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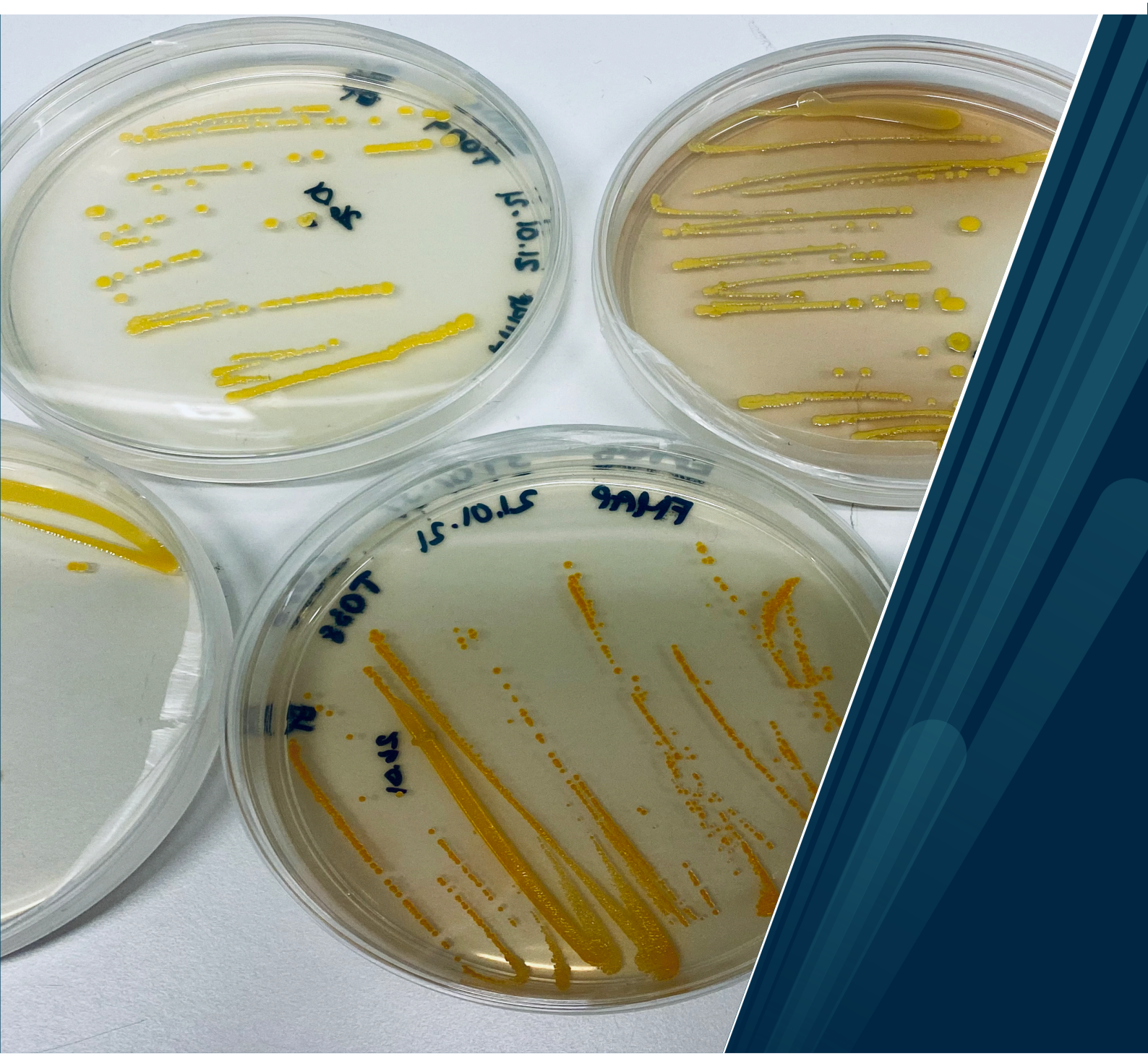
Identification of Bioactive Molecules from Arctic Marine Arthrobacter Isolates

Ella Trosten

Master's thesis in Chemistry and Biotechnology (May 2021)
30 credits



Identification of Bioactive Molecules from Arctic Marine Arthrobacter Isolates

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Master's thesis (30 credits)
The Norwegian College of Fishery Science, Marbio

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Acknowledgment

This thesis was conducted in the period January to May 2021 at Marbio, The Norwegian College of fisheries science. This thesis concluded my master's degree in Chemistry and Biotechnology at the Norwegian University of Life Sciences (NMBU).

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The Covid-19 pandemic has unavoidably set its mark on this thesis. Through short timelines, lockdowns, social distancing and personal loss, this thesis still came together with a result I hope could be beneficial in the future. For this I would like to thank my friends, loved ones and my significant other that stood by me in this challenging time.

Abstract

More than two thirds of the Earth's surface is covered by water and that area is estimated to be more biological diverse than a tropical rain forest [1]. This makes the marine environments an interesting potential field for finding new and novel bacterial compounds that could lead to a new drug against antibiotic resistant pathogens, and diseases such as cancer and diabetes.

Arthrobacter sp. are known producers of the antibiotic arthrobacilins and have been shown to produce different variants of this antibiotic under different conditions [2]. The motivation for this thesis was to discover new types of arthrobacilins produced by Arctic marine *Arthrobacter* isolates and identify novel secondary metabolites with potential bioactivity. Another motivation was to gain experience with the genus *Arthrobacter,* and *Actinobacteria* as a phylum in general.

In this study, five isolated Arctic marine bacteria of the genus *Arthrobacter* were cultivated under different temperatures and growth media. Employing the "One Strain-Many Compounds" (OSMAC) approach, in an attempt to produce novel and interesting compounds with potential bioactivity by triggering different metabolic pathways. Of the five strains T009, T011, T024, T038 and T040, three were selected for the OSMAC approach. These showed different yield in biomass and metabolite production, thus the application of the OSMAC approach was deemed successful.

The cultures were extracted, fractionated, and tested for bioactivity against human cancer cell-lines (A2058 and MRC5) and the human pathogenic bacteria *Staphylococcus aureus, Enterococcus faecalis*, *Streptococcus agalactiae*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*. The bioactivity screening resulted in 26 hits in the primary screening, and there was conducted a secondary screening only for the cell-line A2058 and *S. agalactiae*, resulting in 13 hits.

The three fractions deemed the most promising for the identification of potential bioactive compounds were dereplicated using a UHPLC-HR-MS/MS. Dereplication showed a large quantity of media and modified media components in Arthrobacter medium that originates from soybean meal, and showed antibacterial activity. It is not recommended as cultivation media in bioassay-guided identification. This thesis was a valuable starting point for further research on cultivation of Arctic marine *Arthrobacter,* and the *Actinobacteri*a phylum in general, with the purpose of producing bioactive secondary metabolites.

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Abbreviations:

ArtG	*Arthrobacter Medium with Glycerol*
ArtM	*Arthrobacter Medium*
BLAST	Basic Local Alignment Search Tool
B.C.	*Before Christ*
CFU	*Colony Forming Units*
DMSO	*Dimethyl Sulfoxide*
DNA	*Deoxyribonucleic Acid*
et al.	*et alii*
etc.	*et cetera*
EtOH	*Ethanol*
ESI	Electron Spray Ionization
g	*Gram*
HPLC	*High-performance Liquid Chromatography*
ID	*Identification*
kd	*Kilo Dalton*
L	*Liter*
m	*Milli*
MeOH	*Methanol*
MIC	*Minimum Inhibition Concentration*
MS	*Mass Spectrometry*
m/z	*Mass-to-charge ratio*
NP	*Natural Product*
OD	*Optical Density*
OSMAC	*One Strain Many Compounds*
PBS	*Phosphate Buffer Saline*
PCR	*Polymerase chain reaction*
rpm	*Rounds Per Minute*
RT	*Retention Time*
SM	*Secondary Metabolite*
sp.	*Species*
TSB	*Tryptic Soy Broth*
UV	*Ultraviolet*
µ	*Micro*
°C	*Degree Celsius*
nm	*Nano Meter*

1 Introduction - Bioprospecting in Marine Environments

1.1 Natural Products: Secondary Metabolites

The search for and utilization of resources found in nature is a key factor in the evolution and survival of the human race. It has been a source for food, shelter, clothing and medicine. Natural products (NPs) as medicine have been used by mankind throughout history and respective knowledge has been acquired by trial and error with the resources at hand. The earliest recording of NPs used as medicine was in Mesopotamia around 2600 B.C., using herbal oils which are still in use today for treating coughs and colds [3]. Studies of these traditional medicines have provided the knowledge that led to the isolation of their respective active principle. This led to the development of most early drugs, such as aspirin and morphine (figure 1.1) [4]. Alexander Flemings discovery of the antibacterial effect of the fungus *Penicillium notatum* in 1928, and its synthetic versions led to a paradigm shift in drug discovery [4]. While most of the early drugs have been discovered from macroorganisms, particularly plants, the publication on the clinical data of penicillin caused research groups and drug companies to assemble collections of microorganisms in order to discover new antibiotics or bioactive NPs [3].

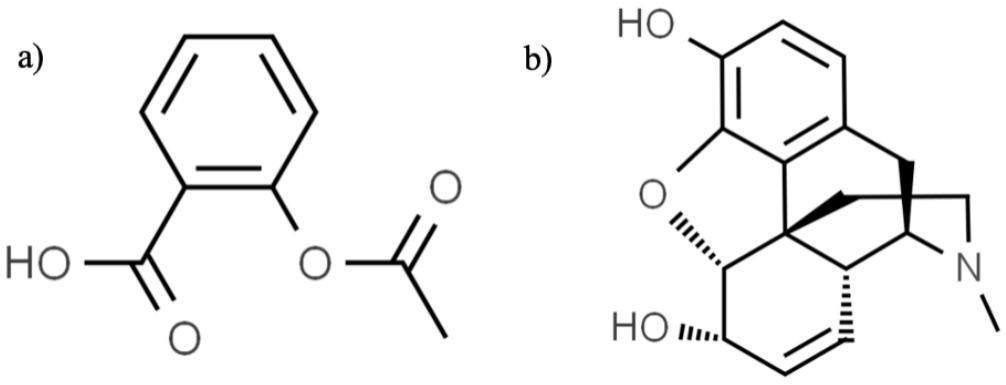

Figure 1.1: Chemical structure of a) aspirin and b) morphine.

NPs are all molecules produced by organisms in nature, and include any compound or molecule that originates from either animals, plants or microorganisms [5, 6]. The molecules that are produced by a given organism can be divided in two sub-groups: primary and secondary metabolites. The former consists of carbohydrates, proteins, lipids, nucleic acids and more, all essential for an organism to live, grow and reproduce. These metabolites are, with a few exceptions, universal for all organisms and is either synthesized by the organism itself or acquired through external sources [5].

Secondary metabolites (SMs) are small molecules that are non-essential for the organisms survival, have a molecular weight of less than 2 kDa [7] and are an *"expression of the individuality of species"* [5]. Both energy and resources are required to produce SMs, and it is therefore costly for the organism. The expression of SMs is a tightly regulated process thought to be activated in response to environmental changes and to increase the competitiveness of the producing organism [8]. SMs are for instance used by the organism as a defense mechanism or to adapt to its surroundings [3]. This can be done by suppression of competing organisms or predators, intra- or interspecific signaling, quorum sensing, inhibition of microbial invasion, protection against radiation, heat or pressure *etc*. [9]. Primary metabolites are the general building blocks and essential for the organism to survive, while SM are optional molecules that mostly have an advantageous function for the producing organism.

The bewildering diversity of secondary metabolites makes them the most medically relevant NPs [8], and SMs are a source for unique structures that have potential novel properties. Natural selection has caused compounds to evolve in order to acquire a maximal effect and stability out of a minimal of material and energy [6]. Since only about 10 % of the biodiversity on Earth is evaluated for potential bioactivity [3], NPs composes an immense untapped reservoir for potential novel compounds that can lead to advances in both industrial and medical fields [8].

1.1.1 Diversity in marine environments

A great number of known drugs derive from living organisms, but most of these are of terrestrial origin [1]. Since the discovery of penicillin in the late 1920s the search for microorganisms that produce novel SMs has been widely accepted, but most of these have originated from soil [10]. Marine microbes and organisms harbor an overwhelming unexplored diversity, and the ocean is estimated to be more biological diverse than a tropical rain forest [11]. *William Fenical* said "*It seemed ridiculous to me that the ocean – with such a vast habitat – had escaped anyone's notice. But there are good reasons. People fear the ocean; it has been considered a very hostile, inhospitable place.*" [1]. The ocean was assumed to be a poor and infertile environment because of its high concentrations of salt [12]. This mindset, that the ocean was infertile and dangerous, together with the fact that up to the point of scientific advances like the SCUBA (1970s), manned submarines (1980s) and remotely operated vehicles (ROVs) (1990s) it was also deemed inaccessible [3]. Thus, the marine environments were not even considered as possible sources for new bioactive compounds.

Two thirds of the Earth's surface is covered by water [1] and harbors extreme environmental conditions [9]. These conditions differ from terrestrial, and have these characteristics: low temperatures, low concentration of organic material, high salinity and hydrostatic pressure [13]. It also contains niches of all kinds, for example deep hydrothermal vents with temperatures up to 350°C, light ranging from absolute darkness to photic zones [9] and varies from shallow coastal waters to deep trenches. To adapt, and even thrive, in these harsh and wide spectrum of conditions marine organisms produce SMs with unique chemical structures and bioactivity [14]. Compounds released into water need to be much more potent to have an effect due to the rapid dilution, which gives the possibility of finding possible drugs with higher efficacy and specificity [11]. This realization opened the eyes of pharmaceutical companies and research groups to the potential for marine environments to be a source for finding novel chemical compounds.

This led to marine bioprospection, which is the systematic search for genes or novel, biological active compounds from marine sources that have potential commercial and scientific value [15]. These can be for pharmaceutical, cosmetic, agricultural or other commercial applications. This has caused countries like Norway to develop national strategies, and the Norwegian government has defined marine bioprospecting as: *"a source of new and viable wealth creation"* [15], and has resulted in funding of research and infrastructure development for NP discovery in marine environments.

1.1.2 The "one train many compounds" approach

Secondary metabolites from microbes are mainly expressed from gene clusters, and a major part of these are considered silent under standard conditions [16]. There is thus an inconsistency between the number of secondary metabolites expressed under laboratory conditions and the potential number discovered by bioinformatic approaches in the genome [8]. This may be due to unsuitable analytic methods, that the genes are not functional or activated. The triggering of these silenced clusters could lead to the discovery of novel chemical compounds with new and unique properties. The "one strain many compounds" (OSMAC) approach is a powerful tool in the triggering of these clusters and is based on the principle that different environmental conditions can induce the production of different compounds or molecules from the same stain [8]. It can impact both the metabolic profile of an organism and the quantity of which the compounds are produced [17].

Secondary metabolites are biosynthesized trough the metabolic pathway and their formation is, with a few exceptions, catalyzed by enzymes [5]. These enzymes stem from mRNA, that themselves originates from complementary DNA located in gene clusters [8]. This offers many targets through which the environmental conditions can influence the formation of secondary metabolites. Transcription, translation, activation or inhibition of enzymes are all points in the biosynthesis that can be manipulated by the OSMAC approach [18] and induce the production of new or modified secondary metabolites.

1.1.3 The genus *Arthrobacter*

Arthrobacter is a genus of the phylum *Actinobacteria*, which is recognized as one of the biggest phylums within the bacterial domain [19]. *Actinobacteria* is a known producer of secreted SMs and compounds with many medicinal properties, such as anticancer and immunosuppressant [20]. 12 of 24 novel natural products leading to drugs between 1981 and 2006 originated from this phylum, and it accounts for two-thirds of all known antibiotics [20, 21].

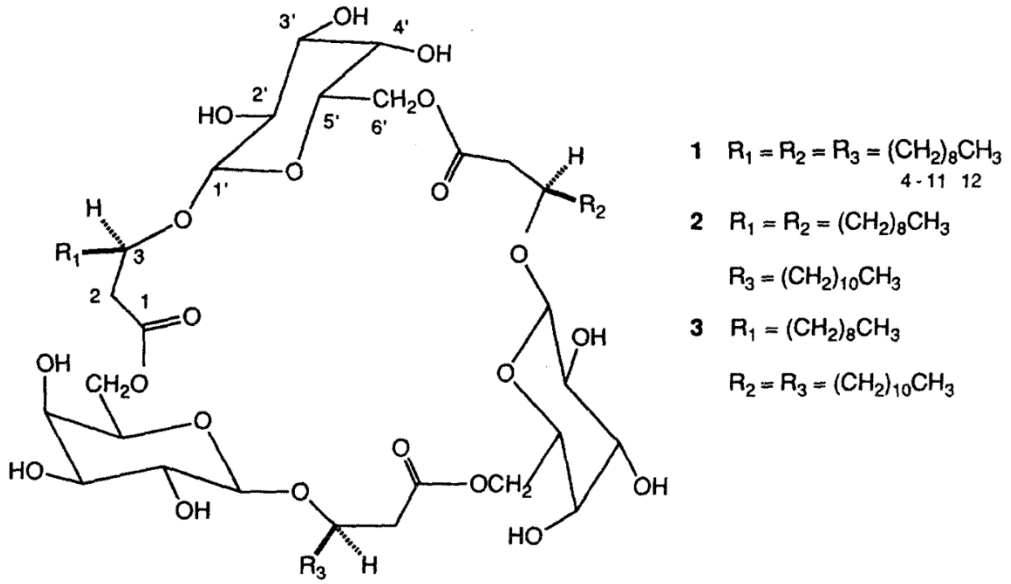

Figure 1.2: The core structure of the antibiotic arthrobactilins, and the side chains (R_1, R_2 and R_3) for arthrobacilins A (1), B (2) and C (3) [2].

Arthrobacter sp. is a gram-positive bacteria that has been found in many different environments, including soil, fresh water, oil, air, sewage, sea water and under terrestrial subsurface sediments down to 220 m [22]. They are known producer of secondary metabolites and different *Arthrobacter* strains has been shown to produce different variants of the antibiotic arthrobacilins (figure 1.2) under different growth conditions [2, 23]. The motivation for this thesis is to discover new types of arthrobacilins produced by Arctic marine Arthrobacter isolates.

1.2 Drug discovery pipeline

The need for new drugs is a pressing problem to be solved. Pathogenic microbes and cancer cells are ever evolving and gaining resistance against current modern medicine [8]. Antimicrobial resistance alone is predicted to cause the death of approximately 100 million by 2050 [24], and infectious diseases, such as tuberculosis, pneumonia and gonorrhoea, are becoming harder to treat [25]. This will lead to an economic burden on society due to the hospitalization caused by illness earlier treated easily by antibiotics, use of longer and more expensive treatments, increased duration of illness etc. [25].

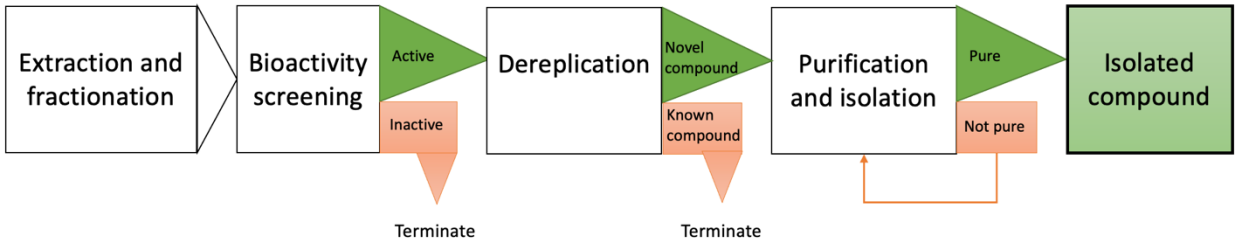

Figure 1.3: Workflow of bioassay-guided purification/isolation (adopted and changed from [26]).

