

BIOACTIVE SECONDARY METABOLITE PRODUCTION AND IDENTIFICATION FROM *STREPTOMYCES*

Submitted in partial fulfilment of the requirements for the award of

Bachelor of Science Degree in Biotechnology

By

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DEPARTMENT OF BIOTECHNOLOGY

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SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY

(DEEMED TO BE UNIVERSITY)

Accredited with Grade “A” by NAAC

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **Angel Alisha (40770004)** who carried out the project entitled "Bioactive secondary metabolite production and identification from *Streptomyces*" under our supervision from October 2022 to March 2023.

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DECLARATION

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ABSTRACT

Streptomyces is well known for its ability to produce wide range of bioactive secondary metabolites. This study aims to isolate bioactive secondary metabolites from *Streptomyces* and characterized for their antimicrobial potential. A preliminary screening of antimicrobial activity of *Streptomyces* UP2A9 was performed against 21 set of bacterial and fungal pathogens. It showed maximum antimicrobial activity against *Klebsiella pneumoniae* and *Candida albicans* with the zone of inhibition of 17.6 ± 1.2 mm and 16.6 ± 1.7 mm, respectively. The bioactive secondary metabolites produced from the potential *Streptomyces* strain were then extracted through solid and liquid phase extraction. In the secondary screening, the crude ethyl acetate extract produced from liquid phase fermentation showed maximum antimicrobial activity against *K. pneumoniae* and *C. albicans* with the zone of inhibition of 24 mm and 20 mm, respectively. Then, the production media was optimized using 15 different carbon and nitrogen source and the maximum antimicrobial metabolites were produced in the media with the combination of lactose as the carbon source and peptone as the nitrogen source. Further, the crude metabolites were evaluated for metabolite profiling using LC-MS/MS analysis and it showed number of bioactive secondary metabolites which can be the reason of their antimicrobial activity. Then, antibiofilm activity was performed against 4 sets of pathogens via, *Acinetobacteria*, *E. coli*, *K. pneumoniae* and *Pseudomonas* and it showed a broad range of activity against *K. pneumoniae*. Based on the morphological, cultural and molecular analysis, the potential strain UP2A9 was identified as *Streptomyces maritimus*. Finally, this study shows that the secondary metabolism of the potential *Streptomyces* strain UP2A9 may serve as an important source for antimicrobial drug development on further investigation.

Keywords:

Streptomyces, preliminary screening, antimicrobial potential, bioactive secondary metabolites

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LIST OF ABBREVIATIONS

μl	Micro litre
BLAST	Basic Local Alignment Search Tool
ESI	Electrospray ionization
HPLC	High performance liquid chromatography

ISP	International <i>Streptomyces</i> Project
LCMS/MS	Liquid chromatography tandem mass spectrometry
MS	Mass spectrometry
SEM	Scanning electron microscopy
TLC	Thin Layer Chromatography

CHAPTER-1

INTRODUCTION

Antimicrobial resistance is an international public health concern that is becoming increasingly alarmingly widespread. Infections become difficult or impossible to cure when bacteria and fungus learn how to resist the medications meant to destroy them. Our ability to cure widespread infectious illnesses is being threatened by the emergence and worldwide dissemination of new resistance mechanisms. The current predicament has been exacerbated by the improper use of antibiotics in the human, animal, food, and agricultural sectors. Increased mortality, longer hospital stays, and greater medical expenses are all consequences of antimicrobial resistance. It is a worldwide issue that has to be addressed by many different industries and all countries (Martha and Catherine, 2018).

Antimicrobial resistance happens when parasites, viruses, fungus, and bacteria learn to resist the medications meant to kill them. When bacteria adapt as a result of the usage of antibiotics, antibiotic resistance is one kind of antimicrobial resistance that develops. Antibiotic abuse and overuse, inadequate infection prevention and control, and the availability of antibiotics and antifungals that force both fungi and bacteria to adapt are all factors that lead to antibiotic resistance. The current predicament has also been exacerbated by the improper use of antibiotics in the human, animal, food, and agricultural sectors. Increased mortality, longer hospital stays, and greater medical expenses are all consequences of antimicrobial resistance (WHO 2020).

There are a number of causes for the emergence of new antimicrobial-resistant strains, including the overuse and misuse of antibiotics, poor infection control procedures, insufficient sanitation and hygiene, and the use of antibiotics in agriculture and animal husbandry. The emergence of new antimicrobial-resistant strains is a major concern because it reduces treatment options and can cause the spread of infections that are difficult to control (Economou *et al.*, 2015).

AMR is a complex issue that has an impact on food safety and quality in addition to human health. Humans can become infected with antibiotic-resistant bacteria through direct interaction with livestock, the food chain, and wastewater from farms, hospitals, and drug manufacturing facilities (Musoke *et al.*, 2021). Antibiotic resistance may be avoided in a number of ways, including by avoiding infections, enhancing the use of antimicrobial agents and antibiotics to impede the emergence of resistance, and halting the spread of resistance when it develops.

Agriculture, drug development, human and veterinary care, and other fields have benefited greatly from the use of natural goods. They have helped create novel medications, particularly those that treat cancer and infectious disorders. New versions and chemical reactions can be developed using inspiration from natural items. They are useful in a circular economy and inspire key ideas and directions in research, development, and business. Furthermore, natural compounds have a biological past that might shed light on their prospective therapeutic applications. In general, natural goods are valuable sources for expanding scientific understanding and creating novel medicines (Atanasov *et al.*, 2021).

In terms of drug development, natural ingredients have various benefits. First of all, they have a biological background that might shed light on prospective therapeutic applications. Second, natural products have been crucial in the development of new drugs, particularly for the treatment of cancer and infectious disorders. Thirdly, natural goods offer fundamental bioactive components that are more potent and less harmful. Fourthly, natural products may shed light on protein expression, function, metabolism, and proteomic strategies for novel drug development. Finally, the creation of novel variations and chemical reactions can be greatly influenced by natural ingredients. In general, natural goods are valuable sources for expanding scientific understanding and creating novel medicines (Mathur *et al.*, 2017).

Bioactive substances generated by a variety of species, including bacteria, fungus, and plants, are known as microbial natural products. Antibiotics, anticancer medications, immunosuppressants, antivirals, and anthelmintics are just a few of the many uses for these compounds. The majority of beneficial natural compounds are produced by microbes, and their synthesis takes up a sizable percentage of microbial genomes. For thousands of years, natural goods have been integral to human life and have been used as a source of medicine, particularly in underdeveloped nations. In medicine, microbial natural products, their semisynthetic derivatives, and synthetic counterparts are often utilised to treat a variety of illnesses (Pham *et al.*, 2019).

Numerous structurally unique specialised metabolites produced by microorganisms have a wide range of biological functions and are used in a wide range of medical procedures, including the treatment of cancer and infectious disorders. Natural compounds produced by bacteria can be utilised as antibiotics to kill other bacteria. Drug development greatly benefits from the use of microbial natural compounds (Rommasi, 2021).

The bacterial phylum known as actinobacteria is one of the most varied and has the greatest metabolic adaptability. In addition to a wide range of other all-natural goods with

medicinal or agricultural uses, it produces the majority of the antibiotics that are clinically utilised (van Bergeijk *et al.*, 2020). Actinobacteria have been used to make numerous significant natural goods, such as antibiotics, cancer-fighting chemicals, and immunosuppressants. The potential for these bacteria to produce novel secondary metabolites is very high, and they are especially prevalent in soil environments. Numerous natural products finding initiatives have been directed towards actinobacteria, and the genomes of these organisms have been sequenced and searched for biosynthetic gene clusters. Additionally, actinobacteria can now be modified to create novel or altered secondary metabolites with desired properties thanks to synthetic biology methods (Xie *et al.*, 2021).

Due to their capacity to create bioactive secondary metabolites, such as antibiotics, antifungal, antiviral, and antitumor agents, actinobacteria have a significant effect on the pharmaceutical industry. Actinobacteria are thought to create a large number of bioactive substances, and their secondary metabolites are a promising source for the creation of novel medications (Lee *et al.*, 2020). Actinobacteria are present in a variety of habitats, including saline regions, tropical jungles, and other ecosystems with a high biodiversity. Actinobacteria from various habitats, such as deserts, marine environments, and the gut, have been studied recently for their variety, functionality, and biotechnology. Numerous strains have been isolated and characterized, and a great deal of research has been done on the variety of Actinobacteria in saline environments (van Bergeijk *et al.*, 2020).

Actinobacteria are abundant producers of bioactive natural products, and numerous techniques have been used to analyse and identify the secondary metabolites of these organisms. These substances are involved in a number of physiological, cellular, and biological processes and have been discovered to have antimicrobial characteristics (Sarmiento-Vizcaino *et al.*, 2021). Antibiotics are widely recognized to be produced in large quantities by the bacteria *Streptomyces*. It generates about 80% of all antibiotics derived from actinobacteria, making it the most significant natural source of antibiotics. *Streptomyces* has the potential of producing bioactive secondary compounds, including antifungals, antivirals, anticancer agents, and anti-infectives. It is still regarded as the leading manufacturer of revolutionary natural medicines (Maiti *et al.*, 2020). The family *Streptomyces* is widely recognized for producing large quantities of medicines as well as numerous signalling molecules that control antibiotic production (Kong *et al.*, 2019).

Bioactive secondary compounds are analyzed and visualized using molecular networking. It entails the identification and comparison of compounds using mass spectrometry, which is followed by the development of a framework to show the connections

between them. Drug creation and the acquisition of natural products have both used this method. A promising approach for finding bioactive substances in complicated combinations is molecular networking. It enables a visual representation of relationships between metabolites and the analysis of mass spectrometry data from multiple samples. Molecular networking can assist in the dereplication of known constituents and the finding of new bioactive compounds by grouping similar compounds together. Natural product discovery, where complicated mixtures of compounds are frequently present, benefits greatly from the implementation of this strategy. Overall, molecular networking is a useful method for locating and describing reactive substances in a range of situations (Dotsha *et al.*, 2019).

In addition to producing a substantial amount of antibiotics, *Streptomyces* additionally yields a broad spectrum of other beneficial compounds, including immune-suppressive, anti-fungal, anti-bacterial drugs and antiparasitic medications. Beginning simultaneously with the aerial hyphal formation from the matrix of mycelium, *Streptomyces* commences synthesizing almost all of the compounds that are bioactive.

The liquid chromatography-mass spectrometry (LC-MS) processes are utilized for identifying secondary compounds. For the determination and quantization of all metabolites of it in biological materials, LC-MS is a formidable tool. With the LC-MS technique, metabolic products undergo initial separation via liquid chromatography, subsequently detected and characterized by applying mass spectrometry. Microbial extracts that are natural are capable of being employed to detect secondary metabolites utilizing LC-MS. Secondary substances are identified using the liquid chromatography-mass spectrometry (LC-MS) procedures (Bin *et al.*, 2012). There are many advantages to using LC-MS for metabolite detection. One benefit is that it can act as a platform for clinical diagnostics as well as an instrument for biomarker discovery. LC-MS has a higher yield than GC-MS because it requires less chemical derivatization and runs more quickly. LC-MS is helpful for *in-vivo* profiling of drug metabolites because it also lowers sample complexity and offers high sensitivity. A multi-stage strategy is used, starting with study design, preanalytical steps, LC-MS analysis, and data processing, to guarantee fact-based metabolite identification in LC-MS.

There are several advantages to identifying secondary compounds. A more potent instrument for study on the production and discovery of secondary metabolites is metabolomics, the global characterization of metabolite profiles. Secondary metabolites are low-molecular-weight compounds with a variety of biological functions. They are crucial for pharmaceutical and ecological research. Targeted or untargeted methods to the study of

microbial secondary metabolites can aid in the identification and characterization of these compounds in both natural and artificial biosystems.

Studies have revealed that a number of variables, including the genetic make-up of the *Streptomyces* strains, the composition of the growth medium, and culture conditions, all have an impact on the generation of these beneficial secondary metabolites. Additionally, modifications to these chemicals' biosynthesis processes have been made possible by developments in molecular biology and genetic engineering, which have allowed researchers to find novel bioactive compounds and increase manufacturing yields (Liu *et al.*, 2019).

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CHAPTER-2

2. REVIEW OF LITERATURE

2.1 IMPORTANCE OF INFECTIVE DISEASES

The public health and economic stability of societies around the globe are significantly impacted by infectious diseases. Since ancient times, they have been among the main causes of mortality and disability and have posed increasing threats to both human advancement and health security. The ongoing emergence of new, unrecognized, and historic epidemics of infectious diseases with global effects intensifies the threat presented by infectious diseases. At least 30 new infectious diseases that impact humans have appeared in the past 35 years; the majority of these diseases are zoonotic, and research has shown that socioeconomic, environmental, and ecological factors are strongly correlated with the origins of these diseases (Nii-Trebi, 2017).

Because they can harm an individual and even result in mortality if improperly prevented and treated, infectious diseases are significant. They are brought on by pathogenic organisms that can infiltrate the body from the outside, including viruses, bacteria, fungi, and parasites. Vaccinations and frequent hand washing are effective ways to avoid infectious diseases. Effective infection control and prevention are crucial for patient safety and high-quality healthcare service. In modern medicine, specialised knowledge and abilities in the administration and research of infectious diseases are becoming more and more crucial (Infection Prevention and Control GLOBAL, 2022).

Table 2.1: Major infectious diseases and their estimated global burden of disease

ESTIMATION OF GLOBAL BURDEN OF DISEASES
38 million people living with HIV/AIDS
Malaria 229 million cases, 409,000 deaths
Tuberculosis 10 million cases, 1.4 million deaths
Hepatitis B 257 million people chronically infected
Hepatitis C 71 million people chronically infected
Influenza 3-5 million cases of severe illness, 290,000-650,000 deaths
Antimicrobial resistance estimated to cause 700,000 deaths annually

Source: (WHO, 2021).

2.2 SIGNIFICANCE OF ANTIMICROBIAL RESISTANCE PATHOGENS

Resilience to antimicrobials the term "pathogen" refers to microorganisms like bacteria and fungi that have evolved the ability to withstand the drugs intended to eradicate

them. Therefore, it may be challenging or even impossible to cure infections brought on by these microbes. Antimicrobial resistance is a naturally occurring process that poses a threat to the health and growth of the entire world and necessitates immediate action. Antimicrobial resistance is one of the top 10 global public health threats facing humankind, according to the World Health Organization (2022).

Antimicrobial resistance (AMR) is an evolving problem that brings the risk of rendering many of the currently available treatments for infections ineffective. In order to solve this issue, it is essential to conduct research in the field of novel antimicrobial substances derived from sources that are naturally occurring (Ed Peile *et al.*, 2020).

When microorganisms create mechanisms that make antimicrobial drugs ineffective, this is known as antimicrobial resistance (AMR). This may result in persistent infections, greater rates of morbidity and mortality, and more expensive medical care. AMR is a serious public health concern everywhere, and we urgently need novel, powerful antimicrobial medications. The search for novel antimicrobial compounds from natural sources, especially from microbes, has become increasingly essential in the fight against AMR. Natural products have historically been a rich source of bioactive compounds with antimicrobial activity (Sameer Dhingra *et al.*, 2020).

A top ten worldwide public health threat that affects people, animals, plants, and the environment is antimicrobial resistance (AMR). AMR has a greater effect in low- and middle-income nations. Around 700,000 people globally pass away every year as a result of HIV, TB, malaria, and drug-resistant infections of common bacterial strains. AMR makes investing in novel antibiotics less appealing and increases the financial burden on the overall healthcare system. AMR is transmitted through the improper use or overuse of antimicrobials in farming and agriculture. AMR is a complex issue that has an impact on food safety and quality in addition to human health. Humans can become infected with antibiotic-resistant bacteria through direct interaction with livestock, the food chain, and wastewater from farms, hospitals, and drug manufacturing facilities (Musoke *et al.*, 2021).

