KARADENIZ TECHNICAL UNIVERSITY
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

BIOTECHNOLOGY DEPARTMENT

ISOLATION AND IDENTIFICATION OF ACTINOMYCETES FROM BLUE LAKE AND DETECTION OF BIOACTIVE SECONDARY METABOLITES

MASTER'S THESIS

HUSSAM LAITH JAFAR AL-SHAREFI

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TRABZON

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This thesis is accepted to give the degree of

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PREFACE

This thesis is the result of the search carried out as part of the requirements for the degree of Master of Science by The Graduate School of Natural and Applied Sciences at Karadeniz Technical University. The study focused on the isolation and characterization of Actinomycetes from Blue Lake and Goksu Travertines in Giresun, Turkiye, with a special emphasis on their ability to produce bioactive secondary metabolites.

I would like to express my sincere gratitude to my supervisor, Prof. Dr. Zihni DEMİRBAĞ for his help and support from planning to the conclusion of this thesis, and to my co-supervisor, Dr. Mehtap USTA for her advice and support. I also extend my appreciation to Prof. Dr. Remziye NALÇACIOĞLU for her guidance, encouragement, and expertise throughout this work. I also would like to thank Prof. Dr. Hatice KATI for her big support in collection and identifying the samples. I am also deeply grateful to Dr. Paolo MONCIARDINI and other members of Naicons team in Milan, Italy for the knowledge they shared during my training program there. My deepest thanks go to my colleagues in the laboratory for their endless help and support.

My greatest gratitude goes to my family for their unwavering love and support.

Hussam Laith Jafar AL-SHAREFI

Trabzon November-2025

THESIS ETHICS STATEMENT

I declare that I have completed this study titled " Isolation and Identification of Actinomycetes from Blue Lake and Detection of Bioactive Secondary Metabolites", which I submitted as a master's thesis, under the responsibility of my advisor Prof. Dr. Zihni DEMİRBAĞ and my co-supervisor Dr. Mehtap USTA from the beginning to the end, collected the data/samples myself, conducted the experiments/analysis in the relevant laboratories, shown the information I received from other sources in the text and references, and acted in accordance with scientific research and ethical rules during the study and accept any legal consequences in case the opposite occurs. 04/11/2025

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Hussam Laith Jafar AL-SHAREFI

Karadeniz Teknik Üniversitesi
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Aktinomisetler, biyolojik olarak aktif sekonder metabolitlerin en önemli üreticilerinden biri olarak kabul edilen, ipliksi yapılı Gram-pozitif bakterilerdir. Son yıllarda, klinikte kullanılan antibiyotiklerin çoğunluğunun kaynağını oluşturmuşlardır. Antibakteriyel bileşiklerin yanı sıra, aktinomisetler aynı zamanda antifungal, antiviral ve antikanser ajanların da kaynağı olarak bilinmektedir. Doğal kaynaklardan yeni terapötik bileşiklerin keşfi; çoklu ilaç dirençli patojenler, ölümcül viral enfeksiyonlar ve kanserin oluşturduğu küresel sorunlar ile devam eden mücadelede en önemli silahlardan biridir. Bu nedenle, yeni ve keşfedilmemiş habitatlarda aktinomisetlerin araştırılması ve potansiyel yeni biyoaktif bileşikler açısından taranması her zaman önem taşımaktadır. Bu çalışmada önemli aktinomiset sekonder metabolitlerinin elde edilmesi amaçlanmış ve bu doğrultuda Türkiye'nin Giresun ilinde yer alan Mavi Göl ve Göksu Travertenleri'nden toprak, su ve çamur örneklerinden toplam 17 aktinomiset izole edilmiştir. Bu izolatların biyoaktif potansiyelleri değerlendirilmiştir. İzolatların, katalaz aktivitesi, nişasta hidrolizi ve kazein hidrolizi gibi biyokimyasal ve morfolojik analizler tipik aktinomiset özelliklerini doğrulamıştır. Moleküler analizler, 13 suşun *Streptomyces* türlerine, diğerlerinin ise nadir aktinomiset türlerine ait olduğunu göstermiş, bu da sınıf içerisinde *Streptomyces* cinsinin baskınlığını desteklemiştir. Tüm izolatların antibakteriyel ve antifungal aktiviteler için test edilmiş, seçilen izolatların kaba (kuru) ekstraktları ise antiviral ve antikanser özellikleri açısından taranmıştır. Antibakteriyel testler, Gram-pozitif ve Gram-negatif bakterilere karşı geniş bir aktivite yelpazesi ortaya koymuştur. Ayrıca *Candida albicans*'a karşı antifungal aktivite de gözlenmiştir. Kaba ekstraktlar, HIV-1 RT enzimine karşı çok yüksek düzeyde *in vitro* inhibisyon göstermiş ve MTT testlerinde belirgin sitotoksisite sergileyerek antikanser potansiyel ortaya koymuştur.

Anahtar Kelimeler: Aktinomisetler, sekonder metabolitler, antibakteriyel, antifungal, HIV-1 RT inhibisyonu, antikanser.

Master's Thesis

ABSTRACT

ISOLATION AND IDENTIFICATION OF ACTINOMYCETES FROM BLUE LAKE AND DETECTION OF BIOACTIVE SECONDARY METABOLITES

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2025, 67 pages

Actinomycetes are filamentous, Gram-positive bacteria that considered one of the most important producers of bioactive secondary metabolites. Over the last decades, they have been the source of the majority of clinically used antibiotics. Beside antibacterial compounds, Actinomycetes are also known for being a source of antifungal, antiviral, and anticancer agents. The search for novel therapeutic compounds from natural sources is one of the most important weapons in the ongoing battle against multidrug-resistant pathogens, fatal viral infections, and the global burden of cancer.

Thus, it is always important to look for new resources of Actinomycetes in new, undiscovered habitats and scan them for potential new bioactive compounds.

In this study, we aim to find valuable Actinomycete secondary metabolites, and in line with this aim, 17 Actinomycete isolates were collected from the soil, water, and mud of Blue Lake and Goksu Travertines, Girusun, Turkiye, and evaluated for their bioactive potential. Morphological and biochemical analysis confirmed the typical Actinomycete traits, including catalase activity, starch hydrolysis, and casein degradation. Molecular analysis showed that 13 strains belong to *Streptomyces* species, while the others belong to rare Actinomycetes, which support the dominance of *Streptomyces* in this class. Whole (liquid) extracts of all isolates were tested for antibacterial and antifungal activities, while crude (dry) extracts of selected isolated were screened for antiviral and anticancer properties. The antibacterial assays revealed wide range of activity against Gram-positive and Gram-negative bacteria. Also antifungal activity was registered against *C. albicans*. Crude extracts demonstrated a very high *in-vitro* inhibition of HIV-1 RT, and considerable cytotoxicity in anticancer MTT assays.

Key words: Actinomycetes, secondary metabolites, antibacterial, antifungal, HIV-1 RT inhibition, anticancer.

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ABBREVIATIONS

°C	: Celsius degree
µg	: Microgram
µL	: Microliter
µm	: Micrometer
AIA	: Actinomycetes isolation agar
AMR	: Antimicrobial resistance
atm	: Standard atmosphere
C	: Cytosine
CFS	: Cell-free supernatant
DMEM	: Dulbecco's Modified Eagle's Medium
DNA	: Deoxyribonucleic acid
dNTPs	: Deoxynucleoside triphosphates
FDA	: US Food and Drug Administration
G	: Guanine
g	: Gram
HIV	: Human immunodeficiency virus
IAA	: Indole-3-acetic acid
IAV	: Influenza A virus
L	: Liter
LC/MS	: Liquid chromatography/mass spectrometry
mg	: Milligram
MHA	: Mueller Hinton agar
mL	: Milliliter
mm	: Millimeter
NCBI	: National Center for Biotechnology Information
OD	: Optical density
PCR	: Polymerase chain reaction
pH	: Potential of hydrogen
rpm	: Round per minute
RT	: Reverse transcriptase
Sab	: Sabouraud Dextrose Agar
SCA	: Starch casein agar
SM	: Skim milk
TSIA	: Triple sugar iron agar
WHO	: World Health Organization

1. GENERAL INFORMATION

1.1. Introduction

Microorganisms, plants, and even animals can produce special organic compounds called secondary metabolites. These compounds are not associated with the growth, maturing, or propagation of the organism. They often play crucial ecological roles such as defense, competition, and signaling. They include antibiotics, pigments, and toxins. Many secondary metabolites have been utilized in medicine, agriculture, and industry.

Actinomycetes are among the most prolific producers of secondary metabolites. They can generate different types of bioactive compounds, including antibiotics, antitumor agents, and immunosuppressants that are vital to modern medicine.

Actinomycetes are Gram positive bacteria that considered among highly heterogeneous groups of microorganisms in nature. They can be found in marine, terrestrial, and eukaryotic habitats (Van Bergeijk, 2020).

1.2. Taxonomy of Actinomycetes

Actinomycetes are large class of Gram positive bacteria that belong to the phylum Actinomycetota (formally known as Actinobacteria). According to NCBI Taxonomy Browser, there are more than 20 orders under the class Actinomycetes such as Actinomycetales, Bifidobacterials , Micrococcales, Micromonosporales, Mycobacterials, Kitasatosporales and Streptosporangiales (Figure 1).

Streptomyces, the largest and most valuable genus of Actinomycetes goes under the family Streptomycetaceae that belong to the order Kitasatosporales. The genus name *Actinomyces* comes from Greek origins; *aktina* means ray, and *mykis* means fungus (Urban and Gajdacs, 2021) which expresses the similarity between fungi and Actinomycetes. Beside that all Actinomycetes share the following morphological and biochemical features:

- High G+C content in their DNA.
- Mycelium like (filamentous) structure.
- Ability to produce antibiotics or decompose organic matter.

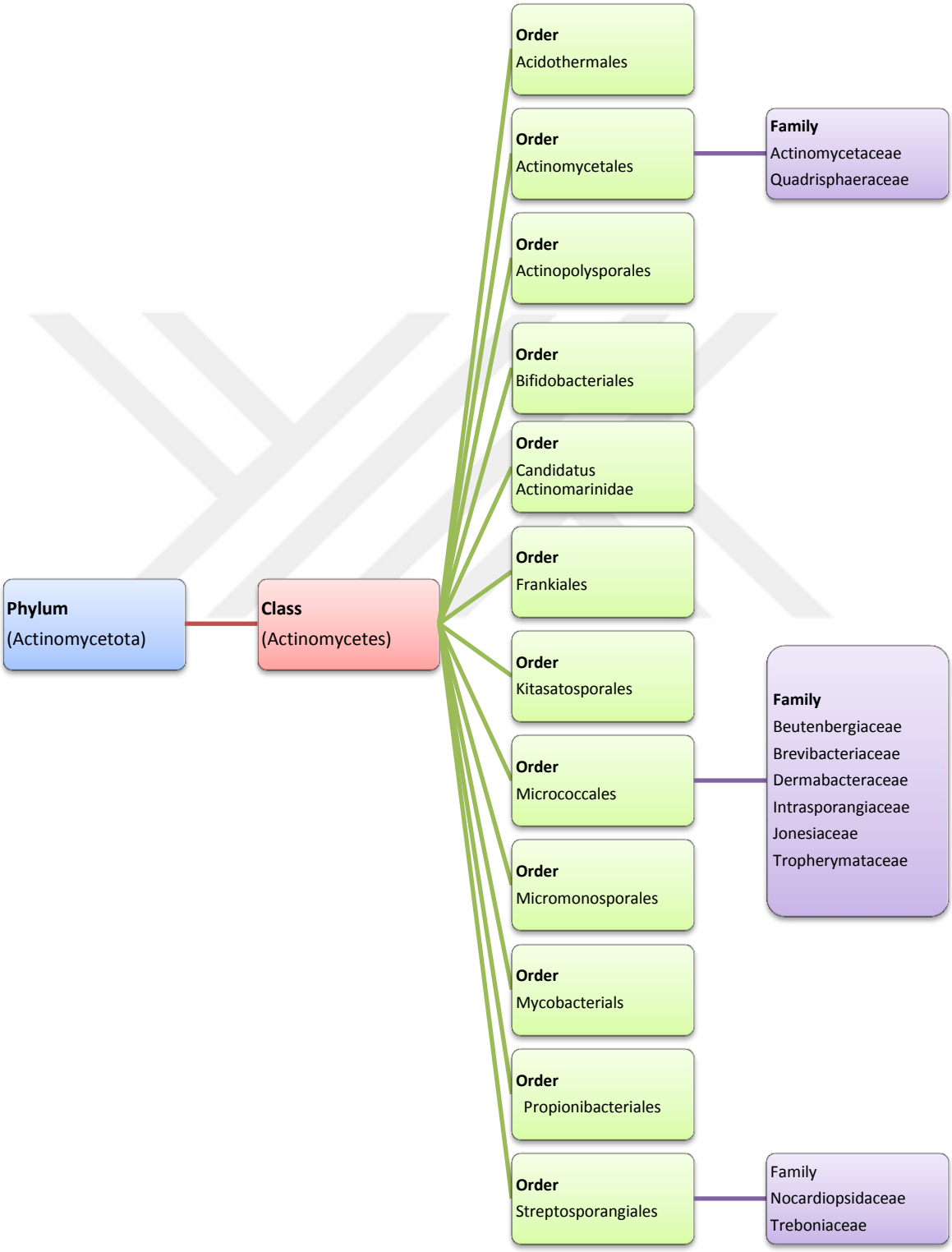

Figure 1. Taxonomy of Actinomycetes

1.3. Morphological Characteristics of Actinomycetes

Among Gram positive bacteria, Actinomycetes have the most considerable morphological differentiation. Not to forget that the cell structure of Actinomycetes is completely different from fungi as they are classic prokaryotes. They have well distinguished radial mycelium, and these mycelia are split into aerial mycelium and substrate mycelium depending on their function and the difference in morphology (Figure 2). A lot of Actinomycetes have the ability to form special forms, from simple ones like spore and spore chains to more complicated ones like sporangia and sporangiospore. The growth levels of substrate mycelium, the number and position of spores, and the shape of sporangia can be different among Actinomycetes. Some sporangiospores have flagella while others don't. All of these factors can play big role in the morphological classification of Actinomycetes (Li et al., 2016).

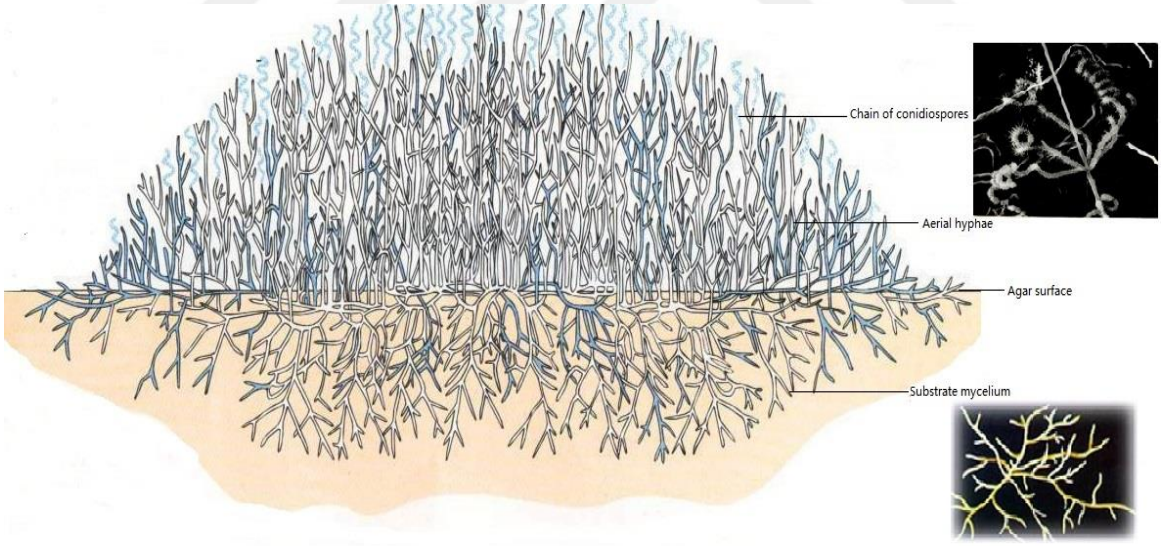

Figure 2. Actinomycetes colony on agar. Cross section of single Actinomycete colony showing the substrate mycelium and aerial mycelium with chains of spores (Li et al., 2016).

1.3.1. Substrate Mycelium

It is also called vegetative or primary mycelium; represent the growth phase of Actinomycetes that extends into or spread across the exterior side of the medium. This mycelial form is primarily responsible for the absorption of nutrients, supporting cellular growth and metabolic activity.

Under microscopic examination, substrate mycelia appear slender, transparent and phase-dark. They are usually more branched than aerial hyphae. One hyphae range from 0.4 to 1.2 µm in diameter, and are generally non-septate. However, they may show some fragmentation or branching (Li et al., 2016).