The discovery of novel compounds through bioassay-guided purification (figure 1.3) is a way of selecting for potential candidates at an early stage of the process and prioritizing the ones deemed especially promising. The need for an early selection is essential when the source of natural products is vast, and the cost and time is estimated to be $50 000 and three months for the isolation and characterization of one NP [27].

The first step in figure 1.3 is the extraction and then fractionation of the crude extract. The selection of extraction method depends on the source (plants, microorganisms, animal tissue, etc.) of the material and the target of extraction (unknown or known, a group of compounds or all metabolites present, etc.) [28]. The crude extract consists of many compounds and fractionation is used to roughly separate these, by separating them based on similar polarities or molecular weight [28]. The fraction or the crude extract is tested for activity in different bioactivity screens, such as anticancer or antibacterial assays. Samples selected based on pre-determined cut-off values are submitted to dereplication. This is a method for identifying if the activity is due to known or novel compounds before proceeding with purification and isolation [3]. Isolated compounds are usually retested for bioactivity, to confirm if the activity really is caused by the selected compound and at which concentrations it is effective. Some discovered novel molecules are approved as drugs unaltered from its original form and some are modified before attaining the desired potency, selectivity or effect [29].

1.2.1 Bioactivity screening: Anticancer, antibacterial and biofilm formation inhibition activity

The assessment of bioactivity trough different high-throughput screens is an important step in the discovery pipeline and is the first indication of potential bioactivity. High-throughput screening is a method for testing the bioactivity of compounds in a large quantity of samples, and is an effective way of preforming parallel assays for a preliminary evaluation of activity [30]. This is a way of identifying the compounds that should be included in further processing and isolation. The assays can be categorized into groups based on their targets: lower organisms (bacteria, fungi, *etc*.), live cells in culture (such as cancer cell or normal lung fibroblasts), cell tissues of animals or humans as a whole, isolated vertebrate organs, and subcellular systems (such as enzymes, receptors, antibodies, *etc*.) [7]. These assays utilize biological systems, such as cells, bacteria, etc., to detect properties like antibacterial, anticancer, antidiabetic, etc. [28] for samples (crude extracts, fractions, isolated compounds or mixtures).

1.2.1.1 Anticancer activity - Viability assay

Cancer consists of a large group of diseases that is caused by abnormal cell growth and distribution in the tissues and organs of the body [31]. It is the second leading cause of death globally and was in 2018 the reason for every sixth death [31].

To identify bioactivity against cancer or selected cell-lines a cell viability assay is used, and can be done for primary human cell-lines, immortalized cell-lines or cells differentiated from pluripotent stem cells to create specific cell types [32]. The samples in this thesis were screened for anticancer activity against the cell-lines A2058 (human melanoma) and MRC5 (normal lung fibroblast) in an Aqueous One Solution Cell Proliferation assay. The assay measures the cell growth of the respective cell-lines over time to determine the effect of the compounds present in the samples on cell growth. The Aqueous One Solution contains a yellow-coloured tetrazolium salt that living, metabolically active, cells can reduce to formazan product with a dark purple colour, and the amount of formazan product is proportional with the number of surviving cells [33]. Formazan absorb photonic radiation at 490 nm, and the amount of reduced tetrazolium salt is measured spectrophotometrically.

1.2.1.2 Antibacterial activity - Minimum inhibition concentration

Pathogenic bacteria are the cause of many infectious diseases and there is an ever-rising resistance against antibiotics used to treat these infections [34]. This rise in resistance will not only make it harder to treat life threatening infectious diseases, such as pneumonia and tuberculosis, but also conditions, such as cancer, and surgical procedures that requires antibiotics to avoid infections after treatments [24]. This has led to a need for re-prioritizing the use of antibiotics [25], as well as an increased interest in discovering and developing new and effective antibiotics.

In this thesis a minimum inhibition concentration (MIC) assay was conducted to assess the antibacterial activity of the samples. This method determines the lowest concentration of a sample needed to observe growth inhibition of the selected bacterial strains, and was, in this thesis, conducted against five known human pathogens: *Staphylococcus aureus* (gram-positive), *Enterococcus faecalis* (gram-positive), *Streptococcus agalactiae* (gram-positive), *Escherichia coli* (gram-negative) and *Pseudomonas aeruginosa* (gram-negative).

1.2.1.3 Biofilm formation inhibition assay

Biofilm formation is a cause of diseases in both animals and humans, and the attachment of biofilm to different surfaces or systems can be the cause of infection [35]. The gram-positive bacteria *Staphylococcus epidermidis* is a common cause of bloodstream infections due to catheter use on hospitalizes patients, and its ability to form biofilm plays a crucial role in its pathogenesis [36]. Polysaccharide intracellular adhesin is a polymeric substance secreted by *S. epidermidis* to the environment and is important to the formation of biofilm [36]. The removal and treatment of biofilm forming bacteria is more difficult than that of free-living cells, due to their increased tolerance against anti-infection agents [35].

The samples in this thesis were screened for biofilm formation inhibition activity against the bacteria *S. epidermidis* in a spectrophotometric assay, using crystal violet to dye the biofilm and measuring the absorbance at 570 nm to determine the inhibition of biofilm [36].

1.2.2 Dereplication with high performance liquid chromatography and mass spectrometry

To avoid using unnecessary time and resources, and to identify if the compound(s) that are responsible for the activity observed in bioactivity assays is caused by an already known compound, the process of dereplication is used [3]. It is a method for identifying if the activity is due to known or novel compound before proceeding with bioassay-guided isolation [3]. The combination of high-performance liquid chromatography (HPLC) and mass spectrometry (MS) is the most common method of dereplication of NPs, giving a method for calculating the elemental composition of the compound [37]. The elemental composition, together with MS/MS fragmentation, is used to search both molecule and fragment databases to identify the molecule.

HPLC is a technique for separating analytes according to their polarity [26]. It consists of a stationary phase inside a column and a mobile phase with a changing polarity gradient. The stationary phase, called the column material, is comprised of non-polar functional groups, such as C_{18}-hydrocarbons [38], and has affinity to the rather a-polar analytes in the sample. The mobile phase, consisting of a gradient ranging from polar (ddH_2O) to non-polar (acetonitrile), is pumped through the column. The analytes are eluted through the column when the solubility to the mobile phase is higher than the affinity for the column material, and the different chemical properties of the analytes causes them to elute at different mobile phase polarity and thus separate. The time at which the analyte elutes is called retention time (RT).

MS provides molecular weight and structural information of a compound with high sensitivity [30] by analyzing the mass-to-charge ratio (m/z). The coupling of HPLC (separation) to electrospray MS (analyzation) enables the separation of samples containing a complex mixture of analytes and the separation of ions according to their mass spectral data. The method that allows for this coupling is electron spray ionization (ESI), and is used to ionize organic analytes in the sample (figure 1.4). It can ionize in either positive (ESI^+) or negative (ESI^-) ionization mode, depending on if the molecule consists of proton accepting or donating groups respectively [39].

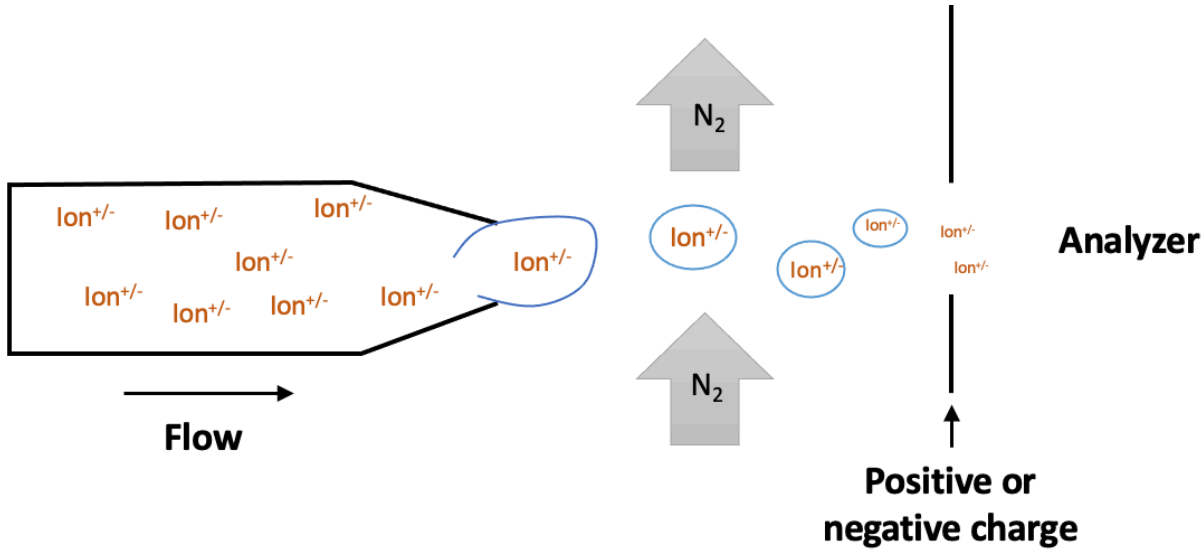

Figure 1.4: Electrospray ionization (adopted and changed from [26]). ESI transforms analytes in a liquid solvent into gaseous ions that can be analyzed. The solvent is pumped through an either positive (ESI^+) or negative (ESI^-) charged capillary, resulting in ions with the same charge. The liquid containing the ions are becoming smaller due to the evaporation solvent because of the N_2 drying gas and the repulsion of charge. The ions then accelerate due to an oppositely charged electric field and move as gaseous ions through the analyzer.

2 Objectives

This master's thesis was conducted within The Norwegian College of Fishery Science, Marbio, with the overall objective to discover potential bioactive compounds in selected species of *Arthrobacter,* and to gain experience with the *Actinobacteria* in general, and with *Arthrobacter* in particular.

The specific objectives of this master's thesis are to:

I. Cultivate selected strains of Arthrobacter under different conditions to investigate the effect of cultivation-conditions on biomass and metabolite production
II. Screen the different extracts for biofilm formation inhibition, antibacterial and anticancer activity.
III. Dereplicate the extracts deemed active to propose potential active compound for isolation.

3 Material and Methods

3.1 General remarks

During this thesis the appropriate chemicals with appropriate quality has been used. Methanol (MeOH) from VWR International S.A.S (France) (Product ID: 20864), ddH_2O was produced with the in-house Milli-Q® system and the filtrated seawater was prepared by filtrating seawater through a Millidisk® 40 Cartridge with Durapore® 0.22 µm filter membrane (Millipore, Burlington, MA, USA). These chemicals are used throughout this thesis, if not otherwise indicated.

3.2 Isolates of Arctic marine *Arthrobacter*

Five isolates from freeze stock from Marbios research expedition in the Barents Sea in August 2020 were used. Isolates T009, T011, T024, T038 and T040 were collected from surface sediment at a depth of 3200 m (77.44N, 2.25E), soil on Bjørnøya (74.33N, 20.11E), stone covered by algae in intertidal zone on Bjørnøya (74.31N, 18.59E), and the two last in cave sediment in the intertidal zone on Bjørnøya (74.31N, 18.59E) respectively.

3.3 Characterization of bacterial strains

Table 3.1: Material, equipment, their product ID and supplier, used for characterization of bacterial strains.

Material/equipment	Product ID	Supplier
DreamTaq Green PCR Master Mix (2X)	K1081/82	Thermo Fisher Scientific (MA, USA)
Forward primer, 27F	-	Sigma-Aldrich (MO, USA)
Reverse primer, 149R	-	Sigma-Aldrich (MO, USA)
Gel Red (10,000x)	41003	BioTium, Cat no 41003
UltraPure™ Agarose	15510-027	Life technologies
10 × TAE	15558042	Thermo Fisher Scientific (MA, USA)
DNA ladder	10787-018	Life technologies
Owl B1 Electrophoresis System	-	OWI separation system Inc.
GeneFlash®	-	SYNGENE Bio imaging
Mastercycler® Nexus	-	Eppendorf
BigDye 3.1	-	University Hospital of North Norway (Tromsø, Norway)
5x sequencing buffer	-	University Hospital of North Norway (Tromsø, Norway)

The bacterial strains T009, T011, T024, T038 and T040 were streaked out on plates of FMAP-agar (table 3.5) and grown for five days at room temperature (20-25°C). Single colonies from the plates were suspended in 100 µL ddH_2O as a template for colony polymerase chain reaction (PCR). It was then stored in a freezer at -23 °C for minimum 20 minutes to break up the cells and store until further use.

3.3.1 Colony PCR to amplify bacterial DNA

A master-mix for the Amplification PCR reaction containing 12,5 µL Dream Taq Green PCR Master Mix, 1 µL forward primer (27F, AGAGTTTGATCMTGGCTCAG), 1 µL reverse primer (149R, CGGTTACCTTGTTACGACTT) and 9,5 µL ddH_20 for each sample was made. This was added together with 1 µL bacterial template in individual PCR tubes, and then amplified with the PCR program shown in table 3.2.

Table 3.2: Cycle scheme of colony PCR, containing the steps, their duration and temperature.

Initial Denaturation		95 °C	5 minutes
Cycle ×35	Denature	95 °C	30 seconds
	Annealing	47 °C	30 seconds
	Elongation	72 °C	1 minute
Final Extension		72 °C	10 minutes
Hold		4 °C	∞

3.3.2 Determination of PCR product by gel electrophoresis

A 1 % agarose solution was made by melting 1 g agarose in 100 mL of 10×TAE buffer. 10 µL 10 000x Gel Red was added before the solution was cast for 20 minutes in a B1 model agarose gel electrophoresis system. 1 kd Plus DNA ladder was made by mixing 1 µL DNA ladder, 1 µL 6× gel loading dye and 4 µL of ddH_2O and added to the first well. 5 µL of the DNA samples for the amplification PCR was added to the remaining wells. The gel was run for 15-30 minutes at 150-200 V and then photographed under exposure of UV light.

3.3.3 Sequencing PCR to prepare bacterial DNA for sequencing

Two parallels containing 1 µL of each of the bacterial templates created by amplification PCR was added with 1 µL BigDye 3.1, 2 µL 5x sequencing buffer and 5 µL ddH_2O. In the first parallel 1 µL forward primer (27F) was added and 1 µL reverse primer (149R) was added in the second parallel. It was then amplified with the PCR program shown in table 3.3.

The PCR products were sequenced at the University Hospital of North Norway (Tromsø, Norway). The online Basic Local Alignment Search Tool (BLAST, www.ncbi.nlm.nih.gov/BLAST) was used for sequence homology comparison and the strains were identified based on their phylogenetic interference.

Table 3.3: Cycle scheme of sequencing PCR, containing the steps, their duration and temperature.

Initial Denaturation		96 °C	1 minutes
Cycle ×30	Denature	96 °C	10 seconds
	Annealing	47 °C	5 seconds
	Elongation	60 °C	2 minutes
Hold		4 °C	∞

3.4 Inoculation of cultivation culture in different growth media

Table 3.4: Material, equipment, their product ID and supplier, used for inoculation of cultivation cultures.

Material/equipment	Product ID	Supplier
D-glucose (dextrose)	D9434	Sigma-Aldrich (MO, USA)
Starch	1.01252.1000	Merck KGaA (Germany)
Soybean meal	-	P&B (Foods) Ltd - Heera
Yeast extract	09182	Sigma-Aldrich (MO, USA)
Calcium carbonate	C5929	Sigma-Aldrich (MO, USA)
Malt extract	70167	Merck KGaA (Germany)
Yeast extract	09182	Sigma-Aldrich (MO, USA)
Peptone from casein, enzymatic digest	82303	Sigma-Aldrich (MO, USA)
Iron(II)sulphateheptahydrat (8 g/L stock solution)	1.03965.0100	Merck KGaA (Germany)
Potassium bromide	221864	Sigma-Aldrich (MO, USA)
Glycerin (≥99 %)	444485B	VWR, Radnor (PA, USA)
Labo Autoclave	-	Panasonic
Agar	20767.298	VWR, Radnor (PA, USA)
Universal Shaker SM 30	-	Edmund Buhler GmbH (Germany)
Infors HT Multitron Pro Incubator	-	Infors HT (Switzerland)

3.4.1 Preparation of growth media

Table 3.5: Chemical composition and amount in the media Arthrobacter medium (ArtM), DVR2, Arthrobacter medium with glycerol (ArtG) and FMAP.

Medium	Chemical	Amount
Arthrobacter medium (ArtM)	D-Glucose (dextrose) Starch Soybean meal Yeast extract Calcium carbonate ddH_2O Filtrated sea water	2 g 2 g 2 g 0,5 g 0,32 g 300 mL 700 mL
DVR2 medium	Malt extract Yeast extract Peptone from casein, enzymatic digest Iron(II)sulphateheptahydrat (8 g/L stock solution) Potassium bromide ddH_2O Filtrated sea water	6 g 6 g 10 g 5 mL 5 mL 450 mL 450 mL
Arthrobacter medium with glycerol (ArtG)	D-Glucose (dextrose) Starch Soybean meal Yeast extract Calcium carbonate Glycerol ddH_2O Filtrated sea water	2 g 2 g 2 g 0,5 g 0,32 g 25 mL 275 mL 700 mL
FMAP	Difco – Marine Broth Peptone from casein, enzymatic digest ddH_2O Filtrated sea water 18 g agar per liter medium	15 g 5 g 300 mL 700 mL

The composition of each media used is listed in table 3.5. The media were sterilized at 121°C for 30 minutes in an Autoclave and cooled down to room temperature and stored until use.

3.4.2 Inoculation and cultivation of the isolated strains

The bacterial strains T009, T011, T024, T038 and T040 were inoculate in Arthrobacter medium (ArtM) from freeze stock with a 10 µL inoculation loop. They were cultivated in a total volume of 900 mL in at 10°C and 100 rpm.

Table 3.6: Bacteria, their respective growth medium and culture ID. Growth mediums: ArtM (Arthrobacter medium), ArtG (Arthrobacter medium with glycerol) and DVR2.

Strain	Medium	Culture ID
T009	ArtM	X0865A
T011	ArtM	X0866A
T024	ArtM	X0867A
T038	ArtM	X0868A
T040	ArtM	X0869A
T009	DVR2	X0870A
T011	ArtG	X0871A
T040	ArtM*	X0872A

** Grown at room temperature*

T009, T011 and T040 were also cultivated in DVR2 medium at 10°C and 100 rpm, Arthrobacter medium with glycerol (ArtG) at 10°C and 100 rpm and ArtM at room temperature respectively.

For each bacterial strain under the same conditions there were used two 500 mL Erlenmeyer flasks containing 450 mL culture and all were cultivated for 21 days in a shake incubator to produce biomass for chemical extraction. The cultures were given a culture ID as seen in table 3.6.

3.5 Extraction of biomass and metabolites

Table 3.7 Material, equipment, their product ID and supplier, used for extraction of the bacterial cultures.

Material/equipment	Product ID	Supplier
Cheesecloth filter, fine mesh	-	Dansk Hjemmeproduktion (Denmark)
Whatman® qualitative filter paper, grade 3	1003-090	GE Healthcare Life Sciences (UK)
Whatman® qualitative filter paper, grade 1	1001-329	GE Healthcare Life Sciences (UK)
Rotary Evaporator (Rotavapor)	-	Heidolph Instruments GmbH & Co. (Germany)
Diaion® HP-20	13607	Sigma-Aldrich (MO, USA)
Universal Shaker SM 30	-	Edmund Buhler GmbH (Germany)

Prior to extraction 400 µL from each culture was put in an Eppendorf tube at -20°C if needed for later strain verification. For X0865A, X0866A, X0867A, X0868A and X0869A this was done after the resin was added to the culture.

3.5.1 Chemical extraction using Diaion® HP-20 resin

Diaion® HP-20 resin was added in the cultures four days before extraction. 20 g per 500 mL culture was weighed into 100 mL Erlenmeyer flasks and activated with 75 mL 100 % MeOH for 30 minutes on a shaker. The MeOH was then removed by carefully pouring and replaced with ddH_2O. The resin was soaked for 15 minutes, before most of the ddH_2O was removed, and the resin was added to the cultures.

