THESIS

Ma Jiayi
MSC in Environmental Engineering

GÖDÖLLŐ
2023

Hungarian University of Agriculture and Life Science

Szent István Campus

MSC in Environmental Engineering

Analysis the Antimicrobial Effect of *Streptomyces* Strain Collection Against Resistant Bacteria by using Gene Mapping

Primary Supervisor：	Harkai Péter
Independent Consultant:	Harkai Péter
	Research fellow
Author:	**Ma Jiayi**
	FUDINZ

Institute/Department:

Institute for Aquaculture and Environmental Safety,

Department of Environmental Safety

GÖDÖLLŐ

2023

List of Content

LIST OF MOST FREQUENTLY OCCURRING MARKS AND ABBREVIATIONS

GYM	Glucose, Yeast-extract, Malt-extract
PDA	Potato Dextrose Agar
TGE	Tryptone Glucose Extract agar
MH	Mueller Hinton Agar
MATE	Hungarian University of Agriculture and Life Science
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
PCR	Polimerase Chain Reaction
MQ	Milli-Q water
WHO	World Health Organization
WGS	Whole-genome sequencing
TBE	Tris-Borate-EDTA
dNTP	Deoxyribonucleotide triphosphate
OTC	Oxytetracycline

1. Introduction

With advancements in chemistry and medical technology, an increasing number of antibiotics are becoming integrated into our daily lives. However, when these antimicrobial agents enter the natural environment, they act as persistent pollutants, enduring for extended periods in surface and underground waters, as well as within geological surroundings. Due to their long-lasting antimicrobial properties, they can cause permanent alterations in the natural microbial population of a particular habitat. One significant consequence of antimicrobial treatments is the emergence of resistant or multi-resistant strains. For example, the well-known danger associated with therapeutic antibiotic usage is the development of resistant pathogenic strains. However, even non-threatening microorganisms or facultative pathogenic organisms can contribute to the propagation of resistance. Any strain of microorganisms has the potential to transfer acquired resistance within or between species, resulting in the gradual loss of activity in the antimicrobial substances produced by microorganisms. This extensive spread of resistance severely hampers effective protection against pathogens.

Facultative pathogenic microorganisms, such as *Ps. aeruginosa* species, are commonly found in the environment and can cause severe nosocomial infections, primarily in clinical settings. However, the most pressing issue arises from the ease with which antibacterial agents become multi-resistant against these groups (Morgado et al. 2021). According to the 2017 report by the World Health Organization (WHO), carbapenem-resistant *Ps. aeruginosa* represents one of the most critical challenges worldwide in terms of antibiotic resistance. To prevent the development and proliferation of multi-resistant strains, it is crucial to explore and develop new antimicrobial agents and groups of active compounds.

It is also known that resistant pathogenic strains are often selected for application in different fields of use, leading to strains that produce antibiotics losing their antibiotic activity. Based on this experience, the selection of new active strains and the expansion of the selection according to the spectrum of action become important research and development tasks.

From a biotechnological standpoint, members of the bacterial genus *Streptomyces* hold immense significance as they serve as a valuable source of enzymes for various industries. Additionally, many members of this genus are utilized in the production of antifungal, antiviral,

and anticancer substances. Their application in the pharmaceutical sector is based on their remarkable ability to generate an extensive range of antimicrobial compounds (Quinn et al. 2020). Therefore, it is important to isolate *Actinomycetes* suitable for antibiotic production for use in plant protection, animal husbandry, and human and veterinary medicine, as well as to extract broad-spectrum active antimicrobial substances.

The purpose of this study was to identify the molecular biology of lab-collected strains K189 and K145 and analyze the results of antibacterial activity tests. After molecular identification of the collected K189 and K145 strains, they were tested for their antibacterial activity using three common antibacterial testing methods: cross-steak, agar diffusion, and filter paper diffusion. The data will be analyzed and compared to clarify their antibacterial capacity. Overall, the results of this study will provide a basis for further research into the potential clinical application of *Streptomyces* strains K189 and K145 in inhibiting multidrug-resistant *Pseudomonas aeruginosa.*

2. Literature

2.1. The *Actinomycetes* and the family Streptomycetaceae

2.1.1. History and taxonomy

Actinomycetes are a group of bacteria within the Phylum *Actinomycetes*, renowned for their ability to produce a broad range of bioactive compounds, including antibiotics. The taxonomy of *Actinomycetes* is based on morphological, physiological, and molecular features, with the best-known genera being *Streptomyces*, *Nocardia*, and *Mycobacterium* (Stackebrandt & Schumann, 2006).

In 1877, Ferdinand Cohn (1877), a German physician and microbiologist, described a new type of bacterium that he named Actinomyces and initially classified as part of the fungi kingdom (Salam, 2020). In the early 20th century, research by Niels Finsen (1894), a Danish bacteriologist, and Paul Ehrlich (1909), a German microbiologist, established *Actinomycetes* as a separate group of bacteria.

Streptomyces, one of the largest families of *Actinomycetes*, is renowned for its ability to produce a diverse range of secondary metabolites, including antibiotics, antineoplastic agents, immunosuppressive agents, and also anti-inflammatory agents. The *Streptomyces* genus consists of over 500 different species, each with its unique morphological, physiological, and biochemical features (Dhanasekaran & Jiang, 2016).

The genus *Streptomyces* was first described by British bacteriologist Edwin Lennard Jones in 1911, and since then, many new strains have been isolated and characterized from different environmental sources. Researchers worldwide have identified and characterized many other species of *Streptomyces*, leading to the recognition of Streptomycetaceae as a distinct family of bacteria (Mukerji et al. 2006). Waksman and Henrici (1943) performed one of the earliest taxonomic studies of the *Streptomyces* genus, proposing a classification scheme based on morphological and physiological characteristics (Kämpfer, 2006). This scheme formed the basis of our understanding of the *Streptomyces* genus for many years. However, as molecular techniques advanced, they became used to better understand the diversity and evolutionary relationships within the genus.

Today, molecular techniques such as 16S rRNA gene sequencing, whole-genome sequencing, and phylogenetic analysis are commonly used for the identification and classification of

Actinomycetes. These techniques provide a more accurate and detailed understanding of the genetic relatedness and evolutionary history of different strains within the genus.

It is worth noting that the genetic sequence of Actinobacteria, specifically *Streptomyces*, is indeed quite large. As a group of Gram-positive bacteria, *Streptomyces* possesses a complex genome, typically ranging from 8 to 12 megabase pairs in size, making it one of the largest genomes among bacteria (Tidjani et al.2019). This significant genome size can be attributed to the presence of numerous non-coding DNA sequences and repetitive elements within the Actinobacterial genome.

These genomes harbor thousands of genes, including those encoding proteins, regulatory elements, and other functional sequences. These genes are responsible for synthesizing enzymes, ketones, proteins, and other biomolecules crucial for the growth, metabolism, and production of biologically active compounds in *Actinomycetes* (Zhu et al. 2014). Moreover, the actinomycete genome encompasses regulatory genes that modulate gene expression, ensuring the precise timing and conditions for the production of desired bioactive substances (Ventura et al. 2007).

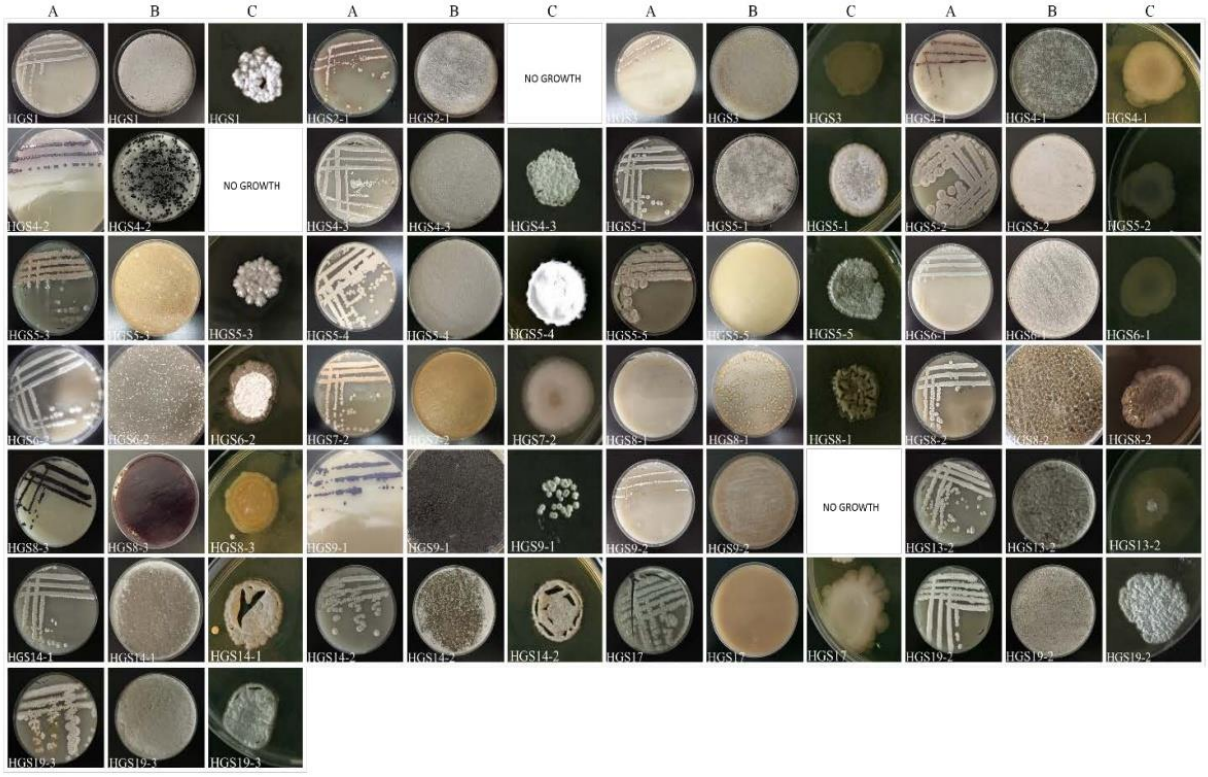
Analyzing and comprehending the Actinobacterial genome pose challenges due to its vast complexity. However, recent advancements in high-throughput sequencing technologies have facilitated in-depth investigations into *actinomycete* genomes, unveiling their organization, function, and regulatory mechanisms (Zhu et al. 2014). These findings hold significant implications for understanding the biosynthetic capabilities of *Actinomycetes* and developing novel bioactive compounds. Furthermore, the abundance of genes and regulatory elements within *Actinomycetes* positions them as valuable microbial resources with diverse potential applications. Recent studies have aimed to improve the taxonomy of the *Streptomyces* genus, including a Chinese study (Zhang et al. 2018) that used whole-genome sequencing data to reclassify the genus into 23 clades, each assigned a unique clade number. Another study proposed a new taxonomic classification based on a combination of genomic and phenotypic data (Klaassen, 2015).

In summary, the Streptomycetaceae family is one of the largest families within the phylum Actinobacteria, known for its ability to produce antibiotics and other important bioactive compounds. The taxonomy of *Actinomycetes* and Streptomycetaceae is based on a combination

of morphological, physiological, and molecular characteristics, which continue to evolve as new species are discovered and characterized.

2.1.2. Cytology and morphology

Streptomyces are filamentous bacteria that form long, branching chains of cells. At the cellular level, *Streptomyces* cells are rod-shaped and measure between 0.5-1.0 μm in diameter and 2-10 μm in length (Mayfield et al. 1972). They have a well-developed cytoplasmic membrane, a thick peptidoglycan layer, and a complex array of metabolic pathways.



Picture 2.1 Colour and morphology of *Streptomyces spp.* that are isolated from house garden soil. The names have been abbreviated to include HG (house garden) S (soil) followed by the sample number (Jalal & Ayad, 2021).

One of the distinctive features of *Streptomyces* bacteria is their filamentous growth, known as mycelium, which is composed of long, branching chains of cells. Mycelium consists of asexual cells involved in growth and metabolism, as well as spore-producing aerial mycelium that produces new spores. These spores are short segments of mycelium that can develop into new, independent colonies, and their production by aerial hyphae is a key factor in the dispersal and settlement of *Streptomyces* in new environments (Dhanasekaran & Jiang, 2016).

In addition to mycelium and aerial hyphae, *Streptomyces* produces a range of secondary metabolites, including antibiotics, antitumor agents, and immunosuppressants, which are synthesized and excreted by special structures called spore chains (Selim et al. 2021). These spore chains are composed of spores of the mycelium that have been modified to contain large amounts of secondary metabolites. Among the most well-known and studied are the antibiotics, which have been used for many years as therapeutics to treat bacterial infections.

Streptomyces produces a range of antibiotics with different modes of action and activity spectra, including tetracyclines, macrolides, aminoglycosides, and beta-lactams, among others (Alam et al. 2022). These antibiotics have been widely used to treat bacterial infections in humans and animals and are a major focus of research in the field of antimicrobial expertise.

2.1.3. Ecology of *Actinomycetes*

Actinobacteria, with their wide distribution in natural environments such as soil and water bodies, are a fascinating group of bacteria. They exhibit remarkable diversity and can be found in diverse habitats, including soil, water, and the surfaces of plants and animals (Goodfellow & Williams, 1983). In soil ecosystems, Actinobacteria thrive and play a pivotal role in nutrient cycling and the decomposition of complex plant polymers, such as cellulose and lignin (Van Der Meij et al. 2017). These microorganisms engage in intricate interactions with other soil microbes, forming complex microbial communities that orchestrate the functions of soil ecosystems.

Moreover, Actinobacteria also make significant contributions to aquatic environments. They actively participate in nutrient cycling and the breakdown of organic matter, making them essential players in the decomposition processes within water bodies (Chen et al. 2021). Furthermore, Actinobacteria are involved in the formation of biofilms on aquatic plants and animals, which serve as critical regulators of water quality and the overall health of aquatic ecosystems.

Among the Actinobacteria, *Streptomyces* stands out as an important genus. Within soil ecosystems, *Streptomyces* plays a vital role in maintaining the equilibrium and well-being of the soil environment through its biocontrol mechanisms (Shanthi, 2021). These fascinating microorganisms have developed sophisticated strategies to ensure the health and functionality

of soils, making them indispensable contributors to the intricate web of life in our natural habitats.

Table 2.2 Changes in the activity of nitrogen metabolizing enzymes in the shoots and roots with and without biologically active *Actinomycete* isolates. *Actinomycetes* fix nitrogen at the tuberous roots of legumes and produce positive effects (AbdElgawad et al. 2020).

	GDH		GS		NR		GOGAT	
Plant-Isolate	**Root**	**Shoot**	**Root**	**Shoot**	**Root**	**Shoot**	**Root**	**Shoot**
Soybean-Cont	0.97 ± 0.09 ^{a}	0.47 ± 0.03 ^{a}	1.99 ± 0.49 ^{a}	0.95 ± 0.21 ^{a}	0.84 ± 0.01 ^{a}	0.22 ± 0.01 ^{a}	1.82 ± 0.06 ^{a}	0.59 ± 0.05 ^{a}
Soybean-I2	0.91 ± 0.08 ^{a}	0.49 ± 0.08 ^{a}	6.56 ± 0.39 ^{b}	1.45 ± 0.04 ^{a}	1.53 ± 0.15 ^{a}	0.20 ± 0.02 ^{a}	2.39 ± 0.04 ^{b}	0.63 ± 0.05 ^{a}
Soybean-I8	0.93 ± 0.09 ^{a}	0.80 ± 0.11 ^{a}	6.70 ± 0.39 ^{b}	2.32 ± 0.08 ^{b}	1.09 ± 0.18 ^{a}	0.27 ± 0.01 ^{a}	1.53 ± 0.02 ^{b}	0.64 ± 0.05 ^{a}
Soybean-I12	1.20 ± 0.11 ^{a}	0.69 ± 0.08 ^{a}	8.31 ± 0.49 ^{b}	1.63 ± 0.09 ^{a}	1.56 ± 0.02 ^{b}	0.32 ± 0.02 ^{b}	3.22 ± 0.10 ^{b}	0.75 ± 0.06 ^{a}
Soybean-I15	0.97 ± 0.09 ^{a}	0.97 ± 0.18 ^{a}	6.99 ± 0.41 ^{b}	1.17 ± 0.11 ^{a}	1.87 ± 0.08 ^{b}	0.28 ± 0.01 ^{a}	3.80 ± 0.25 ^{b}	0.67 ± 0.05 ^{a}
Kidney Bean-Cont	0.92 ± 0.05 ^{a}	0.51 ± 0.00 ^{a}	2.63 ± 0.21 ^{a}	1.67 ± 0.24 ^{a}	0.05 ± 0.01 ^{a}	0.13 ± 0.02 ^{a}	1.59 ± 0.23 ^{a}	6.91 ± 0.65 ^{a}
Kidney Bean-I2	1.07 ± 0.08 ^{a}	0.91 ± 0.13 ^{a}	7.56 ± 0.45 ^{b}	1.69 ± 0.11 ^{a}	0.15 ± 0.00 ^{b}	0.20 ± 0.02 ^{a}	2.54 ± 0.04 ^{a}	9.83 ± 0.76 ^{a}
Kidney Bean-I8	1.10 ± 0.08 ^{a}	1.43 ± 0.23 ^{a}	7.72 ± 0.45 ^{b}	2.81 ± 0.24 ^{a}	0.09 ± 0.01 ^{a}	0.31 ± 0.02 ^{b}	1.62 ± 0.08 ^{a}	10.05 ± 0.78 ^{a}
Kidney Bean-I12	1.36 ± 0.10 ^{a}	1.00 ± 0.12 ^{a}	9.66 ± 0.57 ^{b}	2.50 ± 0.10 ^{a}	0.17 ± 0.00 ^{b}	0.41 ± 0.02 ^{b}	3.38 ± 0.06 ^{b}	10.05 ± 0.78 ^{a}
Kidney Bean-I15	1.14 ± 0.09 ^{a}	0.80 ± 0.02 ^{b}	8.05 ± 0.47 ^{b}	1.73 ± 0.06 ^{a}	0.20 ± 0.00 ^{b}	0.32 ± 0.02 ^{b}	3.38 ± 0.05 ^{b}	10.48 ± 0.81 ^{a}
Chickpea-Cont	1.07 ± 0.08 ^{a}	0.49 ± 0.01 ^{a}	2.37 ± 0.49 ^{a}	1.33 ± 0.13 ^{a}	0.15 ± 0.05 ^{a}	0.15 ± 0.01 ^{a}	0.85 ± 0.15 ^{a}	0.76 ± 0.06 ^{a}
Chickpea-I2	0.90 ± 0.07 ^{a}	0.73 ± 0.17 ^{a}	6.03 ± 0.36 ^{b}	1.15 ± 0.04 ^{a}	0.52 ± 0.01 ^{b}	0.13 ± 0.01 ^{a}	1.83 ± 0.03 ^{b}	0.93 ± 0.07 ^{a}
Chickpea-I8	0.92 ± 0.07 ^{a}	1.03 ± 0.12 ^{a}	6.16 ± 0.36 ^{b}	1.67 ± 0.05 ^{a}	0.36 ± 0.02 ^{a}	0.14 ± 0.01 ^{a}	1.27 ± 0.04 ^{a}	0.95 ± 0.07 ^{a}
Chickpea-I12	1.29 ± 0.10 ^{a}	0.80 ± 0.10 ^{a}	7.90 ± 0.46 ^{b}	1.95 ± 0.08 ^{a}	0.49 ± 0.01 ^{b}	0.18 ± 0.01 ^{a}	2.00 ± 0.03 ^{b}	1.00 ± 0.08 ^{a}
Chickpea-I15	0.96 ± 0.07 ^{a}	0.79 ± 0.09 ^{a}	6.42 ± 0.38 ^{b}	0.97 ± 0.03 ^{a}	0.70 ± 0.01 ^{b}	0.14 ± 0.01 ^{a}	2.48 ± 0.04 ^{b}	0.99 ± 0.08 ^{a}
Lentil-Cont	0.88 ± 0.05 ^{a}	0.47 ± 0.02 ^{a}	2.07 ± 0.29 ^{a}	1.31 ± 0.07 ^{a}	0.39 ± 0.07 ^{a}	0.16 ± 0.01 ^{a}	0.41 ± 0.01 ^{a}	1.43 ± 0.36 ^{a}
Lentil-I2	1.05 ± 0.09 ^{a}	0.91 ± 0.07 ^{b}	5.08 ± 0.30 ^{b}	1.06 ± 0.08 ^{a}	1.43 ± 0.18 ^{b}	0.19 ± 0.04 ^{a}	0.60 ± 0.03 ^{b}	1.23 ± 0.31 ^{a}
Lentil-I8	1.07 ± 0.09 ^{a}	0.96 ± 0.18 ^{a}	5.19 ± 0.31 ^{b}	1.42 ± 0.15 ^{a}	0.72 ± 0.08 ^{a}	0.22 ± 0.03 ^{a}	0.50 ± 0.04 ^{a}	3.25 ± 0.23 ^{a}
Lentil-I12	1.31 ± 0.11 ^{a}	0.58 ± 0.07 ^{a}	6.28 ± 0.37 ^{b}	1.22 ± 0.07 ^{a}	1.73 ± 0.20 ^{b}	0.28 ± 0.04 ^{a}	0.73 ± 0.02 ^{b}	1.74 ± 0.44 ^{a}
Lentil-I15	1.12 ± 0.09 ^{a}	0.80 ± 0.05 ^{b}	5.41 ± 0.32 ^{b}	0.93 ± 0.04 ^{a}	1.34 ± 0.21 ^{a}	0.23 ± 0.03 ^{a}	0.78 ± 0.01 ^{b}	1.43 ± 0.31 ^{a}
Pea-Cont	0.62 ± 0.09 ^{a}	0.55 ± 0.02 ^{a}	2.71 ± 0.44 ^{a}	1.03 ± 0.09 ^{a}	0.30 ± 0.07 ^{a}	0.12 ± 0.01 ^{a}	1.21 ± 0.11^{a}	3.87 ± 0.39 ^{a}
Pea-I2	1.27 ± 0.08 ^{b}	0.89 ± 0.07 ^{b}	6.37 ± 0.38 ^{b}	1.22 ± 0.09 ^{a}	1.33 ± 0.21 ^{a}	0.20 ± 0.03 ^{a}	4.81 ± 0.22 ^{b}	6.70 ± 0.52 ^{a}
Pea-I8	1.30 ± 0.09 ^{b}	0.72 ± 0.03 ^{a}	6.51 ± 0.38 ^{b}	1.66 ± 0.17 ^{a}	0.98 ± 0.19 ^{a}	0.27 ± 0.01 ^{b}	4.02 ± 0.32 ^{b}	6.84 ± 0.53 ^{a}
Pea-I12	1.47 ± 0.10 ^{b}	0.60 ± 0.07 ^{a}	6.76 ± 0.40 ^{b}	1.25 ± 0.07 ^{a}	1.98 ± 0.01 ^{b}	0.29 ± 0.01 ^{b}	5.70 ± 0.18 ^{b}	9.55 ± 0.74 ^{b}
Pea-I15	1.36 ± 0.09 ^{b}	0.86 ± 0.04 ^{b}	6.79 ± 0.40 ^{b}	1.11 ± 0.04 ^{a}	1.82 ± 0.35 ^{a}	0.28 ± 0.01 ^{b}	5.91 ± 0.10 ^{b}	7.13 ± 0.55 ^{b}

Actinomycetes are also found on the surfaces of plants and animals, where they regulate plant and animal health (Li & Guo, 2020). For example, they are essential for the formation of legume root nodules, where they fix nitrogen and make it available for plant growth (AbdElgawad et al. 2020). Recent studies have highlighted the importance of *Actinomycetes* in environmental regulation and their potential for use in developing sustainable environmental management techniques.

