Czech University of Life Sciences Prague

Bachelor Thesis

2020 **Arian MAJID**

Czech University of Life Sciences Prague

Faculty of Agrobiology, Food and Natural Resources

Department of Microbiology, Nutrition and Dietetics

Metabolic Activity of Bacteria Isolated from an Acidic Forest Soil

Bachelor Thesis

Author: Arian Majid

Sustainable Use of Natural Resources ATN

Supervisor: RNDr. Markéta Marečková, Ph.D.

Declaration

I declare that the Bachelor Thesis "Metabolic Activity of Bacteria Isolated from an Acidic Forest Soil" is my own work and all the sources I cited are listed in Bibliography. I also declare that I did not violate any copyrights.

Prague, April 20th, 2020

_____

Acknowledgement:

I would like to acknowledge that this work could not have been done without the help of some people. Marketa Mareckova trusted me enough to give me this project and let me work with her research team in the Crop Research Institute. The tested *Actinobacteria* strains were recovered and isolated by Daria Rapoport, who has been patiently helping me with this research. Tereza Patrmanova and Jan Kopecky were also always there with help during the whole experiment. Thank you.

Metabolic Activity of Bacteria Isolated from an Acidic Forest Soil

Summary:

This study concerns *Actinobacteria* and their production of antibiotics. It also reviews *Actinobacteria* importance in the environment including the production of secondary metabolites. That includes the role of *Actinobacteria* in the carbon cycle, nitrogen fixation, or bioremediation. The study further introduces a short overview of antibiotics and basic antibiotic and resistance mechanisms. Finally, the need for discovering novel antibiotics in order to overcome an increasing bacterial resistance is presented. The theoretical introduction is complemented by an experiment which searches for a new antibiotic produced by *Actinobacteria* living in an acidic littoral soil. For that, 211 *Actinobacteria* strains were grown in laboratory settings on solid media in order to test them against four different microorganisms including a multiresistant *Acinetobacter baumannii* ANC 4097. After the preliminary cultivation, 158 strains were tested further, and 94 of them showed antibiotic activity against *Kocuria rhizophila* while 7 contained antibiotics against *Acinetobacter baumannii* strain ANC 4097, isolated in Czech Republic in 2011 (Krizova et al. 2012). That proved our hypothesis of the potential to find *Actinobacteria* producing compounds active against a multiresistant Gram-negative strain in an unusual environment. Five of those strains were grown in liquid media and the spent medium was successfully extracted by the Solid-phase, while 3 of the extracts retained the antibiotic activity against *Acinetobacter baumannii* ANC 4097. The extracts show the potential for discovery of a new antibiotic and are now subjects for further testing.

Keywords: *Actinobacteria*, soil, low pH, antibiotics, *Acinetobacter baumannii*

Table of Content

1 Introduction

Actinobacteria are microorganisms that play an essential role in the environment. They participate in the carbon cycle through decomposition of dead organic matter because they produce a wide range of decomposing enzymes. They decompose complex compounds and make nutrients available for the ecosystem, while they provide many other ecosystem services. The example includes bioremediation of polluted soils or increase of plant health and productivity. They produce a wide range of secondary metabolites, including many antibiotics, the role of which in nature is not known. However, since the 1940's, these compounds have been used as antibiotics in human, and later also veterinary and plant medicine (Hopwood 2007).

The antibiotics can be harmful also in the natural environment, thus bacteria developed ways to fight them off by resistance. This resistance is transferred to the human environment and generally increases with the use of antibiotics (Monroe & Polk 2000). Over time, bacteria with higher resistance prevail and limit the antibiotics effectiveness. One of the most successful multiresistant pathogens is *Acinetobacter baumannii.* To treat the already resistant bacteria, research of novel antibiotics is crucial. As the most commonly occurring antibiotics have already been discovered, scientists must look at environments with unusual properties with the expectation of finding production strains of compounds with new chemical scaffolds (Butler & Buss 2006).

As the world population increases and the quality of the environment declines, the question of sustaining natural resources becomes a relevant issue. Since food supply is crucial for human existence, finding more sustainable ways of agriculture is equally important. Researching *Actinobacteria* qualities deepens the overall scientific knowledge of the soil and may lead to finding solutions to make agriculture more sustainable.

2 Objectives of the work

The main goal of the literature overview was to highlight the importance of *Actinobacteria* and its secondary metabolites production. It points to the crucial role of *Actinobacteria* in the natural environment as well as in medicine. A big part of the review focuses on the increasing resistance of pathogenic bacteria against commonly used antibiotics and the need for research of novel compounds. The literature overview served mostly as an introduction to the following experiment.

The main goal of the experiment was to prove the hypothesis that *Actinobacteria* inhabiting an acidic environment produce a broad spectrum of antibiotics, potentially even novel antibiotics. This hypothesis was suggested in a study conducted by Sagova-Mareckova et al. (2015). In order to do so, it was needed to imitate their natural environment by growing them on acidic media in Petri dishes and testing them against different microorganisms, including multiresistant *Acinetobacter baumannii* ANC 4097. The main goal was to find strains, which carry biosynthetic pathways of compounds with antibiotic activity against *Acinetobacter baumannii* ANC 4097. Yet, there was also a side goal to map the antibiotic activity against Gram-positive *Kocuria rhizophila*, Gram-negative *Escherichia coli* and *Saccharomyces cerevisiae* and compare their frequency of occurrence to that of *Acinetobacter baumannii*. As a part of the study, four commonly used antibiotics were tested against *Acinetobacter baumannii* ANC 4097. The resistance genes of this strain against these antibiotics were not yet well mapped. Gentamicin, streptomycin and kanamycin were used to map *A. baumannii* resistance genes against aminoglycosides and to check the credibility and uniformity of the results. Chloramphenicol was also used to map its activity against *Acinetobacter baumannii* ANC 4097.

3 Literature Overview

3.1 *Actinobacteria*

Actinobacteria are one of the most represented phylum amongst *bacteria*. They are characterized by a Gram-positive cell wall and a high guanine and cytosine content (Ventura et al. 2007). Their most important habitat is soil, home for *Streptomycetaceae*, a family widely used in antibiotic production (Anandan et a.l 2016). Currently, marine *Actinobacteria* are also gaining attention because of important secondary metabolite discoveries (Ward & Bora 2006). Besides the production of antibiotics, *Actinobacteria* are also an important part of soil because they play a major role in decomposition of dead organic material, while they can produce useful extracellular enzymes (Bhatti et al. 2017; Salwan & Sharma 2020).

3.1.1 Taxonomy of *Actinobacteria*

Actinobacteria represent one phylum of *bacteria* but their phylogenetic separation cannot be estimated, nor which other phylum is their closest relative (Ventura et al. 2007). The taxonomy of *Actinobacteria* is presently based on the 16s rRNA gene phylogeny and is divided into six classes: *Actinobacteria, Acidimicrobiia, Coriobacteriia, Nitriliruptoria, Rubrobacteria, and Thermoleophilia.* Over 80% of known families belong to the class of *Actinobacteria.* As the new gene sequencing is being done and new species are being described, the understanding of the relationships between the families quickly changes; thus, changing the whole *Actinobacteria* taxonomy (Gao & Gupta 2012; Barka et al. 2015). The study done by Verma et al. (2013) suggests that the phylogenetic analyses based on the whole genome sequencing might improve the accuracy of the current taxonomic classification. The most widespread family is *Streptomycetaceae*, which accounts for over 95% of the known *Actinobacteria* strains isolated from soil (Barka et al. 2015).