1.3.2. Aerial Mycelium

During the last stages of actinobacterial development, the aerial mycelium arises and extends vertically over the culture medium. In some cases, it may be difficult to differentiate between aerial and substrate mycelia due to overlapping morphological characteristics. While –as mentioned above- substrate hyphae are typically slender, transparent and phase-dark, the aerial hyphae are coarser, refractive and phase-bright. The ability to form aerial hyphae varies depending on traits of the species, environmental conditions, and the availability of nutrients. In certain genera, the aerial mycelium can differentiate to form spore chains at the terminal ends of hyphae. These chains function as reproductive structures, facilitating spore formation, and dispersal (Li et al., 2016).

1.3.3. Spores and Spore Chains

The aerial hyphae of most Actinomycetes can develop a formation called spores. They lead a process named sporulation which is a key productive mechanism that contributes to the survival, dissemination, and taxonomic identification of Actinomycetes. According to the observations by Lechevalier and his colleagues (1989) spore chains produced on these hyphae can be classified based on their length and the number of spores they contain (Figure 3):

- Monosporous, contains one spore.
- Bisporous (di-sporous) chains, consisting of two spores.

- Oligosporous chains, comprising few spores.
- Polysporous, with many spores.

The shape, color, length and position of these spore chains are fundamental indicators for classification.

The dimensions spores depend on their mode of formation. For example, singly produced spores or those in small chains are typically thicker than the parent hyphae, whereas spores formed in bigger chains generally retain a diameter similar to that of the hyphae. Spores typically range from 1 to 2 µm in diameter and show considerable diversity in form and surface ornamentation. Most usual spore shapes include globose, ovoid, coliform, rod-shaped, allantoid, and reniform. In some species we can observe motile spores which possess flagella that enable active locomotion (Li et al., 2016).

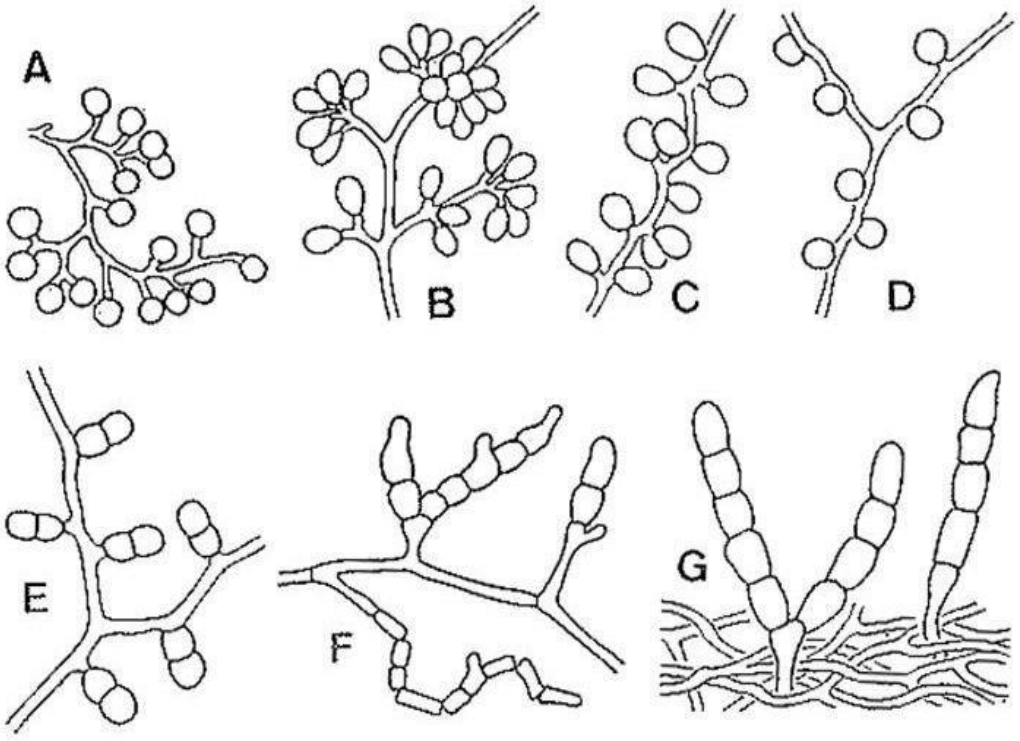

Figure 3. Single spore production and spores in short chains.
1) Monosporous: (A) Micromonospora, (B) Thermomonospora, (C) Saccharomonospora, (D) Thermoactinomyces. 2) Disporous: (E) Microbispora. 3) Oligosporous: (F) Nocardia brevicatena, (G) Catellatospora (Hamada et al., 1997).

1.4. Distribution of Actinomycetes

Actinomycetes are extensively distributed across a wide range of ecosystems, including terrestrial and aquatic habitats, as well as symbiotic and pathogenic associations with various hosts (Figure 4). They occur as free-living saprophytes especially within soil micro pores, where they contribute significantly to organic matter decomposition and nutrient cycling. Additionally, many species can establish endophytic relationships with plants, inhabiting internal tissues without causing harm and often contributing to plant growth and defense.

The ecological diversity of Actinomycetes is reflected in their wide habitat range. For example, genera like *Streptomyces, Micromonospora*, *Rhodococcus* and *Salinispora* are commonly isolated from soil and aquatic environments, whereas *Frankia* species function as nitrogen fixing symbionts in some plants. Other genera like *Corynebacterium, Mycobacterium* and *Nocardia* are known to inhabit animal hosts where they may act as pathogens in humans and other organisms. Further some, Actinomycetes have been recovered from extreme and specialized environments such as desert soils and cold ecosystems like the Antarctic region, indicating their high degree of physiological resilience and adaptability to environmental stressors (Ngamcharungchit et al., 2023).

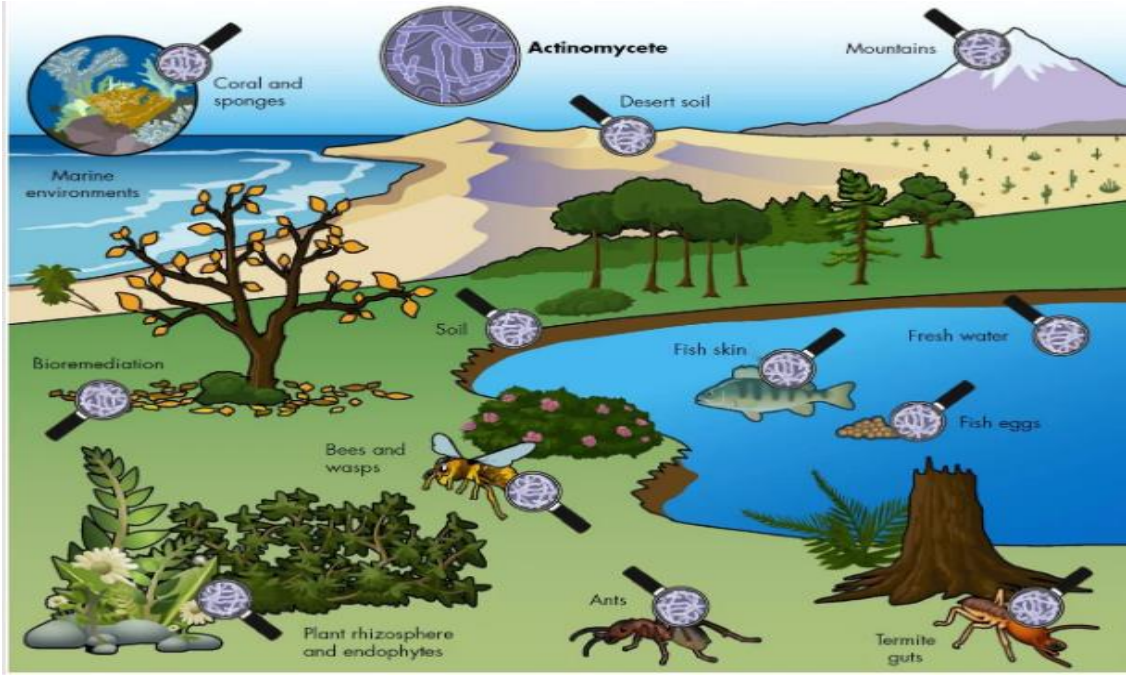

Figure 4. Different ecosystems of Actinomycetes (Gilles P. van Wezel).

1.4.1. Soil Actinomycetes

Due to their filamentous, hyphae forming characteristics, Actinomycetes are responsible of the earthy aroma emitted from freshly disturbed soil. They are most abundant in the top layers of the soil, where oxygen levels are higher, and tend to decrease with the increasing soil depth, reflecting their strict aerobic requirements (Bhatti et al., 2017). Their densities range from 10^4 to 10^8 cells per gram of soil. Soil composition and the ecological conditions play role in determining that density. These organisms are highly sensitive to acidic and waterlogged environments, with an optimal pH range of 6.5 to 8.0. Most Actinomycetes are mesophilic, favoring moderate temperature between 25 and 30 °C for optimal growth and metabolic activity.

Actinomycetes that promote plant growth exert their effects through both direct and indirect mechanisms. Direct mechanisms include the biosynthesis of plant growth regulators, such as indole-3-acetic acid (IAA), gibberellins and cytokinins, which stimulate various physiological processes in plants. Indirectly, they can strengthen plant health by producing antimicrobial compounds, siderophores and cell wall-degrading enzymes that contribute to the suppression of phytopathogenic organisms (Aamir et al., 2020). Moreover, certain Actinomycetes have been isolated from saline soil environments, highlighting their ecological adaptability and potential utility in agricultural systems subjected to abiotic stresses, such as salinity (Guan et al., 2020).

1.4.2. Endophytic Actinomycetes

They are a unique group of bacteria that inhabit the internal tissues inside healthy plants without inducing detrimental effects on their hosts. These microorganisms have emerged as a promising source of novel bioactive compounds, including antibiotics, antifungals, and antitumor agents, making them highly valuable for biotechnological and pharmaceutical applications.

A wide range of plant species have been reported to host endophytic Actinomycetes, including agricultural crops, medicinal plants, halophytes and woody tree species (Rungin et al., 2012).

Although *Streptomyces* species are the most common type of endophytic Actinomycetes, rare Actinomycetes (non- *Streptomyces*) can be found too in the plant samples. Giving the fact that the ratio of *Streptomyces* is low in plant roots, these roots can

be an excellent expected source of rare Actinomycetes and, eventually, a novel secondary metabolite (Ngamcharungchit et al., 2023).

1.4.3. Marine Actinomycetes

Nautical environments are recognized as valuable reservoirs of unique and largely uncharacterized Actinomycetes, offering remarkable chances for discovery novel bioactive compounds. These environments include coastal waters, deep sea sediments, open seawater, and mangrove forests, each providing unique ecological conditions that support microbial diversity.

Mangrove ecosystems, in particular, are biologically complex and dynamic, covering approximately 75% of the world tropical coastlines. Despite their ecological significance, the microbial communities within mangroves remain relatively underexplored. The fluctuating salinity levels, tidal influences and variable redox conditions in mangrove soils create a distinctive habitat that promotes the growth of specialized microorganisms capable of synthesizing unusual and structurally diverse secondary metabolites (Setyati et al., 2021). Mangrove sediments are inhibited by a wide variety of microbial groups, including members of the phyla Pseudomonadota, Actinomycetota and a range of filamentous fungi. Among these, Actinomycetes -particularly species of the genera *Streptomyces* and *Micromonospora*- are considered promising candidates for the isolation of potent bioactive compounds. (Xu et al., 2018). Marine Actinomycetes are valuable sources of novel anticancer compounds. Several notable examples include salinosporamide A, produced by *Salinispora tropica*, actinomycin D from *Streptomyces parvulus*, mitomycin C synthesized by *Streptomyces caespitosus* and rakicidin D, isolated from *Streptomyces* sp. MWW064 (Olano et al., 2009; Subramani and Aalbersberg, 2012).

1.4.4. Actinomycetes in Compost

Composting is a biological process driven by diverse microbial communities including fungi and Actinomycetes, which is indispensable for decomposition of organic matter. This degradation results in production of carbon dioxide, water, heat and humus forming a relatively stable organic product (Hemati et al., 2022). The composting process typically progresses through three distinct phases: the mesophilic phase, the thermophilic phase, the cooling and maturation phase. Each stage is characterized by the activity of

specific microbial consortia adapted to the prevailing environmental conditions (Hemati et al., 2022).

During the mesophilic phase, microorganisms that thrive at moderate temperatures rapidly degrade soluble and easily decomposable compounds. This microbial activity generates heat, leading to a quick expansion in the internal compost temperature. Once temperatures exceed approximately 40 °C, mesophilic organisms become less active and get succeeded by thermophilic microorganisms, which dominate the second phase. This stage facilitates the accelerated breakdown of complex organic molecules, including proteins, lipids, cellulose, hemicellulose, and lignin (Hemati et al., 2021). As these substrates are consumed, the temperature gradually decreases, allowing mesophilic microbes to reemerge and dominate the maturation or curing phase, where the remaining organic matter undergoes further stabilization and humification.

Actinomycetes, particularly thermophilic ones, play a critical role during the thermophilic and maturation phases of composting (de Gannes et al., 2013; Takaku et al., 2006). They are key decomposers in aerobic, high temperature environments, such as compost piles, manure heaps, straw and improperly stored agricultural materials. Their ability to degrade recalcitrant plant polymers makes them indispensable for efficient composting and organic matter recycling (Ngamcharungchit et al., 2023).

1.5. Importance of Actinomycetes

Actinomycetes are well known for their extensive biosynthetic capabilities, particularly in the production of specialized metabolites. These microorganisms have been extensively utilized because they are able to generate an enormous number of bioactive compounds, including antibiotics, anticancer agents, anthelmintics, and antifungals. As a result, Actinomycetes play a pivotal role in biotechnology, medicine and agriculture (Barka et al., 2016). Although few species, such as *Streptomyces scabies*, are known to cause plant diseases like potato common scab (Keinath and Loria, 1989), the majority show antagonistic activity against a broad spectrum of phytopathogenic fungi, offering potential for biocontrol applications (Berg et al., 2001).

The order Actinomycetales comprises more than 80 distinct genera (Stackebrandt et al., 1997); the majority of known bioactive metabolites have been derived from species of the genus *Streptomyces*. However, other families within this order, such as

Micromonosporaceae, Pseudonocardiacea, Streptosporangiaceae and *Thermomonosporaceae*, also contribute significantly to the production of therapeutically valuable compounds, including erythromycin, gentamycin, rifamycin and vancomycin (Okami and Hotta, 1988; Lazzarini et al., 2000). It remains unclear whether the predominance of *Streptomyces*-derived metabolites is due to their superior biosynthetic capacity and their greater strain diversity or simply a result of more extensive screening efforts. The latter may be attributed to the relative ease with which *Streptomyces* strains can be isolated, compared to the other Actinomycete genera (Monciardini et al., 2002).

According to World Health Organization (WHO), it is estimated that by 2050 antimicrobial resistance (AMR) could lead to 10 million deaths per year (Alanis, 2005; Naghavi et al., 2024), thus there is an urgent need to discover novel antimicrobial agents that can fight the rapidly increasing resistant microbial strains. One of the methods is to focus on screening rare types of Actinomycetes that can be found in unusual environments.

1.5.1. Primary and Secondary Metabolites

Bacterial metabolites are generally categorized into two major groups: primary and secondary metabolites, based on their biological roles and production timing. Primary metabolites are fundamental to cellular growth and proliferation, participating in energy metabolism and serving as precursors for macromolecule biosynthesis. These compounds are predominantly synthesized during the logarithmic (exponential) growth phase (Figure 5) and are typically charged intracellular molecules, which limits their diffusion across cellular membranes. In contrast, secondary metabolites are characterized as low molecular weight, non-polar and uncharged compounds that are primary extracellular in nature. These metabolites possess crucial roles in microbial ecological interactions, such as competition, defense and communication. Their production is usually associated with the late exponential or stationary phase, reflecting their non-essential but adaptive functions within complex environments.

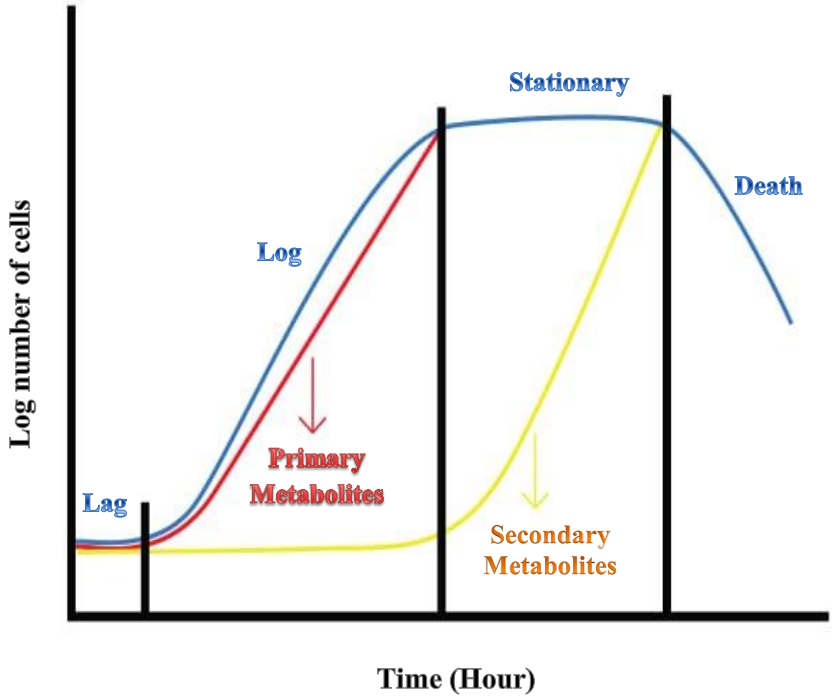

Figure 5. Microbial growth phases and synthesis of metabolites.

