University of South Bohemia České Budějovice

Faculty of Science

Occurrence of the cALAs gene in the BCCO Actinomycetes collection and cultivation improvements for the overproduction of secondary metabolites in soil associated Actinomycetes

Bachelor's thesis

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České Budějovice

2021

Pulferer L., 2021: Occurrence of the cALAs gene in the BCCO Actinomycetes collection and cultivation improvements for the overproduction of secondary metabolites in soil associated Actinomycetes. BSc. Thesis, in English 46 pages, Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

Annotation

Metabolites produced by Bacteria are known to have a wide range of biological activities, which are applied in various fields, like medicine. Most of them are synthesized by members of the group Actinomycetes, in particular from the genus *Streptomyces*.

This thesis deals with the screening process of various *Streptomyces* strains for a specific gene which is involved in the synthesis of a manumycin-like secondary metabolite. Another aspect discussed here is how the addition of agar to the fermentation media and the pH value can influence the synthesis of bioactive metabolites. To determine the effectiveness of the metabolites various experiments were conducted.

Declaration

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Acknowledgements

I want to sincerely thank my supervisor Erika Corretto, Ph.D. from the bottom of my heart for her guidance during all the conducted experiments of PART I and for her time answering all my questions. I am also grateful for the feedback she provided on my thesis. Further, I want to thank my co-supervisor Ana Catalina Lara Rodriguez, Ph.D. for her guidance during the experiments for PART II. Additionally, I want to thank both for their support during these difficult times strongly impacted by the Covid pandemic.

I also want to thank RNDr. Alica Chroňáková, Ph.D. and all the team members, of the Institute of Soil Biology for creating a pleasant work environment and for their help during my lab work. I especially want to thank Ing. František Lorenc, PhD. for his help during the extraction and antibiotic disk analysis.

I appreciate the given opportunity to gain experience in the field of microbiology and some insight in the field of bioinformatics.

List of abbreviations

Abbreviation	Explanation
ACN	Acetonitrile
BCCO	Biology Centre Collection of Organisms
BLAST	Basic Local Alignment Search Tool
cALAs	cyclizing 5-aminolevulinate synthases
DNA	Deoxyribonucleic acid
EtBr	Ethidium bromide
iTOL	Interactive Tree Of Life
NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
TBE	Tris-Borate-EDTA
TLC	Thin layer chromatography

Abstract

Actinomycetes, like *Streptomyces* and *Kutzneria*, are gram-positive bacteria that are predominantly found in soils. They produce a wide range of metabolites, that are used to obtain nutrients and to communicate with other microbes.

This thesis can be separated into two distinct parts. The goal of part I is to determine which strains of the BCCO Actinomycetes collection possess the cyclizing 5-aminolevulinate synthase (*hemA* gene). This gene codes for the C_5N unit, which is present in many bioactive molecules like manumycin. To screen the BCOO Actinomycetes collection, we used a PCR method targeting the *hemA* gene. Among the 71 selected *Streptomyces* strains, only *Streptomyces* sp. BCCO 10_2060 tested positive for the *hemA* gene. Through sequencing and Blast analysis, *Streptomyces cirratus* BCCO 10_1062 is the closest relative. A previous phylogenetic analysis showed that this strain is part of a group of Actinomycetes that harbor a *hemA* gene for the production of annimycin. Based on this result, we predict that also *Streptomyces* sp. BCCO 10_2060 might produce an annimycin-like metabolite.

In PART II, *Kutzneria* sp. BCCO 10_1627 was selected to determine if the synthesis of active secondary metabolites can be influenced by the use of a liquid or semisolid growth medium. During the fermentation process the pH-value was measured every 12 hours. The antibacterial activity of the metabolites extracted from the growth cultures was tested against *Bacillus subtilis* with an antibiotic disk assay and after separation on a TLC sheet. During the fermentation a fluctuation in the pH can be observed. The extracts of two out of three flasks of the liquid medium and one out of three flask of 1% semisolid media showed an antibacterial effect against *Bacillus subtilis*. The TLC plates indicted that the liquid extracts contained three different metabolites which were active against *Bacillus subtilis*; whereas the 1% semisolid medium only contained one active metabolite.

Further chemical analyses are needed to determine the structure of the putative annimycin-like molecule of *Streptomyces* sp. BCCO 10_2060 and of the antibacterial metabolites produced by *Kutzneria* sp. BCCO 10_1627.

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1. Introduction

1.1. Actinomycetes

Actinomycetes is one of the major groups belonging to the Bacteria domain. These bacteria are gram-positive and their DNA contains a high percentage of guanine and cytosine (Barka, et al., 2016). Actinomycetes can be found in aquatic and terrestrial ecosystems. To defend or obtain their food sources, as well as communicate with other organisms they are able to produce a wide range of secondary metabolites. With their mycelial lifestyle Actinomycetes undergo a complex morphological differentiation process (Barka, et al., 2016). The schematic cycle can be seen in Figure 1.

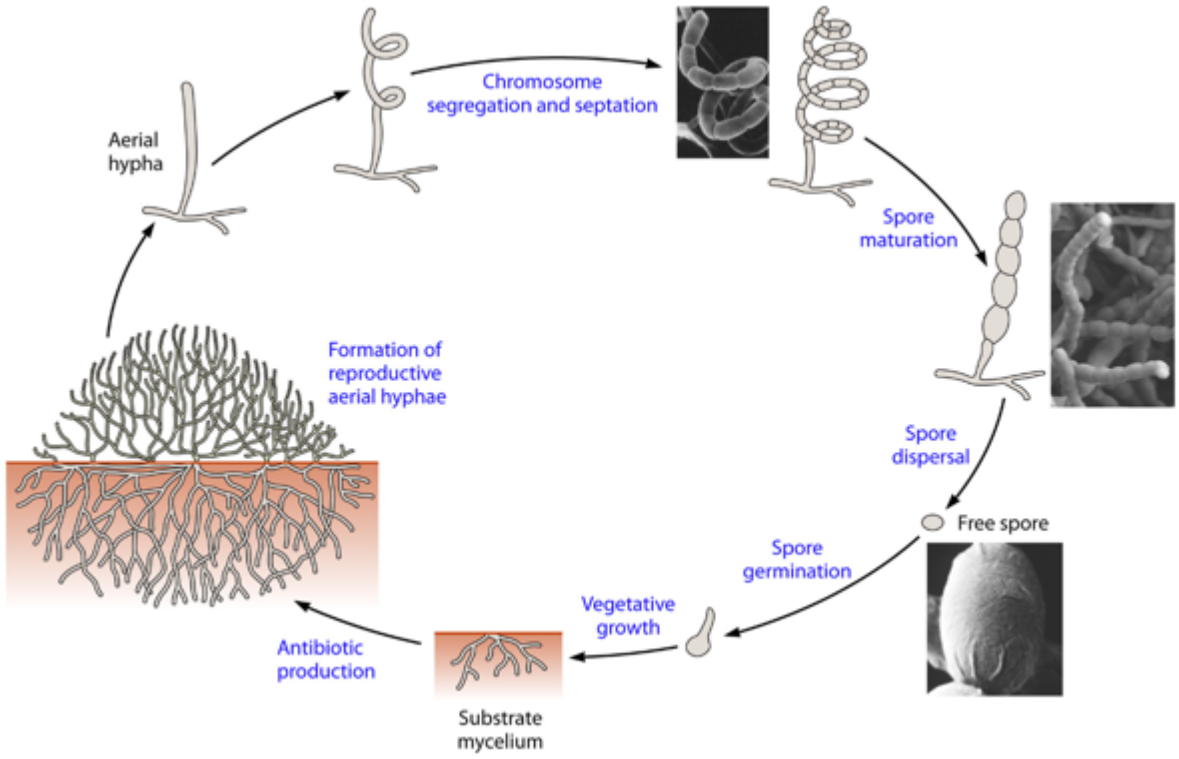

Figure 1: Mycelial lifestyle cycle of Actinomycetes *(Barka, et al., 2016)*

The cycle starts with the germination of spores, which is induced by humidity, carbon dioxide, amino acids and vitamins. The spore swells up and a polarity is established (Kämpfer, et al., 2014). Meanwhile, one or more germ tubes are developed and begin to grow by tip extension and branching. Through this process a network of hyphal filaments is formed in and on the surface of the substrate (Hasani, et al., 2014). When the organism suffers nutrient depletion the process of differentiating the mycelia to form aerial hyphae starts. During this step additional extracellular proteins and

secondary metabolites are produced to protect the remaining nutrient sources and the lysis of vegetative and substrate hyphae recycles nutrients to produce more biomass (Kämpfer, et al., 2014). Sporulation begins with chromosomes in the aerial hyphae being condensed and replicated. Once cell division takes place and a pre-spore, with a single copy of the chromosome, is formed the walls of the compartment in the aerial hyphae where the pre-spore is located thickens and its shape changes. These spores can survive a long time till they find the right conditions to repeat the cycle (Kämpfer, et al., 2014).

Actinomycetes produce secondary metabolites which amount to 2/3 of all naturally derived antibiotics, anticancer, anthelmintic and antifungal compounds available in the market (Barka, et al., 2016). The secondary metabolites produced have various chemical structures which correspond to their bioactivity (Bharti, et al., 2010). A study conducted in the Garhwal region, which is part of the Indian state Uttarakhand, revealed a high unexplored diversity of Actinomycetes. They were able to isolate more than 300 Actinomycetes, of which about 30% had antifungal activity against one or more pathogens (Bharti, et al., 2010) For these reasons they are of high importance in the fields of biotechnology, medicine and agriculture. Thanks to their adaptability, Actinomycetes are also associated with various higher organisms. For instance, some form symbiotic or pathogenic relationships with plants (Barka, et al., 2016).

1.1.1. *Streptomyces* sp.