2.2. Antimicrobial effects of *Streptomyces* species

Streptomyces species are a type of *Actinomycetes* that are known for their ability to produce a wide range of secondary metabolites, including antibiotics. The antimicrobial effects of *Streptomyces* species are due to their ability to produce secondary metabolites that are toxic to bacteria. These secondary metabolites can interfere with various cellular processes in bacteria, such as cell wall synthesis, DNA replication, and protein synthesis. In addition to their

antibacterial activity, some *Streptomyces* species have also been found to produce secondary metabolites with antiviral, antifungal, and antiparasitic activities. This makes them a valuable resource for the discovery and development of new antibiotics and antiparasitic agents (Donald et al. 2022).

Table 2.3 List of some antibiotics produced by *Streptomyces* sp. (Selim et al. 2021).

***Streptomyces* sp.**	**Antibiotic**	***Streptomyces* sp.**	**Antibiotic**
S. orchidaccus	Cycloserin n	*S. erythraeus*	Erythromycin
S. oriantalis	Vancomycin	*S. vensuella*	Chloramphenicol
S. fradiae	Neomycin, actinomycin, fosfomycin, dekamycin	*S. aureofaciens*	Chlortetracycline, dimethylchlor
S. nodosus	Amphotricin B	*S. ambofaciens*	Spiramycin
S. noursei	Nistatin	*S. avermitilis*	Avermicin
S. mediterranei	Rifampin	*S. alboniger*	Puromycin
S. griseus	Streptomycin	*S. niveus*	Novobicin
S. knanamyceticus	Kanamycin	*S. platensis*	Platenmycin
S. tenebrarius	Tobramycin	*S. roseosporus*	Daptomycin
S. spectabilis	Spectinomycin	*S. ribosidificus*	Ribostamycin
S. viridifaciens	Tetracycline	*S. garyphalus*	Cycloserine
S. lincolensis	Lincomycin, clindamycin	*S. vinaceus*	Viomycin
S. rimosus	Oxytetracyclin	*S. clavuligerus*	Cephalosporin

The table 2.3 shows that *Streptomyces rimosus* is responsible for the production of Hygromycin and Oxytetracyclin, which may be a secondary metabolite of their reaction. We look forward to finding out the results of the genome to reveal as a preview.

2.2.1. About antibiotics in general

Antibiotics are a group of compounds produced by microorganisms, such as bacteria, fungi, and *Actinomycetes*, which have the ability to inhibit the growth other microorganisms, including bacteria, fungi, and viruses. Antibiotics have been used for over 80 years to treat bacterial infections and have been essential in reducing the incidence and severity of infectious diseases. However, the widespread use of antibiotics has also led to the development of antibiotic resistance in many pathogenic bacteria, making it increasingly difficult to effectively treat bacterial infections (Davies, 2010).

The history of antibiotics dates back to the turn of the century when Sir Alexander Fleming, a Scottish bacteriologist, discovered penicillin in 1928. Over the next few years, researchers worked to isolate and identify the active compound produced by *Streptomyces*, which was eventually named penicillin (Bennett et al. 2020). In the 1940s, penicillin was mass-produced

for use in World War II, and it proved to be a life-saving tool for the treatment of bacterial infections in soldiers (R. Hare, 1982). Since then, antibiotics have become a milestone of modern medicine, with new antibiotics being developed to treat a wide range of bacterial infections.

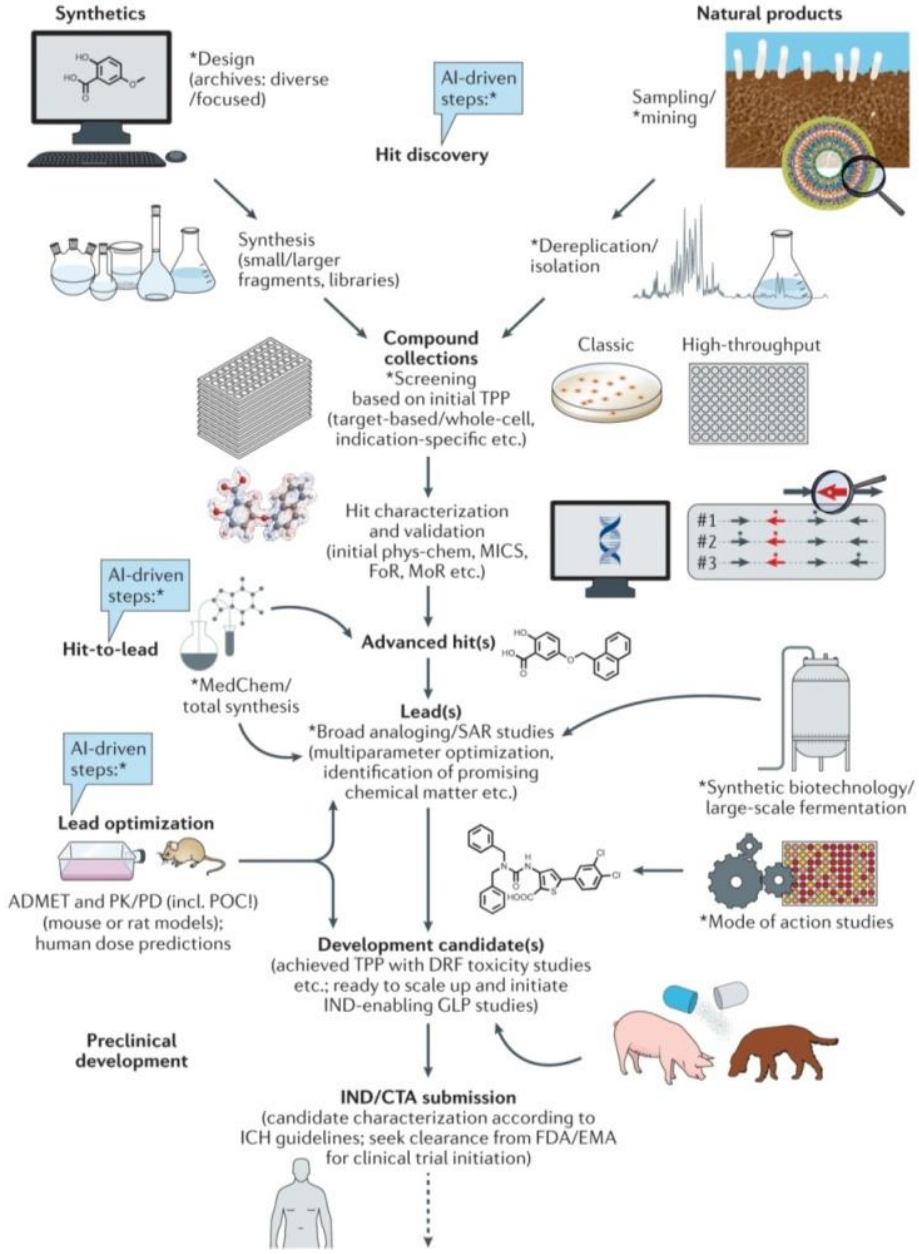


Figure 2.4 Summary of major steps and processes in antibacterial drug discovery and development (Miethke et al. 2021).

Antibiotics are secondary metabolites produced by microorganisms that have the ability to destroy or inhibit the growth of other microorganisms, even at low concentrations. They achieve this by disrupting the enzyme systems of target microorganisms, rendering their reproduction

impossible (Hussain et al. 2012). It is important to note that the production of these secondary metabolites is not directly linked to the cell's energy and material production. Instead, it occurs at a later stage of cultivation, often during the fermentation process.

Currently, scientists have described around 12,000 to 13,000 different antibiotics (Antibiotic Resistance Threats in the United States, 2013). However, out of these known compounds, only about 300 are utilized in human medicine. This selective usage is due to the requirement of antibiotics possessing selective toxicity. In other words, an effective antibiotic must be highly toxic to the pathogen it targets while causing little to no harm to the host (humans) (Purssell, 2020).

Furthermore, antibiotics exhibit different antimicrobial spectrums, meaning they have varying degrees of effectiveness against specific groups of microorganisms. Some antibiotics may be more effective against certain types of bacteria, while others may have a broader spectrum and act against a wider range of microorganisms (Chis et al. 2022).

Understanding the nature and characteristics of antibiotics is crucial for their appropriate use in combating infections and preserving their effectiveness for future medical treatments.

2.2.2. Use of antibiotics

Antibiotics are commonly known for their medicinal use in treating bacterial infections such as respiratory, skin, urinary, and gastrointestinal infections. The appropriate use of antibiotics is crucial to ensure their effectiveness and prevent the development of antibiotic resistance, a phenomenon where bacteria become resistant to the effects of antibiotics. Overuse or misuse of antibiotics can lead to the spread of antibiotic-resistant bacteria, making bacterial infections difficult to treat and control (Tang et al. 2017; Chang et al. 2014).

Aside from their medical use, antibiotics also have various applications in other fields, such as agriculture, food preservation, aquaculture, environmental remediation, and biotechnology. In agriculture, antibiotics are commonly used to prevent and treat bacterial infections in livestock and poultry, as well as to promote growth and feed efficiency in some animals (Oliveira et al. 2020). Similarly, antibiotics are utilized in the food industry to prevent bacterial spoilage and contamination during the processing of certain food products (Mehdi et al. 2018). In aquaculture, antibiotics are used to treat bacterial infections in farmed fish and shellfish (Pepi

& Focardi, 2021; Bojarski, Kot & Witeska, 2020). While in environmental remediation, antibiotics are employed to control bacterial contamination in soil, water, and air, and to clean up contaminated sites. A study found that the use of antibiotics in bioremediation was effective in reducing the levels of bacteria and pollutants in contaminated soil (Liu et al. 2017).

In biotechnology, antibiotics are used to select for or eliminate specific bacterial strains and control bacterial contamination in laboratory cultures (Russell, 2003). Excessive use of antibiotics can lead to the emergence of multidrug-resistant bacteria. These bacteria have developed resistance to multiple antibiotics, making the treatment of infections more challenging (Kraemer et al. 2019). They can spread in healthcare settings, causing severe hospital-acquired infections. Additionally, multidrug-resistant bacteria can also spread in the community, making common infections difficult to treat. This poses a significant threat to public health, as we may no longer rely on conventional antibiotics to combat infections (Van Duin,2016). These multi-resistance refers to bacterial strains that are resistant to two or more antibiotics, making them difficult to treat effectively in clinical therapy. Among Gram-negative bacteria, members of the genera *Acinetobacter* and *Pseudomonas* are known to exhibit multi-resistance (Bojarski et al. 2020). These bacteria can be found in the normal flora of the skin and have occasionally caused minor outbreaks (Morgado-Gamero et al. 2021). The development of resistance can occur through chromosomal or extrachromosomal mechanisms, with plasmids playing a significant role. Plasmids can transfer and disseminate resistance genes not only within the same species but also between different genera. Resistant bacteria have been isolated from various environmental sources, including surface waters, subsurface waters, soil, and plants treated with polluted water (Kraemer et al. 2019; Liguori et al. 2022).

Antibiotics can enter the environment through various sources, including residential antibiotic use and agricultural practices. Residential antibiotic use involves the excretion of antibiotics from the human body, as well as the release of expired drugs into the sewer system, leading to their presence in wastewater. These substances, which are resistant to degradation processes, can ultimately contaminate surface water and drinking water supplies (Liguori et al. 2022). Agricultural antibiotic use is prevalent in veterinary medicine, plant protection, and as yield-increasing feed additives in animal husbandry (Manyi-Loh et al. 2018). In recent years, regulations have been implemented to restrict the use of antibiotic-type feed supplements in the

European Union.

The use of antibiotic resistance marker genes in genetically modified organisms (GMOs) is a topic of scientific debate. Marker genes are used in the development of GMOs to indicate successful gene transfer and integration. However, concerns have been raised about the potential transfer of antibiotic resistance genes from GMOs to the environment or bacterias, leading to the spread of antibiotic resistance (Netherwood et al. 1999; Oraby et al. 2022).

The use of antibiotics is essential in maintaining the health and productivity of different species and systems and controlling bacterial contamination. However, to preserve the effectiveness of antibiotics for future generations, their use in these fields should be carefully monitored to minimize the development of antibiotic resistance (Miethke et al.2021).

2.2.3. Antimicrobial ability of *Streptomyces* genus

The genus *Streptomyces* is one of the largest sources of antibiotics, accounting for over two-thirds of all commercially produced. Some of the most commonly used antibiotics produced by *Streptomyces* species include streptomycin, tetracycline, and erythromycin. *Streptomyces* species produce a wide range of antibiotics that target different aspects of bacterial physiology such as cell wall synthesis, protein synthesis, and DNA synthesis (Quinn et al. 2020). These antibiotics have a broad spectrum of activity and are effective against various bacterial pathogens, including both gram-positive and gram-negative bacteria (Alam et al. 2022).

In conclusion, the *Streptomyces* genus is a crucial source of antibiotics that has significantly impacted the treatment of bacterial infections. While it's essential to avoid the widespread use of antibiotics to prevent the development of antibiotic resistance, the continuous discovery and development of new antibiotics from *Streptomyces* species will be critical in the ongoing fight against bacterial infections and the development of antibiotic resistance.

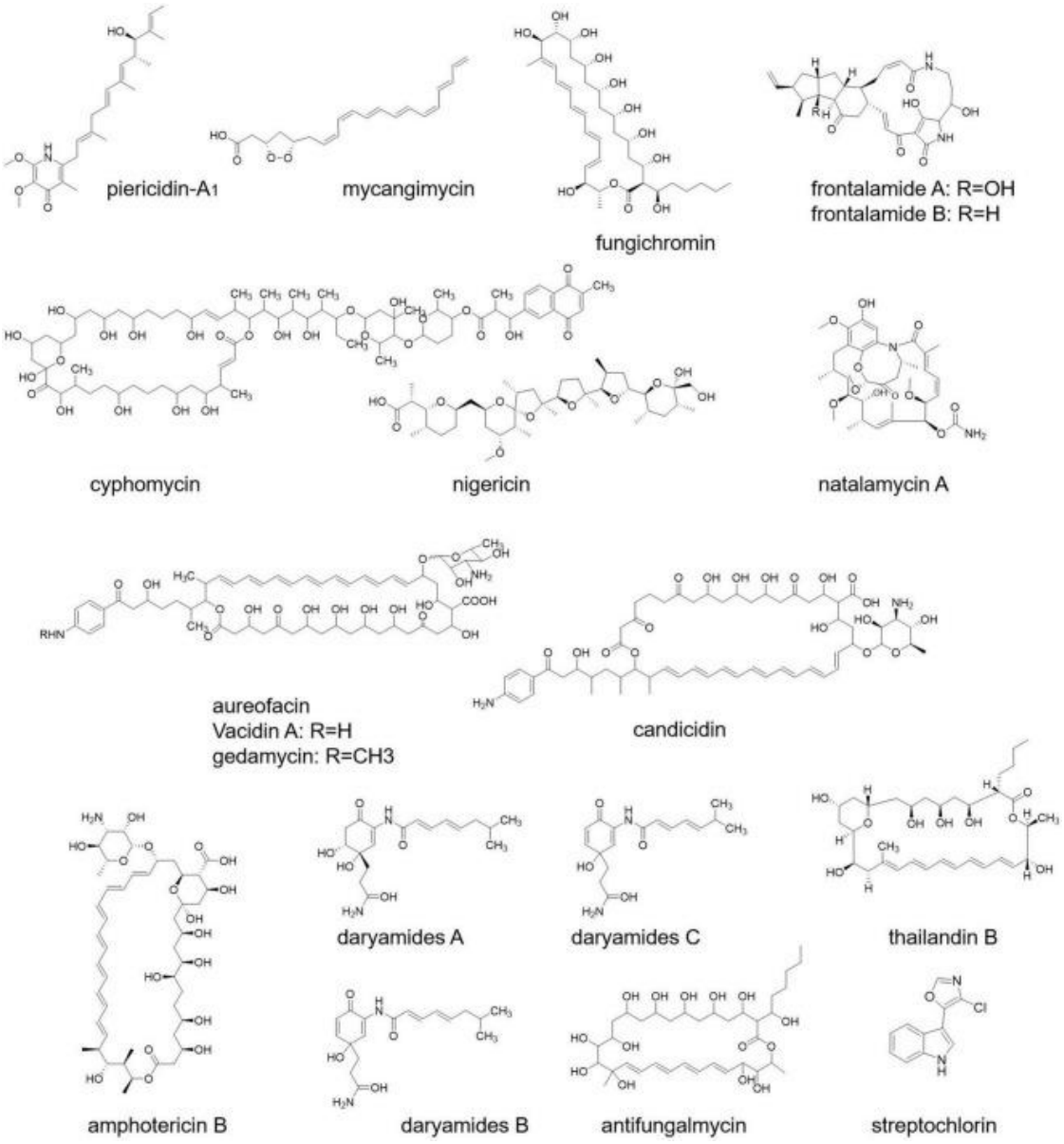


Figure 2.5 Antifungal compounds isolating from *Streptomyces* products (Alam et al.2022).

2.3. Biodegradation as a possible bioremediation method

Biodegradation is a process in which microorganisms break down and mineralize organic compounds, making it a promising method for bioremediation - the use of microorganisms to clean up environmental pollutants in soil, water, or air. These microorganisms can either naturally occur in the environment or be specifically selected for introduction into contaminated sites.

One seminal study by Battersby and Wilson in 1989 provides a comprehensive overview of biodegradation and its potential for treating environmental pollutants. They emphasize the need

for further research and development in this field (Battersby & Wilson, 1989). Recent studies have focused on optimizing biodegradation for bioremediation and developing new techniques to enhance pollutant degradation. Ghorbannezhad's recent study examines biodegradation mechanisms and influencing factors for petroleum hydrocarbons. The authors discuss the potential and limitations of using biodegradation as a method of bioremediation for petroleum-contaminated sites (Ghorbannezhad, 2022).

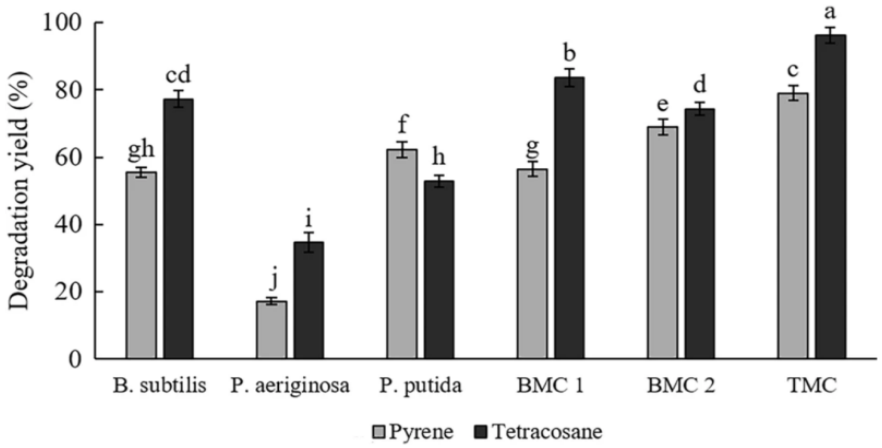


Figure 2.6 Effect of using *Pseudomonas* species mixed-culture with *Bacillus subtilis* HG 01 on pyrene and tetracosane degradation. Using *Ps. putida* ATCC 12633 had more effect on pyrene degradation than tetracosane and increased the degradation yield of pyrene from 50.5 to 70% (Ghorbannezhad et al. 2022).

Meanwhile, Z. Zhang's study investigates using biodegradation for treating per- and poly-fluoroalkyl substances (PFASs) - highly persistent and toxic pollutants. They examine the biodegradation mechanisms and factors influencing the rate of degradation, as well as the potential and limitations of this approach for PFAS-contaminated sites. While bioremediation by biodegradation can be limited by nutrient and oxygen availability, environment pH and temperature, and competing microorganisms, these studies demonstrate the continued interest and importance of biodegradation as a method of bioremediation (Zhang et al. 2022).

