromothymol blue (0.2%) 0.08, agar 2.5, distilled water 1000mL and pH was adjusted to 7.2. The basal medium and glucose were sterilized separately and mixed after cooling. A total of 22 test tubes were prepared and 10mL of the medium was distributed into each of the sterile test tubes using sterile measuring cylinder. Each culture was inoculated into two test tubes by stabbing method with sterile needle, one of the test tubes was covered with paraffin oil and the other left without paraffin cover. Two other test tubes containing the same media without the inoculum (one with paraffin cover and the other without paraffin cover) were used as the

controls. The culture was incubated at 30 ^{o}C for seven days and examined daily and the color change was examined and the result was recorded.

3.3.7.3.4. Starch hydrolysis test

Starch hydrolysis test was conducted following the method described by Williams *et al*. (1983) with slight modification. A loop full of spores from each isolate was inoculated by streak plating method on the surface of Bennett's agar medium plates (glucose was replaced by soluble starch) and incubated at 30°C for 7 days, and growth was examined daily. At the end of incubation period, the plates were flooded with iodine solution. The appearance of the hydrolysed zones around the grown culture was considered as positive test.

3.3.7.3.5. Chitinase test

Colloidal chitin was prepared by adding 100mL of 85% phosphoric acid to 10g of chitin (Sigma) and kept in a refrigerator at (4 °C) for 24h. Subsequently, 2 liter of water was added and the viscous white material formed was separated by suction filtration through filter paper. The retained cake was washed with distilled water until the filtrate had a pH of 7.0. The obtained colloidal chitin had a soft pasty consistency with 90–95% moisture. Isolates were inoculated onto colloidal chitin agar plates prepared in a basal salt medium with the following composition (g/L): K_2HPO_4 0.7, KH_2PO_4 0.3, $MgSO_4$ 0.5, $FeSO_4$ 0.01, $ZnSO_4$ 0.001, $MnSO_4$ 0.001, $(NH_4)_2SO_4$ 0.25 yeast extract 1.0, and 1% (w/v) colloidal chitin and incubated for 7 days at 30 ^{o}C. Observation was made for chitin clearing zones around the colonies and the result was recorded (Nagpure and Gupta, 2013).

3.3.7.3.6. Indole production test

Indole production test was conducted following the method described by Anejia (1993). Test tubes containing 8mL of 1% (w/v) tryptone broth (Oxoid) were inoculated with a loop full of each culture in triplicates and incubated at 30 ^{o}C for 7 days and examined daily. 1mL of Kovac's reagent (prepared by adding 10g of P-dimethylaminobenzyaldhyde into 150mL of amyl alcohol) mixed with 50mL of concentrated HCl) was added to the cultures and shaked for 15min and allowed to stand. The development of cherry (red) color on the top layer was indication of positive test for indole production and the formation of a yellow color is an indication of a negative test.

3.3.7.4. Molecular characterization of selected isolates

3.3.7.4.1. DNA extraction and gel electrophoresis

Genomic DNA was extracted with DNeasy Tissue Kit (QIAGEN) following the instructions from the manufacturer. Sporulated colonies were inoculated in test tubes containing 2mL tryptic soya broth medium and incubated at 30 ^{o}C on a shaker incubator for 48hrs at 200 rpm. Each culture (1mL) was transferred into Eppendorf tubes containing 0.5mL sterile water, mixed and centrifuged at 13,000rpm for 3 minutes. The supernatant was discarded and the pellet was resuspended by pipetting 180µL lysis buffer (20mM Tris HCl, pH 8.0; 10mM EDTA pH 8.0; 1.2% Tritonx100; 20mg/mL lysozyme) and incubated at 37 ^{o}C for 15 minutes. The suspension was mixed by gentle tipping up and down every 5 minutes. Proteinase K (25µL) was added and mixed by pipetting followed by the addition of 200µL of buffer AL and mixed by pipetting up and down several times and the mixture was incubated at 55 ^{o}C for 30 minutes. Ethanol (200µL

of 96%) was added and mixed by pipetting; the mixture was applied on the column sitting in the 2mL collection tube and centrifuged at 13, 000rpm for 2minutes. The column was transferred into the new 2mL tube and 500µL AW buffer was applied, centrifuged at 13,000rpm for 1 minute. The column was transferred into the new 2mL tube and 500µL AW2 buffer was added and centrifuged for 3min at 13,000rpm. The column was transferred into new Eppendorf tube and 125µL of preheated (at 50 ^{o}C) AE buffer was added and incubated at room temperature for 15 minutes; centrifuged at 13,000rpm for 1 min and the column was discarded.

Gel electrophoresis was conducted by preparing 0.8% agarose in gel casting tray, allowed to solidify for 20 minutes and the gel was covered with TAE buffer up to a level of 2mm. The DNA sample of each isolate was prepared in Eppendorf tubes by adding 6µL of sterile distilled water, 5µL of sample DNA and 2µL of loading dye (gel red). A 3µL of the ladder was loaded in the first slot and 10µL of each sample was loaded starting from slot 2-11 and run at 100v for 40 minutes. Gel picture was recorded for genomic DNA with the reference of 1.5kb ladder. In a similar fashion, Gel electrophoresis was conducted to ensure for the presence of amplification in the PCR products, in which samples were prepared in to 11 Eppendorf tubes each consisting of 5µL each of the PCR products, 1µL loading dye (gel red) and 4µL sterile distilled water and the data was recorded.

3.3.7.4.2. Amplification by Polymerase Chain Reaction (PCR) and Sequencing

PCR amplification of the 16S rDNA fragments was performed using primers 27f (AGAGTTTGATC(A/C)TGGCTCAG) and 1492r (TACGG(C/T)TACCTTGTTACGACTT)

designed by Weisburg *et al*. (1991). Reaction mixture consisting of 88µL of sterile distilled water, 44µL buffer, 44µL of reaction solution, 5.5µL of each of the forward and reverse primers, 11µL of the DNTPs and 11µL polymerase enzyme. A 1µL of each of the template DNA was added in to the PCR tube followed by the addition of 19µL of the reaction mixture to each tube mixed by pipetting up and down. Then amplification was performed for 35 cycles of 1min of denaturation at 94 ^{o}C, 1min at 63 ^{o}C for primer annealing, and 2min at 72 ^{o}C for primer extension, followed by a final step at 72 ^{o}C for 10min and cooling to 4 ^{o}C. PCR products were directly sequenced at Eurofins Scientific (Austria) using the same primers. Phylogeny was inferred using the software MEGA7 (Kumar *et al*., 2016). Nucleotide sequences were deposited in GenBank under accession numbers as follows: MF474325 (Ac-146), MF474326 (Go-475), MF474327 (Ac-123), MF474328 (Ed-065), MF474329 (Ru-355), MF474330 (Ac-006).

3.4. Data analysis

All the experiments were conducted in triplicates and the data were analyzed by one-way ANOVA and the mean separation was achieved by the Duncan's multiple range tests, using SPSS (version 20). Numerical differences in the data were considered as significant at $p < 0.05$.

3.5. Results

3.5.1. Isolation of actinomycete cultures

A total of 416 soil actinomycete cultures were isolated from the 13 different unstudied habitats in five geographical regions (central, eastern, northern, rift valley and southern) in Ethiopia (see

Figure 3.1 and Table 3.1 for sample sites clustred into 5 geographical regions). The number of actinomycete cultures obtained varied in each sampling geographical region (Figure 3.2). Among all the sampling sites, soil samples from the central region, contributed the highest number of actinomycete cultures (31%), while the rift valley region yielded the least number of isolates (7%). Isolates obtained from the central region are not only large in number but they were also diverse in colony color, shape and texture (data not shown).

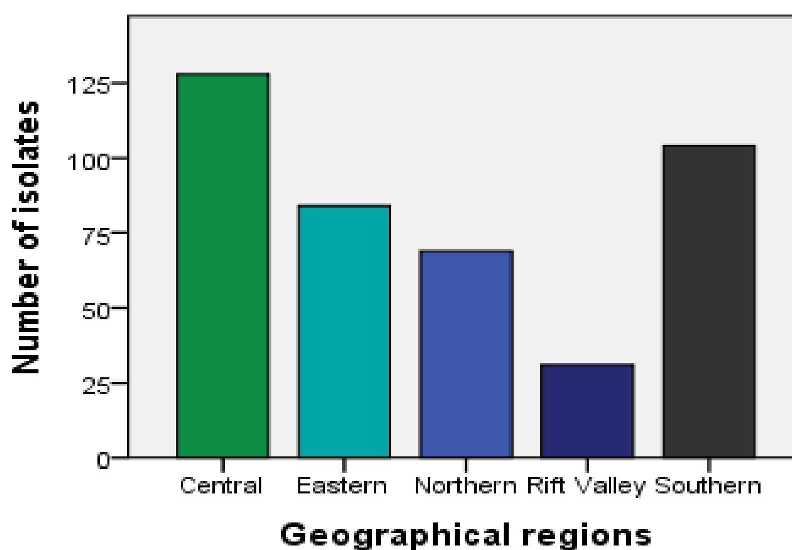


Figure 3.2. Number of actinomycetes cultures obtained from each of the five geographical regions

Table 3.2. Percentage of bioactive isolates obtained from the five geographical regions

Geographical regions	Central	Eastern	Northern	Rift Valley	Southern
Percentage of isolates	30.77%	20.19%	16.59%	7.45%	25%
Percentage of bioactive isolates	10%	3.60%	5.29%	1.92%	3.37%

3.5.2. Primary screening (bioactivity detection)

During the primary antimicrobial activity screening, all the 416 isolates were tested for bioactivity against *Candida albicans* and *Cryptococcus neoformans* (Table 3.3).

Table 3.3. Number of bioactive compound producing isolates obtained during primary screening

Isolates	Active against:		
	C.albicans	*C.neoformans*	Both *C.albicans* & *C. neoformans*
Number of bioactive isolates	101	88	88
Percentage	24.28%	21.15%	21.15%

The primary screening result showed that 101 (24%) isolates inhibited the growth of *C. albicans*, 88 (21%) isolates were inhibited both *C. albicans* and *C. neoformans*. Ten isolates that were most active in the primary screening, designated as Ac-006, Ac-029, Ed-065, Ac-123, Ac-125, Ac-146, Ru-355, Ac-464, Go-466 and Go-475 were selected for production of bioactive secondary metabolites via solid state fermentation (SSF) and further characterized. Among the selected isolates Ac-006, Ac-029, Ac-123, Ac-146 and Ac-464 were obtained from Menagesha suba forest, Ed-065 was obtained from Dengego, Go-466 and Go-475 from Gode and Rv-355 was obtained from Ataye (in Ethiopian rift valley area) (see Figure 3.1).

3.5.3. Bioactivities of extracts from the selected isolates

Methanol extracts from the selected isolates showed promising activities against the target pathogens *Candida albicans* and *Cryptococcus neoformans*. Isolate Go-475 produced the highest inhibition zone diameter followed by Rv-355 and Ac-029 respectively (see Table 3.4) for details.

The bioactivity spectrum of the crude extracts evaluated against bacterial pathogens generally showed better activities against Gram-positive bacteria, with the highest activity detected by the extract from Go-475 against *B. subtilis*, followed by those from Rv-355 and Ed-065. Only a few isolates showed bioactivities against Gram-negative bacteria; most pronounced was that of Rv-355 (Table 3.4). Comparison was made with positive controls (Ketoconazole for yeasts and Chloramphenicol for bacterial targets), and the results revealed that extract from the isolates Go-475 and Rv-355 had comparable activities to those of the control against *Candida albicans* and *Cryptococcus neoformans* respectively. However, crude extract from the rest isolates were less active (Table 3.4).

Table 3.4. Bioactivity profiles of methanol extracts from the selected isolates showing inhibition zone diameters in millimeter against yeasts, Gram positive and Gram negative bacteria using disc diffusion assay method

Isolates	Inhibition zone diameters of extracts from each isolate (in mm) against the target pathogens:						
	C.albicans	*C.neoformans*	*S. aureus*	*B.subtilis*	*E.coli*	*S.typhi*	*S. boydi*
Ac-006	5.67±1.16	5.00±1.00	-	6.33±1.52	5.33±0.58	5.00±0.00	-
Ac-029	15.00±1.00	24.00±1.00	12.67±1.16	15.00±2.0	-	-	10.67±1.16
Ed-065	13.00±0.00	11.00±1.00	12.67±1.16	11.33±1.52	-	-	-
Ac-123	7.67± 0.58	5.66±1.56	-	-	-	-	-
Ac-125	12.00±0.00	14.67±1.56	-	8.67±1.16	-	-	-
Ac-146	6.67± 0.58	8.67±1.56	-	-	-	-	-
Rv-355	27.33±2.08	28.33±0.58	12.67±.58	19.00±1.00	18.33±1.16	17.67±0.58	16.33±0.58
Ac-464	16.67±0.58	14.67±1.56	13.33±1.52	11.33±1.53	3.33±0.58	7.67±1.16	5.00±1.00
Go-466	11.33±1.16	-	13.67±1.16	14.00±1.73	-	-	-
Go-475	31.00±1.73	27.00±1.00	21.00±1.73	21.33±1.16	-	-	10.67±1.16
$^{+ve}$C	29.00±0.00	27.00±0.00	22.00±0.00	23.00±0.00	19.00±0.00	17.00±0.00	18.00±0.00
$^{-ve}$C	-	-	-	-	-	-	-

NB: $^{+ve}$C= positive control (Ketoconazole for yeasts and Chloroaphenicol for bacterial targets),-^{ve}C = negative control (Ethyl acetate) all described in the method (section 3.3.6.2)

3.5.4. Taxonomic identification of selected isolates

3.5.4.1. Cultural and micro-morphological characteristics

Cultural properties of the selected isolates were determined using standard culture media and guidelines recommended by the International *Streptomyces* Project. The data obtained in these experiments are presented in Tables 3.5 and Table 3.6. Depending on the type of media, the age and culture method the isolates displayed various cultural characteristics. As shown in the Table

3.5, isolates cultured on starch casein agar medium revealed intersting ranges of colors in their aerial and substrate mycelium. For instance, the prominent isolates in their bioactivities (Rv-355 and Go-475) exhibited signal white aerial mycelium with substrate mycelium colors of quartz grey and pebble grey respectively. The rest showed, aerial mycelium colors ranging from oyster white (Ac-006, Ed-065), cream (Ac-123), zinc yellow (Go-466) etc. with striking substrate mycelium colors like sand yellow, pastel yellow, traffic grey B, traffic white respectively see Table 9. Furthermore, most of these isolates were characterized by the formation of hygroscopic patches in their sporulating aerial mycelium.

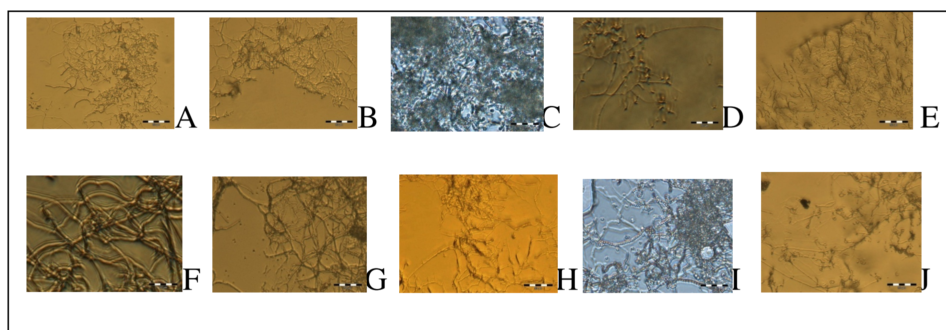


Figure 3.3. Micro-morphological appearance of the selected isolates A) Ac-006, B) Ac-029, C) Ed-065, D) Ac-123, E) Ac-125, F) Ac-146, G), Rv-355, H) Ac-464, I) Go-466, J) Go-475, Bar = 50µm

Micro-morphology analysis revealed that isolates grouped into three distinct spore chain morphology types. Four of the isolates (Ac-006, Ac-029, Ac-125 and Rv-355) showed straight to flexuous (Retctiflexibiles) type, three (Ac-123, Ac-146, Ac-464, and) revealed hooks, loops or spiral with one to two turns (Retinaculiaperti) forms and in the rest three isolates (Ed-065, Go-466 and Go-475) spore chain morphology looks spiral type (Figure3.3).

Table 3.5. Cultural properties and spore diameter of the isolates on starch casein agar medium

Isolates	Morphological and cultural characteristics of the isolates on starch casein agar medium					
	Sampling area	Specific Sites	Aerial mycelium	Substrate mycelium	Spore chain morphology	Mean spore diameter in µm
Ac-006	Central Ethiopia	Menagesha	RAL1013 Oyster white	RAL1002 Sand yellow	Rectiflexibiles	2.61±.27
Ac-029	Central Ethiopia	Menagesha	RAL9003 Signal white	RAL5008 Grey blue	Rectiflexibiles	3.01±.39
Ed-065	Eastern Ethiopia	Dengego	RAL1013 Oyster white	RAL1034 Pastel yellow	Spiral	3.85±.57
Ac-123	Central Ethiopia	Menagesha	RAL9001 Cream	RAL7042 Traffic grey B	Retinaculiaperti	5.92±.02
Ac-125	Central Ethiopia	Menagesha	RAL9016 Traffic white	RAL2010 Signal orange	Rectiflexibiles	1.96±.04
Ac-146	Central Ethiopia	Menagesha	RAL9016 Traffic white	RAL6006 Grey olive	Retinaculiaperti	5.88±.01
Rv-355	Rift valley Area	Ataye	RAL 9003 Signal white	RAL7039 Quartz grey	Rectiflexibiles	2.78±.71
Ac-464	Central Ethiopia	Menagesha	RAL 4009 Pastel violet	RAL5012 Light blue	Retinaculiaperti	3.63±.23
Go-466	Eastern Ethiopia	Godie	RAL1018 Zink yellow	RAL 9016 Traffic white	Spiral	2.90±.00
Go-475	Eastern Ethiopia	Godie	RAL9003 Signal white	RAL7030 Pebble grey	Spiral	2.64±.76

Table 3.6. Cultural characteristics of the 10 selected isolates on various ISP media

Isolates	ISP Media	Substrate Mycelium	Aerial Mycelium	Extent of Growth	Diffusible pigments
Ac-006	ISP 2	RAL 2009 Traffic Orange	RAL7047 Telegrey4	Good	_
	ISP 3	RAL 8024 Beige brown	RAL 1013 Oyster white	Abundant	_
	ISP 4	RAL 1017 Saffron yellow	RAL 7047 Tele grey 4	Moderate	_
	ISP 5	RAL 1015 Light Ivory	RAL 1002 Sand yellow	Moderate	_
Ac-029	ISP 2	RAL 5004 Black blue	RAL7001 Silver grey	Good	Pale green
	ISP 3	RAL 5008 Grey blue	RAL 9002 Grey white	Abundant	Olive green
	ISP 4	RAL 5011 Steel blue	RAL 7035 Light grey	Good	Olive Brown
	ISP 5	RAL 5008 Grey blue	RAL 5021 Water blue	poor	_
Ed-065	ISP 2	RAL 7013 Brown grey	RAL 9003 Signal white	Moderate	_
	ISP 3	RAL 6006 Grey olive	RAL 9003 Signal white	Abundant	Signal Brown
	ISP 4	RAL 2011 Deep orange	RAL 9001 Cream	Good	_
	ISP 5	RAL 2000 Yellow orange	RAL 9001 Cream	Good	_

Ac-123	ISP 2	RAL 8004 Cooper brown	RAL7035 Light grey	Moderate	Clay brown
	ISP 3	RAL 5011 Steel blue	RAL 7047 Tele grey 4	Abundant	Green brown
	ISP 4	RAL 8022 Black brown	RAL 7001 Silver grey	Good	Signal Green
	ISP 5	RAL 1015 Light Ivory	RAL 9001 Cream	Moderate	_
Ac-125	ISP 2	RAL 7039 Quartz grey	RAL 9010 Pure white	Good	_
	ISP 3	RAL 8019 Grey brown	RAL 1013 Oyster white	Excellent	Chrome Green
	ISP 4	RAL 2011 Deep orange	RAL 1013 Oyster white	Moderate	_
	ISP 5	RAL 1017 Saffron yellow	RAL 1013 Oyster white	Good	_
Ac-146	ISP 2	RAL 8000 Green brown	RAL 9016 Traffic white	Moderate	Green Brown
	ISP 3	RAL 7024 Graphite grey	RAL 9002 Grey white	Moderate	Red Orange
	ISP 4	RAL 7002 Olive grey	RAL 9003 Signal white	Good	_
	ISP 5	RAL 1018 Zink yellow	RAL 9010 Pure white	poor	_
Rv-355	ISP 2	RAL 1019 Grey beige	RAL 7035 Light grey	Moderate	_
	ISP 3	RAL 6010 Grass green	RAL 7045 Tele grey 1	Abundant	_

	ISP 4	RAL 7004 Signal grey	RAL 9002 Grey white	Good	_
	ISP 5	RAL 3011 Brown red	RAL 9002 Grey white	poor	_
Ac-464	ISP 2	RAL 5003 Saphire blue	RAL 9003 Signal white	Abundant	Brown grey
	ISP 3	RAL 7021 Black grey	RAL 7004 Signal grey	Very good	_
	ISP 4	RAL 5008 Grey blue	RAL 7004 Signal grey	Good	_
	ISP 5	RAL 1005 Honey yellow	RAL 9001 Cream	poor	_
Go-466	ISP 2	RAL 7013 Brown grey	RAL 9006 White Aluminium	Moderate	_
	ISP 3	RAL 7022 Umbra grey	RAL 1013 Oyster white	Excellent	Chocolate Brown
	ISP 4	RAL 1015 Light Ivory	RAL 8004 Cooper brown	Good	_
	ISP 5	RAL 9002 Grey white	RAL 9007 Grey aluminum	poor	_
Go-475	ISP 2	RAL 7013 Brown grey	RAL 9003 Signal white	Moderate	Green grey
	ISP 3	RAL 8022 Black brown	RAL 7035 Light grey	Excellent	Terra brown
	ISP 4	RAL 3004 Purple red	RAL 9016 Traffic white	Moderate	_
	ISP 5	RAL 9002 Grey white	RAL 9018 Papyrus white	Moderate	_

As presented in Table 3.6, most of the isolates manifested notable aerial and substrate mycelium colors. For instance, isolate Go-475 grown on ISP3 revealed light grey aerial mycelium and blackbrown substrate mycelium; whereas Rv-355 showed Tele grey1 aerial mycelium and grass green substrate mycelium. Furthermore, isolates grown on ISP5 exhibited colors ranging from light ivory (Ac-006), grey blue (Ac-029), brown red (Rv-355), grey white (Go-475) etc. They manifested aerial mycelium colors of sand yellow, water blue, grey white and papyrus white respectively on this medium.

3.5.4.2. Physiological and biochemical characteristics

Growth characteristics of the selected isolates were examined at different temperatures and pH levels. The results showed that most cultures were able to grow well between 20 and 35 ^{o}C and at pH values between 6.5 and 8.5. But, most of them showed optimum growth at 30°C and pH value of 7.5. Mostly the isolates were positive for starch hydrolysis, chitinase, oxidase and catalase tests and showed a negative result for indole production. Besides, Ac-029, Ac-123, Ac-146, Ac-464 and G-475 were found to be positive for melanin production (Table 3.7).

Observation of various carbon and nitrogen utilization capacity revealed that isolates were found to be versatile in their ability to grow and develop on different carbon and nitrogen sources. However, abundant growth was observed on glucose and soluble starch and some grow poorly on sucrose. On the other hand, isolates showed enhanced growth on the basal medium supplemented with peptone and KNO_3 as nitrogen sources (seeTable 3.7).

Table 3.7. Important physiological and biochemical test results of the selected isolates

Isolates	Test for						Growth at various (NaCl %)					Optimum	
	Starch hydrolysis	Chitinase	Oxidase	Catalase	Indole test	Pigment	1.25	2.5	5	7.5	10	pH	Temp.
Ac-006	+	+	-	+	-	-	+	+	+	±	-	7.0	25 ^{0}C
Ac-029	+	+	-	+	-	+	+	+	+	+	±	7.5	30 ^{0}C
Ed-065	+	+	-	+	-	-	+	+	+	+	±	7.5	30 ^{0}C
Ac-123	+	+	+	+	-	+	+	+	+	+	±	7.5	30 ^{0}C
Ac-125	+	+	+	+	-	-	+	+	+	±	-	7.0	25 ^{0}C
Ac-146	+	+	+	+	-	+	+	+	+	±	-	7.5	30 ^{0}C
Rv-355	+	+	+	+	-	-	+	+	+	+	±	7.5	30 ^{0}C
Ac-464	+	+	+	+	-	+	+	+	+	±	-	7.5	30 ^{0}C
Go-466	+	+	+	+	-	-	+	+	+	+	±	7.0	25 ^{0}C
Go-475	+	+	+	+	-	-	+	+	+	+	±	7.5	30 ^{0}C

NB: $^{(+)}$ test positive or the presence of growth, $^{(-)}$ test negative or no growth, $^{(\pm)}$ growth is very scarce or doubtful

Table 3.8. Various carbon and nitrogen source utilization capacity of the selected isolates

Isolates	Utilization of various Carbohydrates sources						Utilization of Nitrogen sources					
	Glucose	Soluble starch	Maltose	Sucrose	Fructose	Glycerol	$(NH4)_2SO_4$	KNO_3	NH_4Cl	Casein	Peptone	Asparagine
Ac-006	+	++	+	+	+	+	+	++	+	+	++	+
Ac-029	++	++	+	+	+	++	+	++	+	+	++	+
Ed-065	++	+	+	±	+	+	+	+	+	+	++	+
Ac-123	+	++	+	+	+	+	+	++	+	+	++	+
Ac-125	++	+	+	±	+	+	+	+	+	+	++	+
Ac-146	++	++	+	+	+	+	+	++	+	++	++	+
Rv-355	++	++	+	+	+	+	+	+	+	+	++	+
Ac-464	++	++	+	+	+	+	+	+	+	+	++	+
Go-466	+	++	+	+	+	++	+	++	+	++	++	+
Go-475	++	++	+	+	+	+	+	++	+	++	++	+

(+) good growth, (++) abundant growth, (±) grow poorly

3.5.4.3. Molecular characterization

Isolates were identified at the genus level using 16S rDNA gene sequences obtained after amplification from genomic DNA. All the sequences were analyzed using the RDP database, which clearly indicated that all the analyzed isolates belong to the genus *Streptomyces*. The phylogenetic relationships of the isolates to type strains and best matches according to the nucleotide BLAST search were inferred using the Maximum Likelihood algorithm in MEGA 7 software (Kumar *et al*., 2016). According to the phylogenetic anlysis, isolates Ac-006, Ac-123, Go-475 and Ru-355 all belonged to distinct clades, while Ed-065 and Ac-146 showed identical 16S rRNA gene sequences and belongs to the same clade.

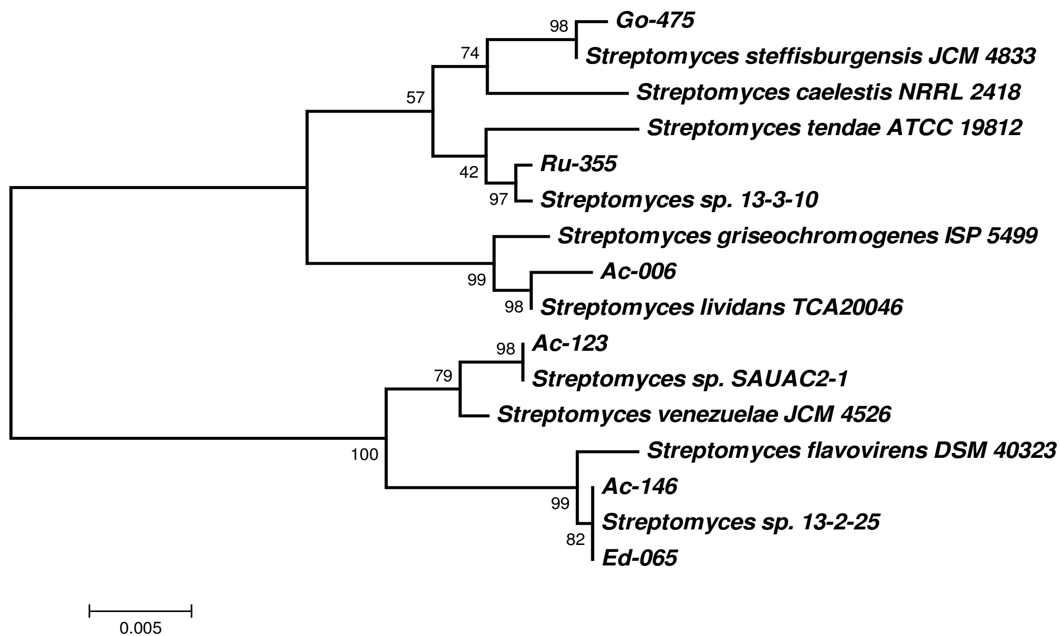


Figure 3.4. Molecular Phylogenetic analysis by Maximum Likelihood method; the evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura & Nei, 1993). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016).

As presented in the phylogenetic tree (Figure 3.4), the most outstanding isolate in its bioactivities namely Go-475 with accession number MF474326, exhibited 99% similarity with *Streptomyces steffisburgensis* JCM4833 and that of Ru-355 accession number MF474329 showed 99% similarity with *Streptomyces* sp. 13-3-10. Isolates, Ac-006 accession number MF474330 and Ac-123 accession number MF474327 exibited 99% similarity to *Streptomyces lividans* TCA20046 and *Streptomyces* sp. SAUAC2-1 respectively. Furthermore, isolate Ac-146 and Ed-065 with accession numbers MF474325 and MF474328 respectively represented interestingly in the same clad with 99% similarity to *Streptomyces* sp. 13-2-25. However, as described earlier, these isolates were obtained from two distinct geographical environments the former from Menagesha suba protected forest and the later from Dengego in the eastern geographical region.

In general, the polyphasic taxonomic identification approach involving the examination of most important phenotypic (cultural, morphological, physiological and biochemical properties) and genotypic characters (partial 16srRNA sequence results) nicely fit with the characters described in Bergaye's Manual of Systematic Bacteriology for the genus *Streptomyces*. Hence, all the selected isolates belong to the genus *Streptomyces*.

3.6. Discussion

The findings in the current study revealed that soil actinomycetes obtained from unstudied habitats at various geographical regions in Ethiopia showed promising and wide spectrum of bioactivity potentials. Similar studies Bull and Stach (2007), Lozupone and Knight (2007), Huang *et al*. (2008) also showed that actinomycetes isolated from untouched niche habitats were

used as important sources of bioactive compounds. For instance, Okoro *et al*. (2009) presented *Amycolatopsis*, *Lechevalieria* and *Streptomyces*, which represent novel taxon and producing new bioactive natural product from Atacama Desert soil (in South America, 600 miles from Pacific coast). Similarly, Bredholt *et al*. (2009) reported the presence of strong potential for promising antibiotic producing actinomycetes from sediments in the Trondheim Fjord, Norway.

Results obtained in this work support the claims that poorly studied habitats have good potentials for drug development. This also points to the fact that environments where the strains were isolated influenced their secondary metabolite biosynthesis potential, most likely via evolutionary adaptations of microbes in response to the particular niche habitats. This is mainly because of the biotic and abiotic dynamics (physicochemical, climatically, microbiological and eco-geographical) factors (Goodfellow and Fiedler, 2010).

Bioactive isolates showed substantial activities against *Candida albicans*, *Cryptococcus neoformans*, Gram positive and some were active against Gram negative bacteria.This indicates bioactive substances from those isolates either have broad spectrum bioactivities or a single isolates have the capacity to produce several active molecules during secondary metabolism. Besides, a single bioactive compound might have several modes of acions. Similar reports by Lee and Hwang (2002) demonstrated that, of the 1510 actinomycete isolates in Korea, 10.26% showed various extent of activity. Furthermore, Boubetra *et al*. (2013) isolated actinomycete strain SA198 from Saharan soil, Tamanrasset, in Southern Algeria, that showed considerable broad-spectrum of antimicrobial activities against Gram positive, Gram negative and filamentous fungi, but they observed no activity against yeasts.

Relatively less activity was displayed by most of the the selected isolates against Gram negative bacteria. This might be due to the structural, physiological and biochemical differences in pathogens. For instance, the presence of impermeable layer (lipopolysaccharides) protective barrier in Gram negative bacteria might enables them to withstand the actions of different antimicrobial substances. On the other hand, lower inhibition zone diameter may not always positively correlate with the lesser potency of the antimicrobial agents. This is because inhibition zone diameter can also depend on the ability of the molecule to diffuse across the solid medium, solubility and other physicochemical properties of the substance which is in concordant with findings by Basilio *et al*. (2003). In this regard, the majority of the publications on the antimicrobial spectra of actinomycetes, (Basilio *et al*., 2003; Hayakawa *et al*., 2004; Bredholdt *et al*., 2009; Koch and Loffler, 2009; Satheeja and Jebakumar, 2011 and Jose *et al*., 2011) reported pronounced activities against Gram positive bacterial pathogens.

Identification study showed that, bioactive isolates were dominated by the representatives of the genus *Streptomyces*. This pronounced antimicrobial production capacity in the genus *Streptomyces* might be related to the presence of multiple secondary metabolite biosynthesis gene clusters in relatively large genomes, ranging in size from 6Mb to 11Mb (Nett *et al*., 2009) and the presence of several metabolic pathways for biosynthesis (Omura *et al*., 2001). For instance the genome size of *Streptomyces coelicolor* A3(2) (the model representative of the genera) is about 8667Mbp, which corresponds to 7825 genes, having 20 gene clusters that encode recognized or expected secondary metabolites, which is greater than the eukaryote *Saccharomyces cerevisiae* (containing only 6203 genes) (Bentley *et al*., 2002). Also *Streptomyces avermitilis* MA-4680T genome has about 9025Mbp, which encompass 30 gene

clusters (Omura *et al*., 2001; Ventura *et al*., 2007). Furthermore, the genome of *Streptomyces* contains about 6500Mbp highly conserved crucial regions related with biosynthesis (Ikeda *et al*., 2003). Previous studies also reported that members of the genus *Streptomyces* isolated from the terrestrial or aquatic environments are responsible for the highest share (in terms of novelty and frequency) of antimicrobial substance production (Basilio *et al*., 2003; Berdy, 2005; Bruntner *et al*., 2005; Demain, 2009; Rao *et al*., 2012).

The phylogenetic relationships of these isolates to type strains and best matches according to the nucleotide BLAST search were inferred using the Maximum Likelihood algorithm in MEGA 7 software (Kumar *et al*., 2016). The analysis of all these data and comparison of all the phenotypic characteristics with those actinomycetes described in Bergey's manual of systematic bacteriology revealed that all of the selected isolates belong to the genus *Streptomyces*. In this regard, the majority of publications used similar approach for taxonomic identification. For instance, Trujillo and Goodfellow (1997) followed polyphasic approach appropriately to determine the taxonomic position of actinomycetes. Likewise Rao *et al*. (2012) identified 4 *Streptomyces* isolates from Mangrove soils, in a similar fashion. The finding generally indicated that high abundance of the genus *Streptomyces* among actinomycetes in soil.

3.7. Conclusion and Recommendation

The study demonstrates that soil actinomycetes isolated from previously unstudied environmental niches in Ethiopia are rich sources of antimicrobial compounds. The results enable us to generalize that actinomycetes could be cultivated using solid state fermentation method for the production of antimicrobial compounds. Antimicrobial compounds produced by the selected isolates are broad spectrum in their bioactivities; and this enables us to conclude that either a single isolate can produce more than one bioactive compound or the bioactive compounds produced by each of the isolates have multiple modes of actions. The findings also enable us to conclude that Streptomyceetes are abundantely found in Ethiopian soils.

Molecular structures of bioactive compounds from all the selected isolates in this study are not yet determined, except the two antimicrobial compounds described from *Streptomyces* sp. Go-475. Besides, the mechanisms of action of the antimicrobial compounds are currently unknown; therefore, further studies must be carried out on the isolation, identification and structural elucidation of all the active components of the antimicrobial compunds and their mechanisms of actions should be determined. Besides, further screening studies are recommended to examine antimicrobial potential of actinomycetes in marine environments and those found in symbiotic association in the bodies of plants and animals.