2.3 INTRODUCTION TO NATURAL PRODUCT DISCOVERY

The process of locating and isolating substances from natural sources, such as plants, animals, and microorganisms, that have possible therapeutic properties is known as natural product discovery. Natural products have historically been important in the search for new medicines, particularly for cancer and infectious illnesses. Natural substances are now frequently used as the basis for drug development, with synthetic modifications added later to help lessen side effects and boost bioavailability. Natural goods come from a variety of

sources, such as plants, microorganisms, and marine organisms. Natural product discovery is a field that is constantly developing, with the genomic age bringing about new developments and opportunities (Atanasov *et al.*, 2021).

For the discovery of new drugs, natural products have been a great supply of bioactive compounds. These substances, which can have a variety of biological activities, are usually produced by microbes, plants, and animals as secondary metabolites. Chemicals created by living things that have therapeutic or pharmacological characteristics are known as natural products. More than half of all drugs authorised by the US FDA between 1981 and 2014 had a natural product origin, demonstrating the important role these compounds have played in drug discovery and development. The main sources of natural products are microbes, plants, and animals. These substances are frequently created as secondary metabolites, which are not necessary but give the producing organism a selective advantage. Natural product development and discovery are continuing processes, and fresh compounds are frequently found and characterised (Zahraa Zaidan *et al.*, 2021).

Drug development has a long tradition of using natural product discovery, particularly for cancer and infectious diseases. Over the past seven decades, natural product discovery strategies have changed, moving from relatively straightforward ones in the first 30 years to more varied and complicated ones in the following two decades, accelerated by advances in science and technology. Natural products have traditionally contributed significantly to pharmacotherapy, and their structural analogues have also been important in the development of new drugs. Natural products continue to be the best sources of novel drug leads and continue to play a crucial role in drug discovery despite the difficulties associated with their discovery, such as technical obstacles to screening, isolation, and characterization (Katz & Baltz, 2016).

Because of their wide range of bioactivities and great structural diversity, natural products continue to be a promising source for drug discovery. It's essential to remember, though, that not all heritable human diseases can be treated with natural remedies. One of the most important rules in the area of discovering natural products is the finding of structurally novel metabolites. Natural product retrospective analysis has shed light on the subject and the possibility for compound novelty. The core of our contemporary drugs is specialised natural products metabolites made by microbes and plants (Zotchev *et al.*, 2021).

Table 2.2: Examples of natural products with antimicrobial activity discovered from microbial sources

S.NO	SECONDARY METABOLITE	MICRO ORGANISM	BIO ACTIVITY
1.	Penicillin	<i>Penicillium chrysogenum</i>	Antibacterial
2.	Streptomycin	<i>Streptomyces griseus</i>	Antibacterial
3.	Vancomycin	<i>Amycolatopsis orientalis</i>	Antibacterial
4.	Cyclosporin A	<i>Tolypocladium inflatum</i>	Immunosuppressive
5.	Echinocandins	<i>Aspergillus</i> species	Antifungal
6.	Bleomycin	<i>Streptomyces verticillus</i>	Anticancer

Source: (Newmann and Cragg *et al.*,2020)

2.4 MICROBIAL NATURAL PRODUCT DISCOVERY

Finding new pharmaceuticals from natural substances produced by microorganisms like plants, fungi, and bacteria is a process known as "microbial natural product discovery." For the treatment of numerous clinical disorders, including infectious diseases, these natural products are extensively used in medicine. Activating dormant biosynthetic gene clusters, via computational techniques, and genome mining are some of the various methods for finding microbial natural products. To locate recognized molecules and investigate the variety of structural series, use the Natural Products Atlas (Abdel-Razek *et al.*, 2020).

Bioactive secondary metabolites are widely produced by microbes, especially bacteria and fungus. These substances exhibit a wide variety of biological activities, such as antibacterial, anticancer, antiviral, and immunosuppressive ones. The range of microbial natural product discovery has considerably increased as a result of the application of numerous techniques, including genome mining, transcriptomics, and synthetic biology. In order to anticipate the structure and biological activity of the encoded secondary metabolites, genome mining entails locating biosynthetic gene clusters in microbial genomes. By analysing how genes are expressed in reaction to environmental cues, a process known as transcriptomics, it is possible to discover novel natural product biosynthetic pathways. The engineering of microbial strains to create brand-new or altered natural products is made possible by synthetic biology (Tushar Mehta *et al.*, 2022).

Many clinical conditions, including infectious diseases and cancer, are treated with microbial natural products in medicine. Penicillin, erythromycin, tetracycline, and streptomycin are some instances of microbial natural products used in medicine. These

antibiotics are made by bacteria. Other examples include the immunosuppressive drug cyclosporine, which is made by the fungus *Tolypocladium inflatum*, and the anticancer drug doxorubicin, which is produced by *Streptomyces peucetius* (Kim *et al.*, 2019).

The use of microbial natural products in health has, many benefits. They are effective for managing a variety of clinical disorders because of their distinctive chemical structures and biological activities, which include antimicrobial, immunosuppressive, anticancer, and anti-inflammatory ones. Additionally adaptable, microbial natural products can be created by a variety of microorganisms, such as bacteria, fungus, and algae. The majority of useful natural products are also produced by microbes, who genomes dedicate a sizable percentage of their space to the synthesis of these beneficial secondary metabolites. A single microbe can create a number of secondary metabolites, and natural products derived from plants and microbes also make excellent medicines (Pham *et al.*, 2019)

Potential use for microbial natural products includes drug discovery, food safety, and horticulture, among others areas. They are a plentiful source of effective vaccines, polymers, lubricants, bioherbicides, immunosuppressants, antivirals, anthelmintics, and enzyme inhibitors. Antibiotic, antifungal, anticancer, immunosuppressive, anti-inflammatory, and biofilm inhibitory actions are just a few of the biological properties that natural products have that are pertinent to human health. The effectiveness of microbial natural products as antibiotics and antifungals has been particularly impressive. Natural antimicrobials have also been crucial to the food industry's efforts to ensure product safety (Tshikantwa *et al.*, 2018)

The discovery of microbial natural products is a potential field of study for the creation of novel medications and other products. New methods for isolating previously uncultured microorganisms and extensive microbial strain collections can aid in the findings of novel natural products. Recombinant proteins and natural products have a wide range of biological functions that have been explored. Nevertheless, there are already significant and widely available microbial natural products, despite the recent findings that microorganisms can serve as a source of medications. To keep moving this field forward, it is necessary to innovate sample gathering and library generation (Atanasov *et al.*, 2021)

2.5 ACTINOBACTERIA: A POTENTIAL BIOACTIVE SECONDARY METABOLITE

The discovery of various significant natural products, such as antibiotics, anticancer agents, and immunosuppressants, can be attributed to the diverse collection of bacteria known as actinobacteria. Future efforts for discovering new drugs may benefit from using these bacteria because they are especially well-known for having the ability for producing bioactive

secondary metabolites. The ability of the Gram-positive bacteria known as actinobacteria to produce bioactive secondary metabolites is well documented. Actinobacteria have been used to make numerous significant natural goods, such as antibiotics, cancer-fighting chemicals, and immunosuppressants. The potential for these bacteria to produce novel secondary metabolites is very high, and they are especially prevalent in soil environments. Numerous natural products finding initiatives have been directed towards actinobacteria, and the genomes of these organisms have been sequenced and searched for biosynthetic gene clusters. Additionally, actinobacteria can now be modified to create novel or altered secondary metabolites with desired properties thanks to synthetic biology methods (Xie *et al.*, 2021).

Due to their capacity to create bioactive secondary metabolites, such as antibiotics, antifungal, antiviral, and antitumor agents, actinobacteria have a significant effect on the pharmaceutical industry. Actinobacteria are thought to create a large number of bioactive substances, and their secondary metabolites are a promising source for the creation of novel medications. The genomic potential of Actinobacteria far exceeds the known chemical space, as shown by contemporary "omics"-based technologies. By fusing knowledge of actinobacterial ecology with cutting-edge computational methods, it is possible to access this untapped reservoir of actinobacterial metabolism. In order to find possibly new and useful compounds for the pharmaceutical industry, actinobacteria have the potential to offer innovative and efficient bioprospecting tools (Lee *et al.*, 2020).

Rare Actinobacteria, untested strains, and extremophilic Actinobacteria that survive in harsh environments are some of the most promising sources of Actinobacteria for the discovery of novel bioactive substances. Rare Actinobacteria are of particular interest because they are a significant source of crucial bioactive compounds but are rarely separated from their environments. If strain divergence is large, exploring unexplored strains is the best way to find novel chemistry. Actinobacteria that have adapted to live in harsh environments and generate special secondary metabolites are also promising sources of bioactive substances. Additionally, new sources of Actinobacteria that may help with the finding of novel compounds include freshwater ecosystems, caves and animals (especially insects) (Hui *et al.*, 2021)

Even though Actinobacteria have the ability to create bioactive compounds, it can be difficult to isolate new compounds from these bacteria. Actinobacteria are challenging to cultivate in the lab because many of these bacteria are slow-growing and necessitate particular growth conditions. The fact that many of the beneficial substances made by

Actinobacteria have already been identified and characterised presents another difficulty. Additionally, thorough screening of numerous Actinobacteria strains is necessary for the discovery of novel compounds, which can be costly and time-consuming. Finally, the incomplete knowledge of the biosynthetic pathways for many secondary metabolites produced by Actinobacteria also hinders the finding of novel compounds (Ribeiro et al., 2020).

The most frequently used strategies for finding novel bioactive substances in Actinobacteria are bioprospecting, investigating novel environments, genome mining, and sophisticated analytical techniques. While exploring new environments like caves, freshwater systems, and marine environments can result in the finding of novel compounds, bioprospecting includes screening numerous Actinobacteria strains for bioactive compounds. Advanced analytical methods like mass spectrometry and nuclear magnetic resonance spectroscopy make it possible to identify and characterise new compounds, while genome mining enables researchers to pinpoint biosynthetic gene clusters that are in charge of producing secondary metabolites (Chan *et al.*, 2020).

Bioprospecting, which entails analysing numerous Actinobacteria strains for bioactive substances, is one of the most popular techniques. Exploring new and uncharted environments, such as caves, freshwater systems, and marine environments, where Actinobacteria may create novel compounds, is an additional strategy. Genome mining is a useful method for locating biosynthetic gene groups that are in charge of producing secondary metabolites, which is another way to find novel compounds. Additionally, improvements in analytical methods like mass spectrometry and nuclear magnetic resonance spectroscopy have made it possible to recognise and characterise novel Actinobacteria substances (Amin et al., 2020).

2.6 DIVERSITY OF ACTINOBACTERIA

A bacterial phylum with a broad variety of metabolic abilities is the actinobacteria. Numerous therapeutically useful antibiotics and other natural products are produced by them, which is well-known. Actinobacteria are present in a variety of habitats, including saline regions, tropical jungles, and other ecosystems with a high biodiversity. Actinobacteria from various habitats, such as deserts, marine environments, and the gut, have been studied recently for their variety, functionality, and biotechnology. Numerous strains have been isolated and characterized, and a great deal of research has been done on the variety of Actinobacteria in saline environments (van Bergeijk *et al.*, 2020).

Extreme habitats like the arctic, boiling hot springs, and salty oceans are just a few of the places where actinobacteria can be located. They can also be found in habitats that are both terrestrial and aquatic, such as mangroves, deep oceans, and deserts. Numerous strains have been isolated and characterized, and a great deal of research has been done on the variety of Actinobacteria in saline environments. Additionally, actinobacteria can be found in our gastrointestinal systems as well as the soil (Narsing Rao & Li, 2022).

2.6.1 Terrestrial Ecosystem:

Actinobacteria are important for the proper operation of terrestrial ecosystems because they increase plant productivity, break down organic matter in the soil, and produce bioactive substances that are vital for both human and animal health. These processes also contribute to the global carbon cycle. It has also been researched how diverse the actinobacterial community is in desert environments (Idris *et al.*, 2017).

The operation of terrestrial ecosystems depends heavily on actinobacteria. They increase plant productivity, aid in the decomposition of organic matter in the soil, and are well known for producing large quantities of bioactive substances that are vital for both human and animal health. The terrestrial environment contains the highest concentration of actinobacteria, and 99% of the diverse species have been identified based on the diversity of actinobacteria in the soil. Actinobacteria are a special class of organisms because they play a role in the development of plants and the cycling of carbon. They establish colonies with the host plants and generate compounds that stimulate plant development. Additionally, actinobacteria have the capacity to create a wide range of bioactive molecules, such as pigments, industrial enzymes, dyes, and antibiotics (Amoroso *et al.*, 2017).

Actinobacteria play an important role in soil ecosystems. Through the breakdown of soil organic matter, which is necessary for nutrient cycling and soil fertility, they add to the global cycling of carbon. Actinobacteria are engaged in the recycling of refractory biomaterials by breaking down intricate polymer mixtures in fungal and animal remains as well as deceased plants and animals. They can produce extracellular enzymes that degrade complicated organic substances like cellulose, chitin and lignin into less complex substances that other microorganisms can utilise. Additionally, auxins, cytokinins and gibberellins, which can improve plant growth and development, are produced by actinobacteria. A broad variety of bioactive substances, such as antibiotics, antifungals and anticancer agents are also known to be produced by actinobacteria. These substances have significant uses in both agriculture and medicine (Araujo *et al.*, 2020).

It is well known that actinobacteria from terrestrial ecosystems generate bioactive secondary metabolites. These organic substances, known as metabolites, are not directly linked to the organism's typical growth. Actinobacteria are abundant producers of bioactive natural products, and numerous techniques have been used to analyse and identify the secondary metabolites of these organisms. These substances are involved in a number of physiological, cellular, and biological processes and have been discovered to have antimicrobial characteristics (Sarmiento-Vizcaino *et al.*, 2021).

7. *STREPTOMYCES* AS A RICH SOURCE OF ANTIBIOTIC

Antibiotics are widely recognized to be produced in large quantities by the bacteria *Streptomyces*. It generates about 80% of all antibiotics derived from actinobacteria, making it the most significant natural source of antibiotics. *Streptomyces* has the potential of producing bioactive secondary compounds, including antifungals, antivirals, anticancer agents, and anti-infectives. It is still regarded as the leading manufacturer of revolutionary natural medicines (Maiti *et al.*, 2020).

The potential of *Streptomyces* to generate bioactive secondary metabolites is what makes it a rich source of antibiotics. These metabolites require expensive biosynthesis and secretion processes, which *Streptomyces* significantly invests in. *Streptomyces* is the most significant bacterial genus for the creation of bioactive compounds, producing around two-thirds of all antibiotics. *Streptomyces* is a possible source of new antibiotics because it has many cryptic secondary metabolite pathways that aren't expressed in typical laboratory settings. The family *Streptomyces* is widely recognized for producing large quantities of medicines as well as numerous signalling molecules that control antibiotic production (Kong *et al.*, 2019).

1. *Antimicrobial Potential:*

The ability of an element to eradicate or prevent the proliferation of microorganisms like bacteria and fungi is referred to as antimicrobial potential. Antimicrobial resistance is a danger to global health that calls for immediate action. The antimicrobial properties of bacteriocins, which have been produced by a wide variety of microbes, have made them useful in medicine, agriculture, and food preservation. Another drug whose antimicrobial potential has been discovered is cannabidiol (Blaskovich *et al.*, 2021).

A genus of bacteria called *Streptomyces* is well-known for its ability to combat infection and as a plentiful supply of antibiotics. Indigenous medicine has made use of *Streptomyces*, and it is still a significant supply of antibiotics in nature. Numerous antimicrobial substances that are powerful against Gram-positive as well as Gram-negative

bacteria are produced by *Streptomyces*. With more than 800 species known, *Streptomyces* continues to be the leading producer of novel natural products (Chevrette *et al.*, 2019).