2.3.1. Biodegradation capacity of *Streptomyces* genus

The *Streptomyces* genus is commonly found in soil environments and is renowned for its ability to produce a diverse array of secondary metabolites through various biosynthetic pathways. Additionally, this genus has demonstrated the capacity to biodegrade a range of organic compounds, such as polycyclic aromatic hydrocarbons (PAHs), chlorinated compounds, and

pesticides, by producing enzymes like hydrolases and oxidases. Research has shown that *Streptomyces* bacteria can break down various environmental pollutants, including PAHs, chlorinated compounds, and pesticides. For example, *Streptomyces* sp. KAIST-BT-01 was able to biodegrade naphthalene, a common PAH, by utilizing it as the sole carbon source (J. H. Lee, 2017); María's research shows that *Streptomyces* has the potential to be a promising tool for lindane biodegradation (Fuentes et al. 2011). The *Streptomyces* genus is also effective in the biodegradation of several pesticides. Déziel found that *Streptomyces fradiae* could break down the pesticide atrazine in soil (Déziel, 2005), while another study showed that *Streptomyces sp.* KY10 could biodegrade the pesticide chlorpyrifos in soil (Park et al. 2019).

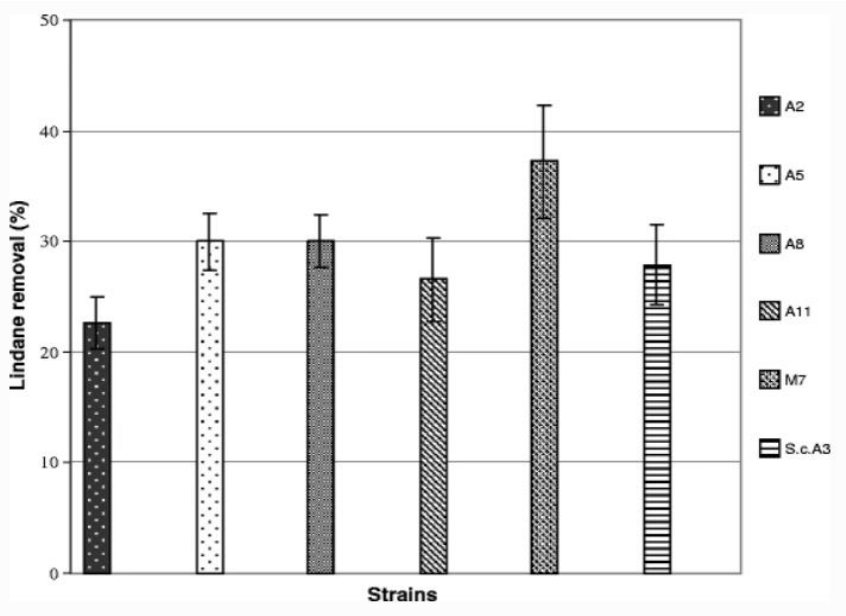


Figure 2.7 Lindane Biodegradation by Defined Consortia of Indigenous *Streptomyces* Strains, *Streptomyces* sp. M7 showed the most effective removal rate of lindane at 37%, while *Streptomyces* sp. A2 exhibited a lower removal rate of 23% (Fuentes et al. 2011).

Streptomyces bacteria achieve the biodegradation of pesticides by producing enzymes such as hydrolases and oxidases, which can break down the chemical structure of the pesticide molecules (Briceño et al. 2018). This process not only reduces the levels of pesticides in the environment but also helps mitigate their toxic effects on living organisms (Cuozzo et al. 2017). The biodegradation capacity of the *Streptomyces* genus has significant implications for bioremediation, as these bacteria have the potential to break down harmful environmental pollutants. Further research is needed to fully understand the biodegradation mechanisms and capacities of these bacteria, but their potential in bioremediation is promising (Ali, 2010).

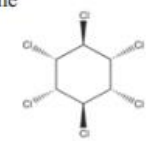
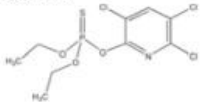
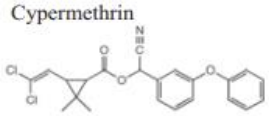
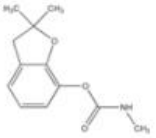
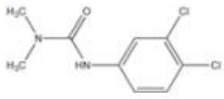
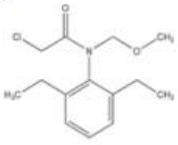
Chemical class	Molecular estructure of pesticide
Organochlorine	Lindane 
Organophosphate	Chlorpyrifos 
Pyrethroids	Cypermethrin 
Carbamate	Carbofuran 
Ureas	Diuron 
Chloroacetanilides	Alachlor 

Figure 2.8 Chemical structures of representative pesticides degraded by *Streptomyces* strains (Ghorbannezhad et al. 2022).

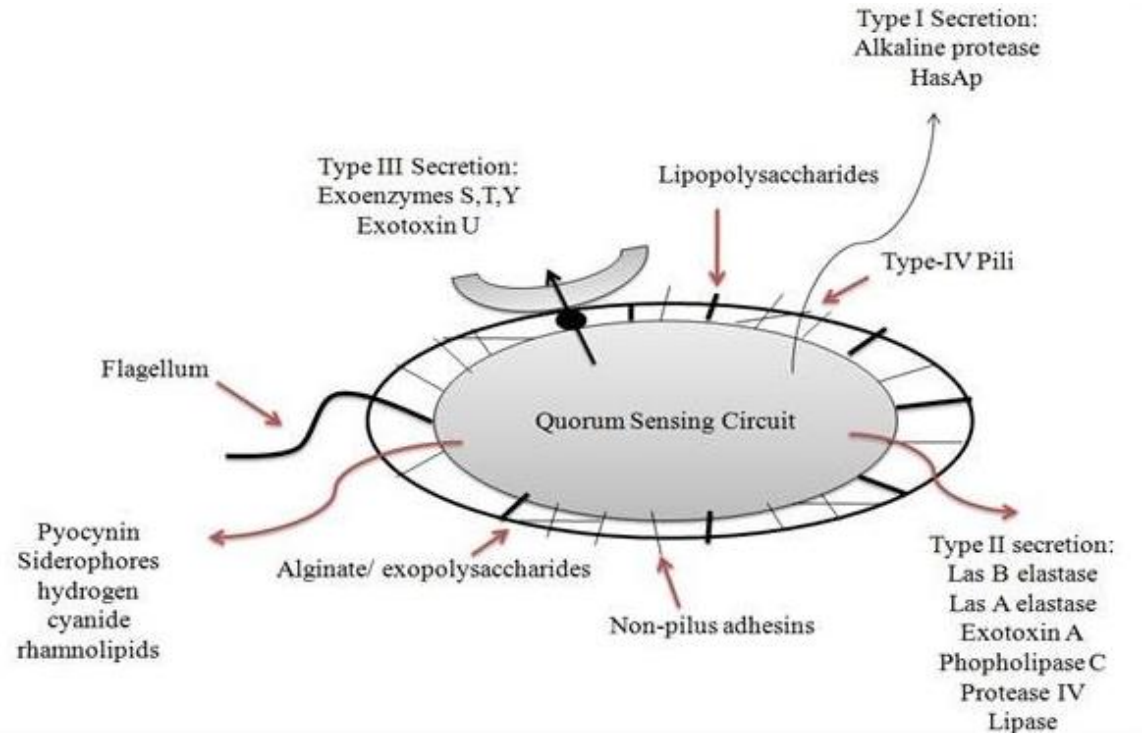
2.4. Presentation of the genus *Pseudomonas*

Pseudomonas aeruginosa is a ubiquitous, gram-negative, facultative pathogenic bacterium that is commonly found in soil, water, and other natural environments. This species has the ability to cause a wide range of infections in humans, including pneumonia, meningitis, bloodstream infections, urinary tract infections, skin infections, and wound infections. *Pseudomonas aeruginosa* is a clinically problematic bacteria that is responsible for many nosocomial and iatrogenic infections, and has been associated with a high mortality rate of approximately 35%. One of the major concerns associated with *Pseudomonas aeruginosa* is its ability to develop antibiotic resistance, including multi-drug resistance, making treatment difficult. In addition to causing disease in healthcare settings, environmental strains of *Pseudomonas aeruginosa* may

also pose a risk to public health due to their ability to develop disease outside of healing institutions. Therefore, *Pseudomonas aeruginosa* is considered an environmental health hazard that requires attention from both medical and public health professionals.

While *Pseudomonas* can cause infections in plants, animals, and humans, certain species have also been found to have beneficial effects on plant growth and protection by producing antibiotics or colonizing plant roots. *Pseudomonas* is a versatile genus, capable of thriving under a wide range of environmental conditions, including challenging ones like high temperatures and low nutrient levels (Palleroni, 2003). This adaptability makes *Pseudomonas* an important target for biotechnology applications, such as the production of biofuels, bioplastics, and other industrial products. Furthermore, some species of *Pseudomonas* have been shown to be effective in breaking down complex compounds like polycyclic aromatic hydrocarbons and other environmental pollutants, making them a valuable resource for bioremediation applications (Peix et al. 2009).

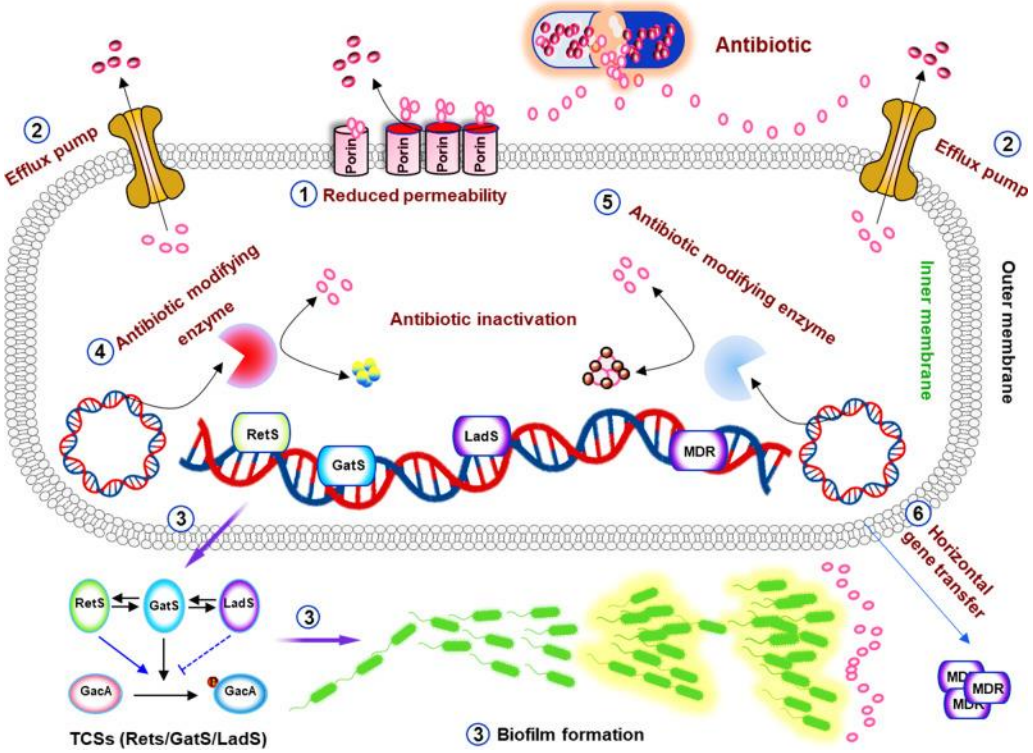
2.4.1. Environmental effects of *Ps. aeruginosa*



Picture 2.9 Morphology of *Pseudomonas aeruginosa* representing cellassociated and extracellular virulence factors (Qin et al. 2022).

Ps. aeruginosa is a bacterium that can cause a range of infections in humans, including urinary

tract infections, pneumonia, and bacteremia. It is also a common cause of hospital-acquired infections, especially in patients with weakened immune systems (Wood et al. 2023). In the environment, *Ps. aeruginosa* can have various effects on ecosystems. For instance, it can contribute to the degradation of organic matter, playing a crucial role in the cycling of nutrients in soil and water systems. Additionally, it can participate in the biodegradation of a variety of pollutants, including hydrocarbons and toxic metals, making it a potential tool for bioremediation (Korshunova T.Y. 2021).



Picture 2.10 Mechanisms of antimicrobial resistance in *Ps. Aeruginosa* (Qin et al.2022).

However, the presence of *Ps. aeruginosa* in the environment can also have negative effects, especially when it infects humans or other animals (Stellato et al. 2015). This bacterium is known to be highly antibiotic-resistant and can also be multidrug-resistant, making it challenging to treat once it has established an infection. Furthermore, the release of antibiotics and other chemicals into the environment can contribute to the spread of antibiotic-resistant strains of *Ps. aeruginosa*, further exacerbating the public health threat posed by this bacterium (Killough et al. 2022). In conclusion, the environmental effects of *Ps. aeruginosa* are complex and can be both positive and negative. While it can play a role in biodegradation and nutrient cycling, it can also pose a severe threat to human and animal health.

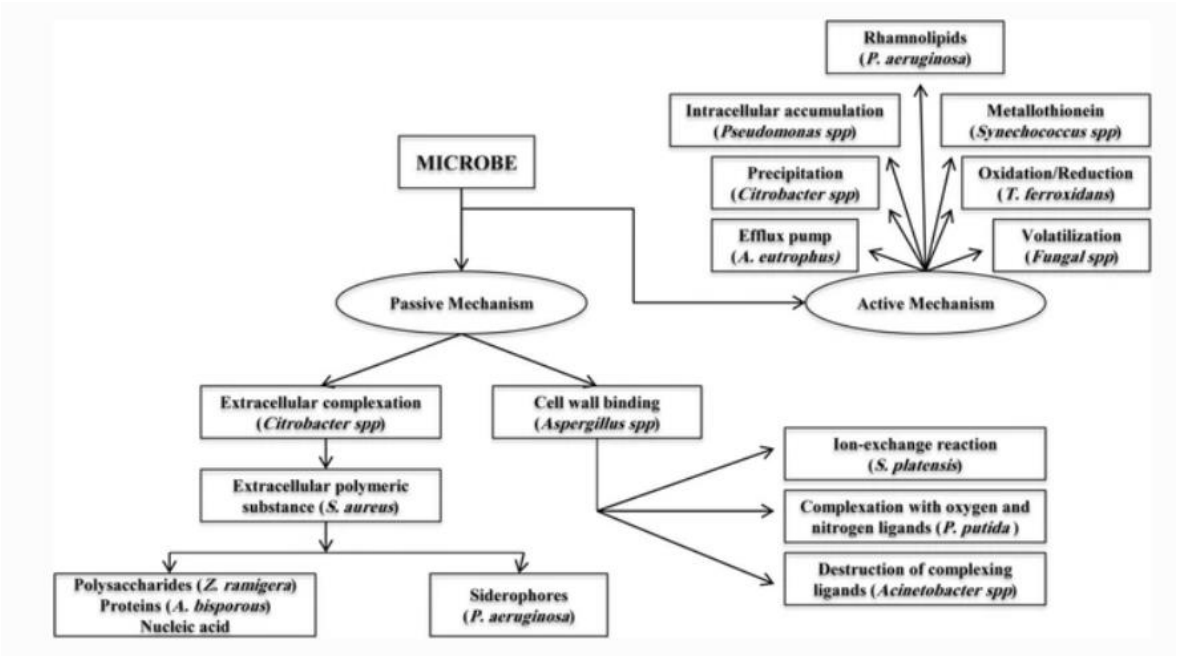


Figure 2.11 General heavy metal sorption mechanisms operating in various microbes (Saxena et al. 2001)

Therefore, it is crucial to carefully manage the presence of this bacterium in the environment, both to minimize its impact on public health and to ensure that it can be effectively utilized for bioremediation purposes like in extremely harmful metal-polluted areas (Sriramulu, 2019).

In 2001 there was reported that *Pseudomonas putida* exhibited resistance to cadmium and likely possessed detoxification capabilities (Lee et al. 2001). Another Chinese scientist Wang also demonstrated the use of *Pseudomonas aeruginosa* for cadmium removal (Wang, 1997). Several studies have shown the ability of microbes to detoxify chromium (McLean & Beveridge 2001; Megharaj et al. 2003; Viti et al. 2003). Indian scientist team, Saxena and Mangala, isolated a strain of *Pseudomonas putida* from copper mines that effectively precipitated copper, making it a promising candidate for copper bioremediation (Saxena et al. 2001). The figure 2.11 illustrates the general mechanisms of heavy metal sorption observed in various microbes.

2.4.2. Environmental risk factors for multiresistance development

The development of multiresistant strains of bacteria is a major public health concern that is largely driven by environmental factors. These factors include the widespread use of antibiotics, poor hygiene practices, and the presence of antibiotic-resistant bacteria in the environment (Gamero et al. 2021). This is because antibiotics can kill off susceptible bacteria, leaving behind

only the resistant strains. Over time, these resistant strains can become more prevalent, leading to the development of multi-resistant bacteria. And some bacteria can persist on surfaces for long periods of time, leading to cross-contamination and the spread of antibiotic-resistant bacteria. Additionally, the presence of antibiotic-resistant bacteria in the environment can also contribute to the development of multi-resistant bacteria (Larsson, 2022). There are several environmental risk factors that contribute to the development of multidrug resistance in microorganisms, including *Pseudomonas aeruginosa*. Some of these risk factors include:

1. Antibiotic pollution in the environment, such as through agricultural runoff or wastewater discharge, can lead to the selection of resistant bacteria (Bondarczuk et al. 2016).
2. Overuse of antibiotics: The overuse of antibiotics in both human and animal populations can contribute to the development of multidrug resistance (Bhardwaj et al. 2021).
3. Lack of infection control measures: Poor infection control measures, such as inadequate hand hygiene or failure to clean contaminated surfaces, can increase the spread of resistant bacteria (Serwecińska, 2020).
4. Antibiotic usage in agriculture, particularly for growth promotion and disease prevention, can contribute to the development of multidrug resistance.
5. Poor access to clean water and adequate sanitation can increase the spread of resistant bacteria, particularly in low-income countries (Mann et al. 2021).
6. Unregulated use of antibiotics: The unregulated use of antibiotics, particularly in low-income countries, can contribute to the development of multidrug resistance (Duarte et al. 2019).

In conclusion, the development of multiresistant bacteria is influenced by a range of environmental factors, including the widespread use of antibiotics, poor hygiene practices, and the presence of antibiotic-resistant bacteria in the environment. To reduce the risk of multiresistant bacteria, it is important to implement measures to reduce the use of antibiotics, improve hygiene practices, and minimize the spread of antibiotic-resistant bacteria in the environment (Larsson & Flach, 2021).

3. Methods and Materials

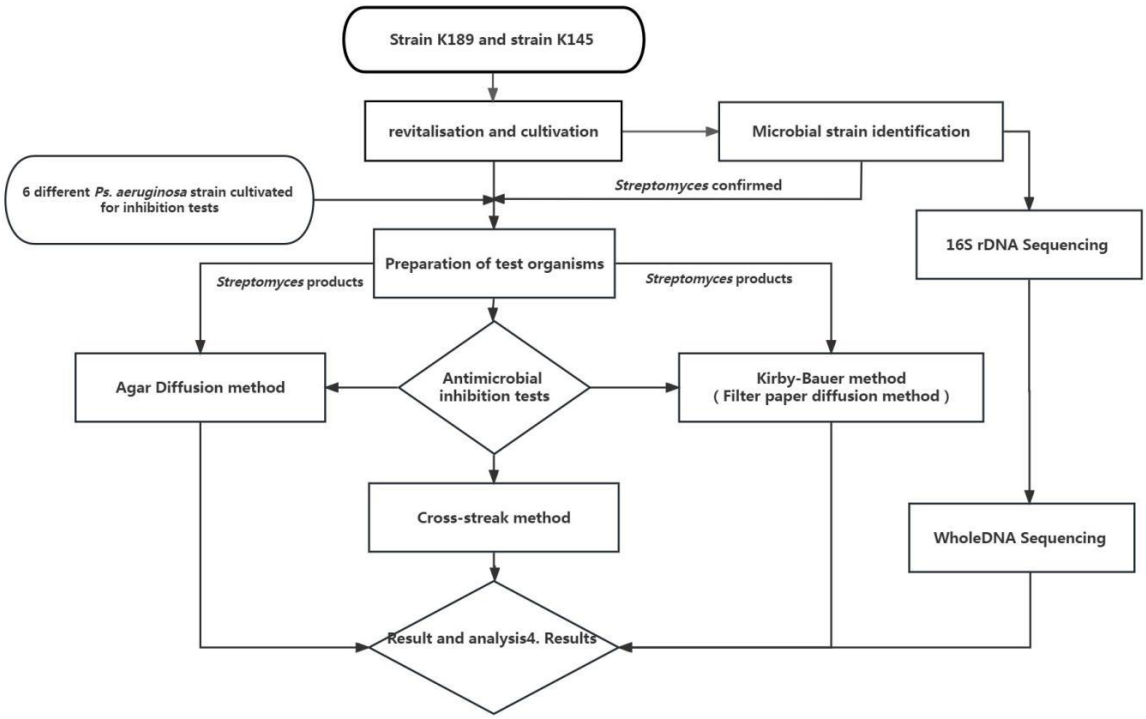


Figure 3.1 Macro flow chart of experimental steps and methods of my thesis

This study is a continuation of a previous research conducted by our department. In prior studies, Harkai (2017) a member of our institution, two of the identified strains K189 and K145 in the collection as having potential for inhibiting against facultative pathogens as the *Pseudomonas aeruginosa*. Building on this foundation, we developed a follow-up research plan and methodology, which serves as a reference point for subsequent experiments and operations （Figure 3.1） outlines the framework for our research and provides a benchmark for my study.

Some of the instruments needed for this study as an equipment:

Autoclave, incubator, freezer, centrifuge, spectrophotometer, PCR thermal cycler, electrophoresis apparatus, pipette, shaker, ultrasonic instrument, sterile box, etc.（Photo 3.2）.