Because primary metabolites are irreplaceable for cellular growth, there is a continuous operation of key metabolic pathways such as glycolysis, tricarboxylic acid and amino acid biosynthesis. Enzymes responsible for these pathways are generally produced in high amounts and are constitutively expressed within the cell. On the other hand, due to secondary metabolites being not essential for cell growth, the biosynthetic pathways responsible for their production are typically silent under normal growth conditions, with corresponding genes remaining unexpressed unless activated by specific environmental signals or stress factors (Seyedsayamdost, 2019).

1.5.2. Actinomyectes as a Source of Secondary Metabolites

The order *Actinomycetales* alone are associated with production of more than 10.000 antimicrobial agents that are in the pharmaceutical market today (Table 1) (Selim et al., 2021). Additionally, out of an estimated 200.000 to 250.000 known bioactive metabolites,

approximately 50.000 have been assigned with Actinomycete fermentation, with the genus *Streptomyces* alone responsible for the production of nearly 35.000 compounds. Actinomycetes can be a lavish origin of structurally diverse secondary metabolites like antibiotics, enzyme inhibitors, antiviral, anti-tumor agents, vitamins and agrochemicals such as pesticides. Several newly identified compounds illustrate their pharmaceutical potential. For example, *Streptomyces* sp. CPCC 203577 produces Lavanducyanin and Napthomevalin, both effective against Gram positive bacteria. Additionally, Shellmycin A–D, derived from *Streptomyces* sp. Shell-016, and Litoralimycins A and B, from *Streptomonospora* sp. M2, have demonstrated notable anticancer activity. Moreover, marine Actinomycetes have risen as a promising source of bioactive compounds including antiparasitic, antiprotozoal and antifouling effects. Notably, crude extracts from endophytic actinomycetes associated with marine seagrass have showed strong antibacterial activity against *Klebsiella pneumoniae* (Kasinathan et al., 2022).

1.5.3. Secondary Metabolite Types

The secondary metabolites of Actinomycetes can be based on their function like antbiotics, anti-viral agents, anti tumor agents and others (Figure 6), or based on their chemical structure as peptides, polyketides, butanolides, polycyclic xanthones and others (Ngamcharungchit et al., 2023). This classification can be different due to the huge diversity of these metabolites. 70% of all known bacterial secondary metabolites are associated with Actinomycetes, while 7% are produced by *Bacillus* spp., 20% by fungi, and 2% by other bacteria (Berdy, 2012).

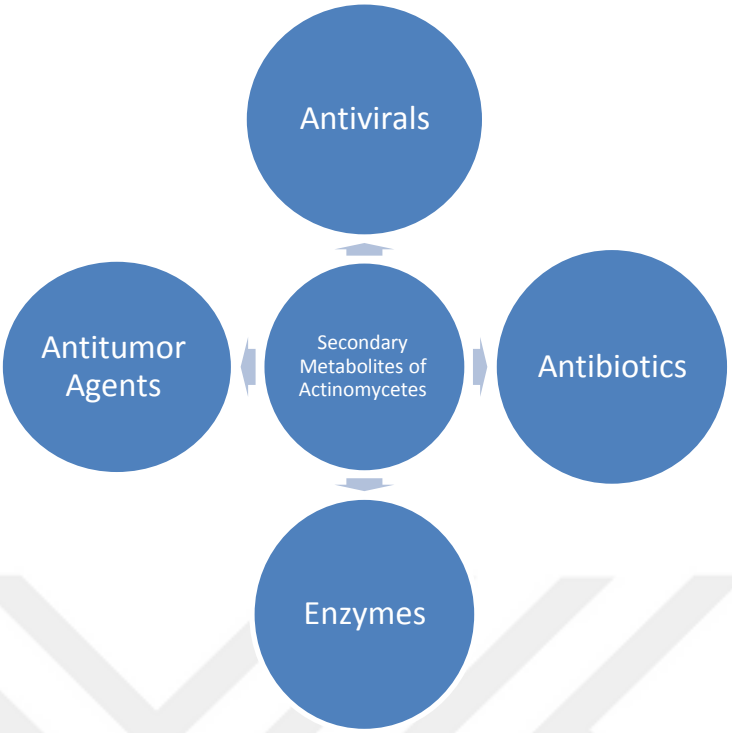

Figure 6. Most important types of Actinomycete secondary metabolites.

- **Antibiotics**

Antibiotics are a class of chemotherapeutic agents that biosynthesized by microorganisms like fungi and bacteria, particularly Actinomycetes. Even in very small concentrations, these compounds are able to inhibit the growth or causing the death of other bacteria. They are organic compounds with low molecular weight produced during the microbial metabolism (Adegboye and Babalola, 2013). About 75% of all antibiotics are derived from Actinomycetes and many of them have a broad-spectrum activity against wide range of Gram-positive and Gram-negative bacteria (Hasani et al., 2014). Most of antibacterial metabolites were derived from *Streptomyces* (Table 2), while others were identified from rare Actinomycetes genera like *Actinoallourus, Actinomadura, Amycolatopsis, Kibdelosporangium, Kitasatospora, Kocuria, Microbacterium, Micromonospora, Rhodococcus, Nocardiopsis, Pseudonocardia, Streptomonospora, Streptosporangium, Thermoactinomyces*, and *Verrucosispora* (Ngamcharungchit et al., 2023).

Table 1. Number of bioactive metabolites produced by Actinomycetes species (Sharma et al., 2014).

Actinomycetes species	**No.**	**Actinomycetes species**	**No.**
Streptomycetaceae		**Mycobacteriaceae (Actinobacteria)**	
Streptomyces	8000	Nocardia	357
Streptoverlicillium	258	Mycobacterium	57
Kitasatosporia	37	Arthrobacter	25
Chainia	30	Brevibacterium	17
Microellobosporia	11	Proactinomyces	14
Nocardioides	9	Rhodococcus	13
Thermomonosporaceae		**Micromonosporaceae (Actinoplanetes)**	
Actinomadura	345		
Saccharothrix	68		
Microbispora	54	Micromonospora	740
Actinosynnema	51	Actinoplanes	248
Nocardiopsis	41	Dactylosporangium	58
Microtetraspora	26	Ampullariella	9
Thermomonospora	19	Glycomyces	2
Micropolyspora-Faenia	13-3	Catenuloplanes	3
Thermoactinomyces	14	Catellatospora	1
Thermopolyspora	1		
Thermoactinopolyspora	1		

Table 2. Some of the antibacterial metabolites derived mostly from *Streptomyces* (Ngamcharungchit et al., 2023).

Organism	Compound Name
Actinomadura sp. KC191	Actinomadurol
Amycolatopsis sp. IRD-009	Pradimicin-IRD
Amycolatopsis sp. M39	Macrotermycins A & C
Amycolatopsis sp. MCC0218	Enceleamycins A–C
Amycolatopsis sp. ML1-hF4	Pargamicins B–D
Streptomyces sp. DSM14386	Svetamycins C, G
Streptomyces sp. FJS31-2	Zunyimycins B,
Streptomyces sp. GKU 220	Rakicidin F
Streptomyces sp. HCCB11876	Quinomycins I, J
Streptomyces sp. HK-2006-1	Aldgamycin O
Streptomyces sp. HN-A124	Cysrabelomycin
Streptomyces sp. Hu103	Anulamycins A-D
Streptomyces sp. HZP-2216E	23-*O*-butyrylbafilomycin D
Streptomyces sp. IB201691-2A	Baikalomycins A–C
Streptomyces sp. ICN19	Ala-geninthiocin
Streptomyces sp. KCB13F003	Ulleungmycin A, B
Streptomyces sp. KCB14A132	Enamidonins B, C
Streptomyces sp. MM168-141F8	Quadoctomycin , Phenol
Streptomyces sp. NA06554	Borrelidin J
Streptomyces sp. NA07423	Nagimycins A, B
Streptomyces sp. SBT345	Ageloline A
Streptomyces sp. SCSIO 41399	Isotirandamycin B
Streptomyces sp. sima1_6	Echinomycin
Streptomyces sp. SN0280	Streptoone A
Streptomyces sp. SM01	Picolinamycin
Streptomyces sp. SS	Sansanmycin Q
Streptomyces sp. Stup16_B49.2	Echinomycin
Streptomyces sp. Stup16_B146	TPU-0037-C
Streptomyces sp. Stup18_J70	Bafilomycin A1 derivative
Streptomyces sp. UICC B-92	4-*O*-glucosyl,1-carboxyl-phenazine
Streptomyces sp. W367A	W367A
Streptomyces sp. XMA39	Strepoxepinmycins A–D
Streptomyces sp. YINM00001	Peperodione, Peperophthalene
Streptomyces sp. ZZ1118	Streptoindoles A–D
Streptomyces sp. ZZ741	Streptoglutarimides A–J
Streptomycete clade MAR4 CNY-960, CNS-284	Marinocyanins A–F

- **Antiviral Agents**

Of all-natural product-based methods approved by the US Food and Drug Administration (FDA), 50% come from microbial origin, including those of antiviral nature. One of the oldest examples of antiviral nucleoside analogue is Vidarabine (Ara-A), isolated from *Streptomyces antibioticus* (Patridge et al., 2016). Another example is a novel compound (an Antimycin A derivative) from *Streptomyces kaviengensis*, which shows antiviral activity against the Western equine encephalitis virus (Jakubiec et al., 2018). Aristromycin isolated from *Streptomyces citricolor* showed potent activity against IAV (Rawal et al., 2016). The supernatant of *Streptomyces chromofuscus* produced a protease inhibitor PISC-2002 that showed antiviral activity against influenza virus A/Rostock/34 (H7N7) (Selim et al., 2021).

As in antibacterial agents, the majority of newly discovered antiviral metabolites have *Streptomyces* sources, with few findings related to rare genera like *Kutzneria*.

- **Antitumor Compounds**

The discovery of novel antitumor agents is increasingly associated with the exploration of marine-derived natural products, specifically marine Actinomycetes. Between 2007 and 2017, genera such as *Micromonospora*, *Salinispora*, and *Verrucosispora* were among the most producers of newly identified secondary metabolites (Subramani and Sipkema, 2019). These bioactive compounds are commonly classified into four major natural product categories: polyketides, alkaloids, peptides, and quinones.

The reason why marine Acinomycetes hold significant anticancer therapeutic potential is the low toxicity profiles of their products compared to traditional chemotherapeutic agents. Salinosporamide A, produce by *Salinispora tropica*, is a good example of marine-derived compound with potent anticancer activity which has reduced side effects (Leo and Jeong, 2020). Moreover, a wide range of marine actinomycetes derived compounds, such as streptochlorin, actinofuranones, aureoverticillactam, chalocomycin B, cyanosporasides, komodoquinones, nonactin, resitoflavine, tetracenomycin D, thiocoraline, butenolides, , and streptokordin have showed antitumor properties (Jagannathan et al., 2021; Igarashi et al., 2010).

Once again, *Streptomyces* are the most dominant genus in producing antitumor compounds (Table 3).

- **Others**

The benefits of secondary metabolites of Actinomycetes are almost unlimited. They cover most of the biotechnological fields in medicine, agriculture, industry, and others. For example, bio-pesticide agents, plant growth hormones, anti-inflammatory compounds, single-cell protein feed, vitamins, pigments, commercial enzymes (Table 4), and enzyme inhibitors.

Table 3. Antitumor compounds identified from Actinomycetes (Ngamcharungchit et al., 2023).

Organism	**Compound Name**
Actinoalloteichus hymeniacidonis 179DD-027	Dokdolipid B
Actinomadura sp. K13-0306	Sagamilactam
Actinomadura sp. K4S16	Nonthmicin, Ecteinamycin
Actinosynnema pretiosum HGF052::asm18	Actinosynneptide A, B
Amycolatopsis sp. IRD-009	Pradimicin-IRD
Amycolatopsis alba DSM 44,262	Thioalbamide
Micromonospora matsumotoense M-412	Paulomycin G
Micromonospora aurantiaca 110B	Isoflavonoid glycosides 1-3
Micromonospora chalcea FIM 02-523	Rakicidins G–I
Micromonospora sp. FIM05328	FW05328-1
Micromonospora sp. HS-HM-036	Naphthalenepropanoic acid analog
Micromonospora yangpuensis DSM 45577	Yangpumicin A
Micromonospora yangpuensis DSM 45577	Yangpumicins F, G
Nocardiopsis alba KM6-1	Isomethoxyneihumicin
Nocardiopsis sp. CG3	Kenalactams A, E
Nonomuraea endophytica GW58/450	Karamomycins B, C
Nonomuraea sp. AKA32	Akazamicin

Table 3. *Cont.*

Organism	Compound Name
Streptomyces sp. AD-3	Nybomycins D
Streptomyces sp. ADI92-24	Tomaymycin
Streptomyces sp. ADI95-17	Naringenin
Streptomyces sp. ADI96-02	Echinomycin
Streptomyces sp. ADI96-15	Antimycin, Lobophorins
Streptomyces sp. ADI97-07	Neocarzilin, Antimycin
Streptomyces sp. ADI98-10	Actinomycin
Streptomyces sp. B9173	Flaviogeranin B1, B, C2, D
Streptomyces sp. BB47	Antarlides F–H
Streptomyces sp. CHQ-64 ΔrdmF	Geranylpyrrol A, Piericidin F
Streptomyces sp. CMAA 1527	Cinerubin B
Streptomyces sp. CMAA 1653	Actinomycin V
Streptomyces sp. EGY1	Sharkquinone
Streptomyces sp. GIC10-1	Bafilomycin M
Streptomyces sp. GKU 220	Rakicidin F
Streptomyces sp. HF-11225	Nivelactam B
Streptomyces sp. HN-A124	Cysrabelomycin
Streptomyces sp. HNA39	Cyclizidines B–I
Streptomyces sp. ICN19	Ala-geninthiocin
Streptomyces sp. IFM 11490	Elmenols G
Streptomyces sp. KCB13F003	Ulleungdin
Streptomyces sp. KCB13F030	Ulleungoside
Streptomyces sp. KIB-H1318	Phenoxazinone-related alkaloid 2
Streptomyces sp. KIB-H714	Actinomycin Z6
Streptomyces sp. M268	Kiamycin B
Streptomyces sp. MSB090213SC12	Neothioviridamide
Streptomyces sp. N1510.2	Strepantibins A–C
Streptomyces sp. NA4286 M	Murayaquinone D
Streptomyces sp. NEAU-L3	Tetracenoquinocin A
Streptomyces sp. OPMA00071	JBIR-150
Streptomyces sp. PU-14G	Puromycins C, D
Streptomyces sp. PU-KB10-4	4-hydroxycinnamide
Streptomyces sp. Q22	Bagremycins C
Streptomyces sp. RAI364	Curromycin A
Streptomyces sp. RK88	Opantimycin A
Streptomyces sp. SBT345	Strepoxazine A
Streptomyces OPOK_MB_A9	Milbemycin-α8
Verrucosispora sp. SCSIO 07399	Kendomycins B-D

Table 4. Some of the enzymes produced by Actinomycetes (Kumar et al., 2014).

Enzyme	**Organism**	**Use**	**Application**
Protease	*S.galbus*	Detergents Cheese making Claricaation- low calorie beer Dehiding Blood clot treatment	Detergents Food Brewing Leather Medicine
Cellulase	*S.actuosus*	Removal of strains, denim nishing, soening of detergent	Deinking, soening of cotton, paper and pulp
Lipase	*S.griseochromo_ genes*	Removal of strains Stability and conditioning of dough Cheese avoring Deinking, cleaning	Detergent Baking Dairy Textile
Xylanase	*S.rameus*	Conditioning of dough Digestibility Bleach boosting	Baking Animal feed Paper and pulp
Pectinase	*S.fradzae* *S.nztrosporeur*	Clarication, mashing	Beverage
Amylase	*S.aureofasciculus* *S.galilaeus*	Soness of bread soness Removal of stains volume Deinking, drainage improvement Production of glucose and fructose syrups Removal of starch from woven fabrics	Detergent Baking Paper and pulp Starch industry Textile
Phytase	*S.ambofaciens* *S.lienomycini*	Phytate digestibility	Animal feed
Glucos oxidase	*Streptomyces* sp	Strengthening of dough	Baking

Table 4. *Cont.*

Lipoxygenase	*Streptomyces* sp	Bread whitening	Baking
β-galactosidase	*Streptomyces* sp	Enzymatic hydrolysis of lactose	Dairy
L-asparaginase	*S.aureofasciculus* *S.canus* *S.hawaiiensis* *S.olivoviridid,*	Reduce the formation of acrylamide, a carcinogen found in starchy food products	Food industry

1.6. Blue Lake and Kuzalan Goksu Travertines

Blue Lake (Mavigöl) comprises four interconnected lakes of varying sizes located along the Göksu Creek in Giresun/Turkiye. It is he only carbonated water body in the Turkish Black Sea region. Because of the basin's rocky geological structure, the lakes display a vibrant turquoise color from June to December as a result of snowmelt. Blue Lake is part of Kuzalan Nature Park, the home of more than 129 plant species belonging to about 60 families; it also hosts 154 vertebrate species.