The culture was removed by vacuum filtrating through a "cheesecloth filter". The filter was soaked in MeOH and washed with ddH_2O before filtration. The filter, with the resin, was transferred back to the bottle and suspended in 150 mL MeOH for one hour under shaking. The suspension was then filtrated through a grade 3 Whatman® filter paper. The filter was transferred back and resuspended in 150 mL MeOH for one hour, and then filtrated again. The filtrate was dried under reducing pressure at 40°C using a Rotary Evaporator. The dry weight was determined (table 4.2) and the filtrate was stored at -20°C until further use.

3.5.2 Extraction from cell pellets

After chemical extraction using resin the bacterial cell pellets were isolated by centrifugation at 4500 rpm for 20 min at 4°C. The containers were balanced by the addition of ddH_2O. The pellets were freeze dried, and the dry weight was determined (table 4.2). The pellets were then re-dissolved in 5 mL ddH_2O. 100 mL MeOH was added, and extracted by shaking for 5 hours before the suspension was filtrated through a grade 1 Whatman® filter paper. The filtrate was dried under reducing pressure at 40°C using a Rotary Evaporator and stored at -20°C until further use.

3.6 Flash purification by Biotage SP4-system

Table 3.8: Material, equipment, their product ID and supplier, used for Flash purification.

Material/equipment	Product ID	Supplier
Aceton, HiPerSolv Chromanorm	20067.320	VWR, Radnor (PA, USA)
Dimethyl Sulfoxide (DMSO)	20B204006	Sigma-Aldrich (MO, USA)
Diaion® HP-20SS	13615-U	Sigma-Aldrich (MO, USA)
Biotage® SNAP Cartridge KP-Sil (10 g)	-	Biotage (Sweden)
Biotage® SP4 Flash Purification System	-	Biotage (Sweden)
Visiprep™ SPE Vacuum Manifold	-	Sigma-Aldrich (MO, USA)
Rotary Evaporator (Rotavapor)	-	Heidolph Instruments GmbH & Co. (Germany)
Büchi Syncore Polyvap	-	Büchi (Switzerland)
Universal Shaker SM 30	-	Edmund Buhler GmbH (Germany)

3.6.1 Preparation of SNAP column

6,5 g column material (Diaion® HP-20SS resin) was weighed and suspended in 75 mL 100 % MeOH for 20 minutes. MeOH was removed by careful pouring and exchanged with ddH_2O. Subsequently, the column material was transferred to a SNAP column using a vacuum manifold and stored at 4 °C until further use.

3.6.2 Preparation of extracts for Flash fractionation

The extracts from section 3.5.1 *"Extraction using Diaion® HP-20 resin"* were dissolved in 20 mL 90 % MeOH in a round 250 mL evaporator flask and 1,5 g Diaion® HP-20SS resin was added. It was subsequently dried under reducing pressure at 40°C using a Rotary Evaporator.

3.6.3 Flash fractionation

The dried samples were loaded to the column and placed in the Biotage® SP4 Flash Purification System. Subsequently, it was eluted with the mobile phase gradient according to table 3.9, flow rate was 20 mL/min and each flash tube consisting of 80 mL. It yielded 27 Flash tubes that were combined into six fractions (1-6) in accordance with table 3.9.

Table 3.9: Overview of flash tubes and their respective mobile phase gradient in the different fractions. Mobile phase consists of varying percentage of ddH_2O, MeOH and Acetone.

Flash tubes	Fraction number	% ddH_2O	% MeOH	% Acetone
1-3	1	95	5	0
4-6	2	75	25	0
7-9	3	50	50	0
10-12	4	25	75	0
13-15	5	0	100	0
16-18	6	0	50	50
19-27	6	0	0	100

3.6.4 Stock solution

The fractions (1-6) were dried under reducing pressure at 40°C using a Syncore Polyvap and weighed (table 4.2). The fractions were then diluted in Dimethyl Sulfoxide (DMSO) to 40 mg/mL, or 80 mg/mL if the final volume was larger than 2 mL. Subsequently, the fractions were placed on a universal shaker at 135 rpm for minimum 12 hours to dissolve properly and transferred to individual 2 mL Cryo tubes.

3.7 Cell viability assay - Anticancer activity screening

Table 3.10: Material, equipment, their product ID and supplier, used for viability assay for anticancer activity screening.

Material/equipment	Product ID	Supplier
A2058	ATCC® CRL-11147™	LGC Standards (Sweden)
MRC-5	ATCC® CCL-171™	LGC Standards (Sweden)
Phosphate buffer saline (PBS)	-	In-house (Appendix 1)
CellTiter 96® Aqueous One Solution Reagent	G3581	Promega (Wisconsin, USA)
Fetal Bovine Serum (FBS)	S1810	Biowest (France)
Gentamycin (10mg/mL)	A2712	Merck KGaA (Germany)
Earle's Minimal Essential Medium, 20 mM HEPES	M7278	Sigma-Aldrich (MO, USA)
Dulbecco's Modified Eagle Medium, 4500 mg/L glucose, 25 mM HEPES	D6171	Sigma-Aldrich (MO, USA)
Glutamine stable (200 mM)	X0551	Merck KGaA (Germany)
Non-essential amino acids (100x)	K0293	Merck KGaA (Germany)
Sodium Pyruvate (100 mM)	L0473	Merck KGaA (Germany)
Sodium Bicarbonate solution (7.5 %)	L1713	Merck KGaA (Germany)
Trypsin (1:250)	X0930	Biowest (France)
Trypan blue 0,4 %	T8154	Sigma-Aldrich (MO, USA)
Dimethyl Sulfoxide (DMSO)	20B204006	Sigma-Aldrich (MO, USA)
Nunc™ Cell Culture Flasks	-	Thermo Fisher Scientific (MA, USA)
96 MicroWell™, Nunclon™	-	Thermo Fisher Scientific (MA, USA)
Heracell™ VIOS 160i Tri-Gas CO_2 Incubator	-	Thermo Fisher Scientific (MA, USA)
Bürker counting chamber	-	VWR, Radnor (PA, USA)
Multimode Detector DTX 880	-	Beckman Coulter, Inc (CA, USA)

3.7.1 Cell culture maintenance and splitting

The cell-lines A2058 (human melanoma) and MRC5 (normal lung fibroblast) were grown in Nunc™ Cell Culture flasks in respectively 15 mL Dulbecco's Modified Eagle Medium (D-MEM) and Earle's Minimal Essential Medium (E-MEM), with the additives in accordance with table 3.11. The cells were grown at 37°C and 5 % CO_2 until a cell density of 70-80 % on the bottom of the cell culture flask was reached.

Phosphate buffer saline (PBS), Trypsin and cell medium was prewarmed to 37 °C. The growth medium was removed from the flask and PBS was used to wash the cells, and then removed. The cells were covered by trypsin and excess was discarded, before it was stored at 37°C until the cells detached (1-10 minutes). They were resuspended in 10 mL of their respective growth medium and split according to table 3.11. The split ratio depends on how long untill the next splitting and the cell density. One part is transferred to a new Nunc™ Cell Culture flask, added 14 mL fresh growth medium, grown at 37°C and 5 % CO_2 to keep the cell-line alive.

Table 3.11: The growth medium, additives in percentages and split ratio for the cell-lines A2058 and MRC5.

Cell-line	Split ratio	Growth medium	Additives in medium
A2058	1:10 – 1:20	Dulbecco's Modified Eagle Medium (D-MEM)	10,0 % (V/V) FBS 0,1 % (V/V) Gentamycin 1,0 % (V/V) Glutamine stable
MRC5	1:3 - 1:4	Earle's Minimal Essential Medium (E-MEM)	10,0 % (V/V) FBS 0,1 % (V/V) Gentamycin 1,0 % (V/V) Glutamine stable 1,0 % (V/V) Non-essential amino acid 1,0 % (V/V) Sodium pyruvate 2,0 % (V/V) Sodium Bicarbonate solution

3.7.2 96- well microtiter plate preparation by cell seeding

100 µL of the remaining cell culture from section 3.7.1. *"Cell culture maintenance and splitting"* was transferred to an Eppendorf tube containing 100 µL trypan blue (0,4 %). Subsequently, 10 µL was transferred to Bürker counting chamber. The living cells were counted and the concentration in 1 mL cell suspension was calculated. The cell cultures were diluted so that each well had approximately 2000 cells for A2058 and 4000 cells for MRC5 when 100 µL from the cell suspention was transferred to the 96 MicroWell™ plates. The plates were incubated for 24 hours at 37°C and 5 % CO_2.

3.7.3 Cell viability assay

All fractions were screened at 100 µg/mL and the pellet extracts at 200 µg/mL for anticancer activity in a primary screening. The fractions considered active or with questionable active were included in a secondary screening with the concentrations 10, 25, 50, 75 and 100 µg/mL. All pellet extracts were tested at 250 and 500 µg/mL in the secondary screening.

The stock solutions from sections 3.6.4 *"Stock solution"* were diluted to 1 mg/mL in ddH_2O with 1 % DMSO. After the cell plates were incubated for 24 hours the growth medium was discarded. It was then replaced by appropriate medium and the diluted stock solution. The final concentration for the flash fractions was 100 µg/mL for the primary screening and a dilution series (10, 25, 50, 75 and 100 µg/mL) for the secondary screening, and for the cell pellet was 200 µg/mL for the primary screening and a dilution series (250 and 500 µg/mL) for the secondary screening. The samples were all screened triplicates. Each plate had negative controls consisting of appropriate growth medium and positive controls consisting of appropriate growth medium with 10 % DMSO. The final volume was 100 µL in each well. Subsequently, the plates were incubated at 37°C and 5 % CO_2 for 72 hours.

After the 72-hour incubation period, 10 µL of Aqueous One Solution was added to each well and put back in the incubator for 1 hour. Subsequently, absorbance at 485 nm was measured with a DTX 880 Multimode Detector.

3.7.4 Interpretation of absorbance in anticancer assay

The average for the samples was calculated based on the triplicates. The negative control was considered 100 % cell survival and the positive control was considered 1 % cell survival. The results were calculated with equation 1. Under 50 % survival was deemed active, 50-60 % was questionable and over 60 % was inactive.

Equation 1: $$\%\,\boldsymbol{Survival} = \frac{(\textbf{Sample} - \textbf{positiv control})\ \boldsymbol{x}\ \textbf{100}}{(\textbf{Negativ control} - \textbf{positiv control})}$$

Samples deemed active or questionable were included in the secondary screening.

3.8 Minimum inhibition concentration - Antibacterial activity screening

Table 3.12: Material, equipment, their product ID and supplier, used for MIC assay.

Material/equipment	Product ID	Supplier
Müller-Hinton broth	275730	BD Biosciences
Brain heart infusion	237500	BD Biosciences
Blood agar	-	SUMP, UNN (Tromsø, Norway)
Gentamicin	E737	Amrescon
Staphylococcus aureus	ATCC® 25923	LGC Standards (Sweden)
Escherichia coli	ATCC® 25922	LGC Standards (Sweden)
Enterococcus faecalis	ATCC® 29212	LGC Standards (Sweden)
Pseudomonas aeruginosa	ATCC® 27853	LGC Standards (Sweden)
Streptococcus agalactiae	ATCC® 12386	LGC Standards (Sweden)
Nunc™ microtiter plate	734-2097	Thermo Fisher Scientific (MA, USA)
Victor Plate Reader	2030-0050	PerkinElmer® (MA, USA)
WorkOut 2.5 Software	-	Dazdaq, England

3.8.1 Preparation of the test-bacteria in 96-well microtiter plate

The bacteria *S. aureus, E. coli, E. faecalis, P. aeruginosa* and *S. agalactiae* were transferred from freeze stock with a 10 µL inoculation loop to blood agar plates. Incubated at 37°C overnight. The bacteria could be kept for one month at 4°C and needed to be re-streaked after 14 days. The bacteria were transferred in 8 mL autoclaved growth medium according to table 3.13 with a 10 µL inoculation loop and was incubated at 37°C overnight. 2 mL of the bacterial suspension was transferred to 25 mL fresh growth medium and incubated in accordance with table 3.13.

Table 3.13: Test bacteria, their growth medium and incubation time.

Test bacteria	Growth medium	Incubation time
S. aureus	Müller-Hinton broth	2,5 hours
E. coli	Müller-Hinton broth	1,5 hours
P. aeruginosa	Müller-Hinton broth	2,5 hours
E. faecalis	Brain heart infusion	1,5 hours
S. agalactiae	Brain heart infusion	1,5 hours

The stock solutions from section 3.6.4 *"Stock solution"* were diluted in ddH_2O with 1 % DMSO. The final assay concentration for the fractions was 100 µg/mL in the primary screening and a dilution series (10, 25, 50, 75 and 100 µg/mL) in the secondary screening. The primary screening of the cell pellet had the concentration 200 µg/mL and two concentrations (250 and 500 µg/mL) for the secondary screening. Duplicates of 50 µL of the samples were added in a 96-well microtiter plate for each of the five bacteria.

The bacterial suspensions were diluted 1:1000 (growth turbidity of 0,5 MacFarland standard) in appropriate fresh growth medium before 50 µL was transferred to the 96-well microtiter plate containing the samples. Each plate included a negative (50 µL appropriate growth medium and 50 µL autoclaved ddH_2O) and positive control (50 µL bacterial suspension and 50 µL autoclaved ddH_2O). The plates were incubated at 37°C overnight.

3.8.2 Gentamicin control

For each antibacterial screening, a gentamicin control was run as a control for the assay and normal growth of the test bacteria. 50 µL from a dilution series of gentamicin and autoclaved ddH_2O with the final assay concentration of 0,01, 0,03, 0,06, 1,12, 0,25, 0,50, 1,00, 2,00, 4,00, 8,00, 16,00 and 32,00 µg/mL was transferred to a 96-well microtiter plate. 50 µL bacterial suspension of each test bacteria was added to a dilution series to determine the minimum inhibition concentration (MIC) of gentamicin for the test bacteria. Reference MIC value for the different test bacteria is ± one titer-step from the values represented in table 3.14. The plates were incubated under the same conditions as the microtiter plates containing the samples.

Table 3.14: Test bacteria and their acceptable MIC-values.

Test bacteria	MIC (µg/mL)
S. aureus	0,06
E. coli	13,00
P. aeruginosa	0,25
E. faecalis	8,00
S. agalactiae	4,00

3.8.3 Plate reading and evaluation of results

After a 24-hour incubation period the plates were visually controlled for growth inhibition by looking at the media turbidity. The optical density (OD) was measured at 600 nm using a Viktor Plate Reader and was processed in the software WorkOut 2.5. OD values under 0,05 were deemed active, 0,05-0,09 were questionable and over 0,09 were inactive. Samples deemed active were included in the secondary screening.

3.9 Biofilm formation inhibition assay

Table 3.15: Material and equipment, their product ID and supplier, used for biofilm inhibition assay.

Material/equipment	Product ID	Supplier
Tryptic soy broth	105459	Merck KGaA (Germany)
Crystal violet (0,1 %)	115940	Merck KGaA (Germany)
Blood agar	-	SUMP, UNN (Tromsø, Norway)
Glucose	D9434	Sigma-Aldrich (MO, USA)
Ethanol (96 %)	20823	VWR International S.A.S (France)
Nunc™ microtiter plate	734-2073	Thermo Fisher Scientific (MA, USA)
Victor plate reader	2030-0050	PerkinElmer® (MA, USA)
Ioculation loops	612-9362	VWR International S.A.S (France)
Staphylococcus epidermis	ATCC® 35984	LGC Standards (Sweden)
Staphylococcus haemolyticus	Clinical isolate 8-7A	UNN
WorkOut 2.5 Software	-	Dazdaq, England

3.9.1 Preparation of the biofilm bacteria in 96-well microtiter plate

The bacteria *S. epidermis* (test-bacteria) and *Staphylococcus haemolyticus* (control for a non-biofilm forming bacteria) were transferred from freeze stock with a 10 µL inoculation loop to blood agar plates. The plates were incubated at 37°C overnight. The bacteria could be kept for one month at 4°C and needed to be re-streaked after 14 days. The test bacteria were transferred to 5 mL autoclaved tryptic soy broth (TSB) with a 10 µL inoculation loop and was incubated at 37°C on shaking overnight. The test bacteria were diluted 1:100 in fresh TSB with 1 % glucose.

The stock solutions from section 3.6.4 *"Stock solution"* were diluted in ddH_2O with 1 % DMSO so that the final concentration for the flash fractions were 100 µg/mL and 200 µg/mL for the cell pellet. Triplicates of 50 µL of the samples were added in a 96-well microtiter plate, and 50 µL of the *S. epidermis* suspension was added to each well. A media blank (50 µL TSB with 1 % glucose and 50 µL autoclaved ddH_2O), positive control (50 µL *S. epidermis* suspension and 50 µL autoclaved ddH_2O) and a negative control (50 µL *S. haemolyticus* suspension and 50 µL autoclaved ddH_2O) was added to a column in each plate. The plates were incubated at 37°C overnight.

The optical density (OD) of the plates was measured at 600 nm using a Viktor plate reader and was processed in the software WorkOut 2.5 (dasdaq, England), to exclude that the analytes inhibit bacterial growth and not the formation of biofilm. The liquid in the plate was poured, the wells were rinsed with water and then poured of. To fixate the biofilm to the bottom of the wells ,the plates were incubated at 55°C for 1 hour.

3.9.2 Reading of plates and result evaluation

After fixation of the biofilm, 70 µL 0,1 % crystal violet was added to each well, incubated for 5 minutes and poured of. Subsequently, the wells were rinsed with water twice. When the wells were dry, 70 µL of 70 % ethanol was added to each well, to get an even distribution of crystal violet, and shook for 10 minutes. The OD was measured at 600 nm using a Viktor plate reader and was processed in the software WorkOut 2.5.

An average for the samples were calculated based on the triplicates. The positive control was considered 100 % biofilm formation and the media blank was considered 1 % biofilm formation. The results were calculated with equation 2. Under 30 % survival was deemed active, 30-40 % was questionable and over 40 % was inactive.

Equation 2: $$\%\,\boldsymbol{Survival} = \frac{(\textbf{Sample} - \textbf{medium blank})\ \boldsymbol{x}\ \textbf{100}}{(\textbf{Positiv control} - \textbf{medium blank})}$$

3.10 Dereplication of active fractions and pellet extracts

Table 3.16: Material, equipment, their product ID and supplier, used for dereplication.

Material/equipment	Product ID	Supplier
HPLC glass vials	-	Waters (MA, USA)
VION® IMS QToF	-	Waters (MA, USA)
Acquity UPLC PDA Detector	-	Waters (MA, USA)
Acquity UPLC Column Manager	-	Waters (MA, USA)
Acquity UPLC I-Class Sample Manager FTN	-	Waters (MA, USA)
Acquity UPLC I-Class Binary Solvent Manager	-	Waters (MA, USA)
ACQUITY UPLC BEH C18 Column, 130Å, 1.7 µm, 2.1 mm X 100 mm	186002352	Waters (MA, USA)
Methanol LC-MS Ultra CHROMASOLV®	14262	Thermo Fisher Scientific (MA, USA)
Formic acid 99 % ULC/MS	069141	Biosolve B.V. (Netherland)
LiChrosolv® Acetonitrile Hypergrade for LC-MS	1.00029	Merck KGaA (Germany)

The samples deemed active in the secondary screening of anticancer (A2058) and antibacterial (*S. agalactiae)* were selected for dereplication, and analyzed using UHPLC-HR-MS/MS with a RPUPLC column. The mobile phase consisted of a gradient beginning at 90 % polar (ddH_2O with 0.1 % formic acid) to non-polar (acetonitrile with 0.1 % formic acid).

5 µL of the stock solutions from section 3.6.4 *"Stock solution"* for the selected samples were transferred to HPLC glass vials and diluted 1:20 in 80 % methanol before injection. The injection volume used was 1 µL. All samples were run in both ESI^+ and ESI^- mode. The parameters used for the VION® IMS QToF are listed in table 3.17.