The *Streptomyces* genus belongs to the group of Actinomycetes. They are mostly soil bacteria but can be found in other environments such as water, high temperature environments such as animal feces, hay, compost, volcano and desert steppe zones to name a few (Kämpfer, et al., 2014). *Streptomyces* are able to cycle the carbon trapped as insoluble organic waste produced by plants or fungi in the soil (Barka, et al., 2016). This characteristic is called chemoorganotrophic. *Streptomyces* have an oxidative metabolism and are also able to grow at different pH ranges (Kämpfer, et al., 2014). *Streptomyces* sp. synthesizes diverse natural secondary metabolites, which cover around 80% of the total antibiotic products compared to the other genera (Bharti, et al., 2010).

The taxonomic identification of *Streptomyces* species using the 16S rRNA gene as molecular marker can be difficult due to a small variation in the nucleotide sequence

for this genus. To archive a higher phylogenetic resolution, the investigation of other single-copy marker gene or genome-based studies are needed (Kämpfer, et al., 2014).

1.1.2. *Kutzneria* sp. BCCO 10_1627

Kutzneria spp. are gram-positive, aerobic bacteria and belong to the family of *Pseudonocardiaceae*. As other Actinobacteria, their way of replication is asexual through the production of spores, which are non-motile, spherical, rode like or ovoid. The sporangia where the spores are formed are globose and have a diameter of 10 to 48 µm (Stackebrandt , et al., 1994). The cell walls consist of N-acetylated muramic acid, meso-diaminopimelic acid and sugars like galactose or rhamnose. *Kutzneria* can be typically found in soil (Stackebrandt , et al., 1994). Their most commonly produced secondary metabolites are called kutznerids and have antifungal and antimicrobial properties (Fujimori, et al., 2007).

Strain BCCO 10_1627 belongs to the genus *Kutzneria*. It doesn't have the cyclizing 5-aminolevulinate synthases (*hemA* gene) involved in the synthesis of manumycin-like compounds (Petříčková, et al., 2014). However, it possesses a short chain length factor associated to a type II polyketide synthase, which is highly likely to be involved in the synthesis of polyene molecules. Strain BCCO 10_1627 has a broad range antibacterial and antifungal activity. For instance, it can inhibit *Alternaria alternata*, *Fusarium oxysporum*, *Saccharomyces cerevisiae*, *Bacillus subtilis* and *Staphylococcus aureus*. The strain is resistant to ampicillin, penicillin, cephalosporin, gentamycin, tetracycline and vancomycin, but is sensitive to ciprofloxacin, erythromycin, rifampicin, streptomycin and chloramphenicol (Jernej, 2019).

1.2. Bacterial secondary metabolites

Secondary metabolites are organic compounds produced by bacteria as signaling molecules and provide an advantage against other microorganisms in the uptake and scavenging of nutrients (Kämpfer, et al., 2014). These compounds have various biological activities which are related to their structure (Hu & Floss, 2001; Pospíšil, et al., 2011). The following example demonstrates their diversity and the important role they play in human wellbeing. Macrolides can affect the signaling pathways of transcription factors, which regulate pro-inflammatory and pro-fibrotic genes (Cecrdlova, et al., 2016). A member of this group used in the medical field is azithromycin. It is used to treat neutrophilic airways disease, since it inhibits the IL-5

release from Th2 cells in asthmatic children. Another member of macrolides is the metabolite clarithromycin, which properties are ani-inflammatory, immunomodulatory and antibacterial (Cecrdlova, et al., 2016).

In Bacteria the genes encoding the biosynthetic enzymes of secondary metabolites are usually organized in clusters, which can either be transmitted vertically or horizontally. Some of these genes have protein domains that are conserved among different species and thus can be exploited to predict the produced metabolite (Kämpfer, et al., 2014; Petrícková, et al., 2015). In this thesis PCR was used to screen for the presence of one key biosynthetic gene, which is part of the cluster for the synthesis of manumycins.

In nature, secondary metabolites are produced as a response to a change in the surrounding environment (Kämpfer, et al., 2014). Taking this into consideration, the production of metabolites under laboratory conditions highly depends on the composition and formulation of the growth medium. For instance, there might be some components that increase or inhibit the production of the metabolite of interest (Hu & Floss, 2001; Pospíšil, et al., 2011). The study, performed by Pospíšil in 2011 revealed the influence of the carbon source on the production of manumycin-like compounds in Actinomycetes. The usual carbon source glucose was replaced with glycerol. The effects of the carbon source change on modified and wild type strains were investigated. It was proven that the modified strains had an increased production of asukamycin congeners (Pospíšil, et al., 2011). Under the same conditions the wild-type synthesized manumycins congeners with a branched-chain and the asukamycin yield dropped from 46 % to 18 %. These results show that by adjusting the feeding mode the ratio of synthesized secondary metabolites can be influenced (Pospíšil, et al., 2011). Another method to investigate metabolites in the lab is by adding to the growth medium the precursor used in the biosynthetic pathway of the desired metabolite (Hu & Floss, 2001). For example, in the case of manumycins by feeding the organisms the ^{13}C-labeled 3-amino-4-hydroxybenzoic acid (3,4-[7 ^{13}C]-AHBA).

1.2.1. Manumycins

The basic structure of manumycins consists of two triene polyketide chains (upper and lower chain), a mC_7N unit and a C_5N unit (Figure 2). To the central mC_7N unit the triene polyketide chains are connected in the meta position and the lower chain

terminates with the C_5N unit (Pospíšil, et al., 2011; Rui, et al., 2010). The upper chain can vary and influences the biological activity. Two major groups have been defined based on the upper chain structure (Pospíšil, et al., 2011). Group A have linear polyene chains, which either have a saturated branched hydrocarbon group or contain a cyclohexane moiety at the end of the chain. A member of this group is asukamycin. Group B have a methyl branched polyene chain, which is terminated with saturated linear hydrocarbon group. Manumycin A is part of group B (Pospíšil, et al., 2011). A further differentiation can be made based on the central mC_7N unit. The molecules with an oxirane at C-5/C-6 (epoxyquinol structure) are categorized as type I manumycins. Type II on the other hand have a hydroxyethylene group (hydroxyquinol structure) at the same carbon positions (Hu & Floss, 2001). Some *Streptomyces* are able to produce both types, but there have been cases were significant difference in the biological activity of the two types have been observed. For example, type I can have an activity against gram-positive and some gram-negative bacteria. However, type II molecules show no sign of activity against them. Therefore, the significance in the structure of the central mC_7N unit plays an important role in the biological activity (Hu & Floss, 2001). As an example, manumycin A is known to be a derivate of the farnesyltransferase inhibitors (Cecrdlova, et al., 2016).

The precursor of the C_5N unit, 5-aminolevulinic acid (ALA), is produced via either the the C5 pathway or Shemin pathway. Then, it is cyclized by the cyclizing 5-aminolevulinate synthase (*hemA* gene). On the other hand, 3-amino-4-hydroxybenzoic acid (3,4-AHBA) for the mC7N is the precursor for the mC_7N (Hu & Floss, 2001; Petříček, et al., 2006). The genes coding for these manumycin characteristic features can be use as targets for the identification of Actinomycin having similar enzymes via a PCR screening.

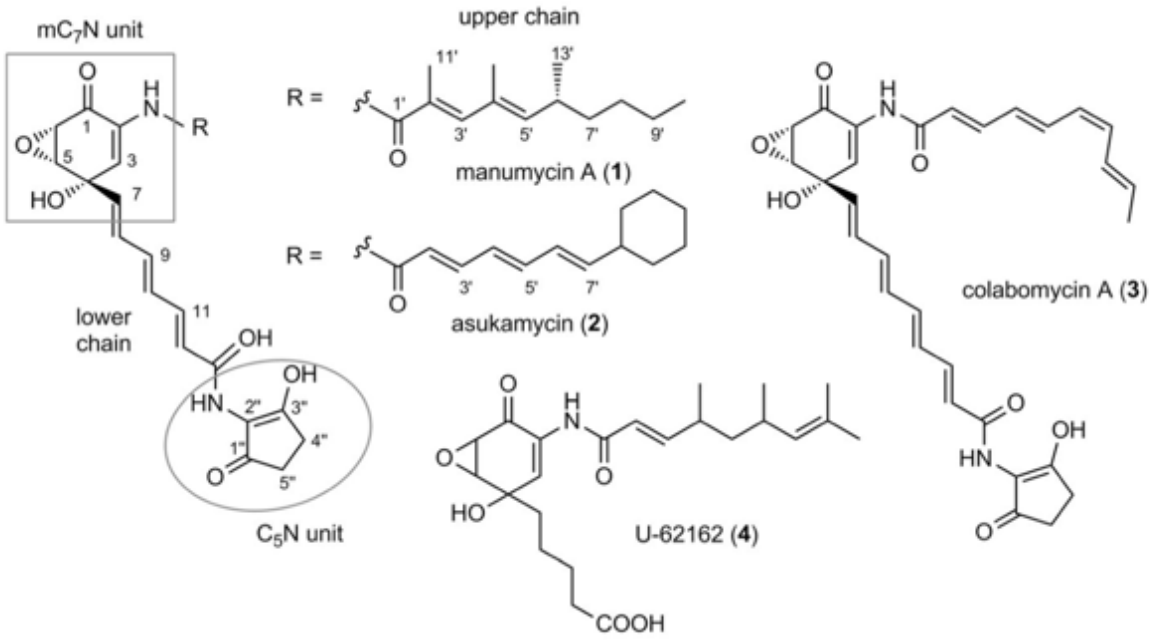

Figure 2: Basic structure of manumycins with a variety of upper chains is shown. *(Petříčková, et al., 2014)*

1.2.2. Annimycin

The biosynthetic gene cluster for annimycin has been characterized in *Streptomyces calvus*, where the complementation of an intact copy of the *bldA* regulator led to sporulation and to the production of annimycin (Figure 3).

