

BIOACTIVE POTENTIALS OF *Streptomyces* AGAINST GRAM-NEGATIVE PATHOGENS

Submitted in partial fulfillment of the requirements for the award of Master
of Technology degree in Biotechnology

by

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SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **Miss. SHARON BEAULA.V** (41810018) who carried out the project entitled "**BIOACTIVE POTENTIALS OF STREPTOMYCES AGAINST GRAM-NEGATIVE PATHOGENS**" under our supervision from January 2023 to May 2023.

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I SHARON BEAULA V (41810018) hereby declare that the Project Report entitled **“Bioactive potentials of *Streptomyces* against gram-negative pathogens”** done by me under the guidance of **Dr. V. Ramesh Kumar (Internal)** and **Dr. K. Manigundan (External)** in Centre for Drug Discovery and Development at Satyabhama Institute of Science and Technology is submitted in partial fulfillment of the requirements for the award of Master of Technology degree in Biotechnology.

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ACKNOWLEDGEMENT

I am pleased to acknowledge my sincere thanks to the Board of Management of SATHYABAMA for their kind encouragement in doing this project and for completing it successfully. I am grateful to them.

I convey my thanks to Dr. V Ramesh Kumar, Dean, School of Bio and Chemical and Head of the Department, Dept. of Biotechnology for providing me with necessary support and details at the right time during the progressive reviews.

I would like to express my sincere and deep sense of gratitude to my Project Guide Dr. V. Ramesh Kumar and Dr. K. Manigundan for their valuable guidance, suggestions and constant encouragement that paved the way for the successful completion of my project work. I would also like to thank Dr. M. Radhakrishnan, Centre for Drug Discovery and Development, Satyabhama Institute of Science and Technology, for continuous support throughout the project.

I wish to express my thanks to all Teaching and Non-teaching staff members of the Department of Biotechnology and Centre for Drug Discovery and Development, who were helpful in many ways in the successful completion of the project.

ABSTRACT

Streptomyces species have produced a variety of bioactive secondary metabolites with intriguing antimicrobial properties. This study aimed to isolate the bioactive potential secondary metabolites from *Streptomyces* and evaluated their antimicrobial potential against gram-negative pathogens. A preliminary screening of the antimicrobial activity of *Streptomyces* strain UP1A1 was evaluated against 20 sets of pathogens. It showed a broad range of activity against *Klebsiella pneumonia* and *Candida albicans* with a zone of inhibition of $18\pm0.2\text{mm}$ and $20.2\pm0.6\text{ mm}$, respectively. Later, the bioactive secondary metabolites were produced from *Streptomyces* UP1A1 through both solid and liquid phase fermentation. In secondary screening, the crude metabolites of the potential strain UP1A1 showed a maximum range of activity against *Klebsiella pneumonia* and *Candida albicans* with the zone of inhibition of 10 ± 0.4 and $12\pm0.6\text{mm}$, respectively. In addition to antimicrobial activity, the crude extract of UP1A1 also exhibited anti-biofilm activity against *K. pneumonia*. Further, large-scale production of bioactive secondary metabolites from *Streptomyces* UP1A1 was achieved through a 10L laboratory fermenter. Totally, 2.21 g of crude extract was produced from 6 liters of fermentation media. Then, the bioactive secondary metabolites were purified by flash chromatography using the gradient solvent system of ethyl acetate and methanol and evaluated for antimicrobial activity against *Klebsiella pneumonia* and *Candida albicans*. The active fraction was further evaluated for molecular networking and metabolite profiling using LC-MSMS analysis. Molecular networking in GNPS showed numerous interesting bioactive secondary metabolites may be the reason for their antimicrobial potential. Based on the cultural, morphological, and molecular analysis, the potential strain UP1A1 was identified as *Streptomyces maritimus*. Our results showed that the robust secondary metabolism of *S. maritimus* UP1A1 may serve as a potential source for antimicrobial drug development upon further investigation.

Keywords: *Streptomyces*, Antimicrobial, Anti-biofilm, Secondary metabolites, Metabolite profiling, Molecular networking.

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CHAPTER 1

INTRODUCTION

Antimicrobial Resistance (AMR) is the result of bacteria, viruses, fungi, and parasites that have evolved through time and have ceased to respond to antibiotics. This makes infections more difficult to cure and raises the risk of disease spread, serious illness, and death. Antibiotics and other antimicrobial medications lose their effectiveness as a result of drug resistance, and infections become harder or impossible to treat (Wanda *et al.*,2018). One of the top 10 risks to world health, according to the World Health Organisation, is antimicrobial resistance (AMR). Its increase poses a risk of returning the globe to a time when fatal infectious diseases, including pneumonia, TB, and gonorrhea, were considered curable. The most expensive price will be borne by underdeveloped nations. The WHO listed the most serious multidrug-resistant microorganisms for which innovative therapies are urgently required. They belonged to the well-known *Klebsiella pneumonia*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. group, which today accounts for the majority of healthcare-associated infections, so this was no surprise. Most of them were gram gram-negative. (Filipa *et al.*,2020).

A microorganism, often known as a microbe, is a type of living thing that cannot be seen with the naked eye but can be observed under a microscope. They can be classified morphologically into gram-positive and gram-negative. Gram-positive bacteria lack an outer lipid membrane and contain a substantial peptidoglycan layer. Gram-positive bacteria are accurately referred to as monograms when referring to their structure rather than their staining abilities because they do not have an exterior lipid membrane. Gram-positive bacteria have a thick peptidoglycan layer but no outer lipid membrane. Because they lack an external lipid membrane, gram-positive bacteria are correctly referred to as monogrammers when discussing their structure as opposed to their staining capacities(Nicole *et al.*,2019).Gram-negative bacteria have a robust, defence-like exterior. They have a peptidoglycan coating that is significantly thinner than gram-positive bacilli. A protective capsule surrounds gram-negative bacteria. This pill aids in preventing germs from entering white blood cells, which fight infection. Gram-negative bacteria have an outer membrane under the capsule that shields them from some antibiotics, such as penicillin. Endotoxins are harmful compounds that are released when this membrane is damaged. When

gram-negative bacteria are involved in an infection, endotoxins increase the severity of the symptoms (Oliver *et al.*,2002) (Patricia *et al.*,2010).

Infections caused by gram-negative bacteria include meningitis, pneumonia, bloodstream infections, wound infections, and infections at surgical sites. Gram-negative bacteria are growing resistant to the majority of existing antibiotics and to a variety of other medications. These bacteria can pass on genetic material that enables other bacteria to develop drug resistance, and they have the innate ability to discover novel methods to be resistant (Junio *et al.*,2022). In 2019, bacterial infections were responsible for more than 1 in 8 deaths worldwide, with five pathogens being responsible for more than half of these deaths. 7.7 million of the 13.7 million infection-related fatalities predicted by experts for 2019 will be attributable to 33 bacterial infections. *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* accounted for 54.9% of the 7.7 million deaths among the pathogens that were examined (Chris *et al.*,2022).

There are several chemicals currently used to treat antimicrobial-resistant pathogens. The choice of chemical will depend on the specific pathogen and the severity of the infection. Some commonly used chemicals include carbapenems, colistin, tigecycline, and polymyxin. The side effects of using chemicals to treat antimicrobial-resistant pathogens will depend on the specific chemical being used and the individual patient. However, there are some general side effects that may occur which include allergic reactions, liver toxicity, kidney toxicity, and many more side effects (Murray *et al.*,2022).

Natural products (NPs) are defined as chemicals created in nature by living things like plants, animals, insects, sea organisms, and microbes that have pharmacological or biological properties. These organic items are priceless gems that Mother Nature has given us and are important sources of medicines for the treatment and prevention of human ailments. As a result, they play a significant and vital role in creating new drugs (Yang *et al.*,2022). Many of these natural products have antimicrobial properties, which means they can kill or inhibit the growth of microorganisms. The use of microbial natural products for treating AMR pathogens is gaining attention as a potential solution. Microbial natural products have been

shown to be effective against a wide range of microorganisms, including those that are resistant to conventional antibiotics. Additionally, the mechanisms of action of microbial natural products are often different from those of conventional antibiotics, making it less likely that microorganisms will develop resistance (Uddin *et al.*,2021). Therefore, researchers are investigating microbial natural products as potential new sources of antimicrobial agents to combat AMR pathogens. These natural products have the potential to provide novel and effective treatments for infectious diseases caused by resistant microorganisms, helping to alleviate the growing threat of AMR (Vikesland *et al.*,2019).

The genetic ability to create 30 secondary metabolites on average makes *Streptomyces* spp. the most prolific makers of antibiotics. Two-thirds of the natural antibiotics currently used by the general public are produced by this genus, which also generates around 80% of all antibiotics from the phylum Actinobacteria. *Streptomyces* produce a lot of secondary metabolites, but when resources are scarce, they create aerial hyphae that split into spores that can endure harsh conditions. Actinomycetes are Gram-positive bacteria with a linear, GC-rich genome that has been shown to be a significant source of secondary metabolites (SMs) with a wide range of structural diversity and biological characteristics (Lin *et al.*,2021 & Atnas *et al.*,2022). Actinomyceaceae family member *Streptomyces* are known to have the capacity to create secondary metabolites, such as antibiotics, anti-cancer, antioxidants, antimicrobials, and immunosuppressants. *Streptomyces* found in uncharted or poorly studied ecosystems are regarded as novel sources of physiologically active compounds (Ramesh *et al.*,2022).

Streptomyces primarily live in aerobic environments. *Streptomyces*, however, produces aerial hyphae that can split to form spores that can endure harsh conditions and are easily transported to new locations and nutrient sources when conditions are restricted. *Streptomyces* develop a number of extremely useful natural products (NPs) during this phase that is not necessary for growth or reproduction but aid in defending the host against infections (Fathima *et al.*,2022). In our present study, we will observe the bioactive potentials of streptomyces against gram-negative pathogens.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Antimicrobial Resistance Pathogens

One of the major public health issues of the twenty-first century is antimicrobial resistance (AMR), which poses a threat to the effective prevention and treatment of an expanding number of infections caused by bacteria, parasites, viruses, and fungi that are no longer susceptible to the conventional medications used to treat them. AMR is a pressing issue, especially in light of bacterial antibiotic resistance. The necessity for an enhanced and coordinated international effort to manage AMR has long been acknowledged by the World Health Organization (WHO)(Annalisa *et al.*,2015).

The Evolving Threat of Antimicrobial Resistance, published by WHO in 2012, proposes a combination of interventions, including improving antimicrobial use in hospitals and the community, infection prevention and control, supporting the development of new drugs and vaccines that are appropriate, and strengthening health systems and surveillance (Patrizio *et al.*,2015).

Antibiotic resistance genes are extensively distributed in a variety of bacterial communities outside of medical facilities. Determinants that confer self-resistance in bacteria that produce antibiotics in the soil and genes encoding intrinsic resistance mechanisms found in all or the majority of bacteria that do not produce antibiotics are two examples of significant reservoirs of resistance genes that need to be taken into account in order to comprehend the development of antibiotic resistance in pathogens. Although the presence of resistance determinants in environmental and soil bacteria does not endanger human health, their mobilization to new hosts and their expression in various contexts, such as their transfer to plasmids and integrons in pathogenic bacteria, can result in a problem of enormous scope (Elizabeth *et al.*,2018).

Bacterial pathogens face a dilemma with significant morbidity and mortality rates: antibiotic resistance. Gram-positive and gram-negative bacteria with multidrug resistance patterns are challenging to treat and may potentially be resistant to standard medications. There are currently few new antibiotics, few viable preventative strategies, and few effective therapeutics available, necessitating the creation of novel therapeutic approaches and alternative antimicrobial therapies (Krishnan *et al.*,2017).

2.2 Significance of gram-negative pathogens

A variety of virulence factors have been developed by human gram-negative bacterial pathogens to help with their mobility, attachment to epithelial or endothelial cell surfaces, avoidance of host immune responses, activation or inactivation of host cellular pathways, and ultimately the development of clinical disease (Daniel *et al.*,2010). The existence of lipopolysaccharide in the outer leaflet of the bilayer structure sets the gram-negative bacteria's outer membrane apart from other bacterial cell envelopes. Numerous variables influence the lipopolysaccharide's pathogenicity. It serves as the hydrophobic anchor for Gram-negative microorganisms. The complex polymer lipopolysaccharide is a four-part system. Numerous antigenic markers can be identified on o-antigen, a polysaccharide with variable structural properties that is found outside of cells. This is connected to a polysaccharide core that is made up of a backbone in two parts and an exterior core. The cores of various bacteria vary. Due to their hydrophobicity and high molecular weights, macrolide antibiotics are essentially ineffective against gram-negative bacteria. Gram-negative bacteria are highly resistant to the majority of drug classes (Sinosh *et al.*,2018).