Chapter 4: Solid State Fermentation for the Production of Bioactive Secondary Metabolites by Selected *Streptomyces* Isolates

Abstract

The fundamental objective of this chapter was to cultivate selected actinomycete isolates via solid state fermentation and evaluate the effects of various solid state fermentation process parameters on crude bioactive secondary metabolite production. Five promising isolates namely Ac-029, AC-125, Rv-355, Ac-464, and Go-475 were chosen for the cultivation and production of bioactive secondary metabolites. Among the four solid substrates evaluated for bioactive secondary metabolites production, wheat bran supplemented with nutrients gave considerably higher bioactive secondary metabolite yield. Depending on the type of the isolate, variations were observed in optimal fermentation process parameters for highest bioactive secondary metabolite production. However, an initial moisture content of 65%, a pH value of 7.5, incubation temperature of 30 ^{o}C, and an inoculums size of $3x10^7$ CFU/mL were the optimal conditions for most of the isolates studied. Besides, the length of incubation period for maximum bioactive secondary metabolite production by most of the isolates was found to be 12 days. Among the different supplemntary carbon and nitrogen sources evaluated, most of the isolates showed their maximum metabolite yield when soluble starch and peptone were supplemented as carbon and nitrogen sources respectively. The isolate named *Streptomyces* sp. Go-475 was the best isolate in terms of both bioactivity and bioactive secondary metabolite yield. In general, the various solid state fermentation process parameters influence microbial growth and metabolite production.

Key words: *bioactive secondary metabolites, Streptomyces, solid state fermentation*,

4.1. Introduction

Solid-state fermentation (SSF) is defined as any fermentation process conducted for growing and cultivation of microorganisms on the surface or at the interior of a solid substrates (solid matrix) in the absence or close to absence of free flowing water. However, the solid substrates must hold sufficient moisture to sustain growth and metabolism of the micro-organisms (Pandey *et al*., 2001; Pandey, 2003; Barrios-Gonzalez, 2012).

SSF has become an attractive option as it gives the opportunity to use cheap and abundant agricultural and agro industrial wastes as substrates and the metabolites obtained are more concentrated and purification methods are inexpensive (Pandey *et al*., 2001; Robinson *et al*., 2001; Barrios-Gonzalez and Mejia, 2008). This process has been used to produce various metabolites in different parts of the world, even though it has been carried out, only on small scale (Ghosh, 1992; Lal and Lal, 1994). Recently, industrial secondary metabolite production by SSF became a reality. As described in Barrios-Gonzalez and Mejia (2008) a company named Biocon, in India, started industrial scale production of some secondary metabolites using SSF technology and approved by Food and Drug Administration (FDA) of the USA for human application.

As described in Pandey *et al*. (2000), quite a lot of studies have been conducted on SSF for the production of various antibiotics including penicillin, cephalosprin, tetracyclines and chlorotetracyclines, by fungi and various actinomycetes. However, SSF still faced with a number of problems. Difficulties in scale-up of the process and nonexistence of standard fermenters are

among the most important challenges in SSF technology. This is mainly because of the inexperience of engineers in western nations in the designing of solid-state fermenters (Pandey *et al*., 2008). Besides, various growth process parameters including the composition of solid substrate medium within a fermentation system can influence the development and yields of secondary metabolites (Nigam, 2009).

Product formation in SSF is significantly affected by biological, physico-chemical and environmental factors (Raimbault, 1998). Hence, any rationally designed bioprocess involving solid state fermentation should consider the most significant parameters such as type of microorganism and substrates, moisture level, inoculum size, pH, temperature, incubation period, and the type of supplementary carbon and nitrogen sources (Pandey, 2003; Barrios-Gonzalez and Mejia, 2008). In general, process factors optimization, downstream processing (recovery) and purification of the product are among the most important aspects that have to be considered to exploite the biotechnological potential of SSF.

In SSF, hyphal growth phenotype is the best adaptation suitable for penetration and colonization of the solid substrate. Thus, the filamentous growth natures make fungi and actinomycetes efficient in SSF processes for bioactive secondary metabolite production (Barrios-Gonzalez and Mejia, 2008). Therefore in the current study, considerable effort was invested on SSF techniques to evaluate the use of cheaper agricultural ingredients (wheat bran, rice, potato peel and coffee husk) from local sources, as solid substrates using five *Streptomyces* isolates. The aim of this particular work was to enhance bioactive secondary metabolites yields through optimization of SSF process parameters. By using such cheap substrates from local agricultural and agro

industrial wastes, the cost of production of valuable secondary metabolites significantly is reduced. The process was chosen because it is easy, economical and environmental friendly.

4.2. Objectives

4.2.1. General objective

The general objective of this chapter is to evaluate the effects of SSF process parameters (biological, physico-chemical and environmental factors) on bioactive secondary metabolite production and improve the yield from selected *Streptomyces* isolates.

4.2.2. Specific objectives

- To evaluate the effects of solid state fermentation process parameters such as moisture level, pH, temperature and inoculum size; and determine the optimal solid state fermentation conditions for maximum secondary metabolite production by selected *Streptomyces* isolates
- To determine the efficiencies of isolates in metabolite production and select the highest producing *Streptomyces* isolate

4.3. Materials and Methods

4.3.1. Actinomycete cultures and maintenance

Five actinomycete cultures (Ac-029, Ac-125, Rv-355, Ac-464 and Go-475) were chosen to evaluate the effects of solid state fermentation process parameters on bioactive secondary metabolite production. Depending on their cultural, micro-morphological, biochemical and physiological characteristics and partial 16S rRNA sequence analysis, all the selected isolates were identified to be a member of the genus *Streptomyces* spp. These *Streptomyces* cultures were maintained on starch casein agar slants and stored at 4 ^{o}C for further experiments.

4.3.2. Substrates pretreatments

Various pretreatment methods were conducted depending upon the nature and type of the solid substrate used. Substrates were pretreated by drying to a constant weight at 50 ^{o}C, grinding with mortar and pestle and passing through various screen sieves 1.5mm and 2.8mm (Fritsch, Idar-Oberstein, Germany). Besides, soaking and boiling was conducted for rice substrate.

4.3.3. Measurement of pH and moisture content of the substrates

In 250mL flasks, 1.0g of each of the solid substrates were mixed thoroughly with 10mL of distilled water separately; after 10min, the pH of the supernatant was measured using a pH meter and adjusted to a desired level by the addition of 1N NaOH or HCl. Moisture level of the solid substrate was adjusted as described in Tunga *et al*. (1998). 10g of the solid substrate was dried to a constant weight at 105 °C and the dry weight was recorded. A desired quantity of water was

added to the substrate and thoroughly mixed and the wet weight was measured. The percentage of each of the initial moisture level was recorded.

4.3.4. Inoculum preparation

The isolates were grown onto the entire surface of yeast malt extract agar plates and incubated at 30 ^{o}C. After 5 days 10mL of sterile physiological saline containing 0.01% Tween 80 was added to each plate and scrapped with sterile scalpel and transferred into five sterile screw caped test tubes. One mL of the suspension from each isolate was inoculated in to each of the 250mL conical flasks containing 50mL sterile broth composed of (g/L): soluble starch 20, tryptone soy broth 20, yeast extract 3, $CaCO_3$ 3, K_2HPO_4 1, $MgSO_4.7H_2O$ 0.025, pH 7.2, and incubated for 7 days on a rotator shaker at 200 rpm at 30 ^{o}C (Vastrad and Neelagund, 2011). At the end of incubation period each culture was harvested by centrifugation for 5min at 2000rpm, the supernatant was discarded and the pellet was washed with sterile physiological saline and resuspended in sterile saline containing 0.01% (v/v) Tween 80 (Murthy *et al*., 1999). The total numbers of cells in the suspension were adjusted spectrophotometrically to contain 2.8 $x10^{6}$ CFU/mL Venkateswarlu *et al*. (2000); confirmed by viable plate count. Unless and otherwise specified, this inoculum preparation method was used for all the SSF processes.

4.3.5. Solid state fermentation

The initial (basic) solid state fermentation were conducted in fifteen 250mL conical Erlenmeyer flasks containing 10g of the solid substrates (wheat bran) supplemented with a solution

consisting of NaCl, 0.1%; $MgSO_4.7H_20$, 0.1%; NH_4NO_3, 0.5% and KH_2PO_4, 0.2 % Namboothiri *et al*. (2004), pH was adjusted to 7.5, and 1% soluble starch was added. The initial moisture content of the medium was adjusted to 65%. The substrates were autoclaved at 121°C for 30minute. The sterilized and cooled media were mixed thoroughly and inoculated with 3mL of the inoculum suspension containing 2.8 $x10^6$ CFU/mL (Venkateswarlu *et al*., 2000) and incubated statically at 30 °C for 15 days. Unless and otherwise specified this basic fermentation conditions was used throughout the experiment.

4.3.6. Analytical methods

4.3.6.1. Extraction and recovery of the products and yield determination

During the downstream processing and recovery of the products, the cultivated solid materials were mixed up with tenfold of organic solvents of various polarities (ethyl acetate, methanol and hexane) for maximum recovery in 500mL Erlenmeyer flasks with beads inside. The mixture was shaked vigorously over night on a rotary shaker at 220rpm to dissolve and release the bioactive substances in the solution (Al-Bari, 2006). The fermented solid materials were separated from the solution by filtration using Whatman filter paper no 1 with a pore size of 11µm. The clear filtrate was used for the antimicrobial bioassay. The yields of crude extracts obtained were determined by concentrating and drying the extracts under vacuum using a rotavapour (pumping motor fitted to it) at a temperature of 40 ^oC. The concentrated crude bioactive substances were kept in small labeled vials in which the weights were predetermined and the substances were further dried to constant weight. The weight of completely dried crude extracts were measured in milligrams and recorded. Finally, the crude extracts were stored at 4 ^oC for further investigation.

4.3.6.2. Bioactivity evaluation

Inoculum preparations for the target test pathogens (described in section 3.3.6) and antimicrobial bioassay for the crude extracts were conducted by using the standardized disc diffusion method as described in section (3.3.6.1 and 3.3.6.2).

4.3.7. Evaluation of cultivation conditions

4.3.7.1. Effect of substrate types

In the current study, four solid substrates (wheat bran, rice, potato peel and coffee husk) were chosen for the solid state fermentation processes. The choice of these solid substrates mainly depended on the availability and cost of the substrates. Basmati rice was purchased from the local market in Addis Ababa, Ethiopia, where as wheat bran and potato peel were obtained from “Misrak flour and bread factory” and local restaurant in Addis Ababa respectively. Ten grams of each of the above solid substrates were placed in 250mL Erlenmeyer flasks and distilled water was added and an initial moisture level of 65% was adjusted. The flasks were then autoclaved at 121°C for 15min. Cooled to room temperature and inoculated as stated above in (section 4.3.2). The substrate that gave the best bioactive secondary metabolite yield was selected for the subsequent experiments.

4.3.7.2. Effects of initial moisture content

The dry wheat bran (10g) was placed in 15 different 250mL cotton plugged Erlenmeyer flasks and various levels of initial moisture levels were obtained ranging from (45%-75%) with 5%

intervals. The moisture content was calculated, as percentage of initial moisture level equal to (amount of water added/total weight of dry substrate and water) ×100. The result was recorded and the moisture content that resulted in the highest production was used in the subsequent experiments.

4.3.7.3. Effect of incubation temperature, pH and length of incubation period

Various incubation temperatures between 20 ^{o}C - 45°C (with 5°C intervals) were evaluated to determine the optimum temperature required by the isolates for maximum crude bioactive secondary metabolites yield. The pH was adjusted using NaOH or HCl as stated in (section 4.3.3). The length of incubation period was evaluated by incubating the culture for different lengths of time ranging from 8-16 days with two days intervals and the data were recorded.

4.3.7.4. Effects of inoculum size

Inoculums for each of the five isolates were prepared by dilution method as described in (section 4.3.4). The total number of cells were adjusted spectrophotometrically and confirmed by viable count, to contain CFU/mL ($3x10^4$, $3x10^5$, $3x10^6$, $3x10^7$, and $3x10^8$) and the optimum inoculum size for maximum yield was determined.

4.3.7.5. Effects of various carbon and nitrogen sources

The effects of various carbon sources on crude metabolite yield were evaluated by replacing starch in the basal medium with 1% of each of the carbon sources (mannitol, glucose, soluble starch, fructose, maltose and glycerol). Nitrogen sources such as $(NH_4)_2SO_4$, $(NH4)_2HPO_4$, and KNO_3 were evaluated as inorganic nitrogen where as malt extract, casein, yeast extract and peptone were used as organic nitrogen sources. In this case 0.5% of the nitrogen sources were supplemented by replacing the 0.5% NH_4NO_3 as described by Bussari *et al*. (2008).

4.4. Data Analysis

The data was analyzed by one way-ANOVA using SPSS (version 20). Multiple comparison analysis after ANOVA (Duncan) test was used for mean comparisons. Numerical differences in the data were considered as statistically significant at $p<0.05$.

4.5. Results

4.5.1. Micro-morphology of cultures and bioactivity

Different micro-morphological appearance of actinomycete cultures used in SSF process optimizations are shown below in Figure 4.1.

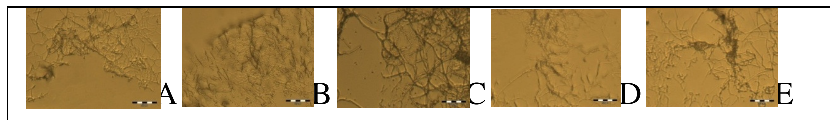


Figure 4.1. Micro morphology of actinomycetes cultures chosen for SSF A) Ac-029, B) Ac-125, C) Rv-355, D) Ac-464, E) Go-475

Table 4.1. Bioactivity profile of extracts using standard bioassay method

Isolates ID	Solvent extracts	*Candida albicans*	*Cryptococcus neoformans*	*Shigella boydii*	*S.typhi*	*Staphylococcus aureus*	*B.sibtilis*
Ac_029	MeOH	++	+	++	na	+	++
	EtOAc	++	+	++	na	+	++
Ac_125	MeOH	+	++	++	na	na	+
	EtOAc	++	++	na	na	na	+
Rv_355	MeOH	+++	++	+++	++	+	++
	EtOAc	+++	++	+	++	+	++
Ac_464	MeOH	+	+	+	+	+	+
	EtOAc	+	+	+	+	+	+
Go_475	MeOH	+++	+++	++	na	+	+++
	EtOAc	+++	+++	++	na	+	+++

NB: $^{(+)}$ has activity, $^{(++)}$ good activity, $^{(+++)}$ very good activity, $^{(na)}$ no activity at all

As presented in Table 4.1, all the selected isolates maintained their bioactivities against yeasts and Gram positive bacteria. The two isolates (Rv-355 and Ac-464), showed activities agains Gram negtive bacteria as similar as in the primary bioactivity detection.

4.5.2. Effects of substrate types

Among the four substrates used in the SSF experiments, all the isolates showed the maximum crude metabolite yield on wheat bran except Ac-125 that revealed its maximum metabolite production on rice substrate. Of all the isolates, Go-475 showed the highest average yield (14.2mg/g) on wheat bran followed by Ac-029 and Rv-355 with an average value of 13.3mg/g and 12.8mg/g respectively (Figure 4.2).

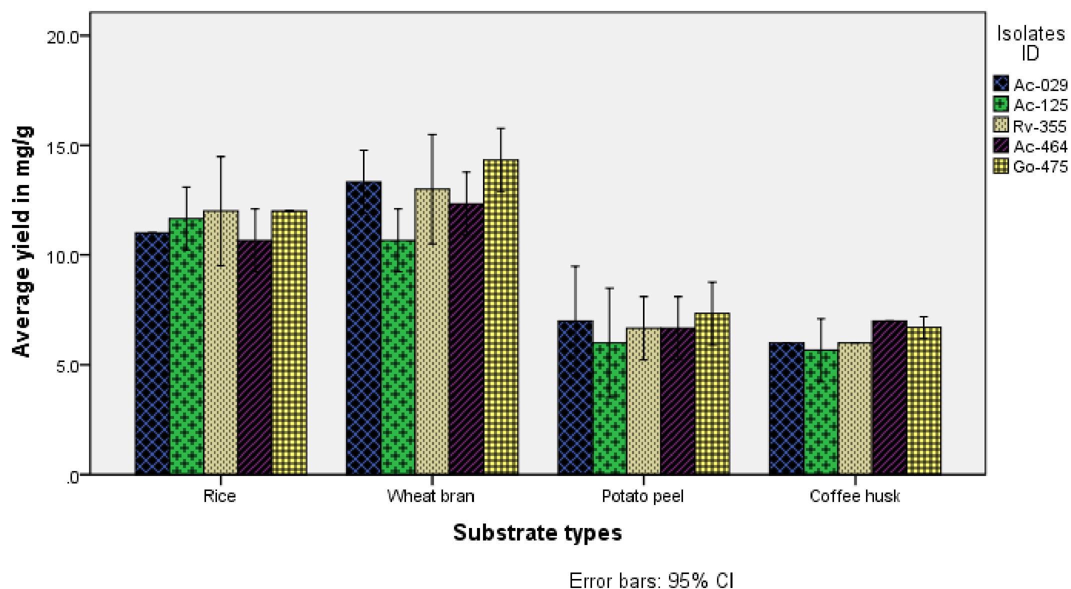


Figure 4.2. Effects of the four substrates on average crude bioactive metabolite yield,

In this experiment the least average yield (5.6mg/g) was observed by the isolate Ac-125 grown on coffee husk. As shown in the Figure 4.2 considerable yields were recorded by all the isolates on rice substrates. For instance, the maximum average yield recorded on rice was 12.5mg/g by the isolate Go-475 and the minimum value was10.6mg/g by the isolate Ac-464.

Cluster analysis of isolates crude metabolite yield obtained by different substrates showed that wheat bran resulted in the highest average yield (12.73±1.38mg/g) and coffee husk gave the least average production (6.27±0.56mg/g). Pair wise comparison analysis, revealed statistically significant difference in yield between the various solid substrates used (coffee husk vs rice, coffee husk vs wheat bran, potato peel vs rice and that of potato peel vs wheat bran) ($p<0.05$). However, the variation in yield, between rice and wheat bran and that of coffee husk and potato peel was not statistically significant ($p>0.05$).

4.5.3. Effects of initial moisture levels

A range of initial moisture levels were used for SSF experiments. The result revealed statistically significant difference ($p<0.05$) between the various levels (45-75%). This shows that an initial moisture level significantly determines the growth and metabolite production in SSF.

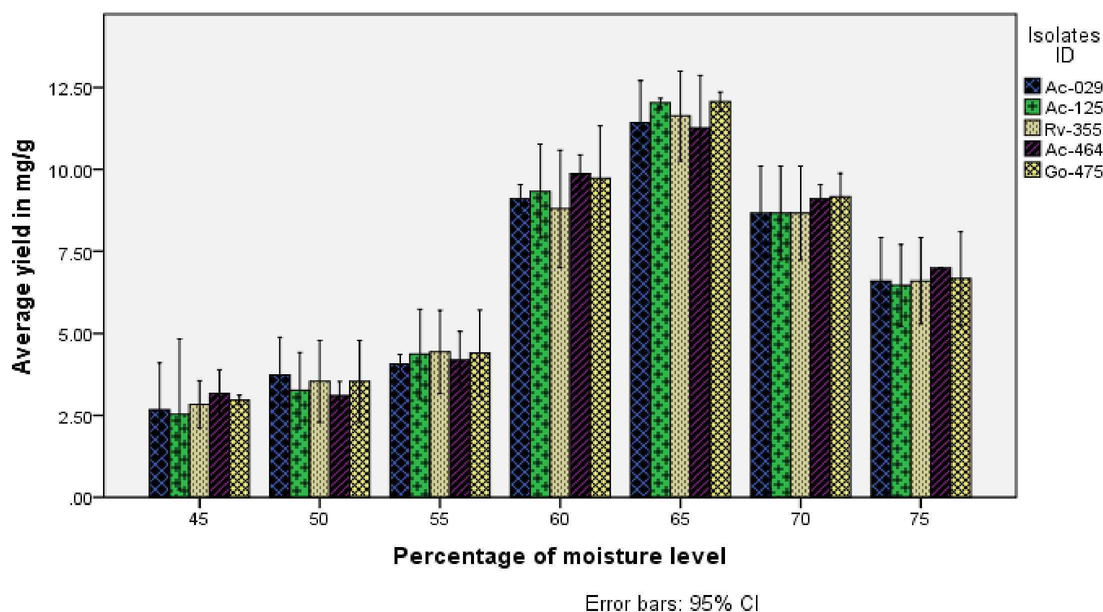


Figure 4. 3. Effect of various moisture contents on metabolite production by the isolates

Good growth and metabolite productions were exhibited by most of the isolates with an initial moisture contents of 60 -70%. However, all the isolates showed their maximum crude metabolite yield at 65% initial moisture level and the least was observed with an initial moisture level of 45%. The highest average yield (12.2±0.58mg/g) was recorded by the isolates Go-475 followed by Ac-125 and Rv-355 with average yields of 12.1±2.51mg/g and 11.6±4.93mg/g respectively.

The findings also showed that, an initial moisture level < 55% resulted in a severe decline in isolates growth and metabolite production. Besides, isolates showed a reduction in growth and metabolite yield with an initial moisture level >70%. However, the reduction in yield was more severe when isolates were grown below the optimum value as compare to their yield obtained above the optimum initial moisture level. All *Streptomyces* cultures showed scanty growth and very low metabolite yield at 45% initial moisture level (Figure 4.3).

4.5.4. Effects of pH

The results of the current investigation revealed that the isolates gave good bioactive secondary metabolite yields when grown in pH values of 7.0-7.5, but most of the isolates exhibited their highest crude metabolite yields at the pH value of 7.5. Among the five isolates, Go-475 gave the highest average yield (13.1±3.22 mg/g) at pH of 7.5; and the least yield (4.2±1.53mg/g) was recorded by the isolate Ac-464 at pH 6.5. At pH 7.0, relatively higher metabolite yields were recorded by all the isolates. At pH 7.0, the foremost isolate Go-475 gave a metabolite yield of (11.6±2.00mg/g). On the other hand, the isolates showed considerable growth and metabolite production at pH 8.0 ranging from 7.5±1.00mg/g-8.9±1.16mg/g). From the analysis, it has been observed that initial pH values above and below the optimum level resulted in reduction in the bioactive secondary metabolite production, but the reduction in yield was found to be more critical in pH values below the optimum as compared to the higher pH values (Figure 4.4).

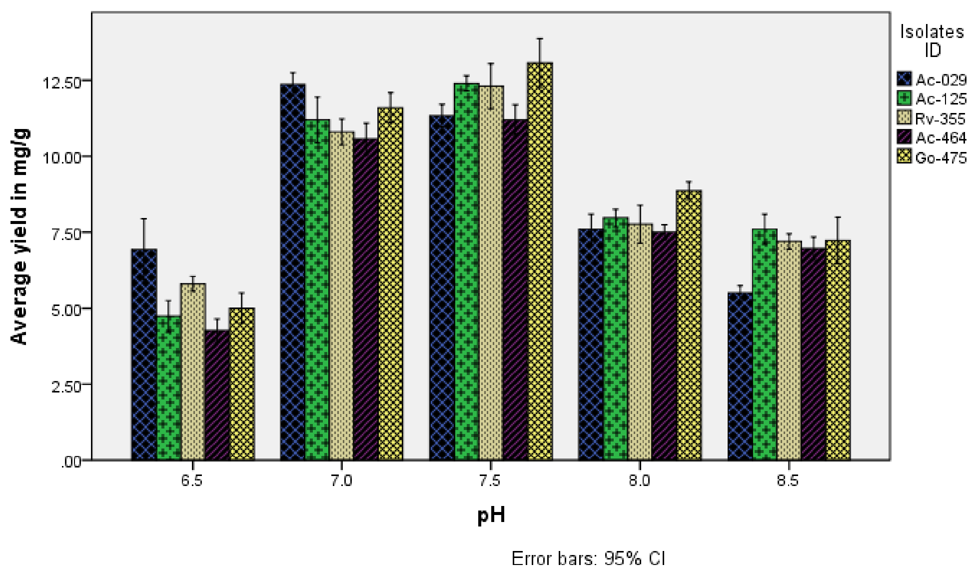


Figure 4.4. Effects of pH levels on isolates metabolite yield

4.5.5. Effects of incubation temperature

The effects of different incubation temperatures on crude bioactive secondary metabolite yields were investigated in ranges of 20 ^{o}C- 45 ^{o}C. Multiple comparison analysis of the data using Post Hoc test after ANOVA showed that, isolates treated with various incubation temperatures revealed statistically significant difference ($p<0.05$) in their metabolite yields. Among the five isolates subjected to the treatment, the highest yield 13.1±3.51mg/g was recorded at an incubation temperature of 30 ^{o}C by the isolate Go-475 and the least (2.0±1.53mg/g) was observed at 40 ^{o}C by isolate Rv-355.

The results indicated that isolates gave good growth and metabolite production within a range of temperatures (25 ^{o}C-35 ^{o}C). However, maximum yields of isolates were exhibited either at 25 ^{o}C or 30 ^{o}C (Figure 4.5) and their least yields were recorded at 40 ^{o}C.

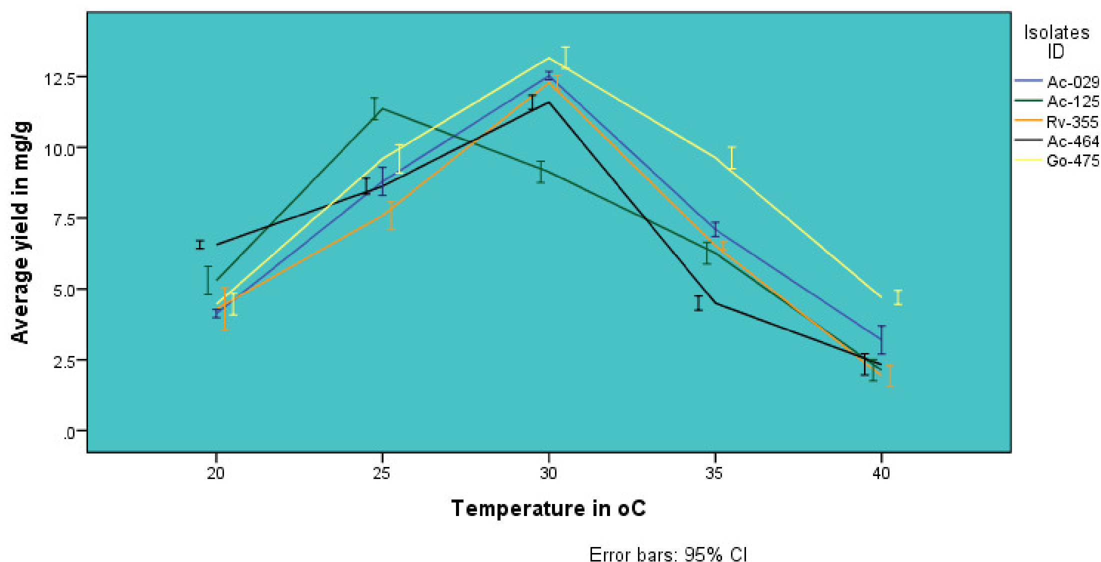


Figure 4.5. Effect of Temperature on metabolite yields

For instance, isolates Ac-029 showed its maximum yields (12.5±0.58mg/g) at an incubation temperature of 30 °C. However, the maximum yield (11.3±1.53 mg/g) for the isolate Ac-125 was recorded at 25 °C. Furthermore, Rv-355 and Ac-464 gave maximum yields of 12.3±2.65mg/g and 12.4±3.79mg/g respectively with an incubation temperature of 30 °C. The result depicted that all the isolates showed minimum metabolite yields at 40 °C. Besides, all the isolates showed a decline in metabolite production with an incubation temperature below 25 °C and above 35 °C. However, the reduction in yield was more pronounced at higher incubation temperature values than the lower temperature.

4.5.6. Effects of incubation periods

In order to determine the length of incubation period for maximum bioactive secondary metabolite yield in solid state fermentation processes, isolates were cultivated for various length

of time ranging from 8-16 days. Analysis of the data revealed statistically significant differences in the yield ($p<0.05$) when cultivated for different lengths of incubation periods.

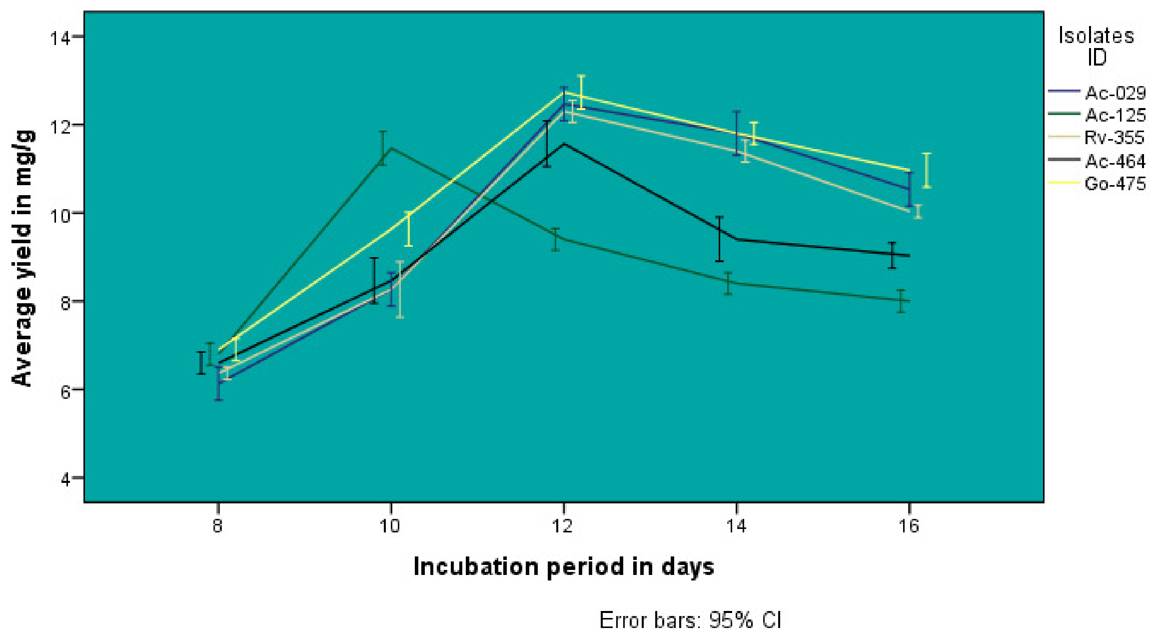


Figure 4. 6. Effects of incubation period on isolates metabolite yield

In the current study, a general increment was observed in isolates crude secondary metabolite yield with increasing the length of incubation periods until isolates attained their maximum production on the 12th day (Figure 4.8). Most of the isolates exhibited their maximum yield ranging from 11.6±2.08mg/g – 12.7±1.53mg/g on the 12th day. As shown in Figure 4.6, a reduction in yield was observed after the 12th day. However, the maximum yield for isolate Ac-125 was recorded at day 10.

Mostly higher levels of bioactive metabolite production were recorded on the 12th day of the fermentation. However, variations were observed in secondary metabolite yields in the course of fermentation periods. During the experiments noticeable secondary metabolite yields were

observed starting from day 8 of fermentation and reached its maximum on the 12th day. Further increase in incubation time resulted in a slight reduction in crude secondary metabolite yield. For instance, the isolate Ac-125 showed its highest yield (11.5±1.53 mg/g) at day10 and 8.0±1.00mg/g was recorded on day 16.

4.5.7. Effect of inoculum size

The effects of inoculum sizes on bioactive metabolites yields were investigated by adjusting the inoculum size ranging from $3x10^4$ to $3x10^8$CFU/mL. Post Hoc multiple comparison analysis indicated the presence of significant difference ($p<0.05$) in yield due to the different in inoculum sizes used in the SSF. The findings depicted that most of the isolates maximum yields were recorded with an inoculum size of 3×10^7 CFU/mL, except isolate Ac-125 that gave its maximum yield with inoculum size of 3×10^6 CFU/mL. On the other hand, the isolate minimum bioactive metabolite yields were observed with an inoculum size of 3×10^4 CFU/mL. However, the difference observed in the yields, with inoculum levels of $3x10^6$ and $3x10^7$were not statistically significant ($p= 0.567$).

The highest yield (13.6±2.00mg/g) was recorded by the isolate (Go-475) with an inoculum size of $3x10^7$CFU/mL followed by Ac-029 and Rv-355 (13.1±1.00mg/g and 12.3±1.53mg/g) respectively with the same inoculum sizes. The least in yield (4.0±2.08mg/g) was obtained by the isolate Rv-355 with an inoculum size of $3x10^4$ CFU/mL. As shown in Figure 4.7, increasing inoculum size resulted in increased metabolite production, but a slight decline in yield was observed as the inoculum sizes were increased.

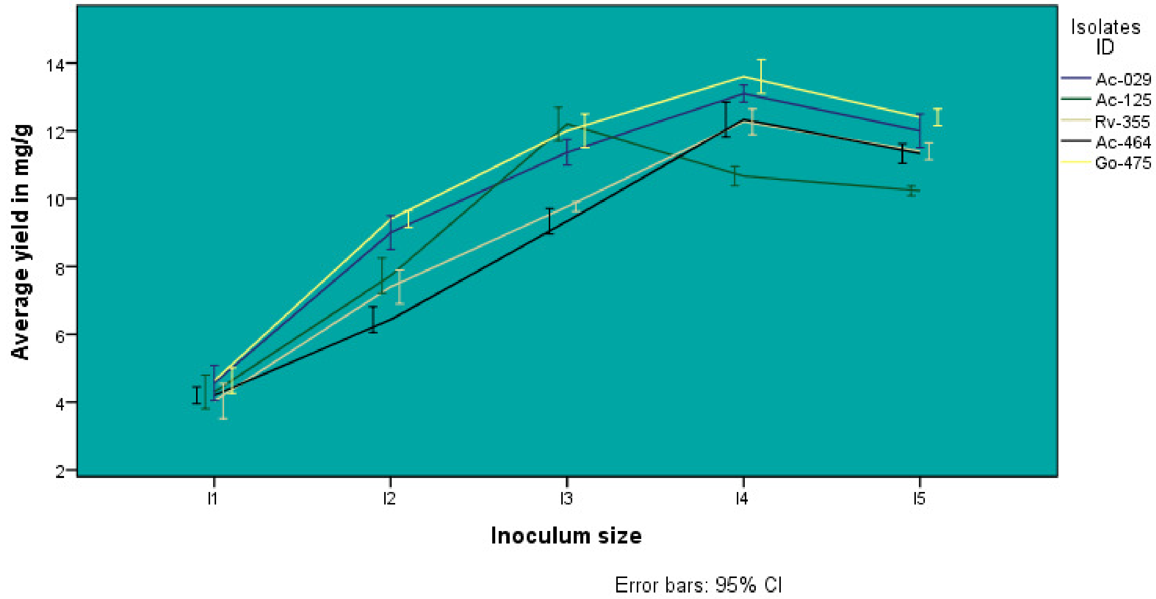


Figure 4.7. Effects of inoculum sizes (I1= $3x10^4$, I2 =$3x10^5$, I3= $3x10^6$, I4= $3x10^7$, I5= $3x10^8$).

4.5.8. Effects of various carbon and nitrogen sources

The effects of supplementing various exogenous carbon sources on isolates metabolite yields were investigated and the findings are as shown in Figure 4.8. The isolates exhibited growth and bioactive secondary metabolite production in all the carbon sources supplemented. However, Post Hoc multiple comparison analysis showed that, the different carbon sources supplemented for production of bioactive secondary metabolites, exhibited statistically significant difference in metabolite yields ($p<0.05$). Among the six carbon sources supplemented in the SSF processes, soluble starch was found to be the best carbon source responsible for better yields by most of the isolates. However, reduction was observed in isolates yield when wheat bran was supplemented with fructose, glucose and glycerol.