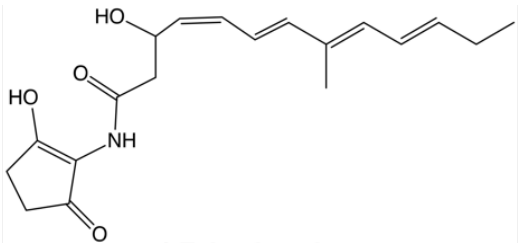

Figure 3: Structure of 4Z-annimycin. *(DulguillenMat, 2018)*

The molecule is synthesized by a type I polyketide synthase and affects the morphological differentiation by inhibiting the formation of the aerial mycelium in other Actinobacteria (Kalan, et al., 2013). Petrícková and colleagues identified a putative annimycin producing strain, *Streptomyces sioyaensis* BCCO 10_981, by a PCR screening for the presence of the cyclizing 5-aminolevulinate synthase (*hemA* gene) involved in the synthesis of the C_5N unit. However, they were not able to detect the molecule in the extracts produced using various growth conditions. The analysis of its genome revealed several deletions and insertions in the key biosynthetic genes that lead that inhibit the annimycin production (Petrícková, et al., 2015).

1.3. Polymerase chain reaction (PCR)

The polymerase chain rection is a molecular biology technique used to amplify a specific DNA region. The PCR technique was invented by Kary B. Mullis in 1983 (Mullis, 1990) and he was awarded the Nobel prize in the year 1993.

To replicate the region of interest, the following items are needed: the targeted DNA, a mixture of the heat stable DNA-polymerase, deoxyribonucleotide triphosphates (dNTP's) and primers. The typically used polymerase is the Taq polymerase isolated from the *Thermus aquaticus* bacterium. Primers are designed to complement the DNA strand at the beginning and end of the sequence of interest. In total the amplification of a DNA fragment via PCR is usually divided into five steps:

1. Initialization step: the reaction is heated up to 94-96°C for 1 to 9 min. This step is used to fully denature the template DNA. Further, some DNA-polymerases require this step for activation (hot-start PCR).

2. Denaturation step: During this step the reaction is heated to 93-98°C for a few seconds. The high temperature desaturates the DNA strains by breaking the hydrogen-bonds between the two strands of the DNA. Two single stranded DNA are obtained.

3. Annealing step: The binding of the primers to their complementary regions of the DNA strains occurs by lowering the temperature to the appropriate annealing temperature, which depends on the length and nucleotide composition of the primers.

4. Elongation step: The optimal operating temperature of the polymerase is used. The time needed for the extension of the newly formed strain depends on the speed of the polymerase and the length of the DNA fragment.

5. Final elongation: The final elongation step ensures that all DNA fragments are fully extended at the same temperature as in the elongating step.

The denaturation, annealing and elongation steps are repeated multiple times for 25-35 cycles. After each cycle the amount of DNA fragments increases exponentially (2^n, n = number of cycles). Figure 4 visualizes the first cycle of PCR.

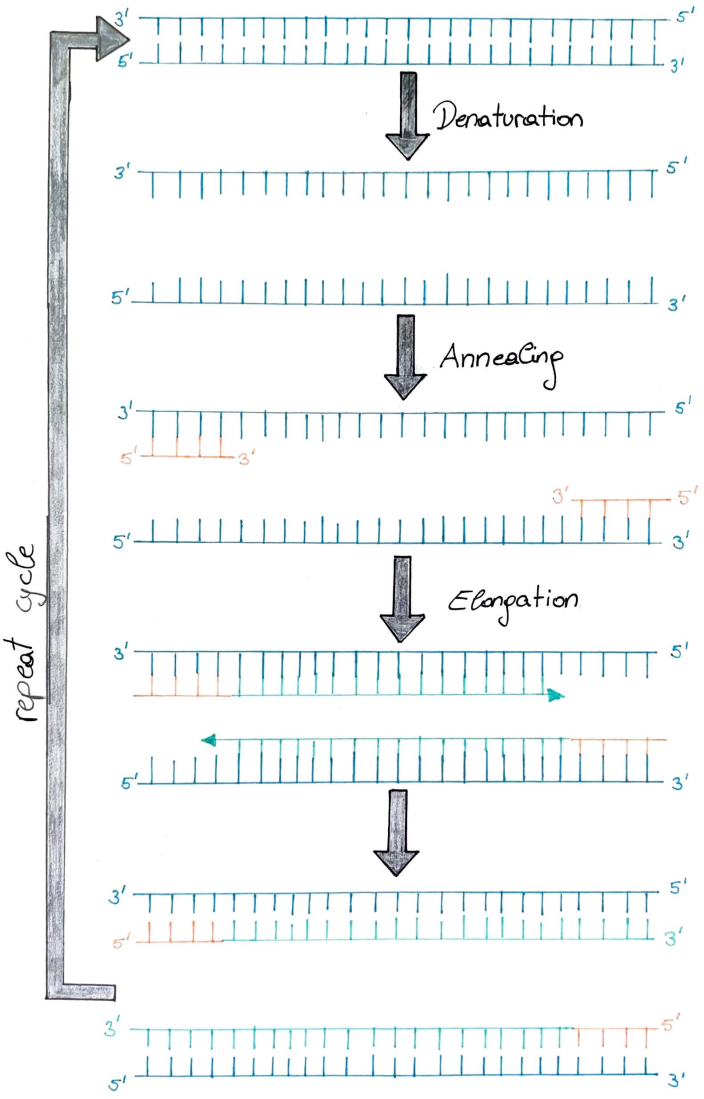

Figure 4: Scheme of the polymerase chain reaction (PCR).

1.4. 16S rRNA gene and phylogenetic analysis

The 16s rRNA gene codes for a part of the small subunit, 30S, of the ribosomal RNA molecule and its important function consists of the conversion process of genetic messengers to functional cell component (Byrne, et al., 2018; Tsiboli, et al., 1994). This process is done via translation of the mRNA to proteins. The RNAs genetic code is self-replicating, therefore the changes in the sequence can be used as an indicator for genetic changes over time (Byrne, et al., 2018). Although there are changes in the sequences the original function is not lost. The nucleotide sequence diversity mirrors the differences between bacterial species. Therefore, the 16S rRNA gene is wildly

used to characterize microorganisms and perform phylogenetic analysis. On average the bacterial 16S rRNA gene has a total length of 1500 bp (Janda & Abbott, 2007). Its gene structure is made of nine highly conserved and nine hypervariable regions (Figure 5). In the hypervariable regions the substitution rate is around 7000 times higher than in the conserved regions (Rosselli, et al., 2016).

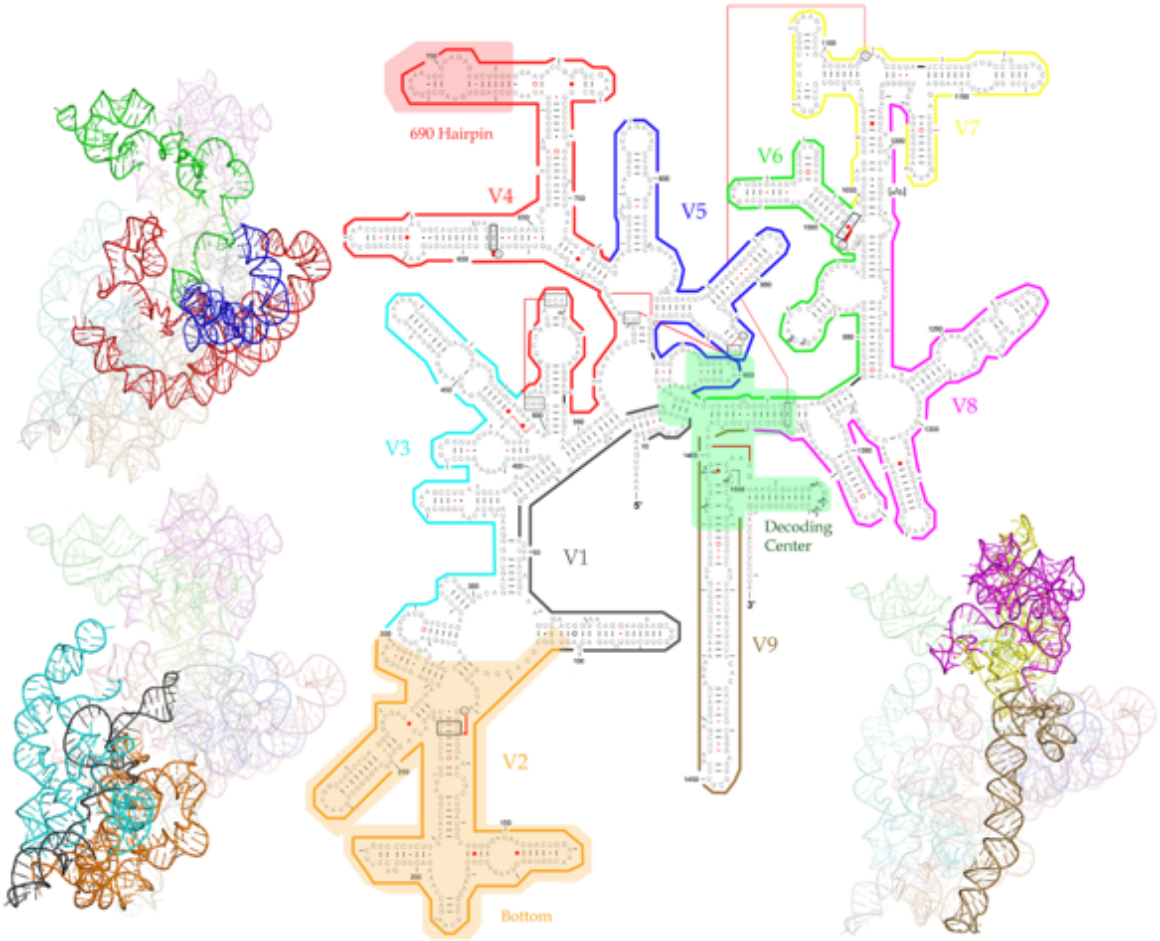

Figure 5: 3D-structure of the 16S rRNA gene, with the assigned subunits (V1, V2, V3, V4, V5, V6, V7, V8 and V9). (Yang, et al., 2016)

The differences in the nucleotide sequence of the 16S rRNA gene can be visualized in a phylogenetic tree. This branching diagram describes the relationship between different organisms, or sequences, and can be used to determine the relationships between them, one of them being evolution. There are various methods to produce a tree, depending on the research question that needs to be addressed. In this study, we used the Maximum Likelihood method, where the tree with the highest compound probabilities of character distribution is selected as best (Hillis, 1997; Nixon, 2001). The branches of a phylogenetic tree resemble the passing of genetic information to the next generation, which are represented as nodes. The ends of the branches are

the recent organisms or sequences. The length of the branches indicates amount of changes or divergence, for better understanding a value can be estimated though the average substitutions of nucleotides per site.