The primary cause of resistance to a variety of antibiotics, such as -lactams, quinolones, colistins, and other antibiotics, is the outer membrane of Gram-negative bacteria. To reach their targets, most antibiotics need to penetrate the outer barrier. Gram-negative bacteria can change the outer membrane in any way, including by mutating porins or changing its hydrophobic characteristics, among other things. This can result in resistance. Gram-positive bacteria don't have this crucial layer, so they are less immune to antibiotics than Gram-negative bacteria (Zeinab *et al.*,2020).

Virulence and resistance have a connection that is influenced by the type of bacterial species, the unique mechanisms of virulence and resistance, the ecological niche and surrounding conditions, and the host's immune system. In addition to immune function, diet, age, and stress also have an impact on a host's susceptibility to infection (Virginio *et al.*,2020). The current need for new antibiotics is especially pressing for drug-resistant Gram-negative bacteria. However, efforts to eradicate these gram-negative bacteria continue (Yu *et al.*,2019).

Small compounds produced spontaneously by an organism, including primary and secondary metabolites, are known as natural products. They comprise both extremely tiny compounds like urea and complicated structures like taxon. Natural product synthesis is an intriguing problem inorganic chemistry because they may only be isolable in small quantities, have interesting biological activity, and have intriguing chemical structures (Yang Tang *et al.*,2022).

The main sources of biologically active natural substances have historically been terrestrial plants and microscopic organisms like fungi and bacteria. Terrestrial and aquatic species of plants and microbes, especially those of marine origin, create a wide variety of helpful treatments and lead structures for potential novel pharmaceuticals. Despite the possibility that they did not coevolve with human proteins, natural substances have acquired the ability to interact with biomolecules. A broad variety of proteins and other biological targets are engaged in interactions with natural products. They also function as modulators of biological processes by preventing challenging protein-protein interactions. (Juergen *et al.*,2013).

Natural products have been crucial in the development of new medications, particularly for the treatment of infectious and cancerous diseases as well as for other the rapeutic conditions including multiple sclerosis and cardiovascular disease. Natural products are characterized by enormous scaffold variety and structural complexity. In the hunt for new medications to address protein-protein interactions, the higher stiffness of NPs may be advantageous. In actuality, NPs are a sizable source of oral medicines (Atnas G *et al.*,2021).

Whole fermentation broths or unprocessed extracts of vegetation and marine life were the most prevalent types of natural product samples examined. After a hit in biological screening¹⁴ has been verified, the extract must be fractionated to isolate

the active chemicals, and this process frequently calls for the execution of bioassays at each stage of purification. As a result, the length of time needed to process a natural product hit depends on the quantity of separation cycles necessary to acquire pure chemicals as well as the time needed to carry out the bioassay and report the findings. For preclinical study, it can be challenging to obtain sufficient amounts of a natural substance. It might be difficult if the substance is made from a plant that only grows in a remote tropical region (John *et al.*,2010).

The inability of other drug discovery methodologies to yield many lead compounds in critical therapeutic areas like immunosuppression, anti-infectives, and metabolic illnesses has lately increased interest in natural product research. The lead structures being studied in natural product research may be used as models by the pharmaceutical business when creating new drugs. There is no question that natural products have been and will continue to be important sources of new pharmacological compounds (Mouhssen *et al.*,2013).

2.3 Need for natural products

Today's most serious public health issue, antimicrobial resistance (AMR), is brought on by resistant pathogenic bacteria, as well as fungi, viruses, and parasites. Multidrug-resistant microorganisms, including certain "superbugs," exacerbate the situation. The emergence of multidrug-resistant microorganisms has dramatically grown during the previous few decades. The main causes of the current rise in antibiotic resistance are the indiscriminate and illogical use of antibiotics, bad cleanliness and sanitation, self-medication often, and a lackluster control and surveillance system. More quickly than new antibiotics are discovered and developed, bacterial resistance mechanisms evolve and spread across bacteria (Arpita *et al.*,2022).

These products, which are included in prescription drug regimens, may enhance the therapeutic effects of traditional therapy. Endogenous natural materials with a variety of medicinal characteristics were the main source of medications in prehistoric society. By conducting research in various areas, the breadth and influence of natural goods will significantly expand, and they will keep growing as long as there are prospects for new discoveries and the distribution of excellent work in this area (Rajya *et al.*,2015).

2.4 Microbial natural products

Microbial natural products are substances made by microorganisms like fungi and bacteria that may have medicinal uses (Atnas *et al.*,2021). Natural products have historically been important in the search for new medicines, particularly for cancer and infectious illnesses. A directory that offers free access to natural bacterial and fungal products is called the Natural Products Atlas (Van *et al.*,2021). Only a few bacterial genera have been mined for the new natural products required to combat the urgent danger of antibiotic resistance, despite the significance of microbial natural products for human health. A variety of clinical disorders, including infectious diseases, cancer, and others, are treated with the help of microbial natural products, their semisynthetic derivatives, and their synthetic counterparts (Arnold *et al.*,2013).

Table 2.4- Biological activities of microbial-derived natural products

Name	Origin	Bioactive Activity	References
Erythromycin(A)1	<i>Saccharopolyspora erythraea</i>	Antibacterial	Cobb <i>et al.</i> ,2013
Cephalosporin	<i>Actinoplanes</i>	Antibacterial	Arnold <i>et al.</i> ,2014
Violacein	<i>Pseudoalteromonas</i>	Antiproliferative	Alem <i>et al.</i> ,2020
Nisin(A)5	<i>Lactococcus lactis</i>	Antimicrobial	Hyo <i>et al.</i> ,2019
Salinosporamide A	<i>Salinispora tropica</i>	Anticancer	Ahmed <i>et al.</i> ,2020
Fumiformamide	<i>Aspergillus fumigatus</i>	Antimicrobial	Abdel <i>et al.</i> ,2020
Clostrubin	<i>Clostridium beijerinckii</i>	Antibacterial	Olga <i>et al.</i> ,2019
Alterochromide	<i>Pseudoalteromonas piscicida</i> JCM 20779	Antibacterial	Ross <i>et al.</i> ,2015

2.5 Actinobacterial natural products

Actinobacteria-derived natural products have significant commercial and biotechnological value. In addition to many other physiologically active products, they play a significant part in the creation of several new metabolites, including enzymes, anti-parasitic drugs, herbicides, pesticides, immunological modifiers, antitumor agents, enzyme inhibitors, and vitamins. Additionally, they are essential for the breakdown of various organic wastes since they can create a variety of enzymes like lignans, xylans, chitinase, and pectinase (Waill *et al.*,2021). Actinobacteria create a large majority of the naturally derived substances that are currently used in therapeutic settings, or roughly 70% of the pharmaceutically active secondary metabolites. Streptomyces leads the pack in this regard due to its abundance of bioactive natural compounds (Kannan *et al.*,2014).

Table2.5- Bioactive natural products from Actinobacteria

Name	Actinobacterial genus	Biological activity	References
Cycloheximide	<i>Actinobacterial</i> sp	Antifungal	Fernando <i>et al.</i> ,2021
Sagamilactam	<i>Actinomadura</i> sp. K13-0306	Antiparasitic	Toru <i>et al.</i> ,2016
staurosporine	<i>Micromonospora</i> sp. L-31-CLCO-002	Anti-proliferative	Lin <i>et al.</i> ,2021
Lupinacidins A and B	<i>Micromonospora lupini</i>	Antitumour	Igarashi <i>et al.</i> ,2007
thiocoraline	<i>Actinobacterial</i> sp. L-13-ACM2-092	Antitumor	Jose <i>et al.</i> ,2010
ZHD-0501	<i>Actinomadura</i> sp.	Anticancer	Gopinath <i>et al.</i> ,2016
Enalapril Diketopiperazine	<i>Actinobacterial</i> sp.VUK-10	Antifungal	Usha <i>et al.</i> ,2015
Nonomuric acid	<i>Nonomuraea rhodomycinica</i> NR4-ASC07	Antibacterial	Nantiya <i>et al.</i> ,2017
Maklamicin	<i>Micromonospora</i> sp. GMKU326	Antimicrobial	Adam <i>et al.</i> ,2018

Nomimicin	<i>Actinomadura</i> sp. TP-A0878	Antimicrobial	Joanna <i>et al.</i> , 2018
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2.5.2 Diversity and distribution of actinobacteria:

Actinobacteria of the class family Actinobacteria are widespread around the globe. They can be found in a variety of habitats, including terrestrial, marine, aquatic, airborne, and severe situations, as well as in close proximity to higher macroorganisms (Kurtbole *et al.*,2017). The uniqueness of the environment determines the diversity of actinobacteria (Dinesh *et al.*,2017).

Table -2.5.2 Actinobacteria isolated from different sources

S.n o	Sample	Actinobacteria/Streptomyces genus	Origin	References
1	Soil sample	<i>Streptomyces</i> sp.PWS52	Nammeri national park	Hridip <i>et al.</i> ,2018
2	Himalayan soil	<i>Streptomyces</i> sp	Rangreth of Kashmir Himalaya, India	Pulak <i>et al.</i> ,2019
3	Volcanic soil	<i>Actinobacteria</i> sp	Deception island, antartic	Cheng <i>et al.</i> ,2015
4	Soil sample	<i>Actinobacteria</i> sp.WND1	wasteland and garden habitats of Ghaziabad	Ravi <i>et al.</i> ,2010
5	Bark sample	<i>Streptomyces</i> sp.OTU4	<i>Melia toosendan</i> trees at seven sites in Sichuan, China	Jing <i>et al.</i> ,2018
6	Soil sample	<i>Streptomyces</i> sp.JS26	niche habitats of Vellore district, Tamil Nadu, India.	Nirmala <i>et al.</i> ,2018

7	Water sample	<i>Streptomyces</i> , sp. MW562814	saline lagoons of northwestern Peru	Rene et al.,2021
8	Soil sample	<i>Streptomyces</i> sp.B2	Mangrove ecosystem of Bhitarkanika , Odisha.	Kishore et al.,2014
9	Leaf sample	<i>Streptomyces</i> sp.KCA1	<i>Phyllanthus niruri</i> plant, Trivandru m, Kerala	Ayswarya et al.,2022
10	Sediment sample	<i>Actinobacteria</i> sp	Sarikum lake, Turkey	Aysel et al.,2021
11	Rhizosphere Soil sample	<i>Actinobacteria</i> sp	black pepper growing areas of Wayanad district	Beenav et al.,2021
12	Virgin soil sample	<i>Streptomyces</i> sp	Rift Valley Province of Kenya, Kericho County.	Mercy et al.,2017
13	Sandy sediment	<i>Nocardia</i> sp.	intertidal regions of Ilha do Mel, Paraná, Brazil.	Patricia et al.,2013
14	Sea sediment	<i>Streptomyces</i> <i>variabilis</i> RD-5	Gulf of Khambhat, Gujarat	Mody et al.,2017
15	Soil sample	<i>Streptomyces</i> sp	Ganga river bed, haridwar	Pallavi et al.,2019

16	Pacific oyster <i>Crassostrea gigas</i>	<i>Streptomyces</i> sp	Wadden sea, Wilhelmshaven, north of Germany	Hani et al.,2022
17	Soil sample	<i>Streptomyces</i> sp	Yelagiri Hills (Long. 78.6°E, Lat. 12.6°N) Vellore district,	Mohanraj et al.,2011
18	Soil sample	<i>Streptomyces</i> SAG -85	East side of the Umm Jirsan cave, Saudi Arabia	Suha et al.,2020
19	Soil sample	<i>Actinobacteria</i> sp	low vegetated sand dunes in Merzouga, southeast of Morocco	Lahcen et al.,2019
20	Marine sponges (<i>Haliclona</i> spp.)	<i>Actinobacteria</i> sp	northern parts of west coast of India	Neha et al.,2020

2.5.3 Terrestrial soil ecosystem:

Actinobacteria are frequently the most prevalent soil microbes. They are crucial for the cycling of several different elements in the soil, including carbon, nitrogen, phosphorus, and potassium. As saprophytes, they create a variety of extracellular hydrolytic enzymes that can break down lignin, cellulose, chitin, and other animal and plant polymers. Numerous perennial woody dicotyledonous plants include root symbionts of the actinomycete genus *Frankia* in their nodules. Actinobacteria play important ecological roles in soils, yet their roles are frequently poorly understood. The identification of novel bioactivities, such as new antibiotics, can be aided by a better understanding of the variety and distribution of actinobacteria (Paul et al.,2019). The abundance of reports and isolates found in inland soil and plant

samples shows that new Actinobacteria are emerging from revitalized terrestrial ecosystems (polpass *et al.*,2021).

Actinobacteria found on land have a variety of intriguing antibacterial potentials. Dessert soil is regarded as a particularly harsh terrestrial habitat where only a few organisms, particularly Actinobacteria, frequently use *Micro coleus* as a food supply. Actinobacteria were found in a variety of soil types, including sandy soil, black alkaline soil, sandy loam soil, alkaline desert soil, and subtropical desert soil. *Streptomyces* sp. was the dominant species, followed by *Nocardia*, *Nocardiosis*, and *Actinomycetes* (Gopinath *et al.*,2016).