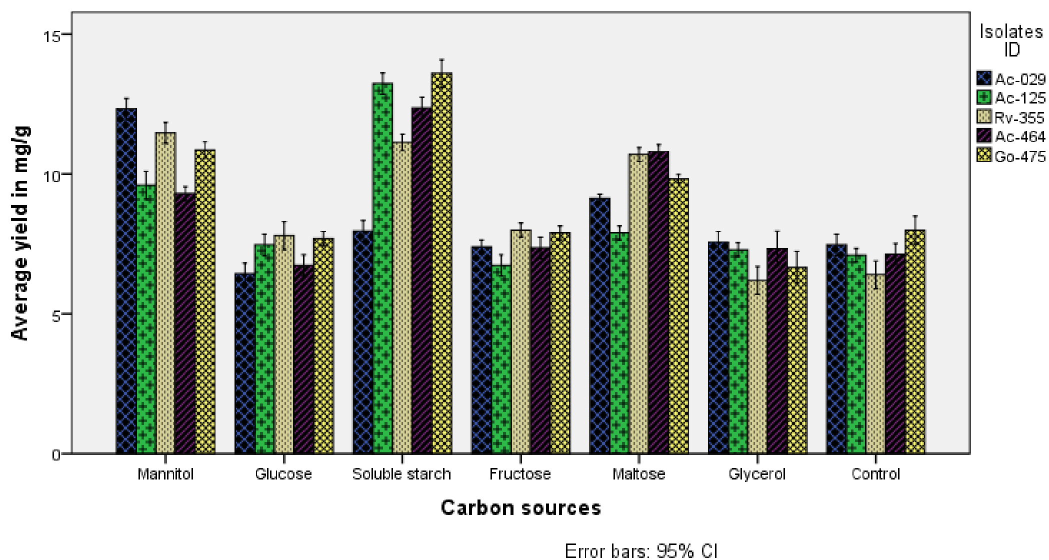


Figure 4.8. Effects of various carbon sources on metabolite yield

The highest average crude bioactive secondary metabolite yield (13.6±2.00mg/g) was recorded by the isolate Go-475 when wheat bran was supplemented with soluble starch; but the least in yield (6.2±2.00mg/g) was recorded on glycerol by the isolate Rv-355. On the other hand, the maximum yield for isolates Ac-029 and Rv-355 (12.3±1.53mg/g and 11.4±1.53mg/g respectively) were recorded on mannitol. Furthermore, considerable metabolite yields were observed by most of the isolates cultivated on solid substrate supplemented with maltose. To this end, isolates Ac-464, Rv-355 and Go-475, supplemented with maltose, exhibited metabolite yields (10.8±1.00, 10.7±1.00 and 9.8±0.58mg/g) respectively.

To determine the effects of different nitrogen sources on growth and metabolite yields by the isolates, the basal solid substrate was supplemented with a variety of nitrogen sources. The analysis of the data indicated that supplementation of exogenous nitrogen sources to the solid medium resulted in statistically significant difference in isolates metabolite yield ($p<0.05$).

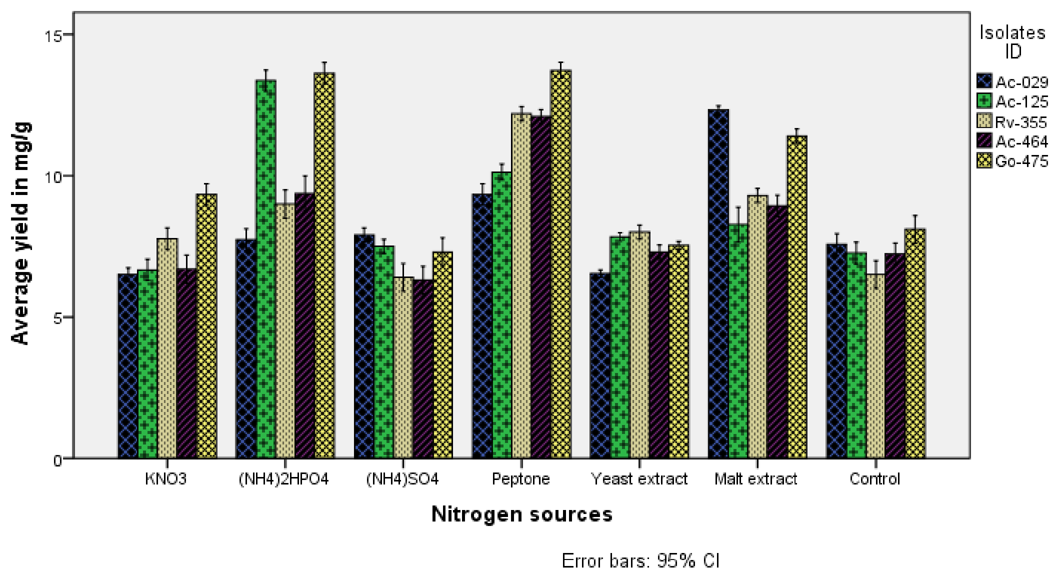


Figure 4.9. Effect of various nitrogen sources on metabolites yield

Among the six different nitrogen sources supplemented to wheat bran in the SSF cultivation processes, peptone was found to be the best nitrogen source which resulted in maximum crude metabolite yield by most of the isolates. This was followed by $(NH_4)_2HPO_4$, and malt extract. The highest average crude metabolite yield (13.7±1.16mg/g) was recorded by the isolate Go-475 when the basal solid medium was supplemented with peptone. The least in metabolite yield (6.4±1.00mg/g) was observed by the isolate Rv-355 cultivated on a solid medium supplemented with (NH_4) SO_4. Two isolates, namely Ac-029 and Ac-125, showed their maximum yield (12.3±0.58mg/g, and13.4±1.53mg/g) respectively, when the solid medium was supplemented with malt extract and $(NH_4)_2HPO_4$ respectively.

The result as presented in Figure 4.9 revealed that different isolates showed different nitrogen source requirement for maximum growth and secondary metabolite production. For instance, $(NH_4)_2HPO_4$ was found to be the best nitrogen source for isolates Go-475 and Ac-125. On the other hand, all isolates supplemented with (NH_4) SO_4 and yeast extract showed no difference in

metabolite yield to that of the control. Whereas isolate Ac-029 showed the maximum production on malt extract as organic nitrogen source. In general, most of the results showed that the addition of various organic and inorganic nitrogen sources to the solid medium stimulates growth, mycelium formation and improved the isolates metabolite yield.

4.6. Discussion

In this work important SSF process parameters were evaluated for crude bioactive secondary metabolite production by selected isolates. The result indicated that, bioactive secondary metabolite production depends on various solid state fermentation conditions.

Among the four solid substrates evaluated, wheat bran exhibited the highest *Streptomyces* growth, proliferation and bioactive secondary metabolite production followed by rice; whereas coffee husk was found to be the least. This might be due to the properties of wheat bran (its physical structure, water retention capacity and nutritional composition) suitable for the growth of *Streptomyces* and metabolite formation. Similar, studies from previous publications, such as Venkateshwarlu *et al*. (2000) who studied *Amycolatopsis mediterranei* VA18 also reported that wheat bran was the best solid support material for the production of rifamycin B under SSF process. Likewise, Sharmila *et al*. (2012) published maximum cyclosporin- A production on wheat bran as solid substrate. This might be due to the ability of *Streptomyces* spp. to produce extracellular enzymes capable of utilizing the carbon and nitrogen sources in wheat bran. A similar result was reported by Sun *et al*. (2008).

A number of authors investigated the nutritional composition of substrates used in SSF processes. For instance, Javed *et al*. (2012), Reisinger *et al*. (2013) and Pruckler *et al*. (2014) studied the nutrient content of wheat bran and reported its composition as, protein ranging from (12-18%), fat (0.5-4.3%) and total carbohydrates (56-60%). However, rice grain is composed of 7.0-7.7 % protein, 0.5-3.5% fat and 73.7-80 % carbohydrates (Oko *et al*., 2012; Koehler, and Wieser, 2013). On the other hand, potato peel is reported to have 14.7% protein, 69.0% carbohydrate and 7.7% ash (Al-Weshahy and Rao, 2012). Coffee husk generally consists of 5.2-11% protein, 0.3-5.0% fats, and 35-85% carbohydrates (Bondesson, 2015). This indicated that, wheat bran has higher protein and intermidate carbohydrate contents. Besides, its physical nature might be appropriate for hyphal penetration for filamentous *Streptomyces* and good quality suitable for the diffusion of gases. It might also be due to its better water retention capacity (related with high fiber content) and supply nutrients slowly but constantly during the microbial growth (Nisha and Ramasamy, 2008). Hence, these qualities of wheat bran might contribute for better bioactive secondary metabolite concentrations obtained during the cultivation of *Streptomyces* spp.

Almost all the isolates gave their maximum bioactive secondary metabolite yields with an initial moisture content of 65%. Similar studies such as Kagliwal *et al*. (2009) also reported maximum cephamycin C production using *Nocardia lactamdurans* NRRL 3802 with an initial moisture content of 65%. In a similar fashion, Basavaraj and Shivayagees war (2011) reported the highest tetracycline production using various strains of *Streptomyces* (*S. aureofaciens* NCIM2417, *S. aureofaciens* NCIM 2614, *S. aureofaciens* NCIM 2615, *S. rimosus* NCIM 2213 and *S. viridifaciens* NCIM 2506) with the same initial moisture content level.

Isolates showed a decline in growth and metabolite yield when the initial moisture content of the substrate was above 70% and below 60%. However, Kota, and Sridhar (1999), Nagavalli *et al*. (2014) reported maximum antibiotics (cephamycin C and Rifamycin SV production respectively) with an initial moisture content of 80% on solid substrates (wheat rawa and ragi bran) respectively. On the other hand, Adams *et al*. (2002) reported maximum biocontrol agent production with 60% initial moisture content on broliler litter substrate. In this regard variation observed in optimum initial moisture content in different SSF processes for maximum growth and metabolite production might be due to the ability of solid substrates to retain water (the water-holding capacity) and the type of isolates employed; and these conditions were also reported by Singhaniaa *et al*. (2009).

From the analysis, it was noted that the initial moisture level remarkably affects the growth and metabolite formation of the cultures in solid state fermentation. This might be because the early circumstances have to create a sufficient environment (preconditions) for microbial rapid growth phase for metabolite production. Moreover, moisture content of the solid particle has an effect on the physical properties of the solid particle. Reduction in yield observed in higher moisture contents beyond the optimum level might be because of the substrate particle agglomeration, reduced porosity of the particles that might retard diffusion and availability of oxygen, and interferes with heat and mass transfer. On the other hand, low moisture level might resulted in inadequate solubility, hampered the diffusion of enzymes and reduced supply of nutrients to the microbial culture which can hinder microbial growth and cause a reduction in the secondary metabolite yield (Pandey, 2003; Barrios-Gonzalez and Mejia, 2008). Furthermore, variation in metabolite yields observed in different initial moisture content might be because the amount of

water available and bounded to the substrate determines the amount of water to take part in biochemical reactions as reported by Pandey *et al*. (2008).

Most of the isolates exhibited their maximum bioactive secondary metabolite yield at a pH level of 7.5, except one isolate (Ac-029) that gave its maximum yield at pH 7.0. The later isolate well agreed with the finding by Nagavalli *et al*. (2014) who reported maximum rifamycin SV production at pH 7.0. However, the yield observed by most of the isolates were consistent with Ellaiah *et al*. (2004) who reported maximum neomycin production by *S. marinensis* NUV 5 at pH 7.5. On the otherhand, Mahalaxmi *et al*. (2010), Vastrad and Neelagund (2012) who studied *Amycolatopsis* strains reported that pH 8.0 was the optimum level for rifamycine B production. These variations reported in optimum pH values needed for maximum production might be due to genetical variation between the different species used under study. Besides, higher or lower pH levels than the optimum value, resulted in yield reduction indicating the sensitivity of microbial physiology to pH changes. This might be because, change in pH modifies and disrupts the hydrogen bonding of enzymes, which in turn promotes changes in the folding of the molecule, promoting denaturation and destroyed their activities. It can also control the growth and metabolic activities particularly the transport of substances across microbial cell membranes (Cuadra *et al*., 2008). However, pronounced yield reduction was observed in lower pH levels as compared to the higher pH values indicating that isolate favored neutral to alkaline pH values.

Incubation temperature was another key factor evaluated in cultivation processes and isolates were cutivated in ranges of temperatures. The highest secondary metabolite yields were recorded at 30 ^{o}C in most of the strains. This might be attributed to key microbial physiological activities

such as, spore germination, growth, sporulation and synthesis of metabolites could be affected by lower or higher incubation temperatures than the optimum. Similarly, Ellaiah *et al*. (2004) and Vastrad and Neelagund (2012) also reported maximum neomycin production at the same incubation temperature level using the isolates *Streptomyces marinensis* and *Streptomyces fradiae* NCIM 2418 respectively. However, Venkateshwarlu *et al*. (2000) reported maximum antibiotic (rifamycin-B) production at 32 ^{o}C, where as Mahalaxmi *et al*. (2010) reported that 28 ^{o}C was the best incubation temperature for the same rifamycin-B production by isolates *Amycolatopsis* sp. RSP 3 and *Amycolatopsis mediterranei* VA18 respectively. Furthermore, Basavaraj and Shivayageeswar (2011) obtained the highest tetracycline yield at 35 ^{o}C. The variability in optimum incubation temperatures for maximum secondary metabolite yield observed in the various reports might be due to the different species employed in the SSF processes; besides the temperature profile might depend on the water holding capacity of the solid substrates used for cutlivation.

The reduction observed when the incubation temperature was below and above the optimum value might be due to the vital role of temperatures in biological process. For instance under high temperatures, structural cell components and biomolecules become denatured and inactivation of heat-sensitive enzymes might occur (Mossel *et al*., 1995) which in turn affects growth and metabolite production in the isolates. On the other hand, at low temperatures, reaction rates for the particular enzymes in the microbes become much slower, and low temperatures decrease the fluidity of the cytoplasmic membrane, thus interfere with transport mechanisms in the cell (Nedwel, 1999).

The effects of incubation period on secondary metabolites yield were determined by cultivating the isolates in ranges of incubation periods (8-16) days. Most of the isolates appeared to produce their maximum titers within 12 days. This is in agreement with Bibb (2005), who stated that secondary metabolite formation occurs during the late growth phase of the producing microorganism. Similarly, previous study by Vastrad and Neelagund (2011) showed that the highest antibiotic production (neomycin) was attained at the 12th day of incubation using *Streptomyces fradiae*. However, Vastrad and Neelagund (2012) reported the highest rifamycin B production at the 10th day using an actinomycete (*Amycolatopsis Mediterranean* MTCC 14). Mossel and Thomas (1988) illustrated that the length of incubation period in fermentation may vary depending on the temperature and pH values used for the fermentation process. According to Mossel and Thomas (1988), a temperature shift from 10 to 25 °C decreased the lag time of a microbe from 60h to 10h. Similarly, a pH increase from 4.5 to 6.5 decreased the lag time from 60h to 5h. Thus, variation in length of incubation period might depend on other factors like pH and temperature in the cultivation processes.

On the other hand, a gradual decline in yield observed after the 12th day might be due to accumulation of toxic end products at late stationary phase which hampers secondary metabolite production or the cells may get ruptured due to the increased toxic metabolites concentration (Mahalaxmi *et al*., 2010). Furthermore, cells might be subjected to death phase in prolonged fermentation period due to the depletion of key nutrients required for survival. According to Bibb (2005), the temporal nature of secondary metabolite formation is certainly genetic, but expression can be influenced greatly by environmental manipulations such as exhaustion of a nutrient, or by a decrease in growth rate.

Analysis of the data revealed that sufficient inoculum can facilitate rapid mycelium expansion, substrate colonization and product development. When the inoculum size was lower or higher than the optimum level, a decline in bioactive secondary metabolite yield was exhbited by all the isolates. However, the reduction in yield was more prominent in lower inoculum size as compare to the higher one. This might be because low inoculum size might cause a sever reduction in biomass which in turn resulted in a very low yield of metabolites (Figure 4.9). A number of authors that cultivated actinomycetes by solid state fermentation for the production of bioactive secondary metabolites reported various inoculum sizes for maximum yield. For example El-Naggar *et al*. (2009) and Vastrad and Neelagund (2011) reported maximum meroparamycin and neomycin yield from *Streptomyces* sp. strain MAR01and *Streptomyces fradiae* NCIM 2418 with an inoculum sizes of 5×10^9 spores/mL and $2x10^6$ CFU/mL respectively.

These variations in optimum inoculum sizes reported by different authors might be because of the isolates genetical variations and the medium used. However, all agreed that considerably larger inoculum size resulted in maximum production which is in consistence with the current findings. This might be because sufficient inoculum size can create rapid colonization, mycelium formation, thereby assure the predominant growth of the desired isolate and hence resulted in better metabolite yield. In addition, the actinomycetes have relatively less capacity to colonize the solid medium than fungi, and hence larger inoculum size is essential for efficient growth. Furthermore, large inoculum size can shorten the lag time in the microbial growth phases (Barrios-Gonzalez and Mejia, 1996).

Among the six types of carbon sources evaluated in the current study, soluble starch resulted in the highest yield by most of the isolates. Similarly, Basavaraj and Shivayageeswar (2011) reported better metabolite yield using soluble starch as supplementary carbon source. However, reductions in yields were observed when other carbon sources such as glucose, fructose and glycerol were used. In this regard different authors reported different results on the effects of carbon sources on secondary metabolite yield. For instance, Vastrad and Neelagund, (2011) cultivated *Streptomyces fradiae* NCIM 2418 and achieved maximum neomycin yield using fructose as a supplementary carbon source to the solid medium. Others like, Ma *et al*. (2008), Mahalaxmi *et al*. (2010), Vastrad *et al*. (2014) reported the highest Rifamycin B yield with glucose using the isolates (*Amycolatopsis mediterranei* S699, *Amycolatopsis* sp. and *Nocardia mediterranei* MTCC14) respectively. However, secondary metabolite yields decreased when glucose, fructose or glycerol were used as supplements in the current study. This might be due to the repression effects of these carbon sources on the secondary metabolite production.

In this regard, Barrios-Gonzalez *et al*. (2004) and Sanchez *et al*. (2010) illustrated the repression exerted by glucose on the production of important antibiotics such as actinomycin, erythromycin, kanamycin, tetracyclin, neomycin, mitomycin and bacitracin. According to Sanchez *et al*. (2010), more than 20 antibiotics are reported to be suppressed by glucose and other carbon source like glycerol and sucroses. Similar information have been described in Barrios-Gonzalez and Mejia (2008). Therefore, better secondary metabolite yields observed in the current study by using soluble starch as a carbon supplement might be because of its polysaccharide nature that can dose sugar to the microbes very slowly and constantly.

Finally the effects of different nitrogen sources were evaluated during the cultivation processes. Most of the isolates showed the maximum yield on a solid medium supplemented with peptone. Similarily, previous studies by Kagliwal *et al*. (2009) and Manikyam *et al*. (2012) reported maximum Cephamycin C and Rifamycin yields from *Nocardia lactamdurans* NRRL 3802 and *Amycolatopsis mediterranei* by using $(NH_4)_2HPO_4$ and peptone respectively.

Ma *et al*. (2008) reported the highest rifamycin B by *Amycolatopsis mediterranei* S699 in solid state cultivation medium supplemented with $(NH_4)_2SO_4$. Variability observed in using various nitrogen sources for optimum yield development, might be due to the variations in metabolic path way intermediates used by the microbes. Thus, the utilization of the key elements (like nitrogen) might depend on the biochemical reaction taking place inside each cell. For instance, ammonia is the form of inorganic nitrogen that can be directly assimilated into amino acids and the ability of microbes to utilize other forms of inorganic nitrogen sources depend on the presence of enzymes or enzyme systems that are able to convert those compounds to ammonia (Basak and Majumdar, 1973).

4.7. Conclusion and Recommendation

The findings enable us to conclude that SSF offer potential advantages to use cheap solid substrates for the production of bioactive secondary metabolites from *Streptomyces* spp. The use of SSF for secondary metabolite production needs minimal expenditure on media and product recovery, suggesting that, it is a promising alternative technology for higher bioactive secondary metabolite yields from actinomycetes. Variations in secondary metabolite yield by using different solid substrates enable us to generalize that solid substrates not only used as solid support but also as a source of nutrients for the cultivated *Streptomyces* spp. The various amounts of bioactive secondary metabolite yields as a result of the difference in physicochemical, environmental and biological factors, allowed us to conclude that SSF process parameters have significant effects on secondary metabolite productions by *Streptomyces* spp.

In the current work the product titers and number of bioactive secondary metabolites detected in SSF are higher than SmF, however there is no clear cut made on these issues. Although such comparisons are challenging tasks, further and detail investigations are essential to make detail contrast on quantitative and qualitative variations between SSF and SmF metabolite yields. It is also vital to find out if there are variations in actinomyctes physiology and gene expressions between SSF and SmF environments. Hence, further studies shall be carried out on these important areas. At present the use of SSF for the production of commercially valuable metabolites is not utilized, therefore, pharmacetical companies and other industries egaged in bioactive compound production in Ethiopa have to work on this vaital area in the future.

Chapter 5: Antimicrobial Potentials of extracts from Submerged Cultures of *Streptomyces* sp. Rv-355

Abstract

The aim of this study was to evaluate the antimicrobial potential of extracts from submerged culture of *Streptomyces* sp. Rv-355 against yeasts (*Candida albicans* (ATCC 62376), *Cryptococcus neoformans* (clinical isolate)), Gram positive bacteria (*Bacillus subtilis* (ATCC6633) and *Staphylococcus aureus* (ATCC25923)) and Gram negative bacteria (*E.coli* (ATTC 25922), and *Sallmonella typhomerium*n (ATCC6539)). The isolate was identified as *Streptomyces* sp. Rv-355 based on cultural, morphological, bio-chemical and 16SrRNA sequence phylogenetic analysis. *Streptomyces* sp. Rv-355 produced antimicrobial compounds with wider spectrum of activities against yeasts, Gram positive and Gram negative bacterial pathogens. The maximum bioactivity was observed against *Candida albicans* when the isolate was cultivated in inorganic salts starch (ISP 4) medium. There was a positive correlation between the biomass production and bioactivity profiles of *Streptomyces* sp. Rv- 355. The isolate exhibited higher bioactivities when the basal medium was supplemented with soluble starch and peptone as carbon and nitrogen sources respectively. TLC, column chromatography, HPLC, LC-MS analysis of the bioactive crude extract showed the presence of potential compounds. The partially purified compound showed the MIC values of 50µg/mL against *Candida* albicans and 100µg/mL against *Bacillus subtilis*. In general, the study has shown the promising potential of *Streptomyces* sp. Rv-355 and has set the stages for further analysis of the crude extract using HR-MS, and NMR methods.

Key words: *Antimicrobial profile*, *bioassay*, *submerged fermentation*, *Streptomyces* spp.

5.1. Introduction

Bioactive compounds from *Streptomyces* have been the chief sources of antibiotics accounting for two thirds of drugs in clinical use; together with many other valuable compounds including anticancer agents, immunosuppressant, antiparastic and insecticidal drugs (Berdy 2005; Hopwood, 2007). Besides, coccidiostatics (used in poultry farming) and compounds affecting blood pressure, enzyme inhibitors that block synthesis of cholestrole etc. have been produced by *Streptomyces* (Behal, 2000). Such vast arrays of bioactive metabolite formation mainly by *Streptomyces* have necessitated the need for a diverse metabolic stock of the bacterium to sustain their extraordinary lifecycles (Arasu *et al*., 2009).

The life cycle phases of *Streptomyces* are comparable to that of filamentous fungi, forming hyphae and mycelia. Recent reports showed that these filamentous bacteria have sporulation and programmed cell death stages and are considered as multicellular prokaryotic models (Yague *et al*., 2013). *Streptomycetes* begin their life as spore germination, from which one or more germ tubes emerge. They showed more distinct asymmetric, polar mode of growth by tip extension and via the formation of new branches. This apical growth is controlled by a polarisome (a dynamic protein complex) including the vital polarity cytoskeletal-like protein (DivIVA) which is found in foci at growing tips (Flardh, 2003). As a component of the polarisome, DivIVA acts as a marker of the future branching site, responsible for recruiting the components of the cell wall biosynthetic machinery that is essential to initiate a new branch (Flardh *et al* ., 2012). The hyphae and emerging branches also develop by tip extension at some intervals, and as the processes recur; the whole branched vegetative mycelium is produced, which can proficiently

explore the existing space (Flardh, 2003; Flardh and Buttner, 2009; Flardh, 2010). At this phase the aerial hyphal filaments that are coated with a hydrophobic protein layer (chaplin), protects them from desiccation. These aerial hyphae are ultimately changed into chains of dormant exospores all the way through a synchronous round of cell division and chromosome segregation (Flardh, 2010).

Due to various environmental stresses, Streptomycetes shift from the vegetative phase (i.e. substrate mycelium) to a reproductive sporulation phase in the form of aerial multinucleated mycelium (Hwang *et al*., 2014). At the transition between these two phases of the life cycle, secondary metabolites are synthesized, which is accompanied by morphological differentiation. This indicates that the onset of morphological differentiation usually coincides with the production of secondary metabolites (Flardh and Buttner, 2009). Their production is a response to different stresses sensed by the colony as it communicates with its environment, exporting enzymes, importing nutrients and monitoring their concentrations by relays of sensory and regulatory signals that lead to the expression of appropriate suites of genes (Gust *et al*., 2003; Flardh and Buttner, 2009; Dyson, 2011).

Genome analysis showed that *Streptomyces* have a linear chromosome, around 8Mb to 10Mb depending on the particular species and a number of plasmids in a linear or circular form (Ventura *et al*., 2007; Hwang *et al*., 2014). One of the typical characteristics of their genome is the possession of biosynthetic gene clusters that encode enzymes meant to induce the production of secondary metabolites with different chemical structures, including polyketides, lactams, nonribosomal peptides and terpenes (Nett *et al*., 2009). In this regard Arasu *et al*. (2009)

described that *Streptomyces* strain fundamentally has the genetic potential to produce 10–20 secondary metabolites. That might be the major reason for this genus to contribute the lion share of the commercially and medicinally valuable antibiotics (Berdy, 2005). However, only 3% of streptomycetes biosynthesis capacities have been reported so far. This leaves huge potentials for new drugs to be discovered from these microbes (Weber *et al*., 2003).

Even though there has been a long history of studies on these microorganisms, they still have numerous biochemical mysteries to be described. Thus, *Streptomyces* species continue to attract attentions as a source of invaluable medicinal compounds that have great socio-economic relevance and with many potential therapeutic applications (Hwang *et al*., 2014). This clearly showed that the biosynthetic ability of streptomycetes is inexhaustible. For instance, recent genome sequencing analysis depicted that this genus have the potential to produce many more bioactive secondary metabolites than had been previously appreciated, many of which have not yet been elucidated (Berdy, 2012). In addition to the vital pathways (for example, polyketide synthase), they can synthesize new types of compounds through extensive branching and in sequences of optional reactions using different enzymes, such as condensations, alkylations, oxidations and isomerizations (Berdy, 2012). While secondary metabolites have been mined widely, genome sequencing has recommended that a number of chemical diversity and bioactivity remains to be discovered and characterized (Craney *et al*., 2013).

Therefore, the current investigation on one of the *Streptomyces* spp. from untouched habitat and characterization of its secondary metabolites is a valuable endeavor. A greater attention has been paid to evaluate the antimicrobial activities of *Streptomyces* sp. Rv-355 which was cultivated via

submerged fermentation and the effects of some fermentation parameters and antimicrobial profiles of the isolate were assessed.

5.2. General objectives

The aim of this study was to investigate the antimicrobial profiles of *Streptomyces* sp. Rv-355 cultivated in submerged culture, to investigate the correlatation between biomass, yields and antimicrobial activities and to analyze the crude extracts

5.2.1. Specific objective: it is specifically intended to:

- investigate the antimicrobial profile of *Streptomyces sp.* Rv-355 in submerged culture
- determine the relationship between the biomass yield and antimicrobial activities of *Streptomyces sp*. Rv-355 and analyze the crude extract

5.3. Materials and Methods

5.3.1. Strains used in the study

The strain used in submerged fermentation was identified as *Streptomyces* sp. Rv-355 based on phenotypic properties such as morphological, cultural, biochemical, physiological and partial genome sequencing methods. The 16SrRNA sequence analysis of this strain showed 99% similarity with *Streptomyces* sp. 13-3-10. Detailed taxonomic identification study is described in (section 3.3.7). The culture was maintained in starch casein agar slants at 4 ^{o}C.

5.3.2. Test Microorganisms

Test pathogens used in these experiments were those microorganisms described in Section 3.3.3.

5.3.3. Inoculum preparation

Inoculum preparation was conducted in a similar way as described in section 4.3.4. Briefly, *Streptomyces* sp. Rv-355 was grown onto the entire surface of starch casein agar plates and incubated at 30 ^{o}C. After 5 days, 10mL of sterile physiological saline containing 0·01% Tween 80 was added to the plates and scrapped with sterile scalpel and kept in sterile screw caped test tubes. A 1mL of the suspension was inoculated into 250mL conical flasks containing 50mL sterile medium composed of (g/L): soluble starch 20, tryptone soy broth 20, yeast extract 3, $CaCO_3$ 3, K_2HPO_4 1, $MgSO_4.7H_2O$ 0.025, pH 7.2, and incubated for 7 days on a rotary shaker (200rpm) at 30 ^{o}C (Vastrad and Neelagund, 2011). At the end of the incubation period, the culture was harvested by centrifugation for 5min at 2000rpm, the supernatant was discarded and the pellet was washed with sterile saline and resuspended into 10mL sterile phsiological saline containing 0.01% (v/v) Tween 80 (Murthy *et al*. 1999). The total numbers of cells in the suspension was adjusted spectrophotometrically and consistes of 2.04 x10^{8}CFU/mL; confirmed by viable plate count. Unless and otherwise specified, this inoculum preparation method was used for all the submerged fermentation processes.

5.3.4. Fermentation and recovery of the products

Submerged fermentation was conducted as described in Boubetra *et al.* (2013), 3mL of inoculum suspension containing about $2.04x10^8$ CFU/mL of *Streptomyces* sp. Rv-355 culture was inoculated aseptically into each of the 500mL sterile flask containing 100mL of the five different sterile media (starch casein , yeast extract malt extract (ISP2), oat meal (ISP3), inorganic salts starch (ISP4) and glycerol asparagines (ISP5)) and pH of the cultures adjusted to 7.5 using 1N NaOH or 1N HCL. The cultures were incubated at 30 ^oC on a rotary shaker at 250rmp for 10 days.

At the end of the incubation period, the fermented material was filtered out using Whatman filter paper number one with a pore size of 11µm and followed by centrifugation at 5000 rpm for 10min. The cell-free supernatant was extracted with an equal volume of chloroform. The organic phase was separated from the aqueous phase by separating funnel and concentrated to dryness under reduced pressure using Rotavapour.

5.3.5. Biomass yield (dry weight determination)

At the end of incubation period, the culture pellet from each of the submerged fermentations was filtered using Whatman filter paper number 1 having a pore size of 11µm. Then the mycelia were washed three times with sterile distilled water under aseptic conditions. The filter papers used in this experiment were dried to constant weight. The weight of each was predetermined and designated as the weight of filter paper (WtFP). The pellet was washed twice with sterile distilled

water and placed at 105 ^{o}C for about 24h, until constant weight. The dry weight of (biomass) was measured in milligrams and designated as total weight (TWt). The net dry weight was calculated by subtracting WtFP from the TWt. (Net dry weight = TWt - WtFP).

5.3.6. Effects of inoculum size, various carbon and nitrogen sources

In order to develop various inoculum sizes, serial dilution was conducted as described in section 5.3.3. The total numbers of cells were adjusted spectrophotometrically and confirmed by viable count, to contain CFU/mL (2.04 $x10^4$, 2.04 $x10^5$, 2.04 $x10^6$, 2.04 $x10^7$, and 2.04 $x10^8$). Each of the inoculum suspensions was inoculated into 500mL Erlenmeyer flasks containing 100mL fermentation medium (ISP4) and incubated at 30 ^{o}C with continuous shaking at 200 rpm for 10 days.

The effects of various types of carbon and nitrogen sources on bioactive secondary metabolite production were tested. The basal medium was ISP4, composed of (g/L): soluble starch 10; K_2HPO_4, 1; $MgSO_4.7H2O$, 1; NaCl 1; $(NH_4)_2SO_4$, 2; $CaCO_3$, 2; distilled H_2O 1000 mL; pH 7.5 (Shirling and Gottlieb, 1966). Each of the carbon sources (glucose, soluble starch, fructose, maltose, and mannitol) was added to the basal media at 1% concentration (Sanghvia *et al.*, 2014). The choice of carbon sources was mainly based on the accessibility and to represent mono-, di-, or poly-saccharides. Yeast extract, peptone, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, and $NaNO_3$ were provided separately as nitrogen sources into the production medium at 0.42g/L concentration each (Raytapadar and Paul, 2001). Carbon and nitrogen sources were added to the basal medium by replacing soluble starch and $(NH_4)_2SO_4$ respectively. The basal medium

without carbon and nitrogen sources was used as a control. Cultures were incubated on a rotary shaker (200rpm) at 30 °C for 10 days.

5.3.7. Time course of fermentation and bioactivity

The time course of bioactive secondary metabolite production in *Streptomyces* sp. Rv-355 was determined as described by Valanarasu *et al*. (2010) using the test pathogen *C. albicans. Streptomyces* sp. Rv-355 was cultivated in 500mL Erlenmeyer flasks, containing 100mL of ISP4 broth media and incubated at 30 °C with continuous shaking at 200rpm for 14 days. With two days of intervals, 1mL sample of the fermented culture was taken and centrifuged at 5000g for 10min. Then 50µL of the supernatant was adsorbed onto paper disks 6mm in diameter. The disks were placed onto Muller Hinton agar (Sigma Aldrich) previously seeded with the test organism. At each test procedure, inhibition zone diameters were measured in millimeters (Valanarasu *et al*., 2010).

5.3.8. Antimicrobial bioassay

Inoculum preparations for the target test pathogens and antimicrobial bioassay of the crude extract was conducted by using the standardized disc diffusion method as described in section 3.3.6.1 and 3.3.6.2. The inhibition zone diameters were measured in millimeter (mm).

5.3.9. Analysis of the crude extract

5.3.9.1. Thin layer chromatography (TLC) analysis

Crude extract from *Streptomyces* sp. Rv-355 was analyzed by thin layer chromatography (TLC) method using silica gel plates 60 F254 (Merck). About 0.01mL of the crude substance to be analyzed was spotted on a single line placed 1cm from the edge of the silica gel plate using small capillary tube and dried. The solvent system was Chloroform: Methanol: water (2:3:1 v/v/v). The solvent mixture was made to cover the bottom of the jar to a depth of 1.0cm and the jar was capped and allowed to stand for 15min so as to reach liquid-vapor equilibrium. After the TLC development and drying, the plate was sprayed with 10% H_2SO_4 followed by vanillin, heated and visualized with ultraviolet light at 254nm and 366nms. The different bands observed in the ultraviolet light as bright spot were outlined with pencil. Retention factors (Rf values) were calculated by dividing the distance traveled by the compound by the distance traveled by the mobile phase (Guangying *et al*., 2005; Atta, 2010).

5.3.9.2. Column chromatography

Partial fractionation and purification of the crude extract was conducted by column chromatography using silica gel (particle size 0.063-0.200nm). The eluting solvent system was Chloroform and Methanol 95:5 (v/v). The column packed with silica gel slurry and left overnight so as to settle silica gel slurry completely. A 100mg of the crude extract to be fractionated was mixed with the mobile phase and loaded to the top of the silica gel column surface carefully. A total of 42 fractions (10mL each) were collected and tested for their bioactivities (Dharumaduari *et al*., 2008).

5.3.9.3. Analytical HPLC and LC-MS profiles

The analysis of the bioactive compound was conducted as described by Snyder *et al*. (2010) using an auto sampler analytical HPLC (SHIMADZU, Japan) reverse phase HPLC with PDA detector monitored with a variable wave length of 190-400nm using a column (Phenomenx, type Synergy: 4μ, MAX_RP80A (150 x 4.6 mm) with a pre-column filter. Before the sample injection the column was equilibrated with solvent B and all the mobile phases were degassed by purging. Instrument method (LC-Time program) was adjusted, injection volume was10μL and a pressure of 300bar with a flow rate of 0.8mL/minute. Elution was carried out with solvent gradient system as described in the LC-Time program (in section 6.1). Mass spectra were recorded on H-class UPLC Acquity (Waters, USA). The LC-MS analysis was conducted using the same column in which QDA from positive and negative ion scans, ELSD and UV-detectors were used.