2. Aims of the study

The overall goal of this thesis was to screen the BCCO Actinomycetes collection (CCSACB, www.actinomycetes.cz) and investigate the metabolic potential of selected strains using different approaches. The study can be separated into two different sections:

1. PART I: screening of the BCCO Actinomycetes collection (CCSACB, www.actinomycetes.cz) for the identification of strains containing the cyclizing 5-aminolevulinate synthase (*hemA* gene), which is involved in the synthesis of C_5N unit present in different bioactive molecules like manumycins and annimycins. This gene was chosen as target for the identification of BCCO Actinomycetes that potentially produce new manumycin-like molecules.

2. PART II: previous study showed that *Kutzneria* sp. BCCO 10_1627 has a strong antifungal and antimicrobial activity against a broad range of microorganisms. In this thesis, we tested whether the use of a liquid or semisolid growth medium influenced the production of active secondary metabolites. The antibacterial potential of the extracted metabolites was tested against *Bacillus subtilis*.

3. Materials and Methods

3.1. Actinomycetes used in PART I

All Actinomycetes (total 71) used in this study belong to the Culture Collection of Soil Actinomycetes České Budějovice (CCSACB, www.actinomycetes.cz). The 16S rRNA gene PCR analysis and the PCR screening for the *hemA* gene were performed using DNA which was previously extracted and stored at 4°C. The complete list of organisms is shown in Table S1.

3.2. 16S rRNA gene PCR analysis

The quality and quantity of the previously extracted DNA was first assessed with the NanoDrop spectrophotometer. Additionally, to verify if the DNA was suitable for PCR, we performed a PCR with the universal primers for the 16S rRNA gene. The primer sequence, the PCR mix and thermal conditions are reported in Table 1, 2 and 3 respectively. For every PCR reaction we had a positive and negative control. As a positive control we used the DNA of strain BCCO 10_322, which harbors the *hemA* gene.

Table 1: Primers used in the 16S rRNA gene PCR

Primer	Primer sequence 5' to 3'	Preference
pAf	5' AGAGTTTGATCCTGGCTCAG 3'	(Edwards, et al., 1989)
pHr	5' AAGGAGGTGATCCAGCCGCA 3'	(Edwards, et al., 1989)

Table 2: PCR mixture composition. The reaction was carried out in a total volume of 25µL.

	Stock concentration	Final concentration	Volume [µL] for one sample
2x Fast Start Master mix	2x	1x	12.5
pAf	100 µmol/L	10 µmol/L	0.75
pHr	100 µmol/L	10 µmol/L	0.75
DNA		50-300 ng/µL	2
MilliQ water			9.7

Table 3: Thermal protocol for 16S rRNA gene PCR.

Temperature [°C]	Time	
95	3 min	
94	1 min	
66	30 sec	35 cycles
72	1 min 30 sec	
72	5 min	
12	∞	

To analyze the PCR products, gel electrophoresis was performed with a 1% agarose gel in 1 x TBE. On the gel we loaded 3 µL of "1kb" DNA ladder (Thermo Scientific 0.5 µg/µL; 50µg), as well as 5 µL of PCR product together with1.5 µL 6x Loading Dye (Thermo Scientific). After running for 45 min at 100 V, the gel was stained in an EtBr bath for approximately 45 to 60 min. A picture of the gel was taken with the Azure Biosystems c280 device using the UV 320 transilluminator function and automatic exposure. If the bands were not sufficiently visible the exposure time was adjusted.

The PCR products of the bacteria, which had not been identified yet, were sent to SEQme s.r.o for sequencing with both primers. First, the obtained forward and reverse

sequences were cleaned in ChromasLite 2.1 and then assembled in a consensus sequence in BioEdit. Finally, they were analyzed with BLAST to identify the closest related organism. The strains sent for sequencing are highlighted in gray in Table S1.

3.3. Screening for the *hemA* gene via PCR

Strains listed in Table S1 were screened for the presence of the hemA gene. The primer sequences, the PCR mix and the thermal conditions are reported in Table 4, 5 and 6 respectively. For every PCR reaction we had a positive and negative control. As a positive control we used the DNA of strain BCCO 10_322, which harbors the hemA gene.

Table 4: Primers used in *hemA* gene PCR

Primer	Primer sequence 5' to 3'	Preference
HemA1a	5' GTSTGGTGYTCSAACGACTACCT 3'	(Petrícková, et al., 2015)
HemA1b	5' GTSTGGTGYRGSAACGACTACCT 3'	(Petrícková, et al., 2015)
HemA3	5' GTACATSCCSACSGCGTGSACCTCGTC 3'	(Petrícková, et al., 2015)

Table 5: PCR mixture composition. The reaction was carried out in a total volume of 25µL.

	Stock concentration	Final concentration	Volume [µL] for one sample
2x Fast Start Master mix	2x	1x	12.5
HemA1a	100 µmol/L	10 µmol/L	0.2
HemA1b	100 µmol/L	10 µmol/L	0.2
HemA3	100 µmol/L	10 µmol/L	0.4
DNA		50-300 ng/µL	2
MilliQ water			9.7

Table 6: Thermal protocol for *hemA* gene PCR

Temperature [°C]	Time	
94	2 min	
94	30 sec	
60-55	30 sec	touchdown 30 cycles
72	1 min	
72	5 min	
10	∞	

The gel electrophoresis conditions were identical to the ones described in the previous paragraph. The positively identified strain was sent to SEQme s.r.o for sequencing. The obtained sequence was analyzed with BLAST to identify the most similar sequence, which provides an indication of the putative produced metabolite.

3.4. Phylogenetic analysis

We created a phylogenetic tree using: the 16S rRNA gene sequences of the strains analyzed in this study, the closest identified organisms, some known metabolite producers from Petříčková et al., 2015 and the closest organism to the *hemA* positive

strain, *Streptomyces* sp. BCCO 10_2060. The sequence alignment was done with muscle (Edgar, 2004) in MEGA X (version 10.2.4) (Kumar, et al., 2018). The webserver RAXxML BlackBox version 1.0.0 (Kozlov, et al., 2019) was used for the construction of the Maximum Likelihood tree with GTR substitution matrix and 100 bootstrap replicates. The phylogenetic tree was visualized and edited with the online tool Interactive Tree of Life (iTOL) (Letunic & Bork, 2021).

3.5. *Kutzneria* sp. BCCO10_1627 used in PART II

Kutzneria sp. BCCO 10_1627 is part of the Culture Collection of Soil Actinomycetes České Budějovice (CCSACB, www.actinomycetes.cz). It was isolated on YEME agar medium from forest soil of mount Cameroon, Cameroon.

3.6. Growth and fermentation media

The different growth media for *Kutzneria* sp. BCCO 10_1627 are listed in Table 8 and 9.

Table 7: liquid media used to grow *Kutzneria sp.* BCCO 10_1627

	Reagent	**Amount for 1L**
LB **(Lysogeny Broth/ Luria Bertani)**	Trypton	10 g
	Yeast extract	5 g
	NaCl	5-10 g

Table 8: solid media used to cultivate *Kutzneria sp.* BCCO 10_1627

	Reagent	Amount for 1L
MS **(Murashige and Skoog medium)**	Manitol	20 g
	Soja extract	20 g
	Agar	20 g
Nutrient agar	Peptone	5 g
	Beef / Yeast extract	3 g
	Sodium chloride	5 g
	Agar	15 g

For the optimization of the fermentation medium composition, we prepared three versions of the manumycin fermentation medium (Table 10) having different amounts of agar: two semisolid media with 0.5 % and 1 % agar and one liquid medium without agar.

Table 9: manumycin fermentation media

	Compounds	Amount for 1L
Manumycin fermentation media	Glucose	20 g
	Pentane	5 g
	K_2HPO_4	0.25 g
	$MgSO_4$	0.25 g
	$(NH_4)_2Mo_7O_{24} * 4H_2O$	0.5 mg
	$FeSO_4 * 7H_2O$	50 mg
	$CuSO_4 * 5H_2O$	5 mg
	$ZnSO_4 * 7H_2O$	5 mg
	$MnCl_2 *4H_2O$	10 mg

3.7. Fermentation of *Kutzneria* sp. BCCO 10_1627 and extraction of metabolites

As described above for the fermentation, we used different versions of the manumycin fermentation medium: liquid and semisolid (0.5 – 1 % agar). Four flasks were prepared for the three types of media. Pre-cultures were prepared from a glycerol stock of BCCO 10_1627. After 72 h at 150 rpm at 28°C, a total of 10 mL from the pre-cultures were used to inoculate the flaks used for the fermentation process. After adding the pre-culture the total volume during the fermentation process amounted to 100 mL. The flasks were incubated for 72 h at 150 rpm at 28°C. Every 12 h the pH-value of the growing cultures was measured form one of the four flasks for each media.

After 72 h the fermentation medium of the other three flasks was transferred into a separate 500 mL round bottom flask and freezed at -20°C overnight. The flasks were transferred to -80°C for another night and freeze dried for 2-3 days. The solution for the metabolite extraction consisted in 200 mL of ACN:H_2O (1:1) mixed with 200 µL formic acid. A total of 20 mL of this solution was added to the flasks containing the freeze-dried cultures grown in the liquid medium; whereas 45 mL were added to the cultures grown in 0.5 % and 1 % semisolid agar media. The flasks were shaken for 1 h in the cold room. Afterwards, the liquid transferred into a syringe and it was filtered through a cellulose filter with a pore size of 0.45 µm into a 10 mL glass vial. The filtrate was stored at -20°C and sent for analysis to the analytical lab of Dr. Roman Grabic at the Faculty of Fisheries and Protection of Waters at the University of South Bohemia for chemical analysis.