2.5.4 Aquatic ecosystem:

Actinobacteria are abundant in aquatic areas and can occasionally be brought in by nearby terrestrial habitats through runoff. It is crucial that the types and numbers of Actinobacteria are understood in the context of knowledge about organisms like *Thermoactinomyces* and *Rhodococcus coprophilus*, which are well-known to be reliable indicators of the terrestrial component of Actinobacterial propagules in water and sediments. *Thermoactinomyces* produce its resistant endospores in self-heating composts, overheated feeds, and surface soil. However, these endospores are also capable of being washed into aquatic habitats where they are deposited in mud and sediments. In most aquatic settings, it has been considered that these thermophiles cannot develop at ambient temperatures (Gopinath *et al.*,2016).

There are several actinobacteria in the bacterial populations of different freshwater settings. There is currently a paucity of knowledge on their variety and variation, distribution, use, and ecology. Additionally, it is crucial to look into the variety of Actinobacteria that live in freshwater aquatic habitats in order to find new isolates that may be promising sources of novel biomolecules like antibiotics and other industrial agents (Sahar *et al.*,2021).

2.5.5 Marine ecosystem:

Actinobacteria's distribution in seawater is mostly unknown, and the existence of native marine Actinobacteria in the oceans is still a mystery. This is partly because marine Actinobacteria have not received as much attention as terrestrial Actinobacteria, which until recently were successful sources of new bioactive

chemicals. The fact that resistant spores produced by terrestrial bacteria are known to travel from land to sea, where they can stay available but latent for years, raises further doubts about the presence of native populations of marine Actinobacteria. It is not unexpected that diversification has taken place and distinct marine species are now being recognized in actinobacteria living in the ocean as opposed to their terrestrial counterparts, who endure drastically different environmental conditions. It is yet unknown how diverse marine actinobacteria are and how these organisms have adapted to life in the water by producing secondary metabolites (Jayachandran *et al.*,2013).

The ocean's nutritional content and fluctuating salinity make the marine environment a favorable place to find new actinobacteria. One could anticipate that the organisms that live in the marine environment would be quite diverse given the diversity of nutrients found in the oceans. Diverse nutrients and minerals can be found in the various ocean zones. As a result, the microorganisms that live in different zones typically have metabolites and functions that are advantageous to that zone. It suggests there's a good chance that the marine environment's microbial variety will result in the synthesis of new antibiotics and enzymes (Vaishali *et al.*,2022).

2.6 Streptomyces-derived natural products

Gram-positive, filamentous, spore-forming, and non-motile bacteria belonging to the Phylum Actino-bacteria are found in the genus *Streptomyces* in various environments. *Streptomyces* are the source of about 80% of the bioactive natural compounds that have been investigated for their potential use in medicine or agriculture. Only a few of the natural products with biotechnological significance that are derived from *Streptomyces* include insecticides, pesticides, herbicides, vasoactive substances, neurological agents, immunomodulators (immunosuppressants and immunostimulators), antineoplastics, antifungal, antiviral, and antiparasitic agents. More than half of all antibiotics have reportedly been made by *Streptomyces*. Their reproductive cycle is complex (Rajeev K *et al.*,2021).

In contrast to deep-sea habitats, desert habitat has gained tremendous significance over the past ten years due to the search for such abundant *Streptomyces* sp. in the context of natural product discovery. Due to the unusual environment, it has been

hypothesized that the desert is home to uncommon potential *Streptomyces*, the majority of which have not yet been researched for their potential to treat cancer and newly emerging infectious diseases that are resistant to antibiotics. There is no doubt that volcanic springs are some of the harshest ecosystems on Earth and that they are home to unusual bacteria that could provide novel therapeutic leads (John *et al.*,2019).

Table 2.6 - Bioactive natural products from *Streptomyces*

Name	Origin	Biological activity	References
Cosmomycins	<i>Streptomyces</i> sp. CNT-302	Anticancer	Ahmed <i>et al.</i> ,2020
Nystatin	<i>Streptomyces noursei</i>	Antibacterial	Abdel et al.,2020
Streptomycin	<i>streptomyces griseus</i>	Antibacterial	Shama <i>et al.</i> ,2018
fumiformamide	<i>Streptomyces peucetius</i>	Antimicrobial	Zainab et al.,2021
Kitamycin C	<i>S.antibioticus</i> strain 200–09	Antibacterial	Polpass <i>et al.</i> ,2020
Kiamycin B	<i>Streptomyces</i> sp. M268	Antibacterial	Anjisha <i>et al.</i> ,2020
Actinorhodin	<i>Streptomyces albus</i> S4	Antioxidant	Hitesh <i>et al.</i> ,2021
Antimycin A2b	<i>Streptomyces</i> sp. RJA 3265	Antifungal	William <i>et al.</i> ,2013
Anthraquinone	<i>Streptomyces Aquilis</i>	Cytotoxic	John <i>et al.</i> ,2012
Caboxamycin	<i>Streptomyces</i> sp. NTK 937	Antitumour	Kui <i>et al.</i> ,2019
Avermectins	<i>Streptomyces avermitilis</i>	Anthelmintics	Zheng <i>et al.</i> ,2022

Streptovitacin	<i>Streptomyces</i> sp.ZZ741	Antiproliferative	Binet <i>et al.</i> ,2019
Chaxamycin	<i>Streptomyces</i> sp. strain C34	Antibacterial	Kuiet <i>et al.</i> ,2019
Cineromycin	<i>Streptomyces</i> sp. TN262	Antibacterial	Lobna <i>et al.</i> ,2010
Nyastin	<i>Streptomyces</i> sp. SA32	Antagonistic	Wu <i>et al.</i> ,2021

2.7 Application of streptomyces-derived bioactive compound

2.7.1 Antimicrobial

Six different commercial formulations for the Biocontrol of bacterial and fungal soil diseases contain four *Streptomyces* strains: *S. lydicus* WYEC108, *S. violaceus* niger YCED9, *S. griseoviridis* K61, and *S. saraceticus* KH400. These strains frequently have a wide range of options for managing phytopathogens. They are able to prevent because of their chitinases and glucanases, which have a wide range of effects and are both antifungal and antibacterial (Sandra *et al.*,2021).

The antimicrobial potential of marine actinobacteria has received substantial research. A marine *Verrucosispora* strain produces the new polycyclic polyketide antibiotic known as abyssomicin C. Strong antibacterial activity is exhibited by abyssomicin C against Gram-positive bacteria, including clinical isolates of vancomycin- and multiple-resistant *Staphylococcus aureus*. The development of abyssomicin C or a related compound as an antibacterial agent against infections with medication resistance is possible (Kannan *et al.*,2013). The *Actinomadura* sp. TP-A0878 has the ability to produce the spirotetrone substance nomimicin, which originates from polyketides. Nomimicin had strong antimicrobial action against *Kluyveromyces fragilis*, *Candida albicans*, and *Micrococcus luteus* (Akash *et al.*,2022).

Streptomyces also secrete compounds with antibacterial action, such as bis anthraquinone, carbomycin, glaciapyrroles, and tirandamycins (Harir *et al.*, 2018). Recent research has discovered that the thermophilic *Streptomyces werraensis* MI S.24 3 possesses both antibacterial and cytotoxic activities. It was isolated from an

extremely hostile environment in Egypt. *Streptomyces lanatus* AR2 was able to adapt to a harsh environment, such as a salty wetland, by producing unique polyketides and phenolic compounds with observable bioactivities. These natural products were then produced and have useful biotechnological, pharmaceutical, and medical applications (Saiful *et al.*,2022).

For technical exploitation and commercialization, formulations with a broad spectrum of antimicrobial action, stimulating plant development, a longer shelf life, and disease control under field circumstances would be very appealing (Raul *et al.*,2021)

2.7.2 Anticancer

A study with Malaysian mangrove forests revealed the presence of Malaysia phenolic and pyrrolopyrazine compounds in *Streptomyces* having anticancer activities. *Streptomyces* sp. 211726, isolated from soil, also exerts anticancer compounds, with seven azalomycin F analogs and macrocyclic lactones. *Streptomyces* also produces some well-known anticancer drugs, such as doxorubicin and bleomycin (Arpita *et al.*,2022).

Intense efforts have been made to develop cancer therapeutics, and natural products have been proven to be promising sources of novel anti-cancer drugs. In the past few decades, there have been many reports of anticancer activity in actinobacteria isolated from marine environments. The metabolites of marine actinobacteria are the source of many anticancer chemicals found in marine medications, and they are crucial for identifying medicinal molecules. Many different types of malignant cell types have responded negatively to pure active substances isolated from the marine actinobacterium *Salinispora tropica* (Akash *et al.*,2022, Kannan *et al.*,2013). An important molecule from the anthracycline class is cinerubin B. Daunomycin, doxorubicin, adriamycin, and aranciamycin anhydride are members of a unique class of antibiotics known as anthracyclines that are frequently utilized as antitumor drugs (Gilen *et al.*,2020).

2.7.3 Anti-inflammatory

A *Streptomyces* secondary metabolite called anhydroexfoliamycin has demonstrated antioxidant activities in primary cortical neurons that lessen the

symptoms of neurodegenerative disorders in both in vitro and in vivo models. In the central nervous system, activated microglia play a significant part in neuroinflammation and are linked to neurodegeneration. This substance's effects were comparable to those of the immunosuppressive medication cyclosporine A. As a result, it suggests that anhydroexfoliamycin has the potential to treat neuroinflammation and is a viable strategy for reducing microglia-driven inflammation (Nadia *et al.*,2021).

The host's reaction to a variety of stimuli, such as injury, microbial invasion, and immunological responses, comprises numerous biochemical pathways that are influenced by both internal and external inputs. *Streptomyces* sp. CNB-091 produces the bicyclic dissipation known as salinamides A and B from the jellyfish *Cassiopeia xamachana*. These metabolites are effective anti-inflammatory and antibacterial compounds. Alginate, porphyrin, fucoidan, chitin, and chitin derivatives are examples of marine polysaccharides that are used to reduce allergic reactions. Due to their structure and physicochemical properties, polysaccharides extracted from algae that are predominantly sulfated demonstrate anti-inflammatory action in vitro and in vivo (Abey *et al.*,2022; Se- kwon *et al.*,2013).

2.7.4 Anti-oxidant

To be "antioxidant" is to be "against oxidant." Any agent that delays or prevents deterioration, damage, or destruction brought on by oxidation is considered an antioxidant. By using HPLC-DAD analysis, it was possible to identify a new family of secondary metabolites with an alkyl side chain and a carbazole moiety in *Thulamela pseudospumae* strain Acta 1857 cell extracts. According to their chemical makeup, metabolites were given the name lipo carbazoles and showed potent free radical scavenging abilities (Jayachandran *et al.*,2013; Abey *et al.*,2022). A reddish-pink xanthophyll carotenoid with increased antioxidant capabilities, astaxanthin has two additional oxygenated gatherings on each of its rings. Astaxanthin has a stronger biological activity than other antioxidants because of its capacity to connect across the whole-cell membrane from the inside to the outside. It is mainly got from marine animals. The beneficial health effects of astaxanthin, including photoprotection of the skin and eyes, anti-inflammatory qualities, improved

heart health, and cancer prevention properties, may be due to its potent antioxidant activity (Cedric *et al.*,2020; Ambathi *et al.*,2014).

Diabetes, cardiovascular disease, inflammation and arthritis, cancer, and neurodegeneration are among the illnesses linked to excessive levels of ROS in the body. Additionally, it appears that the food industry is becoming more interested in using antioxidants that are naturally derived rather than artificial antioxidants. With ABTS, DPPH radical scavenging, metal chelating, and ferric-reducing activity, *Streptomyces* sp. strains MUSC 14 is a very good generator of broad-spectrum antioxidant metabolites. Further evidence reveals that the antioxidant activities of extract MUSC 14 were caused by its phenolic content because there was a high association between the phenolic concentration and the antioxidant activities (Jodi *et al.*,2020).

2.7.5 Enzyme inhibitors

On the basis of HAase activity screening, the antibiotic hygromycin, a novel member of the puromycin family, was discovered in the culture extract of a marine-derived *Streptomyces* sp.(Takeshi *et al.*,2014).

Recently, it has been revealed that the chemical butenolide, which was isolated from the bacterium *Micromonospora izumensis*, is a selective inhibitor of β -lactamase, particularly those enzymes produced by Gram-negative bacteria (Bush *et al.*,2010).

By cultivating specific bacteria of the genus *Streptomyces*, particularly *Streptomyces griseoaurantiacus* SANK 63479, a novel chemical with the name "Griseolic Acid" and its pharmaceutically approved salts can be made. Because it inhibits the activity of the cyclic adenosine monophosphate phosphodiesterase enzyme, griseolic acid, and its pharmaceutically approved salts have a variety of pharmacological actions and uses (Akira *et al.*,2020).