5.3.9.4. Minimum inhibitory concentration (MIC) determination

MIC determination for the partially fractionated antimicrobial compound from *Streptomyces* sp Rv-355 was conducted for the standard pathogen *Candida albicans* following the method, CLSI (2002) as described in section 6.3.3. Whereas, the MIC for *Bacillus subtilis* was determined following the protocols described in CLSI (2012). An antibacterial assay was performed in Muller Hinton Broth (MHB) whereas Roswell Park Memorial Institute (RPMI) was used for the yeast. Two fold serial dilutions of the partially fractionated extracts were conducted in the media stated in test tubes. The inoculum suspensions of the overnight grown *Candida albicans* and *Bacillus subtilis* were prepared in sterile normal saline with direct colony suspension method and adjusted to 0.5 Mcfarland standards at a wave length of 530nm and 625nm respectively. The

culture for *Bacillus subtilis* was further diluted to a final working inoculum size of $2x10^8$ CFU/mL whereas for yeast *Candida albicans* $2.5x10^3$ CFU/mL was used. The cultures were incubated at 37 ^{o}C for 24h and observed visually. The lowest concentration showing no visible turbidity was considered as MIC.

5.4. Data analysis

Multiple comparisons analysis was carried out using Duncan test and correlation analysis was conducted by using SPSS (version 20). The presence of statistically significant difference was reported at $p<0.05$.

5.5. Results

5.5.1. Colony appearance and micro-morphology of *Streptomyces* sp. Rv-355

Streptomyces sp. Rv-355 (Figure 5.1) was isolated from soil around rift valley area in specific place called Ataye with longitude of 40° 13'12'' and latitude of 10° 14'11'', see Figure 3.1 and Table 3.1 for sampling sites and their coordinate values.

Figure 5.1. Colony appearance of *Streptomyces* sp. Rv-355 on starch casein agar plate: A) colony (aerial mycelium) B) substrate mycelium C) micro-morphology

5.5.2. Antimicrobial activities of *Streptomyces* sp. Rv-355

Various media (ISP2, ISP3, ISP4 ISP5 and starch casein broth) were used to evaluate the bioactivity potential of *Streptomyces* sp. Rv.355 under SmF and the fidings are indicated in Table 5.1. From the result presented in Table 5.1 *Streptomyces* sp. Rv-355 cultured in the various ISP media exhibited various levels of bioactivities against a wide ranges of pathogens. Broad spectrum bioactivities were observed against pathogenic yeasts (*Candida albicans* ATCC 62376, *Cryptococcus neoformans*), Gram positive bacteria (*Staphylococcus aureus, Bacillus subtilis*) and Gram negative pathogens (*E.coli* ATTC 25922, *Salmonella typhi*).

Table 5.1. Bioactivities of *Streptomyces* sp. Rv-355 cultivated in five different fermentation media (inhibition zone diameters measured in mm)

Media	D. Wt. g/L	Average inhibition zone diameters (in millimeter) against the target pathogens:					
		Candida	*Cryptococcus*	*Staphylococcus*	*Bacillus*	*E.coli*	*Salmonella*
SCM	2.03	21.00±0.00	19.00±0.00	21.00±0.00	12.00±0.00	5.67±0.58	8.00±0.00
ISP 2	1.93	20.67±0.58	18.00±0.00	10.67±0.58	13.00±0.00	6.67±0.58	11.00±0.00
ISP 3	2.25	22.00±0.00	20.00±0.00	15.00±0.00	14.00±0.00	8.67±0.00	13.00±0.00
ISP 4	3.26	23.67±0.58	21.67±0.58	17.00±0.00	15.00±0.00	9.33±1.16	16.00±0.00
ISP 5	1.50	19.00±0.00	18.00±0.58	11.67±0.00	11.00±0.00	7.00±0.00	12.00±0.00

The highest bioactivity was observed against *Candida albicans* with mean inhibition zone diameter of 23.67±0.58mm and the least was exhibited against *E.coli* (with inhibition zone diameter of 5.67±0.58mm). Among the five different media used in the fermentation processes, *Streptomyces* sp. Rv-355 showed the highest bioactivity when cultivated on ISP4 medium.

To determine the relationship between bioactivity of *Streptomyces* sp. Rv-355 and biomass yield, bivariate correlations analysis was conducted. The result showed that significant positive correlation existed between dry weight (biomass) and bioactivity ($r = 0.969$, $p=0.007$) and ($r=0.952$, $p<0.05$) against *Candida albicans* and *Cryptococcus neoformans* respectively. Besides, significant positive correlation was observed between biomass and bioactivity against Gram positive bacterial pathogens such as *Staphylococcus aureus* ($r=0.879$, $p<0.05$) and *Bacillus subtilis* ($r=0.902$, $p<0.05$). On the other hand, the positive correlation between biomass and bioactivity against Gram negative bacterial pathogens is not statistically significant in which the correlation coefficients for *E.coli* was $r=0.733$, $p=0.16$ and for *Salmonella typhi* $r=0.686$, $p=0.20$. However, there existed a general positive correlation between biomass of *Streptomyces* sp. Rv-355 and its antimicrobial activities.

5.5.3. Effects various carbon and nitrogen sources on antimicrobial activities

Streptomyces sp. Rv-355 was cultivated in the basal fermentation medium, inorganic salts starch (ISP4) supplemented with different carbon and nitrogen sources and their effects on antimicrobial activities were tested. The result showed that *Streptomyces* sp. Rv-355 is a versatile in carbon and nitrogen utilzation. However, the antimicrobial activities showed variation depending on the carbon and nitrogen sources used. The isolate showed the highest mean inhibition zone diameters of 25.00±0.00mm against *Candida albicans* when cultivated in a basal medium supplemented with soluble starch. The least inhibition zone diameter (3.33±0.58mm) was recorded when glycerol was used as carbon source (Table 5.2).

Table 5.2. Effects of different carbon sources on antimicrobial activities

Carbon sources	Antimicrobial activities (inhibition zone diameter in mm)					
	Candida	*Cryptococcus*	*Staphylococcus*	*Bacillus*	*E.coli*	*Salmonella*
S. starch	25.00±0.00	22.00±1.73	19.33±1.52	16.33±2.08	13.33±0.58	10.33±0.58
Glucose	20.67±2.08	18.33±1.53	14.33±0.58	15.33±0.58	8.00±1.00	9.33±0.58
Glycerol	13.00±1.00	12.33±0.58	9.33±1.53	10.33±0.58	5.67±0.58	3.33±0.58
Mannitol	13.67±0.58	12.00±1.00	10.00±1.00	10.67±0.58	8.33±0.58	6.67±0.58
Fructose	9.33±1.16	8.67±1.53	9.00±1.00	8.33±0.58	6.67±0.58	5.67±0.58

Table 5.3. Effects of different nitrogen sources on antimicrobial activities

Nitrogen sources	Antimicrobial activities (inhibition zone diameter in mm)					
	Candida	*Cryptococcus*	*Staphylococcus*	*Bacillus*	*E.coli*	*Salmonella*
Yeast extract	16.67±1.53	15.00±1.00	13.00±0.00	12.00±1.00	12.00±2.00	10.00±1.00
Peptone	23.67±0.58	19.33±0.58	15.00±1.00	13.00±1.00	12.33±0.58	10.67±0.58
$(NH_4)_2SO_4$	21.00±1.00	20.00±0.00	13.00±1.00	12.00±1.00	9.33±1.16	9.00±1.00
$(NH_4)_2HPO_4$	18.33±0.58	18.00±1.00	16.67±0.58	15.00±1.00	9.00±1.00	8.33±0.58
$NaNO_3$	16.33±1.53	14.67±1.1	11.00±1.00	7.33±0.58	5.00±1.00	4.00±0.58

On the other hand, organic nitrogen (peptone) suplemnted to the basal medium (ISP4) showed better effect in antimicrobial metabolite production with mean inhibition zone diameters ranging from (9.00mm - 21.00mm) followed by the inorganic nitrogen sources such as $(NH_4)_2SO_4$ and $(NH_4)_2HPO_4$. As shown in the Table 5.3, *Streptomyces* sp. Rv- 355 showed the lowest activities when $NaNO_3$ was used as the nitrogen source.

5.5.4. Effects of incubation period and inoculum sizes

The time course of the antimicrobial metabolite production by *Streptomyces* sp. Rv-355 was monitored on ISP4 medium by testing the bioactivity against *Candida albicans*. It has been observed that *Streptomyces* sp. Rv-355 switched to antimicrobial metabolite production at a certain time between the 4th and 6th days of incubation period. It exhibited bioactivity starting from the 6th day and the maximum bioactivity was observed on the 10th day (late in the growth cycle) with mean inhibition zone diameter of 24.83±0.29mm and the least inhibition zone diameter (9.58±0.72mm) was observed at the sixth day of incubation period. The bioactivities continue during the subsequent production stages with a slight decline afterwards (Figure 5.2).

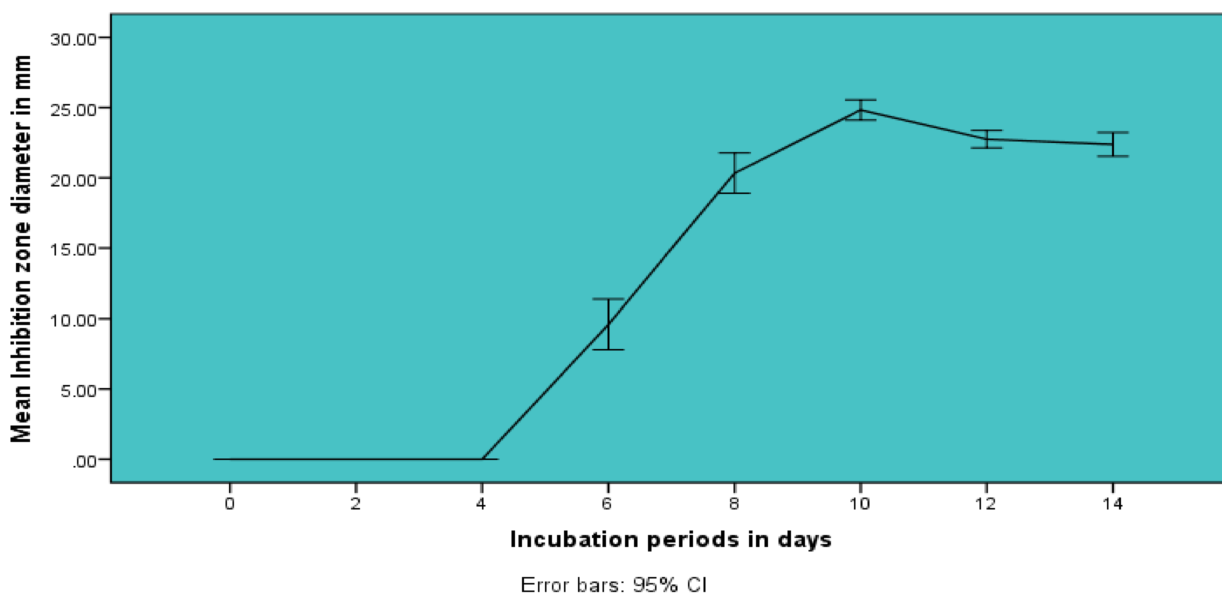


Figure 5.2. Bioactivity profile of *Streptomyces* sp. Rv.355 against *Candida albicans* tested in different incubation period (inhibition zone diameter measured in mm)

Extracts from *Streptomyces* sp. Rv-355 at different incubation periods were subjected for bioassay and analysis of the data using one-way ANOVA showed statistically significant difference in mean inhibition zone diameter ($p<0.05$). However, multiple comparison analysis

revealed that mean inhibition zone diameter at day 12 and day 14 exhibited no statistically significant difference (p>0.05) (Figure 5.2).

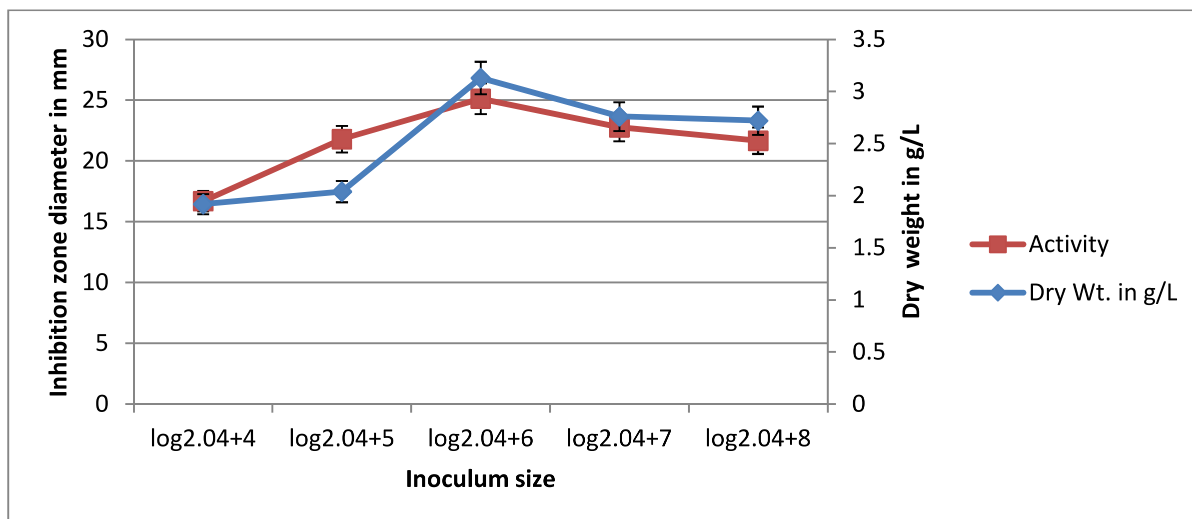


Figure 5.3. The relationship between inoculum sizes, dry cell weight and bioactivities

Multiple comparison analysis was conducted to evaluate the effects of inoculum size on the mean inhibition zone diameter of antimicrobial compounds from *Streptomyces* sp. Rv-355 treated with various inoculum sizes. The result showed that the mean inhibition zone diameter of *Streptomyces* sp. Rv-355 showed statistically significant difference (p<0.05). However, the culture treated with an inoculum size of $2.04x10^5$ CFU/mL and $2.05x10^8$ CFU/mL exhibited no statistically significant difference in inhibition zone diameter (p>0.05). The highest mean inhibition zone diameter (25.13±0.42mm) was recorded at the inoculum size of $2.04x10^6$ CFU/mL and the least (16.70±0.300) was observed at $2.04x10^4$ CFU/mL. Likewise, LSD analysis revealed that the cultures treated with various inoculum sizes showed statistically significant difference in dry cell weight (biomass production) (p<0.05). The maximum dry cell

weight (3.13±0.04g/L) was obtained in the culture treated with inoculum size of $2.04x10^6$ CFU/mL and the least (1.93±0.01g/L) was recorded at the inoculum size of $2.04x10^4$ CFU/mL.

The relationship between inoculum sizes, dry cell weight (biomass production) and that of antimicrobial activities were analyzed; the result showed that *Streptomyces* sp. Rv-355 grew and produced the antimicrobial substances in submerged cultures treated with all the five different inoculum sizes. The analysis showed that inoculum size and bioactivity of *Streptomyces* sp. Rv-355, are positively correlated (r=0.56), but the correlation is not statistically significant (p=0.33). Similarly, positive correlation existed between inoculum size and dry weight yield (r=0.71), but it is not statistically significant (p=0.18). As the result presented in (Figure 5.3) average dry weight (biomass) yield was ranging from 1.93g/L- 3.13g/L, whereas the antimicrobial activities vary from 16.70mm-25.13mm inhibition zone diameters.

5.5.5. TLC and column chromatography analysis

Thin layer chromatography analysis of the crude extracts from *Streptomyces* sp. Rv-355 showed different bands (Figure 5.4) with different retention factors (Rf values) (Table 5.4).

Table 5.4. Retention factors of the different bands in the TLC analysis

Isolate	Rf values in the TLC developing solvent system Dichloromethane: Methanol (9:1, v/v)					
	Rf1	Rf2	Rf3	Rf4	Rf5	Rf6
Rv-355	0.29	0.39	0.62	0.81	0.87	0.91

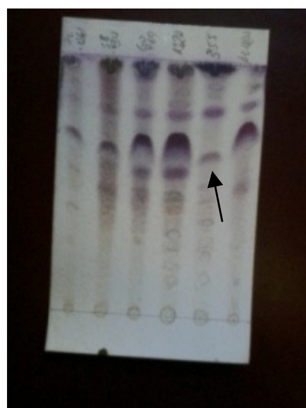


Figure 5.4. TLC bands of the extract from *Streptomyces* sp. Rv-355 shown in blak arrow, the solvent system was a mixture of (Dichloromethane: Methanol 9:1, v/v)

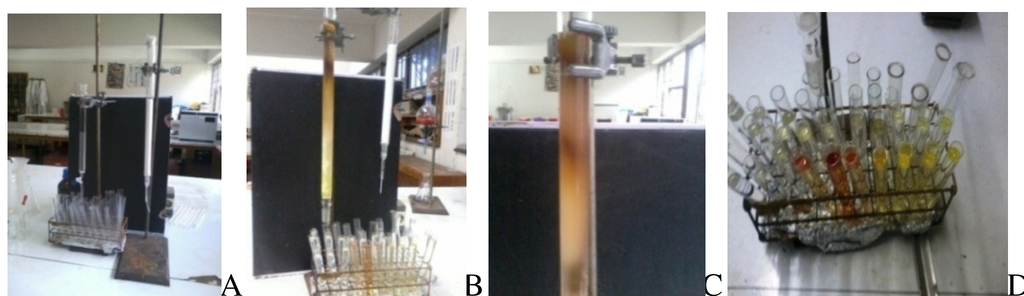


Figure 5.5. Column chromatography separation, A) setup preparation, B) at the beginning of elution, C) in the middle of elution D) collected fractions

The presence of different bands with various colors ranging from pink to red-brown (Figure 5.5), were observed in the crude extracts from *Streptomyces* sp. Rv-355. TLC plate visualized with 254nm and 366nm exhibited different bands with various retention factors ranging from 0.29-0.91 (Table 5.4). During column chromatographic separation, 42 fractions (Figure 5.5) were collected and all were tested for bioactivities. The result revealed that fraction 24 exhibited considerable bioactivity. This fraction was concentrated and dissolved in methanol for further analytical HPLC and LC-MS analysis.

5.5.6. Analytical HPLC profile of partially fractionated extract

HPLC profiles of partially fractionated bioactive compounds were analyzed using analytical HPLC SHIMADZU, with a column Phenomenx, type Synergy: 4μ, MAX_RP80A (150 x 4.6 mm). Elution and detections were monitored at a wave length of 190-400nm. As presented in Figure 5.6 three significant peaks were detected with different intensities at closer retention times. The most significant peak eluted at the retention time of 32.5 minutes has an absorbance (or intensity of 2700mAU at 190nm). On the other hand, the two other peaks eluted at retention times of 31.5min and 33.5 min with intensities of 800mAU and 650mAU respectively.

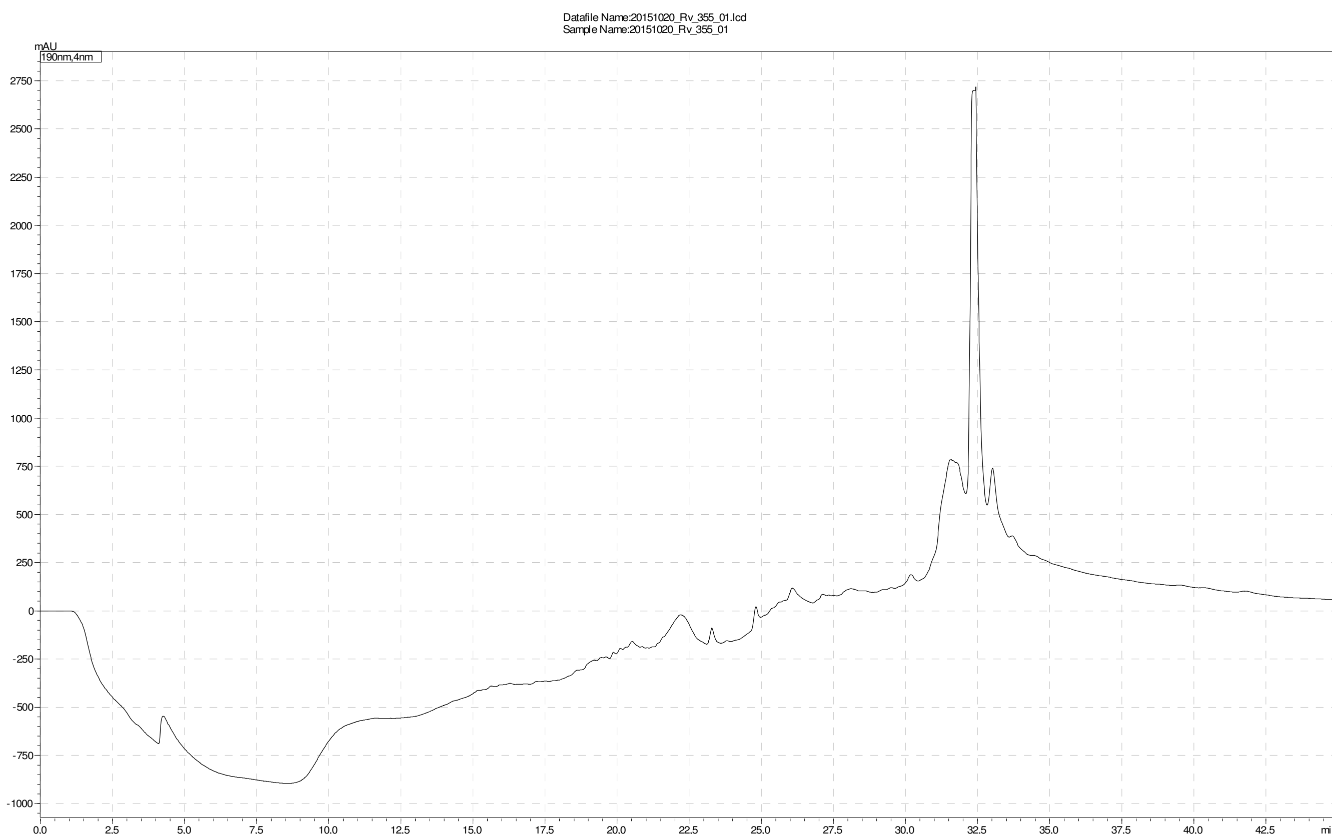


Figure 5.6. Chromatogram or analytical HPLC profile of fraction 24 (partially purified bioactive substance) with analytical HPLC, SHIMADZU at the wave length of 190nm

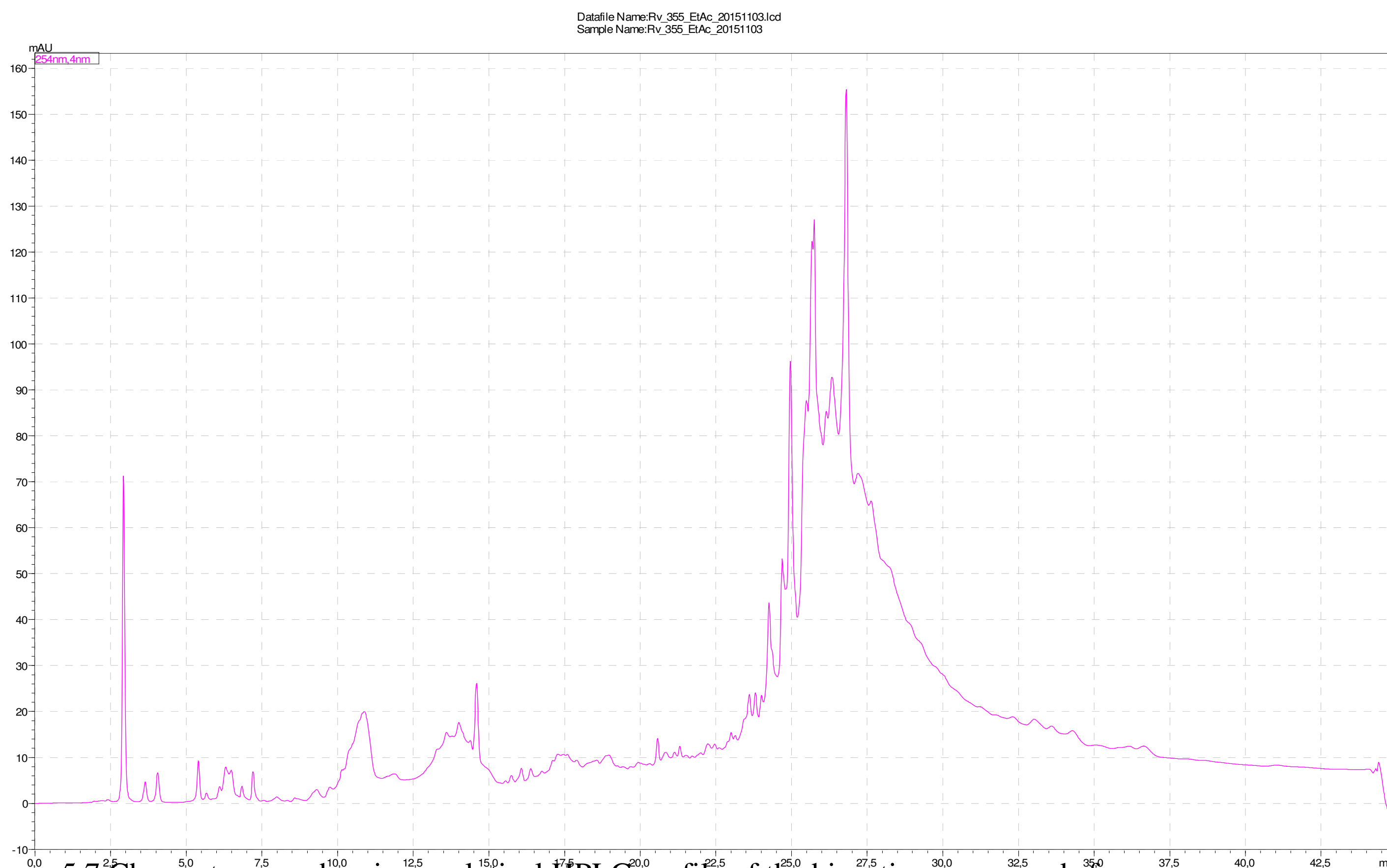
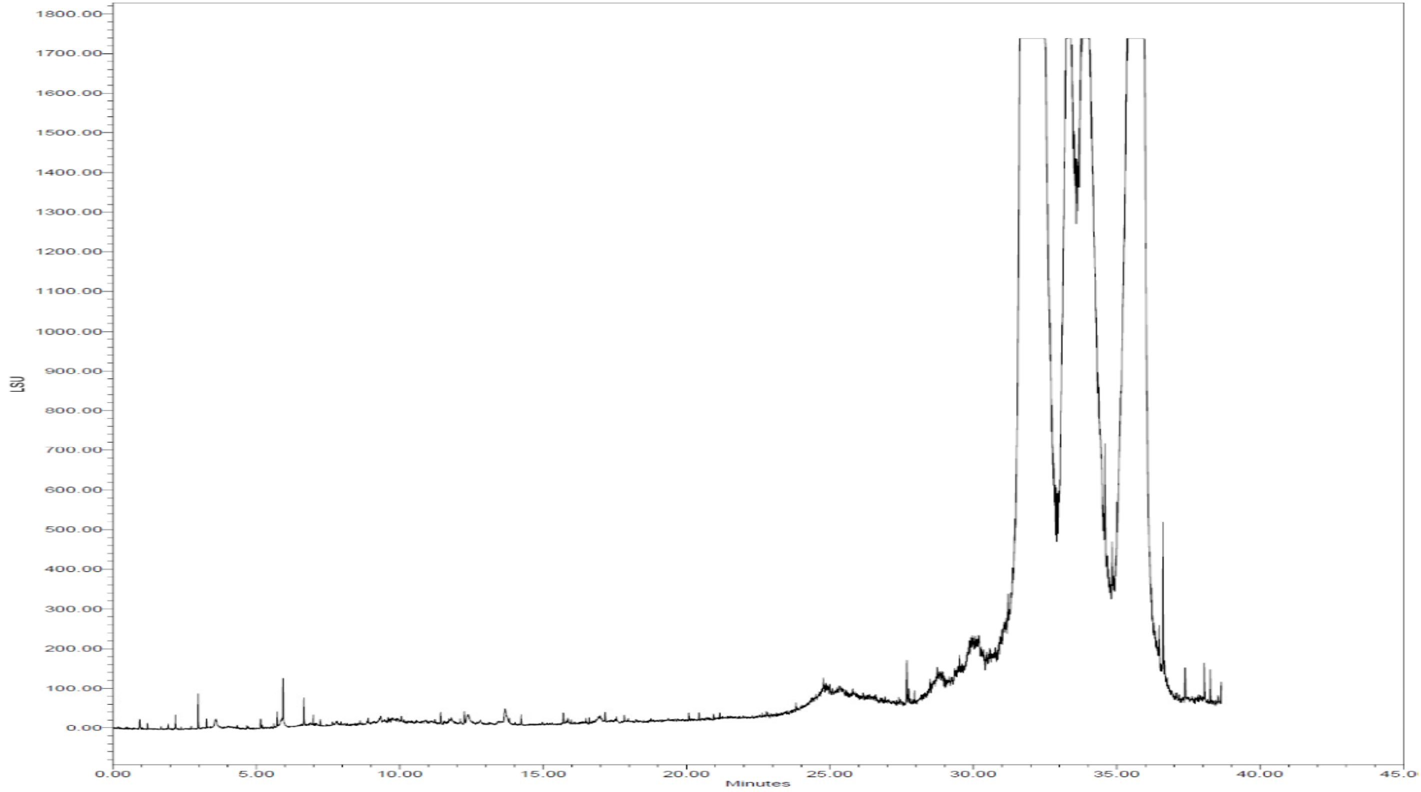
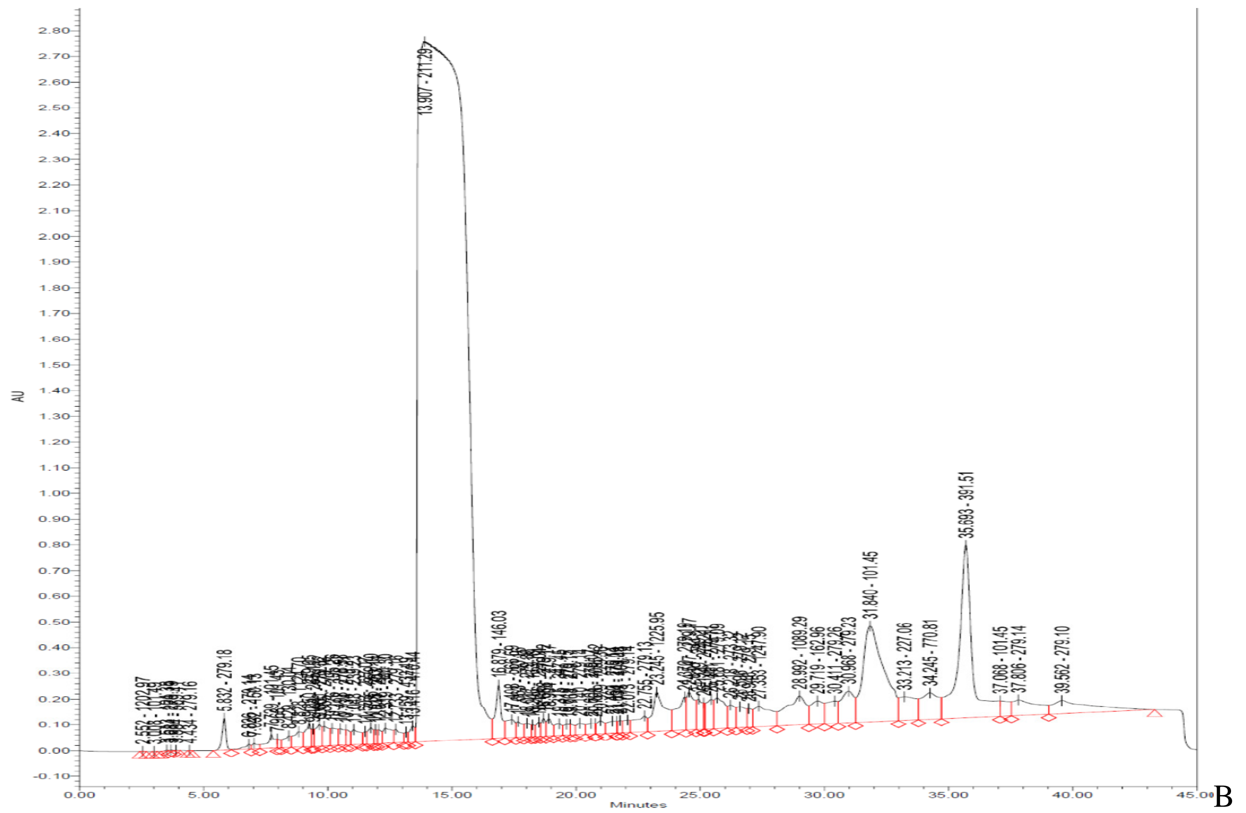


Figure 5.7.Chromatogram showing analytical HPLC profile of the bioactive compounds from *Streptomyces* sp. Rv- 355 at a wave length of 254nm

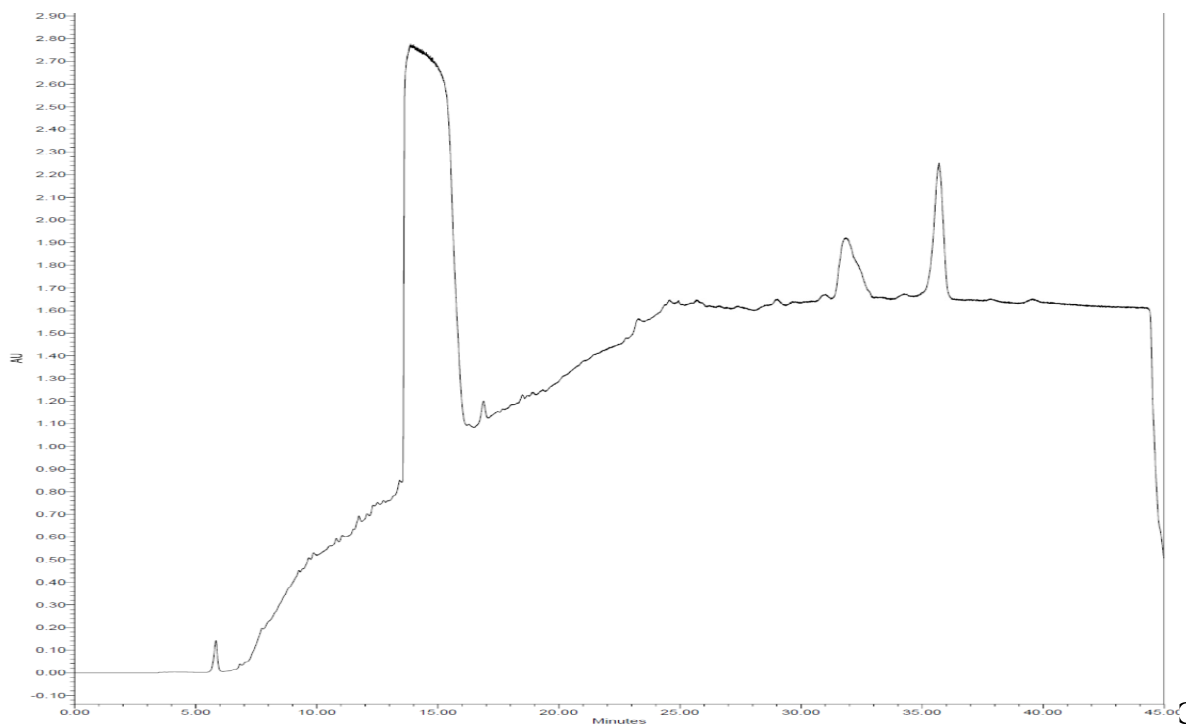
On the other hand, the chromatogram manually integrated at the wave length of 254nm revealed several peaks with different retention times. For instance, some of the significant peaks eluted at wave length of 254nm with a retention time in minutes (3, 11, 14.5, 25, 26, and 27) with various ranges of intensities in mAU (72, 20, 26, 98, 126 and 156 respectively). Based on the chromatogram, more substances were detected with 254nm as compared to the 190nm wave length. This indicates the presence of a number of different compounds, but the compound eluted at retention time of 27minute was shown to be with significant intensity (Figure 5.7).