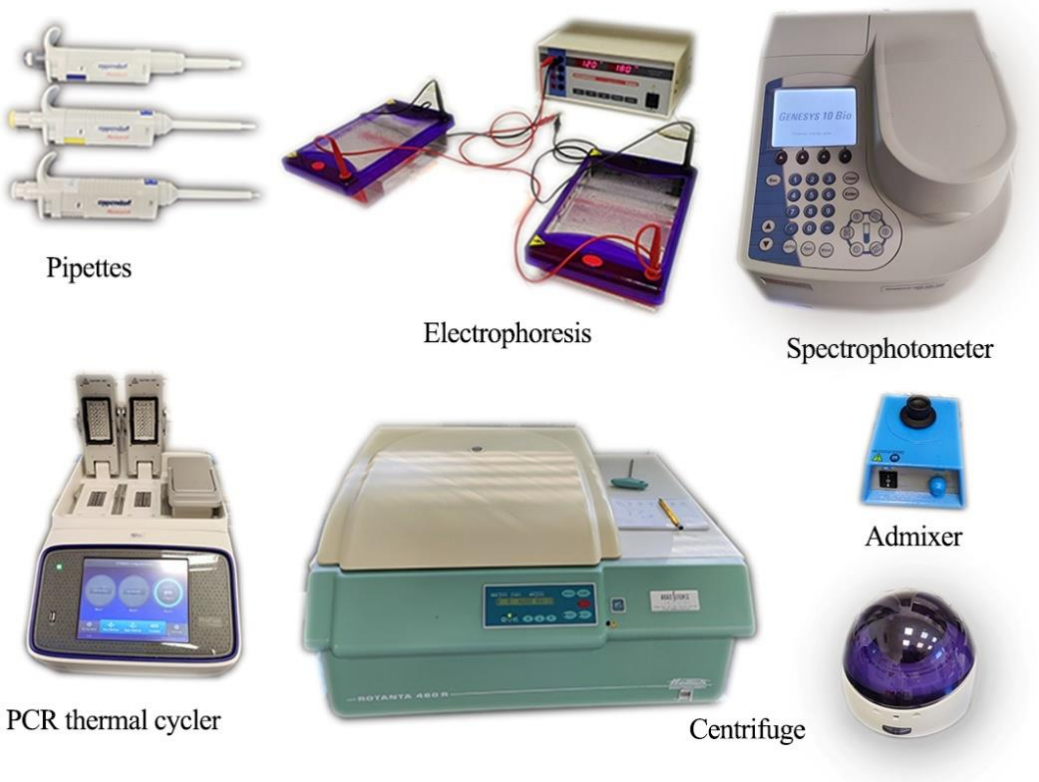


Photo 3.2 The instruments needed for strain selection and identification (Lab of Environmental Safety Department, MATE, Hungary, 2022)

3.1. Previous study Related Work

Actinomycetes are a group of gram-positive, rod-shaped bacteria that are widely distributed in soil and water environments. The Environmental Safety Department of the Hungarian University of Agriculture and Life Sciences isolated a microbial population from soil, decaying plant debris, peat bogs, also peat moss and compost samples in the 1970s, which were designated as "K" in their naming system. This name (“K”) pays homage to the German microbiologist named as Küster, who was also one of the leading researchers in the field of *Streptomyces*. All the samples were provided as individual freeze-dried copies and were mostly labeled or marked only with codes. In 2017, Harkai Peter revived, molecularly identified, and phenotypically tested 494 strains, of which 407 were from the family of *Actinomycetes*, and 123 different species of the genus *Streptomyces* were identified. Subsequently, Harkai conducted preliminary screening of the antibacterial and biodegradation abilities of some strains and found that K234 but from these all especially K145, and K189 showed potential for further research in these areas (Harkai, 2017).

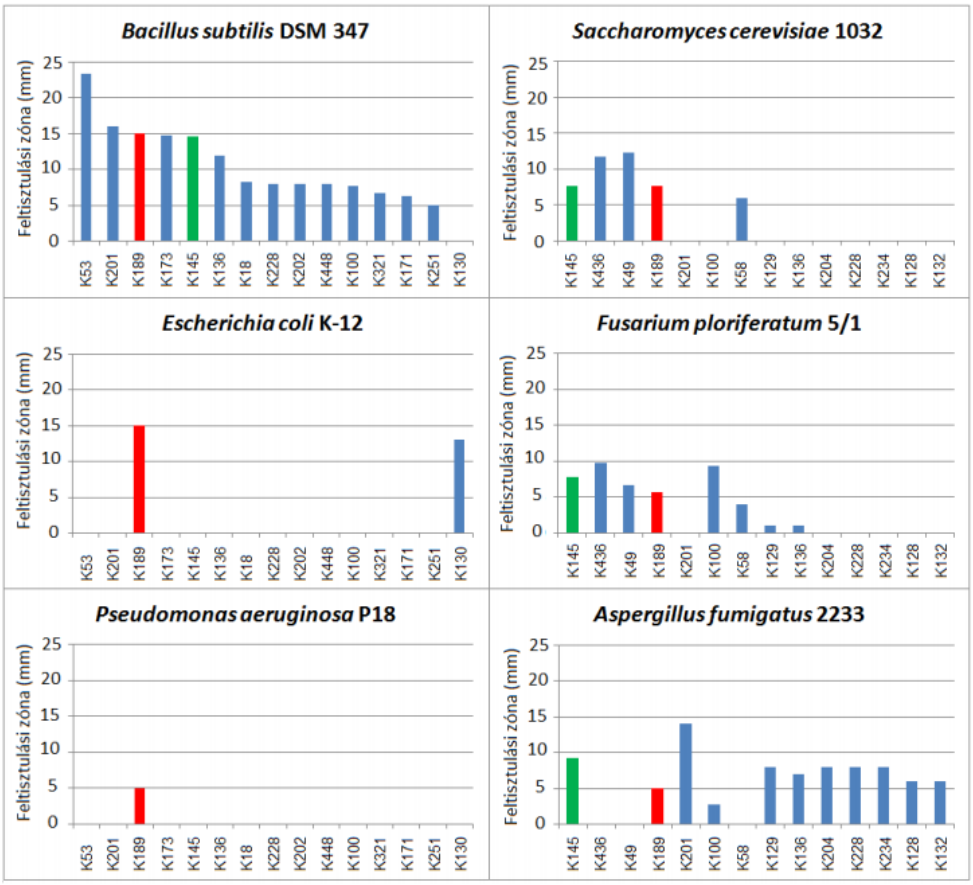


Figure 3.3*Streptomyces* Strains Inhibit Clean Zone (mm) in Agar diffusion test (Harkai, 2017)

3.2. Origin of the strain

The strains used in this study were obtained from a collection of microorganisms labeled with the letter "K" in the departmental nomenclature. The majority of the "K" labeled strains in the department belong to the *Actinomyces* genus. The collection was originally sourced from a variety of environmental habitats, such as soils, decaying plant matter, peat bogs, and compost samples, primarily during the 1970s. These strains were selectively isolated by *Streptomyces* and deposited by previous legal predecessors due to their unique properties, which may be of interest for biotechnological research.

The relevant previous experiments were conducted by Peter Harkai in 2017, and achieved certain results. Strains K189 and K145 were identified as *Streptomyces rimosus*. The experiments showed they have antibacterial potential (Harkai,2017). My specific strains are *Streptomyces* spp. numbered K189 and K145. I will revitalize the strains labelled K189 and K145 obtained from storage in -80°C freezers and freeze-dried vials in the laboratory.

3.3. Revitalisation

The experiment's initial strain revitalization process involved reactivating strains from a collection that had been stored in lyophilized ampoules at room temperature in boxes, protected from light and heat. The procedure included scratching the surface of the ampoules with a diamond-tipped cutting tool, followed by heating the ampoules with an open flame and cooling them. Next, 200-200 µl of 5% peptone contained water was measured into the ampoules and spread onto solid agar media. From there, 50 µl of the ampoule contents were pipetted and spread onto sterilized GYM agar poured into Petri dishes, as recommended by the German National Collection of Microorganisms and Cell Cultures (DSMZ). And I also cultured strains with the same numbers which were stored at -80°C in the same media to ensure that a pure and single target strain could be obtained.

GYM agar contains glucose, malt extract, and $CaCO_3$ as a pH buffer, which are ideal for *Streptomyces* strains, promoting till spore formation appears. Finally, the Petri dishes were inverted and incubated for 120 hours upside down at 28°C in a Memmert-type heated thermostat and designed to prevent condensation.

Table 3.4 Composition of GYM medium

GYM medium	Solid	Liquid
Glucose	4.0 g	4.0 g
Yeast extract	4.0 g	4.0 g
$CaCO_3$	10.0 g	10.0 g
Agar	2.0 g	-
Malt extract	15.0 g	-
Distilled water	1000.0 ml	1000.0 ml
PH	7.2	7.2

3.4. Microbial strain identification

Microbial strain identification is a crucial step in any microbiological study, as it allows researchers to accurately identify the microorganisms under investigation. Traditional methods of strain identification, such as phenotypic and biochemical characterization, have limitations, as they are often time-consuming, subjective, and prone to errors. In recent years, molecular

methods based on the analysis of specific DNA sequences, such as the 16S rRNA gene, have become increasingly popular for strain identification, as they offer several advantages over traditional methods, including greater accuracy, speed, and reproducibility (Rainey & Stackebrandt, E. 2000). In the 2017 article, it was proposed that the K189 and K145 strains were identified as *St. rimosus* species. However, in many cases, they may be reclassified to exclude the possibility. Therefore, we would like to verify the two strains by species to determine if they are still representatives of the *St. rimosus* species. In this study, we used 16S rDNA sequence analysis to identify the microbial strains under investigation.

3.4.1. Phenotype examination

To begin with, it is essential to conduct a thorough observation of the morphology and characteristics of the strains. This is done to compare the current findings with previous experimental records and establish whether they meet the initial diagnostic criteria for *Streptomyces rimosus.*

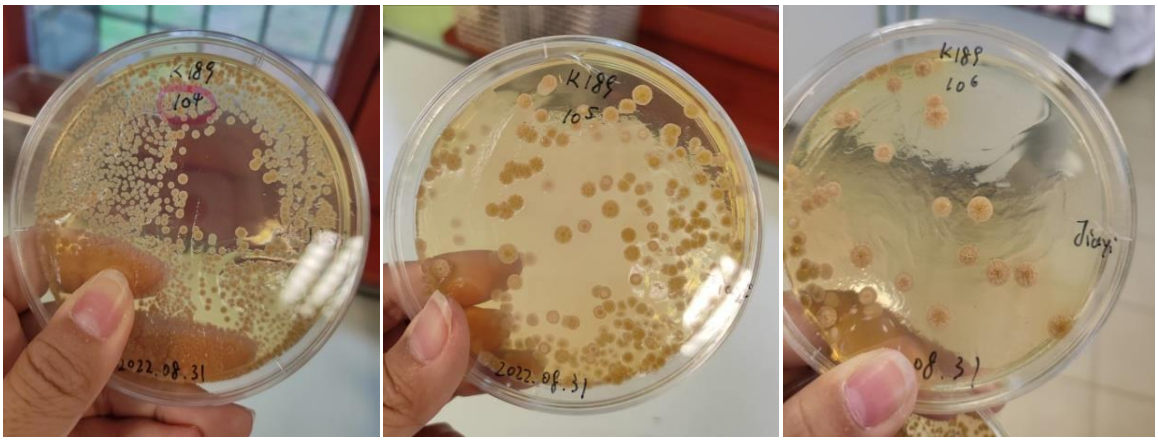


Photo 3.5 Revitalisation effect of K189 strains of different dilutions from -80°Cafter 7 days incubation. Left: diluted to10^4; Mid: 10^5; Right: 10^6 Colony Forming Unit/mL. (Lab of Environmental Safety Department, MATE, Hungary, 2022)

As part of the phenotyping process, I conducted a detailed examination of the colonies' colour, surface morphology, and arrangement, as well as the presence of pigment production, its colour, and the formation of spore production. The appearance of spore production was confirmed by a distinct greyish-white colour change on the upper surface of the colony, known as 'powdery spore mats,' after 96-120 hours by using a constant temperature set at 28°C. Following this

period, I performed a macroscopic description of the strains and documented their characteristics through photographic means.

3.4.2. 16S rDNA-based strain identification

Advancements in molecular biology have made it clear that identification procedures based solely on phenotyping, such as morphology, culture parameters, and biochemical tests, may produce erroneous or imprecise results. As a result, molecular taxonomic studies have become crucial in species identification. Therefore, I have used molecular taxonomic methods, specifically 16S rDNA sequence analysis, to examine the revitalized members of this strain collection. The steps of this analysis are outlined below.

3.4.2.1. DNA isolation

At first, I cultivated the revitalized strains by incubating them for 120 hours at 28°C in a Memmert-type heated thermostat using liquid GYM medium. I obtained the latest viable strains by culturing them in two sterile flasks with a constant temperature shaker set at 28°C and 160 rpm for 96 hours. Due to the thick cell walls of the strains that are difficult to break down, I used the Zymo Quick-DNA Miniprep Plus Kit for this operation. I chose this kit because enzymatic digestion is more effective than other methods for facilitating DNA extraction. The kit contains lysozyme, which degrades the cell wall and aids in the release of DNA during the cell lysis process.

The DNA isolation was performed according to the manufacturer's protocol. I added the samples to ZR Bashing Bead Lysis Tubes along with 750 µl of ZymoBiomics Lysis Solution and tightly capped them. I then subjected them to bead beating at maximum speed for 20 minutes. After that, I centrifuged the tubes at ≥10,000x g for 1 minute. The supernatant was transferred to Zymo-Spin III-F Filter Collection Tubes and centrifuged again at ≥10,000x g for 1 minute. I discarded the filter and added 1200 µl of ZymoBIOMICS DNA Binding Buffer to the filtrate, mixing it well. The mixture was transferred in two batches to Zymo-Spin IICR Columns and centrifuged at ≥10,000x g for 2 minutes, discarding the flow-through each time.

Next, I added 400 µl of ZymoBIOMICS DNA Wash Buffer 1 to the Zymo-Spin IICR Column in a new Collection Tube and centrifuged it at ≥10,000x g for 1 minute. I discarded the flow-through and repeated the process with 700 µl of ZymoBIOMICS DNA Wash Buffer 2,

centrifuging at ≥10,000x g for 1 minute, and discarding the flow-through again. Finally, I transferred the Zymo-Spin IICR Column to clean 1.5ml microcentrifuge tubes and added 100 µl of ZymoBIOMICS DNase/RNase-free water directly to the column matrix. I incubated it for 1 minute and then centrifuged it at ≥10,000x g for 1 minute to elute the DNA. The DNA should be stored at -20°C and kept for future use. I used the NanoDrop DNA test instrument to measure the DNA concentration and ensure its validity.

3.4.2.2. DNA detection by agarose gel electrophoresis

To detect the genomic DNA isolated with the kit described above, I performed agarose gel electrophoresis.

Materials used:

Agarose powder, TBE buffer, DNA samples, loading buffer (with tracking dye), distilled water, ECO Safe Nucleic Acid Staining Solution.

Specifically, I prepared a 1% agarose gel supplemented with 3.5 µl of ECO Safe Nucleic Acid Staining Solution to allow for visualization of the DNA bands. Next, I poured the mixture into a gel tray with precast wells formed by comb lines. As a control, I used 3µl Gene Rule DNA ladder for size reference. The DNA samples "K189" and "K145" were taken out from -20°C, and 3 µl of each sample was mixed with 1 µl of loading buffer containing the tracking dye for visualization during electrophoresis. The sample was loaded into the wells of the agarose gel using a micropipette, with the agarose gel acting as a filter for the nucleic acid molecules during electrophoresis.

Table 3.6 Materials and dosages used in agarose gel electrophoresis

	Dosage
Agarose powder	0.7 g
10×TBE	7.0 ml
DNA samples	3.0 µl
Loading buffer	1.0 µl
MQ water	63.0 ml
ECO Safe Staining Solution	3.5 µl

I connected the gel electrophoresis apparatus to a power supply and ran it at 110V for 20 minutes. During electrophoresis, the negatively charged DNA molecules migrated towards the positively charged electrode, with smaller fragments migrating faster than larger ones.

When electrophoresis is complete, carefully remove the gel from the electrophoresis apparatus and place it on a UV transilluminator due to observing the DNA bands under UV light. Capture images of the gel with gel imaging software. Analyse the DNA bands with gel imaging software. Determine the size and intensity of each band.

3.4.2.3. Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a laboratory technique used to amplify a specific segment of DNA in vitro. It involves several steps, including denaturation, annealing, and extension, which are repeated in cycles to amplify the target DNA exponentially. This process is facilitated by a heat-stable DNA polymerase enzyme, such as Taq polymerase, which extends the primers by adding new nucleotides. PCR has revolutionized molecular biology and is widely used for a variety of applications, including DNA sequencing, genetic testing, forensics, and disease diagnosis. The PCR's master mix was prepared by mixing Taq buffer, primers, dNTP, DNA sample, Taq polymerase and MQwater (Table 3.7) in a sterile microcentrifuge tube at room temperature. The mastermix was vortexed briefly and then aliquoted into PCR tubes containing the DNA template.

Table 3.7 Preparation of 16S rDNA PCR Solution and Melting Curve Analysis

Mastermix:		Hear profile:			
Taq buffer	5.0 µl	95°C	3 min		
27f forward primer	0.5 µl	94°C	30 sec	}	32
1492r reverse primer	0.5 µl	52°C	30 sec		x
dNTP	10.0 µl	72°C	1 min		
DNA sample	1.0 µl	72°C	10 min		
Taq polymerase	0.25 µl	4°C	∞		
MQ water	32.75 µl				
Final volume	50.0 µl				

Polymerase Chain Reaction (PCR):

The PCR amplification was performed in a thermal cycler with the following cycling conditions (Table 3.7). The PCR product was then analyzed by gel electrophoresis, and the resulting data was analyzed by using gel imaging software.

3.4.2.4. Detection of PCR products

I used agarose gel electrophoresis to detect the amplified PCR products.

I prepared the agarose gel by following the same protocol in **3.3.2.2**. I used a pipette to load 3µl Gene Rule DNA ladder and each of my samples of the PCR product 5 µl into the wells of the agarose gel. I ran the gel electrophoresis at a voltage of 110V for 30 minutes. After the run was complete, I checked the gel under UV light and compared the results to the Gene Rule standard to determine the quality of the samples.

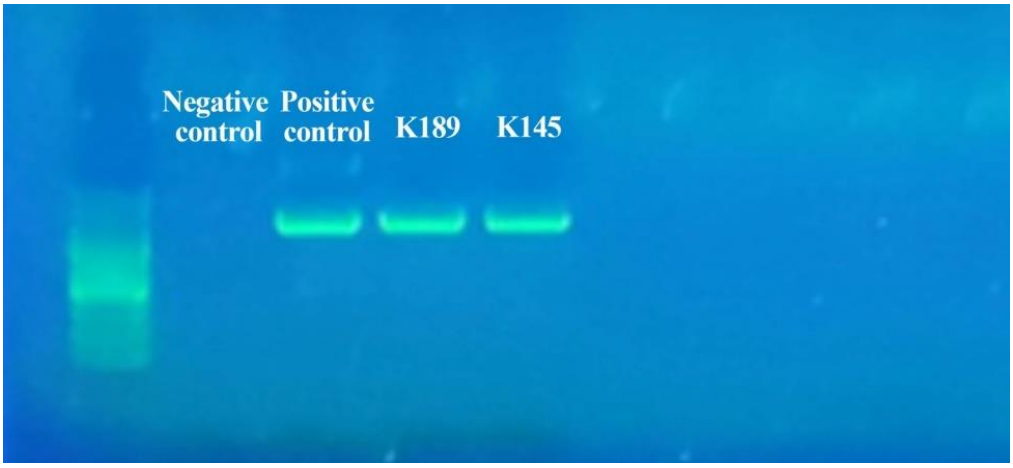


Photo 3.8 DNA Fluorescent Reaction Under UV Light After Electrophoresis (Lab of Environmental Safety Department, MATE, Hungary, 2022)

3.4.2.5. Purify PCR products

After performing a PCR reaction, it is often necessary to purify the PCR products to remove any unwanted impurities such as unincorporated primers, nucleotides, and enzymes. One common method for purifying PCR products is using a PCR purification kit. This time I used the NucleoSpin Gel and PCR Clean-up to do the PCR clean-up.

I followed its protocol to do these steps. To adjust the DNA binding conditions, I added 90 µl of Buffer NTI to each of my two samples (approximately 45 µl per sample) and mixed them well. I then placed a NucleoSpin Gel and PCR Clean-up Column into a Collection Tube and

loaded up to 700 µl of sample. Following the manufacturer's instructions, I centrifuged the column for 30 seconds at 11,000 x g and discarded the flow-through. I repeated this step for the remaining solution.

After that, I added 700 µl of Buffer NT3 to the NucleoSpin Gel and PCR Clean-up Column, and centrifuged it for 30 seconds at 11,000 x g. Again, I discarded the flow-through and placed the column back into the collection tube. I repeated this step for the remaining solution. To ensure complete removal of Buffer NT3, I centrifuged the column for an additional 1 minute at 11,000 x g.

Finally, I placed the column into a new micro-centrifuge tube and added 30 µl of Buffer NE. I incubated the sample for 1 minute and then centrifuged it for 1 minute at 11,000 x g to obtain the purified PCR products.

3.4.2.6. Sequencing Reaction and analysis

The PCR products were sequenced using the Big Dye Terminator v3.1 Cycle Sequencing Kit (ThermoFisher Scientific). The sequencing reaction was set up using 0.8 µl of purified PCR product, 0.25µl of 27f primer, 2.7 µl of MQ water, 0.5 µl of Big Dye Terminator mix, and 0.75 µl sequencing buffer in a final volume of 5 µl. The reaction was subjected to thermal cycling using the following conditions: 96°C for 1 min, followed by 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. (Table 3.9).