3.8. Antibiotic disk assay of the raw extracts against *Bacillus subtilis*

A suspension with 300 µL liquid *Bacillus subtilis* culture and 3 mL sterile tap water was prepared. The cell density was adjusted to 0.5 McFarland turbidity value (approx. $1.5*10^8$ CFU/mL). A total volume of 200 µL of this suspension was spread on MS agar plates with a Drigalski spatula. The filter disks with the extracted metabolites were prepared by adding 25µL of the extract. A disk 25µL of 100 % ACN was used as negative control. The filter disks were placed onto the MS agar plates with *Bacillus subtilis* and incubated 37°C. The inhibition zones were measured on the next day.

3.9. Separation of the extracted metabolites using TLC and antibiotic assay against *Bacillus subtilis*

The TLC assay and Bioassay with the TLC sheet, which is described below, was carried out by Ana Lara.

A gaslight syringe was used to transfer 20-25 µL of the extract onto the designated dot on the TLC sheet. A benzene:acetone (3:2) solution was used as solvent for the run. Two pictures were taken at two different UV-light wavelengths: 254 and 365 nm. The exposure time for both wavelengths amounted to one minute.

The same TLC sheet was used for the antibiotic assay against *Bacillus subtilis*. In a square petri dish, a first layer of nutrient agar was poured and left to solidify. Then, we poured a second layer (10 mL) containing 100 µL of a fresh *Bacillus subtilis* culture. After 10 min, the TLC sheet was printed on the agar plate, which was incubated overnight at 37°C. Photos of the inhibition zones were then taken.

4. Results and Discussion

PART I

4.1. Identification of BCCO strains via 16S rRNA gene PCR analysis

Before starting the screening process, the concentration and quality of the isolated DNA was checked with the Nanodrop spectrophotometer. The DNA absorbance was measured at the following wavelengths: 230, 260 and 280 nm. The measured ratio at 260/280 nm should ideally lie between 1.8 to 2.0. For the wavelength ratio 260/230 nm the detected value should be around 2. The DNA concentration was determined in ng/µL. For further analysis, the DNA was diluted if the measured concentration exceeded 300 ng/µL. In Table 12 an example of gathered data can be seen. All measured values are summarized in the appendix.

Table 10: Example of measured DNA concentration and values at 260/280nm and at 260/230nm

Isolation source	Identification	BCCO_10	ng/µL	260/280	260/230
Palava	*Streptomyces* sp.	1499	3401.6	1.85	1.98
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1543	87.1	1.89	0.74
Unknown habitats, Antarctica	*Streptomyces* sp.	2060	3708.5	1.86	1.74

The reason why the DNA concentration was measured at the three different wavelengths was to determine the presence of potential contaminants in the samples. At the wavelength of 260 nm and 280 nm the absorption of nucleic acids and proteins is measured, respectively. Other contaminations, such as ethanol, phenol and salts absorb at a 230 nm.

Figure S1 in the appendix shows a gel used to visualize the PCR products of 16S rRNA gene PCR. As expected, the band around 1500 bp represents the correct PCR product. From the entire list of analyzed strains (Table S1) one 16S rRNA gene PCR

resulted negative. To screen this strain again the DNA extraction process and PCR must be repeated.

The 16S rRNA gene sequences of strains that were not identified yet were processed in Blast (strains highlighted in gray in Table S1). All strains belong to the *Streptomyces* genus and the identification details are summarized in Table S2.

4.2. Screening of BCCO Actinomycetes collection for the presence of the *hemA* gene

When screening for the *hemA* gene, only one strain had a band at the expected size of 500 bp (Figure 6). The only positive strain is *Streptomyces* sp. BCCO 10_2060, which was isolated from an unknown habitat in the Antarctic.

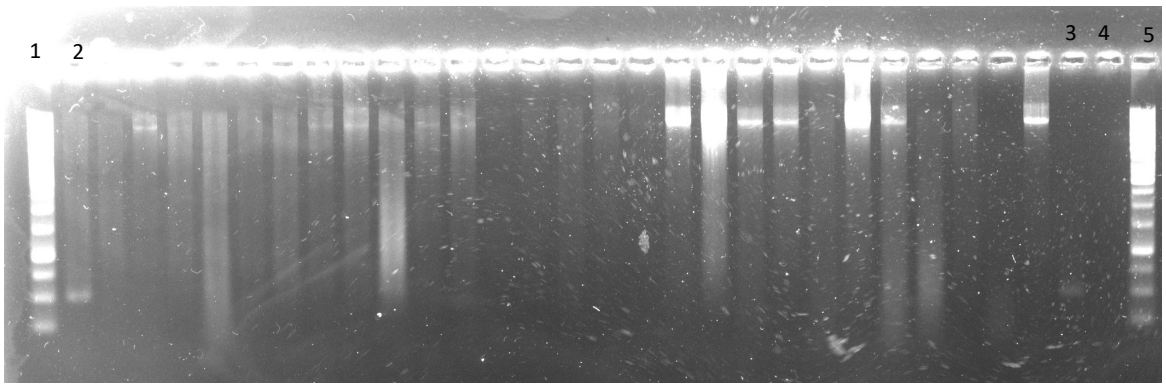

Figure 6: Image of the gel electrophoresis of the *hemA* gene PCR. (**1, 5**) 1kb DNA ladder; (**2**) BCCO 10_2060; (**3**) positive control BCCO 10_322; (**4**) negative control

The obtained PCR product was sequenced and the most similar *hemA* gene deposited in the GenBank database was identified using Blast (Table 13). Results show that *Streptomyces lunaelactis* MM109 and *Streptomyces cirratus* BCCO 10_1062 have a *hemA* gene sharing 94 % nucleotide identity with the one of BCCO 10_2060. This information can be exploited to predict which kind of C_5N unit containing metabolite might be produced by BCCO 10_2060. Based on the phylogenetic tree published by Petříčková, etc. 2015, *Streptomyces cirratus* BCCO 10_1062 is part of the annimycin cluster, where *Streptomyces calvus* ATCC13382 is the reference strain. Given the high sequence similarity, we expect that also BCCO 10_2060 might produce a compound with a similar structure.

Table 11: Closest relatives hits for the *hemA* gene of BCCO 10_2060 from BLAST

Closest hit (Blast)	bp	%	acc number	isolation source
Streptomyces lunaelactis MM109	423/449	94	CP026304.1	cave, Belgium: Comblain-au-Pont
Streptomyces cirratus BCCO 10_1062	406/434	94	KP404514.1	Vyklantice, soil from potato field

4.3. Phylogenetic analysis based on the 16S rRNA gene

When looking at the phylogenetic tree (Figure 7) based on the 16S rRNA gene, the closest strains to *Streptomyces* sp. BCCO 10_2060 are BCCO 10_2063, BCCO 10_2073, BCCO 10_2061, BCCO 10_2062, BCCO 10_2071, BCCO 10_2070 and BCCO 10_2064.They form a group together with *Streptomyces avidinii* IHBA 9319. Even though it seems that these strains are closely related based on the 16S rRNA gene, only BCCO 10_2060 possesses the *hemA* gene. In order to clearly separate and fully identify these strains, we would need to investigate other single-copy marker genes. Other studies showed that the 16S rRNA gene does not provide enough phylogenetic resolution and thus recommend the use of other gene like *atpD* (Laskaris, et al., 2012), *rpoB* (Kim, et al., 2004), *trpB* (Egan, et al., 2001), *recA* and *gyrB* gene (Guo, et al., 2008).

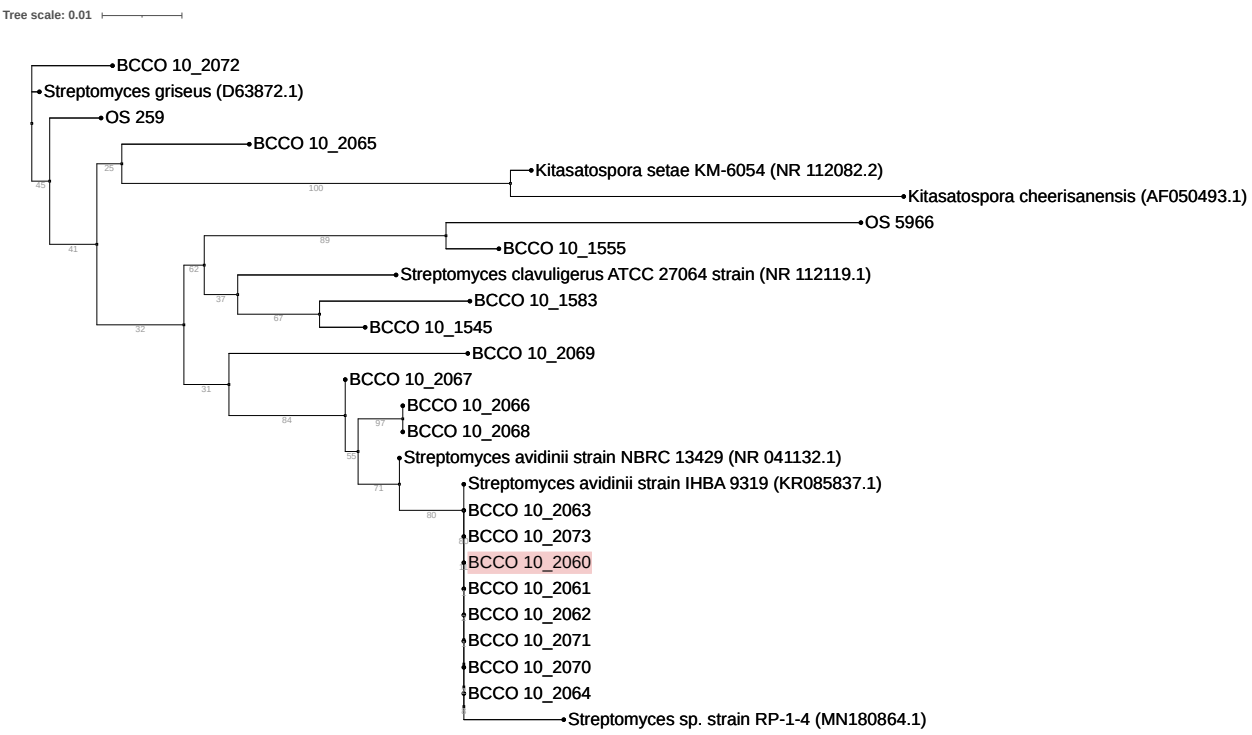

Figure 7: Phylogenetic tree based on 16S rRNA gene sequences calculated in RAxML-NG *(Kozlov, et al., 2019)*. iTol was used for visualization *(Letunic & Bork, 2021)*.