Table 3.17: Parameters and their specifications used for the VION® IMS QToF in $ESI^{+/-}$ mode during the dereplication process.

Parameters	Specifications
Mass range	150-1500 m/z
Capillary voltage	3,00 kV
Source temperature	100°C
Desolvation temperature	250°C
Cone gas flow	50 L/h
Desolvation gas flow	600 L/h
High collision energy	20-60 eV
Scan time	0.20 s

4 RESULTS

4.1 Characterization and identification of bacterial strains

The comparison of sequence homology and the identification based on phylogenetic interference in standard nucleotide BLAST of the 16s sequence for the strains T009, T011, T024, T038 and T040 resulted in a query cover of 90 % with *Arthrobacter* sp., 86 % with *Arthrobacter* sp., 92 % with *Arthrobacter* sp. and *92* % with *Arthrobacter* sp. respectively. For T040 the query cover was 89 % for both *Glutamicibacter* sp. and *Arthrobacter* sp.. Results are shown in table 4.1. The three *Arthrobacter* sp. isolates with the lowest query cover with known bacteria (T009, T011 and T040) were selected for the OSMAC approach to be grown in different media or culture conditions.

Table 4.1: The identified strains with query cover for the cultivated strains with their cultivation media and extracts.

Strain	Identified strain	Query cover (%)	Culture ID	Cultivation media
T009	*Arthrobacter* sp.	90	X0865A X0870A	ArtM DVR2
T011	*Arthrobacter* sp.	86	X0866A X0871A	ArtM ArtG
T024	*Arthrobacter* sp.	92	X0867A	ArtM
T038	*Arthrobacter* sp.	92	X0868A	ArtM
T040	*Arthrobacter* sp. *Glutamicibacter* sp.	89 89	X0869A X0872A	ArtM ArtM*

** Grown at room temperature*

4.2 Extraction and fractionation yield

The cultures of the bacteria that were cultivated under different conditions and in different growth media were extracted to capture secreted metabolites with Diaion® HP-20 resin and MeOH. This resulted in 8 extracts: X0865A, X0866A, X0867A, X0868A, X0869A, X0870A, X0871A and X0872A (growth conditions listed in table 4.1). The dry weight of the resin extracts varied between 350 and 1080 mg (table 4.2), and the extracts of the strains grown in ArtM medium varied between 350 and 420 mg. There was no significant difference in extraction yield for strain T040 grown in ArtM at room temperature compared to the same strain grown in ArtM at 10°C, 409,6 and 513,1 mg respectively . The extracts of the strain T009 and T011 grown in DVR2 and ArtG respectively (table 4.1), gave higher yields compared to their counterparts grown in ArtM (table 4.2).

Table 4.2: The yield in mg for the individual fractions, total fraction yield, resin and pellet extraction. Shown for the different extracts: X0865A, X0866A, X0867A, X0868A, X0869A, X0870A, X0871A and X0872A.

Culture ID	Fraction	Resin extraction yield [mg]	Fraction yield [mg]	Total fraction yield [mg]	Cell pellet dry weight [mg]	Pellet extraction yield [mg]
X0865A	F1 F2 F3 F4 F5 F6	447,2	272,4 15,3 20,2 23,4 26,8 8,3	366,4	1415,0	441,7
X0866A	F1 F2 F3 F4 F5 F6	445,2	133,1 129,1 21,0 27,9 20,3 10,0	341,4	1850,0	599,6
X0867A	F1 F2 F3 F4 F5 F6	472,3	156,1 170,1 73,0 33,6 6,9 8,3	448,0	1319,0	245,2
X0868A	F1 F2 F3 F4 F5 F6	356,3	112,4 135,8 34,1 20,3 8,7 8,1	319,4	743,0	215,0
X0869A	F1 F2 F3 F4 F5 F6	513,1	180,0 135,5 39,9 32,7 10,5 23,6	422,2	754,0	182,7
X0870A	F1 F2 F3 F4 F5 F6	1076,1	390,4 327,8 231,9 42,0 6,1 11,5	1009,7	3343,0	525,1
X0871A	F1 F2 F3 F4 F5 F6	874,6	437,6 340,2 46,1 25,0 9,1 10,7	868,7	1524,0	1058,0
X0872A	F1 F2 F3 F4 F5 F6	409,6	209,2 62,3 25,5 18,3 2,8 7,6	325,7	316,0	117,5

The extract cultures were centrifugated and the pellets were freeze dried, resulting in eight isolated bacteria pellets. The pellets were subsequently extracted for secreted metabolites with MeOH. This extraction had a lower yield in X0867A, X0868A, X0869A, X0870A and X0872A than in the resin extraction, 117,5 - 525,1 mg for pellet extraction and 356,3 - 1076,1 mg for resin extraction (figure 4.1). For X0867A and X0871A the extracts from pellets (599,6 and 1058,0 mg) had a little higher yield than that of the resin extraction (445,2 and 874,6 mg). For X0865A the yield was approximately the same for both extraction methods.

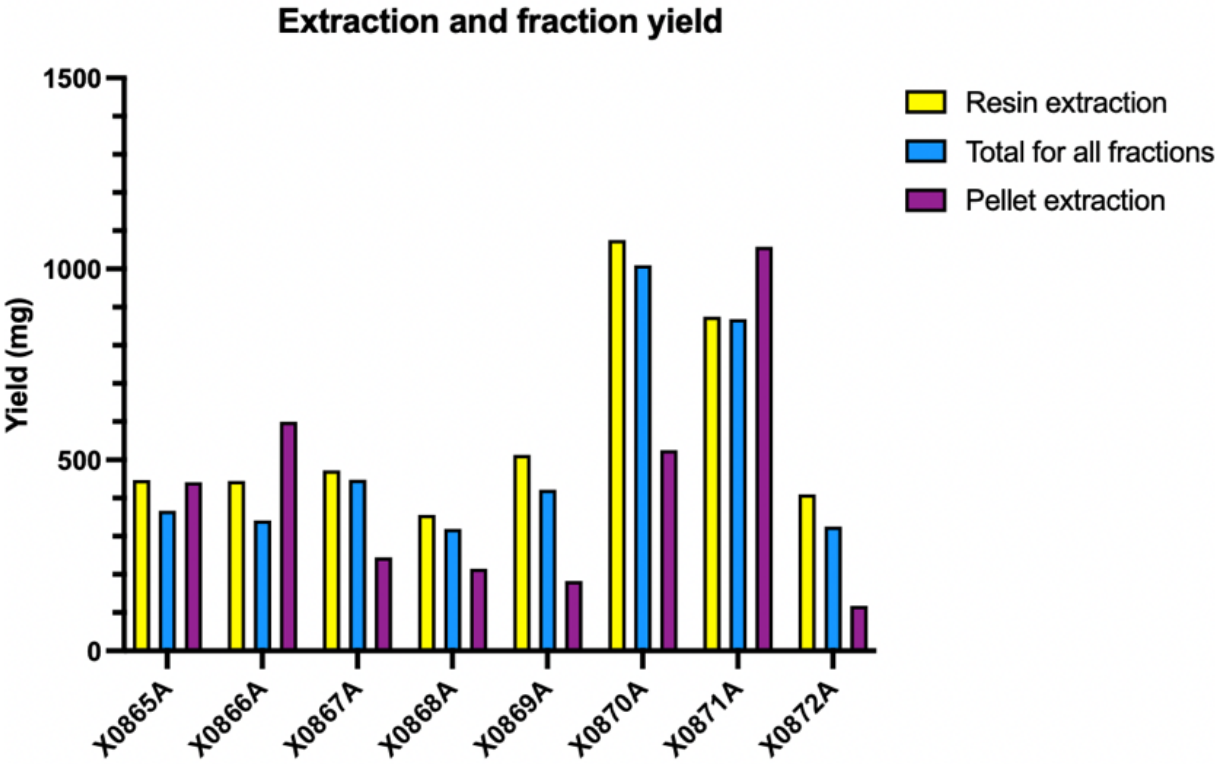

Figure 4.1: Yield in mg for the dry weight after resin extraction (yellow), fractionation (blue) and pellet extraction (purple). Shown for the different extracts: X0865A (ArtM at 10 °C), X0866A (ArtM at 10 °C), X0867A (ArtM at 10 °C), X0868A (ArtM at 10 °C), X0869A (ArtM at 10 °C), X0870A (DVR2 at 10 °C), X0871A (ArtG at 10 °C) and X0872A (ArtM at room temperature).

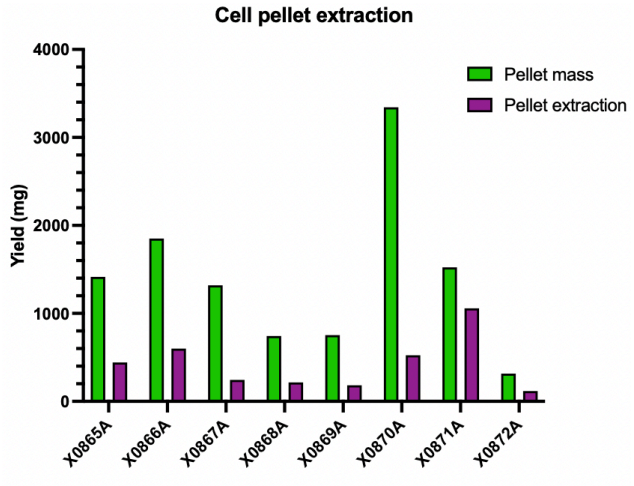

Figure 4.2: Dry weight of cell pellets (yellow) and their extracts (blue) in mg from the extract cultures: X0865A, X0866A, X0867A, X0868A, X0869A, X0870A, X0871A and X0872A.

Figure 4.2 shows that the pellet isolation yielded in between 743 and 1850 mg pellet mass and between 182,7 and 599,6 mg pellet extraction yield for the extracts from the cultures grown in ArtM at 10°C (X0865A, X0866A, X0867A, X0868A and X0869A). The cell culture grown in ArtM at room temperature (X0872) resulted in 316 mg pellet mass and 117,5 mg extract, which is the lowest yield for all the cultures. X0870A, grown in DVR2, resulted in the highest cell pellet mass, but only the third highest pellet extraction yield (525,1 mg). The culture grown in ArtG (X0871A) resulted in 1524 mg in pellet mass, which is around the same as the cultures grown in ArtM, but with a significantly higher extraction yield (1058 mg).

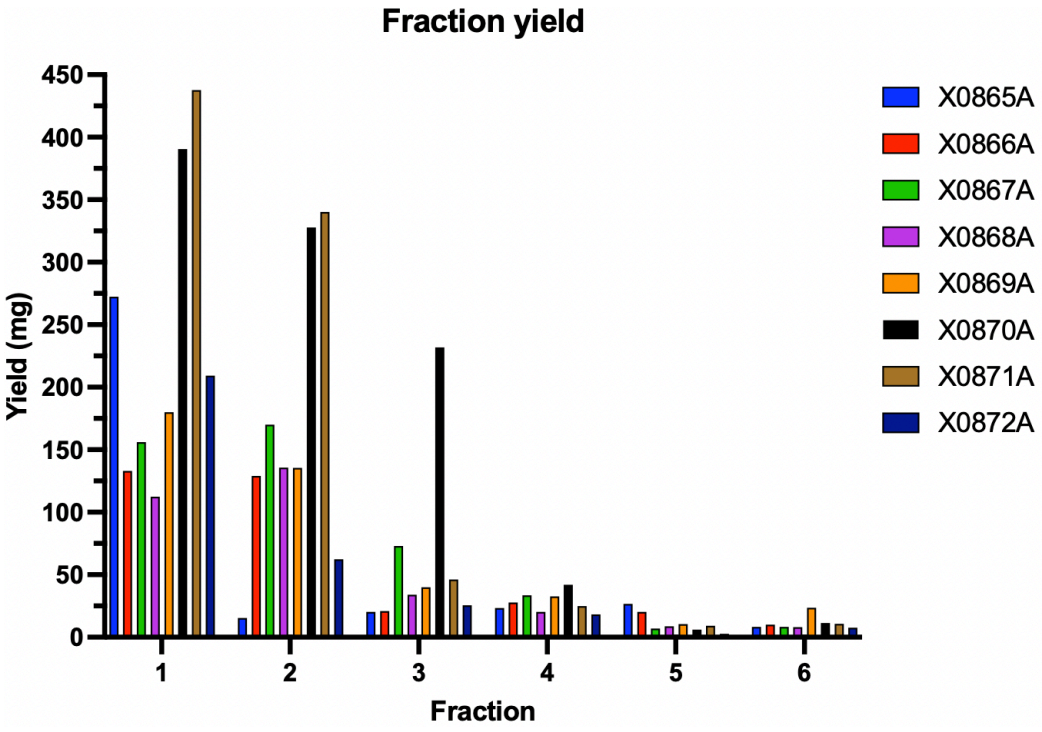

Figure 4.3: Yield in mg for the dry weight of the different fractions of the resin extracts (X0865A, X0866A, X0867A, X0868A, X0869A, X0870A, X0871A and X0872A). The mobile phase had a gradient ranging from polar (ddH2O) in fraction 1 and decreasing polarity until absolute non-polarity (acetone) in fraction 5.

The resin extracts were fractionated with a Biotage® SP4 Flash Purification System, resulting in six fractions per extract for further bioactivity testing and potential dereplication. The stationary phase consisted of Diaion® HP-20SS resin and the mobile phase had a gradient ranging from polar ddH_2O to non-polar acetone (table 3.9). The dry weight for the six fractions combined is generally lower than that of the extracts (figure 4.2).

The 1st and 2nd fractions are the most polar fractions eluting in 95 % and 75 % ddH_2O respectively, with the remaining percentage consisting of MeOH. These have generally a higher fraction yield than the remaining fractions (figure 4.3), especially for X0870A and X0871A. For X0872A there was a higher yield only in the first fraction, and approximately the same in the remaining fractions. The 3rd and 4th fractions are less polar and elute at 50 % and 25 % ddH_2O, with 50 % and 75 % MeOH respectively. They had a lower yield than in fraction 1 and 2. Fraction 3 for X0870A, with its 231,9 mg yield, was significantly higher than fraction 3 for the other extracts, and if excluded, makes fraction 3 vary between 20,2 and 73,0 mg. Fraction 5 had a mobile phase with 100 MeOH, and had yields between 2,8 - 26,8 mg. The remaining fraction 6 had a mobile phase from 50/50 % MeOH and acetone, to 100 % acetone, and is thus the most non-polar fraction. The fraction yield varied between 8,1 to 23,6 mg.

4.3 Bioactivity screening of fractions and unfractionated pellet extracts

The 48 fractions and eight unfractionated pellet extracts were screened for antibacterial, anticancer and biofilm formation inhibiting activity. The antibacterial assay was conducted for the bacteria *S. aureus, E. coli, E. faecalis, P. aeruginosa* and *S. agalactiae*. The anticancer assay was conducted for the cell-lines A2058 (human melanoma) and MRC5 (normal lung fibroblast). The biofilm formation inhibiting assay was conducted for the bacteria *S. epidermis.* In the primary screening the fractions were tested at a concentration of 100 µg/mL and the pellet extracts at 200 µg/mL. The results are shown in table 4.3.

The fractions and pellet extracts deemed active in the primary screening were submitted to a secondary screen, where the fractions were tested at concentration of 10, 25, 50, 75 and 100 µg/mL and the pellet extracts at 250 and 500 µg/mL. Secondary screening was only conducted for the cancer cell-line A2058 in the anticancer assay and for the bacteria *S. agalactiae* in MIC assay. The results are displayed in table 4.4. This selection is based on the cut-off values set at Marbio and are values that are meant to select and limit the number of samples included in further bioactivity screening and dereplication. It is mainly a guidance and are listed under the sections concerning the results of the individual bioactivity screens

Table 4.3: Overview of the results from the primary screens conducted for anticancer (A2058 and MRC5), antibacterial (S. agalactiae) and inhibition of biofilm formation (S. epidermis) activity for pellets and fractions 3-6 of the extracts (X0865A, X0866A, X0867A, X0868A, X0869A, X0870A, X0871A and X0872A). Fraction 1 and 2 showed no activity in any of the screens and are thus excluded from the results. Green (X) shows activity, orange (-) shows questionable activity and white (blank) shows inactivity.

(n*)	A2058 – Human melanoma (3)					MRC5 – Lung fibroblast (3)					MIC – *S. agalactiae* (2)					Biofilm – *S. epidermis* (3)				
Fractions:	**3**	**4**	**5**	**6**	**P**	**3**	**4**	**5**	**6**	**P**	**3**	**4**	**5**	**6**	**P**	**3**	**4**	**5**	**6**	**P**
Culture ID:																				
X0865A																				
X0866A													x							
X0867A			x										x					-		x
X0868A													x	x				-		x
X0869A	x		x										x					-		
X0870A			x										x							x
X0871A	x	-	x										x					-		
X0872A	x		x	x				-					x							

** Number of technical replicates the results are based on*

Table 4.4: Overview of the results from the secondary screenings conducted for anticancer (A2058) and antibacterial (S. agalactiae) activity for pellet extracts and fractions of the extracts (X0865A, X0866A, X0867A, X0868A, X0869A, X0870A, X0871A and X0872A) deemed active in the primary screening. The numbers show the lowest concentration (µg/mL) at which activity of the respective fraction or pellet extract was observed. Fractions not showing activity or included in the secondary screening show the concentration at which activity was recorded in the primary screening (yellow). Fraction 1 and 2 showed no activity in any of the screens and were thus excluded from the results.

(n*)	A2058 – Human melanoma (3) [µg/mL]					MIC – *S. agalactiae* (4) [µg/mL]				
Fraction:	**3**	**4**	**5**	**6**	**P**	**3**	**4**	**5**	**6**	**P**
Culture ID:										
X0865A										
X0866A								100		500
X0867A			100					50		
X0868A								75	100	
X0869A	100		75					100		
X0870A			25					50		
X0871A	100	100	75					75		
X0872A	100		50	50				50		

** Number of technical replicates the results are based on*

4.3.1 Cell viability assay – Anticancer activity screening

The cell-lines A2058 (human melanoma) and MRC5 (normal lung fibroblast) were exposed to fraction 1-6 of the resin extracts and pellet extracts to indicate anticancer activity and to select samples for further investigation. Samples with 50 % or less cell survival were deemed active and samples with 50-60 % were deemed questionably active.

The primary screening resulted in nine active and one questionably active sample for A2058 (figure 4.4). Three of these were from fraction 3, one from fraction 4, four from fraction 5 and one from fraction 6. The questionably active sample was from fraction 4. No active fractions were observed for the extracts X0865A, X0866 and X0868A or from the pellet extracts. Activity screening against the normal lung fibroblast (MRC5) cell-line resulted in only one questionably active sample from fraction 5 (table 4.3) and was, due to the urge of focus on the promising hits and limited time, not included in the secondary screening.