A



B



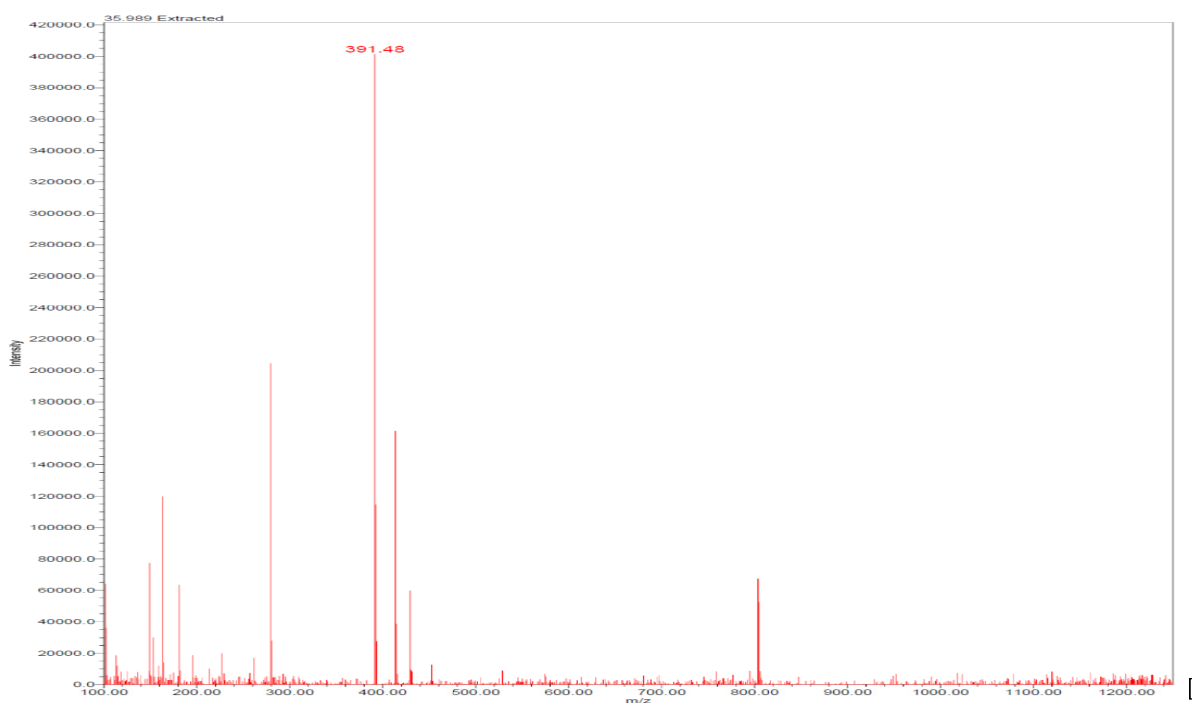


Figure 5.8. Chromatograms of antimicrobial compounds from *Streptomyces* sp. Rv-355, A) Chromatogram in ELSD detector, B) important peaks in PDA detector C), Chromatogram in UV detector D) Mass spectra of significant peaks in QDA detector from the positive ion scan.

The LC-MS analysis of partially purified antimicrobial substances from *Streptomyces* sp. Rv-355 in different detectors (PDA, ELSD, UV, and QDA from positive and negative ion scan) was conducted simultaneously in H-class UPLC, waters. As shown in Figure 5.8, significant peaks were observed with retention time ranging from 31-37 minutes with intensities up to 1760 AU (Figure 5.8A). Similarly, PDA detector revealed the corresponding peak at the same retention time with a fragment ion or molecular ion mass (*m/z*) of 391.51. Besides, QDA+ detector from the positive scan showed at *m/z* value of 391.48 at similar retention time. As shown in the chromatogram (Figure 5.8B) the crude extract chromatogram showed a number of peaks. Some of the significant mass specta observed at various retention time includes *m/z* = 279.18 at Rt 5.83 min, *m/z* = 211.29 at Rt 13.907 min, *m/z* = 146.03 at Rt 16.87 min, *m/z* = 1225.95 at Rt 23.245 min, *m/z* = 101.45 at Rt 31.84 min and *m/z* = 391.51 at Rt 35.693min, which indicate the presence of a number of fragment ion peaks that represent different molecules. The mass spectra of the partially purified active component (fraction 24 during the column chromatography) as shown in the (Figure 5.9D) revealed a base peak *m/z* = 391.48 at retention time of 35.898 minutes. The LC-MS spectra exhibited common positive ion adducts peaks like $[M+H]^+$ at *m/z* = 392, $[M+Na]^+$ at *m/z* = 414.48 and $[M+K]^+$ at *m/z* =430.48.

MICs of the partially purified crude extracts from *Streptomyces* sp. Rv-355 were determined for *Candida albican*s and *Bacillus subtilis*. The results showed that MIC tube treated with 50µg/mL have no visible growth of *Candia albicans*; whereas culture of *Bacillus subtilis* showed no turbidity at the MIC tube treated with a concentration of 100µg/mL. Therefore, partially purified antimicrobial metabolites from *Streptomyces* sp. Rv-355, with concentrations (50µg/mL and 100µg/mL) considered as MIC values for *Candia albicans* and *Bacillus subtilis* respectively.

5.6. Discussion

Streptomyces sp. Rv-355 cultivated in submerged culture showed variation in bioactivities depending on the type of pathogen and the type of medium used. The variation in bioactivities might be due to the difference in the composition of the different media. For instance, the principal carbon source in ISP4 is soluble starch, whereas the carbon source in ISP2 is glucose. On the other hand, ISP3 is a complex medium and in the case of ISP5 the carbon source is glycerol. *Streptomyces* sp. Rv-355 showed highest bioactivities in ISP4 might be due to soluble starch used as a carbon source in this medium which might be assimilitated slowely and having no carbon repressive effect. But the reduction in inhibition zone diameter observed in the presence of fructose, glycerol and glucose might be due to repressive effect of these carbon sources on secondary metabolite production.

Studies carried out by Song *et al*. (2012), Kumar *et al*. (2014) showed that bioactivities of secondary metabolites produced by *Streptomyces* strains depend on the type of fermentation media used. In this regard, wider inhibition zone diameters obviously indicate the potency of the antimicrobial substances. However, it has to be noted that bioactivities were measured using inhibition zone diameters which could be affected by the ability of the molecule to diffuse across the surface of the solid media. Molecules that can diffuse faster could travel in a wider diameter and to inhibit the target pathogen in a wider area, whereas those molecules unable to diffuse (or diffuse slowly) might result in narrow inhibition zone diameters (Driscoll *et al*., 2012).

The wider spectrum of bioactivities observed in *Streptomyces* sp. Rv-355 might be due to the capacity of the strain to biosynthesize multiples of bioactive secondary metabolites induced by domestic gene clusters. These also indicate the presence of large genome to encode a number of bioactive secondary metabolites. It has been noted that *Streptomyces* spp. have the genetic capability to generate as many as 30 distinctive secondary metabolites per strain, including polyketides, nonribosomal peptides and other classes of compounds (Nett *et al*., 2009). Similarly, a study by Arasu *et al*. (2013) identified polyketides related antibiotics from marine *Streptomyces* sp. AP-123 and reported wider spectrum bioactivities against different pathogens (such as *Bacillus subtilis*, *Staphylococcus aureus*, *E.coli* and *Candida albicans*) and different filamentous fungi like *Aspergillus niger*. Likewise, Lahoum *et al*. (2016), Sanjivkumar *et al*. (2016) studied on *Actinomadura* sp. ACD1 and *Streptomyces olivaceus* MSU3, isolated from Saharan soil and mangrove ecosystem in, India respectively reported in a similr fashion. Furthermore, Arasu *et al*. (2009) reported the genetic potential of *Streptomyces* strains to produce 10–20 bioactive secondary metabolites.

In the analysis to determine the relationship between bioactivities of *Streptomyces* sp. Rv-355 and its biomass (dry cell weight), positive correlation was observed between biomass yield and bioactivities when cultivated in different ISP media. This showed that the growth of cells have to reach sufficient quantities (which are directly related with the isolates biomass) to get high secondary metabolite titers. Studies by (Kandula and Terli, 2013; Govindarajan *et al*., 2014; Lahoum *et al*., 2016) also reported a direct relationship between biomass yield and secondary metabolite production. This is because bioactive secondary metabolite production might depend on the availability of precursor molecules for secondary metabolism, and this could be

determined by the growth medium composition, which can affect biomass yield and antimicrobial activities.

Streptomyces sp. Rv-355 showed the highest bioactivities when the basal medium was supplemented with soluble starch as carbon source. This might be due to the polysaccharide nature of soluble starch, assimilated by the cells slowly and constantly, with out repression effects (Sanchez and Demain, 2002). In this regard, Sanchez *et al*. (2010) described the interfering effects of glucose, glycerol and other carbon sources on the biosynthesis of more than 20 antibiotics in *Streptomyces*. In agreement with the current findings, Silva *et al*. (2012) reported soluble starch as effective carbon source for the production of antibiotics by *Streptomyces* spp. Likewise, Yu *et al*. (2008), investigated on *Streptomyces rimosus* MY02 and found higher antifungal activity when the basal medium was supplemented with starch as carbon sources. This indicated that culture environments (types of carbon sources) are very crucial to determine the antimicrobial metabolite titers.

Maximum bioactivity was observed when the basal medium supplemented with peptone. This might be because peptone released the amino acids slowly and has no negative effect on the strain's bioactive secondary metabolite production. It has been noted that ammonium salts are related with negative nitrogen catabolic regulation in many antibiotic production (Shapiro, 1989; Sahnchez and Demain, 2002; Sanchez and Demain, 2008). For instance, Voelker and Altaba (2001) reported that ammonium either directly supplied as a nitrogen source or originating from the breakdown of amino acids play a central role in negative regulation in pristinamycin production by *Streptomyces pristinaespiralis*. Similarly bioactivity reduction observed in yeast

extract might be related with the excess aminoacides released linked with catabolite regulation (Voelker and Altaba, 2001).

Howevere, Atta *et al.* (2010), who worked on the antimicrobial agents by *Streptomyces* sp., reported maximum production with glucose and KNO_3 as carbon and nitrogen sources respectively. Whereas, Sanghvia *et al*. (2014) reported the highest antibiotic production from *Streptomyces werraensis* using sucrose as a carbon source and yeast extract as nitrogen source. On the other hand, Grahovac *et al*. (2014) reported maximum antifungal metabolite production using glycerol as carbon source and yeast extract as nitrogen source. The variations in the carbon and nitrogen requirement indicate that there is no uniform utilization of carbon and nitrogen sources by different *Streptomyces* spp. This might be related with distinct bioactive secondary metabolites have specifc precursors.

The maximum bioactivity observed on the 10th day of incubation period indicated that the strain turn-on the antimicrobial metabolite synthesis during the transition between growth phase and stationary (4th-6th day) and attains highest antimicrobial metabolite titers late in the stationary phase. Bioactivity shown to be slightly constant after the 10th day might be because no more bioactive compund production after the 10th day. The bioactivities are related with the already produced bioactive compounds released in the medium. In this regard, Govindarajan *et al*. (2014) published the highest activity by *Streptomyces* sp. JRG-04 starting from day 7 with a constant production up to the 10th day of incubation period. Unlike to the current result, Yu *et al*. (2008) reported the highest antimicrobial activities by *Streptomyces rimosus* MY02 on the 5th day of incubation period.

Positive correlation between biomass and antimicrobial activities indicated the necessasity of enough biomass (cell factories) to get higher titers of bioactive secondary metabolites. Similar results reported in a non-polyenic antifungal production by a *Streptomyces yatensis* strain isolated from North-East of Algeria (Benouagueni *et al*., 2015). Likewise, Govindarajan *et al*. (2014) reported the positive correlation between biomass production and antimicrobial activities of *Streptomyces* sp. JRG-04. The decline in bioactivities observed in the late stationary phase might be due to the autolysis and disintegration of cells which is related with a decrease in biomass and reduction in metabolite synthesis. Furthermore, reduction in bioactivities might be due to the deactivation of the antibiotics and reduction in concentration of the bioactive metabolites.

TLC result showed different bands that have various retention factors ranging from 0.29-0.91 indicated the crude extract consists of a number of compounds with various polarities. This is because the stationary phase silanol (Si-OH) groups (Dong, 2006) in both TLC and column chromatography, retained the polar molecules more than non polar groups. On the other hand, of the 42 fractions in column chromatography, fraction 24 showed bioactivities indicated that, the bioactivie components have medium polarities. Furthermore, in RP-HPLC analysis, the retention time for the active fraction 32.5minutes indicated that the active component is either medium polarity or non polar compound. Detail information is documented in Synder *et al*. (2010).

5.7. Conclusion and Recommendation

Bioactivity potential of *Streptomyces* sp. Rv-355 maintained in both SSF and SmF suggesting that the strain produce bioactive secondary metabolites irrespective of the types of media and the culturing conditions. Positive correlation observed between biomass and bioactivities of *Streptomyces* sp. Rv-355 enables us to conclude that the isolate's biomass should be large enough to produce its maximum bioactive secondary metabolite titers. It is also possible to suggest the positive relation shipe between biomass and bioactivities of *Streptomyces* sp. Rv-355 culture. Active fraction eluted at various retention times enables us to generalize that the strain has the capacity to produce more than one bioactive compound.

Eventhough *Streptomyces* sp. Rv. 355 showed broad spectrum bioactivities, currently it is impossible to conclude weather the strain produces novel bioactive compounds or not, therefore, further studies are essential to fractionate the bioactive compounds in to pure forme and elucidate the molecular structures of the antimicrobial compounds. At this stage we could not able to generalize about the selective activity of the bioactive secondary metabolites from *Streptomyces* sp. Rv-355, therefore further studies are mandatory to determine the mechanism of action for those bioactive compounds from this isolate. Detail studies are essential to examine weather the bioactivity is related with toxicity properties of metabolites or due to the potential antimicrobial compounds. Besides, further studies should be carried out to examine the potency level of the antimicrobial compunds from *Streptomyces* sp. Rv. 355. Furthermore, a detailed study is recommended to analyze the bisosynthetic gene clusters responsible for the production of bioactive secondary metabolites by *Streptomyces* sp. Rv-355.

Chapter 6: Antibacterial antibiotics: 8-O-methyltetrangomycin and 8-O methyltetrangulol from *Streptomyces* sp. Go-475

Abstract

The aim of this particular chapter was to analyze and determine the molecular structures of antibacterial antibiotics produced by *Streptomyces* sp. Go-475. Various analytical methods including TLC, analytical HPLC, liquid chromatography coupled with mass spectrometry (LC-MS), preparative HPLC, high resolution mass spectrometry (HR-MS) and ^{1}H and ^{13}C NMR were used. *Streptomyces* sp. Go-475 displayed potent activity against both yeasts and Gram-positive bacteria. MIC values of the crude extracts were 100µg/mL and 50µg/mL against *Candida albicans* ATCC62376 and *Bacillus subtilis* ATCC6633 respectively. During SSF, strain Go-475 was able to produce at least three known secondary metabolites that were not detected in the SmF extract. Two major anti-bacterial compounds were purified from methanol extract, and their structures were elucidated by ^{1}H NMR, ^{13}C NMR and HR-MS/MS as 8-O-methyltetrangomycin and 8-O-methyltetrangulol. The three known secondary metabolites produced in SSF but not detected in the SmF extract were 4-Methoxy-1(3H)-isobenzofuranone, 3-Phenylpropionic acid or 1, 2-Benzenedio and Dehydrocineromycin B. Many potentially novel secondary metabolites were detected, the majority of which were produced in SSF method. This suggested the ability of *Streptomyces* sp. Go-475 to produce new secondary metabolites as an untapped potential source for new drug discovery and warrant further investigations. Analyses of complete genome sequence of *Streptomyces* sp. Go-475 confirmed its great potential for the production of chemically diverse secondary metabolites.

Key words: *antimicrobial assays, high resolution mass spectrometry, Streptomyces, solid state fermentation,*

6.1. Introduction

Due to the serious impacts and vibrant nature of antibiotic resistance, the need for novel bioactive compounds has been given considerable emphasis (Thumar *et al*., 2010). This scenario is even worthy in the current times due to the expansion of multidrug resistance pathogens (Alhede *et al*., 2014). Nowadays, more than 70% of pathogenic bacteria became resistant to most antibiotics in commercial use (Berdy, 2012). Hence, antimicrobial resistance is currently an urgent center of attention for research and alternative new bioactive compounds are essential to fight against these pathogens (Talbot *et al*., 2006). In the anti-infective area, the largest shares of drugs available in the market worldwide are natural products mainly from microbial sources or their derivatives (Berdy, 2005; Demain 2009; Berdy, 2012; Newman and Cragg, 2016).

Natural products are characterized by having untapped diversity of chemical structures nearly impractical to imitate, usually open up new therapeutic approaches, proved to contribute precious drugs and constantly continue as a potential source of future drug discovery processes (Grabley and Sattler, 2003; Newman and Cragg, 2007; Newman and Cragg, 2016). Berdy, one of the leading authors in the area, illustrated the advantages of natural products, like structural multiplicity (provide functional diversity), high potency, selectivity, multiple modes of action and host compatibility (Berdy, 2012). Furthermore, they are most excellent leads for successful structural amendment to enhance or alter the therapeutic activity of the original compounds by combining biotechnological and chemical approaches (Berdy, 2005; Berdy, 2012).

Despite the early success, chemical diversity and specific action on target, drug discovery from natural sources has been deemphasized by many pharmaceutical companies in favor of advanced methods based on combinatorial chemistry and genomics (Gurnani *et al*., 2014). However, by using these new methodologies, we still cannot find combinatorial compounds approved anywhere in the world except the two compounds namely Nexavar and Translarna (Newman and Cragg, 2016). On the other hand, Newman and Cragg, in their four compressive analytical reports, that encompassed 34 years data (1981-2014) emphasized that natural product and/or natural product structures keep on to play a highly significant role in the drug discovery and development process (Newman and Cragg 2003; Newman and Cragg, 2007; Newman and Cragg, 2012; Newman and Cragg, 2016).

Out of 33, 500 bioactive microbial natural products described from microorganisms in the years 1940s -2010, actinomycetes account for the production of 13, 700, the biggest share of which (10, 400) were represented by the genus *Streptomyces* mainly isolated from soil samples (Grabley and Sattler, 2003; Berdy, 2012). These bacteria have an outstanding capacity to produce diversity of bioactive secondary metabolites with wide ranges of applications. Their extraordinary bioactive secondary metabolite biosynthesis are related with their large genome size and the availability of a number of gene clusters (20-50) that are devoted for the biosynthesis of an array of secondary metabolites (Nett *et al*., 2009). The detection of novel secondary metabolites from microbial sources requires exploration of untouched niche habitats and efficient screening approach (Berdy, 2012).

Therefore, the current study was aimed at bioassay guided purification, isolation, identification and structural elucidation of antibacterial antibiotics from *Streptomyces* sp. Go-475. A number of standard analytical methods such as TLC, analytical HPLC, preparative HPLC, LC-MS, HR-MS /MS and NMR methods were employed in the analysis.

6.2. General objectives

This particular study was aimed at bioassay guided purification, identification and structural elucidation of potential antibiotics, analyses of secondary metabolite profiles of the crude extract and genome sequence analyses of *Streptomyces* sp. Go-475

6.2.1. Specific objectives: Specifically the study was aims at:

- Analysis, fractionation and identification of bioactive secondary metabolites
- Structural elucidation of promising bioactive compounds
- Identification of some biosynthetic gene clusters from *Streptomyces* sp. Go-475

6. 3. Materials and Methods

6.3.1. Actinomycete culture

The culture used in this particular study was the strain Go-475. Using polyphasic approach, the isolate was taxonomically identified as *Streptomyces* sp. Go-475 with 99% similarity in 16SrRNA sequence to *Streptomyces steffisburgensis* JCM 483 see (section 3.3.7) for details.

6.3.2. Fermentation and preparation of extracts from *Streptomyces* sp. Go-475

SSF was conducted following the method described by Bussari *et al*. (2008) using rice as solid substrate. The detail was as described in section 4.3.5. Bioactive secondary metabolites were prepared from the fermented materials by solvent extraction system following the method described by Venkateswarlu *et al*. (2000). Briefly, the fermented material and solvent mixtures (1:10) were shaken vigorously with glass beads overnight on a rotary shaker at 200 rpm. Two important solvents (ethyl acetate and methanol) were used separately for the extraction processes. The material was filtered through Whatman number 1 filter paper with a pore size of 11µm and the solvent was evaporated using (BUCHI rotavapour, RE 121, Switzerland) under vacuum. Concentrated and dried extracts were stored at 4 ^{o}C for further investigation.

For submerged fermentation (SmF), 15mL of tryptone soya broth (TSB) medium (Oxoid, UK) was inoculated with 200µL of spore suspension of *Streptomyces* sp. Go-475 in a 100mL Erlenmeyer flask and incubated at 28 °C with 200 rpm for 48 hours to produce seed culture. The mycelium was collected by centrifugation and then resuspended in 10mL sterile 20% glycerol solution for preparation of stocks. A 250mL of the medium composed of Soy flour 10g, Glycerol 15g, NaCl 5g, $CaCO_3$ 1g, $CoCl_2.7H_2O$ 1mg, in 1L of distilled water (according to Wink, 2012), was inoculated with 5mL of seed culture stock adjusted to contain about (2.8 x 10^6 CFU/mL) and fermented in 2L baffled Erlenmeyer flasks at 30 °C with 200 rpm for 7 days. The whole culture was freeze-dried and extracted with 50mL of methanol.

6.3.3. Determination of MIC

MIC of the crude extract from *Streptomyces* sp. Go-475 was determined for *Candida albicans* ATCC 62376 using the protocol described in NCCLS (2002) and for that of *Bacillus subtilis* ATCC6633 as described in CLSI (2012). The experiment was performed in Muller Hinton Broth (MHB) for *Bacillus subtilis* whereas; Roswell Park Memorial Institute (RPMI) was used for the yeast. Two fold serial dilutions of the extract were conducted in an appropriate solvent dimethyl sulfoxide (DMSO). Inoculum suspensions of the overnight grown *Candida albicans* and *Bacillus subtilis* were prepared in sterile physiological saline (0.85%) with direct colony suspension method and the optical density was adjusted to 0.5 Mcfarland standards at a wave length of 530nm. The inoculum for *Bacillus subtilis* was diluted to a final working size of $2x10^8$ CFU/mL whereas for *Candida albicans,* $2.5x10^3$ CFU/mL was used. The experiments were conducted with concentrations of the extract ranging from 0.39µg/mL - 800µg/mL. The cultures were incubated at 37 ^{o}C for 24h and observed visually; the lowest concentrations of the extract that showed no visible turbidity (growth) were considered as MIC values.

6.3.4. Thin layer chromatography (TLC) analysis

TLC work was conducted using TLC plates (silica gel $60F_{254}$, MX302619, Merck). About 0.01mL of the samples to be analyzed was placed (spotted) on a line placed 1cm from the edge of the silica gel plate using capillary tubes and allowed to dry. The solvent systems (mobile phase) was allowed to cover the bottom of the jar to a depth of 1.0cm and the jar was capped and allowed to stand for 15min so as to reach liquid-vapor equilibrium. TLC was developed by placing the plate inside a jar with six various solvent systems such as TLC system 1, Dichloromethane: Methanol: Water (2:3:1), TLC system 2, Ethylacetate: Propanol: water (

5:5:1, v/v/v), TLC System 3, Dichloromethane: Methanol (9:1, v/v), TLC System 4, Chloroform: Methanole: Water (5:5:1, v/v/v) and TLC system 5, Chloroform – Methanol (9:1 v/v) and TLC system 6, Methanol-dichloromethane–water (1:1:1 v/v).

In TLC development the mobile phase was allowed to move about 90% of the way up the plate. When the mobile phase reached near the upper end of the plate, the plate was taken out from the jar carefully using forceps; the solvent front was marked right away using a pencil; and allowed to dry. Following TLC development and drying, the plates were sprayed with diluted H_2SO_4 and vanillin and visualized with ultraviolet lamp at 254nm and 366nm. The different bands that have shown ultraviolet light as bright spot were outlined with pencil. Retention factor (Rf value) were calculated by dividing distance traveled by the compound to the distance traveled by the solvent and the result was recorded (Guangying *et al*., 2005; Atta, 2010).

6.3.5. Analytical HPLC analysis

Analyses of bioactive compounds were conducted as described by Snyder *et al*. (2010). The extract was injected into an auto sampler HPLC (SHIMADZU with UV/visible variable wavelength absorbance) and the photodiode array detectors (PDA) with two lamps inside one with 200-400nm and the other 400-800nm. The column Phenomenix Gemini RP C18 (150 x3mm; 5µm) was used. Instrument method, LC-Time program and solvent gradient system were as described in Table 6.1. Injection volume was adjusted to be 10µl with a flow rate of 0.8mL/min.

Table 6.1. HPLC method (LC- Time program), solvent gradient and flow rate during the analysis: (“A” is HPLC grade water, “B” is HPLC grade Methanol)

LC-Time in min.		0.00	20	40	40	45	45
Solvent Gradient	% A	95	2	2	95	95	Stop Time
	% B	5	98	98	5	5	“
Flow rate		0.8mL/min	0.8mL/min	0.8mL/min	0.8mL/min	0.8mL/min	“

6.3.6. LC-MS profiles of the crude extract from *Streptomyces* sp. Go-475

To identify the anti-microbial compounds in the active extracts, an analytical strategy previously established, in the Department of Analytical Chemistry, Mass Spectrometry Center, University of Vienna was employed (Ladurner *et al*., 2017). The crude extracts obtained from both SSF and SmF were diluted to a concentration of 1mg/mL and were first analyzed by high-performance liquid chromatography (HPLC) on an UltiMate 3000 RSLC-series system (Dionex/Thermo Fisher Scientific, Germering, Germany) coupled in parallel to a Corona ultra RS charged aerosol detector (CAD, Dionex/Thermo Fisher Scientific) and high capacity ion trap (HCT) 3D quadrupole ion trap mass spectrometer (MS) equipped with an orthogonal ESI source (Bruker Daltonics, Bremen, Germany). The CAD detector allows the relative quantification of the non-volatile constituents and thus the identification of the main compounds, while the diode-array detector (DAD) and the MS provide qualitative information. Separation was carried out on a Synergi 4u MAX-RP 80Å, 150 x 4.60 mm, 4μm HPLC column (Phenomenex) using water and acetonitrile as mobile phase A and B, respectively. The gradient started from 50 % to 95 % B in 40 min, followed by a washing (10 min at 95 % B) and re-equilibration step (10 min at 50 % B).

The flow rate was 1.0mL/min and the column oven temperature was set at 35.0 °C. After passing the diode-array detector (DAD), the eluate flow was split 4:1 between the CAD and the MS, respectively. The CAD nebulizer temperature was 35 °C and the ESI ion source was operated as follows: capillary voltage: +3.5/-3.7 kV, nebulizer: 26 psi (N_2), dry gas flow: 9 L/min (N_2), and dry temperature: 340 °C. Positive and negative ion mode multistage mass spectra up to MS^3 were obtained in automated data-dependent acquisition (DDA) mode using helium as collision gas, an isolation window of $\Delta m/z = 4$, and a fragmentation amplitude of 1.0 V.

In the next step, high-resolution mass spectra were recorded on a maXis HD ESI-Qq-TOF mass spectrometer (Bruker Daltonics) that was also connected to an UltiMate 3000 RSLC-series system. The separation was performed with the above described HPLC methods. The eluate flow was split approximately 1:8 and the following ESI ion source settings were applied: capillary voltage: ±4.5 kV, nebulizer: 0.8 bar (N_2), dry gas flow: 7.0 L/min (N_2), and dry temperature: 200 °C. The sum formulae of the detected ions were determined using Bruker Compass Data Analysis 4.2 based on the mass accuracy ($\Delta m/z \leq 10$ ppm) and isotopic pattern matching (Smart Formula algorithm).

6.3.7. Purification of antibacterial compounds and structure elucidation

The crude extracts from SSF and SmF were purified repeatedly in separate experiments. Briefly; 500mg of the extract was mixed with Silica gel 60, MERCK (particle size 0.063-0.200nm) in a ratio of 1:2 and packaged in cartridge. For fractionation, the PuriFlash column 15 C18 HQ 35G - 35.0 g (22 bar) was used with a gradient of 5-98% Methanol/H_2O in 50 minutes and a flow rate of 15 mL/min using PuriFlash 420. About 109 fractions were collected (Figure 6.6), freeze dried

and concentrated in methanol for antimicrobial bioassays. The identification of the antimicrobial compounds in the active fractions was similar as described above for the crude extracts, but the HPLC separation was as given in detail in Ladurner *et al*. (2017), whereby the gradient used in this study was: 5 % to 95 % B in 45 min followed by a washing (10 min at 95 % B) and re-equilibration step (10 min at 5 % B).

Further purification was also conducted using Sephadex LH-20 column as described in Praveen and Tripathi (2009), Ebada *et al*. (2008). Briefly, 7.41mg of the active fraction was subjected to Sephadex LH-20 separation using (25-100µm) porous beads (Sigma Aldrich) as a stationary phase and specifically designed for the separation and purification of natural products within a column (45cm in length and 9mm in diameter) with a flow rate of 2mL/min, and MeOH was used as a mobile phase. Fractionation and separation was conducted by the principle of size exclusion chromatography. Compounds with larger molecular diameter excluded from the interior of the bead which elute first. However, those compounds with smaller diameter enter into the beads and elute according to their ability to exit from the small sized pores.

6.3.8. Nuclear Magnetic Resonance Spectroscopy (NMR) analysis

NMR analysis was conducted in the Department of Pharmaceutical Chemistry, University of Vienna, Austria. Briefly, NMR spectra were recorded on a Bruker Avance 500 NMR spectrometer (UltraShield) using a 5mm switchable probe (TCI Prodigy Kryo-probe head, 5mm, tripel resonance-invers-detection probe head) with z axis gradients and automatic tuning and matching accessory (Bruker BioSpin) in the Department of Pharmaceutical Chemistry,

University of Vienna. The resonance frequency for ^{1}H NMR was 500.13 MHz and for ^{13}C NMR 125.75 MHz. All the measurements were performed for a solution in fully deuterated chloroform or methanol at 298K. Standard 1D and gradient-enhanced (ge) 2D experiments, like double quantum filtered (DQF) COSY, HSQC, and HMBC, were used as supplied by the manufacturer. Chemical shifts are referenced internally to the residual, non-deuterated solvent signal for chloroform ^{1}H (δ 7.26 ppm) or methanol (δ 3.31 ppm) and to the carbon signal of the solvent for chloroform ^{13}C (δ 77.00 ppm) or methanol (δ 49.00 ppm).

3,4-Dihydro-3-hydroxy-8-methoxy-3-methyl-benz[*a*]anthracene-1,7,12(2*H*)-trione = 8-O-methyltetrangomycin (**1**):

^{1}H (500 MHz, d_4-Methanol) d = 8.26 (d, 8.1 Hz, 1H, H-6), 7.81 (dd, 8.5 and 7,1 Hz, 1H, H-10), 7.67 (d, 7,1 Hz, 1H, H-11), 7.66 (d, 8,1 Hz, 1H, H-5), 7.51 (d, 8.5 Hz, 1H, H-9), 4.01 (s, 3H, OMe), 3.26 (d, 16.9 Hz, 1H, H-4/1), 3.15 (d, 16.9 Hz, 1H, H-4/2), 3.09 (d, 14.3 Hz, 1H, H-2/1), 2.88 (d, 14.3 Hz, 1H, H-2/2), 1.45 (s, 3H, CH_3).

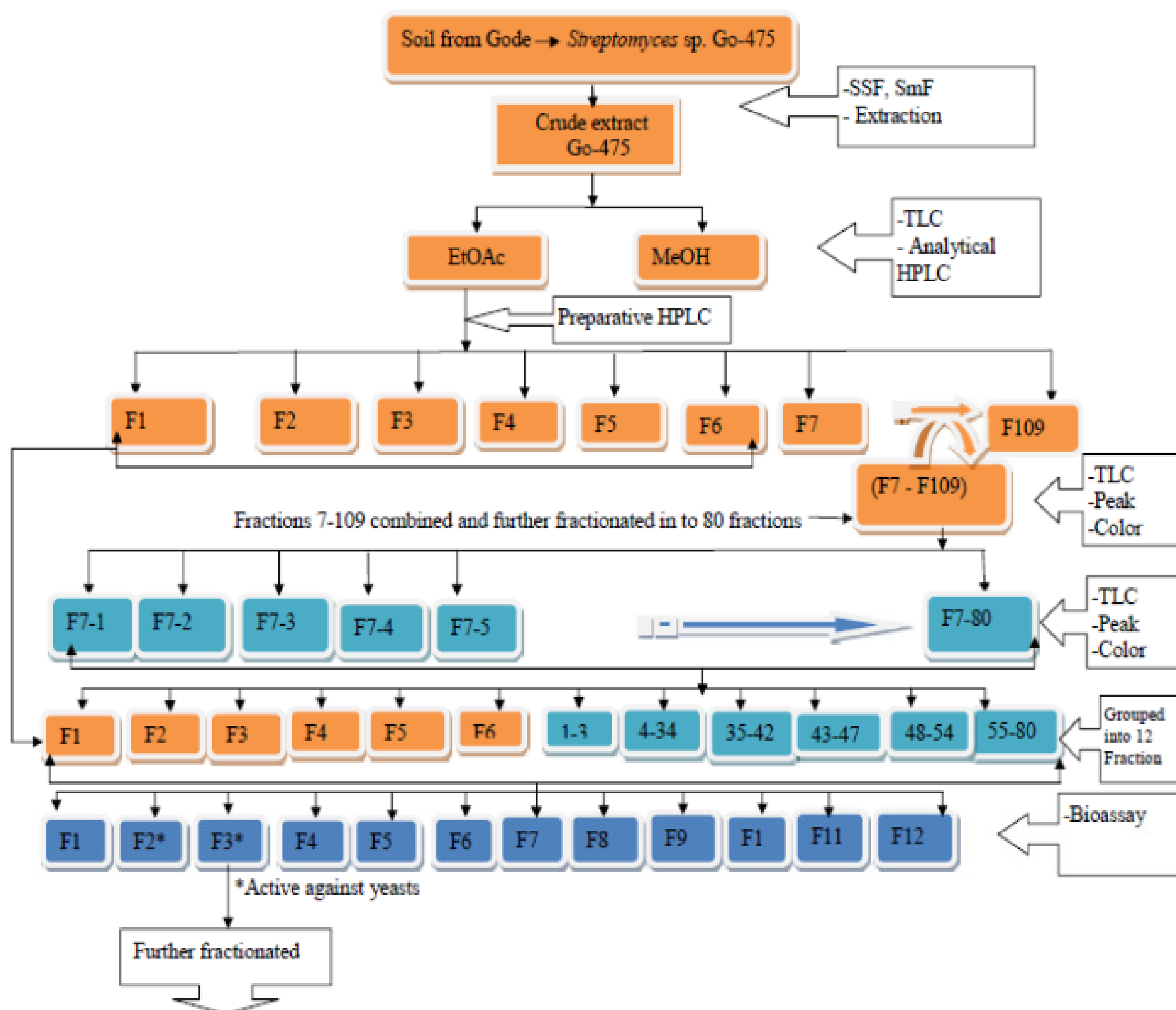
^{13}C (125 MHz, d_4-Methanol) d = 199.36 (C-1), 186.04 (C-12), 182.88 (C-7), 161.33 (C-8), 149.24 (C-4a), 139.02 (C-11a), 137.02 (C-10), 136.48 (C-12a), 136.11 (C-6a), 135.44 (C-12b), 135.30 (C-5), 130.70 (C-6), 121.43 (C-7a), 120.14 (C-11), 118.90 (C-9), 73.21 (C-3), 56.89 (OMe), 54.27 (C-2), 44.64 (C-4), 29.84 (CH_3).