Table 3.9 Preparation of DNA sequencing and Melting Curve Analysis

Sequencing PCR		Hear profile:			
Big dye	0.5µl	96℃	1 min		
Big dye buffer	0.75 µl	96℃	10 sec	}	28x
27f primer	0.25 µl	50℃	5 sec		
DNA sample	0.8µl	60℃	4 min		
MQ water	2.7µl	4℃	∞		
Final volume	5.0 µl				

The sequencing products were then purified using the ethanol precipitation method. Briefly, the sequencing reaction mixture was mixed with 3 M sodium acetate (NaAC), pH 5.2, and 96% ethanol, and incubated at room temperature for 10 min.

Table 3.10 Materials and Unit amounts Required for DNA Ethanol Precipitation

3M NaAC	3.0µl
MQ water	14.5µl
EtOH (96%)	62.5µl
EtOH (70%)	180µl
HiDi solution	20µl

The mixture was centrifuged at 3220g for 20 min at 4°C, and the supernatant was carefully removed. The pellet was washed with 70% ethanol, centrifuged again at 3220g for 20 min at 4°C, and the supernatant was removed. I then inverted the tubes and fixed them on trays and centrifuged them at 4°C for 1 minute at 630g to remove the remaining solution. Finally, 20 µl of HiDi solution was added and left at 4°C for 24 hours to await the laboratory specialist for analytical operations. The amount of reagent is shown in Figure 3.10.

The molecular biological identification of the *Streptomyces* strains was conducted using DNA sequencing analysis of the 16S rRNA gene. The bacterial genomic DNA was extracted using a Zymo quick DNA miniprep plus kit according to the manufacturer's instructions. The 16S rRNA gene was amplified using PCR with universal primers, and the PCR products were purified and sequenced. The obtained sequences were analyzed using BLAST and compared to those available in the NCBI GenBank database. Fix them with the MEGA x software.

3.5. Antimicrobial inhibition studies

3.5.1. Microorganisms utilized for inhibition testing

Table 3.11 Origin of *Pseudomonas aeruginosa* in the inhibition tests

Ps. Aeruginosa	**Origin**
P18	Environment isolation from groundwater
P43	Environment, isolation from a Hydrocarbon-contaminated site
P66	Environment isolation from groundwater
ATCC27853	Reference strain from strain collection
B02600	Lab collection strain
B02040	Lab collection strain

In my study, six different strains of *Pseudomonas aeruginosa* were used as the indicator microorganisms for inhibition testing. They are all *Ps. Aeruginosa* P18, P43, P66, ATCC27853, B02600, B02040 were chosen for its widespread presence in various environments, including soil and water, and its known susceptibility to antibiotics and other antimicrobial agents (Table 3.11).

Based on a list published by the World Health Organization in February 2017, *Pseudomonas aeruginosa* was already listed among the most critical antibiotic-resistant bacterial species (WHO,2017). Environmental strains of *P. aeruginosa* were subjected to antibiotic resistance testing at the department. Strain P43 was isolated from a groundwater sample contaminated with hydrocarbons at a site where a refinery product pipeline was damaged. This strain demonstrated exceptional resistance to various third-generation cephalosporins, broad-spectrum penicillins such as piperacillin, and carbapenems (imipenem) antibiotics. Furthermore, P43 was found to be completely resistant to the applied amino glycosides, specifically gentamicin. The reference strain for *P. aeruginosa* is ATCC 27853. Strains P18 and P66, which I used as test organisms, showed resistance to 14 of 31 tested antibiotics in disk diffusion antibiotic resistance testing and they also had some reaction against Oxytetracyclin on the scale. (Kaszab, 2010).

The main microorganisms of interest were *Streptomyces* strains, which were obtained from laboratory collections, originally from soil samples in nature. To prepare the cultures for inhibition testing, a single colony from each *Streptomyces* strain was inoculated onto TGE-5 medium plates and incubated at 28°C for 24 hours. After culturing for up to 24hours, I used a sterile glass stick to scrape bits into 5 ml of physiological saline to dilute and test for cell density. Using a Genesys Spectro Photometer (Thermo Scientific, GENESYS 10S UV-VIS), the cell density was adjusted to 0.7 per ml for subsequent testing.

3.5.2. Preparation of test organisms

To prepare the *Streptomyces* products for antimicrobial inhibition studies, a single colony from each strain was inoculated into 100 mL of sterile GYM liquid medium which often preferred for the growth of *Streptomyces* because it provides a nutrient-rich environment that supports the growth and sporulation of *Streptomyce*s and incubated at 28°C for 120 hours days with

shaking at 200 rpm. The antimicrobial product has been given just only occur after 120 hours. As we cannot determine whether the antimicrobial substance exists inside the cells, we divided the samples into two groups. One group was sonicated using an ultrasonic device to break the cell walls, allowing any possible enzymes or metabolites present inside the cells to enter the liquid, which was then centrifuged to separate the solid material (Chater et al. 2010). The other group was directly centrifuged to remove the solid material in 4°C and 3220g for 20 minutes. The resulting cultures were centrifuged at 3220g for 20 minutes to pellet the cells, and the supernatant was filter-sterilized using a 0.45 µm sterile syringe filter to remove any residual cells or debris. And I also collected the same sample from the *Streptomyces* which incubated at 28°C for 168 hours with shaking at 200 rpm. We obtained a metabolite sample from 168 hour to observe the differences between the 120 hour and 168 hour samples.

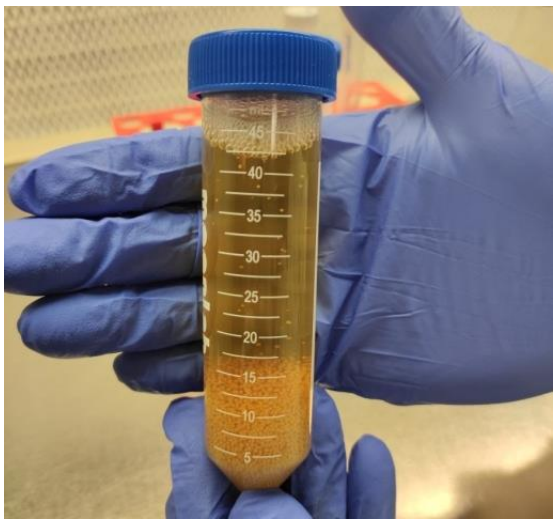


Photo 3.12 *Streptomyces rimosus* K145 cultured in GYM liquid medium for 120 hours for further investigation (Lab of Environmental Safety Department, MATE, Hungary, 2022)

After retaining about 20ml of an original sample, the other 20ml was concentrated. We took an equal number of samples in each case. The filtered supernatant was then concentrated using a rotary evaporator (Biotage) at 40°C to remove the solvent and reduce the volume. The resulting sample is about only 2.5 ml and stored all of them at 4°C until further use.

Initially, we attempted to test the original, unconcentrated samples. But the experimental results cannot find any significant antibacterial activity. So, we attempted to redesign this experimental step by conducting antibacterial experiments again using both the original samples and concentrated samples.

Table 3.13 Preparation conditions for sample collection（+: processed；-: not processed）

Sample name	Concentrated	Sonicated	120 h	168 h
⑤ **K189**	−	−	+	−
⑤ **K145**	−	−	+	−
⑤ **K189-CC**	+	−	+	−
⑤ **K145-CC**	+	−	+	−
⑤ **K189-SCC**	+	+	+	−
⑤ **K145-SCC**	+	+	+	−
⑦ **K189**	−	−	−	+
⑦ **K145**	−	−	−	+
⑦ **K189-CC**	+	−	−	+
⑦ **K145-CC**	+	−	−	+
⑦ **K189-SCC**	+	+	−	+
⑦ **K145-SCC**	+	+	−	+

This will give us total 12 samples, 4 original samples（from 120 hours till 168 hours）, 4 samples that have been concentrated to 10% compared to the original sample, 4 samples that have been sonicated and concentrated to 3ml.

3.5.3. Antimicrobial inhibition methods

The aim of the experiment was to determine the zone of inhibition, which is the area of the agar plate where the *Pseudomonas* strains did not grow due to the inhibitory effect of the product samples. The size of the zone of inhibition reflects the efficacy of the product sample against the test microorganisms. A larger zone of inhibition indicates a stronger inhibitory effect, while a smaller or no zone of inhibition indicates a weaker or no inhibitory effect. By comparing the zone of inhibition of each sample against the control, the experiment can evaluate the efficacy of the product samples against the *Pseudomonas* strains.

3.5.3.1. Cross-streak method

The cross-streak method is a microbiological technique used to investigate the interaction between two different microorganisms. It's history can be traced back to the early 20th century when microbiologists first began using agar as a medium for growing bacteria. This method is commonly used in antimicrobial susceptibility testing to evaluate the effectiveness of a compound against a specific pathogen (Kamat & Velho-Pereira, 2012).

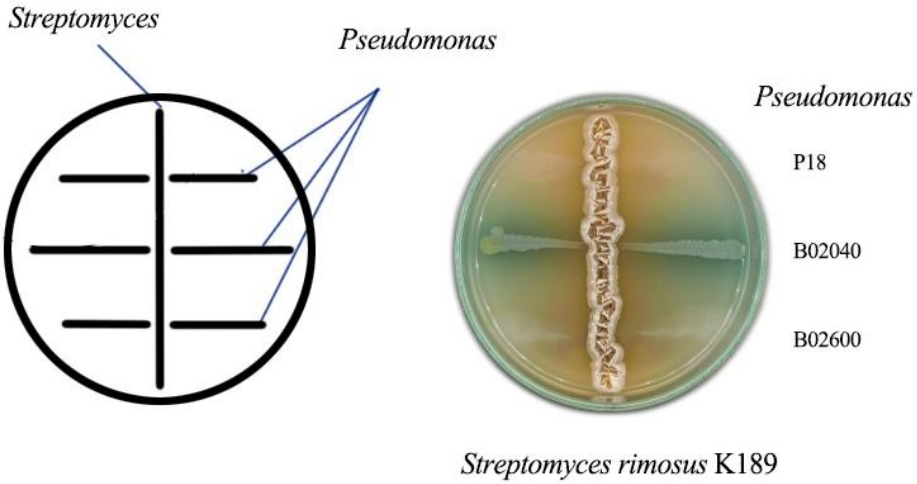
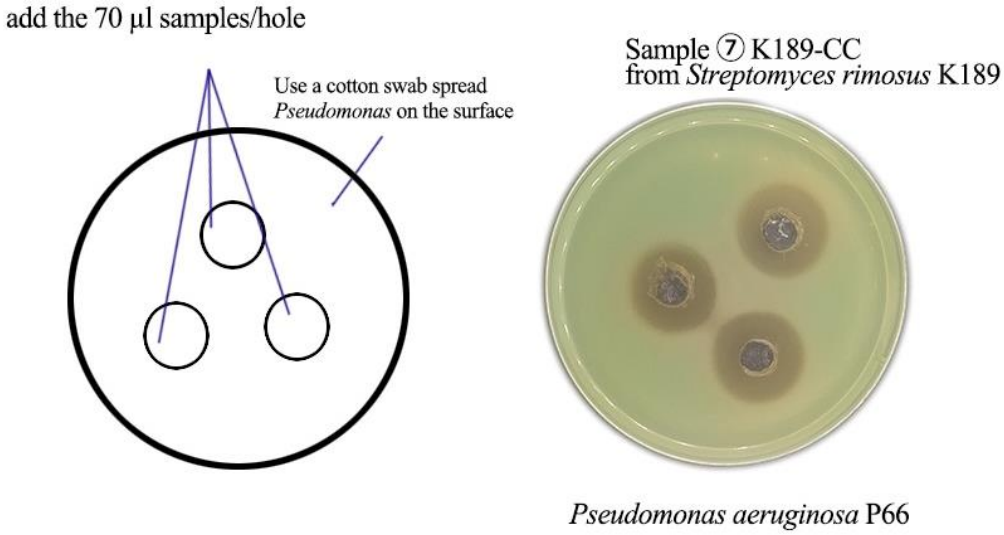


Figure 3.14 Examination of antimicrobial effect using the cross-steak method, photo on the right shows the strain *Streptomyces* K189, which produces an antifungal substance against fungal species and forms small inhibition zones around *Pseudomonas aeruginosa* P18 and B02600. The diagram on the left is a schematic representation of this process, where the central stripe is *Streptomyces*, and the lateral stripes are the test micro-organisms is *Pseudomonas*.

In my experiments, I inoculated two samples of *Streptomyces* onto six separate 22 ml GYM medium Petri dishes using a sterile glass rod. The spores were applied to the middle of the Petri dishes in a straight line from the very end to another edge, 1 cm from the edge in all directions. After incubating the plates in a 28°C thermostat for 24 hours, each of the six species of *Pseudomonas* was inoculated onto three Petri dishes using a glass rod dipped into a laterally scribed form (Figure 3.14). The gap lengths were measured for preliminary observations after the completion of the experiment.

3.5.3.2. Agar diffusion method

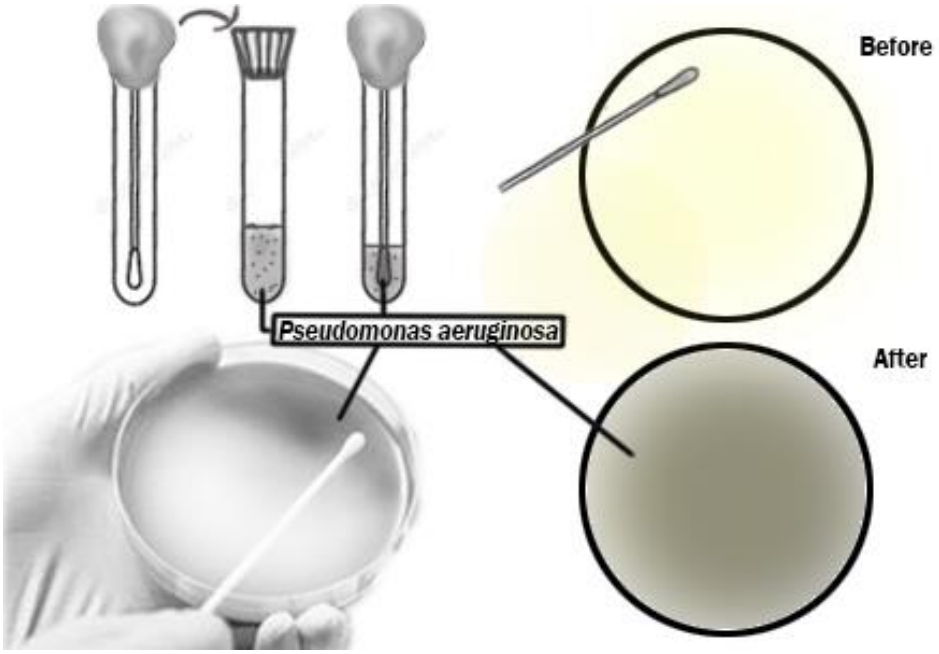
The agar diffusion method is a simple and reproducible technique that involves measuring the zone of inhibition around an antimicrobial agent that diffuses into an agar medium. This method has been used extensively for screening the antimicrobial activity of various natural and synthetic compounds (Bonev et al. 2008). In this study, the method was to utilized assess of the inhibitory activity of 16 product samples obtained from two *Streptomyces* strains against two different *Pseudomonas aeruginosa* strains.



Figue 3.15 Schematic diagram of the agar diffusion test. The right photo shows a sample ⑦K189-CC (Table 3.12) produced from *Streptomyces* K189, which formed a clear inhibition zone on the culture medium of *Pseudomonas aeruginosa* P66.

The *Pseudomonas* strains were cultured on 22 ml MH medium using a cotton swab spread method (Figure 3.16). This technique allowed for uniform growth of the microorganisms on the medium. We selected a total of 6 *Pseudomonas aeruginosa* strains and 12 samples of metabolites for our experiment. Each *Pseudomonas* strain was inoculated in 12 Petri dishes, with a total of 60 dishes for the 6 strains. Subsequently, three holes were made in each medium using a tool, and 70 µl/hole of same sample was added to each hole to ensure that they absorb the same amount of product for observation and analysis. The chosen volume of sample ensured that sufficient amount of the compound diffused through the agar medium and into the surrounding area to exhibit the inhibitory effect. Then I put them in the fridge at 4°C not to incubate, till it gets absorb into the medium. After about 4 hours (it needs to have 4 hours to disappeared from the holes), they were transfered to 28°C for 24 hours.

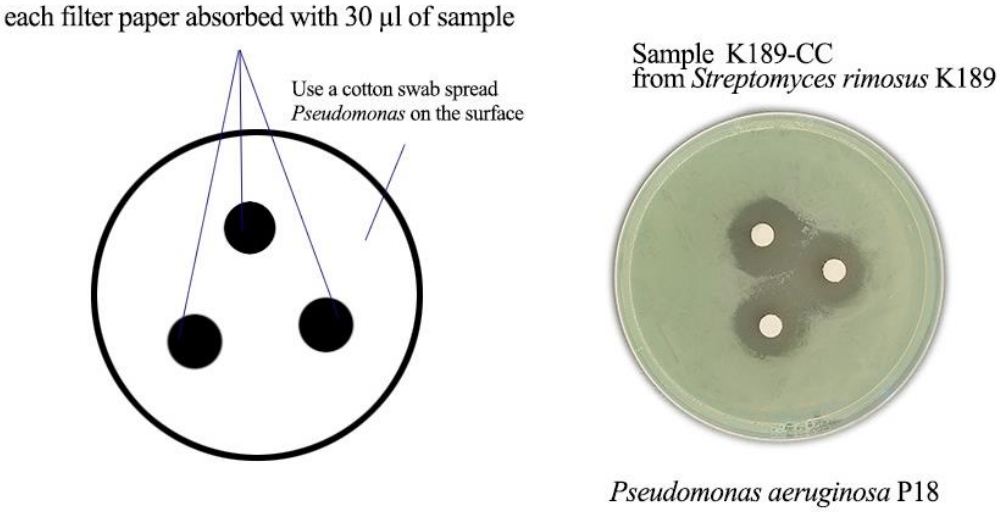
The primary aim of the experiment was to measure in mm-s the zone of inhibition and assess the effectiveness of the product samples against the *Pseudomonas* strains. The zone of inhibition is an indicator of the antimicrobial activity of the tested compounds. It is the area of cleared agar surrounding the well that contains the sample. A larger zone of inhibition indicates a higher level of inhibitory activity, indicating the effectiveness of the product sample against the *Pseudomonas* strains.



Picture 3.16 Schematic diagram of using a cotton swab spread for *Pseudomonas aeruginosa*

3.5.3.3. Kirby-Bauer Method（Filter paper diffusion method）

The Kirby-Bauer method is widely used to evaluate the antimicrobial activity of various compounds. This method is based on the diffusion of an antimicrobial compound from a filter paper disk into and on an agar medium containing microorganisms (Hudzicki, 2009). The zone of inhibition around the disk indicates the degree of inhibition of microbial growth, and its size is influenced by several factors such as the size of the disk, the diffusion coefficient of the antimicrobial agent, and the susceptibility of the microorganism (Biemer,1973).



Figue 3.16 Schematic diagram of the filter paper diffusion test. Right：a sample K189-CC

produced from *Streptomyces rimosus* K189 formed a clear inhibition zone on the culture medium of *Pseudomonas aeruginosa* P18. To avoid the technical implementation, clean zone less than 2 mm won't be account.

We aim to observe and compare experimental findings with each other. We used Petri dishes of the same size and equal amounts of medium, inoculum concentrations of the inhibited strain, and multiple identical control conditions. The samples used as variables in our experiments included different concentrations of samples, number of sample days, and whether the samples were sonicated (Table 3.13). Multiple sets of data were obtained in this way, and the results were analyzed by calculating the standard deviation and mean values.

In my experiment, the Kirby-Bauer method was employed to evaluate the inhibitory activity of 8 concentrated samples produced from two *Streptomyces* strains against 2 different *Pseudomonas* species. Both of these strains were naturally sourced from the environment and possess multiple resistances, allowing us to evaluate the inhibitory efficacy of the product against multiple resistant strains. The experiment included testing samples that were grown for 5 and 7 days, as well as samples that were sonicated and those that were not. Filter paper diffusion method was used, with each filter paper absorbing exactly 30µl of samples. The *Pseudomonas* strains were cultured on 22 ml MH medium.

After the filter papers were placed on the surface of the agar medium, I put them in the fridge at 4°C for 2 hours to incubate and wait for the agar medium to absorb the samples. This step is crucial as keeping the test organism at 4°C prevents premature growth, which can interfere with the accuracy of the results by overestimating the readings. Then the plates were incubated for 24 hours at 28°C. During this period, the antimicrobial compounds from the filter paper disks diffuse into the agar medium and inhibit the growth of microorganisms. The zone of inhibition around each filter paper was measured and compared to the control. The aim of the experiment was to determine the effectiveness of the 8 product samples against the *Pseudomonas* species. There exists a situation where it is difficult to decide whether the clean-up zone is small. Because of the technical implementation it is difficult to decide whether or not there is a clean-up zone. In general, cleaning of less than 2 mm is not taken into account precisely for this reason.