The specific antimicrobial metabolite produced by *Streptomyces* has a different mode of action. However, many of these metabolite function by preventing the target microorganism's development or by actually killing it. For instance, some *Streptomyces* create antifungal and antibacterial substances that damage the target organism's cell wall or membrane, causing cell death. Other substances might obstruct vital enzymes or metabolic pathways, which would stop the target microbe's development or cause it to perish. Each compound's precise mode of action needs to be researched separately. Depending on the strain and the environment it is found in, *Streptomyces* can generate different antimicrobial metabolites. Streptomycin, tetracycline, erythromycin, and chloramphenicol are a few of the antimicrobial metabolites that are most frequently generated. However, numerous other substances have been found and investigated for their antimicrobial qualities, and more substances are continually being found (Donal *et al.*, 2022).

An agent's capacity to eradicate or halt the development of microorganisms is known as antimicrobial activity. Microorganisms such as fungus, bacteria, and others may be a part of this. Numerous elements, including the agent's concentration, the existence of foreign materials, and host defence mechanisms, can have an impact on antimicrobial activity. Antibiotics for bacteria and antifungals for fungi are two examples of antimicrobial compounds that can be categorised based on the microorganisms they combat. Agents that can destroy microorganisms include antimicrobial peptides, which are affected by things like peptide length and net charges. The antibacterial action of silver nanoparticles against particular bacterial strains has also been investigated (Urnukhsaikhan *et al.*, 2021).

2.7.2 Antioxidant Potential:

Antioxidants are chemicals that can stop or slow down some kinds of cell damage brought on by free radicals. Free radicals are unstable molecules that have the potential to harm cells and play a role in the emergence of illnesses. By donating some of their own electrons to neutralise free radicals, antioxidants can prevent a chain reaction that might otherwise impact other molecules within the cell and other cells throughout the body. Numerous foods, particularly fruits and vegetables, contain antioxidants, and studies have shown that diets rich in these foods are healthy. Antioxidant supplements do not, however, appear to be helpful in preventing disease, according to studies (Antioxidants: In Depth, n.d).

Numerous investigations have revealed the antioxidant potential of the bacterial genus *Streptomyces*. It has been demonstrated that various *Streptomyces* species have antibacterial

and antioxidant properties. *Streptomyces* strains have been investigated as a possible source of antioxidant-active photoprotective metabolites. *Streptomyces* sp. strain MUSC 14's antioxidant capacity was evaluated by comparing its methanolic extract to a variety of antioxidant assays. *Streptomyces* strains have been shown in other research to have antibacterial and antioxidant properties (Rammali *et al.*, 2022).

Antioxidants derived from *Streptomyces* have a high antioxidant activity and may have health benefits. Due to their high productivity in the production of bioactive compounds that are advantageous to human health, *Streptomyces* strains have been widely studied. Strong antioxidant activity against ABTS, DPPH free radicals, and metal chelating activity have been observed in *Streptomyces* sp. strain MUSC 14. A possible source of antioxidants and UV-protective agents, metabolites derived from *Streptomyces* have been found to have the ability to scavenge DPPH radicals. As a result, antioxidants produced from *Streptomyces* are a source of antioxidants and UV-protective agents and can be used therapeutically (Teng-Hern Tan *et al.*, 2019).

Superoxide radical scavenging, ABTS, DPPH free radicals, and metal chelating activity assays are just a few of the antioxidant assays that have been used to evaluate *Streptomyces*-derived antioxidants. By comparing the methanolic extract of the *Streptomyces* sp. strain MUSC 14 to various antioxidant tests, such as ABTS, DPPH free radicals, and metal chelating activity assays, the antioxidant potential of this strain of *Streptomyces* was identified. Due to their ability to scavenge DPPH radicals, metabolites derived from *Streptomyces* have the potential to be used as sources of antioxidants and UV-protective compounds. In order to ascertain their antioxidant capacity and bioactivity, *Streptomyces*-derived antioxidants have been examined using a variety of antioxidant assays (2020).

2.7.3 Anticancer:

Numerous organic substances have been identified as having potential anticancer effects. For instance, it has been discovered that natural bark products have strong antioxidant activity, block proteins that activate cancer cells, activate DNA repair processes, and promote the production of defensive enzymes. An antioxidant called quercetin has also been discovered to play a part in lowering certain malignancies in humans. Additionally, although these discoveries were accidental, certain non-oncology medications have been shown to have anticancer properties. It has also been discovered that several alkaloids, including vinblastine, vincristine, and colchicine, may have anticancer properties. Another class of substances known as furanocoumarins has been shown to trigger numerous signalling pathways that result in apoptosis, autophagy, and antioxidant effects (Winkiel *et al.*, 2022).

Species of *Streptomyces* may possess anticancer effects. It has been shown that *Streptomyces* from mangrove habitats may synthesise a variety of bioactive metabolites, including anticancer substances. *Streptomyces* extracts have been proven in in vitro tests to be cytotoxic to cancer cells. Additionally, *Streptomyces* are a viable source for novel medications due to their ability to create a variety of therapeutically significant bioactive compounds. To completely comprehend the anticancer potential of *Streptomyces* and create efficient therapies, additional study is necessary (Woan-Fei Law *et al.*, 2020).

Different anticancer substances produced from *Streptomyces* have different mechanisms of action. While certain substances have been demonstrated to have antibacterial characteristics that can be strengthened by conjugation or combinatorial treatment, others have been proven to induce apoptosis and cell cycle arrest in cancer cells. Numerous bioactive metabolites, such as antibiotics, immunosuppressive medications, and other physiologically active substances, are known to be produced by *Streptomyces*. To completely comprehend each compound's anticancer capabilities, its unique mode of action must be examined separately. However, the low risk assessment of chemicals generated from *Streptomyces* is optimistic for their prospective application in the treatment of cancer (Pattnaik *et al.*, 2022).

There are various difficulties in turning chemicals from *Streptomyces* into cancer treatments. The intricacy of the biosynthesis pathways for substances generated from *Streptomyces*, which require the expression of several genes and enzymes, is one of the major difficulties. To fully comprehend the potential of these chemicals in the treatment of cancer and to design efficient medicines, more study is required. The pharmaceutical sector also has difficulties in sorting through the numerous *Streptomyces* strains and metabolites to find and isolate the most promising drugs. However, *Streptomyces* is frequently employed as a drug research scaffold, and small molecule therapy has demonstrated promise in the treatment of cancer. To overcome these obstacles and fully realise the promise of molecules generated from *Streptomyces* in cancer treatments, more study and development are required (Christy *et al.*, 2022).

Due to their many bioactivities, which include anticancer qualities, substances produced from *Streptomyces* have the potential to be employed in the treatment of cancer. *Streptomyces* is frequently utilised as a drug research scaffold, and small molecule therapy has showed promise in the treatment of cancer. Compounds produced from *Streptomyces* have been shown to offer therapeutic promise against cancer cells and to have cytotoxic effect against cancer cells. Furthermore, chemicals produced from *Streptomyces* have been

classified as low risk, which is encouraging for their prospective application in the treatment of cancer. The utilisation of substances generated from *Streptomyces* in the treatment of cancer may open up fresh possibilities for cancer treatments and medication repurposing. To fully appreciate the potential of these chemicals and create efficient therapies, more study is necessary (Teng-Hern Tan *et al.*, 2019).

2.7.4 Immunomodulatory:

The capacity to excite human peripheral blood mononuclear cells makes secondary metabolites derived from *Streptomyces calvus* strong immunomodulators. Numerous natural chemicals that can affect immune cell function and control the immune system are also known to be produced by *Streptomyces*. The immunomodulatory potential of *Streptomyces* is an interesting field of research even if there are other kinds of immunomodulators accessible, such as those employed in cell therapy (Sarsenova *et al.*, 2022).

Due to its capacity to create secondary metabolites with immunomodulatory function, *Streptomyces* can be employed in immunotherapy. These metabolites have the ability to modulate immune system function and affect immune cell activity, making them suitable for the development of new medicines. Additionally, the *Streptomyces* bacteria is the source of certain medications used in cancer treatment, including anthracyclines. *Streptomyces* is frequently used as a manufacturing base for pharmaceuticals. *Streptomyces* may thus be employed in the creation of immunotherapeutic medicines for a variety of disorders (Barbuto Ferraiuolo *et al.*, 2021).

Streptomyces is used in immunotherapy as a result of the bacteria's secondary metabolite production. These metabolites can modulate the immune system by affecting the release of antibodies or the activity of immune cells. Bioactive substances generated from *Streptomyces* can boost the immune system's activity or favour one of its components' activities. For instance, the bacteria *Streptomyces* sp. CK4412 produced the immunosuppressive substance tautomycin (TMC), which has a mechanistically different immunosuppressive effect that is 100 times stronger than cyclosporine A. Additionally, it has been demonstrated that some cancer treatment medications, including anthracyclines, which are produced from the *Streptomyces* bacteria, might enhance the body's ability to fight tumours. Consequently, the secondary metabolites of *Streptomyces* are used in immunotherapy to modify the immune system and treat illnesses (Alam *et al.*, 2022).

Due to the secondary metabolites that *Streptomyces* creates, it possesses a number of distinct immunomodulatory activities. These metabolites act as potent immunomodulators

and can activate human peripheral blood mononuclear cells. Bioactive substances generated from *Streptomyces* can boost the immune system's activity or favour one of its components' activities. Tautomycetin (TMC), a substance specifically produced by *Streptomyces* sp. CK4412 to inhibit the immune system, has a mechanistically different immunosuppressive effect that is 100 times stronger than cyclosporine A. *Streptomyces* also generates a wide range of naturally occurring substances with a wide range of structural compositions, including as terpenes, aminoglycosides, glycopeptides, macrolides, tetracyclines, and ansamycins, which have antibacterial, antifungal, antiviral, anti-immune, and anticancer activities. As a result, *Streptomyces* is a prospective option for the creation of new medications due to its diverse array of immunomodulatory capabilities (Mahmoudi *et al.*, 2016).

2.7.5 Enzyme inhibitor/ Inducer activity:

Streptomyces can create substances with glycolytic enzyme inhibitory action as well as -lactamase inhibitors like clavulanic acid as examples of enzyme inhibitors. However, more investigation might be required to identify other instances of enzyme inhibitors made by *Streptomyces* (Viana *et al.*, 2018).

Enzyme inhibitors made by *Streptomyces* have potential uses across several industries. For instance, -lactamase inhibitors made by *Streptomyces*, such as clavulanic acid, are frequently used in clinical settings to lessen the consequences of resistance to -lactam antibiotics. Other bioactive substances with antibacterial, enzyme-inhibitory, antioxidant, and antifungal properties are also known to be produced by *Streptomyces*. The entire scope of possible uses for the enzyme inhibitors generated by *Streptomyces* may require more study (Araújo Viana Marques *et al.*, 2018).

Enzyme inhibitors produced by *Streptomyces* may potentially be used as antifungal, anticancer, and anticancer substances. Additionally, the development of novel antibiotics and bioactive metabolites as well as the enhancement of endogenous product yields may be possible uses for the enzyme inhibitors generated by *Streptomyces*. The entire scope of possible uses for the enzyme inhibitors generated by *Streptomyces* may require more study. As opposed to conventional antibiotics, which directly kill bacteria or other germs, *Streptomyces* enzyme inhibitors target and selectively disrupt the action of enzymes. For instance, -lactamase inhibitors, like clavulanic acid made by *Streptomyces*, function by attaching to and obstructing the activity of -lactamase enzymes, which are produced by some bacteria and may break down -lactam antibiotics, leaving them useless. Neomycin, streptomycin, and chloramphenicol, among other antibiotics made by *Streptomyces*, function

by eradicating bacteria directly, in contrast. Additionally, the development of novel antibiotics and bioactive metabolites as well as the enhancement of endogenous product yields may be possible uses for the enzyme inhibitors generated by *Streptomyces* (Zong *et al.*, 2021).

6. Antiviral Activity:

According to certain investigations, extracts of the genus *Streptomyces* contain antiviral qualities and may one day be used to make antiviral medications. Nevertheless, a study of clinical data on the antiviral effects of macrolides, such as erythromycin, clarithromycin, and azithromycin, did not uncover any conclusive proof that they directly reduce the risk of most viral infections. The *Streptomyces* bacteria generate ara A and tunicamycin among other antiviral substances. *Streptomyces*-derived analogues of antimycin A exhibit antiviral action by impeding the mitochondrial electron transport chain in living things. Beta-lactams, polyketides, glycopeptides, macrolides, tetracycline, aminoglycosides, polyenes, and actinomycins are just a few of the many antimicrobial metabolites that *Streptomyces* is capable of producing. Various chemical compounds, including as butanolites, cyclopeptides, acetyltyramines, hydroxybenzoic acid, sesquiterpenoid, and antimycins, have been discovered in extracts of *Streptomyces* sp. SMU 03, many of which show anti-influenza A virus activity. The antiviral substances produced by *Streptomyces* sp. 1647 include antipain, chymostatin, and -MAPI, all of which have been shown to exhibit a variety of antiviral properties (Al-Ansari *et al.*, 2019).

Different antiviral substances generated from *Streptomyces* work in different ways. For instance, antimycin A analogues from *Streptomyces* block the cellular mitochondria's electron transport chain, which prevents the creation of new pyrimidines. *Streptomyces* also makes ara A and tunicamycin, both of which have been shown to have antiviral effects. *Streptomyces* sp. SMU 03 extracts were chemically profiled, and it was discovered that several of the chemical structures they contain, such as butanolites, cyclopeptides, acetyltyramines, hydroxybenzoic acid, sesquiterpenoid, and antimycins, had anti-influenza A virus activity. By preventing viral attachment, entry, uncoating, genome replication, and several viral enzymes including DNA polymerase, protease, nucleotide reverse transcriptase, and integrase, antiviral substances can target viruses (Raveh *et al.*, 2013).

7. Other biological activity:

A genus of bacteria called *Streptomyces* is renowned for its prodigious capacity to manufacture a wide range of important natural products with substantial biological activity in the fields of medicine, the environment, the food industry, and agronomy. The biggest

producer of antibiotics, *Streptomyces* also produces a broad variety of other bioactive substances, including immunosuppressants, in addition to antibacterial, antifungal, and antiparasitic medications. The generation of lipopeptides with a variety of biological roles, as well as antibacterial and antioxidant capabilities, are only a few of *Streptomyces*' biological activities. *Streptomyces* have a variety of biological properties, including the ability to be cytotoxic, insecticidal, antibiotic, and pancreatic lipase inhibitory. They are also a significant source of bioactive substances for the biotechnology sector, including medicines against common illnesses like cancer and antibiotics utilised in clinical settings. *Streptomyces* are crucial in the creation of rich soil for agriculture because they are engaged in the breakdown of complex organic components in the soil. *Streptomyces* also produces bioactive volatile substances that have anti-inflammatory, antibacterial, and antioxidant effects (Rammali *et al.*, 2022).

Untapped metabolic potential from the *Streptomyces* genomes has recently been discovered thanks to advances in genome sequencing technology. Non-ribosomal lipopeptides are one of the many active natural compounds that may be found in *Streptomyces*. More antimicrobial-resistant organisms have been discovered to be inhibited by insect-associated *Streptomyces* than by soil *Streptomyces* (Otani *et al.*, 2022).

Streptomyces is a significant producer of a wide range of sophisticated natural goods. These bioactive substances are prospective candidates for drug discovery and development because they display a wide variety of biological actions, such as antibacterial, anticancer, anti-inflammatory, and immunosuppressive effects.