PART II

4.4. Fermentation of *Kutzneria* sp. BCCO 10_1627 using a liquid and semisolid medium

In order to compare the effect of a traditional liquid medium versus a semi-solid one on the production of bioactive metabolites, we prepared different versions of the manumycin fermentation medium with the agar concentration ranging from 0 to 1%. For instance, studies showed that when some fungi had a solid surface as support, the production of metabolites was improved (Hölker, et al., 2004; Ellaiah, et al., 2004). During the fermentation process, we monitored the pH value along the 72 h of incubation (Figure 8) It is assumed that during the fermentation process the pH value will change and could influence the production of the secondary metabolites.

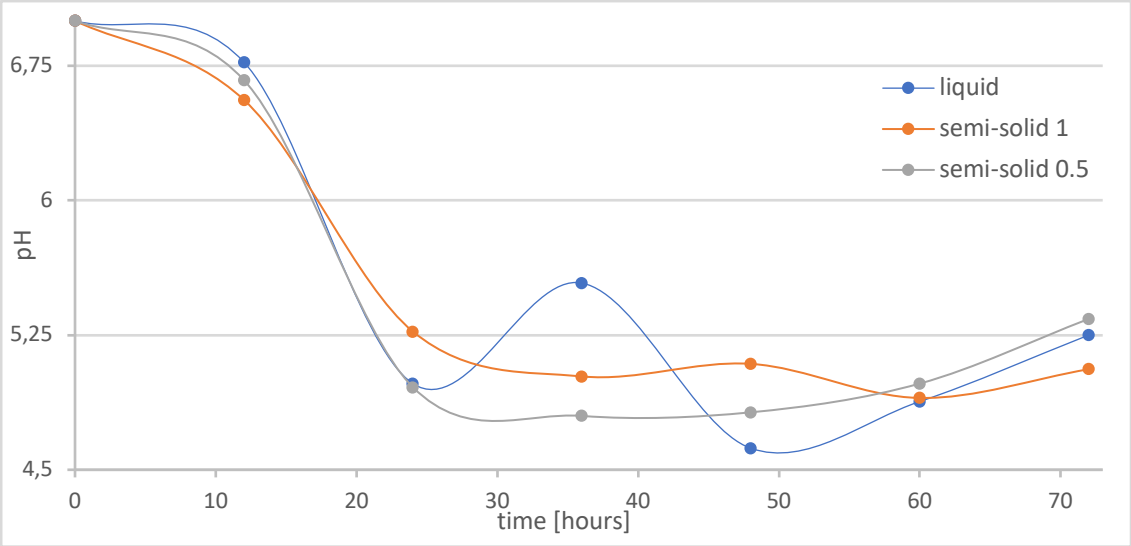

Figure 8: The pH changes plotted over the time span of 72 hours. The interval of the pH was measured was 12 hours. The lines describe: (**blue**) pH change of the liquid media; (**orange**) pH change of the 1% semi-solid media; (**gray**) pH change of the 0.5% semi-solid media.

At the beginning a decrease of the pH can be observed for all the three types of. After 24 h the liquid medium pH starts to increase. After a small increase around 36 h, the pH of the liquid medium decreased again and then followed the same trend as the semi-solid media. Unfortunately, we were not able to obtain reliable measurements for the biomass produced during fermentation due to the interference of the semisolid media.

Previous studies showed that depending on what strain of *Streptomyces* is used the optimal pH can vary slightly. Khattab and his collages isolated *Streptomyces* from marine soil sediment and determined a maximum production rate of metabolites at a pH range between 7 and 7.5 (Khattab, et al., 2016). Another study determined the optimal pH fermentation conditions of *Streptomyces* sp. RUPA08PR to lie around 8, while extreme pH values are not favored for the production of secondary metabolites (Ripa, et al., 2009). Taken these studies into consideration, the optimal pH range seems to be around 7-8, whereas under acidic or basic conditions the production of secondary metabolites is negatively affected. Considering these previous studies, the acid pH observed here could be a reason for a low yield in active secondary metabolites as shown in the next sections. To determine the optimum pH value for BCCO 10_1627 additional fermentations at different pH values must be done. Moreover, during these experiments the pH has to be kept constant for a longer period.

After 72 h, the metabolites were extracted and sent for a chemical analysis to the analytical lab of Dr. Roman Grabic at the Faculty of Fisheries and Protection of Waters at the University of South Bohemia. The obtained chromatographs indicated that the semi-solid extracts were too contaminated with the agar to obtain valuable results. On the other hand, we were able to analyze the raw extract of the liquid extract. Unfortunately, no new peak/compound was discovered (data not shown).

4.5. Antibiotic disk assay of *Kutzneria* sp. BCCO 10_1627 raw extracts against Bacillus subtilis

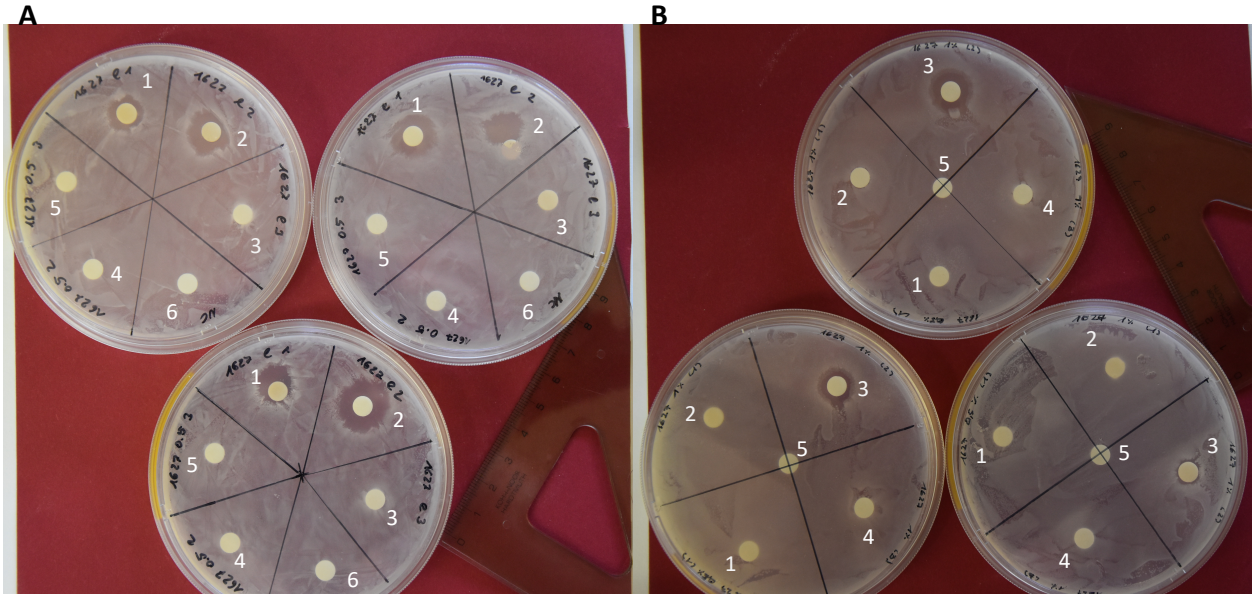

Figure 9: Antibiotic disk assay to test the antibacterial activity of BCCO 10_1627 raw extracts against *Bacillus subtilis*. **Picture A: (1, 2, 3)** extracts obtained from the liquid cultures; **(4,5)** extracts obtained from the 0.5% cultures of the second and third flask; **(6)** negative control. **Picture B: (1)** extract obtained from the 0.5% cultures of the first flask; **(2,3,4)** extracts obtained from the 1% cultures; **(5)** negative control.

The antibiotic activity of the raw extracts produced with the three types of fermentation medium was tested against *Bacillus subtilis* (Figure 9). The the measured inhibition zones are summarized in Table S3. In the Table 12 below the mean range of the triplicates was calculated.

Table 12: The average diameter values of the measured inhibition zones of the conducted antibiotic disk assays. The number in the brackets correspond to the numbering of the fermentation flasks from which the extracts originated from.

Extract from	Average inhibition zone [diameter mm]
liquid (1)	10.9
liquid (2)	12,0
liquid (3)	0.0
0.5% (1)	0.0
0.5% (2)	0.0
0.5% (3)	0.0
1% (1)	0.0
1% (2)	9,5
1% (3)	0.0

Two of the three flasks with the liquid medium and one flask with the 1% semisolid medium contained some active metabolites (Figure 9). On the other hand, neither of the raw extracts from the 0.5% semisolid cultures contained active metabolites against *Bacillus subtilis*. By comparing the inhibition zones of the liquid and the 1% semisolid media, the metabolites from the 1% cultures produced a much smaller inhibition area. This can either be because the synthesized amount or the purity of the bioactive metabolites were insufficient.

4.6. Separation of the extracted metabolites using TLC and antibiotic assay against Bacillus subtilis

We used thin layer chromatography, TLC, to determine approximately how many compounds are present in the raw extracts (Figure 10A, B, C). Then, the antibiotic activity of the separated compounds was determined with an antibiotic assay against *Bacillus subtilis* (Figure 10D).