3.6. Sequencing the whole DNA

3.6.1. Identification of Acting Genes within the Sequences

Whole-genome sequencing (WGS) is a powerful tool used in the field of genetics to study the complete DNA sequence of an organism's genome. In this study, WGS was carried out by BIOMI Ltd. (Hungary) using the Illumina MiSeq platform. To identify the two isolates, the KmerFinder 3.2 program was applied, which is a widely used method for bacterial identification (Clausen et al. 2018; Hasman et al. 2014; Larsen et al. 2014). Gene annotation and genome analysis were performed using the MicroScope platform (Vallenet et al. 2020). MicroScope is a user-friendly and comprehensive platform that allows for the annotation and comparative analysis of prokaryotic genomes. Additionally, whole-genome-based phylogenetic analysis was performed using the TYGS (https://tygs.dsmz.de/). This platform is a reliable and robust tool for phylogenetic analysis of prokaryotic genomes, using a standardized pipeline for taxonomic classification and genome-based phylogenetic tree construction.To obtain the genomic DNA of the strains, the DNeasy® UltraClean® Microbial DNA isolation Kit (Qiagen, Germany) was used. The whole-genome sequencing was carried out at BIOMI Ltd. (Gödöllő, Hungary), using a previously described method on an Illumina MiSeq platform (Német et al. 2020). De novo assembly and scaffolding were performed with SPAdes version 3.13.0 (Nurk et al. 2013).

To identify the two isolates, the KmerFinder 3.2 program was applied (Clausen et al. 2018; Hasman et al. 2014; Larsen et al. 2014). The complete genome of the investigated strain was automatically annotated using the NCBI Procaryotic Genomes Automatic Annotation Pipeline (PGAP) v4.5 (Tautsova et al. 2016). The genome was annotated using the Genoscope platform MaGe (Magnifying Genomes) (Vallenet et al. 2006; 2009). The analysis was then performed by combining automated annotation from MaGe and manual curation using information from MetaCyc (Caspi et al. 2014), KEGG (Kanehisa et al. 2000), and also UniProt (Bateman et al. 2019). Whole-genome-based phylogenetic analysis was performed by using the TYGS (https://tygs.dsmz.de/) (Meier-Kolthoff & Göker, 2019). This study employed a range of powerful genomic tools and techniques to identify and analyze the genomes of two isolates. In order to identify gene clusters involved in the degradation of aromatic hydrocarbons, CLC Genomics Workbench Tool v21 (Qiagen) will be used.

4. Result

4.1. Revitalisation and Strain Characteristics

The revitalisation of the *Streptomyces* strains "K189" "K145" used in this study was successful, as evidenced by their growth on GYM media and their ability to produce metabolites with antimicrobial activity. The following characteristics were observed for each strain:

Photo 4.1 Left: *Streptomyces rimosus* K145 growing in GYM liquid medium for 72 hours. Right: *Streptomyces rimosus* K189 growing in TGE-5 solid medium for 48 hours. (Lab of Environmental Safety Department, MATE, Hungary, 2022)

Strain K189

Morphology: Strain K189 displayed typical *Streptomyces* morphology, forming filamentous colonies with a rough surface and a powdery shape appearance. The cell formations are yellow and spherical in the liquid medium.

Pigmentation: Strain K189 produced light-yellow aerial mycelium and brownish-yellow substrate mycelium.

Growth rate: Strain K189 grew well on GYM and TGE-5 (GYM) solid media and produced visible colonies after 72-96 hours of incubation at 28°C.

Observation of Metabolites (Naked Eye): brownish-yellow in the liquid medium with flocculent precipitation.

Strain K145

Morphology: Strain K145 also displayed typical *Streptomyces* morphology, forming filamentous colonies with a rough surface and a powdery appearance. The cells are yellow and spherical in liquid medium, much larger than strain K189.

Pigmentation: Strain K145 produced yellow aerial mycelium and yellow-brown substrate mycelium.

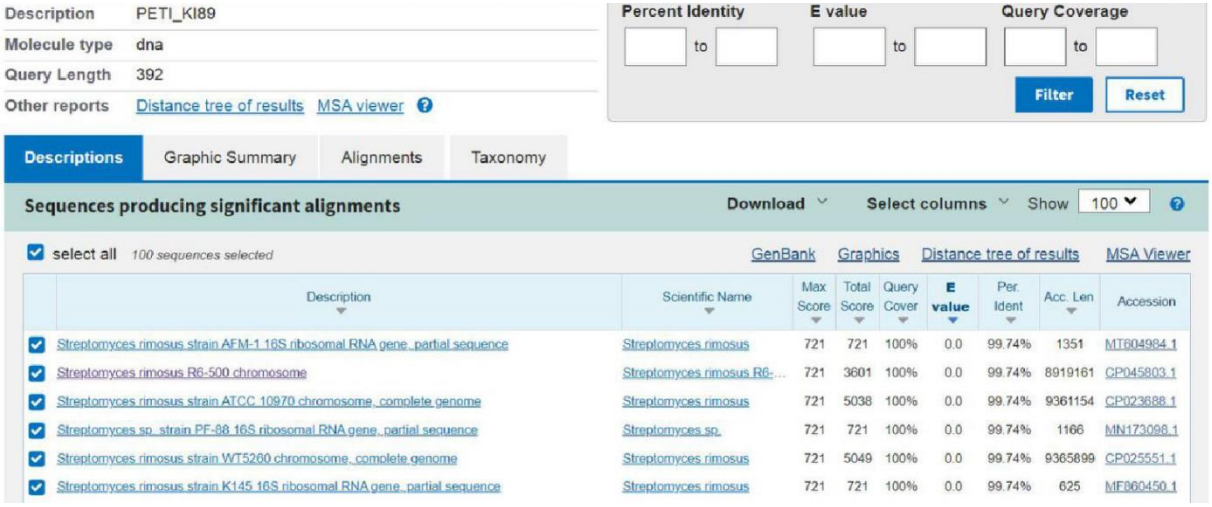
Growth rate: Strain K145 grew well on GYM and TGE-5 solid media and produced visible colonies after 72-96 hours of incubation at 28°C.

Observation of Metabolites (Naked Eye): clean and yellowish-brown in the liquid medium.

In summary, the revitalisation process was successful for both *Streptomyces* strains, and their characteristic morphology, pigmentation, growth rate, and antimicrobial metabolites were consistent with previous reports in the literature.

4.2. Molecular Biological Identification

The results of the molecular biological identification revealed that the *Streptomyces* strains used in this study were closely related to the reference strains in the GenBank database, with identities ranging more than 99%. The strains used in this study were authentic *Streptomyces rimosus* strains by telling the 16S rRNA gene sequencing, and the molecular biological identification method was reliable for strain identification.



Picture 4.2 Molecular identification showed high similarity (≥99%) between the *Streptomyces rimosus* strains used in this study and the reference strains in the GenBank database.

Table 4.3 Identification and morphological summary of *Streptomyces* strains both K189 and

K145 (Color and morphology are observed through naked eye.)

	Scientific names	Per. Ident	Morphology	Pigmentation	Metabolites colour
K145	*Streptomyces rimosus* (MT604984)	99.74%	filamentous colonies rough surface thicker cell wall	Yellowish-brown	Yellowish-brown
K189	*Streptomyces rimosus* (FJ799182)	99.8%	filamentous colonies rough surface	Yellow	Yellowish-brown

4.3. Results of Antibacterial Activity Tests

4.3.1. Cross-streak Method

The cross-streak method was performed to evaluate the inhibitory activity of the sample against a panel of *Pseudomonas aeruginosa* strains, they are P18, P43, P66, ATCC27853, B02600, B02040. The results showed that only four *Pseudomonas* strains (P18, P66, B02600, B02040) were significantly inhibited, while since less than 2mm will not be measured, so the rest of the strains shown as weak or insignificant inhibited effect (photo 4.4).

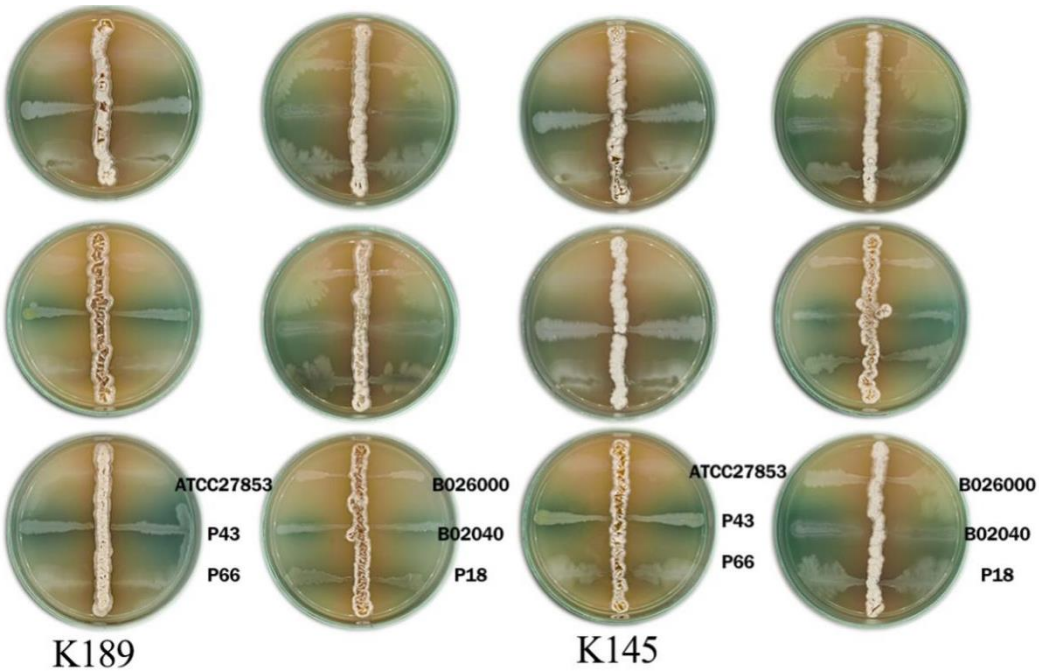


Photo 4.4cross-steak method photos (Lab of Environmental Safety Department, MATE, Hungary, 2022)

The test was performed using the cross-streak method. The plates were incubated for 24 hours at 28°C, and the zone of inhibition was measured in mm's. The experiment was performed in triplicate, and the results were recorded as the mean ± standard deviation. This is important because it allows for better accuracy, precision, and statistical significance of the results obtained.

Table 4.5 K189 cross-streak method data recording and mean ± SD results (due to technical reasons, data < 2 mm will not be recorded)

(mm)		ATCC27853	P43	P66	B02600	B02040	P18
K189	1	2.00	-	5.00	3.00	3.00	2.00
	2	2.00	-	3.00	3.00	3.00	3.00
	3	2.00	-	5.00	4.00	2.00	4.00
	4	3.00	-	4.00	5.00	5.00	5.00
	5	2.00	-	6.00	4.00	4.00	5.00
	6	4.00	-	5.00	5.00	3.00	3.00
Average		2.50	-	4.67	4.00	3.33	3.67
σ		0.84	-	1.03	0.89	1.03	1.21
Average±σ		2.50±0.84	-	4.67±1.03	4.00±0.89	3.33±1.03	3.67±1.21

The results showed that K189 had antibacterial effect on 5 out of 6 *Pseudomonas aeruginosa* bacteria. Numerical results show that K189 has a better inhibitory effect on multi-resistant strains P66 and B02600 the average of clean zones is more than 4 mm. This could also prove that K189 itself or its products have an antibacterial effect. K189 did not show significant inhibitory effect on *Pseudomonas aeruginosa* strain P43.

Table 4.6 K145 cross-streak method data recording and mean ± SD results. Measures are ment to be done in mm's (due to technical reasons, data < 2 mm will not be recorded)

(mm)		**ATCC27853**	**P43**	**P66**	**B02600**	**B02040**	**P18**
K145	1	-	-	7.00	-	4.00	-
	2	2.00	-	5.00	2.00	4.00	2.00
	3	-	-	3.00	4.00	3.00	-
	4	3.00	-	5.00	4.00	4.00	-
	5	2.00	-	3.00	2.00	4.00	2.00
	6	3.00	-	3.00	4.00	3.00	2.00
Average		**1.67**	**-**	**4.33**	**2.67**	**3.67**	**1.00**
SD σ		1.37	-	1.63	1.63	0.52	1.10
Average±σ		**1.67±1.37**	**-**	**4.33±1.63**	**2.67±1.63**	**3.67±0.52**	**1.00±1.10**

K145 has been shown to have the ability to inhibit specific *Pseudomonas* species. Comparing

Photo 4.4, we can prove that K145 still has an inhibitory effect on most of the *Pseudomonas aeruginosa* strains, but the effect is not significant as K189.

Sample K145 showed significant inhibitory effect against *Pseudomonas aeruginosa* P66, with an inhibition zone of from 3 to 7 mms. It also exhibited some inhibitory effect against strains B02600 and B02040. However, it showed no significant inhibitory effect or inhibition against the other tested strains.

Due to the space and raw material limitations of the Petri dishes, we can expect that a certain concentration of K145 or its product may have a significant inhibitory effect. From the figure 4.6, we can see that the *Pseudomonas aeruginosa* on both sides exhibit acute angles and grow outwardly near the midline where the K145 and K189 strains were inoculated. I infer that this phenomenon may be due to insufficient concentration. In support of this view, the K145 and K189 products were concentrated for observation in subsequent experiments.

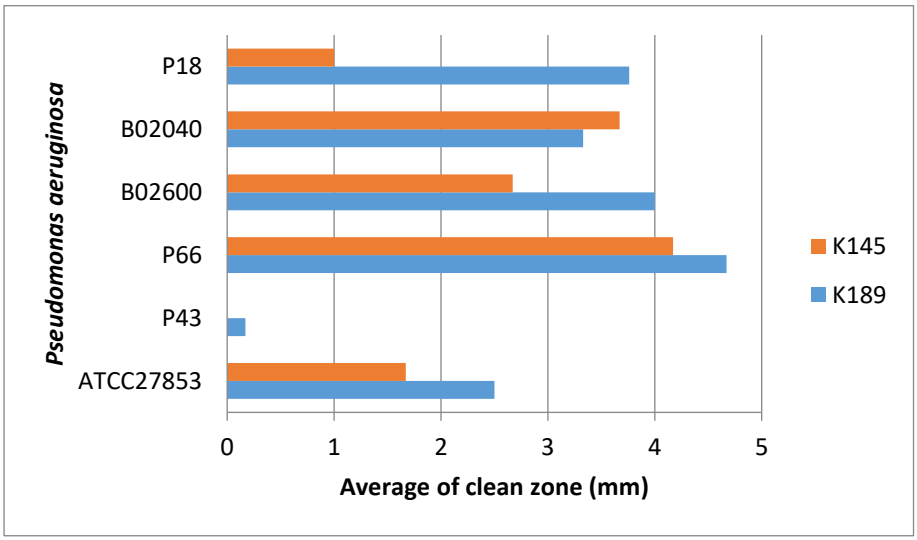


Figure 4.7 K145 and K189 Inhibition clean zone average in mm comparison

The results indicate that the two *Streptomyces rimosus* samples “K189” “K145” have selective inhibitory activity against specific strains of *Pseudomonas aeruginosa* P18, P66, B02040, B02600. The comparison in figure 4.7 clearly shows that the inhibitory capacity and effect of K189 was significantly better than that of K145. For *Pseudomonas aeruginosa* P43, both K189 and K145 showed little or no inhibitory effect.

4.3.2. Agar Diffusion Method

The agar diffusion method was employed to evaluate the antibacterial activity of the samples against a panel of pathogenic bacterial strains from the environment. The results indicated that most samples exhibited antibacterial activity against the tested strains. The zone of inhibition ranged from 14mm to 20mm, indicating that the samples were effective in inhibiting the growth of the tested micro-organisms.

Table 4.8 Recording data of clean zones inhibited by products of *Streptomyces rimosus* K189 and K145against *Pseudomonas aeruginosa* P66 and P18. (due to technical reasons, data < 2 mm will not be recorded)

Unit mm		K189				K145			
		SCC-5	CC-5	SCC-7	CC-7	SCC-5	CC-5	SCC-7	CC-7
P66	1	-	-	14.00	20.00	-	-	20.00	21.00
	2	-	-	14.00	20.00	-	-	21.00	20.00
	3	-	-	16.00	22.00	-	-	20.00	19.00
	Average	**-**	**-**	**14.67**	**20.67**	**-**	**-**	**20.33**	**20.00**
	SD σ	-	-	1.15	1.15	-	-	0.58	1.00
	average±σ	**-**	**-**	**14.67±1.15**	**20.67±1.15**	**-**	**-**	**20.33±0.58**	**20.00±1.00**
P18	1	-	-	14.00	20.00	-	-	22.00	20.00
	2	-	-	16.00	20.00	-	-	22.00	20.00
	3	-	-	16.00	21.00	10.00	-	20.00	20.00
	Average	**-**	**-**	**15.33**	**20.33**	**3.33**	**-**	**21.33**	**20.00**
	SD σ	-	-	1.15	0.58	5.77	-	1.15	0.00
	average±σ	**-**	**-**	**15.33±1.15**	**20.33±0.58**	**3.33±5.77**	**-**	**21.33±1.15**	**20.00±0.00**

As shown in Table 4.8, the concentrated products from K189 and K145 were able to inhibit the growth of several resistant strains of *Pseudomonas aeruginosa*, including P66 and P18. The K145 products, K145 SCC-7 and K145 CC-7, both exhibited significant inhibitory effects, with average clean-zone diameters exceeding 20 mm. Sample K145 SCC-7 even showed a zone diameter of approximately 21.5 mm against the P18 strain. The products from K189 also demonstrated remarkable inhibitory effects against both *Pseudomonas* strains, with zone diameters ranging from 14-20 mm.

According to the results of the study, K189 CC-7 and K145 CC-7, which produced by *Streptomyces rimosus* K189 and K145*,* did not differ significantly in their inhibition of P66 and P18.

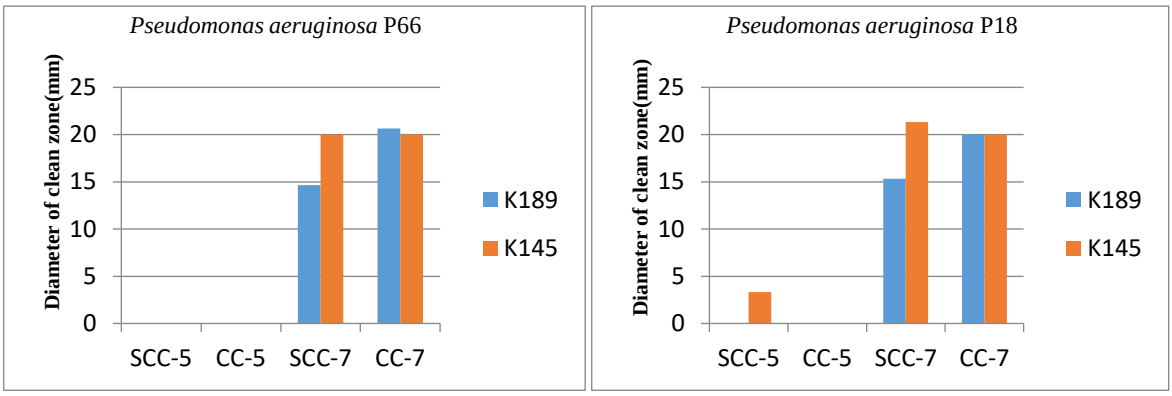


Figure 4.9 Comparative data on the inhibition capacity of secondary metabolite samples (Left: P66 inhibition effect; Right: P18 inhibition effect)

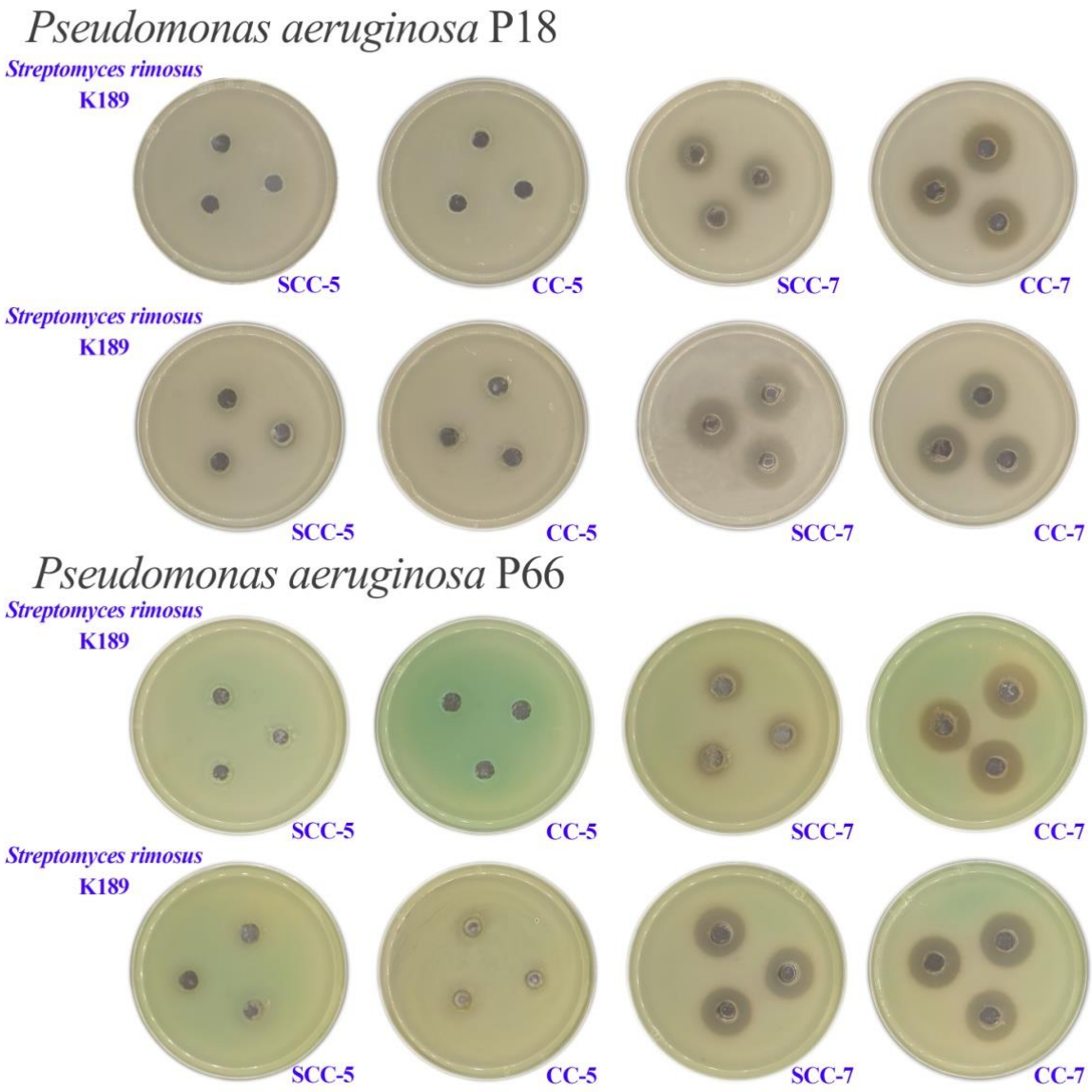


Photo 4.10 Product by *Streptomyces rimosus* K189 and K145 inhibit *Ps. aeruginosa* P66 and P18 growth to form large clean zone in MH solid medium photos (Lab of Environmental Safety

Department, MATE, Hungary, 2022)

From the average diameter of the inhibition clean zone, the metabolites of the strains incubated for 7 days far exceeded the 5 days samples in terms of inhibition capacity. Also comparing the inhibition effect of the sonicated and unsonicated sample, we find the results are not significantly different. The values of K189 SCC-7 and K145 SCC-7 in both groups were not higher than those of K189 CC-7 and K145 CC-7 in the non-sonified samples.