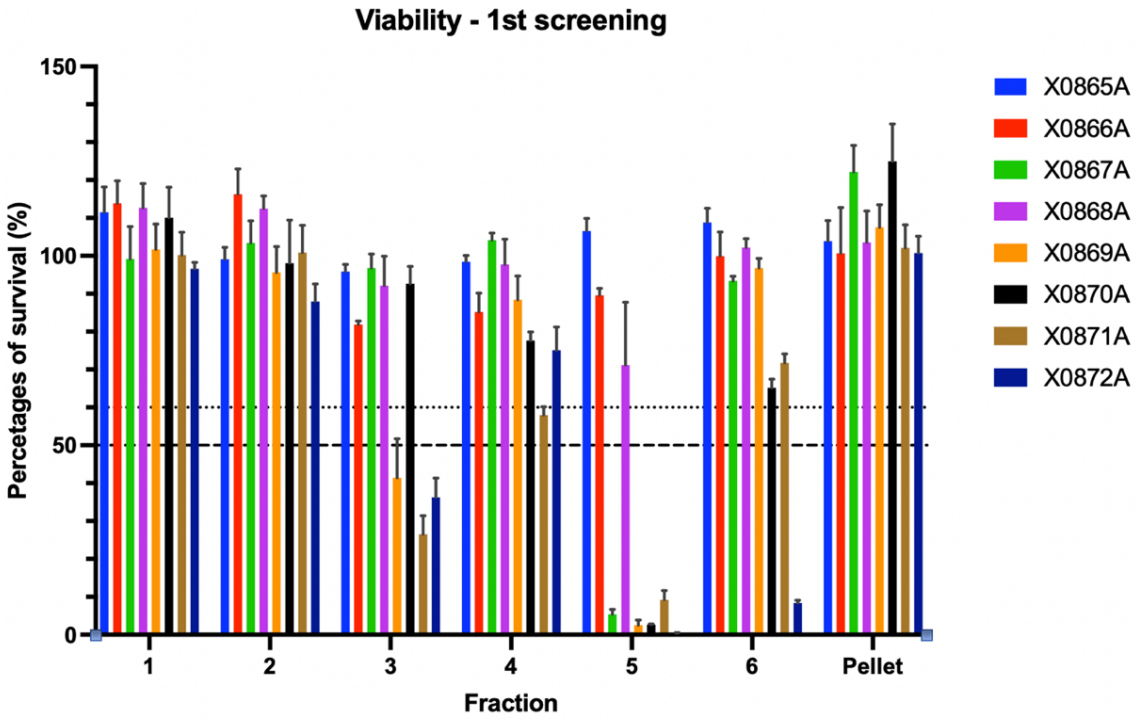

Figure 4.4: Percentages of A2058 cell survival when exposed to 100 µg/mL of fraction 1-6 and 200 µg/mL of the pellet extract from the extracts (X0865A, X0866A, X0867A, X0868A, X0869A, X0870A, X0871A and X0872A). Samples deemed active had a value of 50 % or less survival, and questionable activity was at 50-60 %. The results are based on the mean of three technical replicates.

The fractions X0867A-05, X0869-03, X0869A-05, X0870A-05, X0871A-03, X0871A-05, X0872A-03, X0872A-05 and X0872A-06 showed activity against the Human melanoma (A2058) cell-line in the primary screening (figure 4.4) and were submitted to a retest at different concentrations (figure 4.5). X0872A-05 and X0872A-06 were active down to a concentration of 50 µg/mL, and X0870A-05 was active down to 50 µg/mL and questionably active at 25 µg/mL. Fraction X0867A-05 was active at 100 µg/mL, while X0871A-05 was questionably active at both 75 and 100 µg/mL. Fraction X0869-05 did interestingly not show activity at 100 µg/mL, but activity was recorded at 75 µg/mL. The fractions X0869-03, X0871A-03 and X0872A-03 did not result in activity in the secondary screening at any concentrations (figure 4.5), including at 100 µg/mL where they showed activity in the primary screening (figure 4.4).

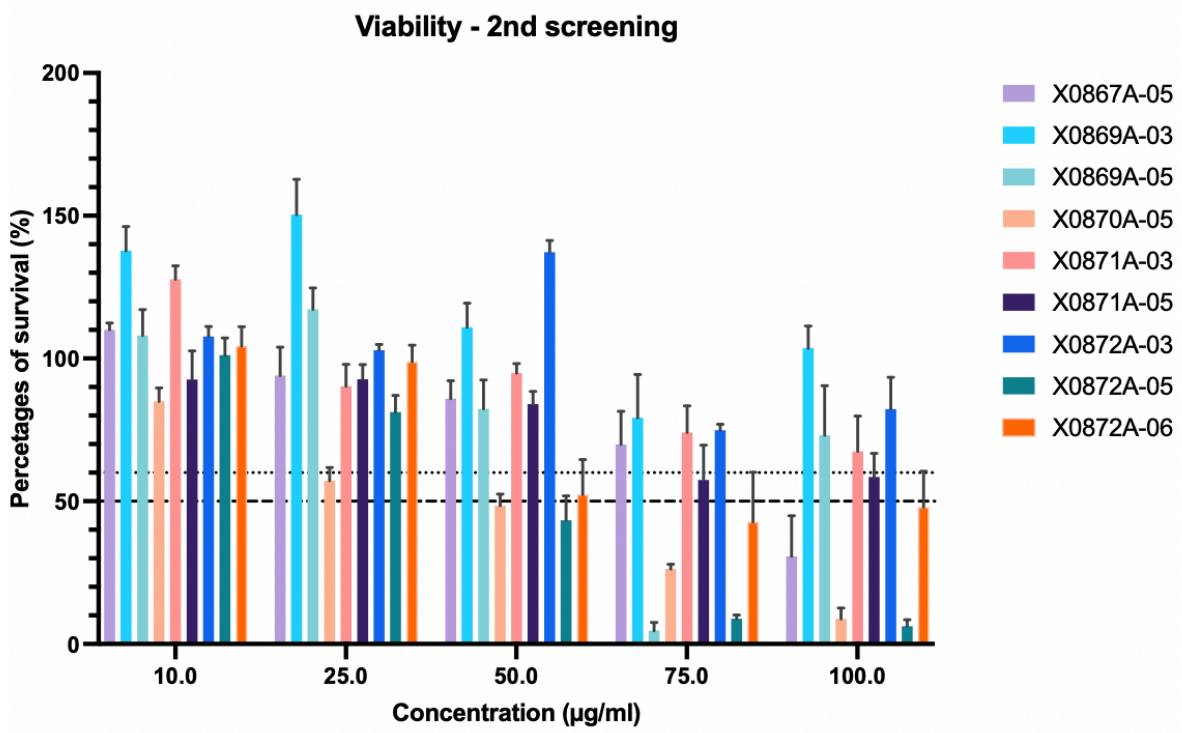

Figure 4.5: Percentages of A2058 cell survival when exposed to different concentrations (10, 25, 50, 75 and 100 µg/mL) of the fractions deemed active or questionably active in the primary screening (X0867A-05, X0869-03, X0869A-05, X0870A-05, X0871A-03, X0871A-05, X0872A-03, X0872A-05, X0872A-06). Samples deemed active had a value of 50 % or less survival, and questionable activity was at 50-60 %. The results are based on the mean of three technical replicates.

The strains T009, T011 and T040 were selected for the OSMAC approach and were cultivated under different conditions. All were cultivated in the ArtM medium, but also at one other condition, such as the different media DVR2, ArtG and ArtM at room temperature (table 4.1).

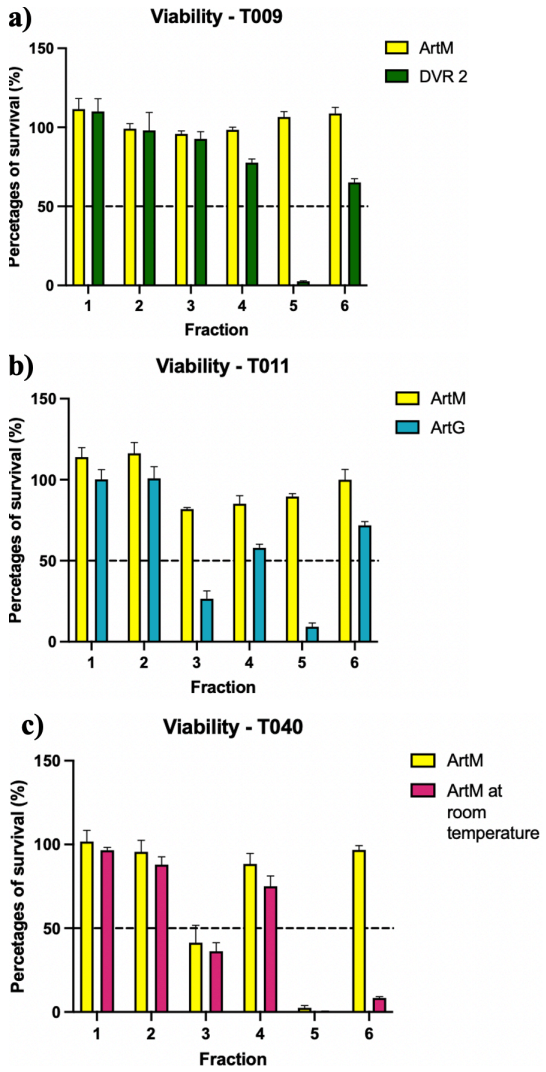

Figure 4.6: Percentages (%) of A2058 cell survival for the six fractions of the strains a) T009, b) T011 and c) T040 in the primary screening. Samples deemed active had a value of 50 % or less survival. The results are based on the mean of three technical replicates.

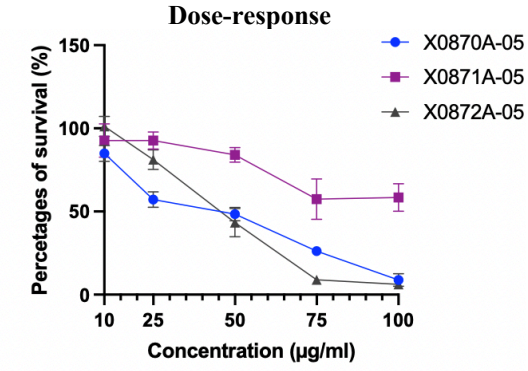

Figure 4.7: Dose-response for the fractions X0870A-05, X0871A-05 and X0872A-05. Plotted as percentages (%) of survival for the cell-line A2050 against concentration (µg/mL) of sample.

Figure 4.6a show that for the strain T009 there was only observed activity in fraction 5 for the extract from the DVR2 medium (X0870A-05), and the percentages of survival was lower for all fractions, especially fraction 4 and 5, compared to the one grown in ArtM (X0865A).

For T011 (figure 4.6b) all activity for the one cultivated in ArtG (X0871A) was lower than that of the one cultivated in ArtM. In X0871A both fraction 3 and 5 showed activity.

Strain T040, shown in figure 4.6c, had activity for both the strain cultivated in ArtM (X0869A) at 10°C and ArtM at room temperature (X0872A). Fraction 3 and 5 was active for both, while fraction 6 was active only for X0872A. The strain grown in ArtM at room temperature had lower activity for all fractions compared to the same strain grown in ArtM.

Dose-response for the fractions X0870A-05, X0871A-05 and X0872A-05 (figure 4.7) show that there is a trend for the cell survival to decrease with increasing sample concentration.

4.3.2 Antibacterial activity screening – Minimum inhibition concentration

All fractions were screened for antibacterial activity at 100 µg/mL and pellet extracts were screened at 200 µg/mL against the five pathogenic bacteria *S. aureus, E. coli, E. faecalis, P. aeruginosa* and *S. agalactiae* in the primary screening. The optical density at 600 nm (OD_{600}) was measured to determine the bacterial growth. OD_{600} values under 0,05 were deemed active and between 0,05 - 0,09 as questionably active.

For the primary screening fraction five for all but extract X0865A from strain T009 were deemed active for the bacteria *S. agalactiae* (figure 4.8). None of the fractions or pellet extracts measured an OD_{600} of less than 0,09 for the four other test bacteria included in the screening. The samples were deemed inactive against these test-bacterias and thus not included in the secondary screening.

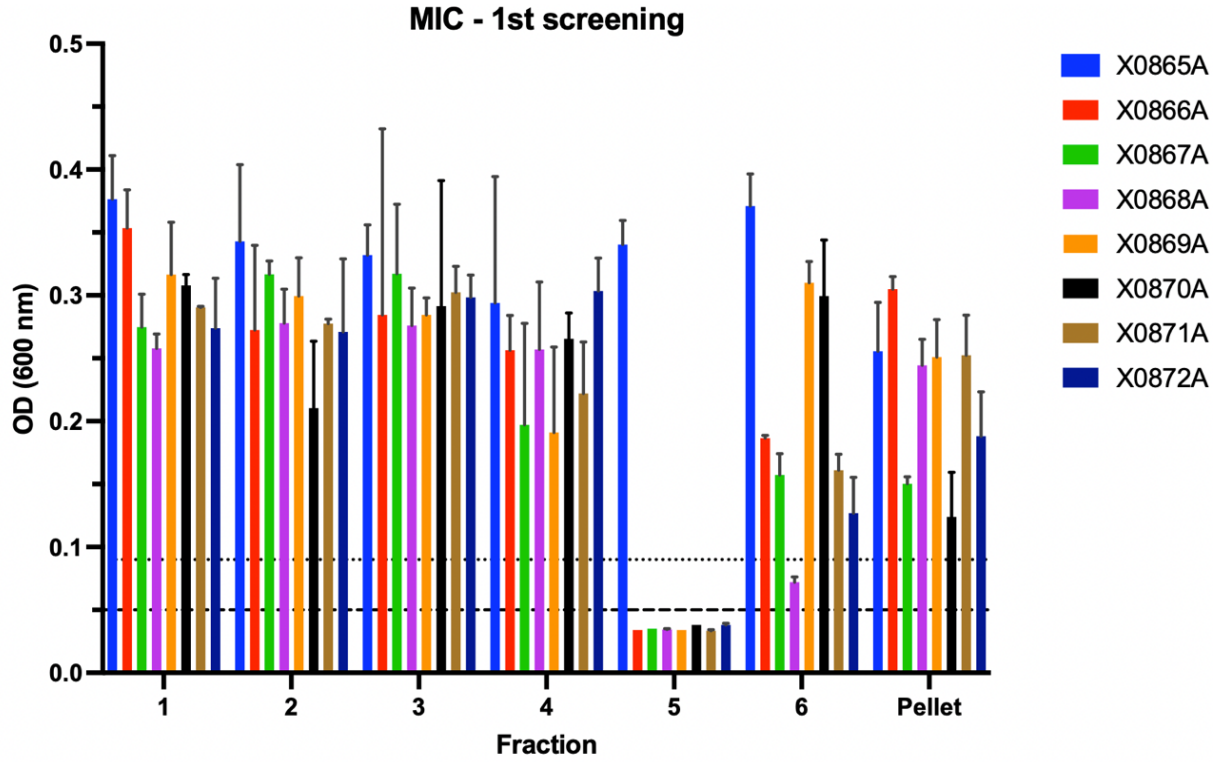

Figure 4.8: Bacterial growth of S. agalactiae (OD_{600}) when exposed to 100 µg/mL of fraction 1-6 and 200 µg/mL of the pellet extract from the extracts (X0865A, X0866A, X0867A, X0868A, X0869A, X0870A, X0871A and X0872A). Samples deemed active had an OD_{600} of 0,05 nm or lower, and samples deemed questionably active had OD_{600} values between 0,05 and 0,09 nm. The results are based on the mean of two technical replicates.

The fractions X0866A-05, X0867A-05, X0868A-05, X0869A-05, X0870A-05, X0871A-05 and X0872A-05 showed activity against the bacteria *S. agalactiae* in the primary screening (figure 4.8) and were retested at different concentrations (figure 4.9). X0866A was active at 100 µg/mL and fraction X0869A-05 was questionably active at this concentration. Fraction X0867A-05 and X0870A-05 were active down to 50 µg/mL. For fraction X0868A-05, X0871A-05 and X0872A-05 were active down to 75 µg/mL, but X0872A-05 was also just above the questionably active line at a concentration of 50 µg/mL. X0868A-06 was not active at any of the concentrations used in the secondary screening, including 100 µg/mL where it showed activity at in the primary screening (figure 4.8).

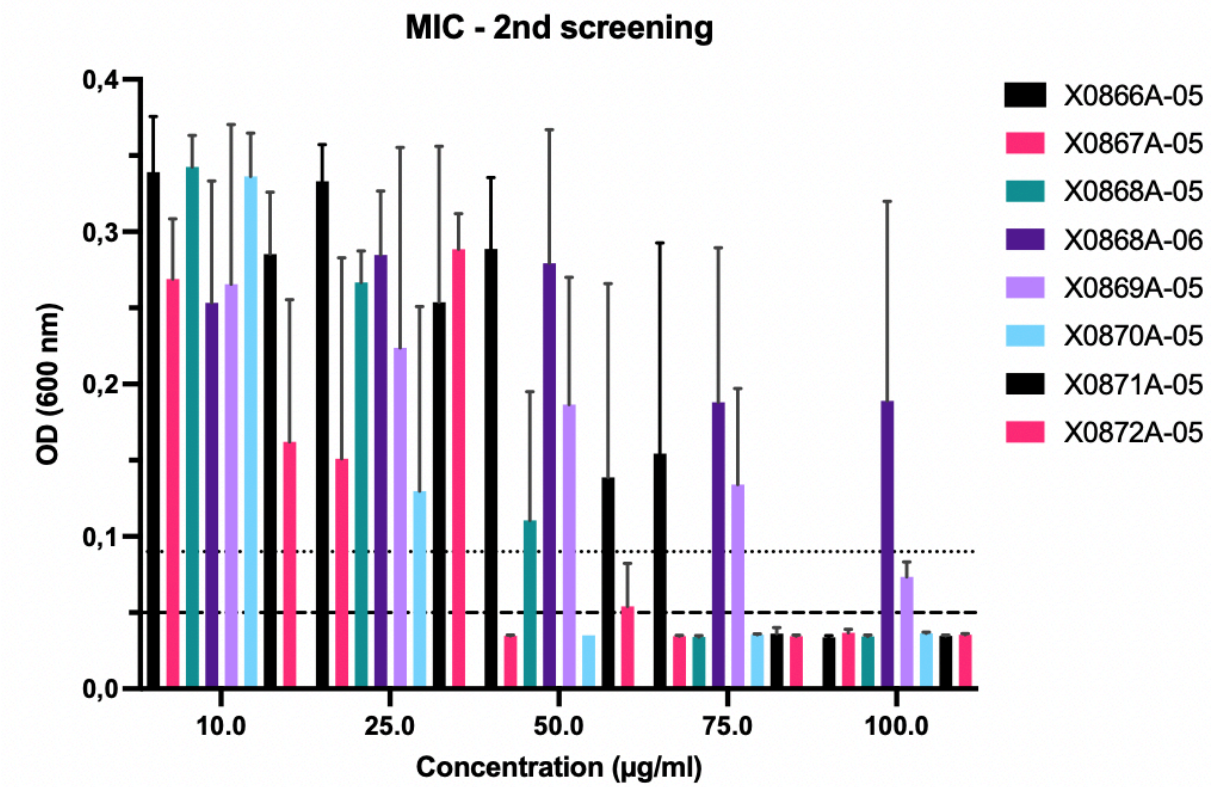

Figure 4.9: Bacterial growth of S. agalactiae (OD_{600}) when exposed to different concentrations (10, 25, 50, 75 and 100 µg/mL) of the fractions deemed active in the primary screening (X0866A-05, X0867A-05, X0868-05, X0868-06, X0869A-05, X0870A-05, X0871A-05 and X0872A-05). Samples deemed active had an OD_{600} of 0,05 nm or lower, and samples deemed questionably active had OD_{600} values between 0,05 and 0,09 nm. The results are based on the mean of two biological replicates consisting of two technical replicates each.

Many of the samples with OD_{600} values around 0,2 had high standard deviation with a lot of uncertainty linked to the result.

4.3.3 Biofilm formation inhibition assay

The biofilm forming bacteria *S. epidermis* was exposed to 100 µg/mL of the fraction 1-6 of the resin extracts and 200 µg/mL of the pellet extracts to indicate biofilm inhibiting activity and to select samples for further investigation. Samples with 30 % or less cell survival were deemed active and samples with 30-40 % were deemed questionable active.