2.8 Pharmaceutical drugs derived from actinobacteria under clinical trials

Streptomyces is the source of a few medications currently being tested in clinical trials, including omadacycline (phase 2 trial), eravacycline, a new cephalosporin siderophore, S649266, and combination formulations of cephalosporin-lactam and this siderophore. In order to compare Fosfomycin to piperacillin/tazobactam in the treatment of acute pyelonephritis and chronic urinary tract infection in hospitalized

adults, fosfomycin, which was previously identified from *S. fradiae* in 1969, is currently conducting comparative phase 3 clinical studies (Hefa *et al.*,2018).

The 9-tert-butyl-glycylamido derivative of minocycline, which was obtained from *Streptomyces aureofaciens* as a semi-synthetic product of chlortetracycline, is tigecycline. The antibacterial properties of tigecycline were similar to those of other tetracyclines, but they were stronger against tetracycline-resistant pathogens. Contrary to the oral dosage forms of tetracyclines presently marketed, tigecycline is only used in an injec formulation for clinical use (Raja *et al.*,2011).

Leconotide is a peptide containing 27 residues containing 3 CYS-CYS bonds. It is undergoing phase 1 clinical trials for the treatment of cancer.

Elisidepsin is a cyclic novel peptide derived from marine sources. It is undergoing phase ii clinical trials with the primary evidence of antitumor potency (Harshad *et al.*,2016).

Eravacycline 57, omadacycline 59, and sarecycline 60 are derived from actinobacteria species. These are currently under Phase 3 clinical trials (Butler *et al.*,2014).

2.9 Challenges in research on actinobacterial natural compounds

Rare actinomycetes are found in both aquatic and terrestrial habitats. Their distribution is influenced by environmental parameters such as soil type, pH, humus concentration, and humic acid content properties. Rare actinomycetes may improve the likelihood of finding novel structures, but little is known about their genetics and physiology. The understanding of the distribution of these underutilized groups of microorganisms must be expanded in order to hasten the isolation process (Kavitha *et al.*,2012).

Compared to their terrestrial counterparts, marine uncommon actinobacteria are typically more challenging to culture because of their unique growth requirements or unidentified culture circumstances. By using traditional plate cultivation, only about 2% of bacterial cells may form colonies. A significant portion of them are "viable but not culturable" strains. In recent years, techniques including imitating the natural environment's pH, oxygen gradient, nutritional contents, etc. have been

used. Some formerly VBNC species can now be farmed more successfully thanks to these modifications (Dipesh *et al.*,2017).

In order to find potentially useful bacteria, researchers have mainly relied on empirical methods and have only sampled a small portion of the microbial population present in natural settings. Therefore, methods that accelerate the growth of desired microorganisms in natural samples (enrichment) or remove the undesirable streptomyces propagules and other contaminants from the primary isolation plate must be created and used for the purpose of focusing on isolating specific, rare genera of Actinobacteria (Ramesh *et al.*,2013).

CHAPTER 3

AIM AND OBJECTIVE OF THE PRESENT STUDY

AIM

To study the bioactive potentials of streptomycetes against gram-negative pathogens.

OBJECTIVES

- Identification and preservation of potential *Streptomyces*.
- Antimicrobial screening and bioactive metabolite production from *Streptomyces*.
- Extraction and optimization of bioactive potential *Streptomyces*
- Large-scale production of bioactive potential *Streptomyces*.
- Purification and structural elucidation of bioactive potential metabolites from *Streptomyces* UP1A1.

CHAPTER - 4

MATERIALS AND METHODS

4.1 Culture vessels

Culture vessel that is glass plates, plastic plates, disposable plates, and conical flasks (250,500,1000ml) were used for in vitro studies. Measuring Cylinders (50,100,1000 ml) and test tube tubes were used for measuring and for inoculation. The culture tubes and conical flasks were plugged with non-absorbent cotton with cotton gauze. The petri-dish was covered with para-film to prevent contamination.

4.2 Culture medium

The following media were used to cultivate streptomyces culture. The following media were prepared from the analytical-based chemicals from (Hi Media & co) (Quinyan et al.,2016).

4.2.1 ISP2 agar

The composition of ISP2 agar is as follows for 1000ml (Mohseni Met al.,2013)

Yeast Extract	4g
Malt Extract	10g
Glucose	4g
NaCl	10g
Agar	22g

4.2.2 ISP2 broth

The composition of ISP2 broth is as follows for 1000ml (Mohseni Met al., 2013)

Yeast Extract	4g
Malt Extract	10g
Glucose	4g
NaCl	10g

4.3 IDENTIFICATION AND PRESERVATION OF POTENTIAL *STREPTOMYCES*:

4.3.1 Morphological characterization:

Morphological characterization of strain UP1A1 was done with respect to the colony color, the surface of the colony, the texture of the colony, and gram staining by streaking the colonies on ISP2 plates after incubation at 30°C for 5 days. Gram's nature of the strain was studied using a standard gram staining procedure which consists of the following steps:

- Fresh cultures of the strains were used for this study.
- 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 rinsed off with tap water and then the slide was flooded with decolorizer (Ethanol 70%) for 30 seconds.
- Then the slide was rinsed with tap water and counterstained with safranin for 30 seconds.
- The slides were rinsed off and let to dry.
- Then the slides were 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. The smear from the bacterial culture was prepared on a slide and heat fixed. It was then observed under the light microscope.

4.3.2 Cultural characterization:

Cultural characteristics were studied by inoculating the growth of streptomyces strain UP1A1 into different ISP media such as yeast extract-malt extract agar (ISP-2), Oatmeal-agar (ISP-3), inorganic salts-starch agar(ISP4),glycerol-asparaginase 54 agar(ISP5), peptone - yeast extract -iron agar (ISP6) and Tyrosine agar(ISP7).All

the media were prepared by following the guidelines described by shirling and gottlieb (1996). Platting was done and plates were incubated for 7-10 days at 30°C. Cultural characteristics recorded include the nature of growth, consistency, aerial mass color, presence of reverse side pigment, and details of soluble pigment production if any.

4.3.3 Scanning electron microscopy:

Morphological characterization of strain UP1A1 was carried out using light and electron microscopy. The sample was prepared using the following standard protocol 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 kept in an oven for drying at 80°C overnight and the samples were then gold coated.

4.3.4 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.

4.3.5 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 10mMdNTPs 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.

4.3.6 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.

4.3.7 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 labeled with four different fluorescent dyes so that only one reaction mix was required. In addition, polymerase enzymes such as dATP, dGTP, and dCTP dTTP were also added. The sequence detection was made by subjecting labeled 'labeled'. 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 sequences.

4.3.8 16S rRNA sequence analysis

The closely related 16S rRNA gene sequence of strain UP1A1 homologs 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 UP1A1.

4.3.9 Phylogenetic characterization

Phylogenetic and molecular evolutionary analyses of potential strains UP1A1 were conducted using software included in the MEGA version 4 (Takahashi and Omura, 2003) package. The 16SrRNA sequence of the strains SACC78 was aligned (<http://www.ebi.ac.uk/clustalw>) (Thompson et al., 1994) against corresponding nucleotide sequences of representatives of the genus *Streptomyces* retrieved from GenBank (Boudjella et al., 2006). Evolutionary distance matrices were generated as described by Jukes and Cantor (1969) and a phylogenetic tree was inferred by the Neighbor-joining method (Saitou and Nei, 1987). Tree topologies were evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 resampling of the neighbor-joining data set.

4.3.10 Nucleotide accession num

The partial 16S rRNA nucleotide sequence of the potential actinobacteria strain UP1A1 was deposited at the GenBank database by using the Bankit tool (<http://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank>).

4.3.11 Preservation of *Streptomyces*:

Colonies showing suspected streptomyces morphology were recovered from isolation plates using sterile L-shaped needles and subcultured on yeast extract malt extract agar (ISP2 medium) plates. The plates were incubated at 28°C for 7 days. Morphologically different *Streptomyces* cultures were selected and preserved using ISP2 agar slants as well as in 30% glycerol.

4.4 ANTIMICROBIAL SCREENING AND BIOACTIVE METABOLITE PRODUCTION FROM *STREPTOMYCES* :

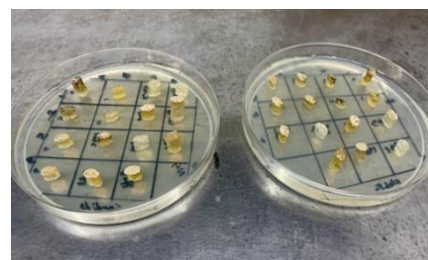
4.4.1 Testing for antimicrobial activity against bacterial and fungal pathogens

The antimicrobial activity of streptomyces culture was tested by adopting well diffusion (Radhakrishnan et al., 2014). *Streptomyces* culture grown on YEME agar plates was taken. An Eppendorf tube was taken and one ml of NA or MHA broth was added to it. Then, a loop full of culture was taken in an inoculation loop and put inside the Eppendorf tube. Then it was centrifuged at 4000 rpm for 2-3 minutes. Test

pathogens were inoculated in NA or MHA broth and vortexed. NA or MHA plates were taken and the test pathogens were swabbed in them using cotton swabs. The antimicrobial agent or extract solution is then introduced into the well after a 6 to 8-mm diameter hole is aseptically punched with a sterile cork borer or tip. The volume (20 to 100 L) of the antimicrobial agent or extract solution is then introduced into the well at the desired concentration. Then, the plates were incubated at 37°C for 24 hours. The zone of inhibition was expressed in millimeters in diameter (Saad *et al.*,2016). The test pathogens used in this study are as follows:

- a) *Candida Albicans*
- b) *Klebsiella Pneumonia*
- c) *Vibrio cholerae*
- d) *Vibrio Harveyi*
- e) *Vibrio vulnificus*
- f) *Vibrio Parahaemolyticus*
- g) *Staphylococcus aureus* 23 AGS
- h) *Staphylococcus aureus* 13 AGS
- i) *Bacillus cereus* 1 CCU
- j) *Escherichia coli* ESBL U55
- k) *Escherichia coli* AGS
- l) *Escherichia Faecalis* AGS
- m) *Escherichia coli* ARH
- n) *N-acetylcystiene*10
- o) *Candida albicans* C365
- p) *Stenotrophomonas*.Mutant
- q) *Lactobacillus .acidophilus* MTCC
- r) *Aeromonas.Baumannii* MTCC
- s) *Pseudomonas aeruginosa* AGS
- t) *Pseudomonas aeruginosa* ARH

4.4.2 Media optimization



Media optimization is done by the component replacement method. Various media were prepared by replacing the ISP2 media components. The carbon, nitrogen sources, and pH were altered. Then the potential strain is streaked on it and allowed to grow. The antimicrobial activity was performed against *K. pneumonia* and *Candida albicans* using the agar-plug method. The growth of strain UP1A1 was noted on these various components and the media was optimized. The various components and pH altered are listed below:

Characteristics	Variables
Growth on different ISP mediums	ISP2
	ISP3
	ISP4
	ISP5
	ISP6
	ISP7
Utilization of carbon source	Glucose
	Cellulose
	Lactose
	Peptone
	Mannitol
	Raffinose
Utilization of Nitrogen source	Soyabean
	Asparaginase
	Glutaminase
	Potassium nitrate
Ph	0
	1
	5
	7
	9
	11

4.4.3 Production and optimization of bioactive metabolite

The effect of solid-state and submerged fermentation on bioactive compound production by UP1A1 was investigated. Spores of the streptomyces strain UP1A1 were inoculated into 10 ISP2 agar plates and 100 ml of ISP2 broth. YEME agar plates were incubated at 28°C for 12 days. YEME broth containing flasks was incubated in a rotary shaker with 95 RPM for 12 days. For every 24 hours, agar plugs from YEME agar plates were taken and tested against *Klebsiella pneumonia* and *Candida albicans* (Eccleston *et al.*, 2008). Each 2 ml of YEME broth was taken and centrifuged at 10000 RPM for 10 minutes. The cell-free supernatant was also tested against *Klebsiella pneumonia* and *Candida albicans* by adopting a well-diffusion method (Avya *et al.*, 2011; Gebreyohannes G *et al.*, 2013). A volume of the microbial inoculum was dispersed across the whole surface of the agar plate to inoculate it. The antimicrobial agent or extract solution was then added to the well after a hole measuring 6 to 8 mm in diameter had been made aseptically with the use of a sterile cork borer or tip. The agar plates were then incubated at 37 degrees Celsius for 24 hours. The antibiotic ingredient spreads across the agar media and inhibits the tested microbial strain from growing (Saad *et al.*, 2016).

4.5 EXTRACTION AND SOLVENT OPTIMIZATION OF BIOACTIVE METABOLITES

4.5.1 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 UP1A1 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 (UP1A1) 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.