4.3.3. Filter Paper Diffusion Test (Kirby-Bauer Method)

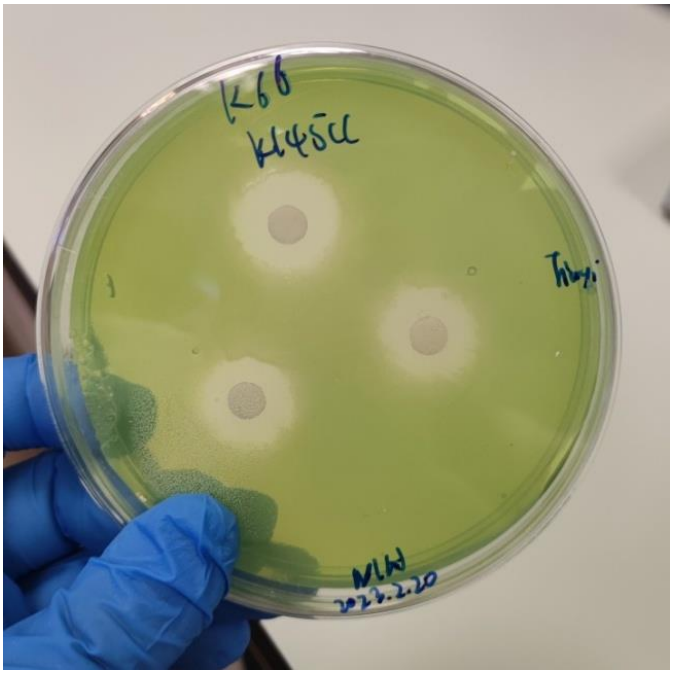


Photo 4.11 The product of K145 inhibited the growth of P66, and a clean zone of inhibition appeared**.** (Lab of Environmental Safety Department, MATE, Hungary, 2022)

The filter paper diffusion test is based on the diffusion of an antimicrobial compound from a filter paper disk into and on an agar medium containing micro-organisms, expecially on the surface region. It was utilized to evaluate the antibacterial activity of the samples against *Ps. aeruginosa* P66 and P18. Compared to the previous method, this approach has a greater impact on the growth of *Pseudomonas aeruginosa*, especially for strains inoculated using the cotton swab method. This allows us to more clearly observe the antibacterial abilities of each sample against the strains and make a more intuitive comparison.

Table 4.12 Diameter of the inhibition cleaning zone and inhibition effect of the filter paper method.

Unit mm		K189-CC	K189-SCC	K145-CC	K145-SCC
P66	1	24.00	18.00	16.00	10.00
	2	22.00	16.00	18.00	12.00
	3	22.00	15.00	16.00	10.00
	Average	22.67	16.33	16.67	10.67
	SD σ	1.15	1.53	1.15	1.15
	average±σ	**22.67±1.15**	**16.33±1.53**	**16.67±1.15**	**10.67±1.15**
P18	1	22.00	22.00	22.00	18.00
	2	22.00	20.00	20.00	20.00
	3	20.00	22.00	18.00	18.00
	Average	21.33	21.33	20.00	18.67
	SD σ	1.15	1.15	2.00	1.15
	average±σ	**21.33±1.15**	**21.33±1.15**	**20.00±2.00**	**18.67±1.15**

The results indicated that all samples exhibited antibacterial activity against the tested strains. The zone of inhibition average ranged from 16 mm to 23 mm, indicating that the samples were effective in inhibiting the growth of the tested strains. Each filter paper absorbs 30 µl of products, so we can see more visually how the samples compare with each other.

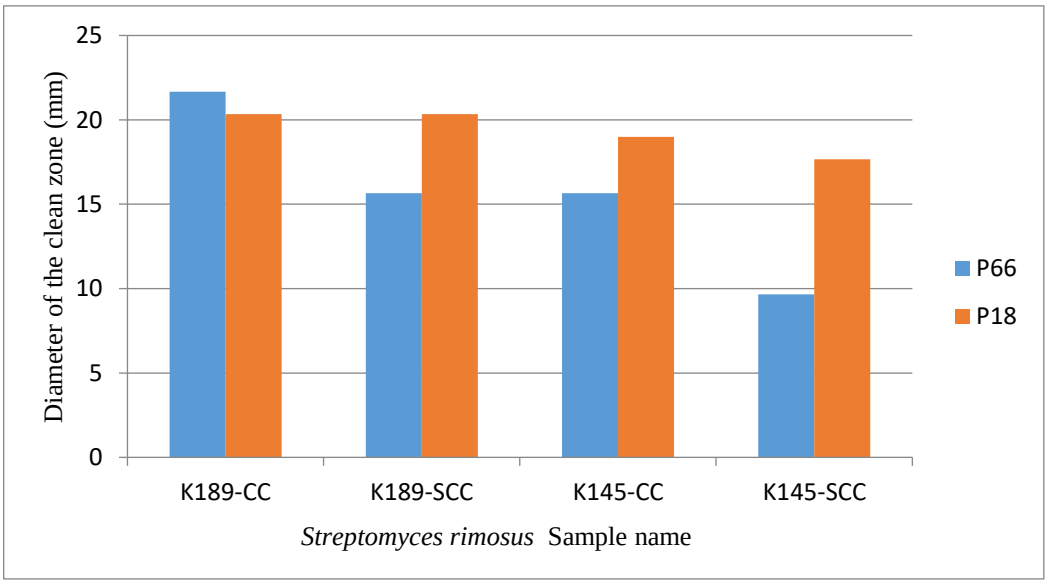


Figure 4.13 Comparative data on the inhibition clean zone average of samples produced by *Streptomyces rimosus* K189 and K145 on *Ps. aeruginosa* P66 and P18

Figure 4.13 shows that the product samples from K189 exhibited good inhibition ability against *Pseudomonas aeruginosa* P18 and P66, with inhibition zones of over 20 mm. Among them, the K189-CC sample performed the best in inhibiting for both strains. The product samples from K145 showed better ability to inhibit P18 compared to P66, with a maximum average inhibition

zone of 20 mm and a common range of from 10 to 20 mm. Overall, the product of strain K189 was more effective than that of K145 in inhibiting bacterial growth.

4.4. Analysis of Effective Gene Fragments

Eurofins BIOMI Kft. provided the complete sequences of two samples, K189 and K145. The results showed that both samples exhibited the highest similarity with NZ_CP094298.1 *Streptomyces rimosus* subsp. *rimosus* strain R7 chromosome, complete genome (NZ_CP094298). Therefore, K189 and K145 belong to the species *Streptomyces rimosus*.

Whole-genome sequencing was carried out in the sequencing facility of BIOMI Ltd. (Gödöllő, Hungary), according to a previously described method on an Illumina MiSeq platfom (Német et al. 2020). *De novo* assembly and scaffolding were performed with SPAdes version 3.13.0 (Nurk et al. 2013). The complete genome of the investigated strain was automatically annotated using the NCBI Procaryotic Genomes Automatic Annotation Pipeline (PGAP) v4.5 (Tautsova et al. 2016). To identify gene clusters involved in the degradation of aromatic hydrocarbons, CLC Genomics Workbench Tool v21 (Qiagen) was used.

CLC Genomics Workbench Tool was used to the genome analysis revealed that strain K145^{T} and K189^{T} possesses OTC (otr) genes genes encoding resistant determinants. An ORF encoding *otrB* gene was found in the genomes of both strains. The efflux of OTC from the cell is carried out by an integral membrane protein enconding by *otrB* gene (McMurry et al. 1998). Besides, several genes responsible for the OTC biosynthesis were found downstream of the *otrB* gene. As described by Petrovic et al. (2006), pathway genes are clustered together and are flanked by the resistance genes as the *otrB* and *otrA* protecting the ribosome from translational arrest by tetracylines (Doyle et al. 1991). The third OTC resistance gene is *otrC*, a putative ATP-binding cassette (ABC) transporter with multidrug resistance function (Yu et al. 2012). The *otcA* and *otcC* genes were not identified or revealed using automatic annotation. Detailed analysis of the genome predict show that strains K145^{T}and K189^{T} presumably have the potential to produce oxytetracycline.

5. Conclusion and Suggestion

Based on the results obtained in this study, it can be concluded those *Streptomyces* strains K189 and K145 have antimicrobial activity against *Pseudomonas aeruginosa*, even some of them are multi-resistance strains. The revitalization processes were successfully done in the case of both strains, and their characteristic morphology, pigmentation, growth rate, and antimicrobial metabolites were consistent with previous reports in the literature. The molecular biological identification method was reliable for strain identification, and the strains used in this study were authentic *Streptomyces rimosus* strains.

The cross-streak test showed that K189 had a better inhibitory effect on multi-resistant strains P66 and B02600 than K145. However, K145 still has an inhibitory effect on 5 of the *Pseudomonas aeruginosa* strains, which suggests that it could be useful in combination with other antibiotics to increase their effectiveness. Both strains had no significant inhibitory effect on strain P43. Further studies are needed to investigate the mechanism behind this selectivity and to determine the potential applications of the sample against these specific strains. These results suggest that *Streptomyces* strains K189 and K145 have the potential to be developed as a new antibiotic for the treatment of multi-resistant *Pseudomonas aeruginosa* infections.

As we previously used unconcentrated samples for the agar well diffusion and paper disc methods and did not observe any inhibition zones, we speculated that higher concentrations of the samples may achieve the desired antibacterial effect and redesigned this experiment. As a result, the concentrated metabolites from K189 and K145 showed significant antibacterial activity in both agar well diffusion and paper disc methods. Among them, the concentrated metabolites from K189 had stronger inhibition ability against multi-drug resistant *Pseudomonas aeruginosa* strains.

Additionally, we found that the inhibition ability was significantly enhanced when the samples were incubated for 7 days compared to 5 days. While most *Streptomyces* incubation products are expected to appear by the 5th day, some strains may require a longer incubation period. Therefore, I presume that for strains K189 and K145, we need to cultivate them for 6-7 days in order to expect more inhibition products. Further studies are needed to explore the metabolite production of these two strains.

Because the experiments showed that K189 and K145 possessed significant ability to inhibit multi-resistant *Pseudomonas aeruginosa*. In our comprehensive analysis of the strains' complete sequences, we successfully identified the presence of OTC (*otr*) genes, which encode the crucial resistant determinants responsible for the production of the inhibitory substance. Notably, the genomes of both strains contained an open reading frame (ORF) encoding the *otrB* gene. Moreover, we discovered several genes downstream of the *otrB* gene that are directly involved in OTC biosynthesis. However, our automatic annotation process did not reveal the presence of the *otcA* and *otcC* genes. These findings strongly suggest that $K145^T$ and $K189^T$ may possess the capacity to produce Oxytetracycline.

The study conducted on two known *Streptomyces rimosus* K189 and K145 showed that they had antibiotic activity against a test organism. However, summarizing the results of the three different methods used, it was found that not all cases of a specific *Streptomyces* strain showed antibody production in the presence of a specific test organism in all three experiments. These differences can be attributed to the different physical and chemical properties of the two experimental systems.

During the cross-streak method, a stationary culture was utilized, which is known to promote the production of secondary metabolites, including antibiotic substances, during active growth in the stationary phase or towards its end. The coexistence of test organisms and the *Streptomyces* strain on a thin agar plate created a competitive environment where limited nutrient availability induced a stress situation, triggering antibiotic production in response.

Conversely, the agar diffusion well test involved the use of an antibiotic filtrate derived from a shaken culture. In this case, the bacterial strains did not directly produce antibiotic metabolites in the presence of the test organism but responded to the shaking conditions by initiating antibiotic production.

From the results of the experiments, it can be concluded that optimal growth conditions, adequate amounts of available food and oxygen, and lack of nutrient competition do not necessarily promote antibiotic production. Under laboratory conditions, spore formation takes place in the stationary phase, similar to antibiotic production. Antibiotic production on a solid culture medium is related to the formation of aerial mycelia, i.e. the stage of spore formation. Accordingly, during the cross-stripe test, the phenomenon of spore formation was experienced

in almost all cases.

And based on the experimental results, we find it is crucial to preserve and maintain the bioactivity of strains exhibiting effective antibiotic effects. This necessitates determining appropriate storage durations and conditions. Conducting optimization experiments for nutrient medium composition is recommended, including defining the ideal carbon-to-nitrogen (C-N) ratio and favorable pH range.

Refining the extraction methods for the antimicrobial substances produced by these strains is important. Solubility tests, such as experiments aimed at determining concentration gradients, can provide valuable insights into the nature and types of antibiotics produced by the strains. Further experiments focusing on antibiotic production could reveal whether multiple antibiotics are produced simultaneously and provide insights into potential cross-reactions among them. Additionally, it is advisable to include other members of the strain collection in experiments to assess their ability to induce antibiotic effects using the described methods.

Further experiments aimed at the antibiotic production of the strains can determine whether they produce several antibiotics simultaneously and provide information about their mutual effects (cross reactions). It is also recommended to include the other members of the strain collection in an experiment to test their ability to induce an antibiotic effect using the described methods.

This study sheds light on the antibiotic activity of the investigated *Streptomyces rimosus* K189 and K145 and provide a foundation for further research in the treatment of multi-resistant *Pseudomonas aeruginosa* infections, and highlights the importance of understanding the influence of different experimental systems on antibody production.

If there is a desire to develop *Streptomyces* strains K189 and K145 for medicinal or agricultural purposes, it is essential to conduct further research to thoroughly evaluate their safety, toxicity, and pharmacokinetics. These evaluations will provide crucial insights and pave the way for their potential application in various fields.

6. Summary

In recent years, the emergence of microorganisms with multiple resistance to antibiotics has posed a significant threat to public health. Multi-drug resistant strains (MDRs) of bacteria have made it increasingly difficult to treat infections and have increased the risk of severe infections and death. The overuse of antibiotics in both humans and animals has contributed to the development of MDRs, which is now a global health concern. To address this issue, it is crucial to discover new substances that can effectively inhibit the growth of MDR strains.

One potential source of new drugs is natural products, such as those produced by *Streptomyces*. These soil-dwelling bacteria are known to produce a wide range of secondary metabolites, including antibiotics and other bioactive compounds. These metabolites have the potential to be developed into new drugs for the treatment of MDR infections.

To explore this potential, a study was conducted to identify the molecular biology of lab-collected *Streptomyces rimosus* strains K189 and K145 and analyze their antibacterial activity. The study employed three common antimicrobial testing methods: the cross-streak method, the agar diffusion method, and also the filter paper diffusion method.

The results of the molecular biological identification using 16S rRNA gene DNA sequencing revealed that K189 and K145 were genuine *Streptomyces rimosus* strains. The results of the antibacterial activity tests demonstrated that both strains exhibited selective inhibitory activity against specific strains of *Pseudomonas*. K189 displayed superior inhibitory effects against P66 and P18 when compared to K145, while neither strain had any inhibitory effects on *Pseudomonas aeruginosa* P43. Additionally, an effective DNA fragment that generates antibacterial products was identified by sequencing the entire DNA of the K189 and K145 strains.

These results suggest that *Streptomyces rimosus* strains K189 and K145 have potential as a source of new drugs for the treatment of multidrug-resistant *Pseudomonas* infections. Further research is needed to fully understand the mechanisms of action of these compounds and to evaluate their safety and efficacy in clinical trials.

In the future, it will be essential to continue exploring natural sources of antibiotics and other bioactive compounds to combat MDRs. The development of new drugs is a complex and time-

consuming process that requires significant investment, but the potential benefits for public health are enormous. The use of natural products has several advantages, including their long history of use in traditional medicine, their low toxicity, and their potential for producing a wide range of bioactive compounds.

As we continue to face the challenges posed by MDRs, it is crucial to develop new strategies for the responsible use of antibiotics in both humans and animals. This includes reducing unnecessary antibiotic use, promoting the development of new antibiotics, and investing in research into alternative therapies, such as bacteriophages and probiotics. It is also crucial to improve surveillance systems to monitor the spread of MDRs and to implement effective infection control measures to prevent their transmission.

In conclusion, the study of *Streptomyces rimosus* strains K189 and K145 highlights the potential of natural products as a source of new drugs for the treatment of multidrug-resistant infections. Further research is needed to fully understand the mechanisms of action of these compounds and to evaluate their safety and efficacy in clinical trials. As we continue to face the challenges posed by MDRs, it is crucial to develop new strategies for the responsible use of antibiotics and to invest in research into alternative therapies. By working together, we can develop new treatments to combat MDRs and ensure that antibiotics remain effective for generations to come.

7. Acknowledgements

First and foremost, I would like to express my heartfelt gratitude to my advisor, Péter Harkai. He has been an exceptional mentor, demonstrating kindness, patience, and a strong sense of responsibility. His guidance and assistance have been invaluable in both my thesis writing and laboratory work. Without his support, completing this thesis would not have been possible. I consider myself incredibly fortunate to have had such a remarkable teacher who has had a positive impact not only on my academic journey but also on my future.

I would like to extend my thanks to Dr. Balázs Kriszt for providing me with the opportunity to conduct experiments. I am also grateful to all the staff members at the Department of Environmental Safety, especially, Dr. Benséné Ildikó Fekete. Their unwavering support has been instrumental in the successful completion of the majority of my laboratory work. Ildikó, in particular, has been like a nurturing grandmother to me, always kind, helpful, and caring towards everyone. I also would like to acknowledge the contributions of other teachers and fellow students in the lab, including Dr. Gyula Szabó, Dr. Edit Kaszab, Dongze Jiang, and many others.

Furthermore, I am grateful to the RET staff for their invaluable help and support, as I have learned new laboratory procedures from them over the past few months. Special appreciation goes to Dr. András Táncsics, Anna Bedics, Márton Pápai, and the entire team of laboratory technicians.

Last but not least, I would like to express my deepest gratitude to my parents, my friends, and loved ones for their unwavering assistance, support, and encouragement throughout the process of writing my thesis. Their contributions have been instrumental in my academic journey.

8. Reference

AbdElgawad, H., Abuelsoud, W., Madany, M. M., Selim, S., Zinta, G., Mousa, A., & Hozzein, W. N. (2020). Actinomycetes Enrich Soil Rhizosphere and Improve Seed Quality as well as Productivity of Legumes by Boosting Nitrogen Availability and Metabolism. Biomolecules, 10(12), 1675.

Alam, K., Mazumder, A., Sikdar, S., Zhao, Y. M., Hao, J., Song, C., Wang, Y., Sarkar, R., Islam, S., Zhang, Y., & Li, A. (2022). *Streptomyces*: The biofactory of secondary metabolites. Frontiers in microbiology, 13, 968053.

Ali, H. (2010). Biodegradation of synthetic dyes - a review. Water, Air, & Soil Pollution, 213, 251-273.

Battersby, N. S., & Wilson, V. (1989). Survey of the Anaerobic Biodegradation Potential of Organic Chemicals in Digesting Sludge. Applied and Environmental Microbiology, 55(2), 433–439.

Bennett, Joan W; Chung, King-Thom (2001), "Alexander Fleming and the discovery of penicillin", Advances in Applied Microbiology, Elsevier, 49:163-184, Oct.17,2020

Bondarczuk, K., Markowicz, A., & Piotrowska-Seget, Z. (2016). The urgent need for risk assessment on the antibiotic resistance spread via sewage sludge land application. Environment International, 87, 49–55.

Biemer, J. J. (1973). Antimicrobial susceptibility testing by the Kirby-Bauer disc diffusion method. Annals of Clinical & Laboratory Science, 3(2), 135-140.

Bhardwaj, S., Bhatia, S., Singh, S., & Franco, F. C. (2021). Growing emergence of drug-resistant *Pseudomonas aeruginosa* and attenuation of its virulence using quorum sensing inhibitors: A critical review. Iranian Journal of Basic Medical Sciences, 24(6), 699–719.

Bojarski, B., Kot, B., &Witeska, M. (2020). Antibacterials in Aquatic Environment and Their Toxicity to Fish. Pharmaceuticals (Basel, Switzerland), 13(8), 189.

Bonev, B. B., Hooper, J., & Parisot, J. (2008). Principles of assessing bacterial susceptibility to antibiotics using the agar diffusion method. Journal of Antimicrobial Chemotherapy, 61(6), 1295–1301.

Bateman, A. (2019) UniProt: A worldwide hubof protein knowledge. Nucleic Acids Res.47, D506–D515

Briceño, G., Fuentes, M. S., Saez, J. M., Diez, M. C., & Benimeli, C. S. (2018). *Streptomyces* genus as biotechnological tool for pesticide degradation in polluted systems. Critical Reviews in Environmental Science and Technology, 48(10–12), 773–805.