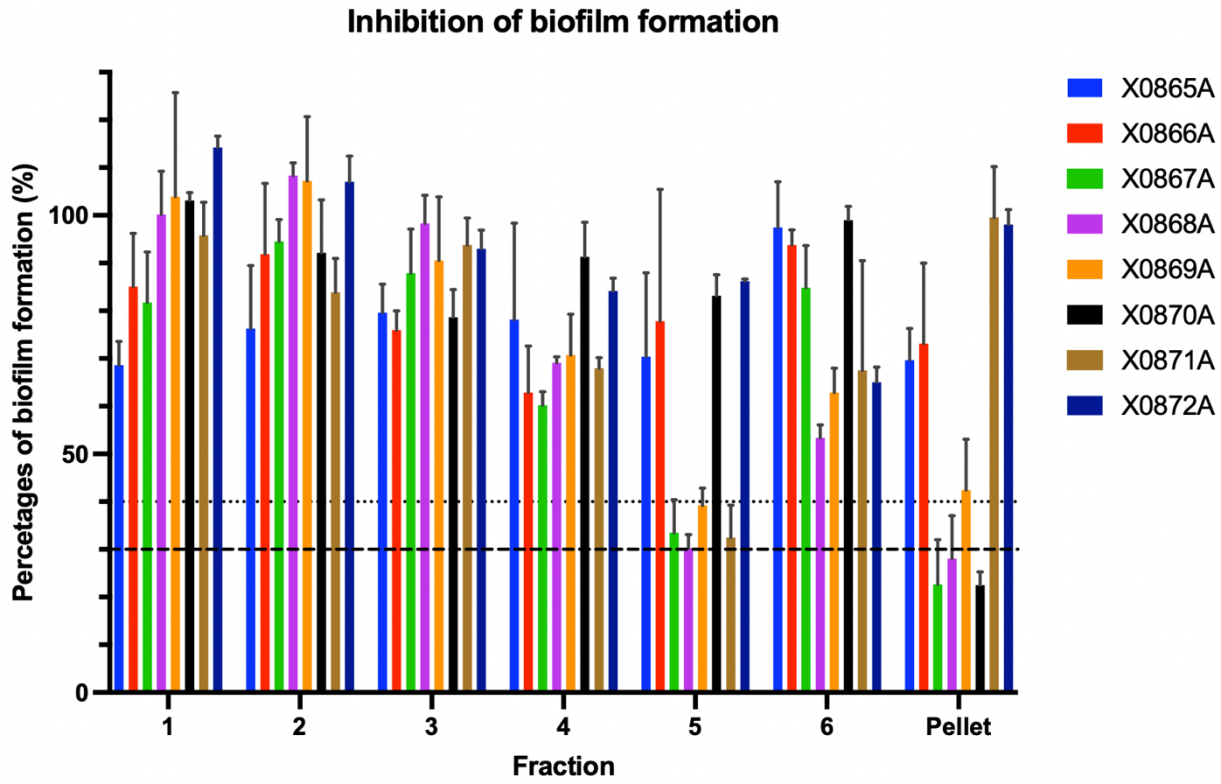

Figure 4.10: Percentages of biofilm formation of S. epidermis when exposed to the 100 µg/mL of fraction 1-6 and 200 µg/mL of the pellet extract from the extracts (X0865A, X0866A, X0867A, X0868A, X0869A, X0870A, X0871A and X0872A). Samples deemed active had a value of 30 % or less survival, and questionable activity was at 30-40 %. The results are based on the mean of three technical replicates.

None of the fractions were deemed active in the biofilm inhibition screening, but fraction five for the extracts X0867A, X0868A, X0869A and X0871A were questionably active. For the pellet extracts X0867A, X0868A and X0870A were active. There was not conducted a secondary screening for biofilm inhibiting activity due to the urge of focus on the most promising hits and time limitation.

4.4 Dereplication of active fractions and unfractionated pellet extracts

Due to time constraints a selection of fractions for dereplication had to be done, and was based on the results of activity in the bioactivity screening and for further investigation into the OSMAC approach. Fraction 5 of the extracts X0870A, X0871A and X0872A, from the isolates T009, T011 and T040 respectively, were selected and subjected to dereplication by HPLC-MS analysis, yielding in chromatograms that were comparable to each other and the media references of ArtM and DVR2. The pellet-extracts were only checked for the presence of arthrobacilins since they were initially discovered by ethanol extraction of *Arthrobacter* cell-biomass, ensuring that the presence of them was not missed due to the use of resin-extraction.

Up to the 30 biggest peaks in the chromatograms for the fractions were compared to the chromatograms of the media references of ArtM or DVR2. This led to the exclusion of the peaks caused by compounds from the growth media that were not produced by the bacteria and are marked with a star (*) in figure 4.13, 4.15 and 4.16. For the biggest peaks the number of signals caused by media components were highest for the two fractions that came from extracts X071A and X072A, that were grown in ArtM and ArtG (modified ArtM with glycerol). These had 29 and 25 signals in ESI^+, and 14 and 18 signals in ESI^- recognized as media components (figure 4.13 and 4.15). This is substantially more than that found in X0870A, grown in DVR2, with 14 signals in ESI^+ and 7 signals in ESI^- (figure 11).

The exclusion of media and modified components resulted in 11 potentially bioactive peaks in ESI^+, 7 peaks in ESI^- and three areas containing lipids assumed to have unspecific bioactivity.

4.4.1 Examples of chromatogram interpretation

The mass (M, equation 3) of a molecule appearing as signal in mass spectrometry can be concluded from the mass-to-charge ratio (*m/z*) which is the measured output signal for a given ion in a MS-detector. m/z is the ions mass (m) over the ions charge (z). The operator is mostly able to conclude the ion's charge from C^{13} isotope satellites and can therefore calculate the mass of the ion.

Equation 3: $\mathbf{M = m \times z}$

An example of a double charged ions is the signal of 532,89 m/z which contains one C^{13} atom compared to the 532,38 m/z signal (figure 4.11). If these were single charged ions ($[M + H]^+$) the difference would be 1 M (there is no half "unit" or half neutron in nature), consequentially the observed difference of 0,5 M needs to be caused by z = 2. This indicates that this is a double charged ion ($[M + H]^{2+}$), with z = 2 in equation 3. The C^{13} containing variants of a molecule always appear in natural numbers (n) and this restriction allows the identification of the correct mass, enabling the calculation of the ions correct elemental composition in high resolution MS. In addition the mass 1063.77 m/z in figure 4.11 indicates the single charged ion $[M+H]^+$ of the molecule.

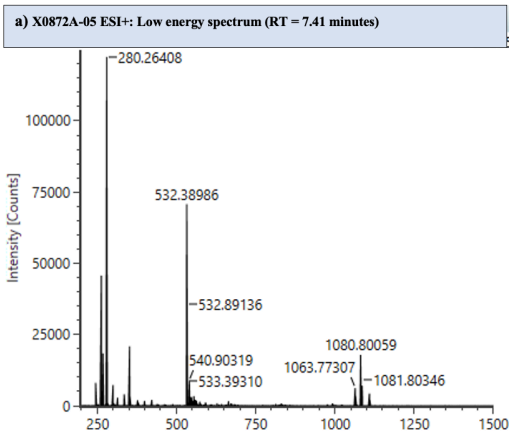

Figure 4.11: Example of double charged ion in X0872A-05.

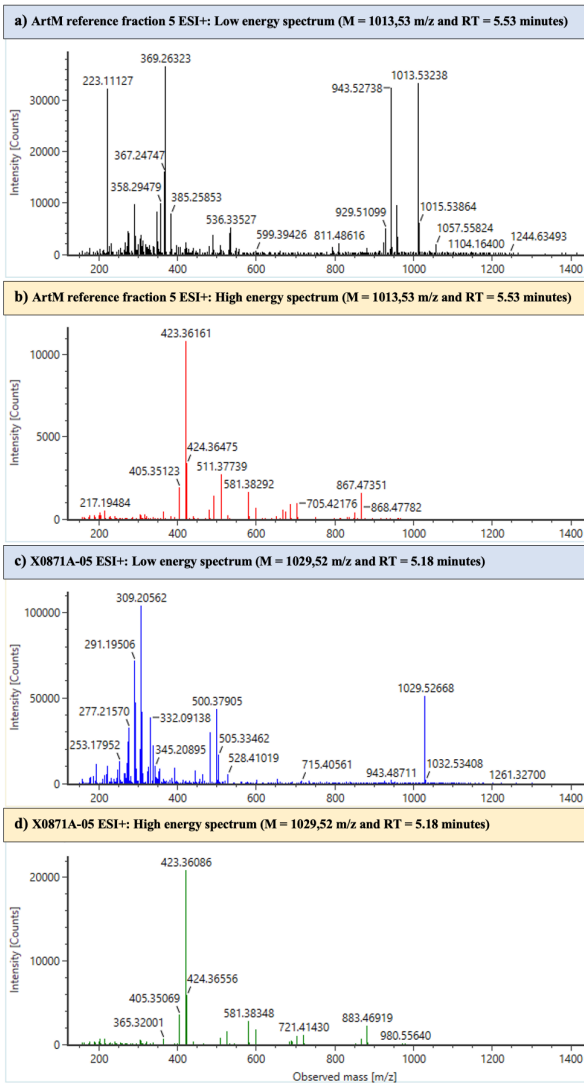

Figure 4.12: High (yellow header) and low (blue header) energy spectrum in ESI^+ of the signals 1013,53 m/z in ArtM reference fraction 5 and signal 1029,54 m/z in X0871A-05.

The only signal above 1000 m/z of noticeably quantity observed in ArtM reference fraction 5 was the signal 1013,53 m/z and thus all signals observed with a higher m/z in the extract fractions were deemed as produced by the bacteria and not media components. These were seen as potential bioactive compounds with a mass corresponding with arthrobactilins. An example of a signal like this was 1029,52 m/z in X0871A-05 (figure 4.12). The low energy spectrum of this compound (figure 4.12c) did not have any dominant peaks corresponding with the low energy spectrum of the media component 1013,53 m/z (figure 4.12 a). The comparison of the high energy spectrum of these compounds revealed that the dominant peaks of 581,38, 423,36 and 405,36 m/z were identical. This led to the assumption that the signal 1029,52 m/z was a modified media component. This way the compounds detected in the respective fraction were checked for being either a media component or a modified media component, and such were excluded from further investigation.

4.4.2 Signals of eventual bioactive compounds

After the compounds were determined not to be media or modified media components, their elemental composition was investigated to determine candidates responsible for the bioactivity. Elemental composition and fragmentation were used to investigate if the signals were caused by known or unknown compounds using the database search tool ChemSpider, and if there was any connected literature. The selection of elemental composition was based on the accuracy of the fit of the predicted elemental composition to the m/z (i-Fit) and the number of common fragments. The peaks or areas mentioned are shown in table 4.5 and contains the information about retention time (RT), m/z, if it is identified as media or modified media component and from which extract it originates.

Table 4.5: Information about the signals marked with arrows and numbers from 1-21 in figure 4.13, 4.15 and 4.16. m/z marked with a line (-) describes an area of peaks and not a specific peak.

Peak number:	From extract:	m/z	Retention time (minutes):
1	X0870A, Grown in DVR2	262,09	2.01
2		265,09	2.57
3		428,34	5.61
4		442,36	5.71
5		358,36	9.31
6*		280,26	9.66-9.90
7*		282,27	10.52-10.63
8		-	9.31-12.33
9		341,28 683,57	5.99
10		350,24 701,50	7.42
11		849,50	9.95-1.98
12	X0871A, Grown in ArtG	295,22	5.44-5.50
13		-	9.20-12.33
14		277,04	3.72
15		301,20	4.59
16	X0872A Grown in ArtM**	285,20	4.40
17		288,25	6.94
18		340,28	7.31
19		-	9.20-12.33
20		301,20	4.57
21		285,20	5.78

* Identified as media or modified media component
** at room temperature

For fraction X0870A-05 (grown in DVR2) the peaks 1 to 11 were identified. These contained seven potentially bioactive peaks and one area in the ESI^+ chromatogram, and three peaks in the ESI^- chromatogram (figure 4.13).

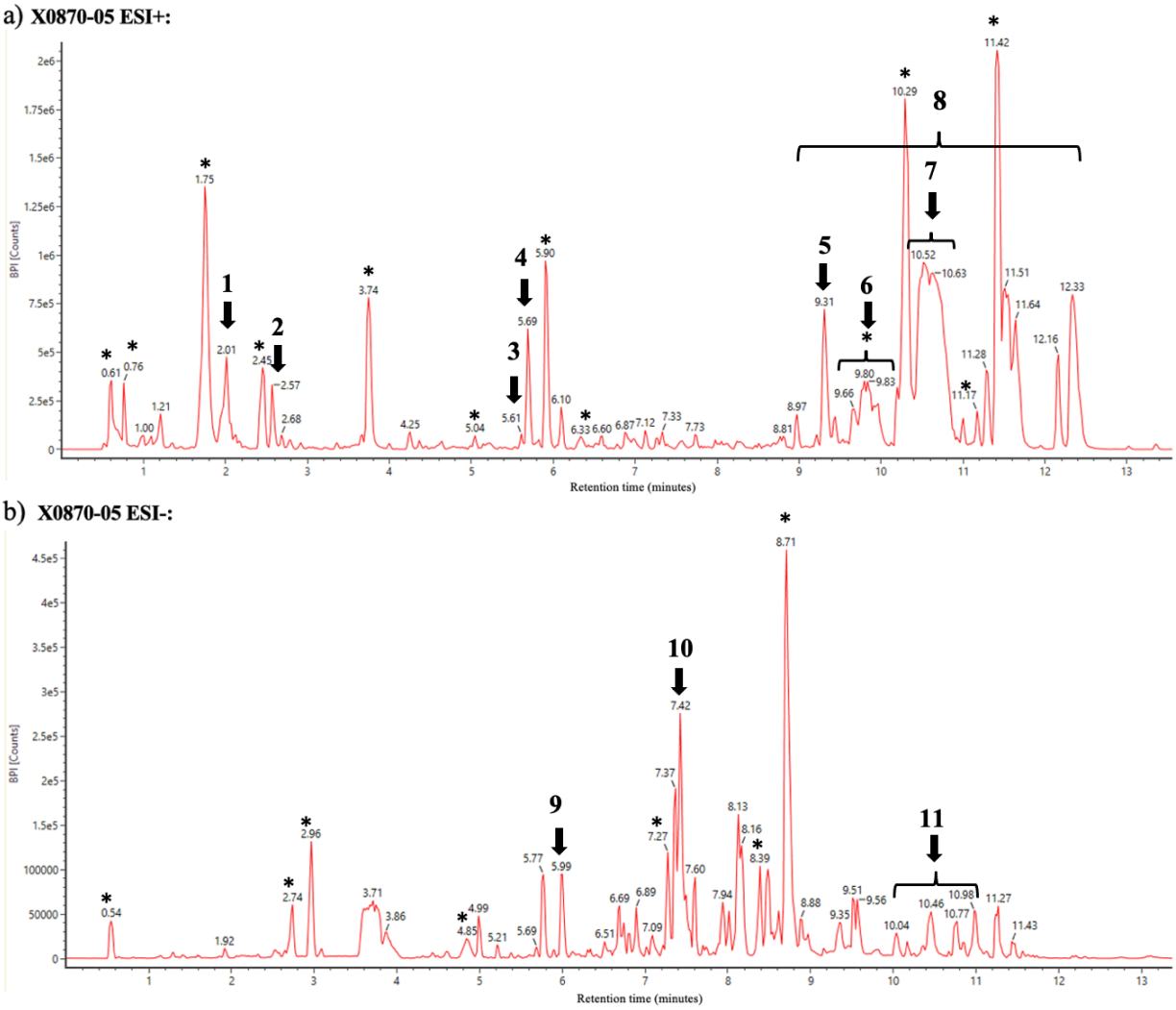

Figure 4.13: BPI chromatogram of a) ESI^+ and b) ESI^- (m/z 50-2000) for fraction X0870A-05. Star (*) indicates media components, numbered arrows indicate signals for compounds with potential bioactivity or examples, and only numbers indicate areas discussed further. More detailed information of the marked signals is shown in table 4.4.

For peak 1 (RT: 2.01 minutes) the signal 262,09 m/z gave an elemental composition of $C_{16}H_{11}N_3O$ (100 % i-Fit). The two hits for this signal in ChemSpider with most common fragments, 6 and 4 respectively, was 4-(Cyano-2-pyridylmethyl)quinoline 1-oxide (molecule **1.**, figure 5.4) and 2-(1H-Indol-3-yl)-4(3H)-quinazolinone (molecule **2.**, figure 5.4).

For peak 2 (RT: 265,09 m/z and 2.57 minutes) the elemental composition given was $C_{16}H_{12}N_2O_2$ (100 % i-Fit), with the two hits 2,3,9,10-Tetrahydro[1]benzofuro[6,5-h]furo[2,3-b][1,5]naphthyridine and Bitolylene diisocyanate (molecule **3.** and **4.**, figure 5.4) in ChemSpider (7 and 8 common fragments respectively).

The 3rd peak (RT: 5.61 minutes and 428,34 m/z) the elemental composition calculated for the signal was $C_{23}H_{45}N_3O_4$ (100 % i-Fit) and the 4th peak (RT: 5.71 minutes and 442,36 m/z) had the elemental composition of $C_{24}H_{47}N_3O_4$ (100 % i-Fit). The proximity in RT and the difference of one carbon and two hydrogens suggest it differs in one methylene group (CH_2).

Peak 5 (RT: 9.31 minutes and 358,36 m/z) gave an elemental composition of $C_{22}H_{47}NO_2$ with a 100 % i-Fit. The ratio between carbons and hydrogen, the number of oxygen and nitrogen, and the suggested molecule (6 common fragments) indicates an aliphatic molecule with a polar head.

The peaks at 6 (RT: 9.66 - 9.90 minutes) and 7 (RT: 10,52 -1 0,63 minutes) in figure 4.13a had the signals of 280,26 and 282,27 m/z respectively. This was a difference of 2 m/z which is, with high probability, a difference of two hydrogens, indicating an extra double bond. Peak 6 was identified as a media component, indicating that signal 7 is a modified media component.

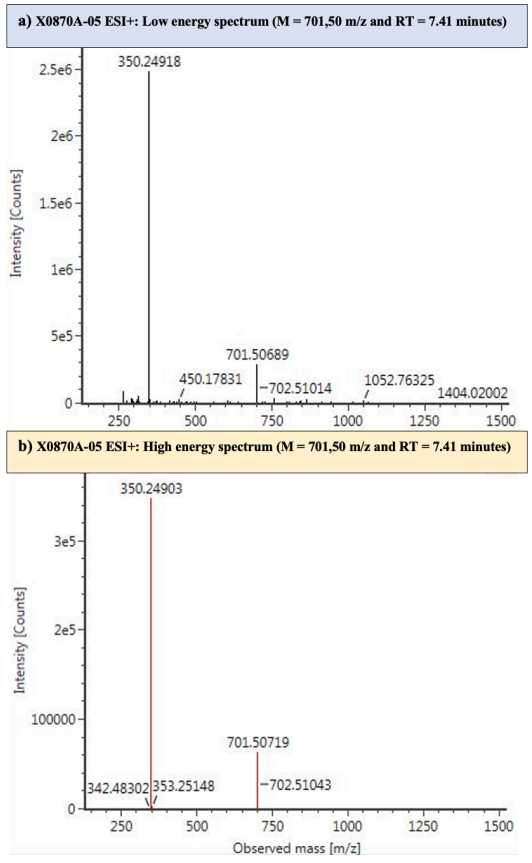

Figure 4.14: a) High and b) low energy spectrum in ESI+ of the signal 701,50 m/z at RT: 7.41 in fraction X0870A-05.

The first peak in ESI- for X0870A (figure 4.13b), peak 9 (RT: 5.99 minutes), had two signals of 341,28 and 683,57 m/z. High energy MS spectrum showed that 341,28 m/z was a fragment of 683,57 m/z and had the elemental compositions of $C_{24}H_{38}O$ (100 % i-Fit) and $C_{48}H_{76}O$ (100 % i-Fit) respectively. A suggested molecule for the signal 341,28 m/z was a steroid.

Peak 10 (RT: 7.42 minutes) also consisted of two signals, 350,24 and 701,50 m/z. Figure 4.14 shows the low and high energy spectrum of the signal 701,50 m/z, where signal 350,24 m/z was observed as a fragment of it. 350,24 m/z had an elemental composition of $C_{24}H_{33}NO$ (100 % i-Fit) and for the 701,50 m/z signal the elemental composition could not be determined accurately (79 % i-Fit).