3.1.2 *Actinobacteria* in soil

Despite belonging to *bacteria*, *Actinobacteria* often resemble fungi. They can grow in morphologies like coccoid, rod-coccoid or hyphal forms, but in soil, they usually grow as a highly branched filamentous mycelium (Ventura et al. 2007, Anandan et a.l 2016). Alongside fungi, they are the most important decomposers. In spring, they represent around 20 % of all microbial organisms in soil and over 30 % in autumn. In winter, the number drops to 13 %, as the fresh organic matter in the soil decreases (Barka et al. 2015). According to Bentley et al. (2002), *Actinobacteria* such as thermophilic *Saccharomonospora viridis* and *Thermobifida fusca* or mesophilic *Micrococcineae* participate in decomposition of lignocellulosic materials. Other thermophilic *Actinobacteria* found in composts, *Streptomycetes* spp are capable cellulose degraders. The same study suggests that adding heat to compost; thus, increasing the percentage of *Actinobacteria* species, speeds up the decomposition. Another study (Větrovský et al. 2014) shows that a single *Actinobacteria* strain, *Streptomyces coelicolor,* is able to produce 7 cellulases and 5 chitinases, proteins important in decomposition of organic compounds. Generally, *Actinobacteria* are known to create many extracellular enzymes that are able to decompose complex polysaccharides. These compounds are then used in other processes in soil, leading to dissolved carbon available to plants; therefore, *Actinobacteria* play an important role in the carbon cycle (Větrovský et al. 2014).

Furthermore, some *Actinobacteria* participate in important processes in soil such as nitrogen fixation or bioremediation of anthropogenic pollution. Nitrogen is an important macronutrient, since it is the building block of amino acids, but as an industrial fertilizer, it appears in places where it becomes a major pollutant for water (Singh & Sekhon 1979). Symbiosis between some plants and species from the genus *Frankia* are believed to be responsible for 15 % of the world's naturally fixed nitrogen (Bhatti et al. 2017). The use of these species could reduce the need for industrial nitrogen for fertilization. Yet, organic pollutants entering soil from industry are degraded by *Actinobacteria*, which seem to be an ideal solution for bioremediation because they are able to remove substances such as pesticides or heavy metals from the soil, making it once more safe for plant production (Alvarez et al. 2017). Through those processes, *Actinobacteria* can indirectly improve plant growth, but *Actinobacteria* can

also increase the plant production directly. Some of them live in the plant rhizosphere, where they increase the availability of nutrients, minerals and plant growth regulators (Bhatti et al. 2017). In recent study, Elgawas et al. (2019) proved that the addition of *Actinobacteria* to the date palms overall increased their health and yield. The same result was obtained in another recent study (Solá et al. 2019), but this time under heavy pollution of Cr(VI) and lindane. The study also highlights the importance and complexity of relationships between plants, pollutants, soil, and *Actinobacteria.*

3.1.3 Secondary metabolites production by *Actinobacteria*

While decomposing complex compounds, *Actinobacteria* have to fight over the sacred resources and their place in the soil. This leads to a production of secondary metabolites, often found with antibiotic activity (Raja & Prabakarana 2011). *Actinobacteria* are responsible for around 10,000 out of 23,000 bioactive metabolites used in pharmacy and produced by all the microorganisms combined. Out of these 10,000, around 7,600 are found in *Streptomyces* (Salwan & Sharma 2020). As they live in different environments, they have to fight against different bacteria, creating a wide range of antibiotics (Sagova-Mareckova et al. 2015).

In a study conducted by Elbendary et al. (2018), where the scientists obtained 100 strains of *Actinobacteria* from a single farming soil in Egypt, 12 of them showed a wide range of antibiotic activity. Another study showed that out of 150 *Actinbacteria* found in soil in Bangladesh, 20 of them showed antibiotic activity (Rahman et al. 2011). The same results concluded a study evaluating *Actinobacteria* separated from soil in western Iran (Dehnad et al. 2010). These and similar studies demonstrate the high frequency of *Actinobacteria* producing antibiotics, living in various types of soil over a large geographical distance.

A relatively new source of *Actinobacteria* with high production of antibiotics is a marine environment. In the past, researches thought that antibiotics produced by marine and soil *Actinobacteria* are very similar, and since it is easier to obtain soil samples, marine *Actinobacteria* remained neglected. But recent studies show that different aquatic environments contain *Actinobacteria* producing novel antibiotics (Manivasagan et al. 2014).

Since it has been suggested that *Actinobacteria* living in different environments can yield different antibiotics, researchers started looking at very specific places. In one study, *Actinobacteria* were isolated in Antarctica, and out of 39 strains, 15 produced bioactive compounds, there (Lee et al. 2012). Another study looked at *Allomerus* ant colonies, where they found *Actinobacteria* producing novel antibiotics, helping ants fight against unwanted microorganisms (Seipke et al. 2012).

The first antibiotics derived from *Actinobacteria* were discovered in the 1940s by Waksman and his team. Those included actinomycin D, streptothricin and streptomycin (Hopwood 2007). Those three antibiotics are ones of the most frequently occurring in the soil and they are isolated from *Streptomyces* (Barka et al. 2015). These discoveries were followed by the discovery of chloramphenicol, chlortetracycline, oxytetracycline, and nystatin in the late 40's. The 50s and 60s are referenced to as the golden age of antibiotics, when many important antibiotics were discovered, such as neomycin, gentamicin, rifamycin or kanamycin. Since then, the number of important discovered antibiotics is constantly declining (Spížek et al. 2016).

3.2 Antibiotics

Antibiotics can be translated as "opposing life." Since the 1940s, they have been used by physicians to treat bacterial infections and various bacterial diseases. At the beginning, their importance was crucial in winning World War II, and since then they have saved an uncountable number of people (Hopwood 2007).

3.2.1 Antibiotics classification and mechanism

Antibiotics kill or prevent bacterial growth by targeting certain parts of their cells. Most of them attack DNA replication, RNA synthesis, cell wall synthesis, or protein synthesis (Procópio et al. 2012). A thin layer around the cell wall in most bacteria is called peptidoglycan. It is an essential part of these cells, because it protects it from the outside environment. Most of the antibiotics that prevent the cell wall synthesis do so by inhibiting the peptide bond creation. This prevents the cross-linking of two enzymes, transglycosylases and transpeptidases, which then prevents the synthesis of the peptidoglycan and it kills the cell.

Important antibiotics that kill the bacteria by destroying its cell wall are Vancomycin or Beta-lactams such as penicillins, cephalosporins, or carbapenems. A group of antibiotics called quinolones prevents DNA replication by interfering with the helicase enzyme, which is responsible for unwinding DNA double helix. Most of these antibiotics are fluoroquinolones. Other representatives from this group can also affect enzymes that are responsible for RNA polymerase; thus, preventing RNA synthesis. Since proteins are responsible for all the processes in the cell, antibiotics preventing their synthesis are effective against many bacteria, and they are largely represented. Proteins are synthesized in ribosomes, which are made out of rRNA. The rRNA is divided into small and large subunits. Antibiotics preventing protein synthesis are divided into 30S inhibitors, included in the small subunit and 50S, representing large subunits. The 50S inhibitors prevent the protein synthesis by blocking one of the first two stages of turning mRNA into proteins, translation and elongation. These antibiotics include clindamycin, lincomycin, chloramphenicol, or linezolid. The 30S inhibitors work by restricting aminoacyl-tRNA in the ribosome and they include tetracyclines or aminoglycosides such as gentamicin or streptomycin (Etebu & Arikekpar 2016; Moore 2020).

3.2.2 Bacterial resistance

Bacterial resistance naturally occurs when bacteria are exposed to antibiotic compounds. This behavior has been observed ever since antibiotics came to use. In a report from 1948, M. Demerec observed a series of experiments of penicillin interacting with *Staphylococcus aureus* and streptomycin interacting with *S. aureus* and *Escherichia coli*, strain B. Based on those observations, he created survival curves, which prove that over time, those bacteria found ways to survive the antibiotics application.

Three major types of bacterial resistance mechanism are recognised (Todar). Firstly, efflux pumps in the cell membrane transport antibiotics out of the cell. Secondly, specific enzymes modify the antibiotic, so it is no longer harmful to the cell, and thirdly enzymes that destroy the antibiotic.

Two types of bacterial resistance are recognized by their origin, inherited and acquired (Todar). Inherited resistance is a naturally occurring resistance. Strains

producing a particular antibiotic are also inheritably resistant to it. When antibiotics are used, these bacteria remain unaffected and can pass down their resistance genes, just like in Darwinian Theory (Livermore 2003).