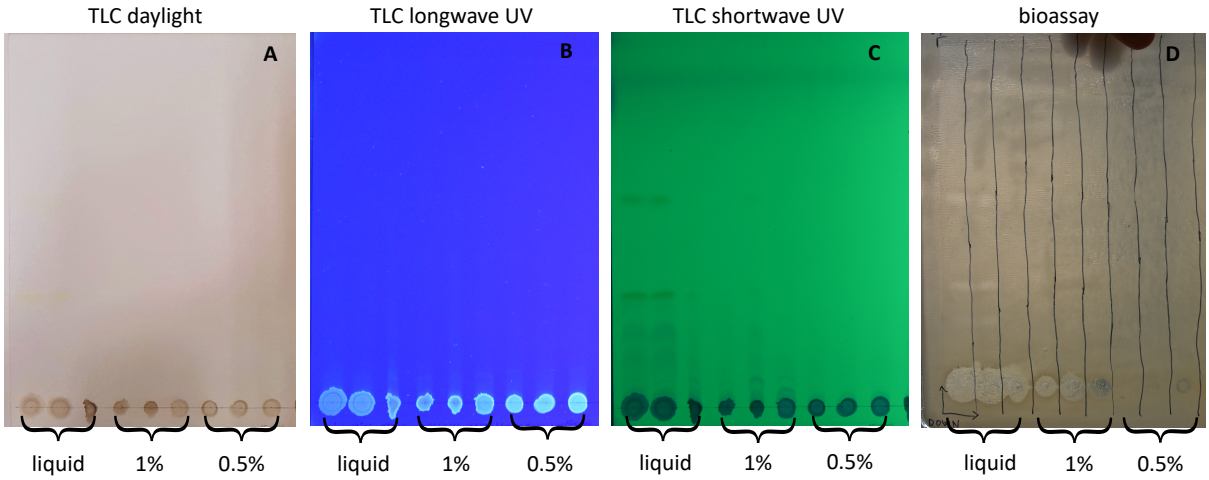

Figure 10: The raw extracts were applied on a TLC plate and after separation the TLC sheet was imprinted onto an agar plate with *Bacillus subtilis*: **(A)** TLC daylight; **(B)** TLC longwave UV; **(C)** TLC shortwave; **(D)** bioassay against *Bacillus subtilis*. Three replicates per type of medium (liquid; 1% and 0.5%).

Three spots can be observed on the TLC plate under the shortwave UV light for the first two replicas of the raw extracts obtained from the liquid medium (Figure 10C). All three spots have an antibiotic activity against *Bacillus subtilis* (Figure 10D). Both liquid replicates had a visible inhibition zone in the previously conducted experiment, the antibiotic disk assay (Figure 9). The other inhibition zone measured originated from the second flask of 1% semisolid media. When determining the composition through TLC, only one very light spot can be seen under the shortwave UV light (Figure 10C). The dots height is around the same height as the first spot of the liquid separated compounds. This corresponds to the smaller diameters of the bioassays and suggest either contamination through the agar or the metabolites were not extracted properly out of the semisolid media.

Combining the information obtained from the different experiments, the use of a semi-solid medium did not increase the production of metabolites with antimicrobial activity against *Bacillus subtilis*. The pH-value of the liquid flask have fluctuated the most and the pH of the 1 % agar semisolid medium was higher most of the time compared to the 0.5 % agar medium. It is highly likely that the secondary metabolites, which were detected in the extracts, were synthesized in the acidic environment ranging between 5.5 and 5.0. This range is assumed since no metabolites were produced in the semisolid media 0.5%, which had its lowest pH value at 4.8 where the other two were above a pH of 5. To prove this assumption another experiment must be conducted to measure the pH-value in a shorter interval and also take samples to determine when

the production of active metabolites begins. Another critical point was the agar contamination in the extracts obtained from the semisolid media, which led to a dirty chromatogram in the chemical analysis. Therefore, future experiments should establish an efficient method for the extraction of metabolites from an agar-containing medium.

5. Conclusions

The PCR screening for the cyclizing 5-aminolevulinate synthase (*hemA* gene) resulted in the identification of one positive strain, *Streptomyces* sp. BCCO 10_2060. The strain is closely related to another Actinomycetes of the BCCO collection, *Streptomyces* sp. BCCO 10_1062, which might produce annmycin as a secondary metabolite. Further analysis is needed to confirm the production of annimycin in these two strains.

During the fermentation of *Kutzneria* sp. BCCO 10_1627, a fluctuation of the pH was measured over a time span of 72 hours. To gain an in-depth knowledge about the optimum pH range for synthesizing bioactive metabolites further experiments are needed. These should gather more data by reducing the measurement intervals of the pH from every 12 hours to every 6 hours. Simultaneously, samples for metabolite extraction should be taken and then analyzed with an antibiotic disk assay and TLC followed by a chemical analysis of the interesting samples. In this thesis the measured raw extracts with the previously mentioned methods showed, that either no secondary metabolites were produced or the extracts were contaminated with the agar of the semisolid medium. No new compound was discovered in the liquid media extracts.

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7. Appendix

Table S1: list of all Actinomycetes with isolation source, number assigned in BCCO collection, measured NanoDrop values, results of 16SrRNA and *hemA* PCR. Strains highlighted in grey were sequenced in this study.

Isolation source	**Identification**	**BCCO 10_**	**ng/µL**	**260/280**	**260/230**	**16S rRNA gene PCR**	**cALAS/ *hemA* gene PCR**
Palava	*Streptomyces* sp.	1499	3401.6	1.85	1.98	+	-
Palava	*Streptomyces* sp.	1501	293.2	1.70	1.83	+	-
Palava	*Streptomyces* sp.	1502	3433.6	1.84	2.12	+	-
Palava	*Streptomyces* sp.	1503	1156.3	1.89	1.94	+	-
Palava	*Streptomyces* sp.	1504	690.9	2.00	1.47	+	-
Palava	*Streptomyces* sp.	1505	804.7	1.93	2.17	+	-
Palava	*Streptomyces* sp.	1506	8.0	1.62	2.41	+	-
Palava	*Streptomyces* sp.	1507	145.9	1.81	1.59	+	-
Palava	*Streptomyces* sp.	1508	189.1	1.83	0.97	+	-
Palava	*Streptomyces* sp.	1509	1497.3	1.91	2.26	+	-
Palava	*Streptomyces* sp.	1510	4194.9	1.83	1.97	+	-
Palava	*Streptomyces* sp.	1511	203.7	1.79	1.57	+	-
Palava	*Streptomyces* sp.	1512	184.5	1.81	1.83	+	-
Palava	*Streptomyces* sp.	1513	1720.7	1.95	2.06	+	-
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1539	373.1	1.93	1.76	+	-

Tblisi botanical garden, Georgia	*Streptomyces* sp.	1540	1689.3	1.86	1.97	+	-
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1541	2575.8	1.79	1.63	+	-
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1542	10762.8	1.85	1.99	+	-
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1543	87.1	1.89	0.74	+	-
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1544	4512.0	1.77	2.19	+	-
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1545	1164.6	1.84	1.79	+	-
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1546	295.4	1.86	1.31	+	-
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1547	425.4	1.83	1.84	+	-
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1550	934.7	1.93	1.79	+	-
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1551	6315.7	1.84	2.09	+	-

Tblisi botanical garden, Georgia	*Streptomyces* sp.	1553	1068.3	1.89	1.99	+	-
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1554	863.8	1.88	1.64	+	-
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1555	124.8	1.88	0.83	+	-
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1556	530.3	1.85	1.77	+	-
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1557	2178.5	1.77	0.56	+	-
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1557	191.8	1.57	0.58	-	-
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1558	287.7	1.80	1.46	+	-
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1559	1158.8	1.87	2.17	+	-
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1560	486.9	1.82	1.56	+	-
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1561	147.9	1.81	1.41	+	-

Tblisi botanical garden, Georgia	*Streptomyces* sp.	1562	1234.1	1.80	1.38	+	-
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1563	754.0	1.80	1.85	+	-
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1564	1796.7	1.78	2.25	+	-
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1565	239.4	1.88	1.36	+	-
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1566	1881.1	1.86	1.98	+	-
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1567	2641.6	1.86	2.21	+	-
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1568	528.2	1.81	1.84	+	-
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1570	20501.9	1.42	1.42	+	-
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1571	9270.7	1.78	1.65	+	-
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1572	1270.0	1.87	1.89	+	-

Tblisi botanical garden, Georgia	*Streptomyces* sp.	1573	223.0	1.91	1.44	+	-
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1574	4462.7	1.91	1.95	+	-
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1575	1597.8	1.80	2.36	+	-
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1576	829.8	1.81	1.75	+	-
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1577	81.0	1.79	1.95	+	-
Tblisi botanical garden, Georgia	*Saccharotrix* sp.	1578	1734.2	1.88	1.76	+	-
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1579	521.4	1.87	1.80	+	-
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1580	3639.5	1.85	1.53	+	-
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1581	2275.2	1.83	1.91	+	-
Tblisi botanical garden, Georgia	*Streptomyces* sp.	1583	1.8	0.86	0.14	+	-

Tblisi botanical garden, Georgia	*Streptomyces* sp.	1584	5570.6	1.78	1.99	+	-
Unknown habitats, Antarctica	*Streptomyces* sp.	2060	3708.5	1.86	1.74	+	+
Unknown habitats, Antarctica	*Streptomyces* sp.	2061	3837.1	1.86	1.79	+	-
Unknown habitats, Antarctica	*Streptomyces* sp.	2062	1467.3	1.89	1.75	+	-
Unknown habitats, Antarctica	*Streptomyces* sp.	2063	71.8	1.84	1.40	+	-
Unknown habitats, Antarctica	*Streptomyces* sp.	2064	447.4	1.80	1.52	+	-
Unknown habitats, Antarctica	*Streptomyces* sp.	2065	1113.9	1.92	1.53	+	-
Unknown habitats, Antarctica	*Streptomyces* sp.	2066	56.6	1.80	1.36	+	-
Unknown habitats, Antarctica	*Streptomyces* sp.	2067	2431.3	1.86	1.97	+	-
Unknown habitats, Antarctica	*Streptomyces* sp.	2068	146.9	1.80	1.28	+	-
Unknown habitats, Antarctica	*Streptomyces* sp.	2069	177.9	2.01	1.26	+	-
Unknown habitats, Antarctica	*Streptomyces* sp.	2070	3229.5	1.90	1.80	+	-
Unknown habitats, Antarctica	*Streptomyces* sp.	2071	3149.0	1.87	1.75	+	-

Unknown habitats, Antarctica	*Streptomyces* sp.	2072	790.6	1.90	1.38	+	-
Unknown habitats, Antarctica	*Streptomyces* sp.	2073	1013.9	1.87	1.82	+	-
Unknown habitats, Antarctica	*Streptomyces* sp.	2074	1217.8	1.83	1.44	+	-

Table S2: The closest relative obtained from blast for BCCO 10_ strains. The bp, similarity in %, acc. number and the isolation source are listed.