In the area marked 11 in figure 4.13b the signal 849,50 m/z was found in many peaks. The signal had an elemental composition of $C_{57}H_{70}O_6$ (100 % i-Fit) in all the peaks, but the difference in RT indicates that these were isomers, and thus eluting at different times.

In the chromatogram for fraction X0871A-05 (grown in ArtG) the peaks 12 to 15 were identified. These contained one potentially bioactive peak and one area in ESI^+, and two peaks in ESI^- (figure 4.15).

A signal of 295,22 m/z in peak 12 (RT: 5.44 - 5.50 minutes) gave an elemental composition of $C_{18}H_{30}O_3$ (100 % i-Fit). This resulted in many hits in ChemSpider with over 50 common fragments, and all of these were identified as lipids. Peak 14 (RT: 3.72 minutes) in ESI^- had a signal of 277,04 m/z and gave one hit for elemental composition: $C_{17}H_{10}O_4$ (100 % i-Fit). This was suggested to be the molecule fluorescamine (molecule **5.**, figure 5.5) with 8 common fragments.

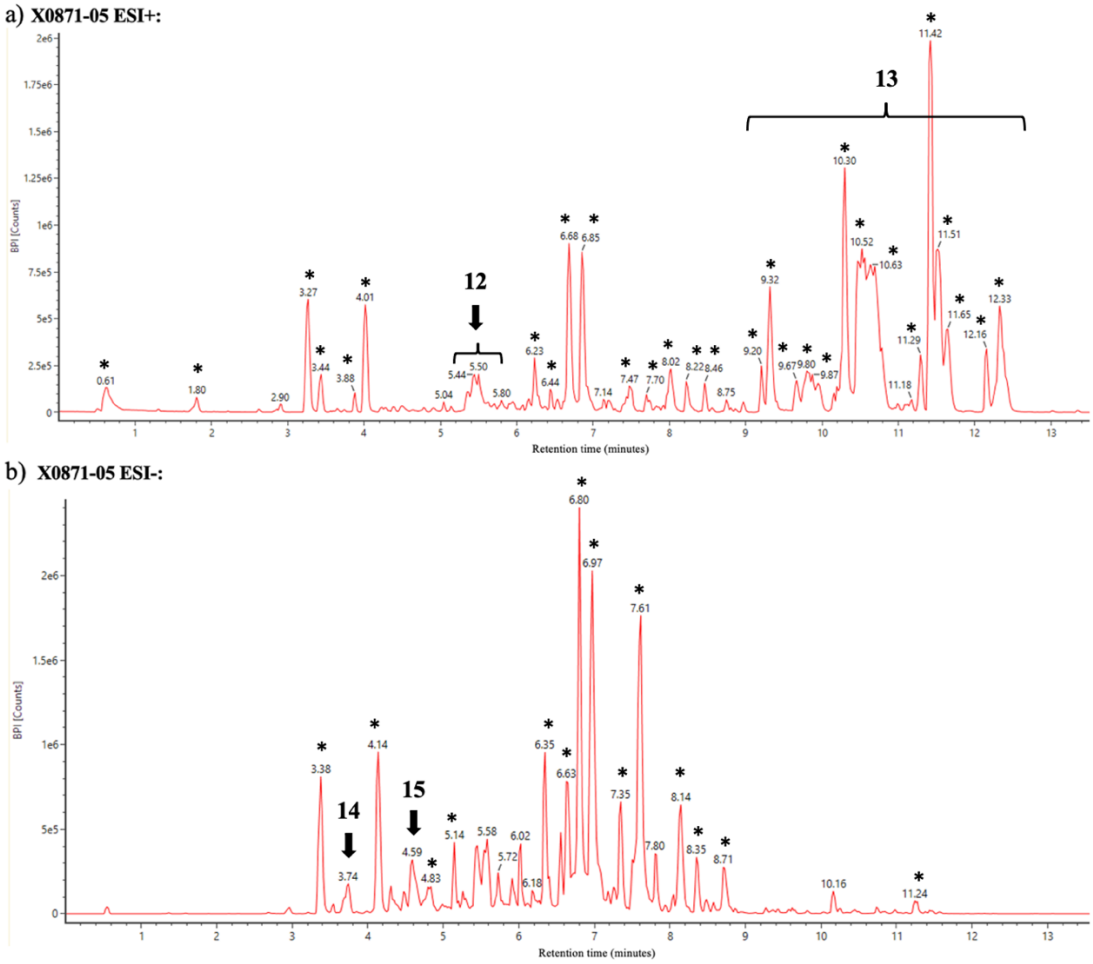

Figure 4.15: BPI chromatogram a) ESI^+ and b) ESI^- (m/z 50-2000) for fraction X0871A-05. Star (*) indicates media components, numbered arrows indicate signals for compounds with potential bioactivity or examples, and only numbers indicate areas discussed further. More detailed information of the marked signals is shown in table 4.4.

Peak 15 (RT: 4.59 minutes) in the ESI^- chromatogram for X0871A-05 (figure 4.15b) and peak 20 (RT: 4.57 minutes) in the ESI^- chromatogram for X0872A-05 (figure 4.16b) both contained the signal 301,20 m/z, but in much higher quantities in X0872A-05, with activity observed at lower concentrations. This signal gave the elemental composition of $C_{17}H_{26}N_4O$ (100 % i-Fit) the suggested molecules were Emedastine and 1-[(4-Methyl-1-piperazinyl)acetyl]-4-phenylpiperazine (molecules **6.** and **7.**,figure 5.5).

The chromatogram for fraction X0872A-05 (grown in ArtM at room temperature) the peaks 16 to 21 were identified. These contained three potentially bioactive peak and one area in ESI^+, and two peaks in ESI^- (figure 4.16).

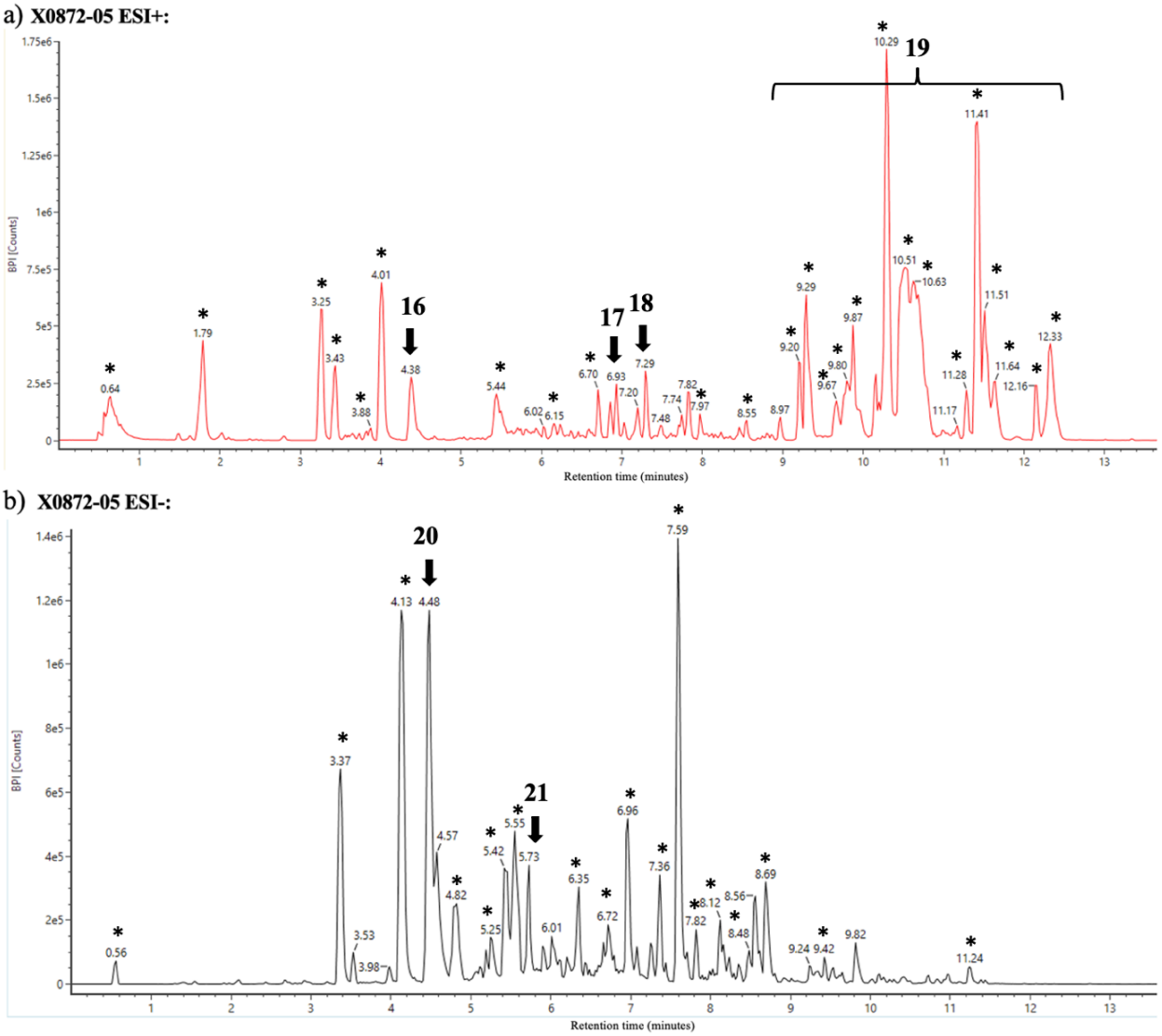

Figure 4.16: BPI chromatogram a) ESI^+ and b) ESI^- (m/z 50-2000) for fraction X0872A-05. Star (*) indicates media components, numbered arrows indicate signals for compounds with potential bioactivity or examples, and only numbers indicate areas discussed further. More detailed information of the marked signals is shown in table 4.4.

Peak 16 (RT: 4.40 minutes) had a signal of 285,20 m/z resulted in the elemental composition $C_{16}H_{28}O_4$ (100 % i-Fit) and many hits in ChemSpider. The two most likely molecules proposed had 25 and 22 common fragments and were (9S,12R)-9,12-Dihydroxy-10-hexadecynoic acid and (5E)-6-[(2S,5S)-5-(1-Hydroxyethyl)-5-methyltetrahydro-2-furanyl]-2,4-dimethyl-5-heptenoic acid (molecules **8.** And **9.**, figure 5.6).

A signal of 288,25 m/z in peak 17 (RT: 6.94 minutes) gave an elemental composition of $C_{16}H_{33}NO_3$ (100 % i-Fit), and some hits in ChemSpider, where the one with most common fragments had three.

For peak 18 (RT: 7.31 minutes) had the signal 340,28 m/z and the elemental composition of $C_{20}H_{37}NO_3$ (100 % i-Fit). The two suggested molecules with the most common fragments, 25 and 17, were MFDO1320532 and Rociverine.

For signal 285,20 m/z at peak 21 (RT: 5.78 minutes), the suggested elemental composition with 100 % i-Fit was $C_{16}H_{30}O_4$. The suggested molecules for this elemental composition were (1R,3aR,4R,7R,8aR)-7-[(2R)-1-Hydroxy-2-methoxy-2-propanyl]-1,4-dimethyldecahydro-1,4-azulenediol and 16-Hydroxy-10-oxohexadecanoic acid (molecules **10.** and **11.**,figure 5.6), with 7 and 8 common fragments respectively.

The areas in ESI^+ marked with 8 (figure 4.13a), 13 (figure 4.15a) and 19 (figure 4.16a) eluted from 9.20 minutes and to the end, and most likely contained many lipids. The peak contained signals for monovalent ions ($[M+H]^+$) and also signals that were two times M – 1 (H) ($[2M+H]^+$) of the monovalent ion. The isotope pattern/C^{13}-satellites and the fact that it was only possible to calculate an accurate elemental composition for the monovalent ion indicated the aggregation of these lipids. An example of this was that at peak 7, where the most dominant signal was 282,27 m/z, there was also found a signal of 563 m/z, which is two times 282 – H. For X0871A-05 and X0872A-05 the lipids were mostly found to be media components. This led to the assumption that there could be unspecific activity caused by the lipids in the growth media, and a MIC assay for *S. agalactiae* with the concentrations 50 and 100 µg/mL was conducted for fraction 5 for the Arthrobacter medium. The OD measured was 0,03 nm for both technical replicates at 50 and 100 µg/mL and determined to be active.

5 DISCUSSION

This chapter include methodological considerations and discussion of the results obtained during this thesis. It will try to answer the overall objective concerning the discovery of potential bioactive compounds in selected species of *Arthrobacter,* and to gain experience with the *Actinobacteria* in general, and with *Arthrobacter* in particular. It will also address the three specific objectives concerning the effect of cultivation-conditions on biomass and metabolite production, screening for bioactivity of the different fractions and extracts as well as dereplicating a selection of active samples in order to discover potential bioactive compounds for further isolation.

5.1 The effect of cultivation conditions and growth media on biomass, extract and fraction yield

This chapter will shed light on methodological considerations and discuss results to answer the first specific objective of this master's thesis concerning the effect of cultivation-conditions on the strains biomass and metabolite production.

Due to the time limitation of five months for this work, three of the bacterial strains were selected for the OSMAC approach. Wietz *et. al* showed that *Arthrobacter* sp. originating from widely different environments in the Arctic ocean contains almost identical 16s sequences [23], and the selection was therefore based on the lowest percentages in query cover from homology comparison of 16s sequences. This would potentially increase the chance of identifying novel compounds.

The selection of the ArtM medium was based on the successful cultivation of *Arthrobacter* sp. by Ohtsuka *et al.* [2], and was thus chosen to be the main growth media during this thesis. The carbon sources available to the microorganism are known to affect both the composition and titration of SMs [8]. Members of the *Actinobacteria* phylum have shown the ability to grow with glycerol as the sole carbon source [40] and it was used by El-Nakeeb *et al.* in an isolation medium to isolate *Actinomycetes* [41]. Following this thought, addition of 1 % glycerol in ArtM, resulting in the growth medium ArtG was decided to be used as one variation within the OSMAC approach. DVR2 is a well-established growth medium at Marbio and was selected based on the established experience with this medium. It also contained ingredients with little variability, unlike the plant derived soybean meal used in ArtM.

The effect of different parameters under cultivation is shown by Bode *et al.* to cause the production of different SMs [18]. The strains in this thesis were thus grown in three different media (ArtM, ArtG and DVR2) to explore if different media triggered the production of SMs and to gain experience with *Arthrobacter* sp..

The dry weight yield from the resin extraction for the bacteria grown in ArtM, both at 10°C and room temperature (cultures X0865A, X0866A, X0867A, X0868A, X0869A and X0872A), did not vary notably (figure 4.2). The resin extract X0871A from the strain grown in modified ArtM with glycerol (ArtG) resulted in a much higher dry weight yield compared to the same strain grown in ArtM (X0866A), suggesting the triggering of metabolite production with the addition of another carbon source. The cell pellet mass for these extracts were about the same, but the yield from the pellet extraction was almost twice as high for the one grown in ArtG compared to the same strain grown in ArtM. Strengthening the assumption that the addition of glycerol as a carbon source has increased the metabolite production in the bacteria presence, and not the growth of the bacteria.

In ArtM there are three sources of carbohydrates (D-Glucose, starch and soybean meal). D-Glucose is an easily accessible carbon source, starch is a more complex polymer, and soybean meal contains a variable source of carbohydrates and nutrients due to it not being an extract or defined mixture. DVR2 does not contain any of these in notable amounts, and its main carbohydrate sources are yeast extract and maltose from malt extract (table 3.5). The cell pellet mass for the strain T009 grown in DVR2 (culture X0870A) is over twice as high as for the same strain grown in ArtM (X0865A) (figure 4.1) and has about the same yield for the pellet extraction. The two resin extracts for this strain resulted in a much higher yield for the one grown in DVR2 compare to the one grown in ArtM (figure 4.2), and thus suggesting that DVR2 is a more suitable growth medium for this *Arthrobacter* sp. in regards of biomass and metabolite production.

Parameters that do not affect the composition of the growth medium are called physical parameters and also have an effect on the growth dynamic and metabolic profile of microorganisms [32]. To explore the effect of temperature, one of the strains (T040) was grown in ArtM at room temperature (culture X0872A) in addition to 10°C. Many bacteria grow faster and have a higher biomass production at higher temperatures, but the results (figure 4.1) showed that the culture X0872A had the lowest cell pellet mass of all the extracts. This suggested that the optimal growth temperature of this strains is closer to 10°C rather than room temperature. These strains were all collected from Arctic marine environments, and the results strengthens the assumption that these strains have adapted to lower temperatures.

Ohtsuka *et al.* isolated the antibiotic arthrobactilins from *Arthrobacter* extracts that were extracted with EtOH from the cell pellet [2]. This extraction method was conducted, alternatively using MeOH, to compare it with resin extraction, a well-established extraction method at Marbio, to check for the presence of arthrobactilins. The resin extraction had approximately the same extraction efficiency as that of pellet extraction, but was easier to perform. The secondary metabolites are usually secreted by the producing organism as a way of adapting to the environment, quorum sensing *etc*. [9], indicating that the medium, and not the inside of the cells, is more likely to contain novel SMs. This makes resin extraction the more suitable extraction method for SMs produced by *Arthrobacter* sp.. The pellet extracts were not fractionated due to the time constraints and because the resin extraction was deemed a suitable extraction method. The unfractionated pellet extracts were used to look into the production of arthrobactilins by *Arthrobacter*.

The sum of the yield of the fractions for one sample is lower than the extraction yield for the same respective sample (figure 4.2), indicating that some of the material is lost during fractionation. Some of the eluent is lost during fractionation, but this does not account for the whole loss. Another plausible reason is the loss of material during transfer of the sample to the column, but it is assumed that this too is too little to account for the remaining loss. It is thus, with high probability, the result of some of the material remaining in the column, and therefore not eluted to the samples.

The extracts of the strains grown in ArtM (cultures X0865A, X0866A, X0867A, X0868A, X0869A and X0872A) have lower fraction yield in fraction 1 and 2 compared to the extracts of the strains grown in DVR2 and ArtG (X0870A and X0871A respectively) (figure 4.3). This suggests that either the bacteria in the growth medium DVR2 or ArtG produce more polar molecules or that there are bigger quantities of these in the growth media compared to ArtM. For X0871A the only difference in growth medium is the addition of glycerol, which is a polar molecule, but it was shown that the extraction yield (figure 4.2) and bacterial growth (figure 4.1) is higher than for the same strain grown in ArtM. This suggests that the glycerol is consumed by the bacteria, and that the higher yield in fraction 1 and 2 is due to the production of polar molecules by the bacteria.

For DVR2 the malt extract is a polar extraction of the molecules from malt, indicating that the high yield in the most polar fractions (1, 2 and 3) is due to leftover media components. In figure 4.1 it was shown that the cell pellet mass was higher for the strain grown in DVR2, suggesting that the compounds from the growth medium were used and that the increase in polar molecules could be due to higher production of polar molecules. For fraction X0865A-01 the observed yield is much higher compared to the others grown in ArtM and less in fraction 2 (figure 4.3), indicating that there were more polar compounds than in the rest of its fractions combined.

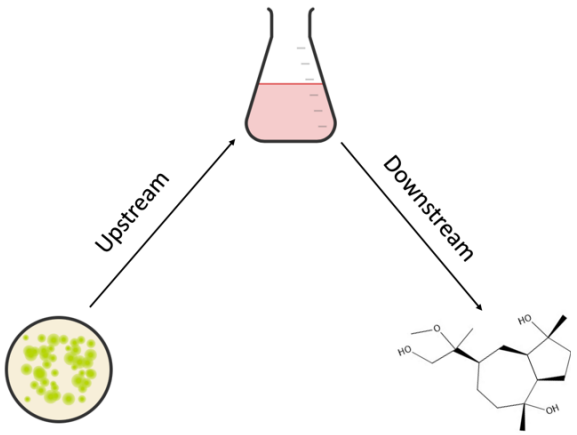

Figure 5.1: The upstream process results in the bacterial culture processed further in the downstream processes.