Acquired resistance is when random mutations cause resistance. Then again, these bacteria survive and pass down the mutated, resistant genes. Mutations usually happen with a small frequency, 10^{-10} to 10^{-9} per base pair replicated (Bridges 2001), but with higher usage of antibiotics, the chance of mutations in bacteria and developing resistant genes rises quickly. When these bacteria reproduce and pass down the resistance genes, it is called a vertical gene transfer (Todar). But they can also pass their genes to unrelated bacteria through horizontal gene transfer. This can happen through transformation, meaning bacteria picking up DNA with resistant genes of dead bacteria, transduction, which is transferred via lysogenic bacteriophages, and most importantly conjugation by plasmides. Since most resistance mechanisms are managed by plasmids, and they are equipped with transposons, the resistant genes are carried between the plasmids, in and out of the chromosome, and some of them in other bacteria, sometimes even different species (Todar; Livermore 2003).

Epidemiologically, we recognize three levels of resistance, local, national, and international (Livermore 2003). First resistance occurred in the 1940s in *Staphylococcus aureus*, after penicillin came to use (Monroe & Polk 2000). Most of the resistance epidemics are local, but with higher use of antibiotics, they can spread widely. Over time, *Staphylococcus aureus* resistance against different antibiotics, methicillin, which falls under the same class as penicillin, reached 30 - 45 % in Spain, Portugal, Italy, France, or the United Kingdom (Livermore 2003). A study conducted by Hill et al. (1998) proves the link between the use of antibiotics and the increase of percentage of *S. aureus* resistant against methicillin. There are many examples of a direct link between an increase of antibiotic usage and an increase of bacterial resistance (Livermore 2003). One of the more recent ones is an increase of fluoroquinolones usage in Canada from 0.8 per person per year in 1988 to 5.5 per person per year in 1997, while an increase of resistance of *Streptococcus pneumoniae* to fluoroquinolones from 0 % in 1988 to 1.7 % in 1993. Until the year 1998, the resistance doubled in adults.

The increase of bacterial resistance is a serious threat to the functionality of antibiotics, and the overuse of antibiotics only speeds it up. In the United States, 30 - 50

% of the average hospital drug budget is spent on antibiotics, but around 50 % of these prescriptions are unnecessary (Hill et al. 1998). However, one Finland study (Seppälä et al. 1997) proved that a decrease in the use of antibiotics leads to a decrease in bacterial resistance. When macrolide use increased from 1988 to 1993, the bacterial resistance against it increased from 5 % to 19 %, but later, as the use dropped by 50 %, the bacterial resistance dropped to 8.6 %. The only ways to prevent the resistance crisis we are facing are reduction of use of antibiotics, prescription of the right antibiotics, prevention of spread of resistant bacteria, and search for new antibiotics (Livermore 2003).

3.2.3 Multiresistant *Acinetobacter baumannii*

Among bacteria resistances, multiresistance is the most feared, because it prevents treatments by many antibiotics. Pathogenic, Gram-negative *Acinetobacter baumannii* carries resistances to most known antibiotics and its rates of resistance are still increasing (Perez et al. 2007). Multiresistant *A. baumannii* is usually found in hospitals in intensive care units, but lately it has been found also in wounded soldiers returning from Iraq or Afghanistan, or in countries with historically low resistance rates like Norway (Onarheim et al. 2000; Davis et al. 2005). One study conducted by Smith et al. (2007) found that A. baumannii, strain ATCC 17978, DNA contains a considerable number of resistance genes. Also, a big proportion of its DNA comes from a foreign source, suggesting that *A. baumannii* can easily pick up resistance genes from other bacteria. Another study conducted in Czech Republic by Krizova et al. (2012), sequenced *A. baumannii,* ANC 4097, and found many resistance genes, including resistance genes against beta-lactams, fluoroquinolones, aminoglycosides, and tetracyclines. Its ability to quickly adopt different resistance genes makes *A. baumannii* a very successful outbreaks causing disease.

Such diseases, usually nosocomial infections, can include mostly ventilator-associated pneumonia and bloodstream infections. Less often, *A. baumannii* can cause community-acquired bronchiolitis, tracheobronchitis, or pneumonia and infections of skin, soft tissues, or urinary tract. However, these diseases are mostly found in patients with weakened immune systems caused by some worsen health

conditions such as diabetes, cancer, or alcoholism (Asif et al. 2018; Harding et al. 2018).

3.2.4 Development of new antibiotics

The bacterial resistance against frequently used antibiotics is rapidly increasing. One of the best ways to overcome this problem is searching for new antibiotics (Donadio et al. 2010). The importance of findings of new antibiotics has been highlighted in the "WHO priority list of antibiotic-resistant bacteria and tuberculosis." This list indicates *A. baumannii*, among those having a critical priority in searching for antibiotics against (Tacconelli et al. 2018). Especially important is to find novel classes of antibiotics, with different resistance mechanisms.

Novel antibiotics can be found either in nature or modified in the lab. For example, lantibiotics have been chemically modified to better serve their original antimicrobial purpose (Donadio et al. 2010). Yet, another way to find antibiotics is to look at unexplored environments as well as unexplored types of organisms. As an example serves the discovery of a new antibiotic called abyssomicin found in an *Actinobacteria, Verrucosispora,* in a deep sea environment (Bister et al. 2004).

When looking for new antibiotics of some novel class, some criteria need to be met. Most importantly, the target of their mechanism has to be clear. This target should be different than in antibiotics that are already in use, so there would not be any bacterial resistance against its mechanism. This target needs to slow the growth of the bacteria, or kill it, and it should be present in a wide range of pathogen bacteria. At the same time, a similar target cannot appear in any human cell, so the antibiotic would not negatively influence human organism (Hughes & Karlen 2014). When the target of an antibiotic is clear and it meets these requirements, it is called a hit. These hits undergo further physical and chemical tests and can potentially become pharmaceutically used antibiotics.

4 Methodology

Antibiotic activity was tested using solid mediums R2A55 and Gauze medium. The tested strains included Gram-positive *Kocuria rhizophila*, Gram-negative *Escherichia coli*, fungus *Saccharomyces cerevisiae* and Gram-negative *Acinetobacter baumannii* ANC 4097, possessing several antibiotic resistances (see below).

Site:

All 211 *Actinobacteria* strains used for the research were collected in 2015 in the littoral of Opatovicky fishpond near the town of Trebon, South Bohemia. They were stored in glycerol preservation at -70 °C.

4.1 Cultivation of *Actinobacteria*

The first goal was to achieve optimal homogenous growth of all strains for further testing on Petri dishes. For this, acidic R2A55 medium with pH 5.5 was used, to imitate their natural habitat. The medium prepared in 0.5l plastic flask consisted of: 0.5 g Bacto Proteose Peptone, 0.5 g yeast extract, 0.5 g casamino acids, 0.5 g glucose, 0.5 g soluble starch, 0.3 g Na-pyruvate, 0.3 g $KHPO_4$, 0.05 g $MgSO_4*7H_2O$, and 1.95 g MES acid. Then, 0.5 l of distilled water was added, mixed well on Variomag Electronicruhrer mixing device (Thermo Scientific, China) and pH was measured by HI 221 Microprocessor pH Meter (HANNA instruments). The pH was adjusted to 5.5. After testing for pH, 0.5 l of the medium was split in two 0.5l autoclavable SIMAX glass bottles “Blue Screw Cap Lab”, so they were only half full to prevent spilling in the autoclave. In a new 0.5l beaker, 3% agar was prepared using 15 g of agar and 0.5 l of distilled water and split into two 0.25l bottles. All four bottles marked with a sterilization tape were sterilized in an autoclave for 20 minutes at 121 °C. After cooling the bottles to 55 °C, they were moved to the laminar flow box Aura vertical S.D.4 (Bioair, EuroClone SpA, Italy), along with plastic gloves, 70% ethanol and 60 mm

GAMA petri dishes and sterilized with UV light for 20 minutes. In the flowbox, the medium was mixed with the agar carefully, so there would not be any bubbles. Petri dishes were filled and stocked to prevent water condensation on the lids.