Goksu travertines, located in Pınarlar Village, approximately 4 kilometers away from Kuzalan Nature Park, resemble a mini Pamukkale with its turquoise and white colors and large and small ponds in the form of terraces created by a project (Figure 8). Travertine is formed with soda water with high mineral value.

Figure 7. The Blue Lake in Giresun (Zenofon tours).

Figure 8. Goksu Travertines (Anadolu Agency).

1.7. Aim of Study

Antimicrobial resistance (AMR) is one of the most dangerous threats of global Public health. It is mainly induced by the overuse or misuse of antimicrobial drugs; not only in humans, but also in animals and plants. According to the World Health Organization (WHO), 1.27 million international deaths were directly caused by bacterial AMR only, and contributed in 4.95 million deaths. Although AMR hits deeper in poor countries, middle-income and rich countries are affected too, this makes infections harder to treat and interacts with life-threaten medical procedures like surgery and chemotherapy. In the battle against AMR there are different strategies include preventing of all infections before they

occur, the education about the deadly outcomes of misusing and overusing of antimicrobials, and the continuous searching for novel antimicrobial agents against stubborn microbes.

Actinomycetes, particularly *Streptomyces* are one of most important sources for novel secondary metabolites that can be helpful not only against AMR, but also in the field of the discovering new anti-cancer therapeutics.

Our aims is to isolate Actinomycetes from a potential rich area of it like Blue Lake and Goksu Travertines, after identifying them the promising ones for antimicrobial and anti-cancer secondary metabolites will be scanned. As we couldn't find any published paper regarding Actinomycetes in that specific area, we hope that our study shows the importance of Black Sea region that is full of caves and mineral water pools.

2. MATERIAL AND METHODS

2.1. Sample Collection

Four soil samples, 5 mud samples, and 2 water samples were collected from Blue Lake (40°38'07.2" N 38°22'56.0" E), while 4 mud sample, 6 water samples were collected from Kuzalan Goksu Travertines (40°65'23.2" N 38°22'56.0" E). *In situ* pH and temperature were measured for water samples then transferred to sterilized bottles. Mud and soil samples were collected by sterile scraper at 20-30 cm depth then transferred to sterile polyethylene bags. All samples were kept cool until arriving to the lab then were put in +4 °C refrigerator for the next step.

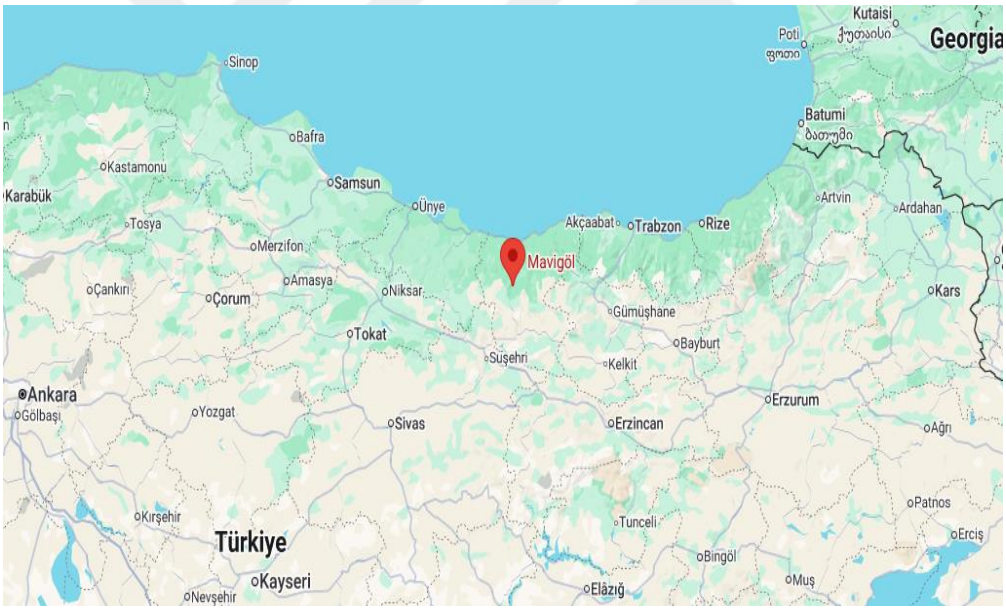

Figure 9. Location of Blue Lake on the map.

2.2. Bacterial Isolation

After drying mud and soil samples for 24 hours, serial dilution method was applied by adding 1g of dry sample to 9 mL of distilled water then diluted up to 10^{-5}. 100 µL of the dilutions 10^{-3} and 10^{-5} were spread on the surface of the Petri dishes containing 15 mL of the solid culture media, while 1 mL of water samples was spread directly.

CM medium (Table 5) with and without adding potassium dichromate was used for bacterial isolation. The benefit of adding potassium dichromate is to limit the growth of other bacteria and fungi (Tan et al., 2009). Next step was to incubate the plates at 30 °C for 2-3 weeks while the growth was checked regularly. After the incubation, Actinomycetes colonies with different sizes and shapes were chosen and transferred to Actinomycetes isolation agar (AIA) medium, and starch casein agar (SCA) medium (Table 6), then incubated at 30 °C for 3-4 days. Pure cultures were kept at +4 °C and preserved in 20% glycerol at -20 °C for later use.

Table 5. The composition of CM medium (Tan et al., 2009)

Ingredient	**g/L**
KNO_3	1
K_2HPO_4	0.5
$MgSO_4.7H_2O$	0.4
Glucose	10
Potassium dichromate	0.025
Agar	18

Table 6. The composition of SCA medium (Yildirim, 2018)

Ingredient	**g/L**
Soluble starch	10
Casein	0.3
KNO_3	2
$MgSO_4.7H_2O$	0.05
K_2HPO_4	2
NaCl	2
$CaCO_3$	0.02
$FeSO_4.7H_2O$	0.01
Agar	18

For both media, all components were dissolved in distilled water to a final volume of 1 L; pH was adjusted to 7.5 then autoclaved at 121 °C (1.1 atm) for 15 minutes.

2.3. Identification of Actinomycetes

Fresh culture of each isolate was brought for identification. Identification of bacterial isolates was made at morphological, biochemical, and molecular levels.

2.3.1. Morphological Identification

- **Colony Morphology**

Actinomycetes were identified by the presence of chalk-like, leathery, and tough colonies that attached to the agar surface (Tan et al., 2009). Moreover, there were examination of the color of the aerial mycelium and diffusible pigments (Sah et al., 2021); the colonies form, elevation, and margin (Figure 10).

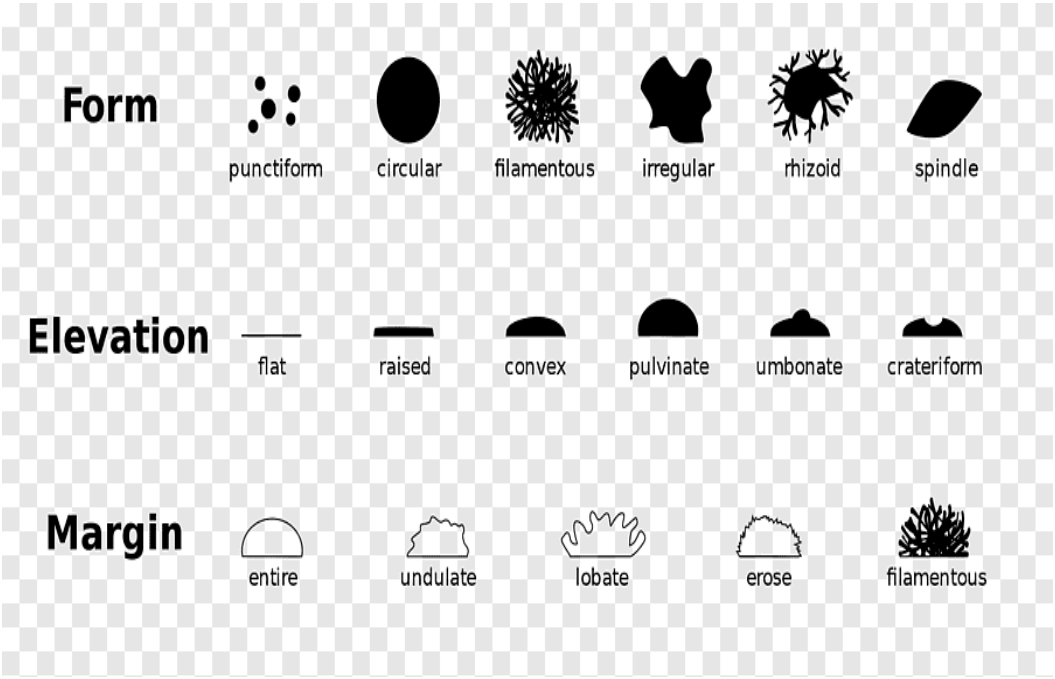

Figure 10. Colony morphology types of Actinomycetes.

- **Microscopic Characteristics**

To determine the general microscopic characteristics of Actinomycete isolates, Gram staining was performed by placing a drop of water on a clean slide, and the isolates were spread onto the slide using a sterile loop. Then the prepared smear was air-dried an passed through a flame 2 or 3 times to fix the sample. Crystal violet was added to the smear and kept for 1 minute, then rinsed with distilled water. After washing, Gram iodine was applied, and it was kept for another minute. After that, it was washed with ethanol until the purple color faded. Finally, safranin was applied for 30-60 seconds, then rinsed with distilled water and the slide was gently blotted with drying paper (Williams et al., 1993). The actinomycetes isolates were examined under a microscope up to x1000.

To examine the spore structure of the samples, the cover slip technique was used (Sahilah, 1991). The samples were grown for about 4 days on a thin layer of SCA poured onto a microscope slide. After incubation, the cultures were stained with crystal violet and kept for 1 minute. After rinsing with distilled water, a cover slip was placed on top of the slide, and the samples were examined under a light microscope up to x1000.

2.3.2. Biochemical Identification

Biochemical assays provide a simple, low-cost, and rapid means of identifying Actinomycetes before proceeding to more complicated and much expensive techniques like molecular ones. These tests can play supportive role in taxonomic differentiation especially in cases where genetic analysis doesn't provide decisive results. In addition to identification, biochemical assays can be helpful to indicate secondary metabolite potential and guide the selection of isolates for bioassay screening (Abdelrahman et al., 2022; Van Bergeijk et al., 2020).

Four tests that usually being used for Actinomycete identification were chosen: catalase enzyme production test, amylase enzyme production test, H_2S production test, and casein hydrolysis test.

- **Catalase Enzyme Production Test**

Test was done according to the American Society for Microbiology (Reiner, 2010). A small amount of fresh culture was transferred to a microscope slide using a sterile

inoculating loop. One drop of 3% H_2O_2 was placed onto the sample on the slide using a Pasteur pipette, then it was observed whether there was immediate formation of bubbles (positive result) or not.

- **The Amylase Enzyme Production Test**

The starch hydrolysis test was done by streaking Actinomycete isolates on Mueller Hinton Agar (MHA) medium then incubating for 48 hours at 37 ^{0}C. After that, 3-4 drops of 10% iodine solution were added directly onto the colony edges. After waiting for 10 minutes iodine solution was discarded from the plates, and the results were taken whether the medium turned dark with the areas around the colonies appeared as clear zone due to the starch had been hydrolyzed by amylase (positive result) or there weren't any zones (negative result) (Ekka and Namedo, 2018).

- **The H_2S Production Test**

Triple sugar iron agar (TSIA) medium was prepared (Table 7). 5-7 mL of the medium was poured into sterile tubes and let to cool and solidify at a slanging position (around 30° inclinations) to form an agar slant. Using sterile inoculation wire, 24 hours old Actinomycetes cultures were stabbed about 4 mm above the butt then streaked over the agar slant (Stab-and-streak inoculation). The tubes were incubated at 37 ^{0}C for 24 hours without fully tightening the screw caps to let air goes in. Tubes were examined to detect presence of blackening inside the medium (H_2S production) or absence of it (Thakur et al., 2021). Besides that, the type of reaction (acidic, alkaline) was determined based on the color of slant and butt of agar slants; red means alkaline reaction while yellow means acidic reaction, red slant and yellow butt indicate that only glucose was fermented, yellow slant and yellow butt mean sucrose and/or lactose was fermented or all three sugars were fermented, while both red slant and red butt shows that none of the three sugars were fermented. Also forming gab in the media, gas bubbles or cracking of the media was detected to check for gas production (Figure 11).

Table 7. The composition of TSIA medium

Ingredient	g/L
Peptone	20
Meat extract	3
Yeast extract	3
Dextrose (Glucose)	1
Lactose	10
Sucrose	10
Sodium chloride	5
Ferric citrate	0.3
Sodium thiosulfate	0.3
Phenol red	0.024
Agar	12

All components were dissolved in distilled water to a final volume of 1 L; pH was adjusted to 7.5 then autoclaved at 121 °C (1.1 atm) for 15 minutes.

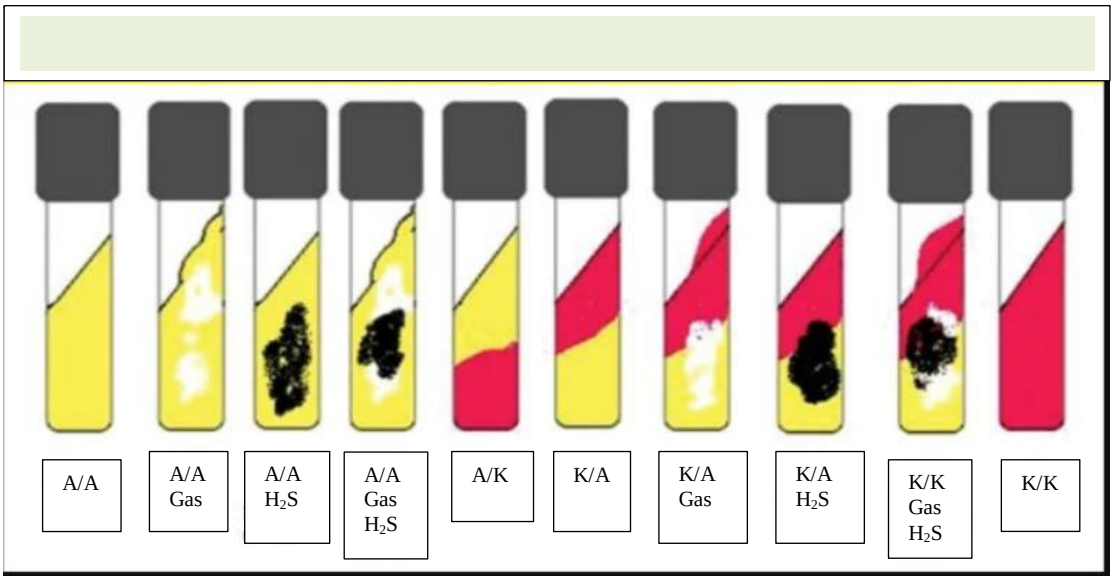

Figure 11. The appearance of TSIA test results; A: acidic, K: alkaline.

- **The Casein Hydrolysis Test**

Skim milk (SM) agar was prepared by adding 28g of skim milk powder, 2.5g of yeast extract, 5g of tryptone, 1g glucose, and 15 g agar to 1L of distilled water. pH was adjusted to 7 then autoclaved at 121 °C (1.1 atm) for 15 minutes. The Actinomycete isolates were streaked on the SM agar plates and incubated at 37 ^{0}C for 24-48 hours. Plates were observed for the formation of a clear zone around the bacterial cultures (positive test) or no zones (negative test).

2.3.3. Molecular Identification

A modified way of the general protocol for *Streptomyces* colony PCR was used (Table 8). After taking a small swap of fresh actinomycetes cultures and mixing it with 100 µL of distilled water in Eppendorf tubes, samples were heated at 95 ^{0}C for ten minutes and centrifuged immediately for 7-8 minutes at maximum speed. The supernatant was used directly for PCR using the kit *FIREPol® DNA Polymerase* from *Solis BioDyne.* Universal primers UNI16SRNA-L (5′-ATTCTTAGAGTTTGATCATGGCTCA-3′) and UNI16SRNA-R (5′-ATGGTACCGTGTGACGGGCGGTGTGTA-3′) (Benagli et al., 2011) were used to amplify the partial 16S rRNA gene sequence. The PCR was performed in a final volume of 50 µL.