4.5.2 Effect of solvents on the extraction of bioactive compounds

After incubation, mycelial growth was removed aseptically using a sterile spatula. The agar medium was cut into pieces and extracted overnight at room temperature using different solvents such as methanol, ethyl acetate, n-hexane, DMSO, and chloroform. The scraped mycelium was suspended in methanol for extraction of intracellular bioactive compounds. The solvent extracts were concentrated using a rotary evaporator and quantified.

The antimicrobial activity of different solvent extracts was tested against *Pneumonia* and *Candida albicans* by well diffusion method (Selvameenal *et al.*, 2009). Bacterial inoculum was prepared using sterile nutrient broth and inoculated onto a nutrient agar plate using sterile cotton swabs. The zone of inhibition was measured after 24 hours of incubation at 37°C and expressed in millimeters in diameter. The solvent extract which showed maximum activity against the test pathogen was used to extract a large quantity of bioactive compounds from 1000 ml of ISP2 agar medium.

4.5.3 Anti-Biofilm

TSB broth with 1% glucose was prepared and autoclaved. Pathogens were inoculated and kept in the incubator for 24 hours.

Day-1:

Antibiofilm activity was performed against 4 biofilm-forming pathogens. The four pathogens are *E.coli*, *Klebsiella*, *Pseudomonas*, and *Acinetobacter*. Four 96 well plates were taken and the pathogens' names were written on it. 100 µl of TSB broth is added to all wells. An extra 100 µl of TSB broth was added to column 2 alone. Water was added to all four sides of the 96-well plate. The extract was added to the first well. 100 µl was taken in a pipette from the first well and transferred to the second well for serial dilution. Again, 100 µl was taken from the second well and transferred to the third well. The same is repeated for well three too. Now, the final concentration is reached upto 25 µl/mL. 50 µL of pathogen inoculum was added to all wells. Then the plates were incubated at 37°C for 24 hours.

Day-2:

The next day the plates were washed with 1X PBS 2 times and let to air dry completely.

Day-3:

150-180µL of 0.1% crystal violet was added to all wells and left for 30 minutes. Then it was rinsed with 1X PBS 2 times. The wells were resolubilized with 150-180µL of 95% ethanol and the OD readings were taken at 570 nm. Biofilm inhibition was calculated using the formula.

% Biofilm inhibition= Control- Test / Control *100

4.6 LARGE SCALE PRODUCTION OF BIOACTIVE POTENTIAL *STREPTOMYCES*

A bioreactor is a distinctively designed vessel that provides controlled physicochemical parameters to support the growth of microorganisms. The design of the fermenter should be such that it is able to provide optimal parameters for the growth of microorganisms.

Impeller: It is used for agitation (mixing up) of the medium to create a uniform environment in which all organisms remain in continuous contact with the medium resulting in maximum uptake of the nutrient. It also increases the air bubble path generated from the sparger (aeration device) and hence more time to dissolve oxygen in the medium.

Sparger: Provides sufficient oxygen to the microorganisms for metabolic requirements.

Baffles: It is used to avoid the vortex formation generated during the agitation of the medium.

Antifoam control: To prevent excessive foaming inside the fermenter, Antifoam control is attached via a pump. The pump may be controlled automatically or manually.

Heating jacket: It is used to maintain a favorable temperature.

Feed ports: These are the tubes connected to the nutrient reservoir and aid the addition of acid/ alkali inside the fermenter before or during the fermentation process.

Probes: Probes like pH and dissolved oxygen are used to monitor pH and dissolved oxygen in the fermenter.

Sampling point: This point is used to obtain samples during the process.

Control unit: This is a Control unit for the fermenter.



Figure 4.6 - production fermenter

Components of the fermentation process:

Inoculum Development: The seed culture was inoculated in 600 ml of ISP2 broth which was incubated in a rotary shaker for 7 days at 28°C at 150 rpm. Its antimicrobial activity was checked against *Klebsiella* and *Candida albicans*.

Table 4.6- Composition of liquid cultivation media

Composition	g/L
Yeast	24
Malt	60
D-glucose	24
NaCL	60

Formulation of production media:

6 L of media was prepared for the batch fermentation process.

Table 4.6- composition of production media

Composition	G/L
Yeast	24
Malt	60
D-glucose	24
NaCL	60

Batch Sterilization of Media, fermenter, and ancillary equipment:

All the inlet pipelines including the harvest line should be sealed with tubing and air filters should be changed after every run of the fermenter to avoid any contamination. Probes like pH, and DO should be placed carefully and the pH should be calibrated at 7.0 ± 0.2 . Antifoam should be added to the media itself and the fermenter should be agitated for approximately 1-2 hours for the proper mixing of the media components. The fermenter along with the media is then autoclaved at 121 °C, 15 psi for 30 min.

Inoculation of pure culture in fermenter:

Inoculum must be in a healthy, active state (lag phase minimized) and should be available in large volume. It is checked for any contamination. 600 mL of pure culture is inoculated in the fermenter under sterile conditions.

Growth of organisms in the fermenter under sterile conditions for product formation

The organism grows in the production media for 4-5 days where it undergoes changes in dissolved oxygen and pH. Parameters like temperature, agitation, pH, and dO₂ are observed after every 2 hours. The gradual decrease in dissolved oxygen marks the growth of bacteria which shows the log phase of growth kinetics.

Extraction of the product

The product was harvested in extraction bottles and it was centrifuged to remove the cells. Cell-free supernatant was taken in a separate beaker and 40 g of resin was autoclaved and added to it and kept in a rotary shaker for 24 hours. The resin was removed and dried and methanol was added to it and left for 24 hours. The next day the resin was removed and the methanol mixture was lyophilized. The weight of the compound was 2.21 grams. The extracts are then used for antimicrobial activity testing against *Klebsiella pneumonia* and *Candida albicans*.

Table 4.6 Parameters maintained during fermentation

STERILIZATION		PROCESS VALUE	
TEMP SP	121.5	TEMP PV	30
TIMER	30.00	Ph PV	0.00
FERMENTATION		SPEED PV	200
TEMP SP	30.00	TIMER ACT	200
Ph SP	7.00	VESSEL PRESSURE GAUGE	1.5 barg



Streptomyces UP1A1



mother Inoculum



Bioreactor



Cell-free supernatant



Centrifugation



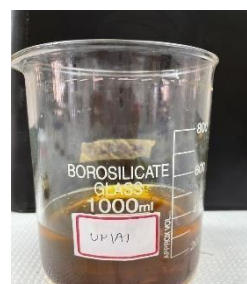
Fermented broth



Resin with supernatant



Resin is removed



Methanol+resin

Powdered crude



Figure 4. 6 - Overview of the fermentation process

4.7 PURIFICATION AND STRUCTURAL ELUCIDATION OF BIOACTIVE POTENTIAL METABOLITES FROM *STREPTOMYCES* UP1A1 :

4.7.1 Thin Layer Chromatography Analysis

A TLC sheet/plate was taken which is generally coated with silica gel and alumina; it was cut into small strips. A small amount of the extract (UP1A1) was spotted on 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 ratios.

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 a few centimeters off 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.

4.7.2 Flash chromatography

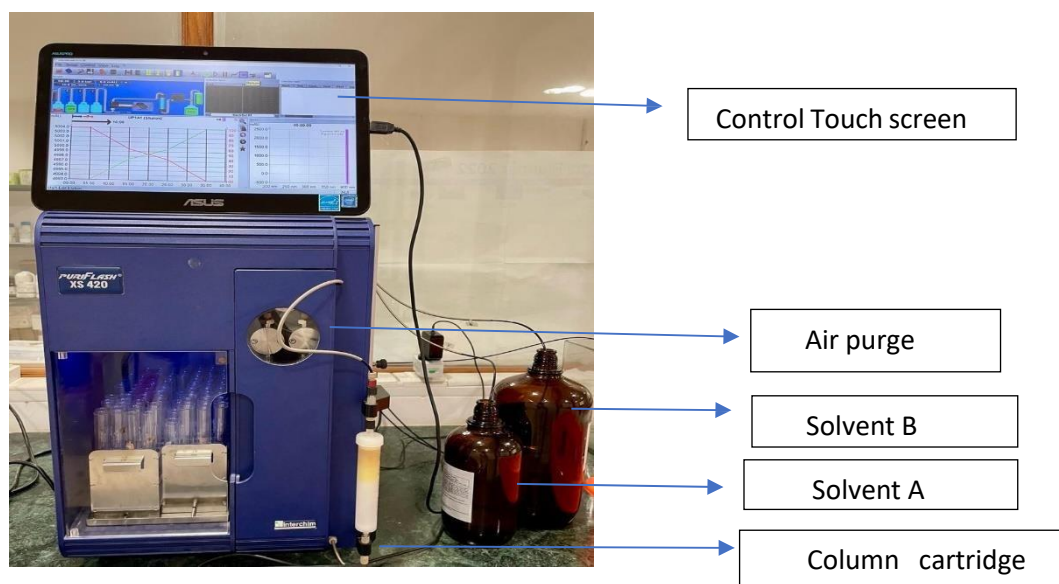


Figure 4.7.2 Flash chromatography

Cartridge:

The cartridge is made up of pharmaceutical-grade Polypropylene material to ensure virtually zero cross-contamination and reliable and reproducible purification. The Distribution Disk on top of the column ensures the best flow distribution path onto the column and the design can withstand pressure up to 300 psi (depending on the size) with no leak. The cartridge body is 85% packed with silica and has 15% empty space to be filled with our sample. And, the cartridge is made ready for purification.

Mobile phase: The gradient of Ethyl acetate and Methanol was used as mobile phase, with gradual increase of 2% methanol to change the polarity for total recovery of the compound.

Purging:

Before starting the process, the column must be purged. The column was purged with hexane. The aim of the purge is simply to flush through all the lines so that any remaining solvent in them from a previous analysis or wash is replaced with the new mobile phase.

Methodology:

Flash chromatography is a technique that makes it easier to separate a compound from a mixture by injecting a small volume of liquid sample into a tube packed with microscopic particles (3 to 5 microns(m) in diameter, known as the stationary phase), where individual components of the sample are moved down the packed tube (column) with a liquid (mobile phase) that is forced through the column by high pressure provided by a pump.

The powdered extract was mixed with methanol, vortexed, and filtered. After the purging process is complete, our extract is added to the column. And the controls were set in the control panel. Sample name was given. Then, the gradients were programmed with 2 solvents: ethyl acetate and Methanol. Flow rate, maximum pressure, and equilibrium time were assigned for the column. Then, either a threshold is set or the fractions were collected manually. Active fractions number 1,2 and waste were collected and dried using a rotary evaporator and their concentration was made up to 1mg/ml, this concentration was then used to test antimicrobial activity.

4.11.3 HPLC-MS/MS Analysis and Metabolites Identification

Thermo LTQ Orbitrap XL mass spectrometer linked to an Agilent model 1100 LC system with a solvent reservoir, an in-line degasser, a binary pump, and a chilled autosampler was used for the LCMS/MS analysis of the crude extract. MeOH served as the solvent for the infusion into the ESI source, which was used to record the spectra. A gradient elution of H₂O and MeOH both with 0.1% formic acid, running at 200 L/min, was used to operate a 5 m Kinetex C18 column (50 mm 2.1 mm), which was kept at a constant temperature of 25 °C. Following was the gradient programme: 10% MeOH for 3 minutes, 10-90% MeOH for 30 minutes, and 90% MeOH for 3 minutes. Acquisition mode that depends on data MS spectra were captured in positive ion mode at spray voltages of 5 kV, 230 °C at the capillary, 12 units of N₂ for the sheath gas, and 5 units of N₂ for the auxiliary gas. Selected ions were subjected to LCMS/MS scans that had fragmentation caused by collision-induced dissociation (CID), isolation width of 2.0, normalized collision energy of 35, activation Q of 0.250, and activation duration of 30 ms.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 IDENTIFICATION AND PRESERVATION OF POTENTIAL STREPTOMYCES

5.1.1 Morphological characteristics of potential streptomyces UP1A1

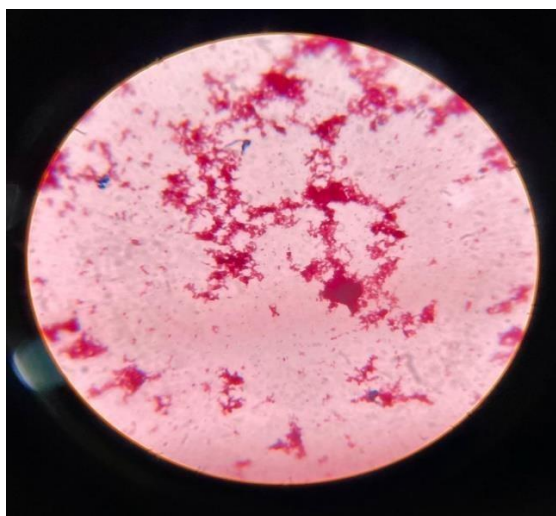


Fig:5.1.1 Microscopic image of potential UP1A1 by gram staining

Figure (5.1.1) shows the morphological characters of the potential *Streptomyces* strain UP1A1 which was attained by gram staining using a light microscope revealing that it is a gram-positive bacterium.