Studies have revealed that a number of variables, including the genetic make-up of the *Streptomyces* strains, the composition of the growth medium, and culture conditions, all have an impact on the generation of these beneficial secondary metabolites. Additionally, modifications to these chemicals' biosynthesis processes have been made possible by developments in molecular biology and genetic engineering, which have allowed researchers to find novel bioactive compounds and increase manufacturing yields. Overall, the literature review emphasises the significance of additional study in this area as well as the enormous potential of *Streptomyces* as a source of bioactive secondary metabolites. The discovery and development of these chemicals provide considerable potential for the creation of novel medications to treat a wide range of illnesses and disorders.

CHAPTER 3

AIM AND OBJECTIVES

AIM:

To produce and identify bioactive secondary metabolite from *Streptomyces* strain UP2A9.

OBJECTIVES:

1. Potential *Streptomyces* selection and identification.
2. Screening of antimicrobial activity of *Streptomyces* against bacterial and fungal pathogens.
3. Production, extraction and optimization of secondary metabolites from potential *Streptomyces* strain.
4. Purification and metabolite profiling of bioactive secondary metabolites from potential *Streptomyces* strain.

CHAPTER 4

MATERIALS AND METHODS

1. SELECTION AND IDENTIFICATION OF POTENTIAL *STREPTOMYCES* ISOLATED FROM RHIZOSPHERE SOIL.

1. *Subculturing of the potential Streptomyces strain (UP2A9)*

For subculturing of the potential *Streptomyces* strain (UP2A9) ISP2 media was prepared. To prepare an ISP2 media 0.4g of yeast extract, 1g of malt extract, 0.4g of dextrose, 1g of NaCl and 2g of agar, was added to 100ml of distilled water and made sure that the pH is 7. The media was sterilized by placing it in an autoclave at 121° C for around 30 to 60 minutes. The media was then taken out of the autoclave and let it cool for some time. After cooling, the media was poured into sterile petri dishes and was allowed to solidify. The ISP2 agar plates were labelled by adding the name, date and other necessary information at the bottom of the plates.

An inoculation loop was taken and sterilized by heating it in a flame until it is red hot; allow the loop to cool down to room temperature. Using the sterile inoculation loop, the culture was streaked onto the ISP2 agar plate by using quadrant streaking method. Make sure not to press the loop too hard while streaking as it may damage the agar surface.

The plates were closed and incubated by placing it in an incubator at an appropriate temperature for the potential *Streptomyces* strain to be cultured (usually around 25-30° C). Monitor the growth of the culture every day and observe the formation of the colonies. The potential *Streptomyces* strain UP2A9 will have aerial and substrate mycelium. The mycelium mat is usually white in colour due to the formation of spores.

2. *Morphological characterization*

Morphological characterization of the strain UP2A9 was done with respect to the colony color, the surface of the colony, the texture and gram staining by streaking the colonies on ISP2 plates after incubation at 25-30°C for 5-7 days. Gram's nature of the strain was studied using a standard gram staining procedure:

For this study fresh cultures of the strains were used. Bacterial smear was fixed on the slide by passing quickly over a spirit lamp repeatedly. The smear was dipped in crystal violet solution for 1 minute and air dried. The slide was rinsed off and then dipped in iodine solution for 1 minute. The slide was again rinsed off using tap water and then flooded with decolourizer (Ethanol 70%) for 30 seconds. The slides were rinsed off and let to dry.

The slides were then placed under the light microscope for microscopic examination. The phenotypic study was carried out using light microscopy to determine the shape and size of bacterial cells.

3. Scanning electron microscopy

The morphological characterization of the potential strain UP2A9 was carried out using light and electron microscopy. The sample was prepared using the following method for scanning under a scanning electron microscope:

- The bacterial cells were picked from the plate by a toothpick and spread on adhesive carbon tape placed over a stub.
- The stub was placed in an oven for drying at 80°C overnight and the samples were then gold coated.

4. Molecular characterization

4.1.4.1 Extraction of chromosomal DNA

The chromosomal DNA of the potential *Streptomyces* strain UP2A9 was extracted using solute ready genomic DNA kit. The spores of the culture UP2A9 were inoculated into YEME broth and incubated at 28°C for 5 days in a rotatory shaker at 95 RPM. About 1ml of the overnight culture was taken in a 1.5ml Eppendorf tube and centrifuged at 6000 RPM for 10 minutes. 320µl of solution A (Tris, EDTA, Guanidine thiocyanate) and 80µl of solution B (10% SOS) were added to the pellet and vortexed well till homogenous suspension was obtained. This was incubated in a water bath at 65°C for 15 minutes. It was then cooled to room temperature and 130µl of solution C (Potassium acetate) was added. The tube was inverted gently and incubated for 5 minutes at room temperature. It was centrifuged for 10 minutes at 10,000 RPM. This makes precipitated cell debris and denatured protein from pellets. The supernatant was transferred into a fresh tube that contains DNA and RNA. PT buffer (isopropanol mix) was added in equal volume, gently inverted five times, and incubated for 10 minutes at room temperature. This makes DNA and RNA precipitate. It was again centrifuged at 10,000 RPM for 5 minutes so that the precipitated DNA and RNA forms a pellet. The supernatant was discarded. 500µl of washed buffer (70% ethanol) was added without disturbing the pellet and kept for 1 minute. This solution removes the co-precipitate salts from the pellet. It was centrifuged at 10,000 RPM for 1 minute and the supernatant was discarded. The pellet was air-dried by inverting it in a paper towel for 10 minutes. The pellet was suspended in 100µl of TE buffer and dissolved completely. The quality of the extracted

DNA was checked by loading in 1% agarose gel and 2µl of extracted DNA was used for PCR amplification.

4.1.4.2 PCR Amplification of 16s RNA

The PCR amplification of the 16s ribosomal RNA gene of strain UP2A9 was performed by using two primers 27F 5'-AGAGGATCMTGGCTCAG-3' (forward) and 1492R 5'-TACGGTACCTTGTTACGACTT-3' (reverse) (Gothwal *et al.*,2007) using a thermal cycler. The final volume of the reaction mixture of 20µl contained 50.0 mg of template DNA (1.0µl), 10 pmol (each 2µl) of primer (forward and reverse separately), 10µl PCR master mix (2µl Taq DNA polymerase 10X Taq reaction buffer, 2mM MgCh, 1µl of 10mM dNTPs and PCR additives) and 5.0µl of double-distilled water. The amplification was performed on Thermo-cycler according to the following profile: an initial denaturation (cycle 1) step at 94°C for 3 minutes, followed by (cycle 2) 30 amplification cycles was carried out at 4 step process, (step 1) denaturation at 94°C for 1 minute, (step 2) annealing the primer at 58°C for 1 minute, (step 3) chain extension at 72°C for 1 minute and 30 seconds and a final extension (step 4) at 72°C for 5 minutes. 2% agarose gel was prepared by dissolving 2g of agarose in 100ml of 1X TAE buffer. About 8µl of 6X gel loading dye was mixed to each PCR vial and 500µl of PCR sample was loaded. This was run in electrophoresis at 50V till the dye reaches three-fourth distance and the bands were observed in UV- transilluminator.

3. Agarose gel electrophoresis

2% of agarose gel was prepared and melted using the micro-oven. When the agarose gel temperature was around 60°C, 5µl of ethidium bromide was added. Now the warm agarose solution was slowly poured into the gel platform and was kept undisturbed till the agarose solidified. 1X TAE buffer was poured into submarine gel tank. The gel platform was carefully placed into the tank and the buffer level was maintained at 0.5cm above the gel. The PCR samples were loaded after mixing with the gel loading dye along with 10µl of 100bp DNA ladder. This was run in electrophoresis at 50V till the dye reaches three-fourth distance and viewed in a UV transilluminator and the band pattern was observed.

4. Sequencing of 16S rRNA

Sanger dideoxy chain terminator sequencing was done in an automated DNA sequence. In this fluorescence-based method of DNA sequencing, four reaction mixers were prepared. Each containing only one ddNTP was labelled with four different fluorescent dyes so that only one reaction mix was required. In addition, polymerase enzymes such as dATP, dGTP, dCTP and dTTP were also added. The sequence detection was made by subjecting labelled ddNTP to a single capillary tube. DNA fragments of different colors were separated

by their respective sizes in a single electrophoretic gel. When current was applied to the gel, the negatively charged 16S rRNA fragments were migrated through the pores of the gel toward the positive end and the rRNA fragments were illuminated with a laser beam. The fluorescent dyes were excited and light emitted from different bases of specific wavelengths was recorded by a special recorder. The sequences were read by determining the sequence of the colors emitted from specific peaks as they pass the detector. This information was fed directly to a computer which determined the sequence. A tracing electrogram of emitted light of the four dyes was generated by the computer. The color of each dye represents the different nucleotides. The computer converted the data of emitted light in the nucleotide sequence.

5. *NCBI BLAST analysis*

The closely related 16S rRNA gene sequence of the strain UP2A9 homologous sequence was determined and compared for similarity level with the reference species of *Streptomyces* contained in genomic database banks, using the NCBI BLAST (www.ncbi.nlm.nih.gov) website. After BLAST analysis, a dataset of potential orthologs was prepared by considering those database sequences which had > 99% sequence identity with the query sequence of strain UP2A9.

6. *Phylogenetic characterization*

Phylogenetic and molecular evolutionary analysis of potential strain UP2A9 were conducted using software included in the MEGA version 4 (Takahasi and Omura, 2003) package. The 16S rRNA sequence of the strain was aligned against corresponding nucleotide sequences of representatives of the genus *Streptomyces* retrieved from GenBank. Evolutionary distance matrices were generated and a phylogenetic tree was inferred.

2. SCREENING OF ANTIMICROBIAL ACTIVITY OF *STREPTOMYCES* AGAINST BACTERIAL AND FUNGAL PATHOGENS

1. Antimicrobial activity of potential *Streptomyces* strain UP2A9 against bacterial and fungal pathogens

Sterile petri plates were prepared containing NA media. In a 2ml Eppendorf tube 1ml of NB media was taken and pathogens were inoculated into it using an inoculation loop. The inoculated pathogen was swabbed using a sterile cotton swab onto the NA plates.

Using a sterile cork borer sterile agar plug was cut out from the potential strain UP2A9 culture, then using sterile forceps the agar plugs were placed on the surface of the inoculated fungal and bacterial pathogens plates. Make sure to name the plates with relevant information. Incubate the plates in an incubator at 25-30° C.

After the incubation period of 24 hours the zone of inhibition was measured around each agar plug using a ruler. Based on the result of agar plug assay we can determine the antimicrobial activity of the potential *Streptomyces* strain UP2A9 against the fungal and bacterial pathogens.

Antimicrobial activity for the potential strain UP2A9 was done against 21 pathogens and the pathogens (i.e., *Klebsiella pneumoniae* and *Candida albicans*) against which the potential strain shows the maximum activity is further selected.

Antimicrobial activity can also be done by agar well diffusion method. For agar well diffusion method, the potential strain UP2A9 was inoculated in an ISP2 broth. 1ml of the inoculated broth (UP2A9) was taken in an Eppendorf tube and centrifuged at around 4000 rpm for 10 minutes. The pathogens *Klebsiella pneumoniae* and *Candida albicans* (B34) were inoculated in 1ml of NB media. The inoculated pathogens were swabbed onto the NA plates each. The agar was plugged from the plates in which the pathogens are swabbed using a cork borer. 100µl of the inoculated broth (UP2A9) was added to the wells. The plates were placed in an incubator at 25-30°C.

After the incubation period of 24 hours the zone of inhibition was measured around the wells using a ruler. Based on the result of agar well diffusion assay we can determine the antimicrobial activity of the potential *Streptomyces* strain UP2A9 against the bacterial and fungal pathogens.

4.2.2 Antibiofilm activity

TSB broth was prepared with 1% glucose and was placed in an autoclave. The pathogens were then inoculated in the prepared media and was placed in an incubator for 24 hours.

DAY 1:

4 biofilm forming pathogens were used to perform the antibiofilm activity. The four different pathogens are *E. coli*, *Klebsiella*, *Pseudomonas* and *Acinetobacter*. Four 96 well plates were taken and was labelled with the name of the pathogens and other relevant information. 100µl of the TSB broth was added to all wells. In addition, an extra 100µl of TSB broth was added to column 2 alone. To all the four sides of the 96 well plates water was added. The extract (UP2A9) was added to the first well. From the first well 100µl was taken in a pipette and was transferred to the second well for serial dilution. Again, 100µl was taken from the second well and transferred to the third well and the same process was repeated for well three too. The final concentration was reached up to 25µl/ml. 50µl of the pathogen inoculum was added to all the wells. The plates were then incubated at 37°C for 24 hours.

DAY 2:

After 24 hours i.e., the next day the plates were washed with 1X PBS 2 times and was allowed to air dry completely.

DAY3:

150-180µl of the 0.1% crystal violet was added to all the wells and was left for 30 minutes. It was then again rinsed with 1X PBS 2 times. The wells were then resolubilized with 150-180µl of 95% ethanol and then the OD readings were taken at 570nm.

Biofilm inhibition was calculated using the formula-

% Biofilm inhibition = $\frac{\text{Control} - \text{Test}}{\text{Control}} \times 100$

3.PRODUCTION, EXTRACTION AND OPTIMIZATION OF SECONDARY METABOLITES FROM POTENTIAL *STREPTOMYCES* STRAIN

1. Solid phase extraction

In around 10 petri plates containing ISP2 media, the potential *Streptomyces* strain UP2A9 was sub-cultured. The plates were then placed in an incubator at 25-30°C. The growth of the of the strain was monitored and the formation of the colonies were observed. The potential *Streptomyces* strain UP2A9 will have aerial and substrate mycelium. The mycelium mat is usually white in colour due to the formation of spores. Take a spatula and scrape out the mycelium sheath and cut the agar into small pieces and transfer it to a beaker. Add ethyl acetate to the beaker containing the agar pieces. Now cover the beaker and keep it for 24 hours [This is done because the secondary metabolite present in the agar pieces is absorbed by ethyl acetate]. After 24 hours separate the agar pieces from the solvent (ethyl acetate) and keep the solvent present in the beaker for air drying. After the solvent is dried out fully, scrape out the metabolites settled in the bottom of the beaker using a pipette and small amount of solvent. Transfer the crude extract in an Eppendorf tube. After the extraction process, antimicrobial activity was performed for the crude extract (UP2A9) obtained from solid extraction to test whether the crude contains the secondary metabolite or not.

2. Liquid phase extraction

In a 1000ml flask around 400ml of ISP2 broth was prepared. The media was then placed in an autoclave for sterilization. After autoclaving the broth was kept to cool down to the room temperature. The strain UP2A9 was then inoculated in the ISP2 broth prepared and was placed in a shaking incubator. The antimicrobial activity of the inoculated broth was checked continuously starting from Day 3. The broth was then poured into 50ml Eppendorf tubes and centrifuged at 4000rpm for 20 minutes. The supernatant of the culture (UP2A9) was then mixed with the solvent ethyl acetate at 1:1 proportion. The separation was then done

in a separating funnel and the upper layer containing the bioactive compounds was collected. Finally, the collected upper layer was concentrated to separate the bioactive compounds from the ethyl acetate.

4.3.3 Media optimization – component replacing method (carbon and nitrogen source)

The ISP2 media was prepared by taking 15 conical flasks and in each flask 50ml distilled water was added. In each flask components of ISP2 media were added to the distilled water. In addition to the components of ISP2 an extra component of carbon and nitrogen source such as: soyabean, cellulose, KNO₃, peptone, glucose, raffinose, ASP, mannitol and lactose were added to the media and for some media the pH was changed to: pH0, pH5, pH7, pH9, pH10, pH11 (This is done to maximize the yield by optimizing the composition of the media). The media which was now prepared after optimizing was then placed in an autoclave for sterilization. After autoclaving the media, it was allowed to cool down and then poured into a petri plate. The media was then allowed to solidify. After the media gets solidified, the potential *Streptomyces* strain was streaked using a sterile inoculation loop on each plate. The plates were then labelled with relevant information and then placed in an incubator at 25-30°C. The growth of the strain was monitored every day and the formation of the colonies were observed. Once the growth was observed antimicrobial activity was performed against clinical pathogens *Klebsiella pneumoniae* and *Candida albicans* for all the 15 optimized media with potential strain UP2A9.