1-Hydroxy-8-methoxy-3-methyl-benz[*a*]anthracene-7,12-dione = 8-O-methyltetrangulol (**2**):

^{1}H (500 MHz, $CDCl_3$) d = 11.15 (S, 1H, OH), 8.30 (d, 8.7 Hz, 1H, H-6), 8.13 (d, 8.7 Hz, 1H, H-5), 7.95 (d, 8.5 Hz, 1H, H-11), 7.75 (dd, 8.5 and 7.8 Hz, 1H, H-10), 7.36 (d, 7.8 Hz, 1H, H-9), 7.24 (s, 1H, H-4), 7.14 (s, 1H, H-2), 4.08 (s, 3H, OMe), 2.49 (s, 3H, CH_3).

^{13}C (125 MHz, $CDCl_3$) d = 190.77 (C-12), 182.25 (C-7), 159.51 (C-8), 154.98 (C-1), 141.16 (C-3), 138.33 (C-12a), 137.58 (C-5), 137.28 (C-11a), 136.69 (C-6a), 135.30 (C-10), 130.67

(C-4a), 122.76 (C-6), 121.16 (C-4), 120.98 (C-11), 119.82 (C-7a), 119.71 (C-2), 119.12 (C-12b), 118.15 (C-9), 56.62 (OMe), 21.24 (CH_3).



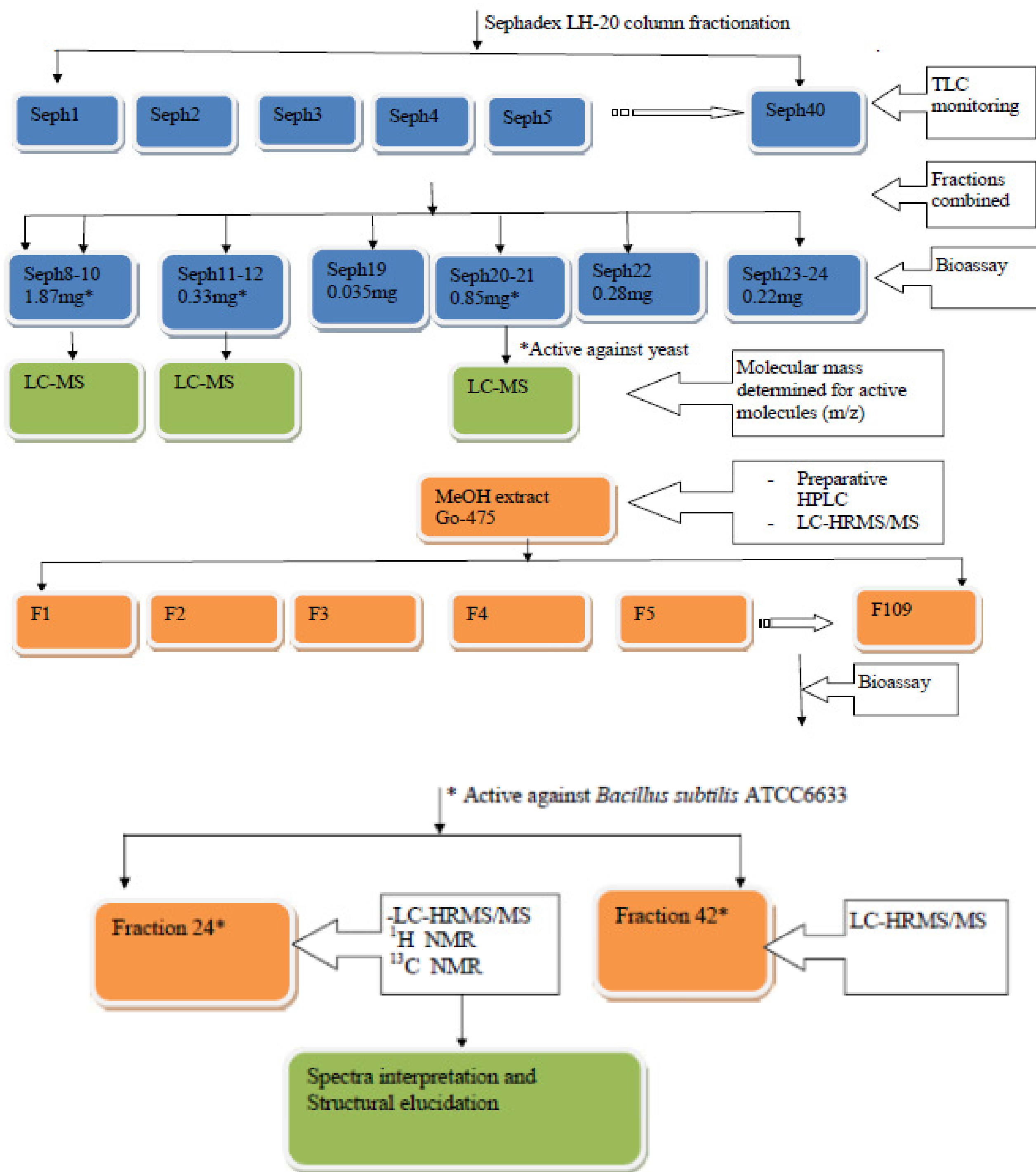


Figure 6.1. Summarized flow scheme for fractionation, purification and structural elucidation of antimicrobial compounds from Streptomyces sp. Go-475

6.3.9. Genome sequencing and analyses for Streptomyces sp. Go-475

Genome analyses for *Streptomyces* sp. Go-475 was conducted at the Center for Biotechnology, Bielefeld University, Germany. Briefly, for sequencing of the genome of isolate Go-475, 1µg of chromosomal DNA was used for generation of a shotgun library (TruSeq PCR-free sequencing library, Illumina Inc.) that was sequenced applying the 2x 300bp sequencing protocol on an Illumina MiSeq system as described previously (Zimmermann *et al*., 2016). The data were assembled using Newbler v.2.8 (Roche), resulting in 423 scaffolds containing 492 contigs. Subsequently, 2µg of the Go-475 genomic DNA was used for generation of a second shotgun library for sequencing on the MinION system (Oxford Nanopore Technologies). Size-selected DNA-fragments of 6 to 50 kb were used to create a 1D^2 sequencing library according to the manufacturer's instructions. Subsequently, the sequencing mix was added to a R9.5 Flowcell for a 24 hours run on the MinION Sequencer. Base calling and data conversion was performed in parallel using Albacore v1.2.4 (Oxford Nanopore Technologies). The sequences were exported in FASTQ format and used for an assembly with CANU v1.5 (Koren *et al*., 2017). After assembly, the resulting 4 contigs were polished with the short Illumina reads using PILON (Walker *et al*., 2014). The final assembly was done manually using CONSED (Gordon and Green, 2013) to combine the contigs of the Newbler and CANU assemblies. Gene prediction and annotation of the finished genome were performed using the program PROKKA (Seemann, 2014). The complete genome sequence was deposited at DDBJ/ENA/GenBank under the accessions no. CP026121. Analysis of the genome for secondary metabolite biosynthesis gene clusters was performed using online version of antiSMASH 4.02 (Blin *et al*., 2017), followed by manual curation.

6.4. Results

6.4.1. Culture of *Streptomyces* sp. Go-475

Culture used in this study was the isolate *Streptomyces* sp. Go-475; colony color and appearance of the culture on starch casein agar was as shown in the Figure 6.2A and 6.2 B.

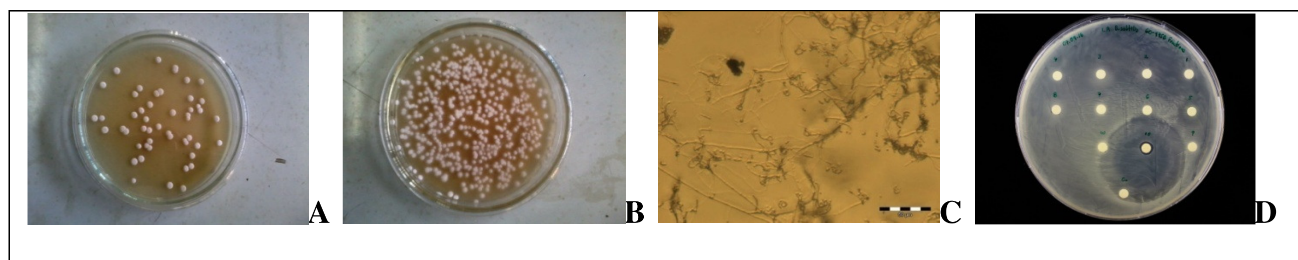


Figure 6.2. Colony appearance on starch casein agar, micro-morphology and bioactivity profile of Streptomyces sp. Go-475 A) from 10^{-3} dilution B) from 10^{-2} and C) micro-morphology (bar = 50µm), D) bioactivity profile against *Bacillus subtilis* using disc diffusion assay

It has been observed that ethyl acetate and methanol extracts showed interesting bioactivities considerably against yeasts and gram positive bacterial pathogens. The minimum inhibitory concentration (MIC) values of the crude extract from *Streptomyces* sp. Go-475 were determined for *Candida albicans* ATCC62376 and *Bacillus subtilis* ATCC6633. Hence, the crude extract from *Streptomyces* sp. Go-475 at concentrations (100µg/mL and 50µg/mL) found to be the MIC for *Candia albicans* and *Bacillus subtilis* respectively.

6.4.2. TLC analysis profile of the extract

During TLC analysis (Figure 6.3) the extract revealed a number of bands with different retention factors (Rf) in the different solvent systems. For instance, with the solvent system Chloroform: Methanol (9:1) bands with Rf values of 0.25, 0.42, 0.57, 0.71, 0.87 and 0.95 were observed.

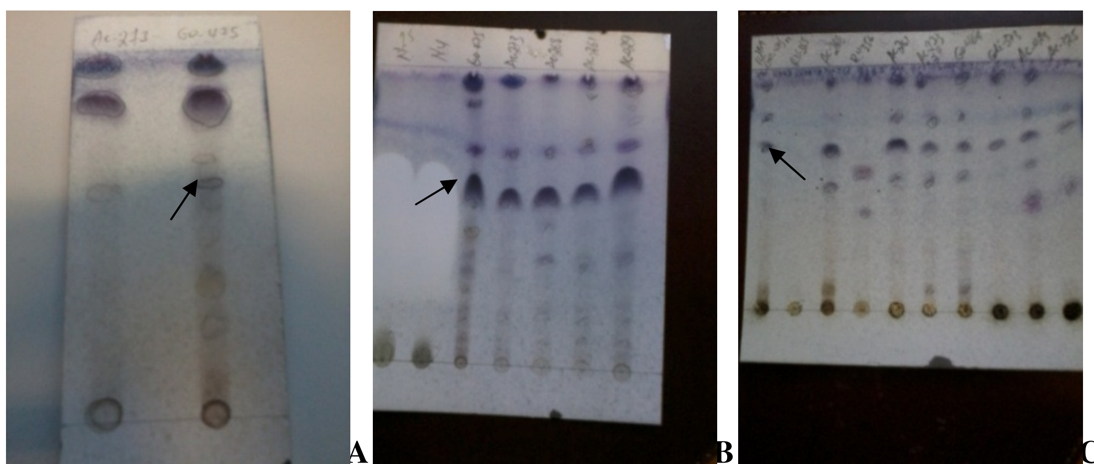


Figure 6.3. TLC analysis showing different bands in three different TLC systems (A) TLC system 1 Dichloromethane: Methanol: Water (2: 3:1), (B) TLC system 2, Ethylacetate: Propanol: water (5:5:1, v/v/v), (C) TLC System 3, Dichloromethane: Methanol (9:1, v/v)

6.4.3. Analytical HPLC profiles of the crude extract

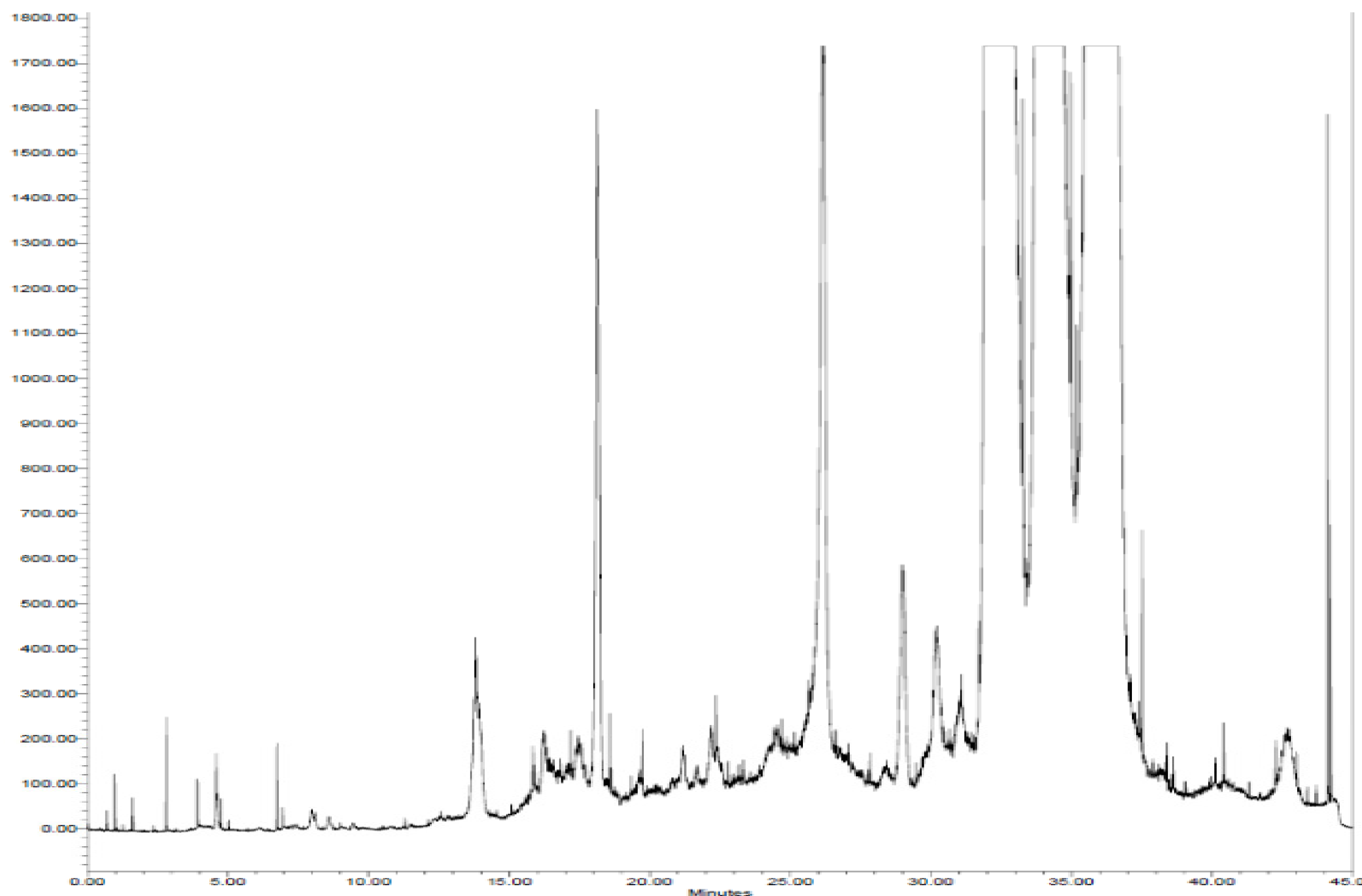
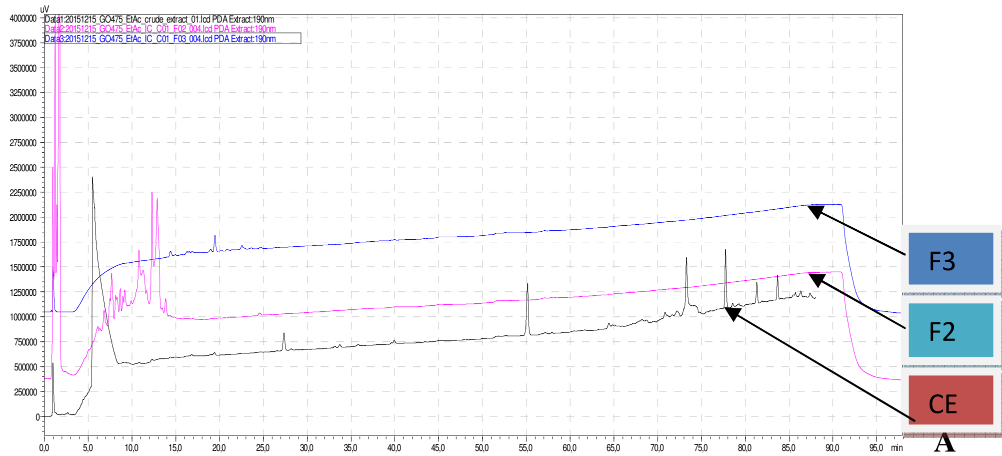
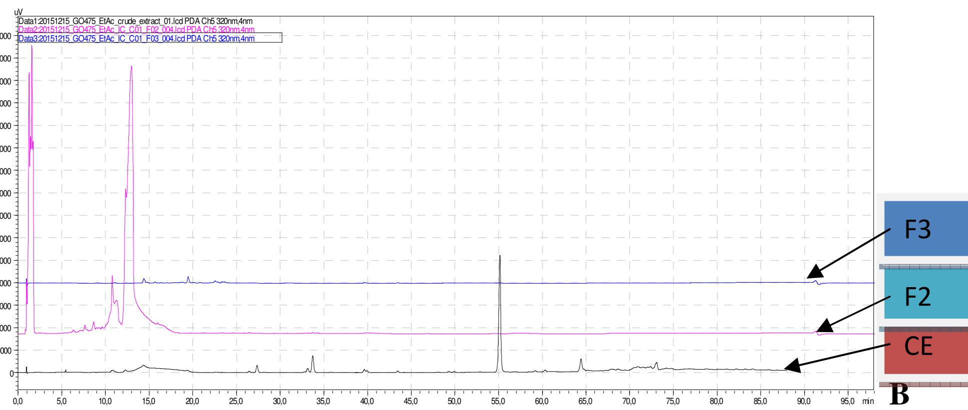


Figure 6.4. HPLC chromatogram profile and significant peaks of the crude extract from *Streptomyces* sp. Go-475 analyzed by H-Class UPLC using ELSD detector

Analytical HPLC profile of the crude extract from *Streptomyces* sp. Go-475 is presented in the Figure 6.4 and Figure 6.5. Significant peaks in different fractions compared with the crude extract are presented in Figure 6.5 at 254nm (A), 190nm (B) and 320nm (C) respectively.





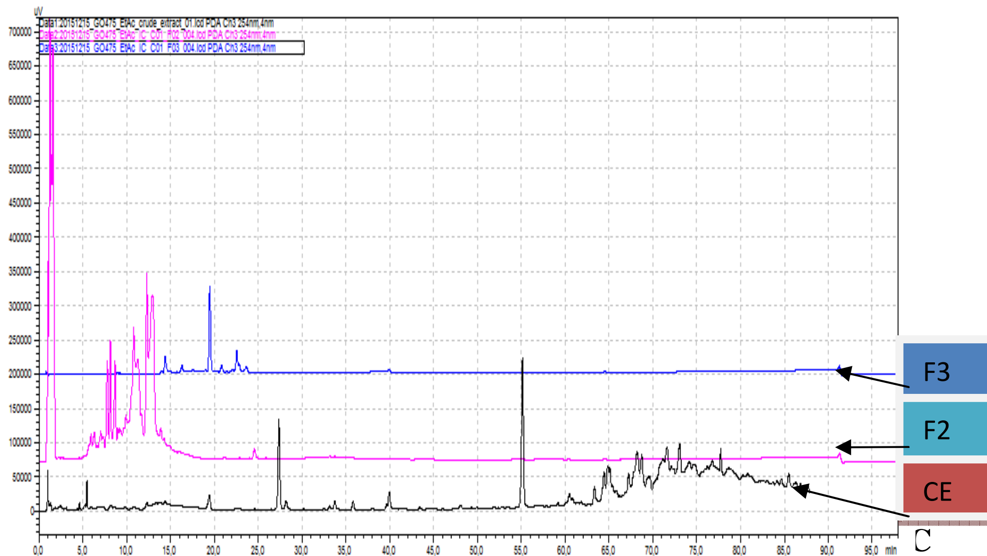


Figure 6.5. Analytical HPLC Chromatogram profiles of the two active fractions (F2 and F3) and that of the crude extract (CE) of *Streptomyces* sp. Go-475 at wave lengths of, 254nm (A), 190nm (B) and 320nm (C) using analytical HPLC, SHIMADZU

6.4.4. Analytical LC-MS profiles of methanol extracts

Since the extracts from *Streptomyces* sp. Go-475 displayed high anti-microbial activities, which differed depending on the mode of fermentation (solid or liquid media), we decided to investigate the corresponding extracts using high-resolution LC-MS. The accurate masses and predicted chemical formulae were queried against the Dictionary of Natural Products (DNP, contains data on ca. 250.000 natural products). The results of this analysis are presented in Table 6.2 and Table 6.3. Both in the SmF and SSF, isolate Go-475 produced betaine (known osmotic stress protectant), derivatives of tetrangulol antibiotics (Kuntsmann and Mitscher, 1966), which are responsible for the activities against Gram positive bacteria. Production of the known secondary metabolite 8-O methyltetrangomycin (Shigihara *et al*., 1988) could only be detected in the SmF extract. However, previous studies showed non-enzymatic conversion of 8-O-methyltetrangomycin (**1**) to 8-O-methyltetrangulol (**2**) (Figure 6.11) under acidic conditions, so that this finding does not necessarily reflect differences in the biosynthesis (Grabley *et al*., 1991).

During SSF, strain Go-475 was able to produce at least three known secondary metabolites that were not detected in the SmF extract (Table 6.2). In addition to the tentatively identified compounds, extracts from both SmF and SSF contained a number of substances that could not be identified using DNP query (data not shown). Notably, there were many more such compounds in the SSF extract compared to those in the SmF extract. Neither of the elucidated compounds could be clearly associated with strong antifungal activities shown for this extract, suggesting that a novel compound might be responsible for antifungal bioactivity exhibited by *Streptomyces* sp. Go-475.

Table 6.2. Compounds identified by high-resolution LC-MS in the crude extracts of *Streptomyces* sp. Go-475 after SSF and SmF. Data presented in the order of the retention time in LC. Compounds in bold font have been confirmed by NMR-assisted structure elucidation.

No	Accurate mass, Da	Compound ID	Type of fermentation	
			SmF	SSF
1	117.0794	Betaine	+	+
2	336.1008	**8-O-Methyltetrangomycin**	+	-
3	164.0474	4-Methoxy-1(3H)-isobenzofuranone	-	+
4	150.0682	3-Phenylpropionic acid or 1,2-Benzenediol; 2-Propenyl ether	-	+
5	318.0897	**8-O-Methyltetrangulol**	+	+
6	292.1674	Dehydrocineromycin B	-	+

Table 6.3. Proposed structure, retention time, HR-MS data and predicted sum formulae of the compounds tentatively identified in the extracts from *Streptomyces* sp. Go-475 cultivated in liquid and solid media using high-resolution LC-MS.

#	**Proposed structure**	**R_t [min]**	**$[M+H]^+$**		**Δm/z [ppm]**	**MW exp. [Da]**	**Predicted sum formula**
			m/z exp.	*m/z* calc.			
1	Betaine	2.6	118.0867	118.0863	3.8	117.0794	$C_5H_{11}NO_2$
2	**8-O-Methyltetrangomycin**	4.0	337.1081	337.1071	3.2	336.1008	$C_{20}H_{16}O_5$
3	4-Methoxy-1(3H)-isobenzofuranone	4.9	165.0547	165.0546	0.5	164.0474	$C_9H_8O_3$
4	3-Phenylpropionic acid or 1,2-Benzenediol; 2-Propenyl ether	6.8	151.0755	151.0754	0.8	150.0682	$C_9H_{10}O_2$
5	**8-O-Methyltetrangulol**	13.7	319.0970	319.0965	1.7	318.0897	$C_{20}H_{14}O_4$
6	Dehydrocineromycin B	22.3	293.1747	293.1747	-0.3	292.1674	$C_{17}H_{24}O_4$

6.4.5. Identification and structure elucidation of the anti-bacterial compounds

Sample : GO475_ETAC	Column : PURIFLASH COLUMN 15 C18 HQ - 35.0 g (22 bar)
Solvent A : H2O	Solvent C :
Solvent B : MeOH	Solvent D :
Channel 1 : UV600:SCAN	Channel 2 : ELSD
Equil+Inject Mode : Simple+Loop+Wait	Stop Mode : Pause
RUN Time : 11/25/2015 2:31:12 PM	

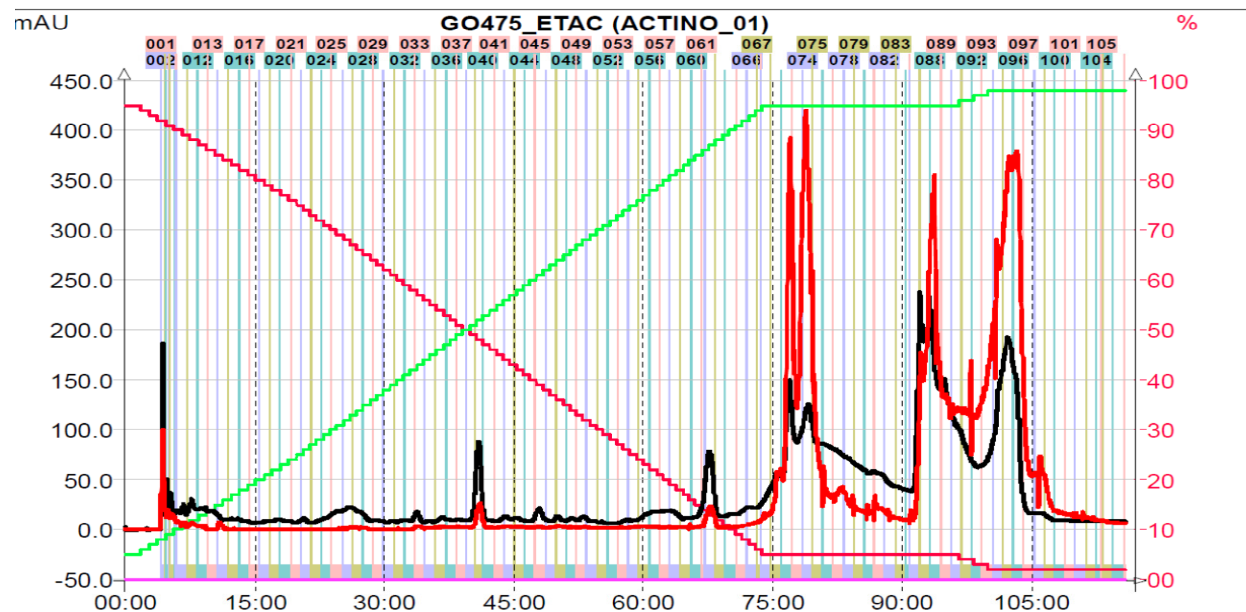


Figure 6.6. Chromatogram during the fractionation and separation of the crude extract by preparative HPLC using PuriFlash 420, Interchim,

The methanolic SmF extract of strain Go-475 showing pronounced activity against *B. subtilis* was fractionated into 109 fractions (Figure 6.6) using Flash chromatography (PuriFlash 420). Fractions 24 (Rt 27.41-28.53 min) and 42 (Rt 49.20-50.32 min) were found to be responsible for the bioactivity against *B. subtilis*. These fractions were analyzed using HPLC-CAD/MS and high resolution LC-MS as described by Ladurner *et al*. (2017). Fraction 42 contained several similarly abundant compounds, which were tentatively identified as branched fatty acids and not further analyzed. In fraction 24, on the other hand, only one major constituent was detected.

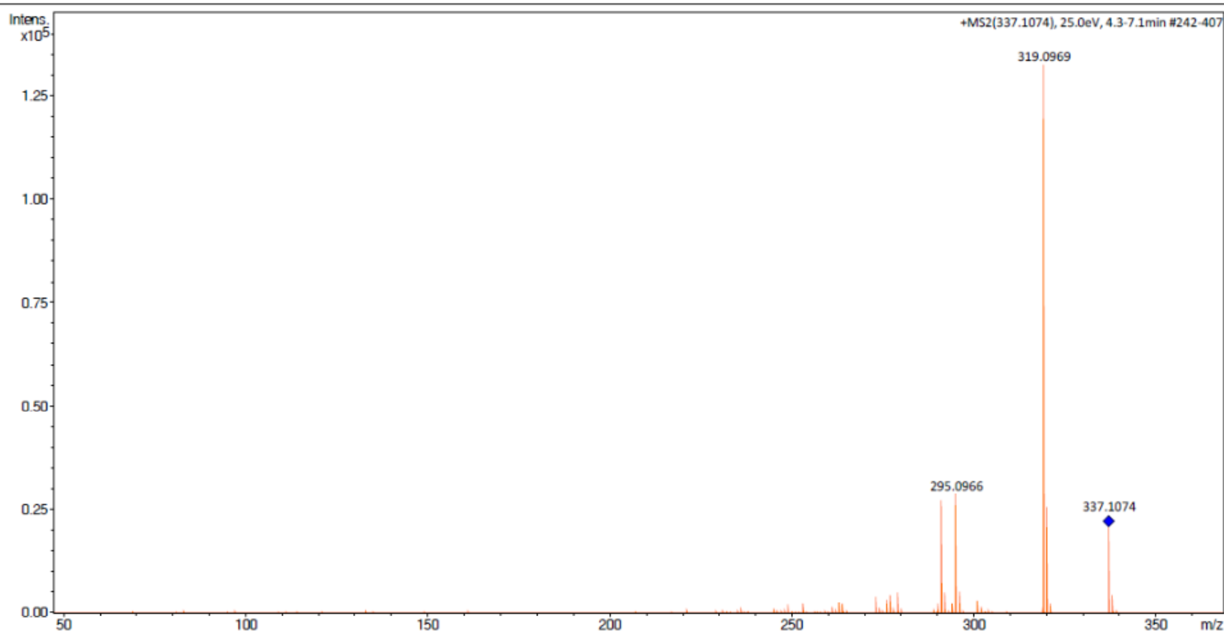


Figure 6.7. MS/MS spectrum of the [M+H]+ ion of 8-O-Methyltetrangomycin (6-deoxy-8-O-methylrabelomycin) at *m/z* 337.1074 obtained on an ESI-Qq-TOF mass spectrometer with a collision energy of 25 eV.

High-resolution ESI-Qq-TOF-MS spectra, obtained by direct infusion of fraction 24, showed that the main compound was $[M+H]^+$ ion at *m/z* 337.1073 (calculated for $C_{20}H_{17}O_5^+$, *m/z* 337.1071, Δ = 0.8ppm) and as $[M+Na]^+$ ion at *m/z* 359.0891 (calculated for $C_{20}H_{16}O_5Na^+$, *m/z* 359.0890, Δ = 0.2 ppm). The HR-MS/MS-spectra of the $[M+H]^+$ ion were not very informative, which is typical for very stable aromatic systems like angucycline antibiotics. However, the abundant loss of H_2O indicated an aliphatic hydroxyl-group (see Figures 6.7 and Figure 6.8). All these data fit nicely to the known compound 8-O-methyltetrangomycin ($C_{20}H_{16}O_5$), but due to the lack of a

reference standard or published MS/MS spectra, this preliminary identification could not be confirmed without further structure elucidation by 1D and 2D NMR spectroscopy.

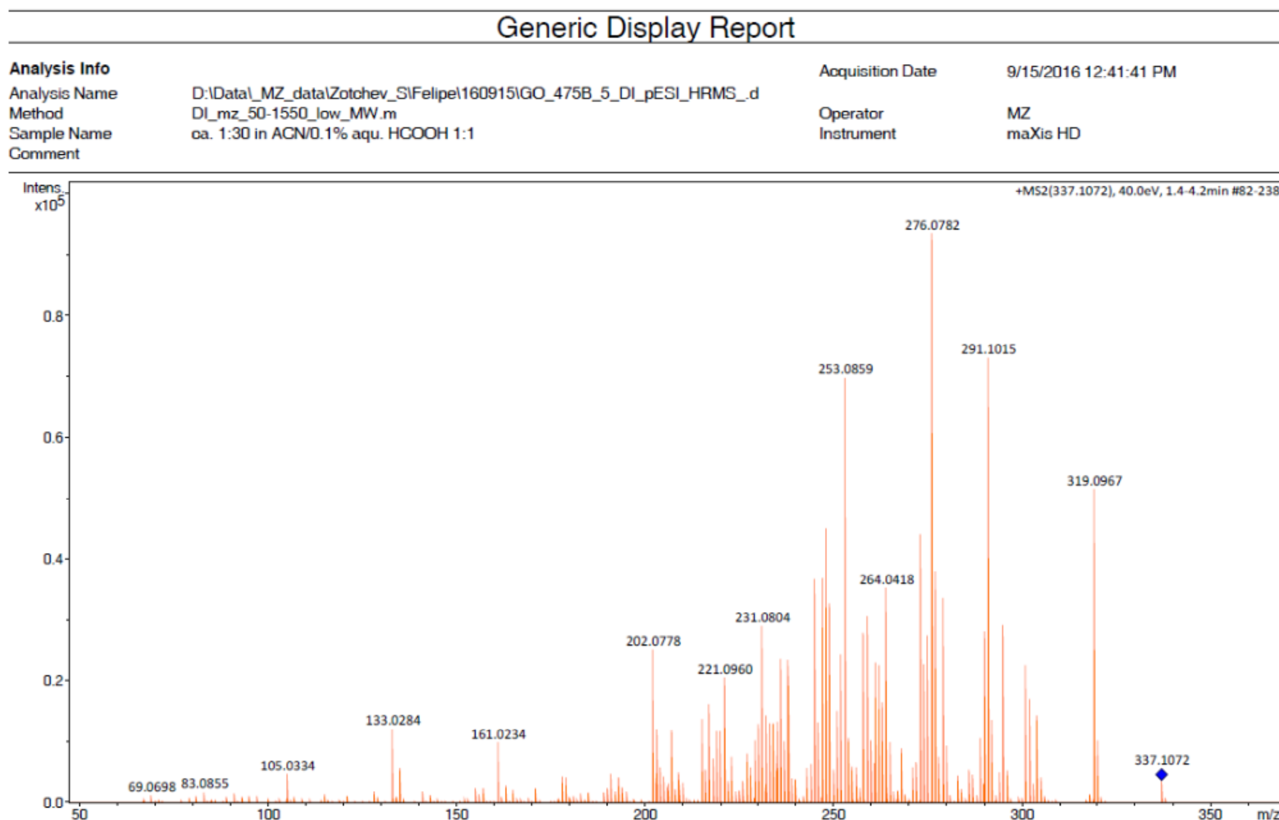


Figure 6.8. MS/MS spectrum of the [M+H]+ ion of 8-O-Methyltetrangomycin (6-deoxy-8-O-methylrabelomycin) at *m/z* 337.1074 obtained on an ESI-Qq-TOF mass spectrometer with a collision energy of 40 eV**.**

When the NMR analysis was executed in deuteromethanol, we obtained 8-O-methyltetrangomycin (**1**) as the result, while measuring the sample in deuterochloroform indicated a complete conversion to 8-O-methyltetrangulol (**2**) (Figure 6.9). Obviously, the conversion of **1** to **2** was catalyzed by traces of hydrocloric acid present in deuterochloroform. Both compounds showed typical signals of a methoxyanthraquinone system. The different

oxidation state of the six-membered ring was indicated in 8-O-methyltetrangomycin by the two methylene carbons C-2 and C-4, while in 8-O-methyltetrangulol two olefinc methine signals were detected for C-2 and C-4. Assignment of the signals was confirmed by COSY, HSQC and HMBC measurements. Comparison of the spectral data of **1** and **2** with literature values confirmed the structure elucidation (Kesenheimer and Groth, 2006; Ding *et al*., 2009).