Table 8. PCR components during 16S rRNA gen amplification

Component	Volume in µL
DNA polymerase	0.5
Buffer	6
$MgCl_2$	3
dNTP	1
Forward primer	1
Reverse primer	1
Colony supernatant	4
Water	33.5

The reaction was done under following conditions: initial denaturing at 94°C for 2 minutes, followed by 34 circles of denaturing at 94 °C for 30 seconds, annealing at 55 °C for 1 minute, extension at 72 °C for 1.25 minute, final extension at 72 °C for 5 minutes, and finally held at 4 °C. The amplified PCR product was examined with 0.7% agarose gel electrophoresis for quality control and the purified samples were sent to *Macrogen Europe* (https://www.macrogen-europe.com) for sequencing.

The sequencing results were analyzed using *BioEdit Sequence Alignment Editor*, and the isolates were identified by comparing similarity with the reference sequences contained in *EzBioCloud* database (https://www.ezbiocloud.net), and submitted at NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank) with the accession numbers. Finally, a phylogenetic tree was created using *MEGA11* application (https://www.megasoftware.net).

2.4. Detection of Active Compounds of the Samples

2.4.1. Primary Screening of Active Compounds by Cultural Methods

- **Plate Streak Method Using Bacterial Isolates**

Cross-streak method was used to scan antibacterial activity (Mondal and Thomas, 2022). Actinomycete isolates were streaked in the center of a Mueller-Hinton Agar (MHA) plates and incubated for one week at 30 ^{0}C. After that, the pathogen cultures inoculated perpendicular to the Actinomycete cultures and incubated at 37 ^{0}C for 24 hours. Growth inhibition zones were written down.

The pathogen samples were provided by Prof. Dr. Hatice KATI at biology department in Giresun University, Turkiye:

P. vulgaris ATCC 13315, *S. typhimurium* ATCC 14028, *K. pneumeniae* ATCC 13883, *E. faecalis* ATCC 51575, *P. aeruginosa* ATCC 9027, *P. mirabilis* ATCC 25933, *S. aureus* ATCC 43300, and *E. coli* ATCC 35218.

- **Agar Diffusion Method Using Whole Extracts of Bacterial Isolates**

The whole extract of the isolates was prepared to test their antibacterial and antifungal activity. This method was applied during Erasmus+ internship program in Milan, Italy at *Naicons* (https://naicons.com) which one of the biggest biotechnological

companies in the world that is specialized in dealing with secondary metabolites of actinomycetes, under the supervision of Dr. Paolo MONCIARDINI.

Whole extract of the samples was prepared following a fast procedure. AF medium (Table 9) was prepared. Using sterile loop, big swaps of fresh cultures were inoculated into sterile flasks containing 10 mL of AF and were incubated for 72 hours at 30 ^{0}C rotary shaker, 200 rpm (Growth phase). Then, 900 µL of the growing cultures in AF medium were transferred to sterile flasks containing 15 mL of M8 medium (Table 10). This step was repeated using SV2 medium (Table 11). All *Streptomyces* samples were incubated for another 72 hours (one week for rare Actinomycetes) at 30 ^{0}C rotary shaker, 200 rpm (Production phase). After that, 500 µL of each sample from both mediums were added to 2 mL Eppendorf tube containing 1 mL of 100% ethanol. Eppendorf tubes were placed horizontally for slight shaking in room temperature for 1 hour then centrifuged for 5 minutes at maximum speed. Finally, Whole extracts were captured by collecting the supernatant of each sample.

These extracts were tested against 6 bacteria: *S. aureus* ATCC 25923, *E. faecalis* ATCC 29212, *B. subtilis* ATCC 6633, *E.coli* ATCC 25922, *Y. pseudotuberculosis* ATCC 911, *P. aerugionsa* ATCC 27853, and against one fungus *C. albiacans* ATCC 10231. These strains were refreshed from -80 °C stocks and grown in LB broth, then incubated for 16 hours for bacteria and 48 hours for fungus at 37 °C. The suspensions of these cultures were standardized using the scale 0.5 to Mac Farland then diluted until the concentration of 10^5 CFU/ mL (Zadra et al, 2013). 100 µL of the diluted cultured were spread on the surface of Petri dishes containing 15 mL of agar media; MHA medium for bacterial samples, and Sabouraud Dextrose Agar (Sab) for *C. albicans*. After waiting for few minutes for the liquid to get dried, 10 µL drops of the Actinmycete whole extracts were placed harmoniously on the surface of the plates. 10 µL of 66% Ethanol was used as negative control, while 5 µL of Kanamycin (5 mg/mL) was used as positive control for bacterial samples, and 5 µL of Amphotericin (1 mg/mL) was used as positive control for fungus. All plates were incubated at 37 °C. Growth inhibition zones were measured after 24 hours of incubation for bacterial plates and after 24-48 hours for fungus.

Table 9. The composition of AF medium

Ingredient	g/L
Glucose	20
Soybean meal	8
Yeast extract	2
$CaCO_3$	4
NaCl	1

pH was adjusted to 7.3.

Table 10. The composition of M8 medium

Ingredient	g/L
Soluble starch	20
Glucose	10
Yeast extract	2
Meat extract	2
Casein hydrolisate	4
$CaCO_3$	3

pH was adjusted to 7.2.

Table 11. The composition of SV2 medium

Ingredient	g/L
Glucose	15
Glycerol	(15) 12 mL
Soy peptone	15
NaCl	5
$CaCO_3$	2

pH was adjusted to 7.

All components of each medium were dissolved in distilled water to a final volume of 1 L; pH was adjusted to its written value then autoclaved at 121 °C (1.1 atm) for 20 minutes.

2.4.2. Detailed Screening of Active Compounds by Chromatographic Methods

Depending on the primary screening results of the isolates and their whole extracts, whole extracts of two samples were chosen for LC/MS analysis. The analysis was done at Naicons labs and the whole extracts of isolates Hs-1 and Hs-5 were used. Results were provided by Dr. Mariana LORIO.

2.4.3. Preparation of Crude Extracts

Depending on LC/MS analysis results Hs-1 and Hs-5 isolates were chosen for antiviral and cytotoxicity tests. Crude extract of these strains was prepared to be used in the tests. After being inoculated into M8 medium, isolates were incubated for seven days at 30 °C and 120 rpm in a rotary shaker incubator until growth became apparent. Centrifugation was carried out for ten minutes at 4 °C and 10,000 rpm. After the cell-free supernatant (CFS) was moved to conical flasks, it was mixed with an equivalent volume of pure ethyl acetate. After then, it was shaken for a full day. The crude extracts were then obtained by collecting the solvent layer and concentrating it using a rotary evaporator. (Mondal and Thomas, 2022).

2.4.4. Anti-viral Tests

To check the antiviral bioactivity and the potential RT (reverse transcriptase) inhibitors of the chosen two samples, the HIV-1 reverse transcriptase assay colorimetric ELISA kit (Roche) was used. The reaction mixture containing the primer complex, dNTPs, and reverse transcriptase (RT) enzyme was supplemented with the inhibitor substance diluted with Lysis buffer and incubated for 1 hour at 37 °C. The final concentration of the extracts after this procedure was 70 µg, while a reaction mixture containing Lysis buffer without HIV-1 RT was used as a negative control. After incubation, the reaction mixture was transferred to a streptavidin-coated microplate (MP) and incubated again for 1 hour at 37 °C. After that, wells were washed and the solution was completely removed, then an anti-DIG-POD (200 mU/mL) was added and incubated for 1 hour at 37 °C. After incubation, the wells were washed again and peroxidase substrate (ABST) was added to the MP and let for about 20 minutes at room temperature for photometric detection. The absorbance of the samples was measured at 405 nm using a microplate ELISA reader. The

inhibitory activity was considered as the inhibition percentage compared to sample without inhibitor (negative control).

2.4.5. Cytotoxicity Tests

The MTT test was used to assess the cytotoxic effect of the selected two extracts. Because of its speed, ease of use, and dependability as a quantitative colorimetric technique, the MTT test is frequently used to evaluate in vitro cytotoxicity. (Mosmann, 1983). The MTT assay relies on the ability of viable cells to convert the yellow, water-soluble tetrazolium salt (MTT) into an insoluble purple formazan precipitate through the action of NADH-dependent succinate dehydrogenase (Riss & Moravec, 1992).

Dulbecco's Modified Eagle's Medium (DMEM) was used to culture human mammary gland cancer cell line MCF7 cells (ATCC: HTB-22) at 37 °C and 5% CO2 atmosphere, including 10% FBS and 100 units/mL penicillin and 100 µg/mL streptomycin.

Stock solutions of Hs-1 and Hs-5 extracts were prepared by dissolving each in ethyl acetate to a concentration of 1000 mg/mL. Logarithmic dilution series (100, 10, 1 mg/mL) were prepared from these stocks. Three concentrations, including the stock solutions, were added to the prepared medium at a final concentration of 0.4% (v/v). The cells were seeded in a 96-well microplate, and after 24h of attachment, the cells were incubated with ethyl acetate extracts with a concentration range (1–1000 mg/mL). Cells treated with 0.4% ethyl acetate were used as a solvent control, while those exposed to 1 µM doxorubicin were used as a positive control. Following a 24-hour treatment period, a final concentration of 0.5 mg/mL of MTT solution was applied to the 96-well plate in DMEM. The plate was agitated on an orbital shaker for ten minutes following three hours of incubation. Using a microplate reader, the absorbance values of the produced formazan crystals were measured at 570 nm and with background adjustment at 690 nm. (Cloos et al., 2011; Mosmann, 1983). Cell viability was expressed as a percentage relative to the solvent control group, and data were calculated as mean % viability ± standard deviation. All experiments were performed in duplicate.

3. RESULTS

3.1. Isolation of Actinomycetes

Seventeen Actinomycete samples were isolated from Blue Lake and Goksu Travertines of Giresun province, Black Sea region in Turkiye (Table 12).

Table 12. Sources of Actinomycete isolates

No.	Name	Location	Source
1	Hs-1	Blue Lake	Soil
2	Hs-2	Blue Lake	Soil
3	Hs-4	Blue Lake	Soil
4	Hs-5	Blue Lake	Soil
5	Hs-7	Blue Lake	Soil
6	Hs-9	Blue Lake	Soil
7	Hs-13	Goksu Travertines	Water
8	Hs-14	Goksu Travertines	Mud
9	Hs-18	Goksu Travertines	Mud
10	Hs-21	Blue Lake	Soil
11	Hs-22	Blue Lake	Mud
12	Hs-25	Goksu Travertines	Mud
13	Hs-27	Blue Lake	Soil
14	Hs-31	Blue Lake	Soil
15	Hs-33	Blue Lake	Soil
16	Hs-34	Blue Lake	Soil
17	Hs-36	Blue Lake	Soil

All isolates showed the classical Actinomycete morphology, with the distinguished chalky appearance of the colonies growing on the proper medium (Figure 12).

The form of the colonies was circular in six isolates (Hs-1, Hs-2, Hs-4, Hs-18, Hs-21, and Hs-36), irregular in the others (Hs-5, Hs-7, Hs-9, Hs-13, Hs-14, Hs-22, Hs-25, Hs-27,

Hs-31, and Hs-33). The isolates showed diversity in the elevation of the colonies; raised in 3 samples (Hs-1, Hs-4, Hs-36), crateriform in 10 samples (Hs-2, Hs-5, Hs-7, Hs-9, Hs-13, Hs-22, Hs-27, Hs-31, Hs-33, Hs-34), flat in two samples (Hs-18 and Hs-21), convex and umbonate in one sample respectively (Hs-14 and Hs-25). The margins were entire in 9 samples (Hs-1, Hs-2, Hs-4, Hs-5, Hs-18, Hs-21, Hs-22, Hs-33, and Hs-36), undolate in 6 samples (Hs-7, Hs-9, Hs-13, Hs-27, Hs-31, Hs-34), and two samples were lobate (Hs-14 and Hs-25). The size of the colonies ranged from small to medium, while there was a distinguished diversity in colonies color (Table 13).

Table 13. Morphological characters of Actinomycete colonies

Name	Form	Elevation	Margin	Color	Reverse Color	Size
Hs-1	Circular	Raised	Entire	Chalk	Pink	Small*
Hs-2	Circular	Crateriform	Entire	Chalk	Yellow	Small
Hs-4	Circular	Raised	Entire	Chalk-grey	Light brown	Small
Hs-5	Irregular	Crateriform	Entire	Light pink	Light pink	Medium**
Hs-7	Irregular	Crateriform	Undulate	Chalk	Light brown	Medium
Hs-9	Irregular	Crateriform	Undulate	Paige	Paige	Small
Hs-13	Irregular	Crateriform	Undulate	Chalk	Orange	Small
Hs-14	Irregular	Convex	Lobate	Chalk-brown	Brown	Small
Hs-18	Circular	Flat	Entire	Creamy white	Creamy white	Small
Hs-21	Circular	Flat	Entire	White	White	Small
Hs-22	Irregular	Crateriform	Entire	Grey	Grey	Medium
Hs-25	Irregular	Umbonate	Lobate	Chalk-brown	Brown	Small
Hs-27	Irregular	Crateriform	Undulate	Brown	Brown	Small
Hs-31	Irregular	Crateriform	Undulate	Chalk-orange	Orange	Medium
Hs-33	Irregular	Crateriform	Entire	Orange	Orange	Small
Hs-34	Irregular	Crateriform	Undulate	Chalk	Paige	Medium
Hs-36	Circular	Raised	Entire	Orange	Orange	Small

*Small: 1-2 mm in diameter.

**Medium: 2-3 mm in diameter.

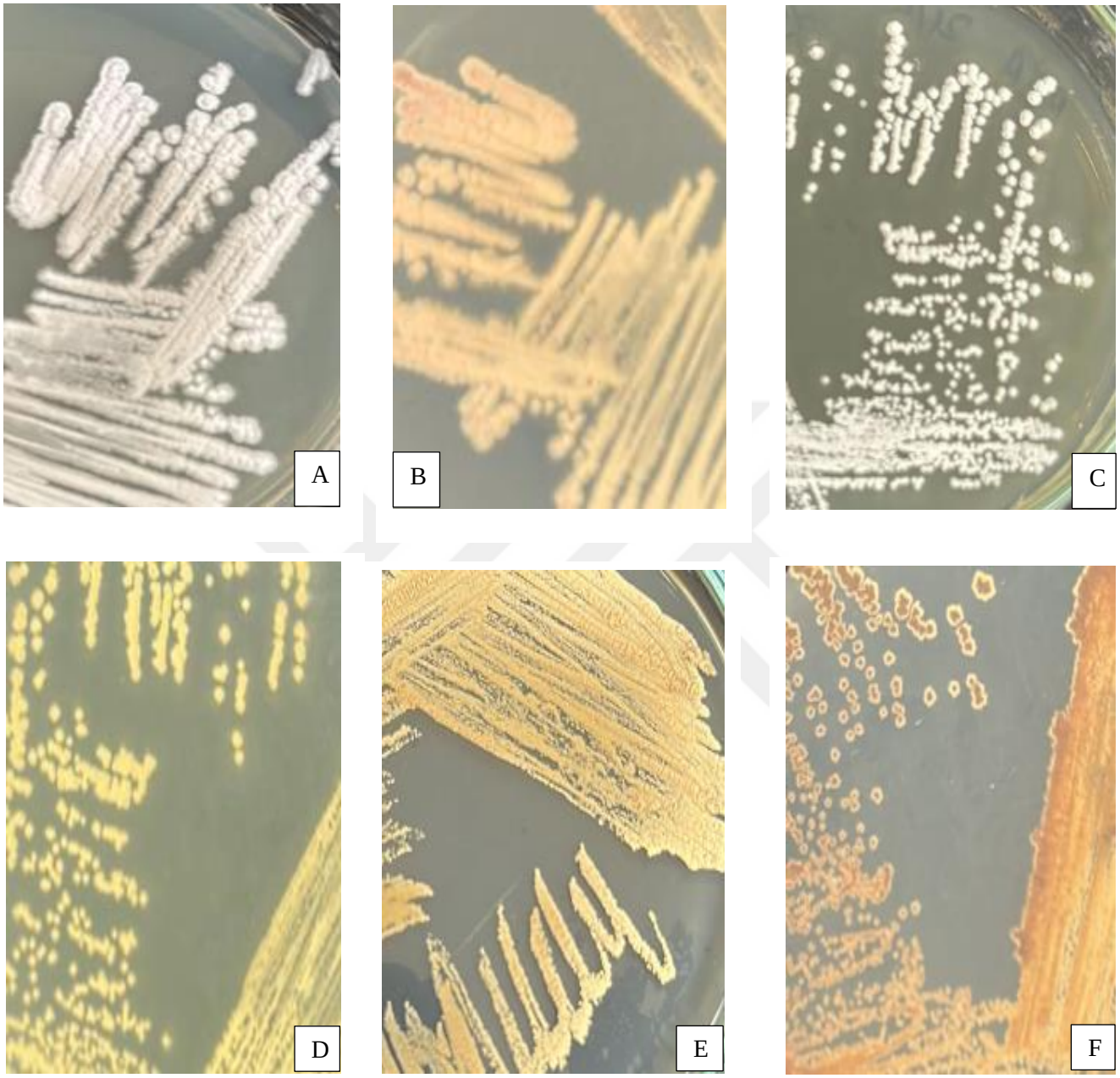

Figure 12. Morphological appearance of some representative isolates
A: Hs-1, B: Hs-1 reverse face of the plate, C: Hs-2, D: Hs-2 reverse face of the plate, E: Hs-14, F: Hs-14 reverse face of the plate.