4. PURIFICATION AND MOLECULAR NETWORKING OF BIOACTIVE SECONDARY METABOLITES FROM POTENTIAL *STREPTOMYCES* STRAIN

1. Purification of the potential *Streptomyces* strain UP2A9

Purification of the potential *Streptomyces* strain was done by using Thin Layer Chromatographic (TLC) analysis.

2. Thin Layer Chromatography analysis

A TLC sheet/plate was taken which is generally coated of silica gel and alumina; it was cut into a small strip. A small amount of the extract (UP2A9) was spotted onto the TLC sheet using a capillary tube. The extract is usually spotted near the bottom of the sheet. Chromatogram was run by using different solvent systems such as chloroform:ethyl acetate, hexane:ethyl acetate and ethyl acetate:DMSO in different ratio.

The TLC sheet was then placed in the beaker containing the mixture of the solvents. The solvent moves up the sheet by capillary action carrying the extract with it. The solvent front was allowed to move to with a few centimetres of the top of the sheet. The TLC sheet

was removed and allowed to air dry. The separated spots were then visualized by placing the sheet in a UV spectrophotometer or by placing them in a reagent such as iodine.

4.4.3 HPLCMS/MS Analysis and Metabolite profiling

A thermo LTQ Orbitrap XL mass spectrometer coupled to an Agilent model 1100 LC system equipped with a solvent reservoir, a binary bump, an in-line degasser and a refrigerated autosampler was used to analyse the crude extract of UP2A9 using LCMS/MS. MeOH was used as a solvent to record the spectrum by infusion into the ESI source. A gradient elution of H₂O and MeOH both with 0.1% formic acid, running at 200µl/min was used to operate a 5µm Kinetex C18 column (50mm x 2.1mm), maintained at 25°C. The gradient program was as follows: 10% MeOH for 3 min, 10–90% MeOH over 30 min, and 90% MeOH for 3 min. A spray voltage of 5 kV, a capillary temperature of 230 °C, a sheath gas rate of 12 units N₂ (ca. 120 mL/min), and an auxiliary gas rate of 5 units N₂ (ca. 50 mL/min) was used to record data dependent acquisition mode MS spectra in positive ion mode. LCMS/MS scans were obtained for selected ions with collision-induced dissociation (CID) fragmentation, isolation width of 2.0, normalized collision energy of 35, activation Q of 0.250, and activation time of 30 ms.

CHAPTER 5

RESULTS AND DISCUSSIONS

1. SELECTION AND IDENTIFICATION OF POTENTIAL *STREPTOMYCES* ISOLATED FROM RHIZOSPHERE SOIL

1. *Cultural morphology of potential Streptomyces strain UP2A9*

Streptomyces isolated from rhizosphere soil have secondary metabolites in abundance which also consists of various biological activities (Peng *et al.*, 2020).



Fig. 5.1 Potential *Streptomyces* strain UP2A9

The figure (5.1) shows the image of the potential *Streptomyces* strain UP2A9 which was been sub-cultured and grown in an ISP2 media. The potential strain shows an aerial and substrate mycelium. The colonies were white in colour. Likewise, 189 different *Streptomyces* species were isolated from 8 different soil sample which showed white, brown, grey and pink coloured colonies with different morphological types (Dharumadurai *et al.*, 2008).

5.1.2 *Microscopic characters of potential Streptomyces UP2A9*

The figure (5.2) shows the microscopic characters of the potential *Streptomyces* strain UP2A9 which was attained by gram staining using light microscope revealing that it is a gram-positive bacterium.

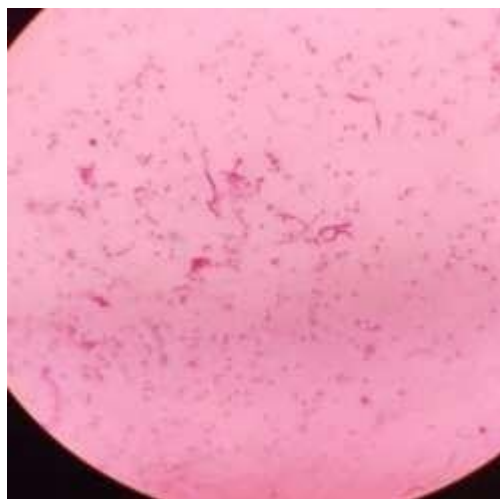


Fig 5.2. Microscopic image of potential UP2A9 by gram staining

5.1.3 SEM analysis of potential *Streptomyces* strain UP2A9

The figure (5.3) shows the SEM image of the potential strain which reveals a long filamentous rod shape bacterium that produced well developed hyphae (between 0.5 μ m-3.0 μ m). It produced aerial and substrate mycelium with spore formation. Also, in a study the *Streptomyces* isolated from banana rhizosphere soil formed spiral chains of rugose ornamented spores and formed aerial mycelia which were straight and long (Chen *et al.*, 2018).

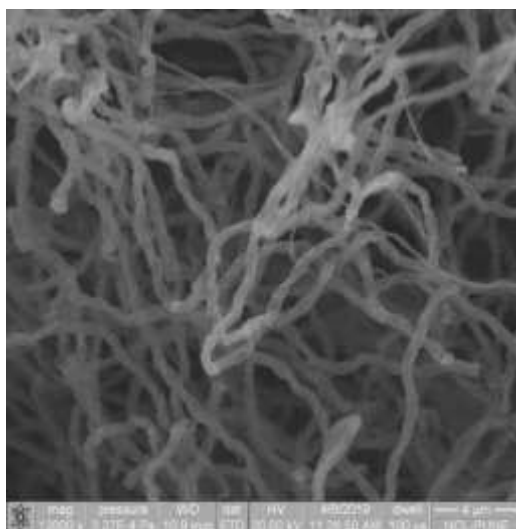


Fig 5.3. SEM image of the potential UP2A9

5.1.4 Contig formation of potential strain sequence

The 16S rRNA sequence was obtained of the potential *Streptomyces* strain. The strain UP2A9 shows closeness to various *Streptomyces* species. Similarly, a study when compared the *Streptomyces* to each other and previously published 16s rRNA sequences of other *Streptomyces* had over 95% identical bases and were highly related.

Streptomycesmaritimus UP2A9 (MT279916)

TTGGATCATGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGA
ACGATGAACCACTTCGGTGGGGATTAGTGGCGAACGGGTGAGTAACACGTGGGC
AATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATAC
TGATCCTCGCAGGCATCTGCGAGGTTTCGAAAGCTCCGGCGGTGCAGGATGAGCC
CGCGGCCTATCAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGACGGGTA
GCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCC
TACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCG
ACGCCGCGTGAGGGATGACGGCCTTCGGGTGTAAACCTCTTTCAGCAGGGAAG
AAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAG
CCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGAATTATTGGGCGTAAAGAGC
TCGTAGGCGGCTTGTACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTG
CAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTA
GCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTG
GGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATA
CCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCAACATTCCACG
TTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCA
AGGCTAAAACTCAAAGGAATTGACGGGGGCCCCGCACAAGCGGCGGAGCATGTG
GCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAA
ACCCTGGAGACAGGGTCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCTG
ACAGCTCGTGTCTGTGAGATGTTGGGTAAAGTCCCGCAACGAGCGCAACCCTTGTC
CCGTGTTGCCAGCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGACCGCCGGGG
TCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGG
GCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGA
GCCAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCAT
GAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCC
GGGCCTTGTACACACCGCCCGTCACGTACGAAAGTCGGTAACACCCGAAGCCG
GTGGCCCAACCCCTTGTGGGAGGGAGCTGTCTGAAGGTGGGACTGGCGATTGGGA
CGAAGTCGTAACAAGGTAACCG

5.1.5 Top BLAST hits

The contig sequence was fed to the NCBI-BLAST, and all the possible hits of the strain along with the name, strain type, accession number and pairwise similarity are viewed (Figure 5.4). The potential *Streptomyces* sp. UP2A9 shows closeness to different *Streptomyces* sp. which retrieved from EzBioCloud.net (Table 5.1).

Table 5.1 Top BLAST hits of UP2A9

S. No	Name	Strain	Accession	Pairwise
1	<i>Streptomyces rochei</i>	NRRL B-2410	MUMD01000370	99.93
2	<i>Streptomyces mutabilis</i>	NBRC 12800	AB184156	99.65
3	<i>Streptomyces djakartensis</i>	NBRC 15409	AB184657	99.44
4	<i>Streptomyces tuius</i>	NBRC 15617	AB184690	99.37
5	<i>Streptomyces violaceorubidus</i>	LMG 20319	AJ781374	99.10
6	<i>Streptomyces tendae</i>	ATCC 19812	D63873	99.03
7	<i>Streptomyces calvus</i>	ISP 5010	AY999780	99.03
8	<i>Streptomyces anandii</i>	NRRL B-3590	AY999803	99.03
9	<i>Streptomyces pilosus</i>	NBRC 12807	AB184161	99.03
10	<i>Streptomyces aurantiogriseus</i>	NBRC 12842	AB184188	98.96
11	<i>Streptomyces virens</i>	NBRC 15901	AB184713	98.96
12	<i>Streptomyces capillispiralis</i>	NBRC 14222	AB184577	98.89
13	<i>Streptomyces aureorectus</i>	NBRC 15896	AB184710	98.89
14	<i>Streptomyces levis</i>	NBRC 15423	AB184670	98.88
15	<i>Streptomyces luteogriseus</i>	NBRC 13402	AB184379	98.88
16	<i>Streptomyces violaceus</i>	NRRL B-2867	KL569104	98.83
17	<i>Streptomyces pseudogriseolus</i>	NRRL B-3288	MUNG01000290	98.83
18	<i>Streptomyces malachitospinus</i>	NBRC 101004	AB249954	98.83
19	<i>Streptomyces tritolerans</i>	DAS 165	DQ345779	98.83
20	<i>Streptomyces rubrogriseus</i>	LMG 20318	AJ781373	98.83

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
✓	Streptomyces maritimus strain UP2A-9 16...	Streptom...	2741	2741	100%	0.0	100.00%	1484	MT279916.1
✓	Streptomyces maritimus strain TIA3 16S r...	Streptom...	2736	2736	100%	0.0	99.93%	1486	MT954062.1
✓	Streptomyces maritimus strain PP3 16S ri...	Streptom...	2730	2730	100%	0.0	99.87%	1489	MH229956.1
✓	Streptomyces maritimus strain MML1710 ...	Streptom...	2728	2728	99%	0.0	99.87%	1493	MH084716.1
✓	Streptomyces sp. strain AN5 16S ribosom...	Streptom...	2726	2726	99%	0.0	99.93%	1479	MH229953.1
✓	Streptomyces maritimus strain SACC-152 ...	Streptom...	2724	2724	100%	0.0	99.80%	1489	MN121692.1
✓	Streptomyces maritimus strain IM20 16S ...	Streptom...	2724	2724	100%	0.0	99.80%	1488	MH021963.1
✓	Streptomyces sp. strain SACC-89 16S ribo...	Streptom...	2724	2724	100%	0.0	99.80%	1495	MW485493.1
✓	Streptomyces maritimus 16S ribosomal R...	Streptom...	2723	2723	99%	0.0	99.80%	1487	AF233338.1
✓	Streptomyces sp. strain PT613 16S riboso...	Streptom...	2721	2721	99%	0.0	99.86%	1498	MZ040755.1
✓	Streptomyces sp. strain PT575 16S riboso...	Streptom...	2721	2721	99%	0.0	99.86%	1495	MZ027494.1
✓	Streptomyces rochei strain UAE1-3 16S ri...	Streptom...	2719	2719	100%	0.0	99.73%	1519	MN795133.1
✓	Streptomyces sp. SGAir0924 chromosom...	Streptom...	2719	16310	100%	0.0	99.80%	7653753	CP027297.1
✓	Streptomyces sp. SS52 chromosome.co...	Streptom...	2719	16310	100%	0.0	99.80%	8184045	CP039123.1
✓	Streptomyces rochei strain APS29 16S rib...	Streptom...	2719	2719	100%	0.0	99.73%	1490	MH021962.1
✓	Streptomyces sp. strain RKBH-B124 16S ri...	Streptom...	2719	2719	100%	0.0	99.73%	1511	KY362382.1
✓	Streptomyces sp. strain KC11 16S riboso...	Streptom...	2719	2719	100%	0.0	99.73%	1497	QQ612716.1
✓	Streptomyces sp. strain F17 16S ribosom...	Streptom...	2719	2719	100%	0.0	99.73%	1495	QQ612715.1
✓	Streptomyces enissocaesilis strain NBF 7...	Streptom...	2719	16305	100%	0.0	99.80%	8343226	CP118027.1
✓	Streptomyces vinaceusdrappus strain AC...	Streptom...	2719	2719	99%	0.0	99.80%	7687527	CP104697.1

Fig. 5.4 BLAST result of the potential strain UP2A9

5.1.6 Phylogenetic characterization of potential *Streptomyces* strain UP2A9

The phylogenetic tree was constructed (Figure 5.5) using the neighbor-joining method using the top 20 closest strains. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value. The analysis involved 13 nucleotide sequences. All

positions containing gaps and missing data were eliminated. There were a total of 1321 positions in the final dataset. Evolutionary analyses were conducted in MEGA11.