Petri dishes with agar media, new gloves, bottle with ethanol, plastic inoculation loops, centropen, and a holder for conserves with *Actinobacteria* strains were left under UV light for twenty minutes. Twenty *Actinobacteria* samples were removed from the freezer. Media on Petri dishes were inoculated from the freezed cultures using a plastic loop. Inoculated strains were placed into a thermostat incubator at 28 °C for 5 days. Some samples were contaminated. Since *Actinobacteria* grow slower than other bacteria, the approach was adjusted by switching to a different flowbox and inoculating 100 µl from the culture conserve using the 20-200 µl pipette and 250 µl FINNTIP tips with cut ends. Finally, all 211 strains were cultivated and kept in the fridge at 4 °C enclosed by Parafilm.

4.2 Antibiotic activity

First, twenty strains grown on petri dishes were picked and inoculated on clean Petri dishes with R2A55 agar medium. They were kept in an incubator set to 28 °C for two weeks. Four microorganisms that would test antibiotic activity in *Actinobacteria* were cultivated. These included Gram-negative *Escherichia coli*, Gram-positive *Kocuria rhizophila*, eukaryotic *Saccharomyces cerevisiae*, and multiresistant *Acinetobacter baumannii* ANC 4097. B1 medium was used to cultivate the three bacteria by mixing the following: 5 g beef extract, 5 g peptone, 2.5 g NaCl, 10 g agar and 0.5 l distilled water, and it was split into two 0.5 l glass bottles. *Saccharomyces cerevisiae* was grown on YPG medium, which consisted of: 5 g yeast extract, 5 g peptone, 35 g glucose, 7.5 g agar and 0.5 l distilled water. Media were sterilized in an autoclave along with toothpicks, a 0.25l glass bottle half filled with distilled water, and two sizes of tips - 250 µl and 1000 µl. Media were then put in a flowbox alongside 90 mm GAMA petri dishes and sterilized with UV light. Media were poured into Petri Dishes and inoculated with the four testing microorganisms, taking 50 µl from conserves using 20-200µl pipette and spread with a plastic loop. Petri Dishes with

Acinetobacter baumannii and *E. coli* were kept in an incubator set to 37 ºC and *Saccharomyces cerevisiae* and *Kocuria rhizophila* for 30 ºC, both for 24 hours.

B1 and YPG liquid media were mixed the same way as solid media, excluding agar from the solution. Sterilized liquid media, 1 ml tips, 200-1000 µl pipette, inoculation loops, and test tubes were put in a flow box and sterilized with UV light. 3 ml of the liquid media were poured in the test tube, and an almost invisible amount of the testing microorganisms from the Petri dishes was added, using the tip of an inoculation loop. Test tubes with liquid media and microorganisms were placed for 24 hours in an Environmental Shaker - Incubator ES-20 set for 37 ºC at 200 RPM (rotations per minute). After 24 hours, distilled water, test tubes, 200-1000 µl pipette, 20-200 µl pipette, 250 µl and 1000 µl tips, sterilized toothpicks, and petri dishes with B1 and YPG media were put in the flowbox. After UV light sterilization, the testing microorganisms in the liquid media, the first 20 tested *Actinobacteria* strains grown for two weeks and McFarland Standard tubes with concentration numbers 0.25, 0.5, and 1 were placed in flowbox. There, 5 ml of distilled water and 50 µl of the testing microorganisms was poured in four test tubes and the concentration was adjusted to 0.5 using McFarland Standards. 1 ml of the solution with testing microorganisms was poured on clean Petri Dishes with B1 (for Saccharomyces cerevisiae YPG), evenly spread, and pipetted out. To transplant *Actinobacteria* on the testing microorganisms, toothpicks and other sides of the 1000 µl tips with 8 mm in diameter were used. The tests were placed in an incubator for 24 hours. *Acinetobacter baumannii* and *E. coli* at 37 ºC and *Saccharomyces cerevisiae* and *Kocuria rhizophila* at 30 ºC. After 24 hours, the samples were observed and all the zones between *Actinobacteria* and the testing organisms and their sizes and shapes were written down. After the first two tests, the method of testing was adjusted by growing *Actinobacteria* on petri dishes three weeks prior to testing on both R2A55 and Gauze media, and making YPG media with glucose, instead of glycerol. Gauze media was mixed by adding following: 0.25 g K_2HPO_4, 0.5 g KNO_3, 0.25 g NaCl, couple of crystals of $MgSO_4*7H_2O$, 10 g soluble starch, 10 g agar, and 0.5 l distilled water. Testing on *E. coli* and *Saccharomyces cerevisiae* was eliminated for the second trial of testing in order to save time and increase the number of successfully tested strains on *Acinetobacter baumannii.* Four commonly used antibiotics were tested against *Acinetobacter baumannii* ANC 4097 to compare their

antimicrobial activity with the *Actinobacteria* strains. Those included: 25 μg per disc of gentamicin and streptomycin and 30 μg per disc of kanamycin and chloramphenicol.

4.3 Extraction of antibiotic compounds from antibiotically active strains

The *Actinobacteria* strains that showed antibiotic activity against *Acinetobacter baumannii* ANC 4097 were grown in liquid media. First, in liquid GYM media, obtained by mixing 4 g glucose, 4 g yeast extract, 10 g malt extract, 2 g $CaCO_3$, and 1 l distilled water. 10 ml of this medium was put in a 50ml Erlenmeyer flask, covered with a sterilization wrap and sterilized in an autoclave. *Actinobacteria* strains were transferred in GYM liquid medium from Petri dishes in the flow box, using inoculation loops. They were put in an Ecotron incubator shaker (Infors HT, Switzerland) set to 28 ^oC and 180 RPM for two days. 1 ml of this media was transferred in a 20 ml of Gauze liquid media and kept in the incubator shaker for 10 days.

The liquid media with grown strains were put in centrifuge tubes, which were inserted in a Universal 32 R centrifuge (Hettich, Germany) set to 4000 RPM and 10 minutes. This liquid sample was once again tested for its antibiotic activity against the testing microorganisms on Petri Dishes. It was also used for the Solid-Phase Extraction. Two types of extraction cartridges were used for this method: HLB and MCX, both 3cc (60 mg) (OASIS, Ireland). These cartridges were put in a SPE vacuum manifold and filled with chemicals in following order: For HLB 3 ml methanol, 3 ml Milli Q water, 6 ml liquid sample, 3 ml Mili Q water and for MCX 3 ml methanol, 3 ml 2% formic acid, 6 ml liquid sample with pH adjusted to 2-3 by adding formic acid, and 3 ml 2% formic acid. The cartridges were filled with 1.5 ml methanol, put in 15ml centrifuge tubes and in a centrifuge set to 1200 RPM for one minute. For MCX, the cartridge was then filled with 1.5 ml methanol + 5 % of ammonium hydroxide (from 29% solution) and centrifuged. The extracts in the centrifuge tubes were tested for their antibiotic properties against *Acinetobacter baumannii* ANC 4097 and *Kocuria rhizophila* on Petri dishes by putting 6 μl of extracts on circle-shaped sterilized pieces of filtration paper (discs) with 6 mm in diameter.