BCCO 10_	Closest hit (Blast)	bp	%	acc number	isolation source
1541	*Streptomyces* sp. ms191	1041/1043	99	MK053660.1	soils in Eastern USA
	Streptomyces fradiae strain YY69	1041/1043	99	MH265962.1	rhizosphere soil from China
	Streptomyces fradiae strain YY68	1041/1043	99	MH265961.1	rhizosphere soil from China
1545	*Streptomyces galilaeus* strain ATCC 14969	1398/1399	99	CP023703.1	soil from South Korea
	Streptomyces sp. strain Mg27	1398/1399	99	MN187453.1	France
	Streptomyces galilaeus strain JCM 4757 (T)	1398/1399	99	MK424308.1	China
1555	*Streptomyces* sp. NEAU-S7GS2	1394/1396	99	CP029541.1	soil from China: Daqing
	Streptomyces sp. ID38673	1395/1396	99	JQ929051.1	soil from Italy
	Streptomyces sp. ID38640	1394/1396	99	JQ929050.1	soil from Italy
1577	sequence was contaminated; therefore no identification could be made				
1583	*Streptomyces ryensis*	1400/1401	99	AB184517.1	strain

2060	*Streptomyces avidinii*	1400/1400	100	KR085837.1	Chandra Tal Lake sediment
2061	*Streptomyces avidinii*	1400/1400	100	KR085837.1	Chandra Tal Lake sediment
2062	*Streptomyces avidinii*	1400/1400	100	KR085837.1	Chandra Tal Lake sediment
2063	*Streptomyces avidinii*	1400/1400	100	KR085837.1	Chandra Tal Lake sediment
2064	*Streptomyces avidinii*	1400/1400	100	KR085837.1	Chandra Tal Lake sediment
2065	*Streptomyces agglomeratus*	1400/1401	99	AB184447.2	strain
2066	*Streptomyces* sp. QLS90	1391/1393	99	JQ838150.1	rhizosphere soils from China
	Streptomyces cirratus strain IHB B 10402	1390/1393	99	KR233768.1	forefield of a glacier from India: Kunzum Pass in Lahaul-Spiti, Himachal Pradesh
	Streptomyces sp. PL75	1390/1393	99	KC789726.1	soil from Czech Republic
2067	*Streptomyces* sp. strain RP-1-5	1395/1399	99	MN180865.1	Arctic soil from South Korea
	Streptomyces sp. strain RP-1-3	1395/1399	99	MN180863.1	Arctic soil from South Korea
	Streptomyces sp. strain RP-1-2	1395/1399	99	MN180862.1	Arctic soil from South Korea
2068	*Streptomyces* sp. QLS90	1390/1392	99	JQ838150.1	rhizosphere soils from China
	Streptomyces cirratus strain IHB B 10402	1389/1392	99	KR233768.1	forefield of a glacier from India: Kunzum Pass in Lahaul-Spiti, Himachal Pradesh
	Streptomyces sp. PL75	1389/1392	99	KC789726.1	soil from Czech Republic
2069	*Streptomyces drozdowiczii* strain IHBA 11040	1397/1400	99	KR085954.1	Suraj Tal Lake in the Indian Trans-Himalayas

	Pilimelia columellifera subsp. pallida strain IHBA 11041	1396/1401	99	KR085949.1	Suraj Tal Lake in the Indian Trans-Himalayas
	Streptomyces drozdowiczii strain PM276A	1394/1400	99	JQ422169.1	Canada: Helmcken Falls Cave, Wells Gray Provincial Park, BC
2070	*Streptomyces* sp. strain RP-1-4	1397/1401	99	MN180864.1	Arctic soil from South Korea
	Streptomyces sp. strain RP-1-1	1397/1401	99	MN180861.1	Arctic soil from South Korea
	Streptomyces avidinii strain IHBA 9319	1397/1401	99	KR085837.1	Chandra Tal Lake in the Indian Trans-Himalayas
2071	*Streptomyces* sp. strain RP-1-4	1379/1393	99	MN180864.1	Arctic soil from South Korea
	Streptomyces sp. strain RP-1-1	1379/1393	99	MN180861.1	Arctic soil from South Korea
	Streptomyces avidinii strain IHBA 9319	1379/1393	99	KR085837.1	Chandra Tal Lake in the Indian Trans-Himalayas
2072	*Streptomyces finlayi* strain CGMCC 4.1436 clone 1	1392/1393	99	JQ924390.1	China
	Streptomyces finlayi strain BCCO 10_740	1391/1393	99	KP718539.1	Borova winter pasture, soil without cattle impact from Czech Republic
	Streptomyces finlayi strain IHBA 11039	1391/1393	99	KR085955.1	Suraj Tal Lake in the Indian Trans-Himalayas
2073	*Streptomyces* sp. strain RP-1-4	1388/1392	99	MN180864.1	Arctic soil from South Korea
	Streptomyces sp. strain RP-1-1	1388/1392	99	MN180861.1	Arctic soil from South Korea
	Streptomyces avidinii strain IHBA 9319	1388/1392	99	KR085837.1	Chandra Tal Lake sediment

2074	*Streptomyces avidinii*	1400/1400	100	KR085837.1	Chandra Tal Lake sediment
OS_259	*Streptomyces atratus* strain T11	1392/1393	99	KU324451.1	earthworm feces from wasteland near the outfall of a sewage treatment plant from China: Sichuan, Chengdu, Qingbaijiang
	Streptomyces yanii strain BCCO 10_1038	1392/1393	99	KP718604.1	Vyklantice, potatoe field from Czech Republic
	Streptomyces sp. S27(2014)	1392/1393	99	KF772635.1	bog from Algeria: Ain Khiar
OS_5966	*Streptomyces* sp. strain NZ2BY 16S	1393/1394	99	MN625921.1	plateau from Turkey
	Streptomyces sp. RBST2-54	1393/1394	99	LC489259.1	compost from Turkey
	Streptomyces sp. RBST2-21	1393/1394	99	LC489251.1	compost from Thailand

Table S3: The measured rounded inhibition zone ranges of the extracts, which contained antibiotic effect against *Bacillus subtilis*. The numbers in the brackets correspond with the fermentation flasks the extracts have been extracted from. Each extract was applied in a total of three times for replication.

Extract	Inhibition zone [diameter mm]
1627 1% (2)	7,5-8,0
1627 1% (2)	10,0-11,5
1627 1% (2)	9,0-11,0
1627 I1	8,5-10,0
1627 I1	10,0-12,0
1627 I1	12,0-13,0
1627 I2	11,0-12,0
1627 I2	14,0-15,0
1627 I2	9,0-11,0

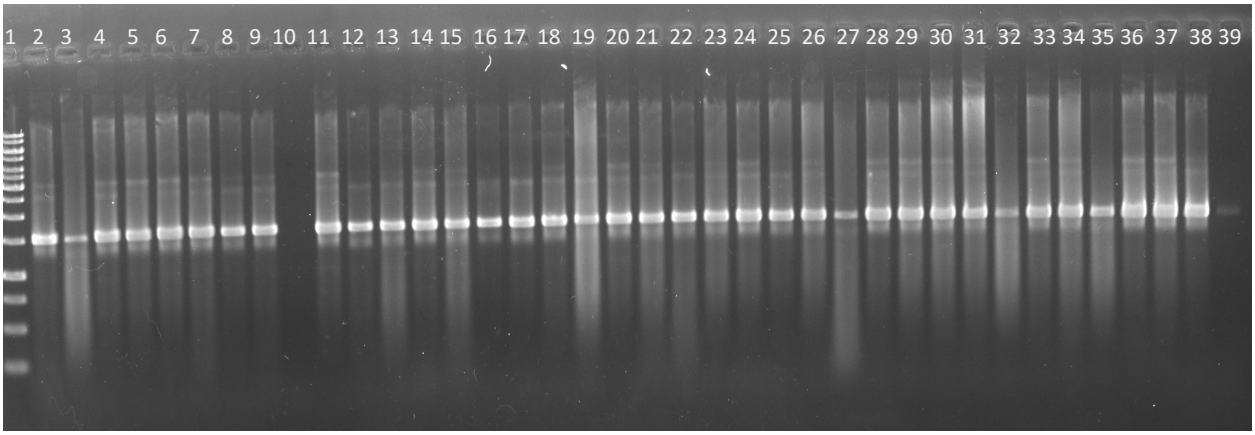

Figure S1: Example gel containing the 16S rRNA gene PCR products. The samples applied in this order: (**1**) 1kb DNA ladder; (**2**) 1562; (3) 1563; (**4**) 1564; (**5**) 1565; (**6**) 1566; (7) 1567; (**8**) 1568; (9) 1570; (**10**) 1571; (**11**) 1572; (**12**) 1573; (**13**) 1574; (**14**) 1575; (**15**) 1576; (**16**) 1577; (**17**) 1578; (**18**) 1579; (**19**) 1580; (**20**) 1581; (**21**) 1583; (**22**) 1584; (**23**) 2060; (**24**) 2061; (**25**) 2062; (**26**) 2063; (**27**) 2064; (**28**) 2065; (**29**) 2066; (**30**) 2067; (**31**) 2068; (**32**) 2069; (33) 2070; (**34**) 2071; (**35**) 2072; (**36**) 2073; (**37**) 2074; (**38**) positive control BCCO 10_322; (**39**) negative control