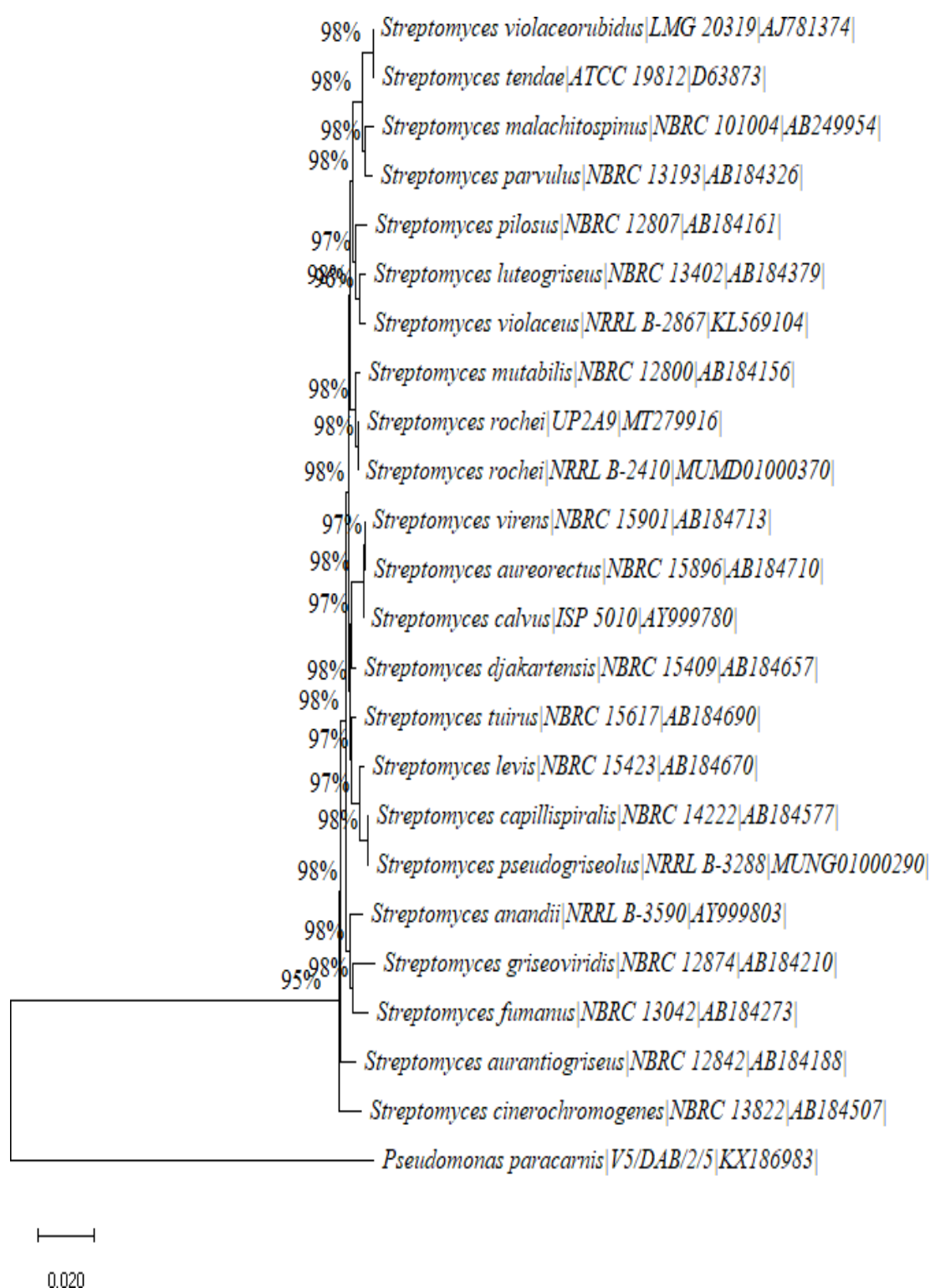


Fig. 5.5 Phylogenetic tree of UP2A9

In a study, Phylogenetic analysis of the 16S rRNA gene data revealed a consistent branching trend. Using 16S rRNA gene sequence data, the phylogenetic tree showed high bootstrap values. Actinobacteria class isolates have been divided into two distinct clusters. Actinobacterial class antagonistic strains were separated out into their own cluster, which had higher bootstrap values of 99% to 100% (Naseer *et al.*, 2017).

2. SCREENING OF ANTIMICROBIAL ACTIVITY OF *STREPTOMYCES* AGAINST FUNGAL AND BACTERIAL PATHOGENS

1. Preliminary antimicrobial screening of potential *Streptomyces* strain UP2A9 against fungal and bacterial pathogens

In this study antimicrobial activity of the potential *Streptomyces* strain UP2A9 isolated from rhizosphere soil was done against 21 sets of pathogens (Table 5.2) for preliminary screening in which the maximum zone of inhibition was obtained for *K. pneumoniae* (figure 5.6) and *Candida albicans* (B34) (Figure 5.7) (bacterial and fungal clinical pathogens) which indicates that the potential strain UP2A9 shows the highest antimicrobial activity against *K. pneumoniae* and *Candida albicans*. Similarly, Al-Ansari *et al.*, 2019 isolated *Streptomyces* sp. from mangrove sediments which produced types secondary metabolites, including antifungal, anticancer, antibacterial and anti-HIV. The study in which the *Streptomyces* was isolated from soil samples from Sammuk mountain, Chonburi province, Thailand had shown antimicrobial activity against human pathogens (Sripreechgasak *et al.*, 2019).

Table 5.2. Antimicrobial activity performed for UP2A9 against 21 pathogens.

S.No	PATHOGENS	ANTIMICROBIAL ACTIVITY(UP2A9) (Zone of inhibition expressed in millimeter in diameter)
1	<i>Escherichia coli</i> ARH	-
2	<i>Klebsiella pneumoniae</i> ARH	19±0.5mm
3	<i>Pseudomonas aeruginosa</i> AGS	-
4	<i>Pseudomonas aeruginosa</i> ARH	-
5	<i>Acinetobacter baumannii</i> MTCC	-
6	<i>Escherichia coli</i> AGS	-
7	<i>Enterococcus faecalis</i> AGS	-
8	<i>Klebsiella pneumoniae</i> AGS	-

9	<i>Staphylococcus aureus</i> 23 AGS	15.6±0.2mm
10	<i>Staphylococcus aureus</i> 13 AGS	-
11	<i>Vibrio harveyi</i> CSU	-
12	<i>Vibrio cholerae</i> CCU	-
13	<i>Bacillus cereus</i> 1 CCU	14.6±0.2
14	<i>Vibrio parahaemolyticus</i> CCU	16.6±0.7
15	<i>Vibrio vulnificus</i> CCU	-
16	<i>Candida albicans</i> (CA C365)	-
17	<i>Candida albicans</i> (NAC10)	-
18	<i>Escherichia coli</i> ESBL U55	-
19	<i>Stenotrophomonas maltophilia</i>	14.6±0.2mm
20	<i>Lactobacillus acidophilus</i> MTCC	-
21	<i>Candida albicans</i> (B34)	18±0.2mm



Fig. 5.6 Antimicrobial activity against *K. pneumoniae*



Fig. 5.7 Antimicrobial activity against *Candida albicans*

5.2.2 Antibiofilm activity

Antibiofilm activity (Figure 5.8) was performed for the potential strain UP2A9 against 4 sets of pathogens i.e., *Acinetobacteria*, *E. coli*, *Pseudomonas* and *K. pneumoniae* in four different concentrations in which the strain showed maximum antibiofilm activity against *K. pneumoniae* (Table 5.3). Similarly, a study performed antibiofilm activity against *K. pneumoniae*, *P. aeruginosa*, *S. aureus*, *C. diphtheriae*, *M. abscessus*, *C. gatti* and *C. neoformans* and their *Streptomyces* sp. demonstrated activity against *P. aeruginosa* (da Fonseca Amorim *et al.*, 2020). Likewise, antibiofilm activity done for the *Streptomyces* sp. GMR22 which was isolated from a soil bacteria showed a high antibiofilm activity against *Candida albicans* which shows that it contained antifungal metabolites (Nirwati *et al.*, 2022).

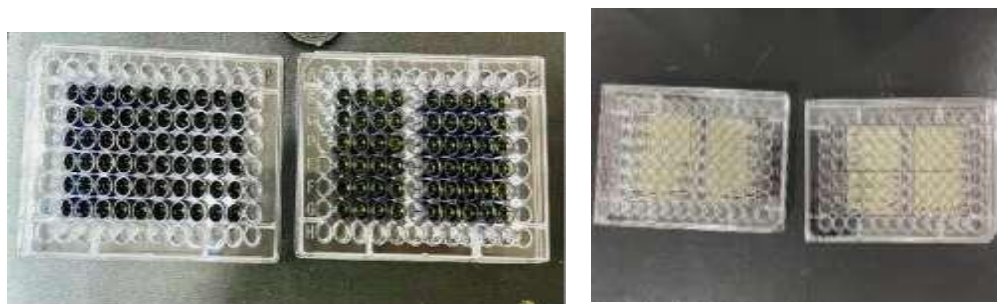


Fig 5.8 Antibiofilm activity

Table 5.3 Antibiofilm activity

<i>E. coli</i>				<i>Klebsiella pneumoniae</i>			
200µl	100µl	50µl	25µl	200µl	100µl	50µl	25µl
-	-	-	53.73%	47.08%	47.3%	50.17%	70.53%

3.PRODUCTION, EXTRACTION AND OPTIMIZATION OF BIOACTIVE SECONDARY METABOLITES FROM POTENTIAL *STREPTOMYCES* STRAIN

1. Effect of solid and liquid fermentation

The extraction of secondary metabolites was done using solid and liquid phase (Table 5.4). The extract obtained from liquid phase fermentation shows the highest antimicrobial activity which depicts that the extract taken from liquid phase fermentation has maximum number of secondary metabolites present in them compared to solid phase extract.

Table 5.4 Antimicrobial activity for solid and liquid phase fermentation

S.No	PHASE	ANTIMICROBIAL ACTIVITY (UP2A9)
------	-------	--------------------------------

		(Zone of inhibition expressed in millimeter in diameter)	
		<i>Candida albicans</i>	<i>Klebsiella pneumoniae</i>
1	Solid phase	19 ±0.5	20.6± 0.5
2	Liquid phase	20.0 ±0.6	24 ±0.5

5.3.2 Media optimization – Component replacing method (Carbon & Nitrogen source)

Optimization of the medium selected for production is generally done to maximize the metabolite yield. During the development of a process, the microbial growth, viability, and total yield of the product can all be considerably improved by the evolution of medium formulations by screening of various carbon and nitrogen sources and their various combinations (Singh *et al.*, 2017).

In this study, Lactose shows maximum antimicrobial activity in the carbon source and peptone shows the highest in the nitrogen source (table 5.5). Likewise, Dextrose was found to be the most suitable carbon source and (NH₄)₂HPO₄ and yeast extract were adequate nitrogen source for antibiotic production for the *Streptomyceskananmyceticus* M27 (Pandey *et al.*, 2011).

Table 5.5 Media optimization antimicrobial activity

S.No	Components added to ISP2 media	ANTIMICROBIAL ACTIVITY(UP2A9) (Zone of inhibition expressed in millimeter in diameter)	
		<i>Candida albicans</i>	<i>Klebsiella pneumoniae</i>
1	pH 0	-	-
2	pH 5	-	-
3	pH 7	-	-
4	pH 9	10.3±0.2	-
5	pH 1	29 ±0.5	29 ±0.5
6	pH 11	20± 0.5	-
7	soyabean	32± 1.3	24.2 ±0.2
8	cellulose	35.6 ±0.2	27.6± 0.2
9	KNO ₃	28 ±0.6	22.2± 0.6
10	peptone	30.2± 0.8	27.6 ±0.2
11	glucose	18.2 ±0.8	15.6± 0.7
12	Raffinose	33 ±2.4	26.3 ±2.4
13	ASP	24.2± 0.2	15.6 ±0.7

14	Mannitol	27.6± 0.2	29.6± 1.0
15	Lactose	36.3± 1.2	32.3± 0.7

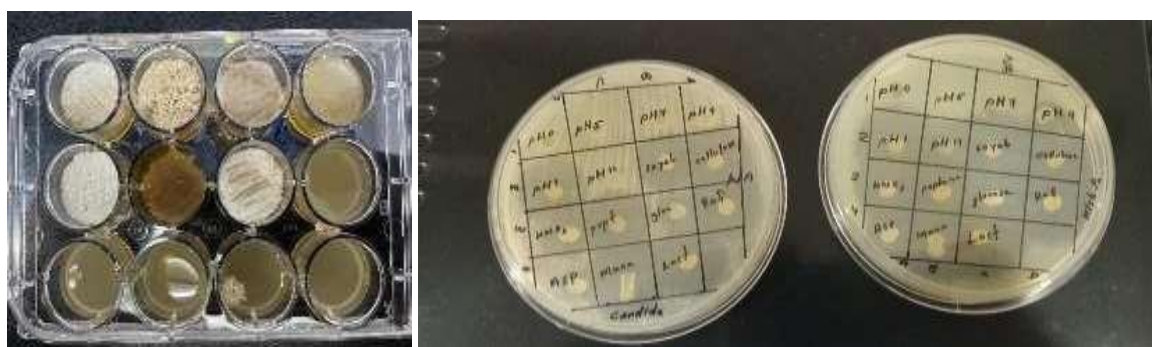


Fig.5.9 Media optimization activity

4.PURIFICATION AND METABOLITE PROFILING OF BIOACTIVE SECONDARY METABOLITES FROM POTENTIAL *STREPTOMYCES* STRAIN

1. TLC analysis

The sample (UP2A9) was tested using various solvent systems in ratios (i.e., hexane : ethyl acetate, ethyl acetate : chloroform using values such as [0.5 : 1.5], [1 : 1], [1.5 : 0.5]) with the aim of getting a solvent system producing a good separation of the extract. The solvent system used for the separation with the mixture of Ethyl acetate and Chloroform in the ratio 1.5:0.5 v/v shows good separation (Figure 5.10). Based on the result we can identify it as a gram-positive bacteria which contains bioactive secondary metabolites which can be used against fungal and bacterial pathogens.

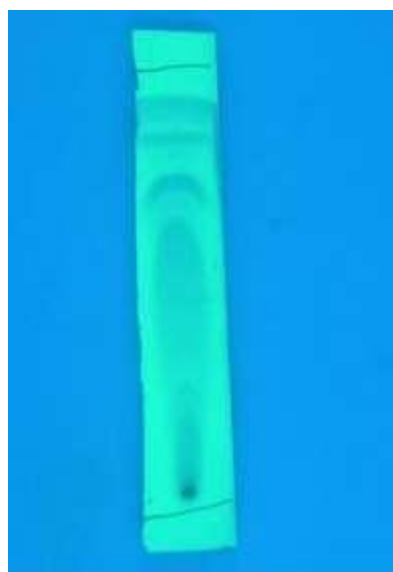


Fig.5.10 TLC Analysis

5.4.2 LCMS/MS Chromatogram for UP2A9

The chromatogram provides a visual representation of the bioactive secondary metabolites that are present in the sample (UP2A9). The ms generated by the mass spectrometer provides additional information on the structure and properties of each compound which includes the molecular weight, formula and other structural features. These can be used to identify and quantify these compounds.

Each peak in the chromatogram represents a specific compound, and the area under the peak corresponds to the quantity of that compound in the sample. The peak height indicates the intensity of the ion detected by the mass spectrometer, which can be used to determine relative abundance of each compound in the sample.