Chen, J., Xu, L., Zhou, Y., & Han, B. (2021). Natural Products from Actinomycetes Associated with Marine Organisms. Marine Drugs, 19(11), 629.

Chiș, A. A., Rus, L. L., Morgovan, C., Arseniu, A. M., Frum, A., Vonica-Țincu, A. L., Gligor, F. G., Mureșan, M. L., & Dobrea, C. M. (2022). Microbial Resistance to Antibiotics and Effective Antibiotherapy. Biomedicines, 10(5), 1121.

Chang, Q., Wang, W., Regev‐Yochay, G., Lipsitch, M., &Hanage, W. P. (2014). Antibiotics in agriculture and the risk to human health: how worried should we be Evolutionary Applications, 8(3), 240–247.

Caspi R., et al. (2014) The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of Pathway/Genome Databases. Nucleic Acids Res. 42, D459–D471.

Clausen, C. H., Dimaki, M., Bertelsen, C. V., Skands, G. E., Rodriguez-Trujillo, R., Thomsen, J. D., & Svendsen, W. E. (2018). Bacteria Detection and Differentiation Using Impedance Flow Cytometry. Sensors (Basel, Switzerland), 18(10), 3496.

Cuozzo, S. A., Sineli, P. E., Davila Costa, J., &Tortella, G. (2017). *Streptomyces* sp. is a powerful biotechnological tool for the biodegradation of HCH isomers: biochemical and molecular basis. Critical Reviews in Biotechnology, 38(5), 719–728.

Chater, K. F., Biró, S., Lee, K. J., Palmer, T., & Schrempf, H. (2010). The complex extracellular

biology of *Streptomyces*. Fems Microbiology Reviews, 34(2), 171–198.

Donald, L., Pipite, A., Subramani, R., Owen, J., Keyzers, R. A., & Taufa, T. (2022). *Streptomyces*: Still the Biggest Producer of New Natural Secondary Metabolites, a Current Perspective. Microbiology Research, 13(3), 418–465.

Davies, J., & Davies, D. (2010). Origins and Evolution of Antibiotic Resistance. Microbiology and Molecular Biology Reviews, 74(3), 417–433.

Dhanasekaran, D., & Jiang, Y. (2016). Actinobacteria: Basics and Biotechnological Applications. IntechOpen.

Doyle, D., K. J. McDowall, M. J. Butler, and I. S. Hunter. (1991). Characterization of an oxytetracycline resistance gene, *otrA*, of *Streptomyces rimosus*: nucleotide sequence, deduced function and promoter analysis. Mol. Microbiol. 5:2923-2933.

Duarte, D. J., Oldenkamp, R., & Ragas, A. M. (2019). Modelling environmental antibiotic-resistance gene abundance: A meta-analysis. Science of the Total Environment, 659, 335–341.

Déziel, E., Nguyen, C., Baillargeon, L., Villemur, R., &Maltz, M. R. (2005). Degradation of the pesticide atrazine in soil by *Streptomyces fradiae*. Applied and Environmental Microbiology, 71(11), 6758-6764.

Ehrlich, P. (1909). Chemotherapeutic Selectivity. Journal of the American Medical Association, 52, 809-816

Finsen, N. (1894). Über Actinomyces. Fortschritte der Medicin, 12, 485-489.

Fuentes, M.S., Sáez, J.M., Benimeli, C.S. et al (2011). Lindane Biodegradation by Defined Consortia of Indigenous *Streptomyces* Strains. Water Air Soil Pollut 222, 217–231

Goodfellow, M., & Williams, S. T. (1983). ECOLOGY OF ACTINOMYCETES. Annual Review of Microbiology, 37(1), 189–216.

Ghorbannezhad, H., Moghimi, H. & Dastgheib, S.M.M. (2022). Biodegradation of high

molecular weight hydrocarbons under saline condition by halotolerant *Bacillus subtilis* and its mixed cultures with *Pseudomonas* species. Sci Rep 12, 13227

Hussain, M. S., Fareed, S., Ansari, S., Rahman, M. A., Ahmad, I. Z., & Saeed, M. (2012). Current approaches toward production of secondary plant metabolites. Journal of pharmacy & bioallied sciences, 4(1), 10–20.

Harkai, P. (2017). Taxonomical and environmental biological studies about a *Streptomyces* strain collection [ph.d. thesis]. Hungarian University Of Agriculture and Life Science.

Hudzicki, J. (2009). Kirby-Bauer disk diffusion susceptibility test protocol. American society for microbiology, 15, 55-63.

Hasman, H., Saputra, D., Sicheritz-Ponten, T., Lund, O. et al. (2014). Rapid whole-genome sequencing for detection and characterization of microorganisms directly from clinical samples. Journal of clinical microbiology, 52(1), 139–146.

Hare, R. (1982). New light on the history of penicillin. Medical History, 26(1), 1-24

Jalal, B. K., & Ayad, H. H. (2021). Molecular and Phenotypic Characterization of Novel Streptomyces Species Isolated from Kurdistan Soil and its Antibacterial Activity Against Human Pathogens. *Jordan Journal of Biological Sciences* , 441-451.

Kämpfer, P. (2006). The Family Streptomycetaceae, Part I: Taxonomy. In Springer eBooks , Springer Nature. pp. 538–604.

Klaassen, C. H. W., Vries, R. P., Reenen, C. A. van, &Wijffels, R. H. (2015). Genome-based classification of *Streptomyces* bacteria highlights the dimensionality of secondary metabolism and the power of horizontal gene transfer. Microbiology and Molecular Biology Reviews, 79(1), 56-77.

Killough, M. G., Rodgers, A. M., & Ingram, R. J. (2022b). *Pseudomonas aeruginosa*: Recent Advances in Vaccine Development. Vaccines, 10(7), 1100.

Kraemer, S. A., Ramachandran, A., & Perron, G. G. (2019). Antibiotic Pollution in the Environment: From Microbial Ecology to Public Policy. Microorganisms, 7(6), 180.

Korshunova, T.Y., Bakaeva, M.D., Kuzina, E.V. et al. (2021) Role of Bacteria of the Genus *Pseudomonas* in the Sustainable Development of Agricultural Systems and Environmental Protection (Review). Appl Biochem Microbiol 57, 281–296.

Kaszab, E. (2010) Environmental safety assessment of *Pseudomonas aeruginosa* in anthropogenic-impact-resistant media. Doktori (PhD) értekezés, Gödöllő.

Kamat, N., & Velho-Pereira, S. (2012). Screening of actinobacteria for antimicrobial activities by modified "Cross-Streak" method. Nature Precedings.

Kanehisa, M., & Goto, S. (2000) KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res. 28, 27–30.

Liu, X., Chen, S., Yan, X., Liang, T., Yang, X., El-Naggar, A., Liu, J., & Chen, H. (2021). Evaluation of potential ecological risks in potential toxic elements contaminated agricultural soils: Correlations between soil contamination and polymetallic mining activity. Journal of Environmental Management, 300, 113679.

Liguori, K., Keenum, I., Davis, B. G., Calarco, J., Milligan, E. D., Harwood, V. J., & Pruden, A. (2022). Antimicrobial Resistance Monitoring of Water Environments: A Framework for Standardized Methods and Quality Control. Environmental Science & Technology, 56(13), 9149–9160.

Lee, J. H., Kim, H. J., Kim, S. H., Kweon, O. H., & Kim, K. S. (2017). Naphthalene degradation by *Streptomyces* sp. KAIST-BT-01. Journal of Microbiology and Biotechnology, 27(7), 1071-1079.

Lee, S. W., Glickmann, E., & Cooksey, D. A. (2001). Chromosomal locus for cadmium resistance in *Pseudomonas putida* consisting of a cadmium-transporting ATPase and a MerR family response regulator. Applied and Environmental Microbiology, 67, 1437–1444.

Larsson, D.G.J., Flach, CF. (2022). Antibiotic resistance in the environment. Nat Rev Microbiol 20, 257–269

Larsson, D. G. J., &Flach, C. (2021). Antibiotic resistance in the environment. Nature Reviews Microbiology, 20(5), 257–269.

Li, Y., Guo, Q., He, F. et al. (2020) Biocontrol of Root Diseases and Growth Promotion of the Tuberous Plant *Aconitum carmichaelii* Induced by Actinomycetes Are Related to Shifts in the Rhizosphere Microbiota. MicrobEcol 79, 134–147.

Larsen, M. V., Cosentino, S., Rasmussen et al. (2012). Multilocus sequence typing of total-genome-sequenced bacteria. Journal of clinical microbiology, 50(4), 1355–1361.

Morgado-Gamero, W. B., Parody, A., Medina, J., Rodriguez-Villamizar, L. A., & Agudelo-Castañeda, D. (2021). Multi-antibiotic resistant bacteria in landfill bioaerosols: Environmental conditions and biological risk assessment. Environmental Pollution, 290, 118037.

Mukerji, K. G., Manoharachary, C., & Singh, J. (2006). Microbial Activity in the Rhizosphere. Springer Publishing.

Mayfield, C., Williams, S., Ruddick, S., & Hatfield, H. (1972). Studies on the ecology of Actinomycetes in soil IV. Observations on the form and growth of *Streptomycetes* in soil. Soil Biology and Biochemistry, 4(1), 79–91.

Mehdi, Y., Létourneau-Montminy, M. et al. (2018). Use of antibiotics in broiler production: Global impacts and alternatives. Animal Nutrition, 4(2), 170–178.

Manyi-Loh, C., Mamphweli, S., Meyer, E., &Okoh, A. (2018). Antibiotic Use in Agriculture and Its Consequential Resistance in Environmental Sources: Potential Public Health Implications. Molecules, 23(4), 795.

McLean, J., & Beveridge, T. J. (2001). Chromate reduction by a *Pseudomonas* isolated from a site contaminated with chromated copper arsenate. Applied and Environmental Microbiology, 67(3), 1076–1084.

Megharaj, M., Avudainayagam, S., & Naidu, R. (2003). Toxicity of hexavalent chromium and its reduction by bacteria isolated from soil contaminated with tannery waste. Current Microbiology, 47(1), 51–54.

Mann, A., Nehra, K., Rana, J. S., & Dahiya, T. (2021). Antibiotic resistance in agriculture: Perspectives on upcoming strategies to overcome upsurge in resistance. Current research in microbial sciences, 2, 100030.

McMurry, L. M., and S. B. Levy. (1998). Revised sequence of OtrB (tet347) Tetracycline efflux protein from *Streptomyces rimosus*. Antimicrob. Agents Chemother. 42:3050.

Miethke, M., Pieroni, M., Weber, T. et al. (2021) Towards the sustainable discovery and development of new antibiotics. Nat Rev Chem 5, 726–749.

Német, Z.; Albert, E.; Dán, Á.; Balka, G.; Szenes, Á.; Sipos, R.; Bódizs, S.; Biksi, I. (2022) Genomic analysis of *Staphylococcus aureus* strains originating from hungarian rabbit farms reinforce the clonal origin of various virulence types. Animals 2020, 1

Nurk, S., et al. (2013) Assembling genomes and mini-metagenomes from highly chimeric reads. In Research in Computational Molecular Biology (eds Deng, M. et al.) 158-170.

Meier-Kolthoff, J. P., &Göker, M. (2019). TYGS is an automated high-throughput platform for state-of-the-art genome-based taxonomy. Nature communications, 10(1), 2182.

Német, Z.; Albert, E.; Dán, Á.; Balka, G.; Szenes, Á.; Sipos, R.; Bódizs, S.; Biksi, I. Genomic analysis of *Staphylococcus aureus* strains originating from Hungarian rabbit farms reinforce the clonal origin of various virulence types. Animals 2020, 1

Nurk, S., et al. Assembling genomes and mini-metagenomes from highly chimeric reads. In Research in Computational Molecular Biology (eds Deng, M., Jiang, R., Sun, F., Zhang, X.) 158-170 (Springer, 2013).

Netherwood, T., Bowden, R., Harrison, P., O'Donnell, A. G., Parker, D. S., & Gilbert, H. J. (1999). Gene transfer in the gastrointestinal tract. Applied and environmental

microbiology, 65(11), 5139–5141.

Oliveira, N. A., Gonçalves, B. L., Lee, S. H., CAF, O., & Corassin, C. H. (2020). Use of Antibiotics in Animal Production and its Impact on Human Health. Journal of Food Chemistry &Amp; Nanotechnology, 06(01).

Oraby, H. A., Aboul-Maaty, N. A., Al-Sharawi, H. A., & Osman, N. M. (2022). Horizontal transfer of antibiotic resistance genes into microflora and blood cells in rats fed on GM-diet. Bulletin of the National Research Centre, 46(1). 268

PurssellE. (2019). Antimicrobials. Understanding Pharmacology in Nursing Practice, 147–165.

Pepi, M., & Focardi, S. (2021). Antibiotic-Resistant Bacteria in Aquaculture and Climate Change: A Challenge for Health in the Mediterranean Area. International journal of environmental research and public health, 18(11), 5723.

Park, S. H., Jeong, Y. K., Kim, H. J., Kim, K. S., &Kweon, O. H. (2019). Biodegradation of chlorpyrifos in soil by *Streptomyces* sp. KY10. Journal of Microbiology and Biotechnology, 29(2), 308-316.

Palleroni, N. J., &Doudoroff, M. (1972). Some Properties and Taxonomic Sub-Divisions of the Genus *Pseudomonas*. Annual Review of Phytopathology, 10(1), 73–100.

Peix, A., Ramírez-Bahena, M. H., & Velázquez, E. (2009). Historical evolution and current status of the taxonomy of genus Pseudomonas. Infection, Genetics and Evolution, 9(6), 1132–1147.

Petkovic, H., Cullum, J., Hranueli, D., Hunter, I. S., Perić-Concha, N., Pigac, J., ... & Long, P. F. (2006). Genetics of *Streptomyces rimosus*, the oxytetracycline producer. Microbiology and molecular biology reviews, 70(3), 704-728.

Quinn, G. A., Banat, A. M., Abdelhameed, A. M., & Banat, I. M. (2020). *Streptomyces* from traditional medicine: sources of new innovations in antibiotic discovery. Journal of Medical Microbiology, 69(8), 1040–1048.

Qin, S., Xiao, W., Zhou, C., Pu, Q., Deng, X., Lan, L., Liang, H., Song, X., & Wu, M. (2022). *Pseudomonas aeruginosa*: pathogenesis, virulence factors, antibiotic resistance, interaction with host, technology advances and emerging therapeutics. Signal Transduction and Targeted Therapy, 7(1).

Russell, A. D. (2003). Biocide use and antibiotic resistance: the relevance of laboratory findings to clinical and environmental situations. Lancet Infectious Diseases, 3(12), 794–803.

Rainey, F. A., & Stackebrandt, E. (2000). rDNA Amplification: Application of 16S rDNA-Based Methods for Bacterial Identification. Nonradioactive Analysis of Biomolecules, 396-406.

Stackebrandt, E., & Schumann, P. (2006). Introduction to the Taxonomy of Actinobacteria. In Springer eBooks, Springer Nature. pp. 297–321.

Selim, M.S.M., Abdelhamid, S.A. & Mohamed, S.S. (2021) Secondary metabolites and biodiversity of Actinomycetes. J Genet EngBiotechnol 19, 72.43141-021-00156-9.

Salam, N., Jiao, J. Y., Zhang, X. T., & Li, W. J. (2020). Update on the classification of higher ranks in the phylum Actinobacteria. International journal of systematic and evolutionary microbiology, 70(2), 1331-1355.

Shanthi, V. (2021). Actinomycetes: Implications and Prospects in Sustainable Agriculture. Biofertilizers, 335–370.

Stellato, G., De Filippis, F., La Storia, A., &Ercolini, D. (2015). Coexistence of Lactic Acid Bacteria and Potential Spoilage Microbiota in a Dairy Processing Environment. Applied and Environmental Microbiology, 81(22), 7893–7904.

Sriramulu, D. (2019). Pseudomonas aeruginosa: An Armory Within. IntechOpen.

Saxena, D., Gowri, P. M., Mago, R., & Srivastav, S. (2001). Removal of copper by *Pseudomonas putida* strain S4 isolated from copper mines. Indian Journal of Experimental Biology, 39(6), 590–593

Serwecińska, L. (2020). Antimicrobials and Antibiotic-Resistant Bacteria: A Risk to the Environment and to Public Health. Water, 12(12), 3313.

Tidjani, A. R., Lorenzi, J. N., Toussaint, M., van Dijk, E., Naquin, D., Lespinet, O., Bontemps, C., & Leblond, P. (2019). Massive Gene Flux Drives Genome Diversity between Sympatric *Streptomyces* Conspecifics. mBio, 10(5), e01533-19.

Tang, K. L., Caffrey, N. P., et al. (2017). Restricting the use of antibiotics in food-producing animals and its associations with antibiotic resistance in food-producing animals and human beings: a systematic review and meta-analysis. The Lancet Planetary Health, 1(8), e316–e327.

Tatusova, T., DiCuccio, M., Badretdin, A., Chetvernin, V., Nawrocki, E. P., Zaslavsky, L., Lomsadze, A., Pruitt, K. D., Borodovsky, M., & Ostell, J. (2016). NCBI prokaryotic genome annotation pipeline. Nucleic acids research, 44(14), 6614–6624.

Ventura, M., Canchaya, C., Tauch, A., Chandra, G., Fitzgerald, G. F., Chater, K. F., & Van Sinderen, D. (2007). Genomics of Actinobacteria : Tracing the Evolutionary History of an Ancient Phylum. Microbiology and Molecular Biology Reviews, 71(3), 495–548.

Van Duin, D., & Paterson, D. L. (2016). Multidrug-Resistant Bacteria in the Community: Trends and Lessons Learned. Infectious disease clinics of North America, 30(2), 377–390.

Van der Meij, A., Worsley, S. F., Hutchings, M. I., & van Wezel, G. P. (2017). Chemical ecology of antibiotic production by Actinomycetes. FEMS microbiology reviews, 41(3), 392–416.

Vallenet, D., Calteau, A., Dubois, M. et al. (2019). MicroScope: an integrated platform for the annotation and exploration of microbial gene functions through genomic, pangenomic and metabolic comparative analysis. Nucleic Acids Research.

Vallenet, D., Calteau, A., Dubois, M. et al. (2019b). MicroScope: an integrated platform for the annotation and exploration of microbial gene functions through genomic, pangenomic and metabolic comparative analysis. Nucleic Acids Research.

Viti, C., Pace, A., & Giovenneti, Y. L. (2003). Characterization of Cr(VI)-resistant bacteria

isolated from chromium-contaminated soil by tannery activity. Current Microbiology, 46, 1–5.

Waksman, S. A., & Henrici, A. T. (1943). The Actinomycetes: their taxonomy, physiology, and biochemistry. Philadelphia: The Blakiston Company.

Wood, S. J., Kuzel, T. M., &Shafikhani, S. H. (2023). *Pseudomonas aeruginosa*: Infections, Animal Modeling, and Therapeutics. Cells, 12(1), 199.

Wang, C. L., Michels, P. C., Dawson, S. C., Kitisakkul, S., Baross, J. A., Keasling, J. D., et al. (1997). Cadmium removal by a new strain of *Pseudomonas aeruginosa* in aerobic culture. Applied and Environmental Microbiology, 63(10), 4075–4078.

World Health Organization: WHO. (2017). WHO publishes list of bacteria for which new antibiotics are urgently needed. Word Health Organization. Retrieved April 25, 2023, from https://www.who.int/en/news-room/detail/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed

Yu, L., Yan, X., Wang, L., Chu, J., Zhuang, Y., Zhang, S., & Guo, M. (2012). Molecular cloning and functional characterization of an ATP-binding cassette transporter *OtrC* from *Streptomyces rimosus*. BMC biotechnology, 12(1), 1-12.

Zhu, H., Sandiford, S. K., & van Wezel, G. P. (2014). Triggers and cues that activate antibiotic production by actinomycetes. Journal of Industrial Microbiology and Biotechnology, 41(2), 371-386.

Zhang, Y., Xu, Y., et al. (2018). Reclassification of the genus *Streptomyces* and proposals of 16 new genera and multiple nomenclatural changes based on phylogenetic analyses of type strains. International Journal of Systematic and Evolutionary Microbiology, 68(7), 2563-2583.

Zhang, Z., Sarkar, D., Biswas, J. K., & Datta, R. (2022). Biodegradation of per- and polyfluoroalkyl substances (PFAS): A review. Bioresource Technology, 344, 126223.

MATE
HUNGARIAN UNIVERSITY OF AGRICULTURE AND LIFE SCIENCES

Szent István Campus, Gödöllő
Address: H-2100 Gödöllő, Páter Károly utca 1.
Phone.: +36-28/522-000
Homepage: https://godollo.uni-mate.hu

STUDENT DECLARATION

Signed below, Ma Jiayi, student of the Szent István Campus of the Hungarian University of Agriculture and Life Science, at the MSc Course of Environmental Engineering declare that the present Thesis is my own work and I have used the cited and quoted literature in accordance with the relevant legal and ethical rules. ! understand that the one-page-summary of my thesis will be uploaded on the website of the Campus/Institute/Course and my Thesis will be available at the Host Department/Institute and in the repository of the University in accordance with the relevant legal and ethical rules.

Confidential data are presented in the thesis: (yes) no*

Date: Gödöllő 2023, 5 month 9 day

Ma Jiayi
Student

SUPERVISOR'S DECLARATION

As primary supervisor of the author of this thesis, I hereby declare that review of the thesis was done thoroughly; student was informed and guided on the method of citing literature sources in the dissertation, attention was drawn on the importance of using literature data in accordance with the relevant legal and ethical rules.

Confidential data are presented in the thesis: yes no *

Approval of thesis for oral defense on Final Examination: approved not approved *

Date: Gödöllő, 2023 May month 09 day

signature

***Please, underline the correct choice**