5 Results

5.1 Cultivation and testing of *Actinobacteria*

In the first trial of the experiment, all 211 strains were grown on R2A55 agar medium, but only 53 were tested, due to high number of contaminations by fungi and bacteria. These contaminations were caused mainly during inoculation or storage due to my low experience. 37 strains were contaminated by bacteria and 68 by fungi. Out of the 54 tested strains, none were active against *Acinetobacter baumannii* ANC 4097. Only two strains were active against *E. coli* and 11 strains showed activity against *Saccharomyces cerevisiae,* though in most parts of the experiment it grew very poorly. 20 strains showed zones against *Kocuria rhizophila* (Figure 2). Due to the low number of tested strains, it was decided to grow all non-tested strains one more time. During the second trial, 138 strains were grown on both Gauze and R2A55 agar media, out of which 105 were tested on at least one of them. Thus, 90 strains were tested on Gauze media and 94 on R2A55. The contamination by either bacteria or fungi was significantly lower in this part of the experiment. Main reason for excluding samples was no visible growth. 42 samples did not show any growth on either one of the media or on both of them. On the R2A55 medium, 51 strains showed some activity against *Kocuria rhizophila*, while 28 of them showed zones 10 mm in diameter or smaller and 23 showed zones in range from 11 to 14 mm. No strains grown on R2A55 medium showed bigger zones or any activity against *Acinetobacter baumannii* ANC 4097 (Figure 4). The strains grown on Gauze medium showed 48 zones against *Kocuria rhizophila*, but they were bigger on average (Figure 5). 27 strains were 10 mm and smaller and 21 strains were 11 mm and bigger. 11 strains out of these 21 were bigger than 15 mm reaching up to 33 mm in diameter. More importantly, 7 strains grown on Gauze medium showed antibiotic activity against *Acinetobacter Baumannii* ANC 4097 (Table 1 and Table 3). The numbers of positive *Actinobacteria* strains against each

testing microorganism are used in Figures 2, 4 and 5, creating pie charts with percentages of their antimicrobial properties.

5.2 Testing frequently used antibiotics on *Acinetobacter baumannii*

Four commonly used antibiotics were tested multiple times against *Acinetobacter baumannii* ANC 4097 - 30 µg per disk of kanamycin and chloramphenicol and 25 µl per disk of streptomycin and gentamicin. All the disks were 6 mm in diameter. The zones were constant throughout the experiment. Gentamicin had a zone of 9 mm in diameter, kanamycin and chloramphenicol had almost invisible zones, and streptomycin had 15 mm zones.

5.3 Testing extracts from the Solid-Phase Extraction

Only 5 strains with antibiotic activity were selected and successfully grown in the liquid media (all of them on Gauze). Those included *Streptomyces* strains 15Tr742, 15Tr784, and 15Tr792 and *Kitasatospora* strains 15Tr67 and 15Tr752. When testing liquid media against *Acinetobacter baumannii* ANC 4097 before the extraction, (circular pits filled with the liquid media had 7 mm in diameter) strain 15Tr742 showed 12 mm zone, strain 15Tr784 showed 11 mm zone, strain 15Tr792 and 15Tr67 showed 14 mm zone and strain 15Tr752 did not show a zone. When testing the same liquid media against *Kocuria rhizophila,* strain 15Tr752 showed an 18 mm zone and the rest of strains showed zones between 30 and 40 mm in diameter. When testing the extracts (on 6 mm discs), the strains that were separated on HLB cartridges did much better than those separated on MCX. The HLB extract from strain 15Tr67 showed 7 mm zone against *Acinetobacter baumannii* ANC 4097 and 20 mm zone against *Kocuria rhizophila,* while the MCX extracts did not show anything against *Acinetobacter baumannii* ANC 4097 and only 14 mm (methanol extract) and 8 mm (second extraction) zones against *Kocuria rhizophila*. Similar results had the extracts from strain 15Tr792. On the other hand, the HLB extract from strain 15Tr742 showed 7 mm zone against *Acinetobacter baumannii* ANC 4097, but the MCX extract #2 (second extraction) showed a 10 mm zone, although against *Kocuria rhizophila* the results were

reversed. The extracts from the strains 15Tr752 and 15Tr784 did not make any zones but the HLB extraction was not executed for strain 15Tr784. Because this strain proved to have some activity during the test of the liquid media, it might have a potential to show a zone on HLB as well.

6 Discussion

6.1 Interpretation of the results

A great difference in cultivation results was observed between the first and the second trial of testing. The first trial provided significantly fewer results. This was caused mainly by the level of my experience. In the first trial, most samples were contaminated and unclear, while the testing microorganisms grew poorly. Also, all the strains showing some antibiotic activity against *Acinetobacter baumannii* ANC 4097 were grown on Gauze media, which was not used during the first trial. Although the first trial provided fewer results, it gave us data about the antibiotic activity against *E. coli* and *S. cerevisiae* in an acidic environment (R2A medium with pH adjusted to 5.5). As shown in the figures 2, 4 and 5, percentages of the strains positive against *Kocuria rhizophila* were considerably close in the first and the second trial, which contributes to the credibility of the experiment. According to Table 2, around 10 % of the samples were antibiotic active against *Saccharomyces cerevisiae.* This could occur because in an acidic forest soil, many decomposing processes are carried out by fungi. *Actinobacteria* have to fight over the resources and they have adopted this antibiotic activity as an instrument to do so. When compared to research works mentioned in the literature review (Dehnad et al. 2010; Rahman et al. 2011; Elbendary et al. 2018), the percentage of *Actinobacteria* producing antibiotic secondary metabolites in this experiment was more than twice higher. This is supported by studies (Lee et al. 2012; Manivasagan et al. 2014) which suggest that environments with unusual characteristics can contain *Actinobacteria* with unusual properties and often with high antibiotic production.

.

6.2 Antibiotic activity by genera

The Table 1 includes a list of genera of each strain. Most zones against *Kocuria rhizophila* (44) were observed in *Streptomyces*, which was also the most represented genus (63 were tested). Second was *Streptacidiphilus* which showed 30 zones out of 55 tested strains. *Catenullispora* showed 11 out of 21 and poorly represented genera

included *Kitasatospora* (5 out of 9), *Nocardia* (3 out of 5), and *Micromonospora* with one active strain out of one tested. Out of the four highest represented genera, *Streptomyces* had the highest percentage of active strains against both *Kocuria rhizophila* and *Acinetobacter baumannii* ANC 4097. This finding corresponds well with the literature, which states that 80 % of all antibiotics are derived from *Streptomyces,* with streptothricin being the first one in 1942. Other examples of antibiotics derived from *Streptomyces* include streptomycin, chloramphenicol, neomycin or tetracycline (Procópio et al. 2012).

6.3 Relevance of the experiment

This experiment was a follow up research of a study conducted by Sagova-Mareckova et al. (2015) and it proved that the acidic environment contained *Actinobacteria* with high antibiotic production that were positive even against the multiresistant *Acinetobacter baumannii* ANC 4097. The strains with these properties were extracted and three of them showed antimicrobial properties against both *Kocuria rhizophila* and *Acinetobacter baumannii* ANC 4097*,* but they were not further tested. It is now known that these extracts contain some kind of an important antibiotic, but it remains a goal for a follow up research to find out their chemical structure and treatment potential. They could be just already known antibiotics, for which the multiresistant *Acinetobacter baumannii* ANC 4097 is now known not to be resistant such as gentamicin or streptomycin. If the extracts prove to be novel compounds with different chemical scaffolds, they could be potentially used in pharmacy in future and they could help the scientists with the fight against bacterial resistance (Butler & Buss 2007).

7 Conclusions

- Further research of *Actinobacteria* secondary metabolites may lead to important discoveries beneficial to sustainable agriculture. Such sustainable solutions include decomposition of dead organic matter, treating polluted soil, or increasing agricultural production.

- *Actinobacteria* from acidic soil located near Trebon proved to produce a wide range of antibiotics and it is a question of more research to figure out if these antibiotics could be novel and useful in treatments of bacterial diseases of plants, animals or even people

- In the first trial of the experiment, 4 % of *Actinobacteria* strains were antibiotic active against *E. coli*, 21 % were active against S. *cerevisiae*, and 37 % were active against *K. rhizophila.* (Figure 2). In the second trial tested on R2A55 media, 54 % were active against *K. rhizophila* and on Gauze media, 53 % were active against *K. rhizophila* and 8% against *Acinetobacter baumannii* ANC 4097 (Figures 4 and 5).

8 References

Alvarez A, Saez JM, Costa JSD, Colin VL, Fuentes MS, Cuozzo SA, Benimeli CS, Polti MA, Amoroso MJ. 2017. Actinobacteria: Current research and perspectives for bioremediation of pesticides and heavy metals. Chemosphere **166**:41-62.