5.1.2 Cultural characterization



Figure5.1.2Potential *Streptomyces* strain UP1A1

Figure (5.1.2) shows the image of the potential *Streptomyces* strain UP1A1 which was been sub-cultured and grown in an ISP2 media. The potential strain shows an aerial and substrate mycelium. The mycelium mat was white in color due to the formation of spores. Likewise, 189 different *Streptomyces* species were isolated from 8 different soil sample and showed white, brown, colored colonies with different morphological types (Dharumadurai *et al.*, 2008). Similarly, 17 different *Streptomyces* species were isolated from 10 different soil samples. The aerial and substrate mycelium appeared to be greyish and white in color, along with no diffusible pigments (Sobika *et al.*, 2022).

Table 5.1.2 - Cultural characteristics of UP1A1

Strain	Growth	Consistency	AMC	RSP	SP	AM	SM
UP1A1	Good	Powdery	White	-	-	+	+

5.1.3 Scanning electron microscope

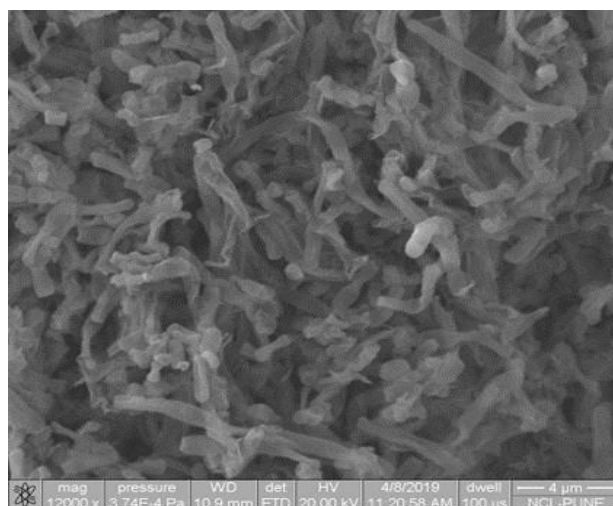


Figure 5.1.3 -Image of streptomyces sp.UP1A1 viewed under a Scanning electron microscope.

The morphological study of strain UP1A1 using a scanning electron microscope revealed long rod-shaped filamentous bacteria that produced well-developed hyphae (between 0.5μm- 3.0μm). 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).

Likewise, *Streptomyces aureovercillatus* isolated from crop soil consisted of cylindrical, and rough-surfaced spores (0.6-0.8 μm $\times$ 0.9-1.6 μm) were produced on aerial mycelia, but spore chains were not observed (Peng *et al.*,2019) .

5.1.4 Contig Sequence of *Streptomyces* sp. UP1A1

*Streptomyces maritimus*_UP1A-1 (MT279914)

The 16S rRNA sequence was obtained of the potential *Streptomyces* strain. The strain UP1A1 shows closeness to various *Streptomyces* species. Likewise, from the soil in Cisolok's geothermal area, 25 chosen thermophilic Actinobacteria isolates were successfully isolated. 25 isolates' sequences revealed similarities to members of the families Streptomycetaceae, Streptosporangiaceae, Thermomonosporaceae, Pseudonocardiaceae, Micromonosporaceae (genus Micromonospora; 2 isolates), and Nocardiaceae (Yabe *et al.*,2019). Similarly, a study that compared the *Streptomyces* to each other and previously published 16s rRNA sequences of other *Streptomyces* had over 95% identical bases and were highly related (Dholakya *et al.*,2017).

```
ACCGTAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACAC
ATGCAAGTCGAACGATGAACCACTTCGGTGGGGATTAGTGGCGAACGGGTG
AGTAACACGTGGGCAATCTGCCgaGCACTCTGGGACAAGCCCTGGAAACGG
GGTCTAATACCGGATACTGATCCTCGCAGGCATCTGCGAGGTTCGAAAGCTC
CGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTAGTTGGTGAGGTAACGG
CTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACAC
TGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATAT
TGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGG
CCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACC
TGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG
GGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTT
GTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATA
CGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTG
AAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGC
CGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATAC
CCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCAACATTCCA
CGTTGTCCGTGCCGACGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGG
CCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAAGCGGCGGA
GCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATA
CACCGGAAAACCCTGGAGACAGGGTCCCCCTTGTGGTTCGGTGTACAGGTGG
TGCATGGCTGTCTGTCAGCTCGTGTCTGTGAGATGTTGGGTAAAGTCCCGCAAC
GAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTTGTGGTGCTGGGGAC
TCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAA
GTCATCATGCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTAC
```

AATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAG
TTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATC
GCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCC
CGTCACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCCTTG
TGGGAGGGAGCTGTCTGAAGGTGGGACTGGCGATTGGGACGAAGTCGTAACA
GGTTAACCGTA

5.1.5 Phylogenetic analysis

The contig sequence is fed to the EZ taxon, and all the possible hits of the strain along with the name, strain type, accession number, pairwise similarity, and completeness are viewed. The *Streptomyces* sp.UP1A1 showed closeness to two strains i.e. *Streptomyces panicles* and *Streptomyces pratensis*. The fasta format of the top 25 hit strains is downloaded and a phylogenetic tree is constructed using maximum likelihood and neighbor-joining method.

The following phylogenetic tree is constructed using the neighbor-joining method using the 25 closest strains.

Table 5.1.5: Top 25 Strains closest to *Streptomyces* sp. UP1A1

Name	Strain	Accession	Pairwise similarity (%)	Mismatch/ Total nt	Completeness (%)
<i>Streptomyces rochei</i>	NRRL B 2410	MUMD0100 0370	100.00	0/1448	100.00
<i>Streptomyces mutabilis</i>	NBRC 12800	AB184657	99.72	4/1442	99.59
<i>Streptomyces djakartensis</i>	NBRC 15409	AB184690	99.51	7/1435	99.10
<i>Streptomyces tuius</i>	NBRC 15617	AJ781374	99.44	8/1438	99.31
<i>Streptomyces violacerorubidus</i>	LMG 20319	D63873	99.17	12/1447	100.00
<i>Streptomyces tendae</i>	ATCC 19812	AY999780	99.10	13/1448	100.00
<i>Streptomyces</i>	ISP 5010	AY999803	99.10	13/1447	100.00

calvus					
Streptomyces anandii	NRRL B- 3590	AB184161	99.10	13/1447	100.00
Streptomyces pilosus	NBRC 12807	AB184188	99.10	13/1448	99.31
Streptomyces aurantiogriseus	NBRC 12842	AB184713	99.03	14/1447	100.00
Streptomyces virens	NBRC 15901	AB184577	99.03	14/1447	99.72
Streptomyces capillispiralis	NBRC 14222	AB184710	99.96	15/1447	99.59
Streptomyces aureorectus	NBRC 15896	AB184670	98.96	15/1438	99.38
Streptomyces Levis	NBRC 15423	AB184379	98.95	15/1432	98.97
Streptomyces luteogriseus	NBRC 13402	AB184380	98.95	15/1425	98.41
Streptomyces violaceus	NRRL B 2867	KL569104	98.90	16/1448	100.00
Streptomyces psedogriseolus	NRRL B 3288	MUNG0100 0290	98.90	16/1448	100.00
Streptomyces malachitospinus	NBRC 101004	AB249954	98.90	16/1448	100.00
Streptomyces tritolerans	DAS 165	DQ345779	98.90	16/1448	100.00
Streptomyces rubrogriseus	LMG 20318	AJ781373	98.89	16/1447	100.00

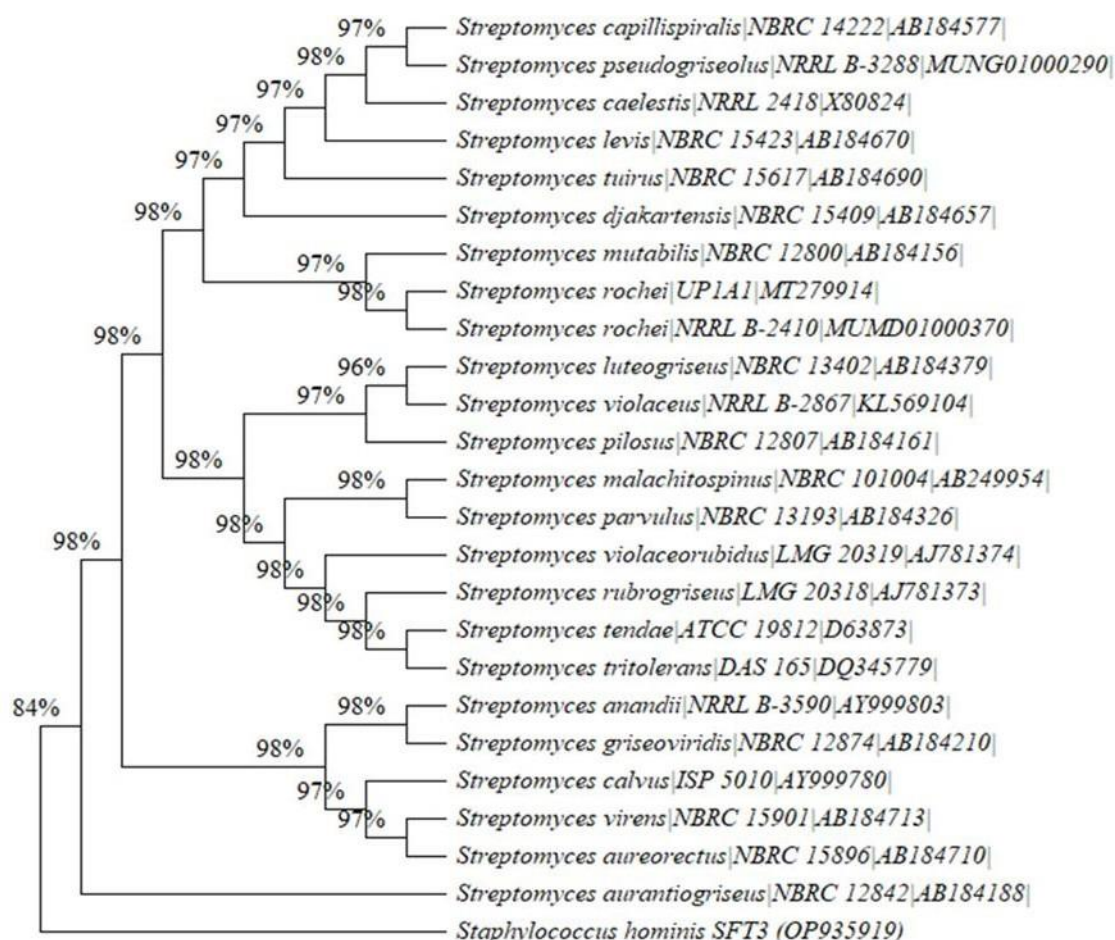


Figure. 5.1.5 Molecular Phylogenetic analysis by Maximum Likelihood method

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 MEGA7.

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). Likewise, in another study Streptomyces strain-329's phylogenetic study was built using a neighbor-joining tree that compared its 16S rRNA sequence to those of other Streptomyces spp. in the NCBI database. Nucleotide sequence information has been entered into GenBank with the entry number HBUM 174206. 99% of the similarities were identical. Streptomyces anulatus was discovered to be the species of the isolated strain 329 (Aung *et al.*, 2019).