3.2. Microscopic Characteristics of the Isolates

Examination under light microscope showed that all samples were Gram positive (Figure 13). Cover slip technique was also used to examine the spore formation of the

isolates where the samples were seen in their natural growing without the disturbance that comes with the preparation of classic Gram staining. The spore formation appears like a connected web which is one of the main microscopic characteristics of most of Actinomycetes (Figure 14).

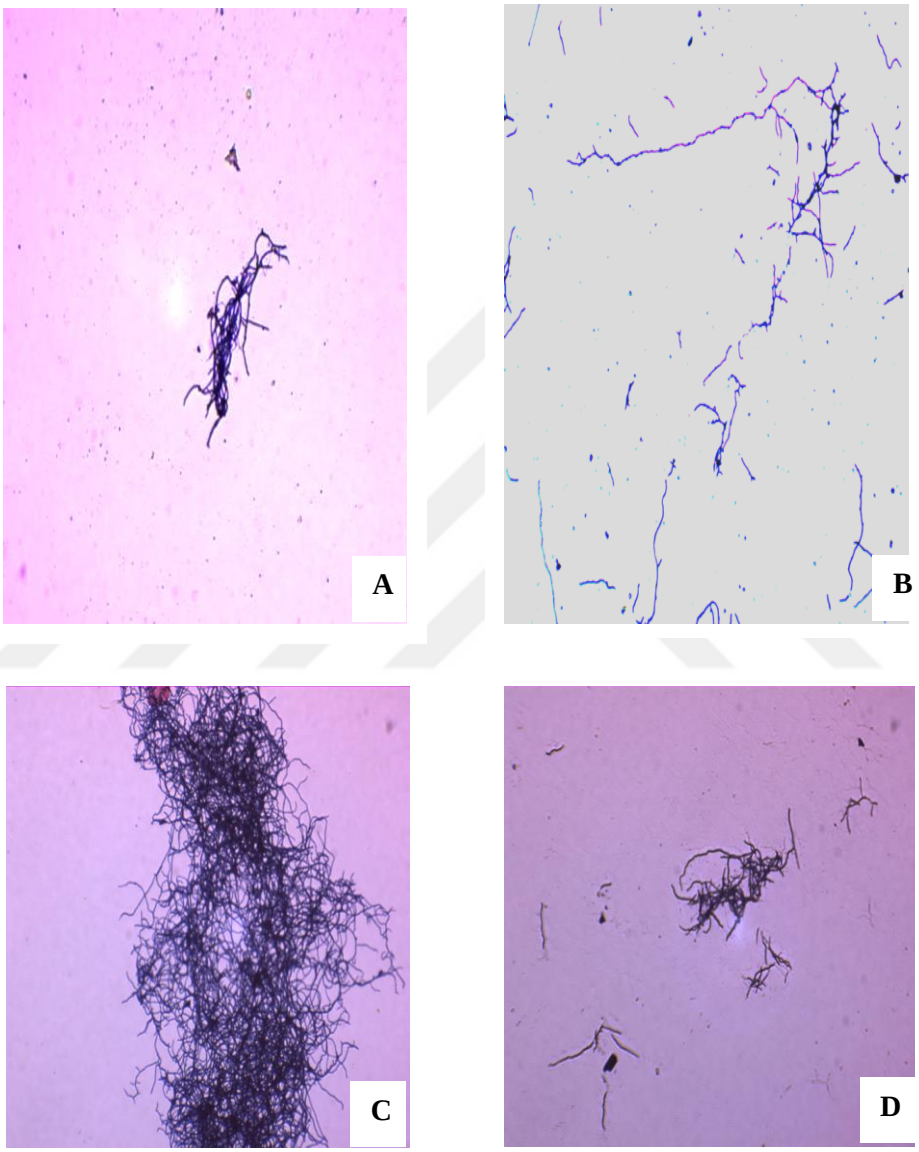

Figure 13. Gram staining of some of the isolates. Magnification: x200
A: Hs-1, B: Hs-9, C: Hs-13, D: Hs-31.

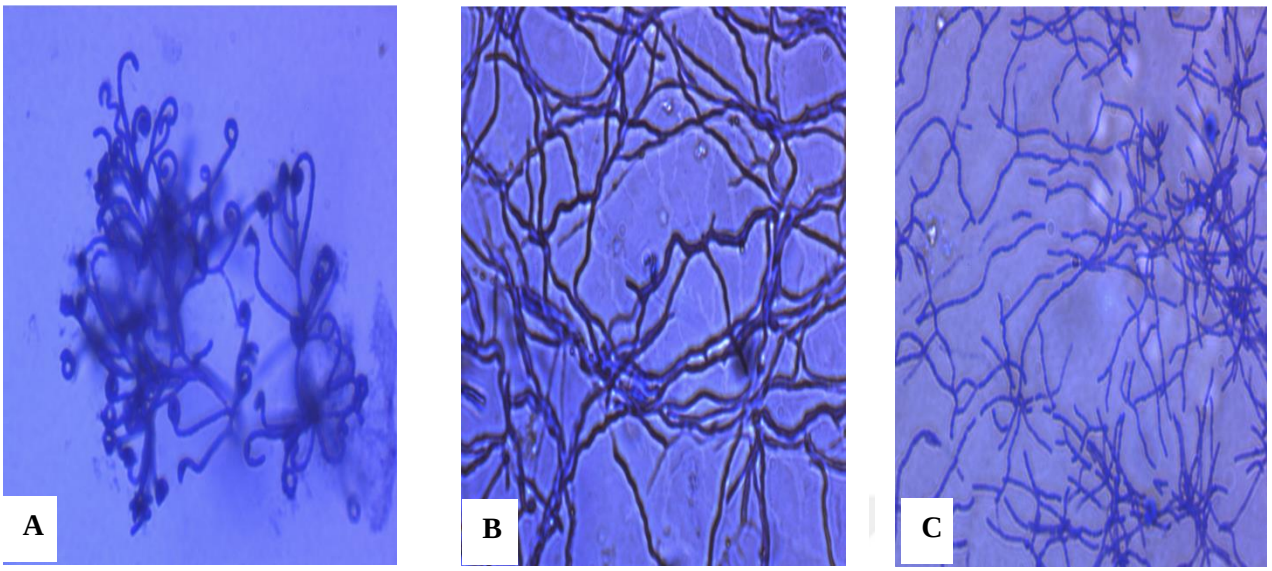

Figure 14. Spore formation of some selected isolates. Magnification: x1000
A: Hs-1, B: Hs-4, C: Hs-33.

3.3. Biochemical Tests Results

Four tests were done to identify the samples, catalase enzyme production test (Figure 15), amylase enzyme production test (Figure 16), H_2S production test (Figure 17), and casein hydrolysis test (Figure 18). All samples produced catalase and amylase enzymes, and were able to hydrolyze casein. Nine samples (Hs-1, Hs-2, Hs-4, Hs-5, Hs-14, Hs-22, Hs-25, Hs-27, and Hs-34) produced H_2S, while eight didn't. There was diversity in acidic (A), alkaline (K) reaction among the slants and butts of the isolates' agar slants (Table 14).

Table 14. Biochemical characteristics of bacterial isolates

Name	**Catalase**	**Amylase**	**H_2S**	**Casein**
Hs-1	+	+	+ (A/A)	+
Hs-2	+	+	+ (K/A)	+
Hs-4	+	+	+ (K/A)	+
Hs-5	+	+	+ (K/A)	+
Hs-7	+	+	-(A/A)	+
Hs-9	+	+	-(A/A)	+
Hs-13	+	+	-(K/K)	+
Hs-14	+	+	+ (K/K)	+
Hs-18	+	+	-(A/A)	+
Hs-21	+	+	-(A/A)	+
Hs-22	+	+	+ (K/K)	+
Hs-25	+	+	+ (K/K)	+
Hs-27	+	+	+ (K/K)	+
Hs-31	+	+	- (A/A)	+
Hs-33	+	+	-(A/A)	+
Hs-34	+	+	+ (K/K)	+
Hs-36	+	+	-(A/A)	+

*A: Acidic

*K: Alkaline

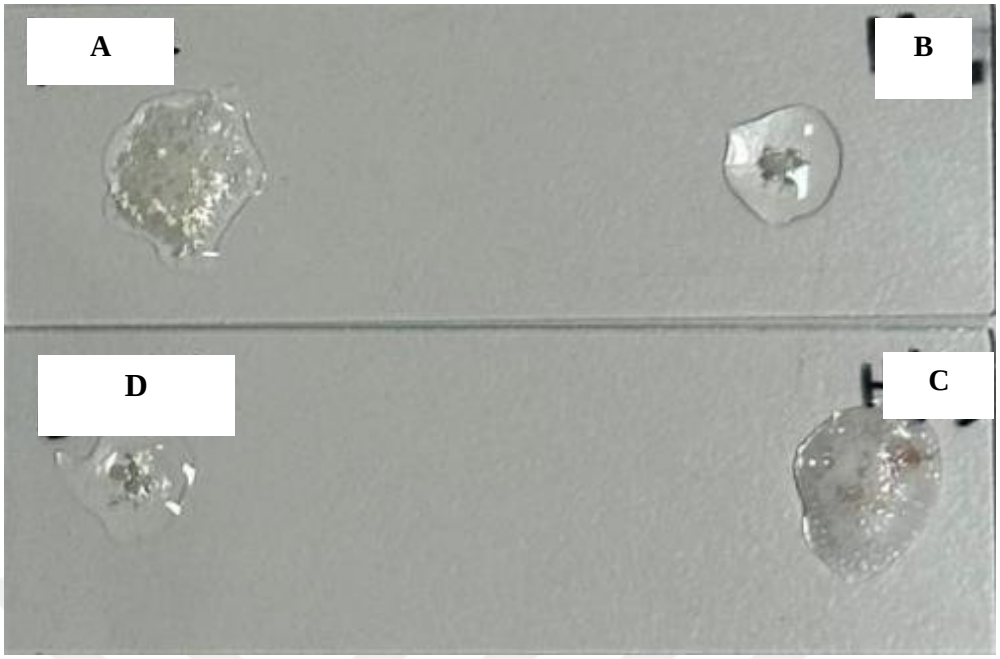

Figure 15. Catalase enzyme production test of some isolates. A: Hs-7, B: Hs-9, C: Hs-13, D: Hs-14

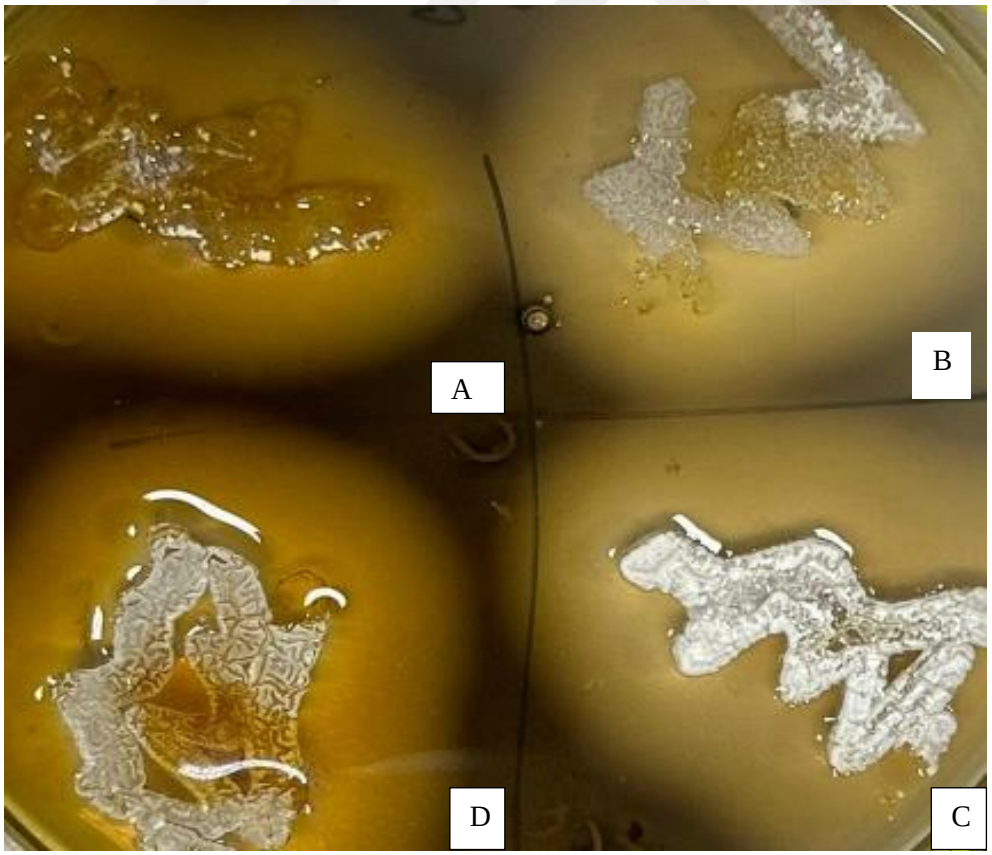

Figure 16. Amylase enzyme production test of some isolates. A: Hs-14, B: Hs-18, C: Hs-21, D: Hs-22.

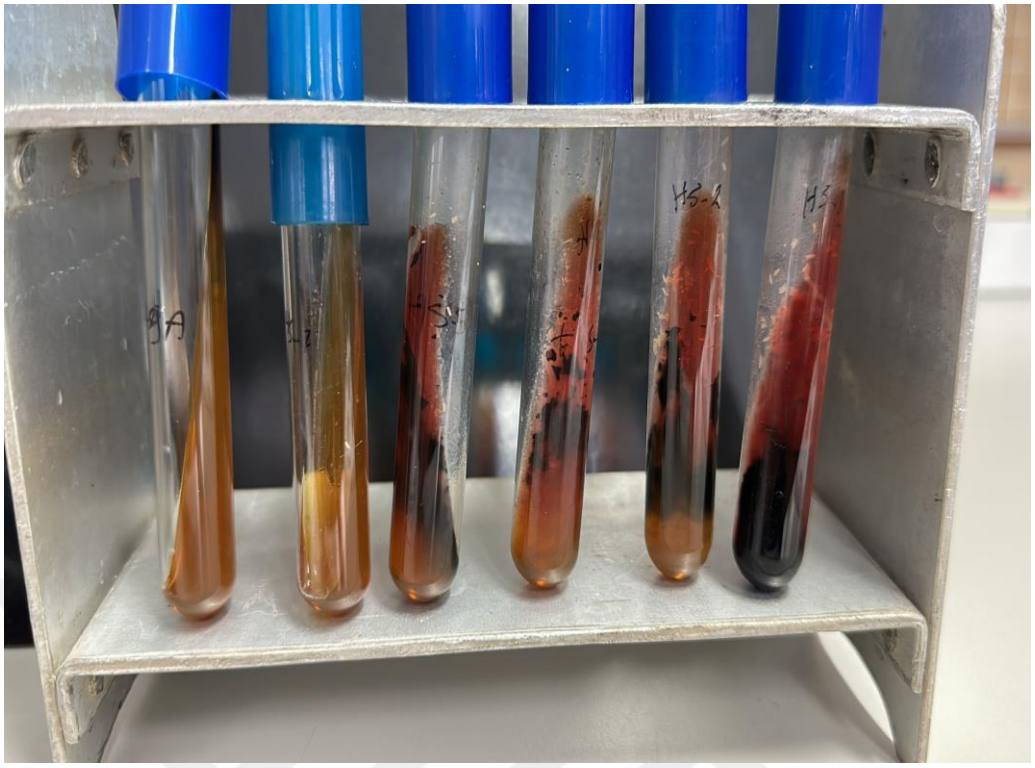

Figure 17. H_2S production test of some isolates. From right to left: Hs-1, Hs-2, Hs-4, Hs-5, Hs-7, Hs-9.

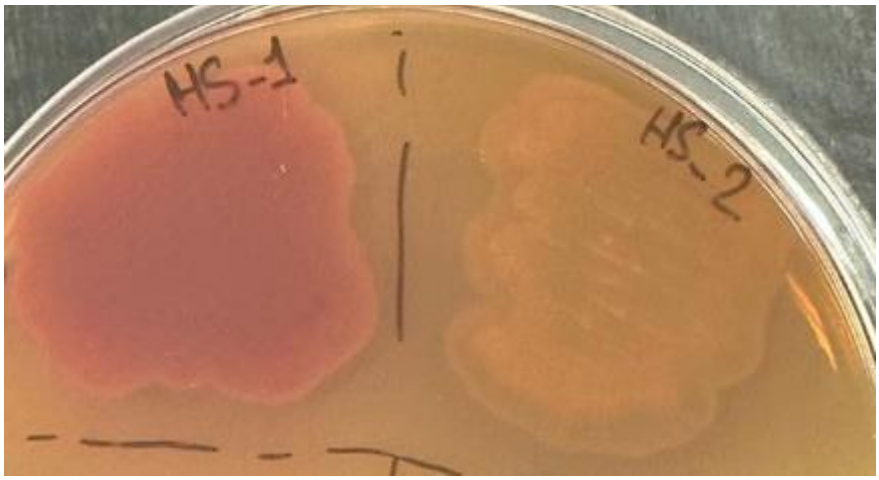

Figure 18. Casein hydrolysis test of Hs-1, Hs-2 isolates.