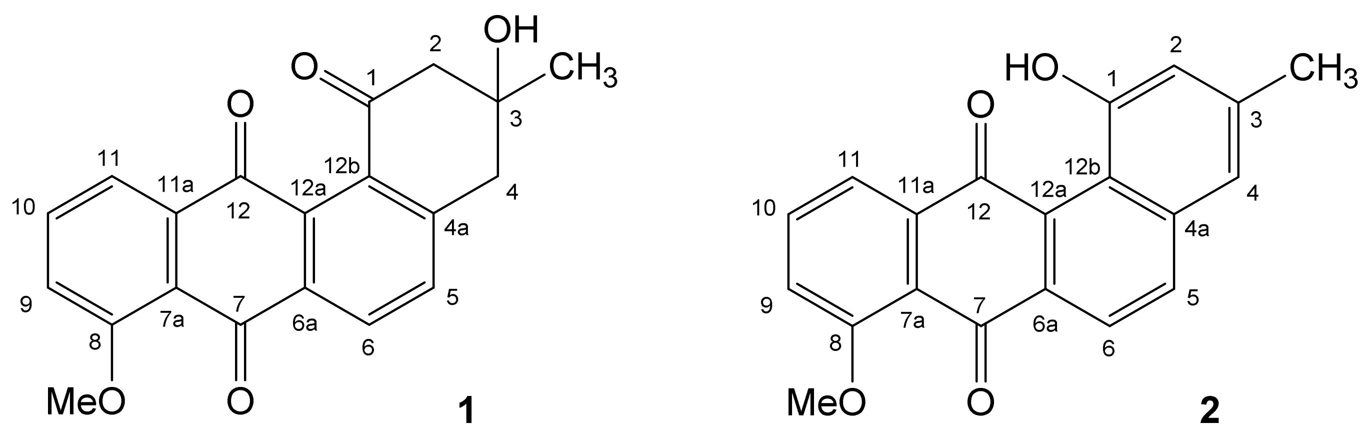


Figure 6.9. Structures of the antibacterial compounds 8-O-methyltetrangomycin (**1**) and 8-O-methyltetrangulol (**2**) produced by Streptomyces sp. Go-475

6.4.6. *Streptomyces* sp. Go-475 genome analyses

The genome of *Streptomyces* sp. Go-475 was completely sequenced (see Materials and Methods for details), revealing a linear chromosome of 8,570,609 bp, featuring a G+C content of 71.96%, contains 7,571 predicted coding sequences, 83 t(m)RNA genes, and six *rrn* operons. In addition, a total of 19 RNA features (5'-UTRs, riboswitches, ncRNAs, etc.) were identified based on hits against RFAM. The inverted terminal repeats of the chromosome were found to be rather short, with a length of just 6,902 bp, which is unusual for *Streptomyces*.

The genome sequence of *Streptomyces* sp. Go-475 shows significant sequence similarity to that of *Streptomyces olindensis* DAUFPE 5622 (GenBank accession number JJOH00000000), which is ca 0.8Mb larger. Apparently, the core genomes of these two strains are very similar; with the ANI (Average Nucleotide Identity) of 94.56% over a length of 6,649,500 bp. Synteny plot of the two genomes (Appndix B9) clearly demonstrates close relatedness of *Streptomyces* sp. Go-475 and *S. olindensis*, despite the genome of the latter being rather fragmented (draft). This fact raises the issue of biogeography, since these strains were isolated on two different continents separated by a vast expanse of water.

Secondary metabolite biosynthesis gene clusters (BGCs) were identified in the genome of *Streptomyces* sp. Go-475 using antiSMASH 4.02 followed by manual curation to split apparently disparate BGCs suggested by the software as being single ones. In total, 36 BGCs were identified (Table 6.4), 27 of which were also present in the genome of *Streptomyces olindensis* DAUFPE 5622 (Rojas *et al*., 2014). The latter streptomycete was isolated in 1960s from a soil sample collected in Brazil, and was shown to produce cytotoxic glycosylated anthracyclines named cosmomycins. The BGC for cosmomycin could be readily identified in the genome of Go-475 (cluster 23, Table 6.4). Cluster 9, of the ectoine type, most likely is responsible for the biosynthesis of ectoine and, possibly, hydroxyectoine and betaine. Beside this, BGCs for geosmin (cluster 28) and the anthraquinones (cluster 31) were also identified. The latter cluster is apparently absent in the *S. olindensis* genome, and contains genes encoding polyketide synthases type II (PKSII) along with some modification enzymes.

Table 6.4. Secondary metabolite biosynthesis gene clusters identified in the genome of *Streptomyces* sp. Go-475 with antiSMASH 4.02 (manually curated).

No	Cluster type	Presence in another bacterium	Putative product
1	PKSI-NRPS	*Streptomyces olindensis* DAUFPE 5622	Glycosylated PK-NRS peptide hybrid
2	Melanin	Multiple *Streptomyces* spp.	Melanin
3	Nrps	*Streptomyces olindensis* DAUFPE 5622	NRS peptide
4	Unknown	*Streptomyces olindensis* DAUFPE 5622	Amino acid-based product
5	Terpene	*Streptomyces olindensis* DAUFPE 5622	Putative sesquiterpene
6	Lantipeptide*	*Streptomyces viridochromogenes* DSM 40736	SapB/AmfS family lantipeptide
7	PKSIII*	*Streptomyces* sp. XY006	1,3,6,8-tetrahydroxynaphthalene
8	Lassopeptide	*Streptomyces olindensis* DAUFPE 5622	Lassopeptide
9	Ectoine	*Streptomyces olindensis* DAUFPE 5622	**Ectoine, betaine**
10	Terpene	*Streptomyces olindensis* DAUFPE 5622	Putative isoprenoid
11	PKSI-NRPS*	*Streptomyces chartreusis* NRRL 12338	PK-NRS peptide hybrid
12	NRPS	*Streptomyces olindensis* DAUFPE 5622	Glycosylated NRS peptide
13	Terpene	*Streptomyces olindensis* DAUFPE 5622	Isoprenoid
14	Melanin	*Streptomyces* sp. S10(2016)	Melanin
15	Lassopeptide*	*Streptomyces chartreusis* NRRL 12338	Lassopeptide
16	Siderophore	Multiple *Streptomyces* spp.	**Desferrioxamine B**
17	Butyrolactone	*Streptomyces olindensis* DAUFPE 5622	Butyrolactone
18	PKSI-NRPS	*Streptomyces olindensis* DAUFPE 5622	PK-NRS peptide hybrid

19	Phosphonate	*Streptomyces olindensis* DAUFPE 5622	Phosphonate metabolite
20	Terpene*	-	Terpenoid
21	Aminocyclitol	*Streptomyces olindensis* DAUFPE 5622	Aminocyclitol
22	NRPS*	*Streptomyces vitaminophilus* DSM 41686	NRS peptide
23	PKSII	*Streptomyces olindensis* DAUFPE 5622	**Cosmomycins**
24	Terpene	*Streptomyces olindensis* DAUFPE 5622	**Albaflavenone**
25	Terpene*	-	Terpenoid (polyprenyl)
26	Siderophore	*Streptomyces olindensis* DAUFPE 5622	Siderophore
27	Bacteriocin	*Streptomyces olindensis* DAUFPE 5622	Bacteriocin
28	Terpene	Multiple *Streptomyces* spp.	**Geosmin**
29	PKSI-NRPS*	*Streptomyces africanus*DSM 41829	PK-NRS peptide hybrid
30	Siderophore	*Streptomyces olindensis* DAUFPE 5622	Siderophore
31	PKSII*	-	**Anthraquinones**
32	Terpene	*Streptomyces olindensis* DAUFPE 5622	Hopanoids
33	PKSI	*Streptomyces olindensis* DAUFPE 5622	PK
34	Bacteriocin	Multiple *Streptomyces* spp.	Bacteriocin
35	Lantipeptide	Multiple *Streptomyces* spp.	SapB/AmfS family lantipeptide
36	PKSI	*Streptomyces olindensis* DAUFPE 5622	PK

Note: NRS – non-ribosomally synthesized; PK - polyketide. * show gene clusters not present in the genome of *S. olindensis* DAUPFE 5622. Products in bold text are inferred with certainty based on >90% identity of the gene products to those of the verified gene clusters or experimental data (e.g. anthraquinones)

Interestingly, although the BGCs for both anthraquinones were previously identified (Hong *et al*., 1997), their products showed less than 80% homology to the corresponding enzymes encoded by cluster 31. This cluster also contains some unique genes not found in other anthraquinone BGCs, suggesting that 8-O-methyltetrangomycin and 8-O-methyltetrangulol could be mere precursors for more complex novel compounds.

The genome of isolate Go-475 harbors several BGCs, homologues of which could not be identified in other genomes available in the public databases. These include the above mentioned anthraquinone BGC, as well as clusters 20 and 25 containing terpene synthase genes. Terpene synthase encoded by cluster 20 is highly unusual in that it contains N-terminal carboxypeptidase regulatory-like domain, while its terpene cyclase domain shares only 76% homology with the best database hit. This BGC also encodes a putative trans-isoprenyl diphosphate synthase, which may provide precursor for the cognate terpene cyclase. Cluster 25 contains genes encoding polyprenyl synthase (only 67% identity to the closest match in the database), IspH homologue (last enzyme in isopentenyl pyrophosphate biosynthesis), methyltransferase and prenyltransferase. It is not clear at the moment, what kind of terpenoid is specified by this BGC.

6.5. Discussion

The findings showed that methanol and ethyl acetate extracts of *Streptomyces* sp. Go-475 exhibited wider spectrums of bioactivities. This suggests that the bioactive components in the extracts are broad spectrum as reported in a number of studies like Lee *et al*. (2016), Shah *et al*. (2017). On the other hand, as indicated in the findings, *Streptomyces* sp. Go-475 have the

capacity to biosynthesize a number of bioactive compounds which are soluble in ethyl acetate and methanol as described in the report by Nett *et al*. (2009).

Minimum inhibitory concentration results were in consistent with Shigihara *et al*. (1988) who reported MIC values > 100µg/mL and > 50µg/mL for 8-O-methyltetrangomycin (**1**) and 8-O-methyltetrangulol (**2**) respectively against *Candida albicans* 3147. They also reported MIC values of 100 µg/mL and 12.5µg/mL respectively for the same compounds against *Bacillus subtilis* NRRLB-558. On the other hand, Grabley *et al*. (1991) reported 100µg/mL and 25µg/mL respectively for the above compounds against *Staphylococcus aureus*. As compared to those reported in the earlier studies (described above) MIC values were found to be larger in the current work (particularly for *Bacillus subtilis*). However, in this study MICs were determined using the crude extract and that might be the source of variations; because the crude extract consists of a number of compounds which resulted in larger MIC values.

Bioassay guided fractionation and analysis of the crude extracts resulted in the detection, identification and structural elucidation of two important antibiotics that belong to the member of benz[a]anthraquinone (Shigihara *et al*., 1988) from the isolate *Streptomyces* sp. Go-475. Both compounds, identified as 8-O-methyltetrangomycin (1) and 8-O-methyltetrangulol (2) were described earlier (Shigihara *et al*., 1988; Grabley *et al*., 1991). LC-MS comparison results showed that metabolite profiles of the extracts from (SSF and SmF) were quite different consistently, and we were able to identify both known and detect potentially unknown metabolites. Notably, considerably more presumably novel secondary metabolites were found in the SSF extract suggesting that this method of cultivation is better option in bioprospecting

efforts and to make use of the biotechnological potentials of *Streptomyces* particularily for antibiotic production.

In addition to 8-O-Methyltetrangomycin and 8-O-Methyltetrangulol, it was also possible to detect many other compounds in the extracts. Such a prominent production of a number of bioactive compounds by this isolate might be due to the large genome size of the isolate which consisting of 36 biosynthetic gene clusters committed for the biosynthesis of several bioactive secondary metabolites. Different authors (Ventura *et al*., 2007; Arasu *et al*., 2009; Nett *et al*., 2009) reported in similar fashion. The analysis of the active fractions also revealed the detection of a number of unknown compounds. These might be an indication that there might be a novel compounds in the bioactive compounds of *Streptomyces* sp. Go-475.

The result showed that, strain Go-475 was able to produce at least three known secondary metabolites in SSF that were not detected in the SmF extract. Most of these compounds were reported from *Streptomyces* spp., and had either antibacterial or cytotoxic activities. However, neither of those could be clearly associated with strong antifungal activity shown by the SSF extract, suggesting that a novel compound might be responsible for the antifungal activities exhibited by SSF extract from *Streptomyces* sp. Go-475. In this regard, the analysis showed that, extracts from both SmF and SSF contained a number of substances that could not be identified using DNP query. Particularly, there were many more such compounds in the SSF extract compared to those in the SmF extract. Although at least 30% of the unidentified compounds in the SSF extract are most likely to be represented by fatty acids and their derivatives, the rest may

represent new compounds that warrant further studies with regard to their origins, bioactivities, identification and structural elucidation.

In this regard, Barrios-González (2012) described the physiology and molecular aspects of SSF and noted clear difference related with higher metabolite yields in shorter periods. It has also been reported that some secondary metabolites (Pyrrocidines and Acremonidins) are produced only in SSF (Bigelis *et al.*, 2006). Furthermore, during Lovastatin production noticeable differences were observed in biosynthetic gene expression level between SSF and SmF (Barrios-Gonzale *et al.*, 2008). These all indicated that SSF method might be responsible for the production of novel products. Besides, it is an economical and best alternative that could be investigated easily for the biosynthesis of bioactive microbial natural products in the drug development processes.

Genome analysis of the isolate Go-475 revealed a linear chromosome, typical for streptomycetes. However, it is noteworthy, that terminal inverted repeats of the Go-475 chromosome are unusually short, ca 7 kb, but in other streptomycetes they range from 30 kb to 1 Mb (Chen *et al.*, 2002). This indicates the presence of distinctive features in the isolate's genome. At least 36 BGCs could be identified in the genome, some of which appear to be unique and may encode novel secondary metabolites. Even seemingly known gene clusters e.g. that for anthraquinone biosynthesis, contain unique additional genes, which may be implicated in modifications never seen before for this kind of molecules. Interestingly, the majority of the BGCs are present in a streptomycete isolated from Brazilian soil, raising interesting questions regarding distribution of a common ancestor of these bacteria.

6.6. Conclusion and Recommendation

The identification of the two important antibacterial antibiotics and detection of several known and unknown bioactive compounds from extracts of *Streptomyces* sp. Go-475 enables us to generalize that the isolate possess a number of secondary metabolite biosynthesis capacity. The results also allow us to conclude that *Streptomyces* sp. Go-475 has the potential to biosynthesize more than one bioactive secondary metabolites. The difference in metabolite profiles of the extracts from SSF and SmF suggests that *Streptomyces* sp. Go-475 use different biosynthesis mechanisms in SSF and SmF cultures. The more known and unknown secondary metabolites detected in the SSF extract than SmF, enable us to conclude that soil *Streptomyces* produce concentrated and wider array of bioactive secondary metabolites in SSF. This might suggest that conditions in SSF are more closely resemble to the isolates native environments as compared to the artificial SmF culture.

SSF method sould be exploited in the future bioprospecting works. Hence, further studies are recommended to utilize its potential for the production of antimicrobial compounds from actinomycetes. Therefore, pharmaceutical companies in Ethiopia have to invest on this important area in the future drug discovery efforts. Further studies must be carried out on SSF extracts from *Streptomyces* sp. Go-475, to describe the unknown bioactive compounds that showed strong antifungal activities. It is also vital to examine the anticancer effects of those bioactive metabolites. The high degree of similarity between the genomes of *Streptomyces* sp. Go-475 and a *Streptomyces olindensis* DAUFPE 5622 isolated in Brazil raises interesting questions about biogeographical distribution of these bacteria. Therefore, furthers studies are recommended to examine their evolutionary relationship and the biosynthesis capacities of these isolates.

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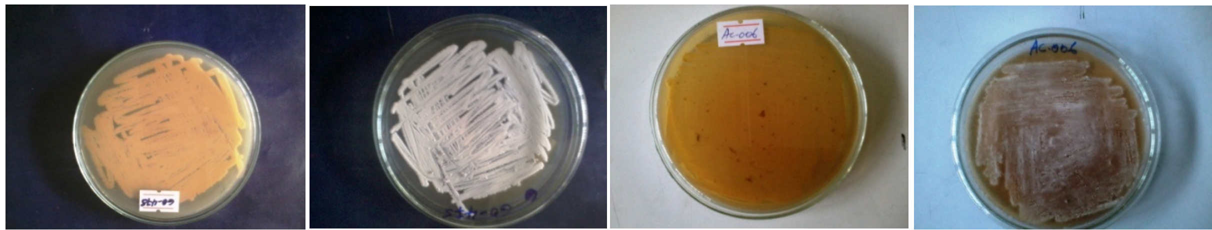
8. APPENDICES

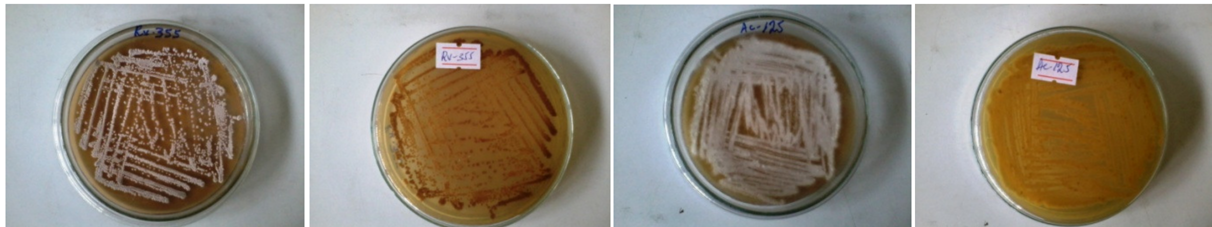
Appendix A1. One of the soil sampling sites (Menagesha Suba forest)

Appendix A2. Some of the isolates grown in starch casein agar

Go-466 Ac-464

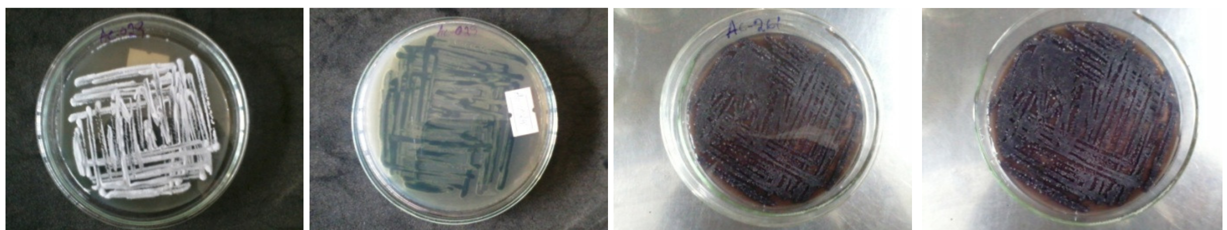


Go-475 Ac-006



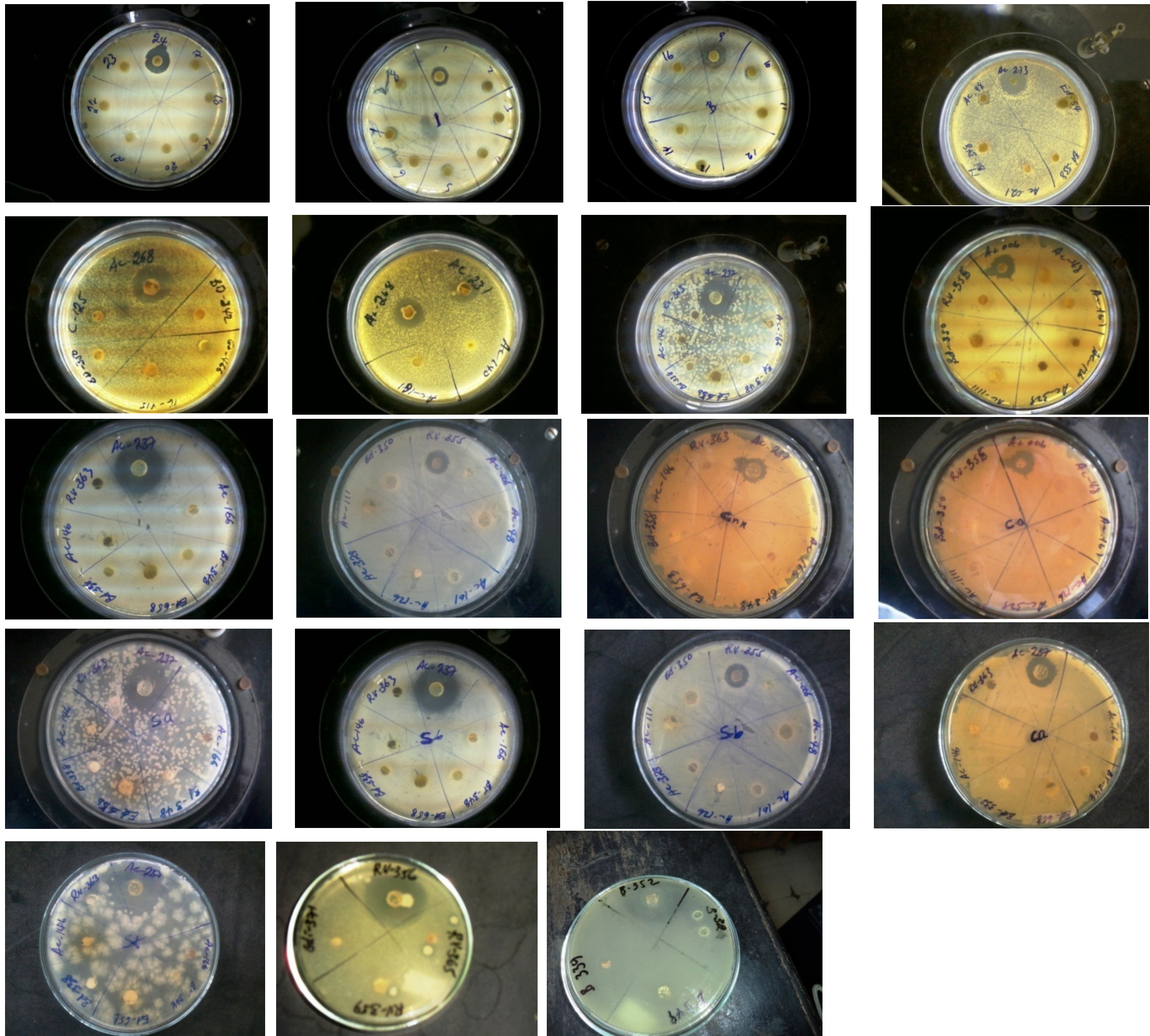
Rv-355 Ac-125

Ac-273 Ac-268



Ac029 Ac-261

Appendix A3. Bioactivity of some of the isolates in primary screening



Appendix A4. Some of the cultures grown in complex media, showing substrate and aerial mycelium

Ac-123 Go-475 Ac-006 Ed-065

Go-466 Ac-125 Rv-355 Ac-146

Ac-464

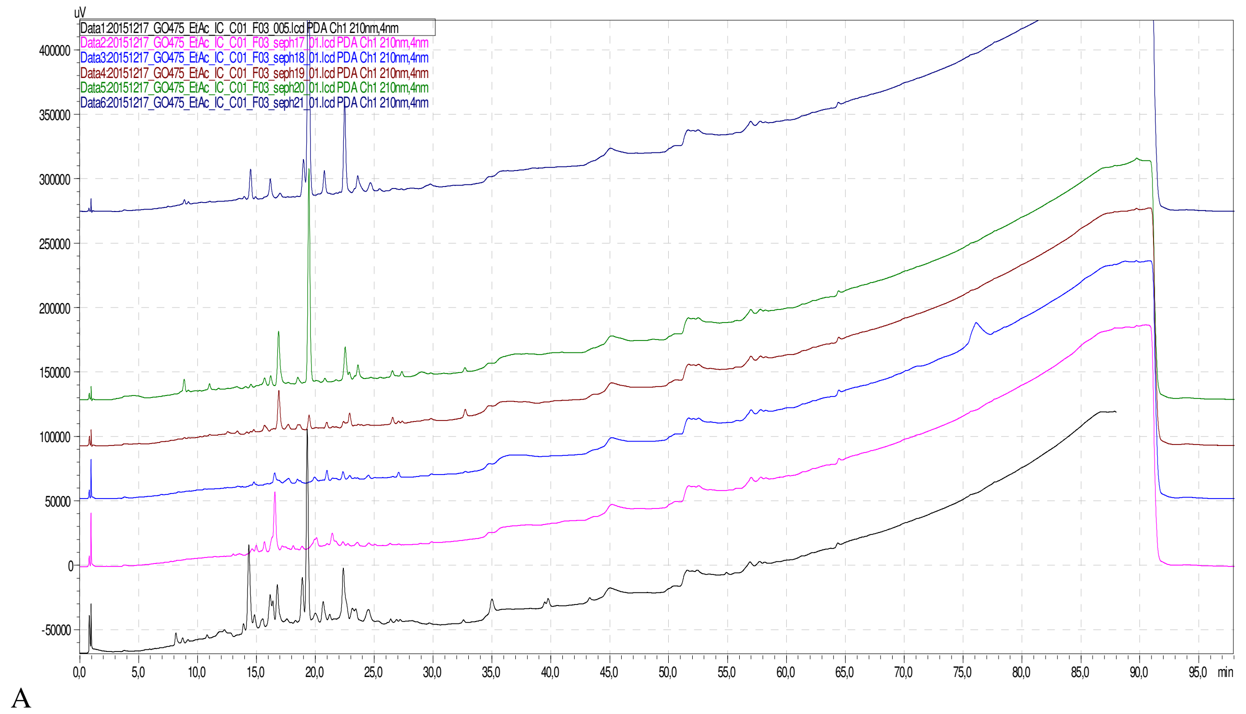
Appendix A5. Cultures in plates and slants partially

Appendix A6. Substrate preparation (A), inoculation (B), incubation (C) and product recovery (D)

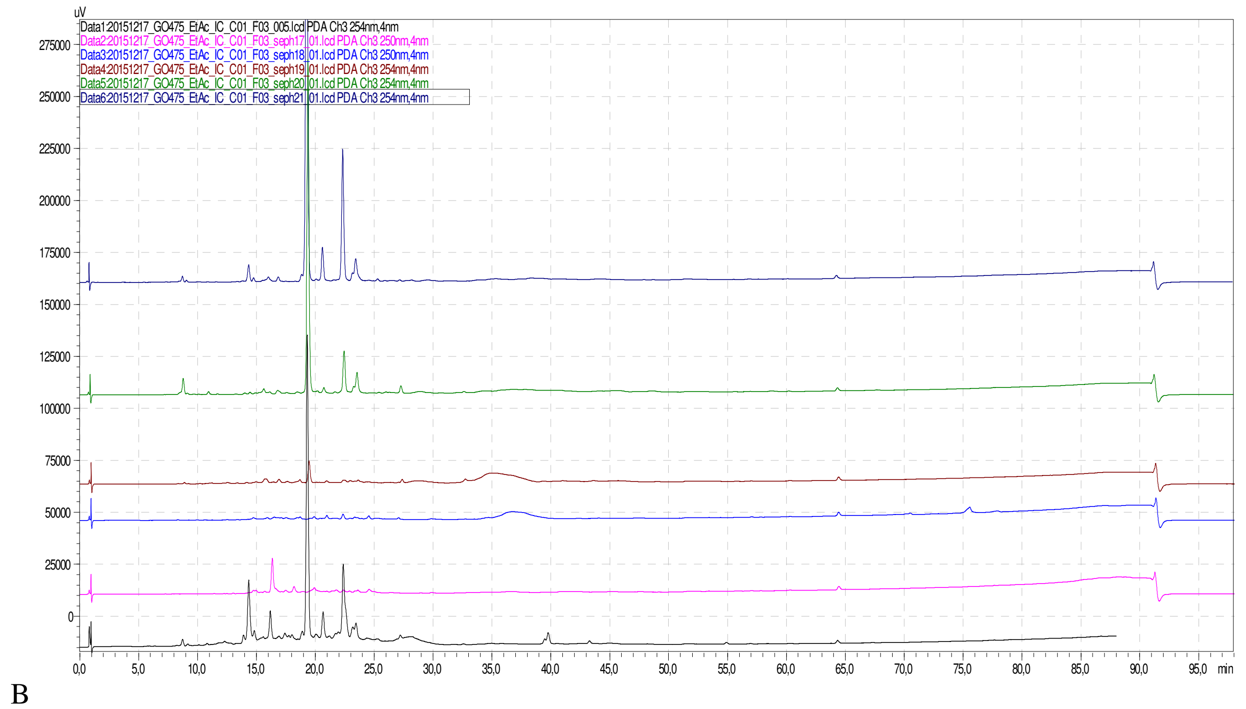
Appendix A 7. Some isolates fermented in SSF

Appendix A8. Extract collections partially

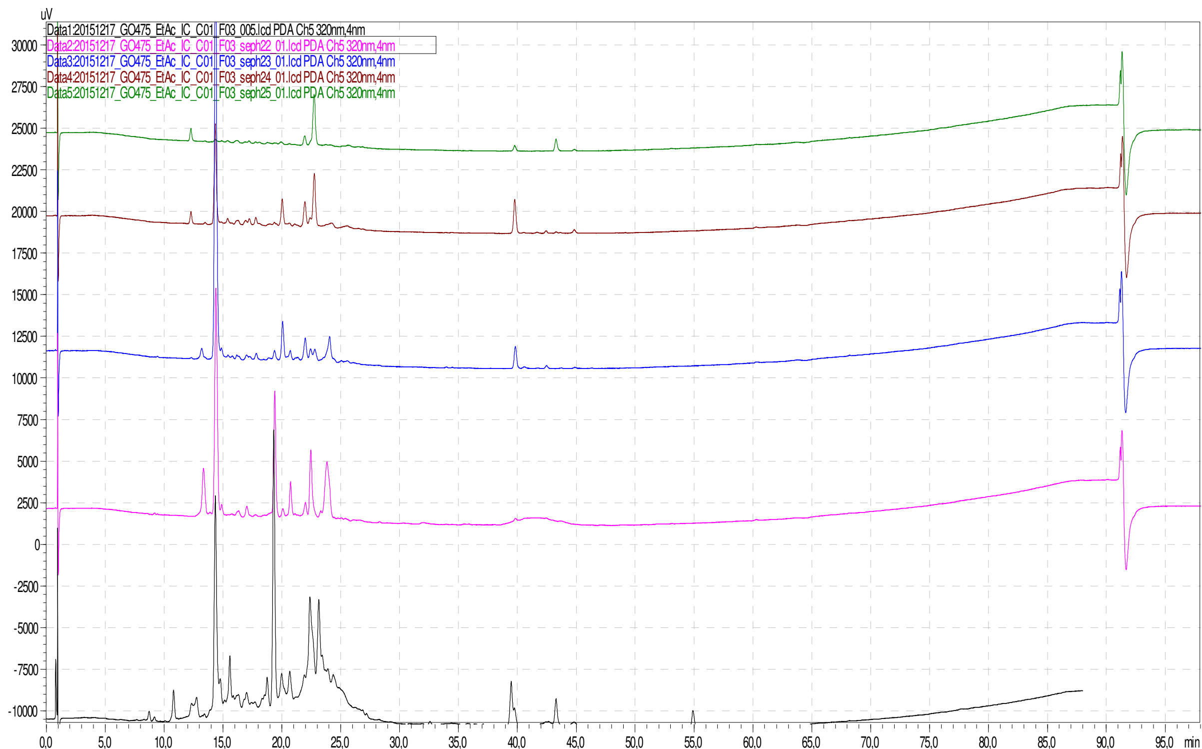
Appendix B1. Analytical HPLC chromatograms of *Streptomyces* sp. Go-475 at 210nm (A), 254nm (C) 320nm and 190nm (D)