5.2 ANTIMICROBIAL SCREENING AND BIOACTIVE METABOLITE PRODUCTION FROM STREPTOMYCES:

5.2.1 Antimicrobial activity of streptomyces sp. UP1A1 by well diffusion method

S.no	Pathogens	Antimicrobial activity (Zone of inhibition expressed in millimeters in diameter)
1	<i>K. Pneumonia</i> ARH	22.6±1.2
2	<i>K. Pneumonia</i> AGS	-
3	<i>E.coli</i> ARH	-
4	<i>E.coli</i> AGS	-
5	<i>S.aureus</i> 23 AGS	-
6	<i>S.aureus</i> 13 AGS	-
7	CA C365	-
8	NAC 10	-
9	<i>V. Harveyi</i> CSU	-
10	<i>V. Cholera</i> CCU	-
11	<i>B. cereus</i> ICCU	14.3±0.7
12	<i>V. Parahaemolyticus</i> CCU	-
13	<i>V. vulnificus</i> CCU	-
14	<i>St. mutant</i> MTCC	20.3±0.7

15	<i>L. acidophilus</i> MTCC	-
16	<i>E. faecalis</i> AGS	-
17	<i>E.coli</i> ESBLU55	-
18	<i>A. baumannii</i>	-
19	<i>Ps. Aeruginosa</i> AGS	-
20	<i>Ps. Aeruginosa</i> ARH	-
21	<i>Candida</i> B34	20.6±1.5

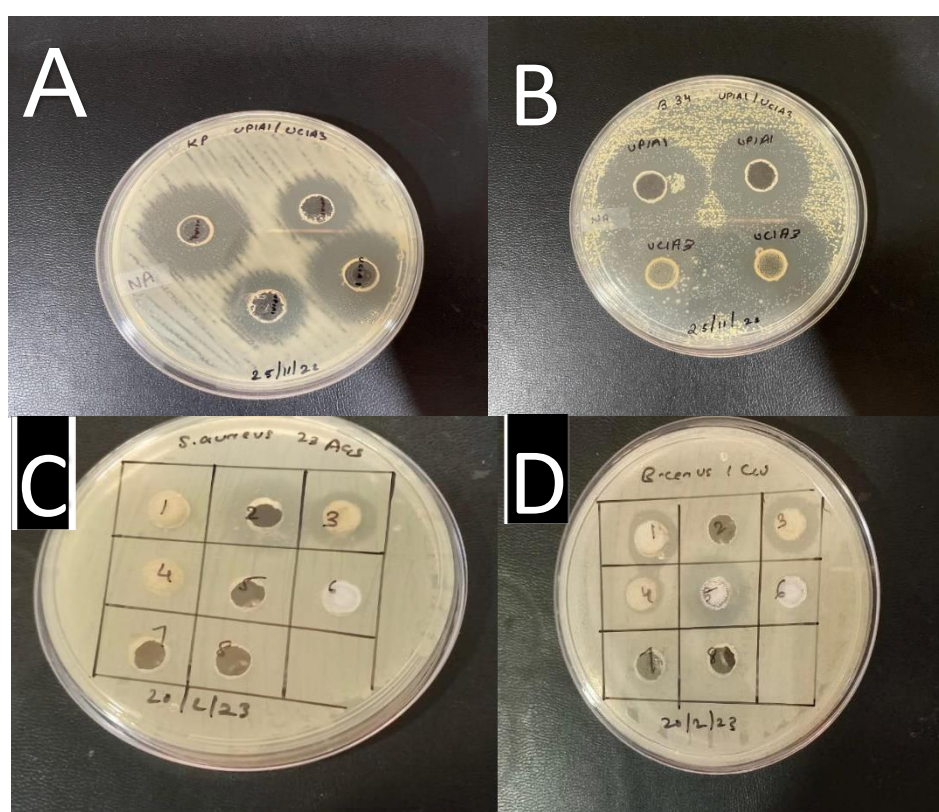


Figure 5.2.1- Antimicrobial activity by well diffusion method against : A.) Klebsiella, B.)Candida albicans C.)Staphylococcus aureus D.)Bacillus cereus

Antimicrobial activity of the potential *Streptomyces* strain UP1A1 was done against 21 sets of pathogens for preliminary screening in which the maximum zone of inhibition was obtained for *K. pneumoniae* and *Candida albicans* which indicates that the potential strain UP1A1 shows the highest antimicrobial activity against *K. pneumoniae* and *Candida albicans*.

In a study, 13 isolates (26.0%) generated secondary metabolites that displayed a wide range of antibacterial activity against yeast and Gram-positive bacteria. As a result, they concluded that the soil of Sammuk Mountain is a significant source of actinomycetes that produce antibiotics (Anan *et al.*, 2019). Similarly, in another study isolated they *Streptomyces* sp. from mangrove sediments which produced types secondary metabolites, including antifungal, anticancer, antibacterial and anti-HIV (Al-Ansari *et al.*, 2019).

5.2.2 Media Optimization (Component replacement method)

Physiological characteristics of UP1A1

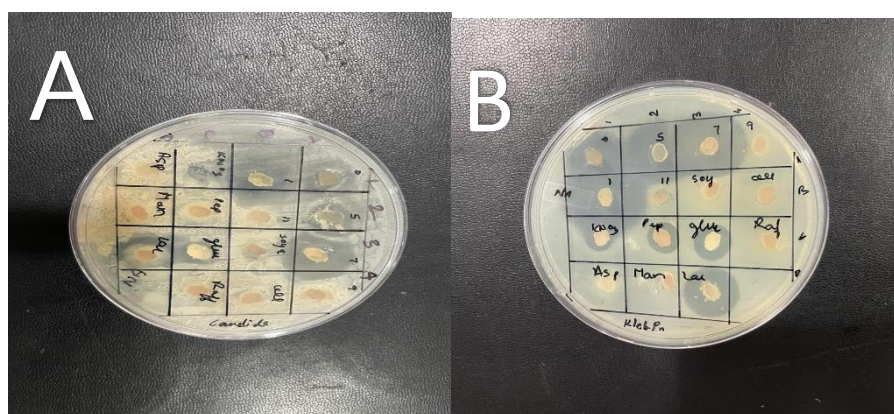


Figure 5.2.2 - Antimicrobial activity against : A.) *Candida Albicans* and B.) *Klebsiella*

Table 5.2.2 - Antimicrobial activity against *Klebsiella* and *Candida* for media optimization

Characteristics	Variables	UP1A1	Inhibition against	
			<i>Klebsiella</i> (mm)	<i>Candida</i> (mm)
Growth on different ISP mediums	ISP2	+	25.6±1.0	23.0 ±0.2
	ISP3	+	23.2± 0.2	21.0 ±0.6
	ISP4	+	-	-
	ISP5	-	-	-
	ISP6	-	-	-
	ISP7	-	-	-
Utilization of carbon source	Glucose	+	9.0 ±0.5	9.0 ±0.5
	Cellulose	+	-	-
	Lactose	+	20.6 ±1.5	9.0 ±0.5
	Peptone	+	9.0 ±0.2	17.6 ±1.2
	Mannitol	+	-	-
	Raffinose	+	-	-

Utilization of Nitrogen source	Soyabean	+	-	-
	Asparaginase	+	-	-
	Glutaminase	+	-	-
	Potassium nitrate	+	9.0 ±0.5	8.0 ±0.2
Ph	0	+	11.3 ±0.3	11.3 ±0.5
	1	+	19.0± 0.5	17.4 ±0.7
	5	+	12.0 ±0.8	10.3 ±0.3
	7	+	11.0± 0.5	11.3± 0.5
	9	+	-	-
	11	+	13.0 ±0.5	14.0 ±0.8

To increase the yield of metabolites, the manufacturing medium is typically optimised. The evolution of medium formulations through the screening of various carbon and nitrogen sources and their various combinations can all significantly increase microbial growth, viability, and the overall yield of the product during the development of a process(Singh et al.,2017).In a study,using statistical techniques based on experimental designs, the culture medium for the strain JAJ13's production of antibacterial compounds was optimised.Then, Streptomyces sp. JAJ13 tested eight different production media for the ability to produce antibacterial compounds. Streptomyces sp. JAJ13 obtained increased production media for the manufacture of antibiotic compounds(Pandiyar *et al.*,2015).

In my study, Lactose showed the highest zone of inhibition and good growth was observed in it.

5.3 Production and optimization of bioactive secondary metabolites



Figure-5.3.1 Solid Extraction



Figure 5.3.1 - Liquid Extraction

Table 5.3.1- Antimicrobial activity of crude got from solid and liquid extraction

Strain	Type	Inhibition against	
		K.pneumonia(mm)	CA B34 (mm)
UP1A1	Surface	10 $\pm$ 0.2	13.2 $\pm$ 0.2
	Submerged	18 $\pm$ 0.6	12 $\pm$ 0.2

Among the solid and liquid extraction, liquid extraction showed maximum antimicrobial activity against *Klebsiella* and *Candida albicans*. Optimization of bioactive secondary metabolites was done to increase the further production of metabolites. In a particular study, Solid and liquid phase extraction for performed for their culture. Similar to my study, antimicrobial activity was observed more in liquid phase extraction(Espen *et al.*,2021).

5.4 EXTRACTION AND SOLVENT OPTIMIZATION OF BIOACTIVE METABOLITES

5.4.1 Liquid extraction

Table 5.4.1 Antimicrobial activity for liquid extraction

Strain	Type	Inhibition against	
		K.pneumonia(mm)	CA B34 (mm)
UP1A1	Liquid	22 $\pm$ 0.2	20.2 $\pm$ 0.2

Further liquid extraction performed showed very good production of secondary metabolites. It showed maximum antimicrobial against *Klebsiella*.

5.4.2 Solvent optimization

Table 5.4.2 - Antimicrobial activity of different solvent extracts

Solvent extracts	Activity (zone of inhibition expressed in millimeters in diameter)	
	<i>Klebsiella</i>	<i>Candida albicans</i>
n-Hexane extract	-	-
Ethyl acetate extract	18.0± 0.2	15.0±0.6
Methanol extract	25.0± 0.8	20.0±0.6
Chloroform	-	-

Optimizing the solvent composition aims to provide a more satisfactory separation of the uncharacterized polyherbal fraction's unknown structure and properties, which is necessary for their ultimate identification (Jayantha *et al.*, 2015).

The solvent extract which showed maximum activity against test pathogens is methanol and ethyl acetate. Whereas, hexane and chloroform showed no activity. Methanol showed maximum activity compared to ethyl acetate and it was further used to extract a large quantity of bioactive compounds.

5.4.3 Anti-Biofilm:

Our potential strain UP1A1 was tested for antibiofilm properties against four biofilm-forming pathogens. Biofilm inhibition was calculated using the formula.

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

Table 5.4.3 - Biofilm inhibition rate against four pathogens

Acinetobacter				E.coli				Klebsiella				Pseudomonas			
200	100	50	25	200	100	50	25	200	100	50	25	200	100	50	25
0	0	0	0	0	0	0	0	75	63	60	18	0	0	0	0

Our potential strain showed Antibiofilm properties against *Klebsiella pneumoniae* alone. 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).

5.4.4 LARGE-SCALE PRODUCTION OF BIOACTIVE POTENTIAL STREPTOMYCES

The yield got from large-scale production of *Streptomyces* UP1A1 is 2.21 grams. Its antimicrobial activity was tested against *Klebsiella* and *Candida albicans*. The zone of inhibition got is mentioned in table (5.4). In another study, they got the yield of their compound to be 3.41 grams and it showed antimicrobial activity against few gram negative and gram positive bacteria (Leumi *et al.*, 2019).

Table 5.4.4 - Yield and day by day antimicrobial activity of the extract got from bioreactor

Strain	Day	Yield(g)	Antimicrobial activity	
			<i>Klebsiella</i>	<i>Candia albicans</i>
UP1A1	3	-	-	
	4	-	-	
	5	-	-	
	6	-	12±0.2	18 ±0.4
	7	2.21	10.0± 0.00	13.0± 0.6

5.5 PURIFICATION AND STRUCTURAL ELUCIDATION OF BIOACTIVE POTENTIAL METABOLITES FROM *STREPTOMYCES* UP1A1

5.5.1 TLC analysis

The sample (UP1A1) 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. It was observed that separation was good in polar solvents. EA: DCM in the ratio of (1.4:0.5).

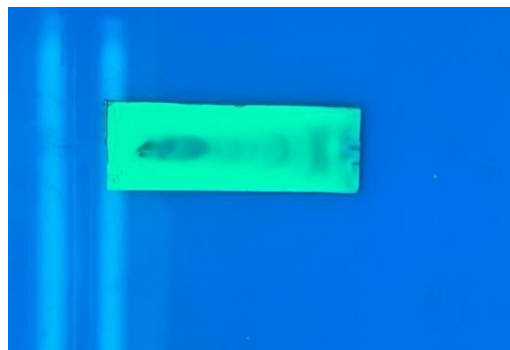


Figure 5.5.1 - TLC analysis

Table 5.5.1 Rf value

Compound	Rf value
1	0.76
2	0.8
3	0.9
4	0.95

5.5.2 Flash chromatography:

During flash chromatography using a gradient of Ethyl acetate and Methanol in total 2 fractions were collected on the basis of the TLC profile. Out of 2 fractions, the active fraction was screened for antimicrobial activity on *K. pneumonia* and *Candida albicans*.



Figure 5.5.2 - Fractions of column chromatography



Figure 5.5.2 - Bioactivity of fraction 2 collected from column chromatography against *K. pneumonia* and *Candida albicans*.

Table- 5.5.2 Inhibition zone of Fraction 2 against *K. pneumonia* and *Candida albicans*.