Anandan R, Dharumadurai D, Manogaran GP. 2016. An Introduction to Actinobacteria, Actinobacteria - Basics and Biotechnological Applications. IntechOpen, DOI: 10.5772/62329.

Asif M, Alvi IA, Rehman SU. 2018. Insight into Acinetobacter baumannii: pathogenesis, global resistance, mechanisms of resistance, treatment options, and alternative modalities. Infection and Drug Resistance **11**:1249-1260.

Barka EA, Vatsa P, Sanchez L, Gaveau-Vaillant N, Jacquard C, Klenk HP, Clément C, Ouhdouch Y, van Wezel GP. 2015. Taxonomy, Physiology, and Natural Products of *Actinobacteria.* Microbiology and Molecular Biology **80**:1-43.

Bentley S, et al. 2002. Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). Nature **417**:141–147.

Bhatti AA, Haq S, Bhat RA. 2017. Actinomycetes benefaction role in soil and plant health. Microbial Pathogenesis **111**:458-467.

Bister B, Bischoff D, Strobele M, Riedlinger J, Reicke A, Wolter F, Bull AT, Zahner H, Fiedler HP, Sussmuth RD. 2004. Abyssomicin C—A Polycyclic Antibiotic from a Marine *Verrucosispora* Strain as an Inhibitor of the *p*-Aminobenzoic Acid/Tetrahydrofolate Biosynthesis Pathway. Angewandte Chemie International Edition **43**:2574-2576.

Bridges BA. 2001. Hypermutation in bacteria and other cellular systems. MRC Cell Mutatio Unit, University of Sussex, Falmer, Brighton, UK

Butler MS, Buss AD. 2006. Natural products — The future scaffolds for novel antibiotics?. Biochemical Pharmacology **71**:919-929.

Davis KA, Moran KA, McAllister CK, Gray PJ. 2005. Multidrug-resistant Acinetobacter extremity infections in soldiers. Emerg Infect Dis. **11**:1218–1224.

Dehnad A, Mokhtarzadeh A. Parsa L, Bakhshi R, Soofiani SA. 2010. Investigation antibacterial activity of Streptomycetes isolates from soil samples, West of Iran. African Journal of Microbiology Research **4**:1542-1549.

Demerec M. 1948. Origin of bacterial resistance to antibiotics. Department of Genetics, Carnegie Institution of Washington, Cold Spring Harbor, New York

Donadio S, Maffioli S, Monciardini P, Sosio M, Jabes D. 2010. Antibiotic discovery in the twenty-first century: current trends and future perspectives. J Antibiot **63**:423–430.

Elbendary AA, Hessain AM, El-Hariri MD, Seida AA, Moussa IM, Mubarak AS, Kalbi SA, Hemeg HA, Jakee JKE. 2018. Isolation of antimicrobial producing *Actinobacteria* from soil samples. Saudi Journal of Biological Sciences **25**:44-46.

Elgawad HA, Saleh AM, Jaouni SA, Selim S, Hassan MO, Wadaan MAM, Shuikan AM, Mohamed HS, Hozzein WN. 2019. Utilization of actinobacteria to enhance the production and quality of date palm (*Phoenix dactylifera* L.) fruits in a semi-arid environment. Science of The Total Environment **665**:690-697.

Etebu E and Arikekpar I. 2016. Antibiotics: Classification and mechanisms of action with emphasis on molecular perspectives. BluePen Journals

Gao B, Gupta RS. 2012. Phylogenetic Framework and Molecular Signatures for the Main Clades of the Phylum *Actinobacteria.* Microbiology and Molecular Biology Reviews **76**:66-112.

Harding CM, Hennon SW, Feldman MF. 2018. Uncovering the mechanisms of Acinetobacter baumannii virulence. Nat Rev Microbiol **16**:91–102.

Hill DA, Herford, Parratt D. 1998. Antibiotic usage and methicillin-resistant Staphylococcus aureus: an analysis of causality. J Antimicrob Chemother **42**: 676–677.

Hopwood DA. 2007. Streptomyces in nature and medicine. Oxford University Press, New York, NY.

Hughes D, Karlen A. 2014. Discovery and preclinical development of new antibiotics. Upsala Journal of Medical Sciences **119**:162-169.

Krizova L, Bonnin RA, Nordmann P, Nemec A, Poirel L. 2012. Characterization of a multidrug-resistant *Acinetobacter baumannii* strain carrying the *bla*NDM-1 and *bla*OXA-23 carbapenemase genes from the Czech Republic. Journal of Antimicrobial Chemotherapy **67**:1550–1552.

Lee L, Cheah Y, Mohd Sidik S, Mutalib NSA, Tang YL, Lin HP, Hong K. 2012. Molecular characterization of Antarctic actinobacteria and screening for antimicrobial metabolite production. World J Microbiol Biotechnol **28**:2125–2137.

Livermore DM. 2003. Bacterial Resistance: Origins, Epidemiology, and Impact. Clinical Infectious Diseases **36**:11–23.

Manivasagan P, Venkatesan J, Sivakumar K, Kim SK. 2014. Pharmaceutically active secondary metabolites of marine actinobacteria. Microbiological Research **169**:262-278.

Monroe S, Polk R. 2000. Antimicrobial use and bacterial resistance. Current Opinion in Microbiology **3**:496-501.

Moore D. 2020. Orthobullets. Antibiotic Classification & Mechanism. Available from https://www.orthobullets.com/basic-science/9059/antibiotic-drugs accessed 1.3.2020

Onarheim H, Høivik T, Harthug S, Digranes A, Mylvaganam H, Vindenes HA. 2000. Outbreak of multiresistant Acinetobacter baumannii infection. Tidsskrift for den Norske Laegeforening : Tidsskrift for Praktisk Medicin, ny Raekke. **120**:1028-1033.

Perez F, Hujer AM, Hujer KM, Decker BK, Rather PN, Bonomo RA. 2007. Global Challenge of Multidrug-Resistant *Acinetobacter baumannii.* Antimicrobial Agents and Chemotherapy **51**:3471-3484

Procópio REL, Silva IR, Martins MK, Azevedo JL, Araújo JM. 2012. Antibiotics produced by *Streptomyces.* The Brazilian Journal of Infectious Diseases **16**:466-471.

Rahman MA, Islam MZ, Islam MA. 2011. Antibacterial Activities of Actinomycete Isolates Collected from Soils of Rajshahi, Bangladesh. Biotechnology Research International **2011**

Raja A, Prabakarana P. 2011. Actinomycetes and Drug-An Overview. American Journal of Drug Discovery and Development **1**:75-84.

Salwan R, Sharma V. 2020. Molecular and biotechnological aspects of secondary metabolites in actinobacteria. Microbiological Research **231**.

Sagova-Mareckova M, Ulanova D, Sanderova P, Omelka M, Kamenik Z, Olsovka J, Kopecky J. 2015. Phylogenetic relatedness determined between antibiotic resistance and 16S rRNA genes in actinobacteria. BMC Microbiology (e81) DOI: 10.1186/s12866-015-0416-6.

Seipke RF, Barke J, Ruiz-Gonzalez MX Orivel J, Yu DW, Hutchings MI. 2012. Fungus-growing *Allomerus* ants are associated with antibiotic-producing actinobacteria. Antonie van Leeuwenhoek **101**:443–447

Seppälä H, Klaukka T, Vuopio-Varkila J, Muotiala A, Helenius H, Lager K, Huovinen P. 1997. The Effect of Changes in the Consumption of Macrolide Antibiotics on Erythromycin Resistance in Group A Streptococci in Finland. The New England Journal of Medicine **337**:441–446.

Singh B, Sekhon GS. 1979. Nitrate pollution of groundwater from farm use of nitrogen fertilizers — A review. Agriculture and Environment **4**:207-225.

Smith MG, Gianoulis TA, Pukatzki S, Mekalonos JJ, Ornston LN, Gerstein M, Snyder M. 2007. New insights into *Acinetobacter baumannii* pathogenesis revealed by high-density pyrosequencing and transposon mutagenesis. Genes Dev. **21**, 601–14 .