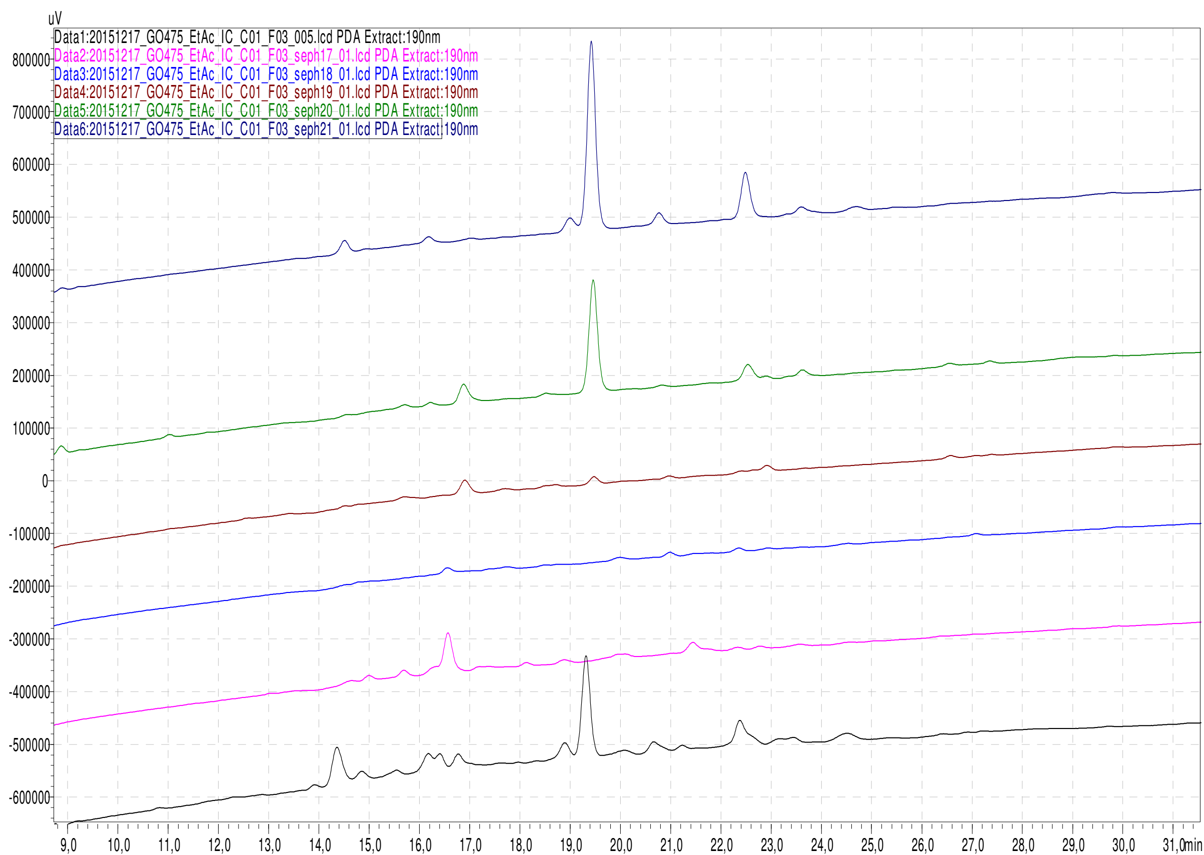
A



B

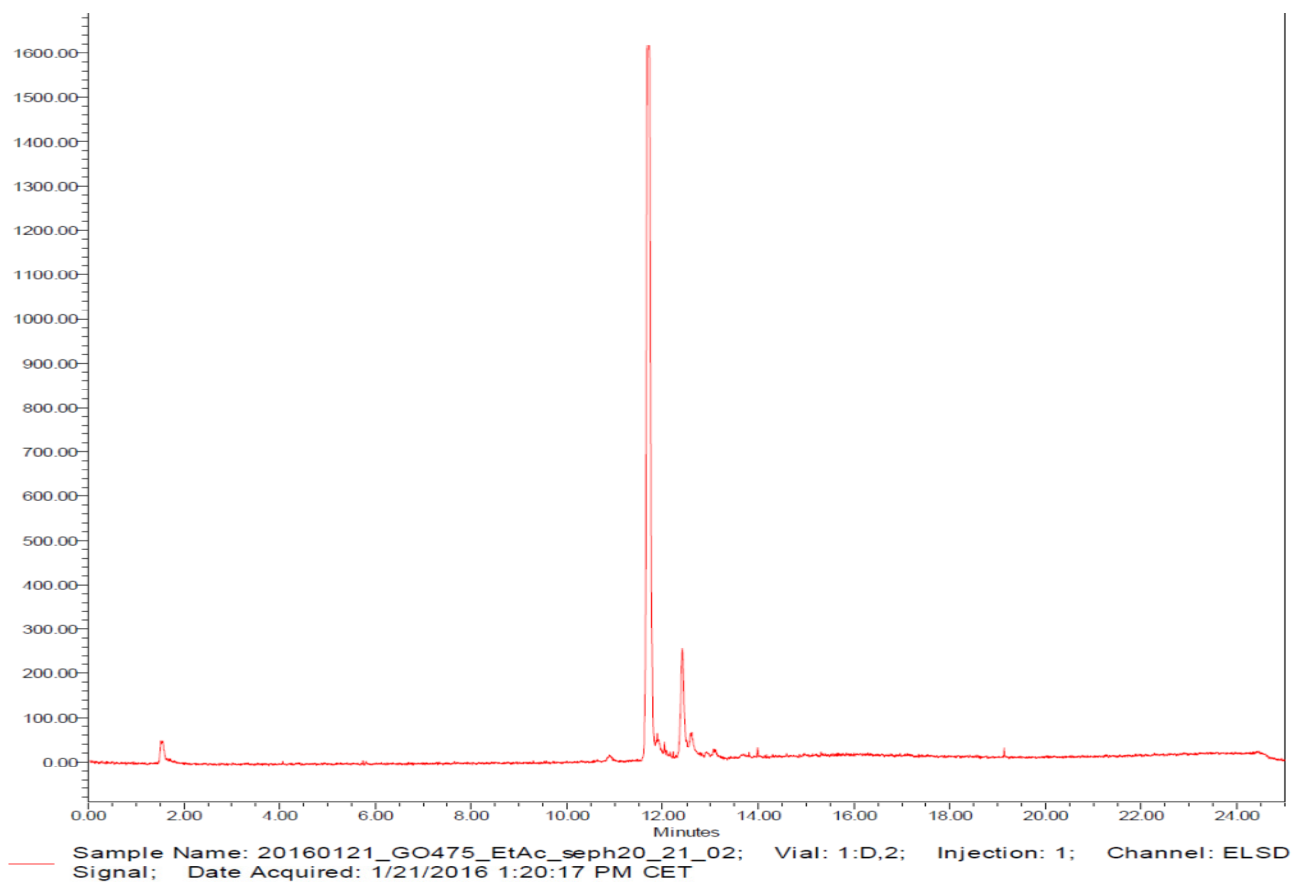


C

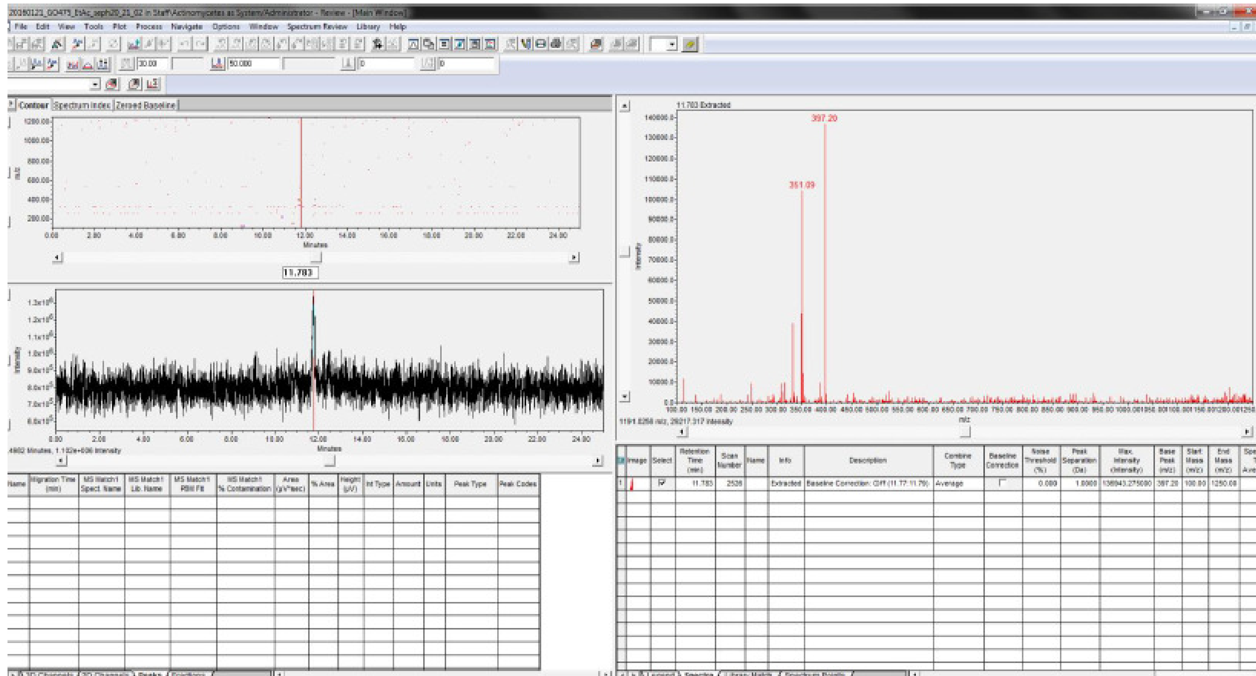


D

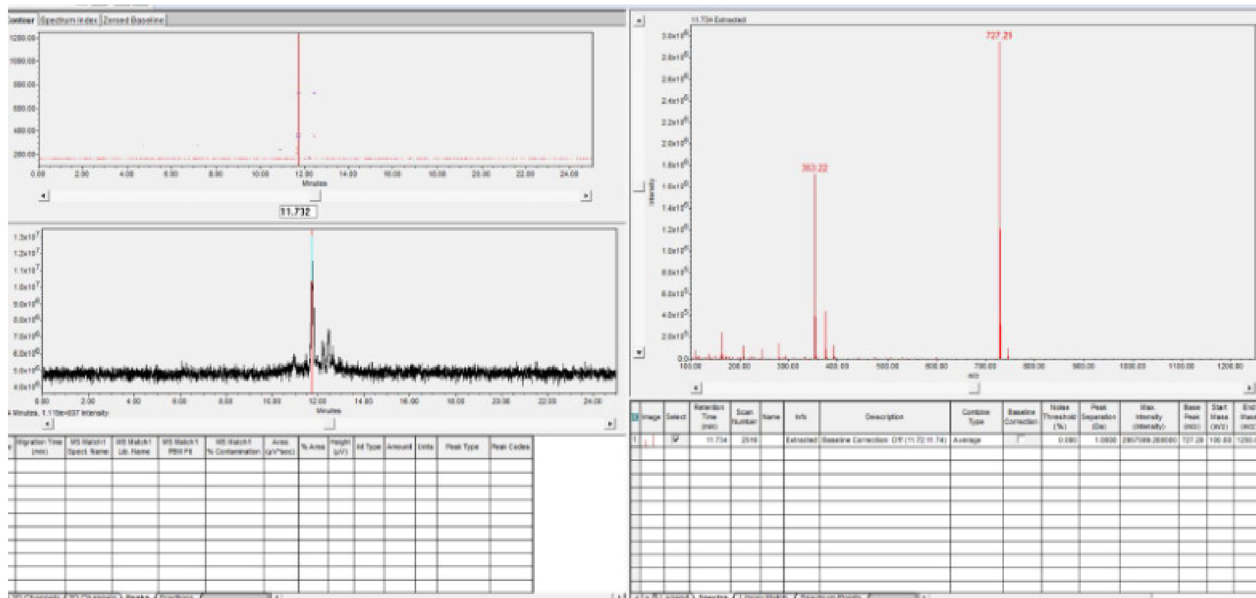
Appendix B2. LC-MS Chromatogram of *Streptomyces* sp. Go-475 in ELSD detector



Appendix B3. LC-MS/ Chromatogram of Streptomyces sp Go-475 showing different base peak with m/z of (A) 337.29 and 727.31 (B)



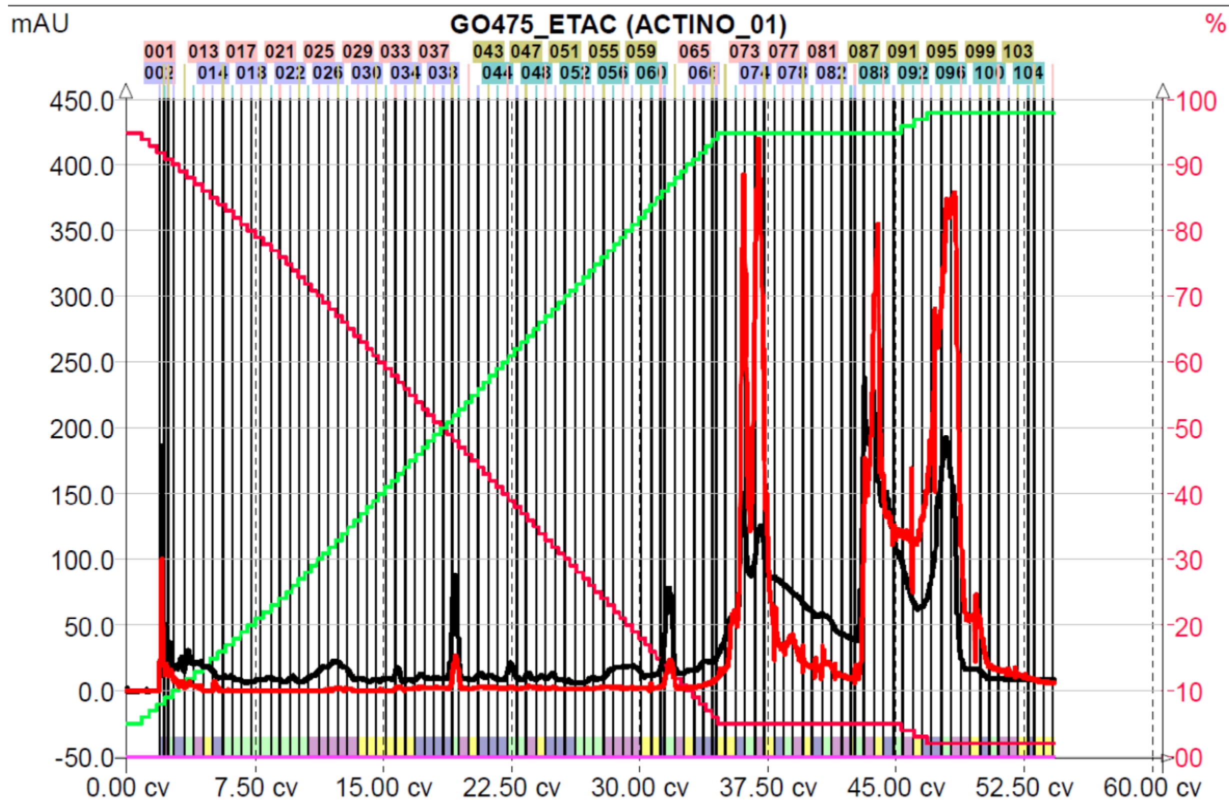
A



B

Appendix B4. Fractionation of crude extract from *Streptomyces* sp. Go-475 using PURIFLASH, (A) peaks (B) elution steps (C) Collection table

Sample : GO475_ETAC	Column : PURIFLASH COLUMN 15 C18 HQ - 35.0 g (22 bar)
Solvent A : H2O	Solvent C :
Solvent B : MeOH	Solvent D :
Channel 1 : UV600:SCAN	Channel 2 : ELSD
Equil+Inject Mode : Simple+Loop+Wait	Stop Mode : Pause
RUN Time : 11/25/2015 2:31:12 PM	



A

Elution Steps

N°	Time	Flow Rate	%A	%B	B.Flush
01	00 s	15.0	60	40	Dir.
02	02:00	15.0	60	40	Dir.
03	17:00	15.0	30	70	Dir.
04	01:02:00	15.0	02	98	Dir.
05	01:06:04	15.0	00	100	Rev.
06	01:07:00	15.0	00	100	Rev.
07	01:12:00	15.0	00	100	Rev.
08	01:17:00	15.0	00	100	Rev.
09	01:22:00	15.0	00	100	Rev.
10	01:27:00	15.0	00	100	Rev.
11	01:32:00	15.0	00	100	Rev.

B

Collection Table

Tube	Peak	Rack	Pos.	RSet	Coll.	Volume	Surface	%Surface	Start Time	End Time
001	001	1	1	1	1	2.3	0.2	0.1 %	00:03:50	00:03:59
002	002	1	2	1	1	9.5	1.6	0.9 %	00:03:59	00:04:37
003	003	1	3	1	1	8.3	0.2	0.1 %	00:05:05	00:05:38
004	004	1	4	1	1	0.5	0.0	0.0 %	00:28:35	00:28:37
005/006	005	1	5/6	1	1	32.5	0.6	0.4 %	00:28:38	00:30:48
007	006	1	7	1	1	0.3	0.0	0.0 %	00:30:48	00:30:49
008	007	1	8	1	1	0.3	0.0	0.0 %	00:30:49	00:30:50
009	008	1	9	1	1	0.8	0.0	0.0 %	00:30:50	00:30:53
010	009	1	10	1	1	2.0	0.0	0.0 %	00:30:53	00:31:01
011	010	1	11	1	1	3.8	0.1	0.1 %	00:31:01	00:31:16
012	011	1	12	1	1	1.0	0.0	0.0 %	00:31:16	00:31:20
013/014	012	1	13/14	1	1	32.3	0.6	0.3 %	00:31:20	00:33:29
015	013	1	15	1	1	1.8	0.0	0.0 %	00:33:29	00:33:36
016	014	1	16	1	1	0.8	0.0	0.0 %	00:33:36	00:33:39
017	015	1	17	1	1	6.0	0.1	0.1 %	00:33:39	00:34:03
018/021	016	1	18/21	1	1	72.0	3.0	1.7 %	00:34:03	00:38:51
022/023	017	1	22/23	1	1	18.8	0.9	0.5 %	00:38:51	00:40:06
024	018	1	24	1	1	1.5	0.1	0.0 %	00:40:06	00:40:12
025	019	1	25	1	1	2.0	0.1	0.0 %	00:40:12	00:40:20
026	020	1	26	1	1	4.5	0.2	0.1 %	00:40:20	00:40:38
027	021	1	27	1	1	1.0	0.0	0.0 %	00:40:38	00:40:42
028	022	1	28	1	1	4.5	0.2	0.1 %	00:40:42	00:41:00
029	023	1	29	1	1	0.3	0.0	0.0 %	00:41:00	00:41:01
030	024	1	30	1	1	6.3	0.3	0.1 %	00:41:01	00:41:26
031	025	1	31	1	1	0.5	0.0	0.0 %	00:41:26	00:41:28
032	026	1	32	1	1	1.8	0.1	0.0 %	00:41:28	00:41:35
033	027	1	33	1	1	2.3	0.1	0.1 %	00:41:35	00:41:44
034	028	1	34	1	1	1.8	0.1	0.0 %	00:41:44	00:41:51
035/042	029	1	35/42	1	1	144.0	18.6	10.6 %	00:41:51	00:51:27
043	030	1	43	1	1	18.0	3.4	1.9 %	00:51:27	00:52:39
044	031	1	44	1	1	18.0	3.8	2.2 %	00:52:39	00:53:51
045	031	2	1	1	1	18.0	5.4	3.1 %	00:53:51	00:55:03
044/045	031	-	-	-	-	36.0	9.2	5.3 %	00:52:39	00:55:03
046	032	2	2	1	1	18.0	4.6	2.6 %	00:55:03	00:56:15
047	033	2	3	1	1	18.0	4.3	2.5 %	00:56:15	00:57:27
048	034	2	4	1	1	18.0	4.2	2.4 %	00:57:27	00:58:39
049	035	2	5	1	1	18.0	4.2	2.4 %	00:58:39	00:59:51
050	036	2	6	1	1	18.0	4.4	2.5 %	00:59:51	01:01:03
051/053	037	2	7/9	1	1	54.0	13.8	7.9 %	01:01:03	01:04:39
054	038	2	10	1	1	18.0	4.6	2.6 %	01:04:39	01:05:51
055	039	2	11	1	1	18.0	3.2	1.8 %	01:05:51	01:07:04
056	040	2	12	1	1	18.0	4.3	2.5 %	01:07:04	01:08:16
057	041	2	13	1	1	18.0	7.0	4.0 %	01:08:16	01:09:28
058	042	2	14	1	1	18.0	9.0	5.2 %	01:09:28	01:10:40
059	043	2	15	1	1	18.0	12.3	7.0 %	01:10:40	01:11:52
060	044	2	16	1	1	18.0	18.4	10.5 %	01:11:52	01:13:04
061	045	2	17	1	1	18.0	17.6	10.0 %	01:13:04	01:14:16
062/063	046	2	18/19	1	1	36.0	11.9	6.8 %	01:14:16	01:16:40
064	047	2	20	1	1	18.0	1.0	0.6 %	01:16:40	01:17:52
065	048	2	21	1	1	18.0	0.8	0.5 %	01:17:52	01:19:04
066	049	2	22	1	1	18.0	0.7	0.4 %	01:19:04	01:20:16
067/068	050	2	23/24	1	1	26.5	1.0	0.6 %	01:20:16	01:22:02
069	051	2	25	1	1	0.8	0.0	0.0 %	01:22:02	01:22:05
070	052	2	26	1	1	18.0	0.6	0.4 %	01:22:05	01:23:17
071/072	053	2	27/28	1	1	36.0	1.2	0.7 %	01:23:17	01:25:41
073/074	054	2	29/30	1	1	36.0	1.2	0.7 %	01:25:41	01:28:05
075/078	055	2	31/34	1	1	58.0	1.8	1.0 %	01:28:05	01:31:57
079	056	2	35	1	1	0.3	0.0	0.0 %	01:31:57	01:31:58
080	057	2	36	1	1	0.5	0.0	0.0 %	01:31:58	01:32:00
-	-	-	-	-	-	408.9	3.2	1.8 %	00:00:00	00:00:00

C

Appendix B5. Some of the collected fractions

Appendix B6. Separation and fractionation of the compounds by Sephadix column

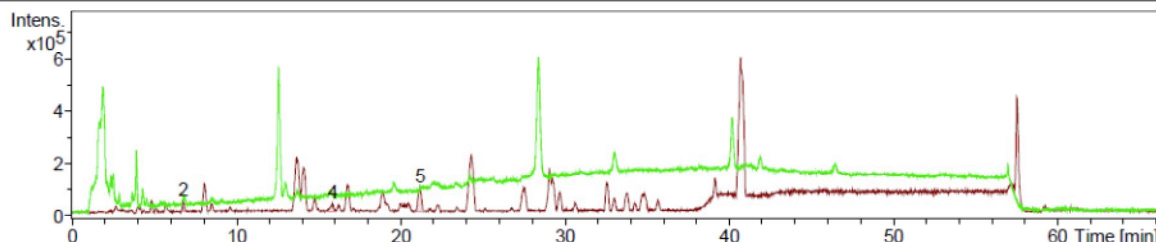
Appendix B7. HRMS analysis of compounds in Streptomyces sp. Go-75 (compounds 1-7) at various retention time

Analysis Info

Analysis Name	D:\Data_TS_data\Valerie\20170522\G0475EtOAc_pMS1_RA2_01_2450.d	Acquisition Date	5/23/2017 10:21:01 AM
Method	lcms_phenomenex_rp80a_50_to_95_in_40min_posms1.m	Operator	JW_DD
Sample Name	G0475EtOAc_pMS1	Instrument	maXis HD 1820881.21300
Comment			

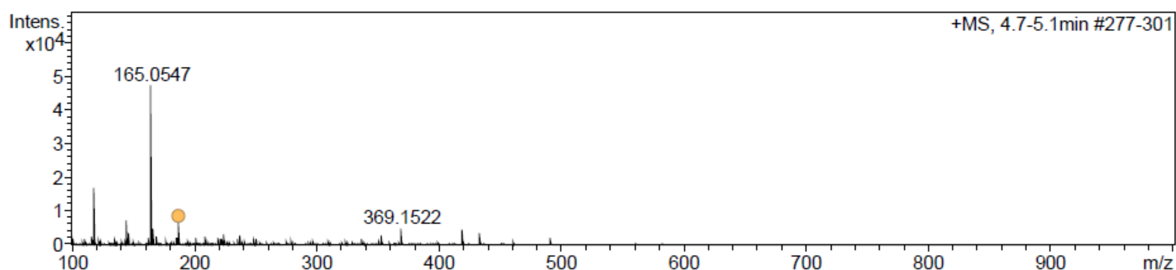
Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.8 Bar
Focus	Active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	7.0 l/min
Scan End	3000 m/z	Set Charging Voltage	2000 V	Set Divert Valve	Waste
		Set Corona	0 nA	Set APCI Heater	0 °C



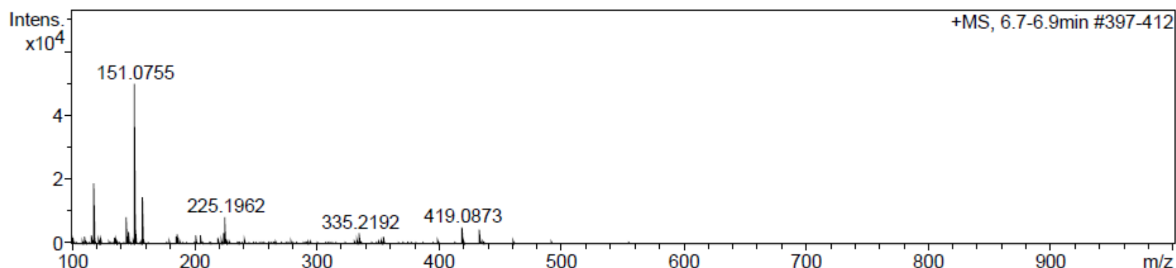
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [min]
1	4.9	603935	Manual	63834	64.7	EIC 165.0545 +All MS	165.0547	
2	6.8	294156	Manual	59570	7.0	BPC +All MS	151.0755	
3	13.7	4714905	Manual	269958	261.1	EIC 319.0969 +All MS	319.0970	
4	15.9	378976	Manual	50112	5.3	BPC +All MS	317.2087	
5	21.2	1234320	Manual	109002	16.1	BPC +All MS	295.1878	
6	22.3	218117	Manual	18358	14.0	EIC 293.1747 +All MS	315.1566	
7	27.5	2306135	Manual	112188	111.7	EIC 353.2669 +All MS	353.2664	

Cmpd 1, 4.9 min

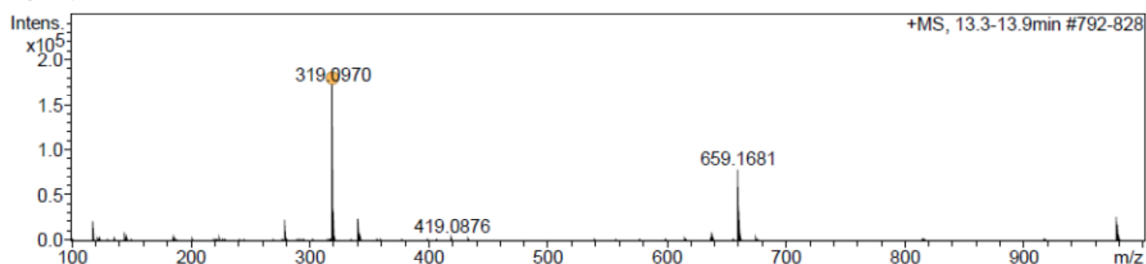


Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e⁻ Conf	N-Rule
165.0547	1	C9H9O3	165.0546	-0.5	3.8	1	100.00	5.5	even	ok
187.0367	1	C9H8NaO3	187.0366	-0.8	16.0	1	100.00	5.5	even	ok

Cmpd 2, 6.8 min

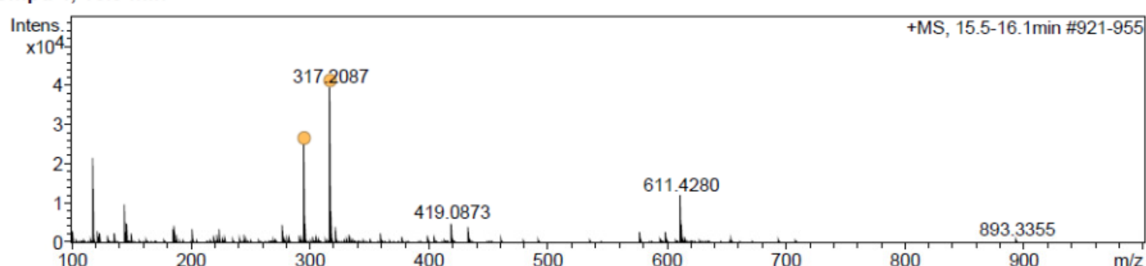


Cmpd 3, 13.7 min



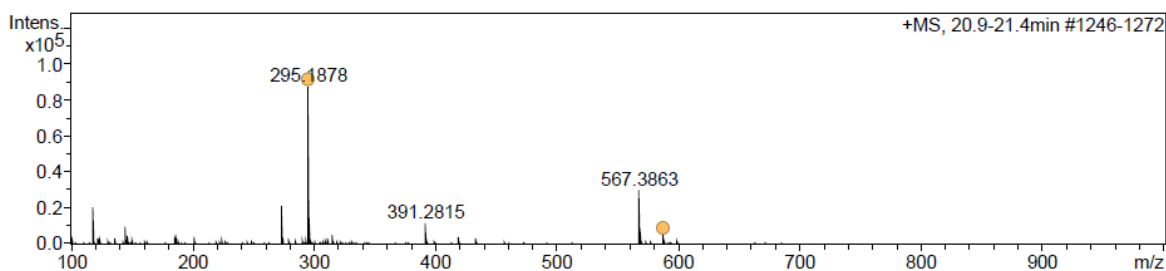
Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e⁻ Conf	N-Rule
319.0970	1	C16H11N6O2	319.0938	-9.9	5.7	1	14.68	14.5	even	ok
	2	C20H15O4	319.0965	-1.5	8.5	2	100.00	13.5	even	ok
	3	C21H11N4	319.0978	2.7	21.9	3	63.57	18.5	even	ok

Cmpd 4, 15.9 min



Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e⁻ Conf	N-Rule
295.2268	1	C18H31O3	295.2268	0.1	1.0	1	100.00	3.5	even	ok
	2	C14H27N6O	295.2241	-9.0	14.8	2	14.33	4.5	even	ok
317.2087	1	C18H30NaO3	317.2087	-0.1	4.4	1	100.00	3.5	even	ok
	2	C14H26N6NaO	317.2060	-8.5	17.5	2	13.88	4.5	even	ok

Cmpd 5, 21.2 min

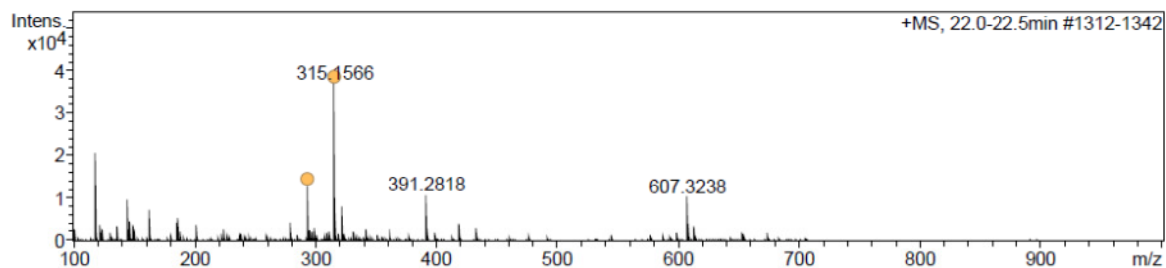


Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e⁻ Conf	N-Rule
295.1878	1	C15H28NaO4	295.1880	0.7	0.7	1	100.00	1.5	even	ok
	2	C16H24N4Na	295.1893	5.3	13.0	2	38.43	6.5	even	ok
	3	C11H24N6NaO2	295.1853	-8.4	14.3	3	18.60	2.5	even	ok
587.3548	1	C32H52NaO8	587.3554	1.0	10.6	1	100.00	6.5	even	ok
	2	C28H48N6NaO6	587.3528	-3.6	12.5	2	37.18	7.5	even	ok
	3	C29H44N10NaO2	587.3541	-1.3	13.8	3	86.57	12.5	even	ok
	4	C25H40N16Na	587.3514	-5.9	19.7	4	6.96	13.5	even	ok
	5	C27H52N2NaO10	587.3514	-5.8	19.7	5	9.38	2.5	even	ok
	6	C33H48N4NaO4	587.3568	3.3	20.2	6	36.01	11.5	even	ok
	7	C24H44N12NaO4	587.3501	-8.1	22.7	7	1.72	8.5	even	ok

G0475EtOAc_pMS1_RA2_01_2450.d

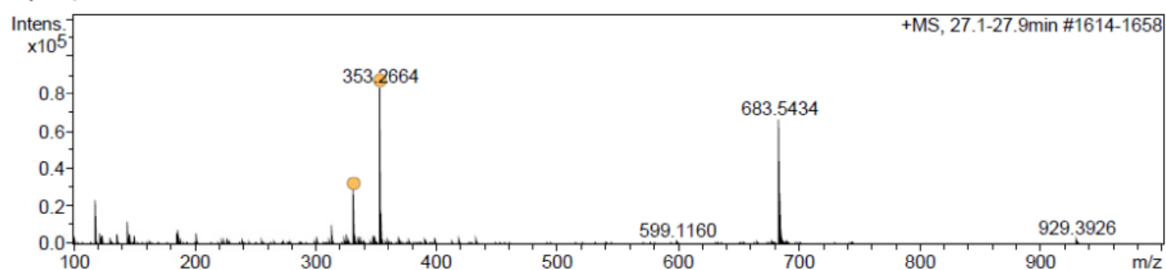
Bruker Compass DataAnalysis 4.2 printed: 7/25/2017 3:33:45 PM by: JW DD Page 2 of 3

Cmpd 6, 22.3 min



Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e⁻ Conf	N-Rule
293.1747	1	C17H25O4	293.1747	0.3	94.0	1	100.00	5.5	even	ok
	2	C18H21N4	293.1761	4.8	97.8	2	41.41	10.5	even	ok
	3	C13H21N6O2	293.1721	-8.9	98.6	3	16.02	6.5	even	ok
315.1566	1	C17H24NaO4	315.1567	0.2	6.8	1	100.00	5.5	even	ok
	2	C13H20N6NaO2	315.1540	-8.3	8.0	2	19.06	6.5	even	ok
	3	C18H20N4Na	315.1580	4.4	20.4	3	37.96	10.5	even	ok

Cmpd 7, 27.5 min

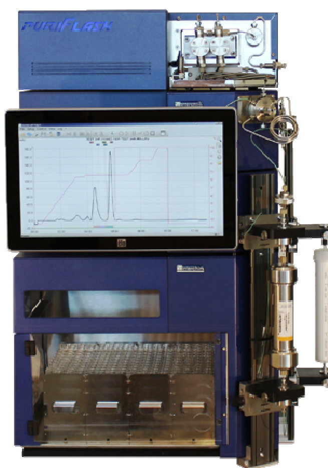


Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e⁻ Conf	N-Rule
331.2843	1	C19H39O4	331.2843	-0.2	4.4	1	100.00	0.5	even	ok
	2	C15H35N6O2	331.2816	-8.3	9.7	2	15.85	1.5	even	ok
	3	C20H35N4	331.2856	3.9	18.0	3	41.39	5.5	even	ok
353.2664	1	C19H38NaO4	353.2662	-0.6	0.9	1	100.00	0.5	even	ok
	2	C15H34N6NaO2	353.2635	-8.2	13.5	2	12.83	1.5	even	ok
	3	C20H34N4Na	353.2676	3.2	14.4	3	49.40	5.5	even	ok

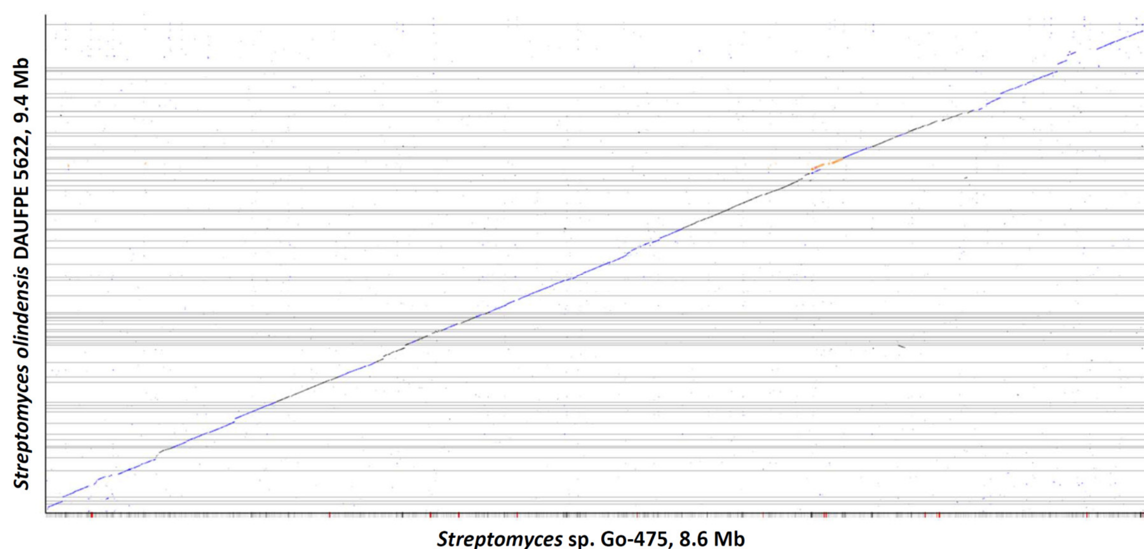
Appendix B8. Some of the machines used for bioactive compound analysis (A) UPLC (B) HPLC, Shimadzu, (C) PURIFLASH, Interchim

A

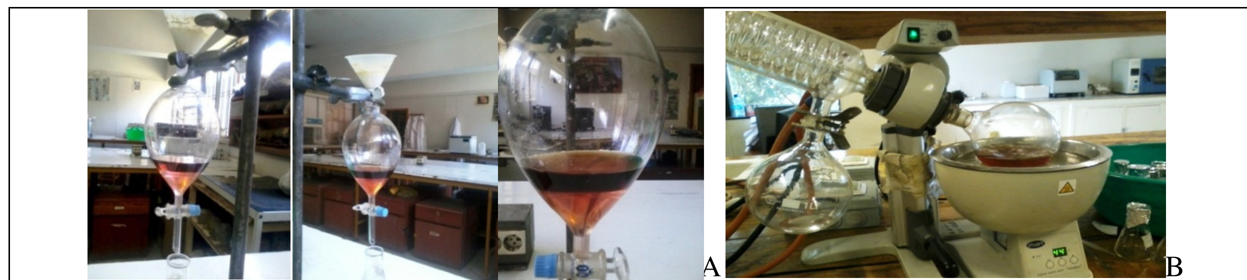
B


C

Appendix B9. Genome synteny of *Streptomyces* sp. Go-475 and *S. olindensis* DAUFPE 5622

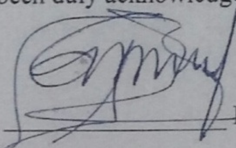


Appendix B10. A) Separation of the organic phase from the aqueous phase of bioactive secondary metabolite using separating funnel B) Concentration and drying of the crude extract using Rota vapor



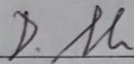
Declaration

I, the undersigned, declare that this PhD Dissertation is my own original work and has not been presented by me or any other person for a similar reason in any other university, and all sources of materials used for this dissertation have been duly acknowledged.



PhD candidate: Moges Kibret Signature ____________ ~~Date~~ ____________

Supervisor: Dr. Dawit Abate

(PhD, Associate Professor) Signature  Date Sept. 14, 2017

Addis Ababa, Ethiopia.