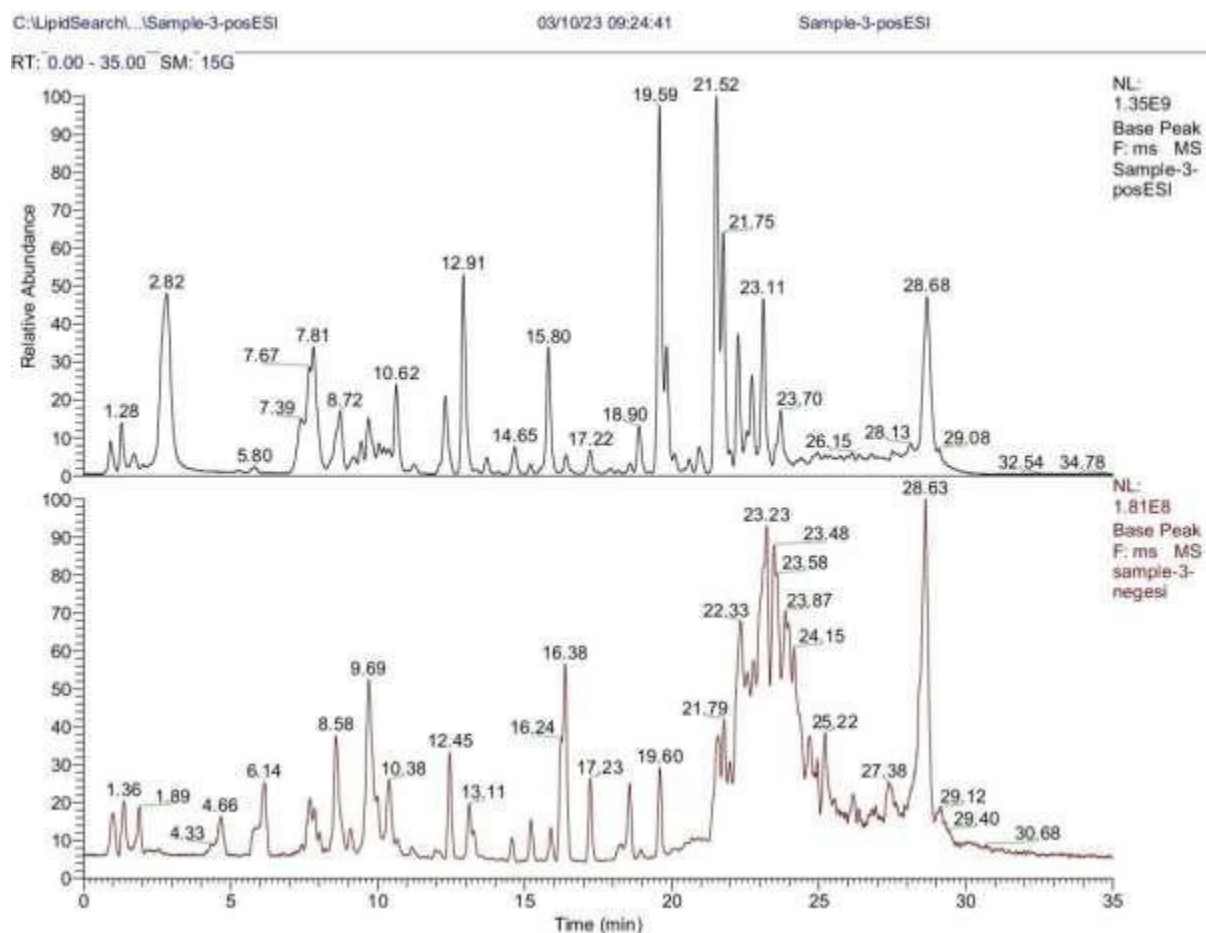


Fig. 5.11 LCMS/MS Chromatogram for UP2A9

5.4.3 List of suspected compounds from potential UP2A9 identified through HRLCMS/MS at negESI mode

The ms data obtained in negESI mode provides information on the molecular weight and fragmentation patterns of the compound in the sample (UP2A9). NegESI mode was used to analyse the compounds that are negatively charged or have acidic functional groups. In this the sample was ionized by removing electrons from the analyte molecules, resulting in

negatively charged ions. Through HRLCMS/MS at negESI mode we could detect more than 90 secondary metabolites in the sample UP2A9. In this study, we obtained compounds such as Deoxycorticosterone 21-glucoside (C₂₄H₄₀O₅) with the highest molecular weight 492.2723 and other compounds such as Cholic acid (C₂₇H₄₀O₅), Prostaglandin F2 β (C₂₀H₃₄O₅), 8-Iso-15-keto-prostaglandin-F2 β (C₂₀H₃₂O₅), etc.

Table 5.6 Compounds from UP2A9 through HRLCMS/MS at negESI mode

NAME	FORMULA	MOLECULAR WEIGHT
Glycolic acid	C ₂ H ₄ O ₃	76.0160
Pyrrole-2-carboxylic acid	C ₅ H ₅ NO ₂	111.0320
Glutaric acid	C ₅ H ₈ O ₄	132.0423
4-Hydroxybenzaldehyde	C ₇ H ₆ O ₂	122.0368
2-Hydroxybenzothiazole	C ₇ H ₅ NOS	151.0092
Salicylic acid	C ₇ H ₆ O ₃	138.0317
Benzoic acid	C ₇ H ₆ O ₂	122.0368
Methylsuccinic acid	C ₅ H ₈ O ₄	132.0423
Cholic acid	C ₂₄ H ₄₀ O ₅	408.2876
4-Methylphenol	C ₇ H ₈ O	108.0575
Azelaic acid	C ₉ H ₁₆ O ₄	188.1049
N-Acetyl-L-tyrosine	C ₁₁ H ₁₃ NO ₄	223.0845
L-(+)-Lactic acid	C ₃ H ₆ O ₃	90.0317
Myristic acid	C ₁₄ H ₂₈ O ₂	228.2089
Stearic acid	C ₁₈ H ₃₆ O ₂	284.2715
16-Hydroxyhexadecanoic acid	C ₁₆ H ₃₂ O ₃	272.2351
trans-10-Heptadecenoic Acid	C ₁₇ H ₃₂ O ₂	268.2402
Ethylmalonic acid	C ₅ H ₈ O ₄	132.0423
Ethyl myristate	C ₁₆ H ₃₂ O ₂	256.2402
Oleic acid	C ₁₈ H ₃₄ O ₂	282.2559
Phenol	C ₆ H ₆ O	94.0419
Palmitoleic acid	C ₁₆ H ₃₀ O ₂	254.2246
Linoleic acid	C ₁₈ H ₃₂ O ₂	280.2402
Suberic acid	C ₈ H ₁₄ O ₄	174.0892
2,4-Dinitrophenol	C ₆ H ₄ N ₂ O ₅	184.0120
3-Hydroxyanthranilic acid	C ₇ H ₇ NO ₃	153.0426

Pentadecanoic acid	C ₁₅ H ₃₀ O ₂	242.2246
Isobutyric acid	C ₄ H ₈ O ₂	88.0524
Apigenin	C ₁₅ H ₁₀ O ₅	270.0528
4-Oxoproline	C ₅ H ₇ NO ₃	129.0426
DL-Mandelic acid	C ₈ H ₈ O ₃	152.0473
Erucic acid	C ₂₂ H ₄₂ O ₂	338.3185
Caffeic acid	C ₉ H ₈ O ₄	180.0423
2-Hydroxycinnamic acid	C ₉ H ₈ O ₃	164.0473
Mono(2-ethylhexyl)phthalate (MEHP)	C ₁₆ H ₂₂ O ₄	278.1518
DL-β-Leucine	C ₆ H ₁₃ NO ₂	131.0946
2-Mercaptobenzothiazole	C ₇ H ₅ NS ₂	166.9863
4-Hydroxy-6-methyl-2-pyrone	C ₆ H ₆ O ₃	126.0317
Prostaglandin A1	C ₂₀ H ₃₂ O ₄	336.2301
3-Methyladipic acid	C ₇ H ₁₂ O ₄	160.0736
Pyruvic acid	C ₃ H ₄ O ₃	88.0160
Resolving E1	C ₂₀ H ₃₀ O ₅	350.2093
5-OxoETE	C ₂₀ H ₃₀ O ₃	318.2195
△17-6-keto prostaglandin F1α	C ₂₀ H ₃₂ O ₆	368.2199
Arachidic acid	C ₂₀ H ₄₀ O ₂	312.3028
Butylparaben	C ₁₁ H ₁₄ O ₃	194.0943
Arachidonic acid	C ₂₀ H ₃₂ O ₂	304.2402
Monobutyl phthalate	C ₁₂ H ₁₄ O ₄	222.0892
α,α-Trehalose	C ₁₂ H ₂₂ O ₁₁	342.1162
2-Hydroxyphenylacetic acid	C ₈ H ₈ O ₃	152.0473
5-Hydroxyindole-3-acetic acid	C ₁₀ H ₉ NO ₃	191.0582
N-Acetyl-L-cysteine	C ₅ H ₉ NO ₃ S	163.0303
Gentisic acid	C ₇ H ₆ O ₄	154.0266
(±)9(10)-DIHOME	C ₁₈ H ₃₄ O ₄	314.2457
(±)9(10)-EpOME	C ₁₈ H ₃₂ O ₃	296.2351
L-Tyrosine	C ₉ H ₁₁ NO ₃	181.0739
L-Phenylalanine	C ₉ H ₁₁ NO ₂	165.0790
Tenofovir	C ₉ H ₁₄ N ₅ O ₄ P	287.0783

Hexadecanedioic acid	C ₁₆ H ₃₀ O ₄	286.2144
3-Hydroxypicolinic acid	C ₆ H ₅ NO ₃	139.0269
Indole-3-acetic acid	C ₁₀ H ₉ NO ₂	175.0633
8-Iso-15-keto-prostaglandin-F2 β	C ₂₀ H ₃₂ O ₅	352.2250
Salsolinol	C ₁₀ H ₁₃ NO ₂	179.0946
15-Keto prostaglandin A1	C ₂₀ H ₃₀ O ₄	334.2144
N-Acetyl-L-phenylalanine	C ₁₁ H ₁₃ NO ₃	207.0895
N-Acetyl-DL-tryptophan	C ₁₃ H ₁₄ N ₂ O ₃	246.1004
N-Acetylvaline	C ₇ H ₁₃ NO ₃	159.0895
Prostaglandin F2 β	C ₂₀ H ₃₄ O ₅	354.2406
Picolinic acid	C ₆ H ₅ NO ₂	123.0320
3-Hydroxybutyric acid	C ₄ H ₈ O ₃	104.0473
Pantothenic acid	C ₉ H ₁₇ NO ₅	219.1107
Glycitein	C ₁₆ H ₁₂ O ₅	284.0685
Ferulic acid	C ₁₀ H ₁₀ O ₄	194.0579
Nordiazepam	C ₁₅ H ₁₁ ClN ₂ O	270.0560
Homogentisic acid	C ₈ H ₈ O ₄	168.0423
Itaconic acid	C ₅ H ₆ O ₄	130.0266
Deoxycorticosterone 21-glucoside	C ₂₇ H ₄₀ O ₅	492.2723
Citrinin	C ₁₃ H ₁₅ O ₅	250.0841
Isovallinic acid	C ₈ H ₈ O ₄	168.0423
N-Acetyl-D-alloisoleucine	C ₈ H ₁₅ NO ₃	173.1052
3-Hydroxybenzoic acid	C ₇ H ₆ O ₃	138.0317
3-Methylglutaric acid	C ₆ H ₁₀ O ₄	146.0579
DL-4-Hydroxyphenyllactic acid	C ₉ H ₁₀ O ₄	182.0579
Hippuric acid	C ₉ H ₉ NO ₃	179.0582
3-Methoxyphenylacetic acid	C ₉ H ₁₀ O ₃	166.0630
(2R)-2,3-Dihydroxypropanoic acid	C ₃ H ₆ O ₄	106.0266
Dodecanedioic acid	C ₁₂ H ₂₂ O ₄	230.1518
2-Isopropylmalic acid	C ₇ H ₁₂ O ₅	176.0685
Citric acid	C ₆ H ₈ O ₇	192.0270
D-(+)-Galactose	C ₆ H ₁₂ O ₆	180.0634

Imidazoleacetic acid	C ₅ H ₆ N ₂ O ₂	126.0429
3,3-Dimethylglutaric acid	C ₇ H ₁₂ O ₄	160.0736
12-oxo Leukotriene B4	C ₂₀ H ₃₀ O ₄	334.2144
Protocatechuic acid	C ₇ H ₆ O ₄	154.0266
N-Acetylalanine	C ₅ H ₉ NO ₃	131.0582
Trolox	C ₁₄ H ₁₈ O ₄	250.1205
DL-Malic acid	C ₄ H ₆ O ₅	134.0215
2-Hydroxyphenylalanine	C ₉ H ₁₁ NO ₃	181.0739
Porphobilinogen	C ₁₀ H ₁₄ N ₂ O ₄	226.0954
Thymidine	C ₁₀ H ₁₄ N ₂ O ₅	242.0903

5.4.4 List of suspected compounds from potential UP2A9 identified through HRLCMS/MS at posESI mode

The ms data generated in posESI mode provides information on the molecular weight and fragmentation patterns of the compounds in the sample. There were more than 150 compounds detected in the potential UP2A9. We obtained Limonin (C₂₆H₃₀O₈) with highest molecular weight and other compounds such as Flavine mononucleotide (C₁₇H₂₁N₄O₉P), Prunin (C₂₁H₂₂O₁₀), Nandrolone glucuronide (C₂₄H₃₄O₈), etc.

Based on LC-MS/MS investigations, substances having antifungal action were found to include N-Acetyl-D, L-phenylalanine, ergotamine, amicomacin, fungichromin, and Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl). These substances exhibited strong all-around antifungal efficacy (Awla *et al.*, 2016).

Table 5.7 Compounds from UP2A9 through HRLCMS/MS at posESI mode

NAME	FORMULA	MOLECULAR WEIGHT
D-(+)-Proline	C ₅ H ₉ NO ₂	115.0633
Hexadecanamide	C ₁₆ H ₃₃ NO	255.2562
3-Pyridinol	C ₅ H ₅ NO	95.0371
Nicotinic acid	C ₆ H ₅ NO ₂	123.0320
4-Methyl-5-thiazoleethanol	C ₆ H ₉ NOS	143.0405
Adenosine	C ₁₀ H ₁₃ N ₅ O ₄	267.0968
2-Mercaptobenzothiazole	C ₇ H ₅ NS ₂	166.9863
5'-S-Methyl-5'-thioadenosine	C ₁₁ H ₁₅ N ₅ O ₃ S	297.0896
Stearoyl ethanolamide	C ₂₀ H ₄₁ NO ₂	327.3137

Trans-4-Hydroxyl-L-proline	C ₅ H ₉ NO ₃	131.0582
Pyrrole-2-carboxylic acid	C ₅ H ₅ NO ₂	111.0320
Isoleucine	C ₆ H ₁₃ NO ₂	131.0946
L-Phenylalanine	C ₉ H ₁₁ NO ₂	165.0790
2-Methyl-S-benzothiazole	C ₈ H ₇ NS ₂	181.0020
N-Acetyl-L-tyrosine	C ₁₁ H ₁₃ NO ₄	223.0845
Crotonic acid	C ₄ H ₆ O ₂	86.0368
Glyciten	C ₁₆ H ₁₂ O ₅	284.0685
2-(Methylthio) benzothiazole	C ₈ H ₇ NS ₂	181.0020
4-Methoxycinnamic acid	C ₁₀ H ₁₀ O ₃	178.0630
Picolinic acid	C ₆ H ₅ NO ₂	123.0320
Leucine	C ₆ H ₁₃ NO ₂	131.0946
Norharman	C ₁₁ H ₈ N ₂	168.0687
Prunin	C ₂₁ H ₂₂ O ₁₀	434.1213
Limonin	C ₂₆ H ₃₀ O ₈	470.1941
Nootkatone	C ₁₅ H ₂₂ O	218.1671
L-Norleucine	C ₆ H ₁₃ NO ₂	131.0946
Flavine mononucleotide	C ₁₇ H ₂₁ N ₄ O ₉ P	456.1046
8-Hydroxyquinoline	C ₉ H ₇ NO	145.0528
L-Isoleucine	C ₆ H ₁₃ NO ₂	131.0946
2-Hydroxybenzothiazole	C ₇ H ₅ NOS	151.0092
Caprolactam	C ₆ H ₁₁ NO	113.0841
Phloroglucinol	C ₆ H ₆ O ₃	126.0317
Oleamide	C ₁₈ H ₃₅ NO	281.2719
Mono(2-ethylhexylphthalate)(MEHP)	C ₁₆ H ₂₂ O ₄	278.1518
N-Acetyl-DL-tryptophan	C ₁₃ H ₁₄ N ₂ O ₃	246.1004
Indole-3-acetic acid	C ₁₀ H ₉ NO ₂	175.0633
Maltol	C ₆ H ₆ O ₃	126.0317
3-Methylhistidine	C ₇ H ₁₁ N ₃ O ₂	169.0851
BiochaninA	C ₁₆ H ₁₂ O ₅	284.0685
N-(-acetamidopentyl)acetamide	C ₉ H ₁₈ N ₂ O ₂	186.1368
D-(+)-Pyroglutamic acid	C ₅ H ₇ NO ₃	129.0426