Solá MZS, Lovaisa N, Costa JSD, Benimeli CS, Polti MA, Alvarez A. 2019. Multi-resistant plant growth-promoting actinobacteria and plant root exudates influence Cr(VI) and lindane dissipation. Chemosphere **222**:679-687.

Spížek J, Sigler K, Řezanka T, Demain A. 2016. Biogenesis of antibiotics—viewing its history and glimpses of the future. Folia Microbiol **61**:347–358.

Tacconelli E, et al. 2018. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. The Lancet Infectious Diseases. **18**:318-327.

Todar K. Online textbook of bacteriology. University of Wisconsin. Available from http://textbookofbacteriology.net/resantimicrobial_3.html accessed 1.3.2020

Ventura M, Canchaya C, Tauch A, Chandra G, Fitzgerald GF, Chater KF, van Sinderen D. 2007. Genomics of *Actinobacteria*: Tracing the Evolutionary History of an Ancient Phylum. Microbiology and Molecular Biology Reviews **71**:495-548.

Verma M, Lal D, Kaur J, Saxena A, Kaur J, Anand S, Lal R. 2013. Phylogenetic analyses of phylum Actinobacteria based on whole genome sequences. Research in Microbiology **164**:718-728

Větrovský T, Steffen KT, Baldrian P. 2014. Potential of cometabolic transformation of polysaccharides and lignin in lignocellulose by soil Actinobacteria. *PLoS One* 9 (e89108) DOI: 10.1371/journal.pone.0089108.

Wang C, Dong D, Wang H, Müller K, Qin Y, Wang H, Wu W. 2016.Metagenomic analysis of microbial consortia enriched from compost: new insights into the role of Actinobacteria in lignocellulose decomposition. Biotechnol Biofuels **9**.

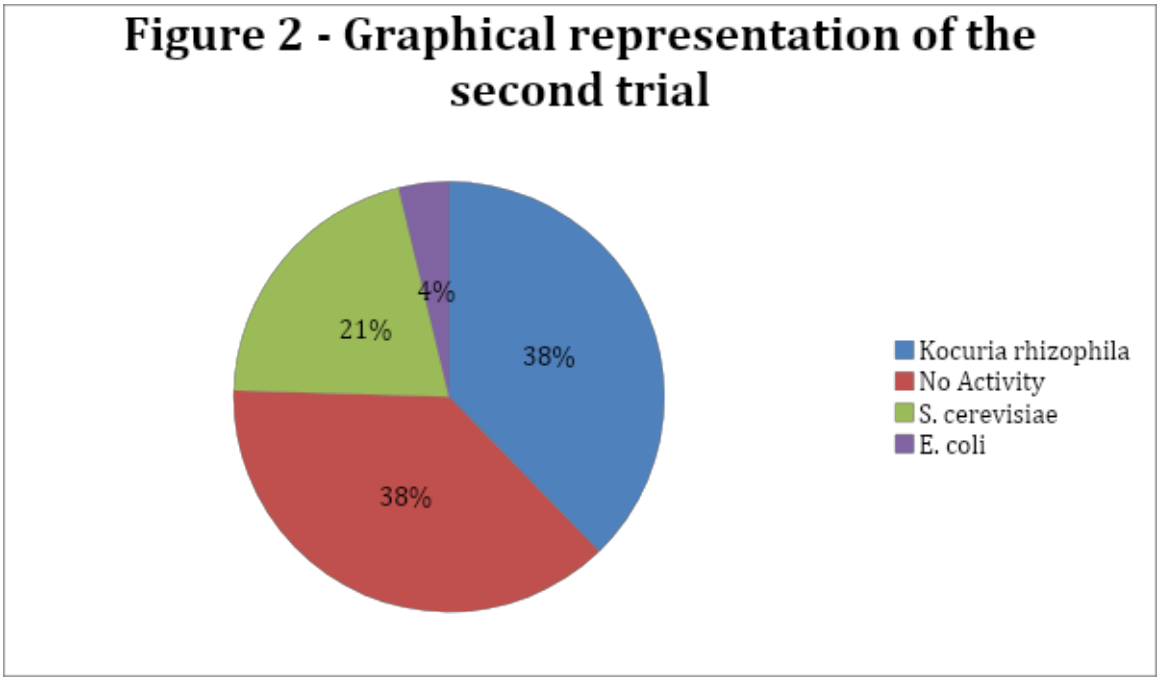
Ward AC, Bora N. 2006. Diversity and biogeography of marine actinobacteria. Current Opinion in Microbiology **9**:279-286.

Enclosures

Table 1 shows all *Actinobacteria* strains tested during the first trial and results of the tests, which are presented by the diameter of the laid zone they produced on a sensitive strain in millimeters.

strain 15TR#	Genus	Results
26	Streptomyces	
45	Catenulispora	
64	Kitasatospora	kocuria Gause 12 mm, kocuria R2A55 mini, S. cerevisiae R2A55 14 mm
40	Catenulispora	
327	Catenulispora	
782	Streptomyces	
762	Streptomyces	
803	Streptomyces	kocuria R2A55 mini
804	Streptomyces	
806	Streptomyces	
109	Streptacidiphilus	kocuria R2A55 mini, S. cerevisiae R2A55 13 mm
13	Streptacidiphilus	
739	Streptomyces	kocuria R2A55 - increased bacterial growth
127	Streptomyces	
139	Streptacidiphilus	
751	Streptomyces	kocuria R2A55 12mm, kocuria Gause 12 mm, S. cerevisiae Gause mini, E. Coli R2A55 increased growth
4	Streptomyces	kocuria R2A55 mini
5	Kitasatospora	
97	Streptomyces	
7	Streptomyces	kocuria R2A55 11 mm, S. cerevisiae R2A55 mini
1163	Nocardia	
1314	Streptacidiphilus	kocuria R2A55 mini, S. cerevisiae R2A mimi, S. cerevisiae Gause 12 mm, E. coli R2A55 mini
175	Streptomyces	kocuria Gause mini
406	Streptomyces	kocuria R2A55 12 mm, kocuria Gause 11 mm, S. cerevisiae Gause mini, E. coli increased growth
1244	Catenulispora	kocuria R2A55 mini, kocuria Gause 11 mm, S. cerevisiae Gause decreased growth 10 mm
11	Streptomyces	

1487	Nocardia	
1427	Streptacidiphilus	
110	Streptomyces	kocuria R2A55 mini
1337	Streptacidiphilus	kocuria R2A55 mini, S. cerevisiae R2A55 mini
214	Streptomyces	kocuria R2A55 17 mm
183	Streptacidiphilus	
211	Catenulispora	some activity against S. cerevisiae R2A55
282	Kitasatospora	kocuria R2A55 14 - 16 mm and 3x increased growth, S. cerevisiae R2A55 11 mm
198	Streptacidiphilus	
222	Streptomyces	kocuria R2A55 mini
227	Streptacidiphilus	kocuria R2A55 15 mm and some activity against S. cerevisiae R2A55
309	Streptacidiphilus	
353	Catenulispora	S. cerevisiae R2A55 mini
302	Streptacidiphilus	
204	Streptomyces	
157	Catenulispora	kocuria R2A55 mini
325	Nocardia	kocuria R2A55 mini
525	Streptacidiphilus	
201	Streptacidiphilus	
351	Streptacidiphilus	
1088	Streptomyces	kocuria R2A55 10 mm, E. coli overgrowth 12 mm probably contamination
413	Streptomyces	
660	Catenulispora	
454	Streptomyces	
339	Catenulispora	
643	Streptacidiphilus	kocuria R2A55 mini
831	Streptacidiphilus	
638	Catenulispora	



The Table 3 shows the results of the second trial of testing.