3.4. 16S rRNA Gene Sequencing Results

The 16S rRNA gene sequencing results showed that 13 of the 17 samples belong to *Streptomyces* species, while two samples (Hs-33 and Hs-36) belong to *Nocardia* species, and two isolates (Hs-14 and Hs-25) go under *Pseudonocardia* species (Table 15). A phylogenetic tree of the samples was created (Figure 19).

Table 15. 16S rRNA gene sequencing results of bacterial isolates

	GenBank accession number	**Most similar strains**	**Percentage**
Hs-1	PQ215831	*Streptomyces thinghirensis* *Streptomyces ambofaciens* *Streptomyces althioticus*	99.29% 99.13% 99.12%
Hs-2	PQ215832	*Streptomyces setonii* *Streptomyces anulatus* *Kitasatospora papulosa*	100% 100% 100%
Hs-4	PQ215833	*Streptomyces sporoverrucosus* *Streptomyces goshikiensis* *Streptomyces colombiensis*	100% 100% 99.91%
Hs-5	PQ215834	*Streptomyces silvae* *Streptomyces durocortorensis* *Streptomyces pratensis*	100% 99.91% 99.91%
Hs-7	PQ215835	*Streptomyces microflavus* *Streptomyces fulvorobeus* *Streptomyces setonii*	100% 100% 99.92%
Hs-9	PQ215836	*Streptomyces fructofermentans* *Streptomyces lacrimifluminis* *Streptomyces phaeolivaceus*	99.59% 98.37% 98.37%
Hs-13	PQ215837	*Streptomyces daghestanicus* *Streptomyces albidoflavus* *Streptomyces violascens*	99.84% 99.84% 99.84%

Table 15 continued

Hs-14	PQ215838	*Pseudonocardia carboxydivorans* *Pseudonocardia alni* *Pseudonocardia Antarctica*	100% 99.92% 99.92%
Hs-18	PQ215839	*Streptomyces avidinii* *Streptomyces xanthophaeus* *Streptomyces nojiriensis*	99.77% 99.45% 99.45%
Hs-21	PQ215840	*Streptomyces decoyicus* *Streptomyces sioyaensis* *Streptomyces caniferus*	99.92% 99.45% 99.45%
Hs-22	PQ215841	*Streptomyces violaceochromogenes* *Streptomyces iakyrus* *Streptomyces collinus*	99.92% 99.68% 99.36%
Hs-25	PQ215842	*Pseudonocardia alni* *Pseudonocardia carboxydivorans* *Pseudonocardia antarctica*	99.82% 99.72% 99.72%
Hs-27	PQ215843	*Streptomyces plumbiresistens* *Streptomyces resistomycificus* *Streptomyces pseudovenezuelae*	100% 98.97% 98.97%
Hs-31	PQ215844	*Streptomyces adustus* *Streptomyces bobili* *Streptomyces phaeoluteigriseus*	99.06% 98.90% 98.75%
Hs-33	PQ215845	*Nocardia salmonicida subsp. cummidelens* *Nocardia fluminea* *Nocardia lasii*	99.42% 99.42% 98.92%
Hs-34	PQ215846	*Streptomyces sannanensis* *Streptomyces mauvecolor* *Streptomyces corynorhini*	99.06% 98.90% 98.83%
Hs-36	PQ215847	*Nocardia fluminea* *Nocardia salmonicida subsp. cummidelens* *Nocardia salmonicida subsp. salmonicida*	99.36% 99.28% 99.04%

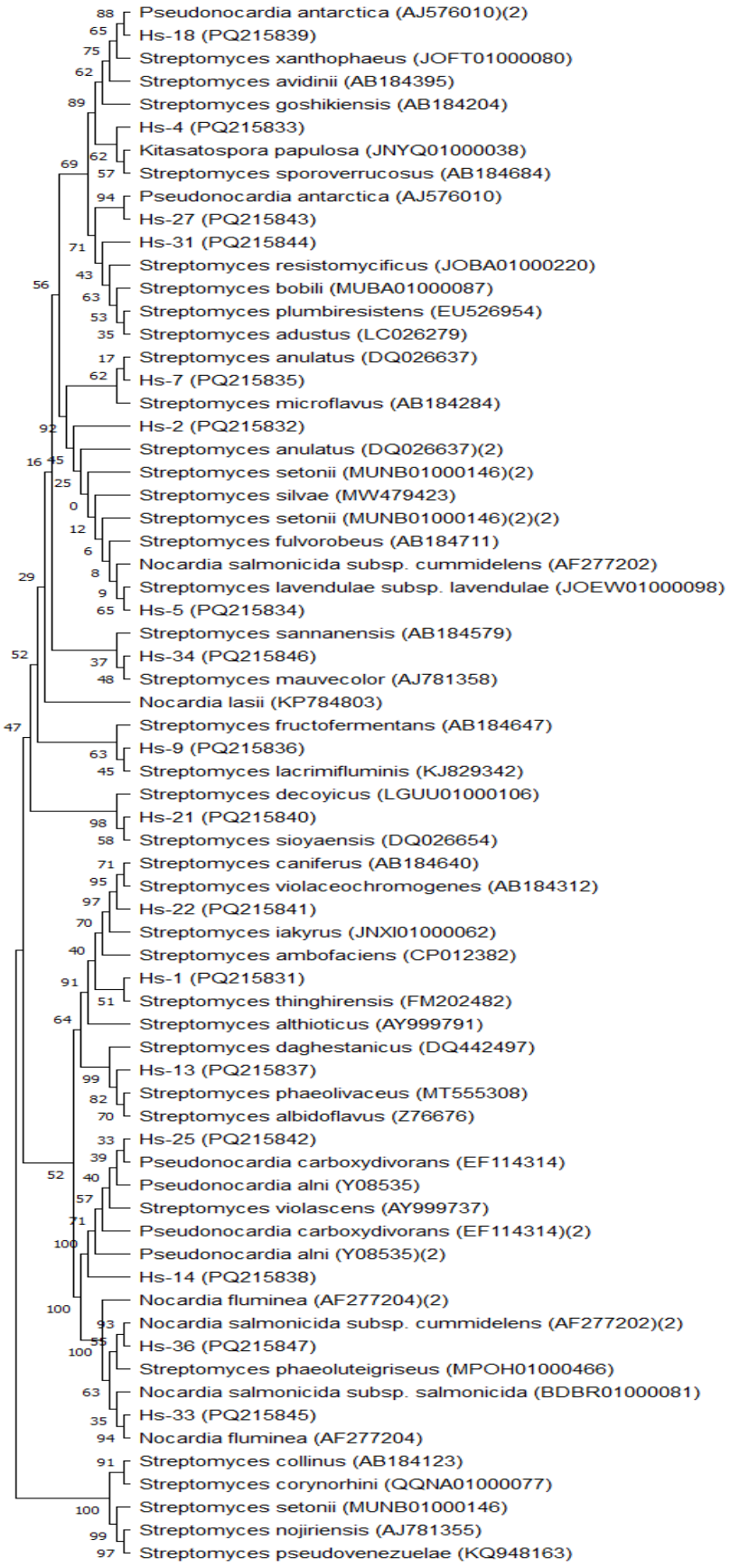

Figure 19. Neighbor joining phylogenetic tree of the bacterial isolates.

3.5. Antibacterial Activity of the Isolates

Cross-streak method (Figure 19) was used to test the isolates bioactivity against 8 different bacterial strains, *P. vulgaris* ATCC 13315, *S. typhimurium* ATCC 14028, *K. pneumeniae* ATCC 13883, *E. coli* ATCC 35218, *E. faecalis* ATCC 51575, *P. aeruginosa* ATCC 9027, *P. mirabilis* ATCC 25933, and *S. aureus* ATCC 43300. Eight Actinomycete isolates (Hs-1, Hs-2, Hs-4, Hs-5, Hs-9, Hs-22, Hs-27, and Hs-34) showed variable levels of activity against all tested bacteria except for *P. mirabilis*. Table 16 shows this activity in details where inhibition zones were measured and written in mm.

Table 16. Growth inhibition zones of active samples

Name	***P.v***	***S.t***	***K.p***	***E.c***	***E.f***	***P.a***	***P.m***	***S.a***
Hs-1	-	16	17	18	13	-	-	10
Hs-2	9	-	-	-	-	-	-	10
Hs-4	-	-	-	10	-	-	-	-
Hs-5	-	23	-	14	25	-	-	20
Hs-9	-	-	-	-	-	20	-	-
Hs-22	-	-	-	-	-	-	-	6
Hs-27	-	-	-	-	-	-	-	16
Hs-34	-	20	10	16	-	-	-	16

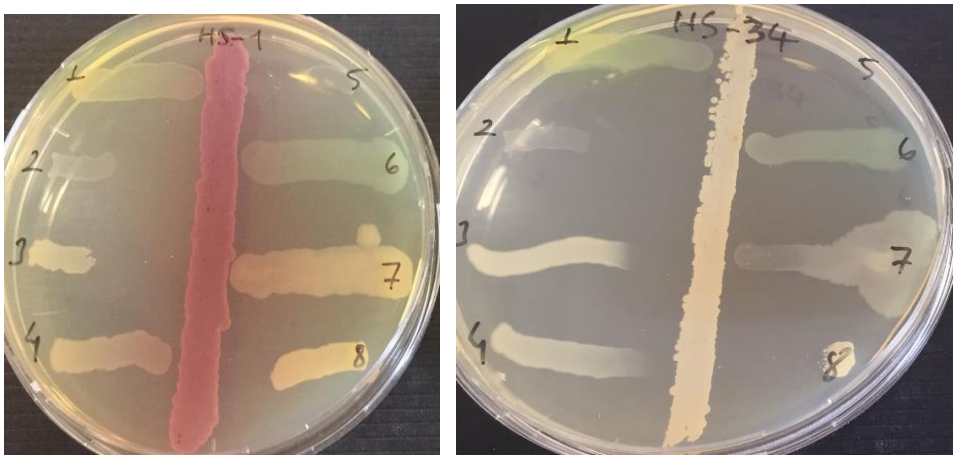

Figure 20. Cross-streak method for isolates Hs-1 and Hs-34.

3.6. Bioactivity of Whole Extracts

The whole extract of all isolates was prepared in M8 and SV2 medium, and then they were tested against 6 strains of bacteria *S. aureus* ATCC 25923, *E. faecalis* ATCC 29212, *B. subtilis* ATCC 6633, *E. coli* ATCC 25922, *Y. pseudotuberculosis* ATCC 911, *P. aeruginosa* ATCC 27853, and against one fungus *C. albiacans* ATCC 10231. The test was repeated three times, growth inhibition zones were measured and the average is written in mm. Highest activity was reported in Hs-1 and Hs-5 isolates, while four isolates (Hs-4, Hs-9, Hs-18, Hs-21) didn't present any kind of activity (Table 17).

Table 17. Growth inhibition zones of whole extracts

Name	***Y.p***	***E.f***	***P.a***	***S.a***	***E.coli***	***B.s***	***C.a***
Concentration µL in 1mL of saline	50	185	50	75	75	55	125
M8 medium: Hs-1		9	4	6	4		5
Hs-2		6			5		5
Hs-4							
Hs-5	5		4	6	5		5
Hs-7	4	6	5		4		4
Hs-9							
Hs-13	5				4		
Hs-14	5	4	5		4		
Hs-18							
Hs-21							
Hs-22	5	7	6		5		
Hs-25	5	8	5		5	4	
Hs-27	6	7	5		5	4	
Hs-31	5	5	5		4	4	
Hs-33	5	5	4		4		4
Hs-34	6	7	5		4		
Hs-36		5	4				5

Table 17. *cont.*

sv2 medium:							
Hs-1		8	5	5	5		
Hs-2	5	8			4		
Hs-4							
Hs-5		7	4				
Hs-7	6	6	5		5		5
Hs-9							
Hs-13	4	8	5		6		
Hs-14		6			5		
Hs-18							
Hs-21							
Hs-22	5	7	4				
Hs-25	4		4		4		
Hs-27	4	7	5		5		
Hs-31	6	6	5		6	5	6
Hs-33			5		5		
Hs-34		6			4		
Hs-36		5	4		5	3	

3.7. LC/MS Analysis Results of Hs-1 and Hs-5 Isolates

After comparing the results of bioactivity of the isolates and their whole extracts, two samples were chosen for LC/MS analysis. The whole extract of samples Hs-1 and Hs-5 were harvested from M8 medium. The analysis was done at Naicon labs in Milan, Italy, and the results were matched with their huge Actinomycete bioactive compounds library.

The analysis results showed that both extracts contain a lot of valuable compounds that may have different bio-effects like antibacterial, antifungal, anti-inflammatory, antiviral, and cytotoxic effects (Table 18).

Table 18. Bioactive compounds of selected isolates

Name	Source	Retention time	Exact mass	Activity
Sannamycin_E	Hs-5	5.069144314	304.2110554	Antibacterial
Saccharopine	Hs-5	0.825332111	276.1321364	Lysine degradation
Terragine_A	Hs-5	6.274422702	518.2740496	Anticancer
Penicillar E	Hs-5	6.224106723	198.1255944	Antibacterial/antifungal
Clavam_2-carboxymethyl	Hs-5	0.871145245	171.0531578	β-lactamase inhibitor (antibiotic enhancing)
Pantothenic acid	Hs-5	1.542190226	219.1106726	Metabolism
Cyclo(l-6-hyp-l-phe)	Hs-5	5.163741959	260.1160924	Antimicrobial/antioxidant
Isokaempferide	Hs-5	7.231204168	300.0633881	Anti-inflammatory
Glucosamine	Hs-5	0.64905248	179.0793725	Anti-inflammatory
Erbstatin	Hs-5	5.578500602	179.0582431	Anticancer
Acetyl-leucyl-valyl-argininol	Hs-5	8.211725792	414.2954537	Antiviral-anticancer
Enaminomycin_B	Hs-5	2.47475005	241.0586371	Antibacterial/antitumor
Bacillusamide_B	Hs-5	1.783556102	212.1160924	Antifungal/antibacterial
FU-10	Hs-1	0.633856939	340.1481803	Antitumor/antimicrobial
2-ethyl-5-(3-indolyl)oxazole	Hs-1	4.244982562	212.094963	Antitumor/antimicrobial
3-trehalosamine	Hs-1	0.655152217	341.1321959	Antibacterial
Pochonicine	Hs-1	2.883253975	260.1372217	Antidiabetic
Trehazolin	Hs-1	0.648366736	380.143095	Insecticidal effects
9,10-Anhydro-daunorubicin	Hs-1	3.928357785	509.1685814	Anticancer (topoisomerase II inhibitor)
Streptcytosine_K	Hs-1	9.043134325	323.1845063	Antiviral/antitumor
Cyclocarbamide_A	Hs-1	1.735538028	280.1423071	Antitumor/Immune effects

Table 18 *cont.*

Daidzein	Hs-1/Hs-5	6.359978043	254.0579088	Antioxidant/anticancer/ anti-inflammatory
Actinoplanone_E	Hs-1/ Hs-5	1.129182209	624.1510728	Antibacterial
Physostigmine	Hs-1/ Hs-5	1.729632363	275.1633769	Neuroactive compound
Gancidin W	Hs-1/ Hs-5	4.737134828	210.1368278	Antimalarial/antitumor
Futalosine	Hs-1/ Hs-5	4.616608754	414.1175489	Antibacterial
Legonmycin_B	Hs-1/ Hs-5	3.617067052	266.1630426	Antimicrobial/anticancer
Citruline - 40.00 eV	Hs-1/Hs-5	0.781416708	175.0956913	Nutraceutical

3.8. HIV-1 RT Inhibition Test Results

According to the LC/MS analysis results that showed some bio-compounds of the isolates Hs-1 and Hs-5 had antiviral activity, a HIV-1 RT inhibition test was done using the crude extract from M8 medium. Beside the two samples, a negative control (without inhibitor), a positive control (without RT), a reverse transcriptase inhibitor control, and M8 control (extract-free medium) were included (Table 19).