D-Sphingosine	C ₁₈ H ₃₇ NO ₂	299.2824
Sphingosine (d18:1)	C ₁₈ H ₃₇ NO ₂	299.2824
Nicotinuric acid	C ₈ H ₈ N ₂ O ₃	180.0535
Skatole	C ₉ H ₉ N	131.0735
N,N-Dimethylsphingosine	C ₂₀ H ₄₁ NO ₂	327.3137
L-Pyroglutamic acid	C ₅ H ₇ NO ₃	129.0426
Wogonin	C ₁₆ H ₁₂ O ₅	284.0685
2-Anisic acid	C ₈ H ₈ O ₃	152.0473
Cis-4-Hydroxy-D-proline	C ₅ H ₉ NO ₃	131.0582
D-(+)-Maltose	C ₁₂ H ₂₂ O ₁₁	342.1162
Adenine	C ₅ H ₅ N ₅	135.0545
3-(2-Hydroxyethyl)indole	C ₁₀ H ₁₁ NO	161.0841
Tropine	C ₈ H ₁₅ NO	141.1154
5-Hydroxyindole-3-acetic acid	C ₁₀ H ₉ NO ₃	191.0582
Trans-3-Hexenoic acid	C ₆ H ₁₀ O ₂	114.0681
6-Methylquinoline	C ₁₀ H ₉ N	143.0735
Trans-3-Indoleacrylic acid	C ₁₁ H ₉ NO ₂	187.0633
Monobutyl phthalate	C ₁₂ H ₁₄ O ₄	222.0892
Proline	C ₅ H ₉ NO ₂	115.0633
Betaine	C ₅ H ₁₁ NO ₂	115.0633
4-Hydroxy-6-methyl-2-pyrone	C ₆ H ₆ O ₃	126.0317
Tenofovir	C ₉ H ₁₄ N ₅ O ₄ P	287.0783
Oleoyl ethanolamide	C ₂₀ H ₃₉ NO ₂	325.2981
(+)-Aphidicoline	C ₂₀ H ₃₄ O ₄	338.2457
Nandrolone glucuronide	C ₂₄ H ₃₄ O ₈	450.2254
Docosahexaenoic acid	C ₂₂ H ₃₂ O ₂	328.2402
2,3-Dinor-11β-prostaglandinF2	C ₁₈ H ₃₀ O ₅	326.2093
2,3-dinor-8-iso ProstaglandinF2	C ₁₈ H ₃₀ O ₅	326.2093
Valine	C ₅ H ₁₁ NO ₂	117.0790
Norfentanyl	C ₁₄ H ₂₀ N ₂ O	232.1576
(+)-Maackiain	C ₁₆ H ₁₂ O ₅	284.0685
Caffeine	C ₈ H ₁₀ N ₄ O ₂	194.0804

Octyl hydrogen phthalate	C ₁₆ H ₂₂ O ₄	278.1518
Lithocholic acid	C ₂₄ H ₄₀ O ₃	376.2977
L-Tyrosine	C ₉ H ₁₁ NO ₃	181.0739
Piceatannol	C ₁₄ H ₁₂ O ₄	244.0736
3-Hydroxyl-L-proline	C ₅ H ₉ NO ₃	131.0582
5-Aminovaleric acid	C ₅ H ₁₁ NO ₂	117.0790
Vasicinone	C ₁₁ H ₁₀ N ₂ O ₂	202.0742
Lapachol	C ₁₅ H ₁₄ O ₃	242.0943
3-(3,4,5-Trimethoxyphenyl)propanic acid	C ₁₂ H ₁₆ O ₅	240.0998
2-Phenylglycine	C ₈ H ₉ NO ₂	151.0633
3-Hydroxy-4-methoxy-9h-xanthen-9-one	C ₁₄ H ₁₀ O ₄	242.0579
Methyl 2-furoate	C ₆ H ₆ O ₃	126.0317
N-(2-hydroxyphenyl)acetamide	C ₈ H ₉ NO ₂	151.0633
Theophylline	C ₇ H ₈ N ₄ O ₂	180.0647
Docosatrenoic acid	C ₂₂ H ₃₈ O ₂	334.2872
Androsterone	C ₁₉ H ₃₀ O ₂	290.2246
DL-Tryptophan	C ₁₁ H ₁₂ N ₂ O ₂	202.0899
Vitexin	C ₂₁ H ₂₀ O ₁₀	432.1056
N-Acetylsphingosine	C ₂₀ H ₃₉ NO ₃	341.2930
Etiocholanolone	C ₁₉ H ₃₀ O ₂	290.2246
Heptadecanoic acid	C ₁₇ H ₃₄ O ₂	270.2559
Kynurenic acid	C ₁₀ H ₇ NO ₃	189.0426
Deisopropylatrazine	C ₅ H ₈ CIN ₅	173.0468
Tyrosylalanine	C ₁₂ H ₁₆ N ₂ O ₄	252.1110
N-Oleoyl dopamine	C ₂₆ H ₄₃ NO ₃	417.3243
Guanidinosuccinic acid	C ₅ H ₉ N ₃ O ₄	175.0593
Cholest-4-en-3-one	C ₂₇ H ₄₄ O	384.3392
11-Oxoetiocholanolone	C ₁₉ H ₂₈ O ₃	304.2038
Pyridoxamine	C ₈ H ₁₂ N ₂ O ₂	168.0899
Alanyltyrosine	C ₁₂ H ₁₆ N ₂ O ₄	252.1110

Indole-3-acrylic acid	C ₁₁ H ₉ NO ₂	187.0633
(+/-)5(6)-EET Ethanolamide	C ₂₂ H ₃₇ NO ₃	363.2773
L-Tryptophan	C ₁₁ H ₁₂ N ₂ O ₂	204.0899
Scopoletin	C ₁₀ H ₈ O ₄	192.0423
Linoleoyl ethanolamide	C ₂₀ H ₃₇ NO ₂	323.2824
7-hydroxy-6-methoxy-2H-chromen-2-one	C ₁₀ H ₈ O ₄	192.423
Methyldienolone	C ₁₉ H ₂₆ O ₂	286.1933
Cortisol	C ₂₁ H ₃₀ O ₅	362.2093
Pyridoxine	C ₈ H ₁₁ NO ₃	169.0739
Estrone	C ₁₈ H ₂₂ O ₂	270.1620
5-Hydroxyindole	C ₈ H ₇ NO	133.0528
Khellin	C ₁₄ H ₁₂ O ₅	260.0685
2-Oxindole	C ₈ H ₇ NO	133.0528
Dehydroepiandrosterone (DHEA)	C ₁₉ H ₂₈ O ₂	288.2089
Androstenedione	C ₁₉ H ₂₆ O ₂	286.1933
Cannabidiol	C ₁₉ H ₂₆ O ₂	286.1933
Arachidonic acid	C ₂₀ H ₃₂ O ₂	304.2402
Glycyl-L-leucine	C ₈ H ₁₆ N ₂ O ₃	188.1161
2-Amino-1,3,4-octadecanetriol	C ₁₈ H ₃₉ NO ₃	317.2930
(+)-ar-Tumerone	C ₁₅ H ₂₀ O	216.1514
1-Stearoylglycerol	C ₁₂ H ₄₂ O ₄	358.3083
2'-Deoxyadenosine	C ₁₀ H ₁₃ N ₅ O ₃	251.1018
phenethylamine	C ₈ H ₁₁ N	21.0891
4-Methylumbelliferyl	C ₁₆ H ₁₆ O ₉	352.0794
Octadec-9-ynoic acid	C ₁₈ H ₃₂ O ₂	280.2402
4,7,8-trimethoxyfuro[2,3-b]quinoline	C ₁₄ H ₁₃ NO ₄	259.0845
Psoralen	C ₁₁ H ₆ O ₃	186.0317
1,2-dihydroxyheptadec-16-yn-4-yl acetate	C ₁₉ H ₃₄ O ₄	326.2457
α-Hydroxyhippuric acid	C ₉ H ₉ NO ₄	19.0532
2-(2-acetyl-3,5-dihydroxyphenyl)acetic	C ₁₀ H ₁₀ O ₅	210.0528

acid		
3,14-dihydro-15-keto-tetranor Prostaglandin E2	C ₁₆ H ₂₆ O ₅	298.1780
13,14-dihydro-15-keto-tetranor Prostaglandin E2	C ₁₆ H ₂₆ O ₅	298.1780
7-(2-aminophenyl) heptanoic acid	C ₁₃ H ₁₉ NO ₂	221.1416
Cytisine	C ₁₁ H ₁₄ N ₂ O	190.1106
Epicatechin	C ₁₅ H ₁₄ O ₆	290.0790
Ethyl oleate	C ₂₀ H ₃₈ O ₂	310.2872
Nicotinamide	C ₆ H ₆ N ₂ O	122.0480
Valylproline	C ₁₀ H ₁₈ N ₂ O ₃	214.1317
Palmitic Acid	C ₁₆ H ₃₂ O ₂	256.2402
Estrone	C ₁₈ H ₂₂ O ₂	270.1620
Catechin	C ₁₅ H ₁₄ O ₆	290.0790
Cordycepin	C ₁₀ H ₁₃ N ₅ O ₃	251.1018
Polygodial	C ₁₅ H ₂₂ O ₂	234.1620
2-Hydroxyphenylalanine	C ₉ H ₁₁ NO ₃	181.0739
2-Isobutyl-3-methoxypyrazine	C ₉ H ₁₄ N ₂ O	166.1106
thymine	C ₅ H ₆ N ₂ O ₂	126.0429
3-benzyl-4-hydroxy-5-(4- hydroxyphenyl)-2,5-dihydroflurane-2- one	C ₁₇ H ₁₄ O ₄	282.0892
Imidazoleacetic acid	C ₅ H ₆ N ₂ O ₂	126.0429
2-Hydroxyhippuric acid	C ₉ H ₉ NO ₄	195.0532
Apocynin	C ₉ H ₁₀ O ₃	166.0630
Kanosamine	C ₆ H ₁₃ NO ₅	179.0794
Pilocarpine	C ₁₁ H ₁₆ N ₂ O ₂	208.1212
Genistein	C ₁₅ H ₁₀ O ₅	270.0528
Northiaden	C ₁₈ H ₁₉ NS	281.1238
Acetophenone	C ₈ H ₈ O	120.0575
Apigenin	C ₁₅ H ₁₀ O ₅	270.0528
9-Oxo-ODE	C ₁₈ H ₃₀ O ₃	294.2195

Picrotoxin	C ₁₅ H ₁₆ O ₆	292.0947
Emodin	C ₁₅ H ₁₀ O ₅	270.0528
Tetranor-12R-HETE	C ₁₆ H ₂₆ O ₃	266.1882
9(S)-HOTrE	C ₁₈ H ₃₀ O ₃	292.2195
3-(4-Hydroxy-3-methoxyphenyl propanoic acid	C ₁₀ H ₁₂ O ₄	196.0736
N-Acetyl-D-galactosamine	C ₈ H ₁₅ NO ₆	221.0899
3-(2,3-dihydroxy-5-methylphenoxy)-5- methylbenzene-1,2-diol	C ₁₄ H ₁₄ O ₅	79.9
B-Asarone	C ₁₂ H ₁₆ O ₃	316.2038
Prostaglandin J2	C ₂₀ H ₃₀ O ₄	334.2144
ProstaglandinH1	C ₂₀ H ₃₄ O ₅	354.2406
Palmitoleic acid	C ₁₆ H ₃₀ O ₂	254.2246
N-Phenylacetylglutam	C ₁₃ H ₁₆ N ₂ O ₄	264.1110
Aloe-emodin	C ₁₅ H ₁₀ O ₅	270.0528
15-epi Prostagladin A1	C ₂₀ H ₃₂ O ₄	336.2301
Tetranor-12(S)-HETE	C ₁₆ H ₂₆ O ₃	266.1882
Leucylproline	C ₁₁ H ₂₀ N ₂ O ₃	228.1474
3,4-Dihydroxyphenylpro pionic acid	C ₉ H ₁₀ O ₄	182.0579
8Z,11Z,14Z-Eicosatrienoic acid	C ₂₀ H ₃₄ O ₂	306.2559
15-Deoxy-12,14-prostaglandin A1	C ₂₀ H ₃₀ O ₃	318.2195
Muramic acid	C ₉ H ₁₇ O ₇	251.1005
L-Valine	C ₅ H ₁₁ NO ₂	117.0790
4-Anisic acid	C ₈ H ₈ O ₃	152.0473
3-phenylpropionitrile	C ₉ H ₉ N	131.0735
Cholecalciferol	C ₂₇ H ₄₄ O	384.3392
L-Threo-3-phenylserine	C ₉ H ₁₁ NO ₃	181.0739
Ferulic acid	C ₁₀ H ₁₀ O ₄	194.0579
WLH	C ₂₃ H ₃₀ N ₆ O ₄	454.2329
N6-Acetyl-L-lysine	C ₈ H ₁₆ N ₂ O ₃	74.7
13,14-dihydro-15-keto Prostaglandin A2	C ₂₀ H ₃₀ O ₄	334.2144

Tetrahydromagnolo l	C ₁₈ H ₂₂ O ₂	270.1620
Anthranilic acid	C ₇ H ₇ NO ₂	137.0477
5,6-Dimethylbenzimidazole	C ₉ H ₁₀ N ₂	146.0844
Zerumbone	C ₁₅ H ₂₂ O	218.1671
Evernicacid	C ₁₇ H ₁₆ O ₇	332.0896
Trigonelline	C ₇ H ₇ NO ₂	137.0477
Sedanolid	C ₁₂ H ₁₈ O ₂	194.1307
Isoferulic acid	C ₁₀ H ₁₀ O ₄	194.0579
5-OxoETE	C ₂₀ H ₃₀ O ₃	318.2195
3,4,5-Trihydroxy-6-methoxy-2-yl 2-(methylamino) benzoate	C ₁₄ H ₁₉ NO ₆	297.1212
Norepinephrine	C ₈ H ₁₁ NO ₃	169.0739
Obscurolide A1	C ₁₅ H ₁₇ NO ₅	291.1107
1,4-dihydroxyheptadec-16-en-2-yl acetate	C ₁₉ H ₃₆ O ₄	328.2614
Prostaglandin E1	C ₂₀ H ₃₄ O ₅	354.2406

CHAPTER 6

SUMMARY AND CONCLUSION

Streptomyces produces over two-third of the naturally occurring antibiotics that are clinically effective. Natural products from members of the phylum actinobacteria are promising source of antibiotics. This study aims on production and identification of bioactive secondary metabolites from *Streptomyces*. The potential strain UP2A9 was sub cultured and the morphology were studied using gram staining and scanning electron microscopy which revealed long rod-shaped filamentous bacteria. Molecular identification of the strain was confirmed the strain UP2A9 identified as *Streptomyces maritimus*. A preliminary screening of antimicrobial activity of *Streptomyces* UP2A9 was performed against 21 set of pathogens and it showed maximum antimicrobial activity against *Klebsiella pneumoniae* and *Candidaalbicans* with the zone of inhibition of $17.6\pm1.2\text{mm}$ and $16.6\pm1.7\text{mm}$, respectively. Antibiofilm activity for the strain UP2A9 was performed for 4 sets of pathogens i.e., *Acinetobacteria*, *E. coli*, *Klebsiella pneumoniae* and *Pseudomonas* and it showed a broad range of activity against *K. pneumoniae*. Production, extraction and optimization was done; the bioactive secondary metabolites were extracted using solid and liquid extraction in which the extract obtained from the liquid extraction shows the maximum antimicrobial activity against *Klebsiella pneumoniae* and *Candida albicans* with the zone of inhibition of 24mm and 20mm, respectively. Then, the production media was optimized using 15 different carbon, nitrogen source and the maximum antimicrobial metabolites were produced in the media with the combination of lactose as the carbon source and peptone as the nitrogen source. Purification of the bioactive secondary metabolite from the potential strain was done using TLC which shows chromatographic separation and the metabolite profiling was done using LC-MS/MS.

In conclusion, there is a lot of medicinal promise in the production and identification of secondary metabolites from *Streptomyces*. The potential strain obtained can be used for further study in this field of drug and discovery and more potent medications can be created.

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