Fraction	Antimicrobial activity against	
	<i>Klebsiella Pneumonia</i>	<i>Candid albicans</i>
1	-	-
2	9 ± 0.5	1.1 ± 0.05
3	-	-

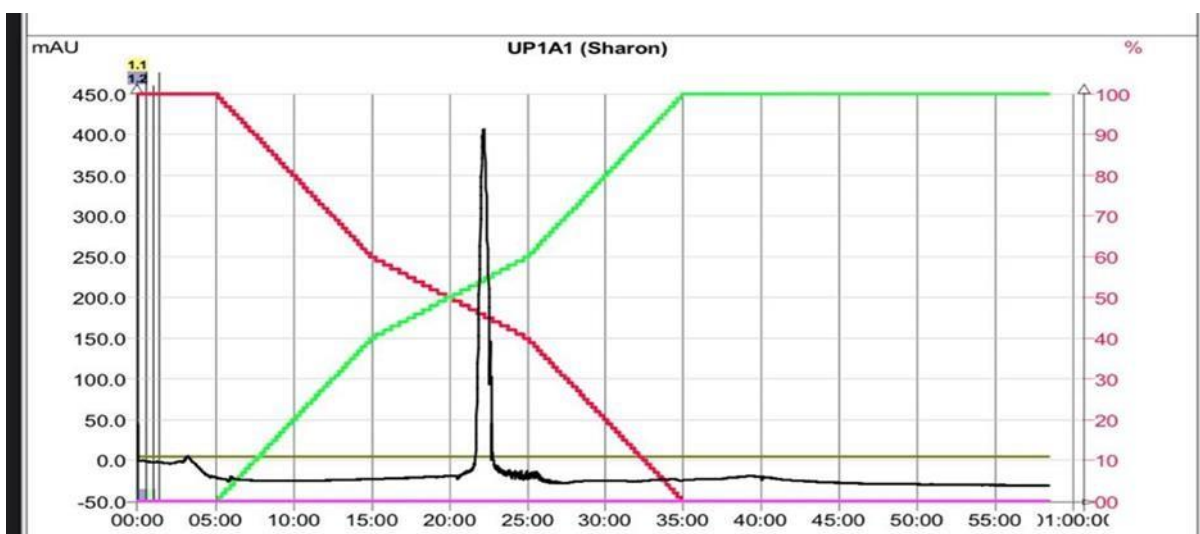


Figure 5.5.2- Flash chromatogram of fraction 2 showing peak

Table- 5.5.2 Flash profile of the purified sample

Fraction	Retention time(min)	Height(mAU)	Area(mAU*min)	Rel. area %
2	23.5	410.1	9.400	90

The active fraction was isolated and it showed good antimicrobial activity. In a study, they isolated and purified oleanolic acid from olive leaf extracts using flash chromatography. It is reported that the yield of the isolate was higher compared to previous isolation methods (Heng *et al.*,2022). Likewise, in another study they isolated the compound causing antimicrobial activity in *Tecoma stans*. And they concluded that flash chromatography is a rapid and efficient method for isolation and purification of isolated compound (Amad *et al.*,2022).

5.5.3 HR-LC/MSMS for structural elucidation

The chromatogram provides a visual representation of the bioactive secondary metabolites that are present in the sample (UP1A1). 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.

LCMS/MS Chromatogram for UP1A1

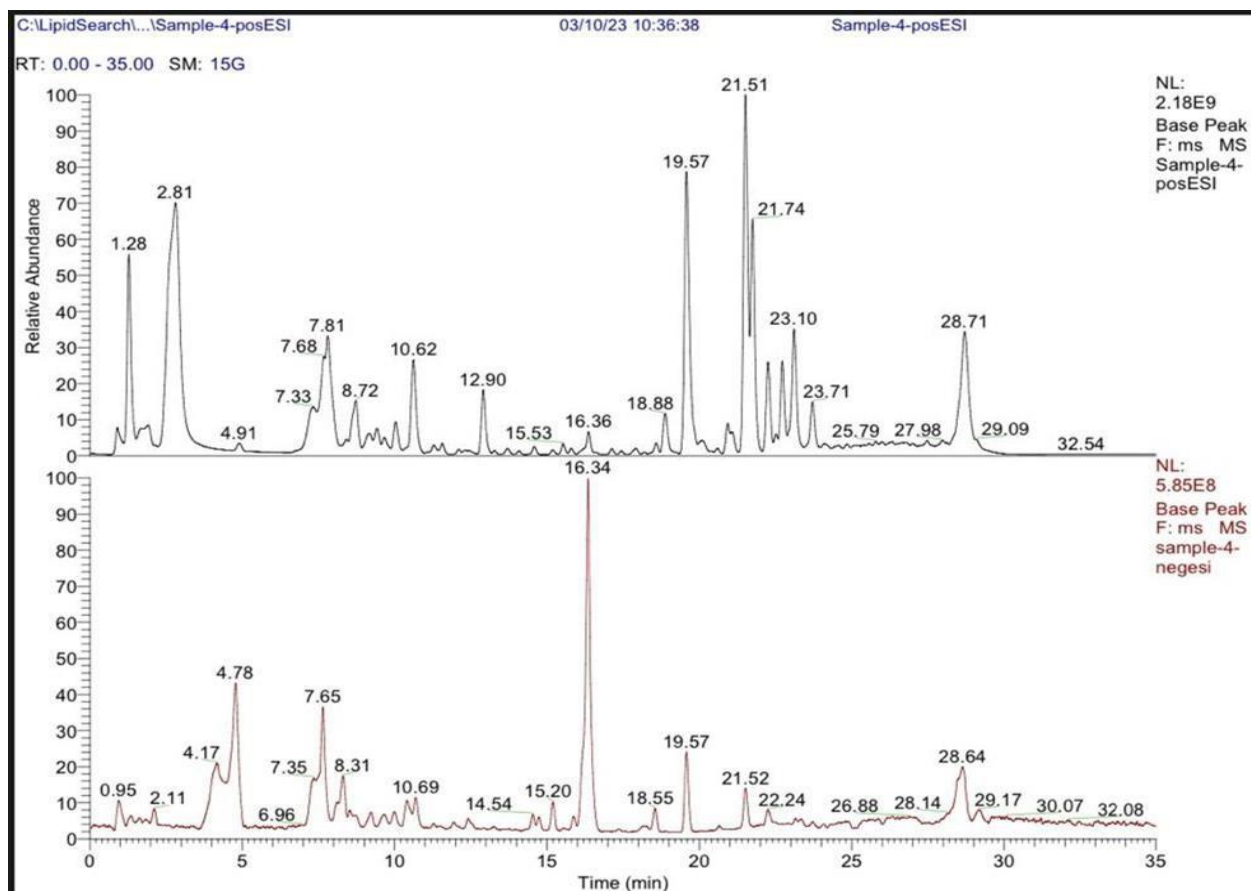


Fig 5.5.3- LCMS/MS Chromatogram for UP1A1

5.5.4 List of suspected compounds from potential UP1A1 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 (UP1A1). 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 UP1a1. In this study, we obtained compounds such as muricholic acid with the highest molecular weight 492.27.

Table 5.5.4 Compounds from UP1A1 through HRLCMS/MS at negESI mode

NAME	FORMULA	MOLECULAR WEIGHT
Glycolic acid	C2 H4 O3	76.01
Glutaric acid	C7 H6 O2	132.04
Pyrrole-2-carboxylic acid	C5 H5 N O2	111.03
4 Methylphenol	C7 H8 O	108.05
Methylsuccinic acid	C5 H8 O4	132.02
Phenol	C6 H6 O	94.04
2-Hydroxybenzothiazole	C7 H5 N O S	151.00
N-Acetyl-L-tyrosine	C11 H13 N O4	223.08
Stearic acid	C18 H36 O2	284.27
Salicylic acid	C7 H6 O3	138.08
Myristic acid	C14 H28 O2	228.20
L-(+)- Lactic acid	C3 H6 O3	90.03
Palmitic acid	C16 H32 O2	256.24
Azelaic acid	C9 H16 O4	188.10
Pentadecanoic acid	C15 H30 O2	242.22
Trans-10-Heptadecenoic acid	C17 H32 O2	268.24
NP-008915	C15 H24 O3	252.12
Oleoyl-L- α -lysophosphatidic acid	C21 H41 O7 P	436.25
α , α - Trehalose	C12 H22 O11	342.11
Arachidic acid	C20 H40 O2	312.30
Oleic acid	C18 H34 O2	282.25
Genistein	C15 H10 O5	270.05
Isobutyric acid	C4 H8 O2	88.05
Pentadecanoic acid	C15 H30 O2	242.22
Palmitoleic acid	C16 H30 O2	254.22
Monobutyl phthalate	C12 H14 O4	222.08
NP-021698	C11 H13 N O4	223.08
Linoleic acid	C18 H32 O2	280.24

11- Deoxy prostaglandin	C20 H34 O4	338.24
Prostaglandin A1	C20 H32 O4	336.23
(+/-)11(12)-DiHET	C20 H34 O4	338.24
Arachidonic acid	C20 H32 O2	304.24
5-OxoETE	C20 H30 O3	318.21
Pimelic acid	C7 H12 O4	160.07
2-Hydroxycinnamic acid	C9 H8 O3	164.04
NP- 014839	C19 H22 O3	298.15
Muricholic acid	C24 H40 O5	408.28
Ferulic acid	C10 H10 O4	194.05
Arachidic acid	C20 H40 O2	312.30
Caffeic acid	C15 H22 O4	180.04
Lignoceric acid	C24 H48 O2	368.36
(±)9(10)-DiHOME	C18 H34 O4	314.24
Nicotinic acid	C6 H5 N O2	123.03
2-Hydroxyphenylacetic acid	C8 H8 O3	152.04
3-Amino-3-(4-hydroxyphenyl) Propanoic acid	C9 H11 N O3	181.07
(±)9-Hode	C18 H32 O3	296.2
8-Iso-15-keto-prostaglandin-F2±B	C20 H32 O5	352.22
Picolinic acid	C6 H5 N O2	123.03
N-Acetyl-DL-norvaline	C7 H13 N O3	159.08
Tenofovir	C9 H14 N5 O4 P	287.07
13,14-Dihydro-15-keto-tetranor prostaglandin	C16 H28 O5	300.19
Sucrose	C12 H22 O11	342.11
Isoferulic acid	C10 H10 O4	194.05
Nordiazepam	C15 H11 Cl N2 O	270.05
Resolvin E1	C20 H30 O5	350.20
NP-002676	C15 H26 O4	270.18

NP-020515	C13 H20 O5	256.13
Lithocholic acid	C24 H40 O3	376.2977
Indole-3-lactic acid	C11 H11 N O3	205.05
Indole-2-2-carboxylic acid	C9 H7 N O2	161.04
3-hydroxy mandelic acid	C7 H7 N O2	137.04
NP-017152	C13 H23 N O2	225.17
NP-001134	C16 H22 N4 O2	302.17
4-Coumaric acid	C9H8O3	164.04
2-Methylhippuric acid	C10 H11 N O3	193.03
Dodecanedioic acid	C12 H22 O4	230.15
Xanthine	C5 H44 N4 O2	152.03
3-Methoxyphenylacetic acid	C9 H10 O3	166.06
D (+)-Maltose	C12 H22 O11	342.11
Homogentistic acid	C8 H8 O4	168.04
2-Anisic acid	C8 H8 O3	152.04
NP-002047	C20 H28 O5	348.19
Porphobilinogen	C10 H14 N2 O4	226.09

5.5.6 List of suspected compounds from potential UP1A1 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. In a study, based on LC-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 5.5.6 Compounds from UP21A1 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
Glycitol	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-11 β -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
Docosatrienoic 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

CHAPTER 6

SUMMARY AND CONCLUSION:

Streptomyces produces over two-thirds of the naturally occurring antibiotics that are clinically effective. Natural products from members of the phylum actinobacteria are promising sources of antibiotics. *Streptomyces* are known producers of secondary metabolites. In this world, there is a pressing need for the development of antimicrobial compounds. Our potential strain UP1A1 was isolated from potato-grown soil from Ooty. This study aimed to isolate the bioactive potential secondary metabolites from *Streptomyces* and evaluated their antimicrobial potential against gram-negative pathogens. A preliminary screening of antimicrobial activity of *Streptomyces* strain UP1A1 was evaluated against 20 sets of pathogens and it showed a broad range of activity against 2 pathogens. In addition to antimicrobial activity, the crude extract of UP1A1 also exhibited anti-biofilm activity against *K. pneumonia*. Large-scale production of bioactive secondary metabolites from *Streptomyces* UP1A1 was achieved through a 10L laboratory fermenter. Then, the bioactive secondary metabolites were purified by flash chromatography using the gradient solvent system of ethyl acetate and methanol and evaluated for antimicrobial activity. The active fraction was further evaluated for molecular networking and metabolite profiling using LC-MSMS analysis. Molecular networking in GNPS showed numerous interesting bioactive secondary metabolites may be the reason for their antimicrobial potential. Based on the cultural, morphological, and molecular analysis, the potential strain UP1A1 was identified as *Streptomyces maritimus*. Our results showed the robust secondary metabolism of *S. maritimus* UP1A1, so we conclude by saying that our potential strain UP1A1 may serve as a potential source for antimicrobial drug development upon further investigation.

CHAPTER 7

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