Table 19. HIV-1 RT Inhibition results of bacterial extracts

Sample	**OD at 405 nm**	**Inhibition %**
Negative control	3.6178	0
Positive control	0.1282	96.4564426
RT inhibitor control	1.6745	53.7163127
M8 control	3.2669	9.70009674
Hs-1	0.1004	97.2257797
Hs-5	0.0945	97.3879393

The measurement was repeated three times and the average was taken. Inhibition percentage was calculated following this equation:

$$\% \text{ inhibition} = 100 - \left(\frac{\text{OD at 405nm with inhibitor}}{\text{OD at 405nm without inhibitor}} \times 100 \right)$$

3.9. Results of Cytotoxic Tests

The LC/MS analysis results showed that the samples Hs-1 and Hs-5 contain a lot of bioactive compounds with potential anticancer activity. To ensure this assumption, a MTT assay was applied where four concentrations of the ethyl acetate solution of the extracts were used; 1000, 100, 10, and 1 mg/mL. According to the duplicate MTT assay results, the Hs-1 extract concentration of 1000 mg/mL significantly reduced MCF7 cell viability compared to the other test groups. The Hs-5 extract concentration of 1000 mg/mL significantly reduced MCF-7 cell viability compared to the other test groups, while the extract concentration of 100 mg/mL significantly reduced MCF7 cell viability compared to the negative control group (Figure 21).

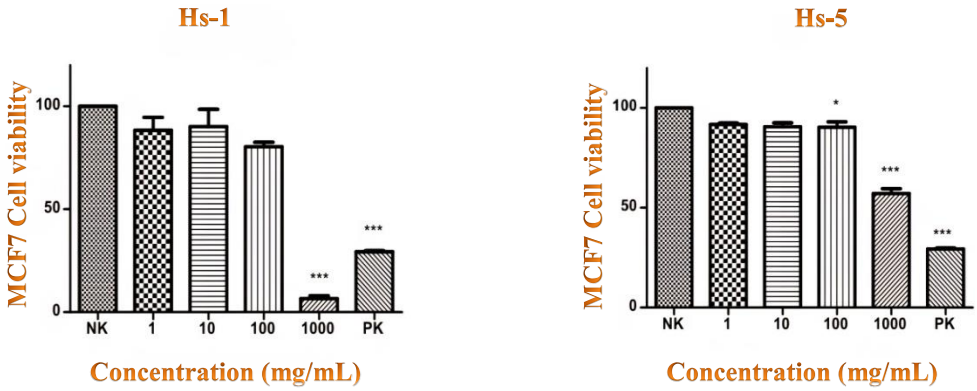

Figure 21. Cytotoxic activity of bacterial isolates. Hs-1 (posttest Tuckey, ***: p < 0,001, R^2=0.9841). Hs.5 (posttest Tuckey, *: p< 0,05,***: p< 0,001, R^2=0.9955). NK: Negative control, PK: Positive control.

4. DISCUSSION

Numerous studies have explored the biological potential of Actinomycetes through enzymatic profiling and bioactivity assays, closely reflecting the present work's approach and findings. For instance, Jassim and Jarallah (2023) isolated 33 Actinomycete strains from soils in Hilla Province, Iraq. They utilized biochemical tests such as amylase, catalase, protease, cellulase, and lipase screening to characterize the isolates. All 13 bioactive isolates were positive for amylase activity, and many exhibited significant antibacterial effects against both Gram-positive and Gram-negative pathogens (Jassim and Jarallah, 2023). In a study of rhizosphere-derived Actinomycetes, researchers isolated *Streptomyces* spp. capable of amylase, pectinase, and protease production. The isolates exhibited notable antifungal and antibacterial activity and promoted plant growth *in vivo* (Elshafie and Camele, 2022). Moreover, Fahmy and Abdel-Tawab (2021) isolated a *Streptomyces* strain (NMF6) from marine sponges and demonstrated its extract exhibited antimicrobial, antioxidant, anticancer, and antiviral activities (Fahmy and Abdel-Tawab, 2021). Gozari and his team (2019) assessed cytotoxicity of marine actinomycete extracts from sediments of the Oman Sea. Besides that, 24% of 168 isolates showed antimicrobial activity (Gozari et al., 2019). Febriansah and his colleagues (2024) isolated an active compound (mannotriose) from *Streptomyces sennicomposti GMY01* and demonstrated potent cytotoxicity against MCF-7 breast cancer cells (Febriansah et al., 2024).

When it comes to Turkish literature, a lot of studies and articles about Actinomycetes can be found, and some of them have promising results. Oksay and his team (2004) collected 50 Actinomycete isolates from the soil of Manisa region; 34% of them showed antibacterial activity against pathogenic bacteria. Uzel and his colleagues (2011) isolated 67 thermophilic Actinomycetes from the hot springs and soils of West Anatolia; 92.5% of them were found to be producers of extracellular protease, and 56.7% of them were found active against MRSA and *E. faecalis*. Demirel and her colleagues (2016) were able to collect 288 soil Actinomycetes isolates from western Turkiye; 30.5% showed fungal inhibition. Tatar (2021) worked near Çorum to isolate 15 halophilic Actinomycetes including *Streptomyces* and *Nocardiopsis* with documented PKS/NRPS gene clusters and antimicrobial activity. Tüfekçi and his team (2023) collected and screened 179 isolates

from the Karst Cave in the eastern Black Sea region (Gümüşhane); 29.6% showed antibacterial activity

Our study highlights the considerable potential of Actinomycete strains isolated from Blue Lake and Goksu Travertines as prolific sources of bioactive secondary metabolites. Through a combination of biochemical characterization, antibacterial and antifungal screening, antiviral assays, and anticancer evaluation, the findings confirm that Actinomycetes remain a cornerstone for natural product discovery.

Among the 17 isolated Actinomycetes in this study, 70.59% of them were collected from the soil of Blue Lake which matches the fact that soil is one of the richest sources of Actinomycetes (Kumar et al., 2010; Shrestha et al., 2021; Devanshi et al., 2021). Although aquatic habitats are no less prevalent than terrestrial habitats as Actinomycete sources (Meenakshi et al., 2024), no sample were found in Blue Lake water and only one sample was isolated from the mud of Blue Lake. This goes along with the reality that most of researches on aquatic actinomycetes focus on marine actinomycetes (Jagannathan et al., 2021; Sarkar et al., 2022; Xu et al., 2025). However, 23.5% of the samples were isolated from water and mud of Goksu Travertines. This variation can be attributed to the chemical nature of the travertines with their high levels of $CaCO_3$, or other factors related to the modification of water stream to construct the artificial pools.

Molecular analysis showed that 76.47% of the samples belong to the genus *Streptomyces*, while 11.76% of them go under the genus *Nocardia*, and 11.76% belong to the genus *Pseudonocardia*. It is not surprising that *Streptomyces* are the most common genus among Actinomycetes (Van Bergeijk et al., 2020; Li et al., 2021; Elias et al., 2022). However, it is also suggested that the dominance of *Streptomyces* over other Actinomycetes can be attributed to their ease of isolation on selective media (Abdelrahman et al., 2022).

The morphological analysis corresponded with the molecular analysis. All isolates showed the typical colony and microscopic characteristics of Actinomycetes. Hs-14 and Hs-25 isolates belonging to *Pseudonocardia* genus had the only lobate colony margins among all samples. Both colonies were irregular in form, and had similar colors. Spore formation was detected in Hs-14, and wasn't detected in Hs-25. Some *Pseudonocardia* species can form spores (Chanama et al., 2018), while others can't (Kampfer and Kroppenstedt, 2004). The classification of *Nocardia* species is unstable due to the major taxonomy changes over time (Traxler et al., 2022). However, the isolates Hs-33 and Hs-36

that were classified under *Nocardia* genus showed similar morphological characteristics with entire colony margins and orange color. *Streptomyces* species are widely diverse (Hungund et al., 2022), which explain the diversity appeared in the morphology of the remaining isolates that were molecularly classified as *Streptomyces*.

For biochemical identification, four tests were chosen in this study; catalase enzyme production test, starch hydrolysis test, casein hydrolysis test, and hydrogen sulfide production test. Typically, all isolates tested positive in the first three tests, with matches the typical biochemical properties of Actinomycetes (Kalimuthu et al., 2022; Meliani et al., 2022), while only 52.94% of the samples produced H_2S. It is widely recorded that some Actinomycetes were not able to produce hydrogen sulfide (Pérez-Corral et al., 2022). In some cases all isolated Actinomycetes didn't produce H_2S (Kurup et al., 2016; Meliani et al., 2022).

The catalase enzyme mediates the breakdown of hydrogen peroxide (H_2O_2) into water and oxygen, which protect the bacterial cell from oxidative damage caused by H_2O_2. Because starch molecules are too big to enter the bacterial cell, Actinomycetes that can hydrolyze starch are producers of exoenzymes; mainly a-amylase and oligo-1, 6-glucosidase (Li et al., 2016). Another exoenzyme is called caseinase responsible of hydrolyzing the phosphoprotein found in milk; casein into smaller soluble amino acids. H_2S production is a sign of the bacterial ability of degrading sulfur-containing compounds that can be harmful to environment.

Needless to say, these biochemical tests are not only important for Actinomycetes identification; they are also considered indicators for their bioactivity and ability to produce valuable secondary metabolites.

In this study, different approaches were applied to investigate the bioactivity of the isolates; testing the isolate cultures for antibacterial activity using cross-streak method, preparation whole extract of all samples and testing their antibacterial and antifungal bioactivity, choosing whole extract of the most bioactive two samples for LC/MS analysis, and finally preparing crude extract of these two isolates for antiviral and cytotoxic tests.

Cross-streak method is one of the oldest methods used for antibacterial tests. It is cheap, fast, and simple to apply. Despite its limitations, it is still can be useful for primary screening (Hossain, 2024). In our study, 47% of the isolates showed different levels of antibacterial activity, with sample Hs-1 inhibiting the growth of 62.5% of the tested pathogens, and sample Hs-5, Hs-34 inhibiting the growth of 50% of them. Surprisingly, the

most affected pathogen in this test was *S.aureus*. The most resistant pathogen was Gram-negative *P. mirabelis* where no isolate could inhibit its growth.

Three media were used for whole extract preparation; AF for growth phase, M8 and SV2 for production phase. They consist of different nutrients with a common component of the three of them; $CaCO_3$ which inhibits Gram-negative bacteria and fungi (Pérez-Corral et al., 2022).

The use of whole extract for antibacterial and antifungal assays is way easier, less expensive and less time-consuming comparing to crude extracts where larger amounts of medium need to be prepared and evaporation of the solvents must be applied. However, crude extracts are more common due to their higher reliability where higher concentration of antimicrobial metabolites can be obtained. However, whole extracts are used for primary screening at *Naicons* where a part of this thesis was carried on. The use of whole extracts is registered in literature where both whole and crude extracts were tested and compared for antibacterial activity (Ozcan et al., 2016).

In this study, 76.47% of the whole extracts from M8 and SV2 media showed antibacterial activity against different both Gram-positive and Gram-negative bacteria. On the contrary of cross-streak method's results, *S. aures* was the most resistant pathogen where inhibition zones were only registered at Hs-1 and Hs-5 isolates. This variation may be attributed to the use of different strains of *S. aureus* in both tests.

The fact that the whole extracts were more effective against Gram-negative bacteria than Gram-positive bacteria is particularly significant, as these organisms are traditionally more resistant due to their impermeable outer membrane. This note can be explained as compounds within the whole extracts may have bypassed typical resistance mechanisms by disrupting membrane integrity or targeting lipid precursors, comparing to crude extracts which may lose hydrophilic or highly labile compounds during solvent partitioning.

Actinomycetes are the main source of antifungal agents (Demirel et al., 2016). 35.29% of M8 extracts showed antifungal inhibition against *C. albicans*, while only 11.76% of SV2 extracts showed this activity. These results correspond with the results of (Rante et al., 2024) where 25% of the isolated Actinomycetes showed antifungal activity against *C. albicans*. M8 medium contains yeast extract and meats extract which seem to be preferable for Actinomycete bioactivity (Ozcan et al., 2016). The variations of the media components may give an answer on the reason M8 extracts were more effective than SV2 extracts.

It has been unmistakable that both isolates Hs-1 and Hs-5 are the most valuable ones regarding antibacterial and antifungal activity. For that, their M8 whole extracts were chosen for LC/MS analysis. The results revealed quite rich bioactive compounds ranging from antimicrobial molecules to cytotoxic agents, and ending in metabolic enzymes. This promising background led to continue with antiviral and anticancer tests.

Although, the LC/MS analysis was done based on whole extracts, we were obligated to switch to crude extracts because the antiviral and anticancer assays must start from dry extracts. M8 medium was used for bacterial fermentation, and ethyl acetate was used as a solvent.

Actinomycetes are one of the main sources of antiviral agents; about 15% of isolated marine microbial secondary metabolites with antiviral activities come from *Streptomyces* (Yi et al., 2020). HIV-1 enzymes, like reverse transcriptase (RT) play a huge role in the viral replication process, especially for retroviruses. HIV-1 RT functions as a convertor of the viral single-stranded RNAs into double-stranded DNAs (Doganci et al., 2022). Thus, HIV-1 RT inhibition assay is one of the important tools for screening antiviral activity.

One of the most significant findings of this study was the notable antiviral activity of both tested isolates. Hs-1 and Hs-5 extracts were able to inhibit HIV-1 reverse transcriptase by 97.22% and 97.38% respectively, taking into account the medium effect (9.7%). These outcomes go along with the recorded HIV-1 RT inhibition by actinomycete compounds (Guo et al., 1998; Lingham et al., 1996).

The crude extracts of Hs-1 and Hs-5 also displayed cytotoxic activity in anticancer assays, suggesting the presence of metabolites with antitumor potential. Actinomycetes are historically significant in oncology, as they are the source of key chemotherapeutic agents such as anthracyclines, enediynes, indolocarbazoles, isoprenoides, macrolides, and non-ribosomal peptides (Olano et al, 2009). Another example is salinosporamide-A which was discovered and developed from marine actinomycete (Fenical et al., 2009). Similarly to the finding in this study, two anticancer compounds ULDF4 and UDLF5 isolated from the extract of *Streptomyces bingchenggensis* ULS14. They showed anticancer activity against different cancer cells including MCF-7 (breast adenocarcinoma) cell line (Davis-Bolorunduro et al., 2019). Finally, Erbstatin, which was one of the metabolites found in Hs-5 extract, has shown to inhibit tyrosine kinases, thereby blocking oncogenic signaling pathways (Imoto et al., 1987).

5. CONCLUSION

This study demonstrates that Actinomycetes isolated from Blue Lake and Goksu Travertines represent a rich source of bioactive secondary metabolites with antibacterial, antifungal, antiviral, and anticancer potential. The predominance of *Streptomyces* (76.47% of isolates) is consistent with global reports that identify this genus as the most abundant among actinomycetes. The recovery of *Nocardia* and *Pseudonocardia* further underscores the ecological diversity of these sites, while morphological traits confirmed molecular classifications.

Biochemical analysis revealed catalase, amylase, and casein hydrolysis activities across all isolates, reinforcing their suitability as producers of extracellular enzymes and secondary metabolites. Hydrogen sulfide production was less consistent, matching prior findings that this trait varies widely among Actinomycetes. These enzymatic patterns serve not only as taxonomic tools but also as indicators of metabolic potential.

Whole extract assays confirmed broad antibacterial activity, particularly against Gram-negative bacteria, which is notable given their intrinsic resistance due to outer membrane barriers.

Antifungal activity against *Candida albicans* was also detected, with M8 medium extracts showing greater potency, consistent with medium-dependent metabolite production reported in earlier work.

Crude extracts from the most active isolates, Hs-1 and Hs-5, demonstrated exceptional antiviral potential, inhibiting HIV-1 reverse transcriptase by more than 97%. These results align with known actinomycete metabolites such as quinoxapeptins and actinomycin D, which are established HIV-1 RT inhibitors. The high inhibition observed here strongly supports further exploration of these isolates for antiviral compound discovery. Similarly, cytotoxicity assays against MCF-7 breast cancer cells revealed promising anticancer potential, reflecting the well-documented contributions of actinomycetes to oncology, including the discovery of anthracyclines and salinosporamide A.

Overall, this research confirms that Actinomycetes from unique Turkish habitats harbor considerable pharmaceutical potential. The complementary use of whole and crude

extracts illustrates two valid strategies: whole extracts enable rapid, low-cost screening, while crude extracts concentrate active metabolites suitable for detailed evaluation. The strong antibacterial, antifungal, antiviral and anticancer activities observed emphasize the continuing importance of Actinomycetes in natural product research. Future studies should prioritize chemical purification, structural elucidation, and genomic analysis of these isolates to fully realize their potential in addressing antimicrobial resistance, viral diseases, and cancer.

6. RECOMMENDATION

As the isolates showed antibacterial, antifungal, antiviral, and anticancer activity, it is highly recommended to isolate more samples from the same area and other areas that have similar environmental characteristics, especially that Black Sea region is considered a natural treasure.

More tests are required to confirm the efficiency of whole extracts, and exploring new methods to use them effectively, especially when dealing with large number of isolates.

It is also recommended to apply chromatographic analysis to the remaining isolates, because of promising results obtained from the two chosen isolates.

Giving the fact that the isolates exhibited a considerable bioactivity, it would be nice to consider testing all isolates for antiviral and anticancer potential in future studies.

Finally, to determine the molecular responsible of bioactivity, it would be favored to do deeper MS analysis by fractioning the extracts and testing them individually.

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