strain 15TR#	Genus	Results
2	Streptomyces	kocuria R2A55 - mini
28	Streptacidiphilus	
48	Kribbella	
10	Streptomyces	kocuria R2A55 10 mm
36	Streptacidiphilus	
37	Streptacidiphilus	Kocuria G- 12 mm, R2A55 - 11 mm but not very visible
38	Streptacidiphilus	
55	Streptacidiphilus	kocuria R2A55 mini
21	Streptacidiphilus	
65	Streptomyces	kocuria R2A55 - 11 mm irregular
42	Actinospica	
74	Streptomyces	kocuria G - 11 mm
348	Streptacidiphilus	
99	Kitasatospora	
197	Catenulispora	
1004	Streptomyces	kocuria R2A55 questionable mini
1007	Streptomyces	kocuria G - 16 mm

340	Streptacidiphilus	
651	Streptacidiphilus	kocuria G mini
343	Streptacidiphilus	kocuria G mini
345	Streptacidiphilus	
779	Streptomyces	
1129	Streptomyces	
372	Streptomyces	
781	Streptomyces	kocuria G mini
734	Streptomyces	kocuria R2A55 mini, G 14 mm
1153	Streptomyces	kocuria R2A mini, G mini
784	Streptomyces	BAUMANNII G 10 mm, kocuria 25 - 27 mm
792	Streptomyces	BAUMANNII G 10 mm, kocuria 27 mm, R2A mini
1207	Streptomyces	kocuria G mini
752	Kitasatospora	BAUMANNII G 12 mm decreased growth, kocuria G 16 mm
802	Streptomyces	kocuria R2A55 mini, G 11 mm
763	Streptomyces	kocuria R2A55 mini, G mini
767	Streptomyces	
14	Streptomyces	kocuria R2A55 mini, G mini
115	Streptomyces	
121	Streptacidiphilus	
33	Catenulispora	
742	Streptomyces	BAUMANNII G 12 mm, kocuria 33 mm
56	Streptacidiphilus	R2A55 mini
766	Streptomyces	kocuria R2A55 mini G 12 mm
1232	Kitasatospora	kocuria G mini, r2a 12 mm
1181	Streptomyces	kocuria G 25 mm
1164	Nocardia	kocuria R2A55 13 mm, G 15 mm
969	Streptosporangium	
261	Polymorphospora	BAUMANNII R2A 12 mm
171	Streptomyces	kocuria R2A55 11 mm
262	Streptomyces	kocuria R2A55 mini
152	Streptacidiphilus	kocuria R2A55 11 mm, G 12 mm
123	Streptacidiphilus	kocuria G mini
32	Streptacidiphilus	kocuria R2A55 mini, G 11 mm

1444	Streptacidiphilus	BAUMANNII R2A55 mini and increased growth, kocuria G mini
172	Streptacidiphilus	kocuria R2A55 11 mm, G mini
49	Streptacidiphilus	kocuria R2A55 11 mm, G mini
140	Streptacidiphilus	kocuria G mini
103	Streptomyces	kocuria R2A55 mini, G mini
189	Streptacidiphilus	kocuria R2A55 mini
1205	Nocardia	kocuria R2A55 11 mm, G mini
192	Streptacidiphilus	
16	Streptomyces	
179	Streptacidiphilus	kocuria G mini, R2A55 11 mm
1433	Kitasatospora	
19	Streptomyces	kocuria G mini, R2A55 11 mm
186	Catenulispora	kouria G increased growth, R2A55 11 mm and increased growth
257	Streptacidiphilus	kocuria R2A55 mini
237	Streptomyces	kocuria G 11 mm, R2A55 11 mm
243	Streptacidiphilus	kocuria R2A55 11 mm and increased growth
233	Catenulispora	kocuria R2A55 11 mm
244	Streptacidiphilus	kocuria R2A55 increased growth
221	Streptacidiphilus	kocuria G mini
191	Streptacidiphilus	kocuria G mini, R2A55 10 mm
271	Streptomyces	
272	Streptomyces	kocuria G mini
209	Catenulispora	kocuria R2A55 13 mm
235	Streptacidiphilus	kocuria G mini, R2A55 10 mm
215	Streptomyces	kocuria G 10 mm, R2A55 11 mm
283	Streptomyces	kocuria G mini, R2A55 mini
254	Catenulispora	kocuria G mini, R2A55 mini
234	Streptomyces	kocuria R2A55 10 mm
352	Catenulispora	kocuria R2A55 11 mm
379	Micromonospora	kocuria G mini
231	Streptacidiphilus	kocuria G mini, R2A55 mini
113	Streptomyces	kocuria G mini
318	Streptacidiphilus	kocuria R2A55 mini

333	Catenulispora	kocuria R2A55 10 mm
324	Streptacidiphilus	kocuria R2A55 mini
404	Streptacidiphilus	kocuria R2A55 mini
67	Kitasatospora	kocuria G 20 mm
248	Catenulispora	kocuria R2A55 12 mm
308	Catenulispora	kocuria R2A55 11 mm
18	Streptacidiphilus	
978	Streptomyces	kocuria G 25 mm
655	Streptomyces	kocuria R2A55 11 mm, G 14 mm
27	Streptomyces	kocuria R2A55 mini, G 12 mm
516	Streptomyces	kocuria R2A55 mini
652	Streptacidiphilus	
1225	Streptomyces	kocuria G 25 mm
996	Streptomyces	kocuria R2A55 10 mm
561	Streptacidiphilus	
644	Streptacidiphilus	
411	Streptacidiphilus	
637	Kitasatospora	kocuria G 17 mm, overgrowth, possible contamination
310	Catenulispora	kocuria R2A55 12 mm
1443	Streptacidiphilus	kocuria R2A55 mini

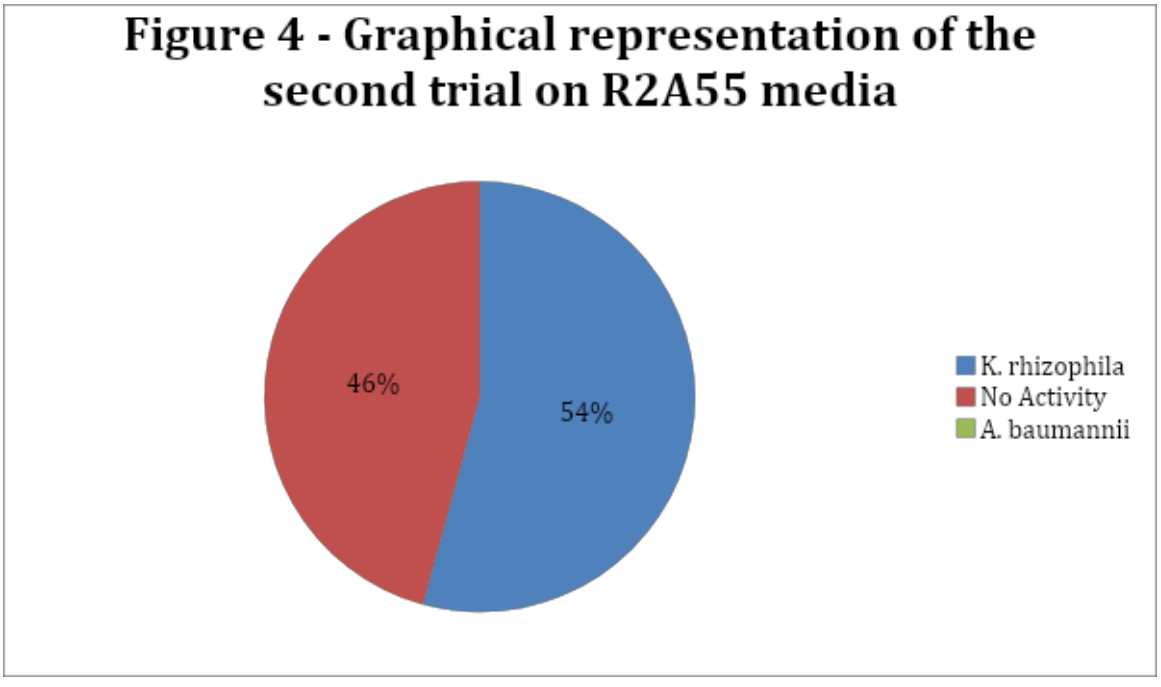


Figure 5 - Graphical representation of the second trial on Gause media