The upstream processes in bacterial NP research results in the bacterial cultures which are further used for the downstream processes of extraction, fractionation, bioactivity screening and dereplication conducted during this thesis (figure 5.1). The choices done during the upstream process will logically affect all the steps in the downstream process positively or negatively. This makes the choice of bacterial strains, growth media and cultivation condition important, since affecting all the results achieved and its optimization is the main goal of the OSMAC approach.

5.2 Bioactivity screening of fraction and unfractionated pellet extracts

This chapter will discuss results to answer the objective concerning the bioactivity screening conducted for the different extracts and fractions for inhibition of biofilm formation, antibacterial and anticancer activity. It will also discuss the main objective of exploring the potential of *Arthrobacter* sp. to produce bioactive compounds by using different cultivation conditions and growth media.

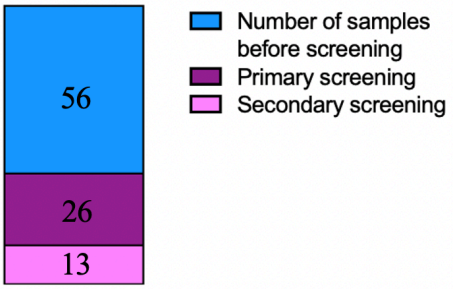

Figure 5.2: Number of samples (fractions and pellet extracts - 56) included in the primary screening, number of active/questionably active after the primary screening (26) and after secondary screening (13).

The primary screening was conducted at 100 µg/mL for the 48 fractions and 200 µg/mL for the eight unfractionated pellet extracts. The pellet extracts were tested at a higher concentration due to them not being fractionated, and thus the concentration of the potential bioactive molecules was much lower in the crude extract. This resulted in 26 hits deemed active or questionably active in the primary screening (figure 5.2) and was used as a selection method for which samples to investigate further.

There were 8 samples that showed activity in the primary screening and not in the secondary screening (table 4.3 and table 4.4) at the same test concentration. A plausible reason for this was that the compound or compounds that caused the activity in the primary screening could be unstable and have degraded so there no longer was observed activity. Kozikowski and colleagues showed that the storage of samples resulted in the degradation of the organic compounds and that the repeated freezing and thawing of the samples increased this degradation [42]. They also observed that it could affects the solubility of the compounds and may cause precipitations that led to loss of activity. There were three samples from fraction 3 with activity only in the primary screening, suggesting that it was the same instable compound responsible the activity. Instable compounds are not interesting for further investigation because of their short degradation time. This makes them harder to isolate, test further and determine their potential commercial value. While they may be interesting, these could give results that were hard to reproduce, and the focus was laid on the so far stable hits. These were thus excluded from any further investigation.

The fractions consist of many compounds, and each compound is thus diluted by the many other compounds present. If the bioactivity observed is the result of a few of these compounds they are present in small quantities, suggesting that they are much more potent when isolated. The low yield in fraction 4-6 could mean that the concentration of the active compound could account for a higher percentage of the sample composition compared to fraction 1-3, that had a much higher yield (figure 4.3). Experience at Marbio has shown that more polar fractions seldom exhibit activity in bioactivity screening. This does not exclude that some novel SMs may be present in these fractions, but are in such small quantities that they are below the analytic detection or masked by the more abundantly produced NPs [8], like lipids or other active metabolites.

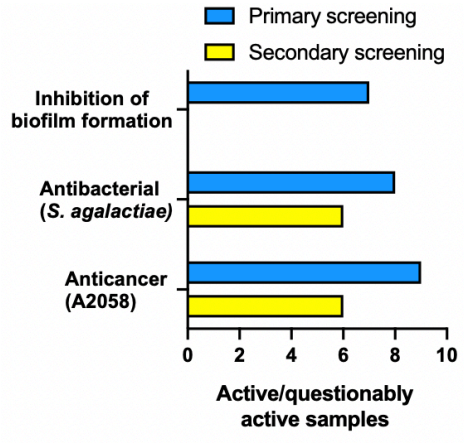

Figure 5.3: Number of samples deemed active/questionably active in the primary (blue) and secondary (yellow) screening of antibacterial (S. agalactiae) and anticancer (A2058) activity. Inhibition of biofilm formation (S. epidermis) was only included in the primary screening.

One important trait of antineoplastic agents (anticancer drugs) is their toxic selectivity towards malignant cells, and not cause damage to the surrounding tissue [43]. In the primary screening of anticancer activity there were nine samples deemed active or questionably active for the human melanoma cancer cell-line A2058 (figure 5.3), while it for the non-malignant, normal lung fibroblast cell-line MRC5 only was observed one questionably active sample (table 4.3). In the secondary screening the active fractions, except X0872A-05, only showed activity for the malignant cell-line A2058, and X0872A-05 showed a higher toxicity for A2058 compared to the non-malignant cell-line MRC5.

Table 4.3 shows that there is some correlation between the determined antibacterial and anticancer activity. This could be due to the production of compounds with an unspecific activity. Both anticancer and antibacterial assays are cell-based, and the activity observed could be due to the disruption of cell membrane. The cell-line A2058 and *S. agalactiae* both showed a higher sensitivity towards lipids compared to the MRC5 cell-line and the other gram-positive test bacteria (*S. aureus and E. faecalis*) respectively [37].

In the secondary screening of anticancer (A2058) and antibacterial (*S. agalactiae*) activity the samples deemed active or questionably active in the primary screening were tested in a dilution series (10, 25, 50, 75 and 100 µg/mL). A correlation for the dose-response of fraction 5 of the samples X0870A, X0871A and X0872A was observed in the anticancer secondary screening, where a decrease in concentration led to a decrease in activity (figure 4.7). The dose-response is an efficient way of assessing the reliability of the assay, but also the toxicity and potency of the samples [44]. Figure 4.7 shows a clear dose-response for the fractions X0870A-05 and X0872A-05. For fraction X0871A-05 there is a weak dose-response that flattens out at higher concentration and suggests that there is a saturation at 75 µg/mL.

For the secondary screening of the bacteria *S. agalactiae* in the MIC assay there was observed a high standard deviation. This suggested that some of the technical replicates could be active, but due to the huge variation the sample as a whole was deemed inactive. These samples could therefore be active in some of the replicates and not in the others.

The inhibition of biofilm formation activity screening only resulted in four of the fractionated samples deemed questionably active and three of the unfractionated cell pellet extracts deemed active. There was only conducted a primary screen for these samples, due to time limitations and prioritization of the more active results from the anticancer and antibacterial screening.

5.3 Dereplication of active fractions and unfractionated pellet extracts

This chapter will address the methodological considerations and discuss results to answer the third specific objective of this master's thesis concerning the dereplication of the extracts deemed active and propose potential active compound for feature isolation. The presence of arthrobactilins was also investigated and discussed.

In the growth media reference of ArtM and in the exclusion of media components in the extracts we identified flavonoids, isoflavonoids and phytosterols *via* MS. Soybean meal is a byproduct of the refining process of soybean oil and is a known source of phytosterols and flavonoids [45, 46]. Activity such as antioxidative, anticarcinogenic and lowering of cholesterol have been recorded for flavonoids, phytosterols and isoflavonoids [45-47]. The presence of these compounds is a problem for dereplication since they may have bioactivity and they increase the analytical effort for bacterial compound identification significantly. In addition, these molecules can be modified by the bacteria and increase the number of metabolites (modified media components) even further. The presence of glycosylated phytosterols in the extract chromatograms is assumed to be such a modified media component. There was also an issue with the presence of many lipids in the fractions, most likely causing an unspecific activity in the cell-based assays. A MIC assay for fraction 5 of the ArtM growth medium was executed due to this high quantity of lipids within the media reference, strengthening the assumption that the activity was a result of the unspecific activity of the lipids. Due to the activity and abundance of media components, further use of the ArtM and soybean meal in growth media in general is not recommended in future cultivations of *Arthrobacter* sp..

The components that originate from the growth media or the media components modified by the bacteria were excluded for further investigation. The elemental composition and common fragments were investigated in the database search tool ChemSpider for known structures or molecules, and connected literature. Steckel *et al.* showed that common fragments indicate a common structure and that fragmentation of a precursor molecule could only result in fragmented elements of that particular molecule [39]. It should also be kept in mind that the dereplication was conducted as a quantitative analysis and not qualitative, meaning that the low quantity observed for some of the compounds could be due to them poorly ionizing. This suggests that some compounds could be present, but not detected or noted due to low recorded amounts.

Ohtsuka and colleagues discovered three different arthrobacilins, all with a m/z above 1000 [2]. The cell pellet extracts and the selected fractions (X0870A-05, X0871A-05 and X0872A-05) were searched for peaks in both ESI^+ and ESI^- for a corresponding m/z, and only resulted in modified media components. The structure of arthrobactilins contains many oxygens/hydroxyl groups (figure 1.2) that should be ionized in ESI^-, and are thus, with high probability, not produced by the selected strains under these conditions.

One general problem encountered throughout the dereplication was the abundant presence of lipids, and these were often observed to aggregated and resulted in single charged double ions. This was observed more in the fractions originating from bacteria grown in ArtM, but was also observed in fractions originating from bacteria grown in DVR2. Some of the fragment-based library searches resulted in many plausible suggested molecules, and because of time limitation, the two with the most common fragments were selected for further investigation.

5.3.1 Fraction X0870A-05 grown in DVR2

Fraction X0870A-05 was a product of the strain T009 cultivated in the growth medium DVR2. The four potential bioactive candidates suggested for this fraction were assigned to the two signals 262,09 m/z and 265,09 m/z in positive ionization mode (ESI^+) and are shown in figure 5.4.

The signals 262,09 m/z and 265,09 m/z resulted in the elemental composition of $C_{16}H_{11}N_3O$ and $C_{16}H_{12}N_2O_2$ respectively, and differ in only 3 m/z. The number of carbons in these compounds suggests that they have a common carbon backbone. Further investigation into their high and low energy MS showed that they shared the low abundant fragments 206,08 m/z and 167,06 m/z, which revealed the elemental composition of $C_{11}H_{11}NO_3$ and $C_{11}H_6N_2$ respectively. They had the double bond equivalence of 7 and 10, indicating small molecules with a complex structure, most likely in a cyclic structure. This suggests that 262,09 m/z and 265,09 m/z have the same predecessor but differ significantly in chemical composition.

For the signal 262,09 m/z there were many suggested molecules with interesting structures, but the two with the most common fragment were molecule **1.** and **2.** shown in figure 5.4. Molecule **1.** was potentially identified as quinoline with a nitrile group. Medically active nitril containing NPs have been shown to derive from marine sources [48] and quinolines have been used for its bioactivity against human diseases such as cancer, bacterial and fungal infections [49]. This suggests that molecule **1.** could be the source of the activity observed. Molecule **2.** was potentially identified as a quinazolinone and literature search led to the discovery that this compound had been isolated from a *actinomycete*, and testes active against monkey kidney fibroblast [50]. This suggests that both molecule **1.** and **2.** could be the source of the bioactivity observed.

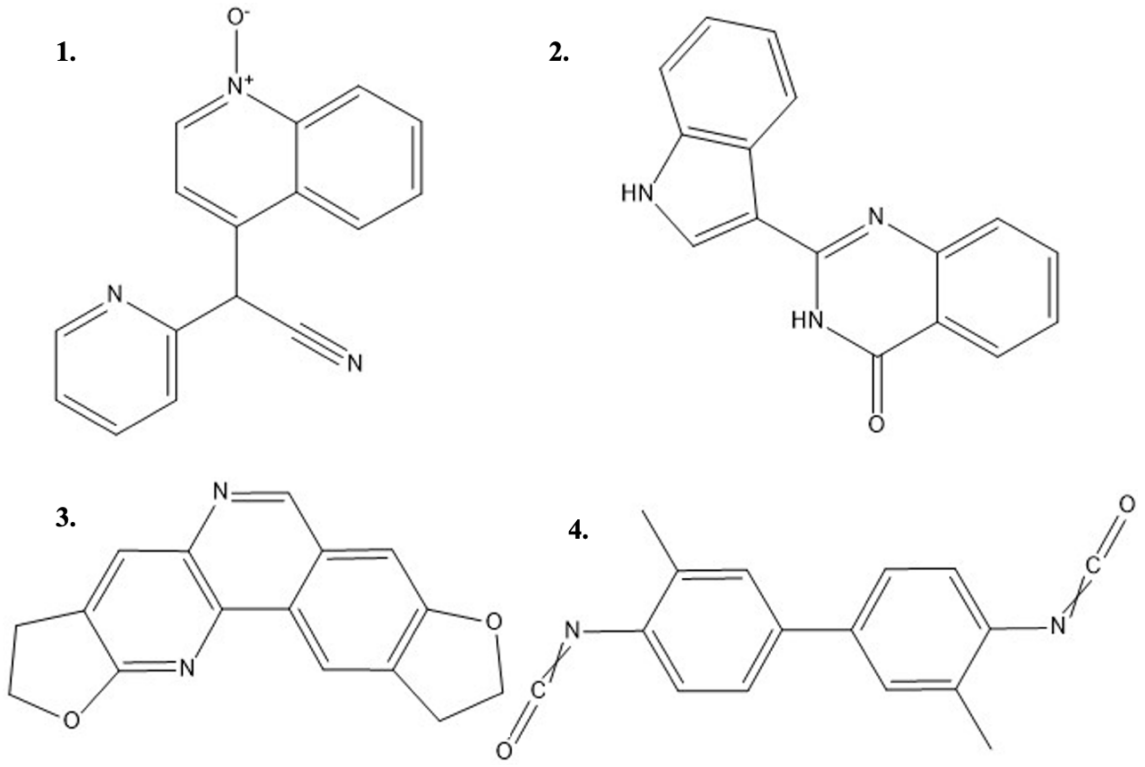

*Figure 5.4: The chemical structure of **1.** 4-(Cyano-2-pyridylmethyl)quinoline 1-oxide (ChemSpider ID: 537556), **2.** 2-(1H-Indol-3-yl)-4(3H)-quinazolinone (ChemSpider ID: 15979111), **3.** 2,3,9,10-Tetrahydro[1]benzofuro[6,5-h]furo[2,3-b][1,5]naphthyridine (ChemSpider ID: 540495) and **4.** Bitolylene diisocyanate (ChemSpider ID: 6805).*

There were several suggested structures for signal 265,09 m/z, and the two selected for further investigation was molecule **3.** and **4.** (figure 5.4). The naphthyridine core in molecule **3.** has been shown to exhibit medically relevant bioactivity against cancer, microorganisms etc. [51], and could be the cause of the bioactivity observed in the screenings conducted for fraction X0870A-05. Molecule **4.** was identified as Bitolylene diisocyanate, and there was not found any connected literature for the structure.

5.3.2 Fraction X0871A-05 grown in ArtG

Fraction X0871A-05 was a product of the strain T011 cultivated in the modified ArtM growth media, ArtG. The three potential bioactive molecules found for this fraction were assigned to the two signals 277,04 m/z and 301,20 m/z in negative ionization mode (ESI^-) and are shown in figure 5.5.

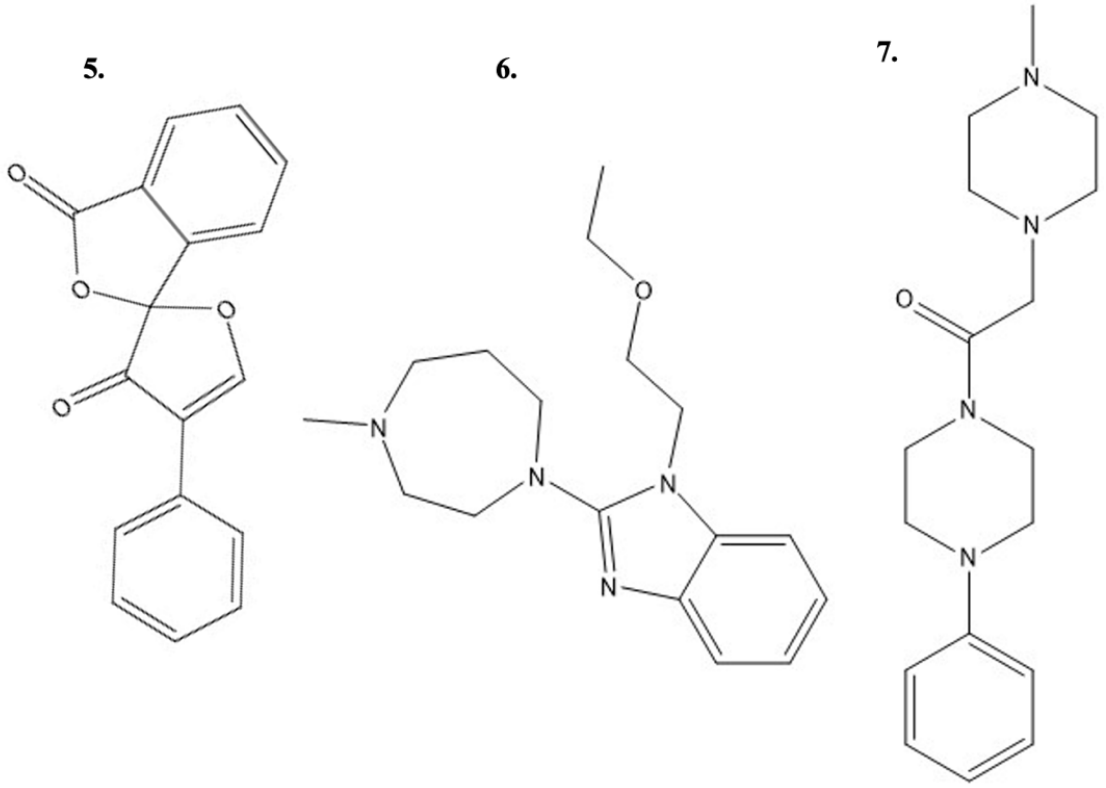

*Figure 5.5: The chemical structure of **5.** Fluorescamine (ChemSpider ID: 34768), **6.** Emedastine (ChemSpider ID: 3106) and **7.** 1-[(4-Methyl-1-piperazinyl)acetyl]-4-phenylpiperazine (ChemSpider ID: 499778).*

For the signal 277,04 m/z the molecule Fluorescamine (molecule **5.**) was identified. It is a molecule used in fluorescent screens by generating florescence when reacting with primary amines [52]. This indicate that activity is, with high probability, not caused by this molecule.

The signal 301,20 m/z was found in the chromatogram for both X0871A-05 and X0872A-05, and will only be discussed here. There were many suggested structures for this signal and the molecules **6.** and **7.** were selected for further investigation. Molecule **6.** was identified as Emedastine, a antihistamic agent that is an antagonist for a histamine receptor [53]. Medina *et al.* stated that histamine has an important role in the biological processes of malignant cells such as cell proliferation, apoptosis etc. [54], and this could indicate that the molecule contributed to the activity observed in the anticancer assay conducted for X0871A-05 and X0872A-05.

Molecule **7.** was identified as a phenylpiperazine, and there was not any accessible information found about that compound. This peak was observed at a higher amount in X0872A-05 compared to X0871A-05, which also could account for the higher activity observed in both antibacterial and anticancer assay (figure 4.4). This peak was also searched for in the unactive fraction X0865A-05, and not found, strengthening the assumption that this peak is the cause of the observed bioactivity.

5.3.3 Fraction X0872A-05 grown in ArtM at room temperature

Fraction X0872A-05 was a product of the strain T040 cultivated in the ArtM at room temperature. There were discovered 6 potential bioactive molecules in this fraction. Two of these were also found in fraction X0871A-05 (molecule **6.** And **7.**) and are already discussed in section *5.3.2 "Fraction X0871A-05 grown in ArtG"*. The remaining signals were 285,20 m/z in positive ionization mode (ESI^+) and 285,20 m/z in negative ionization mode (ESI^-), shown in figure 5.6.

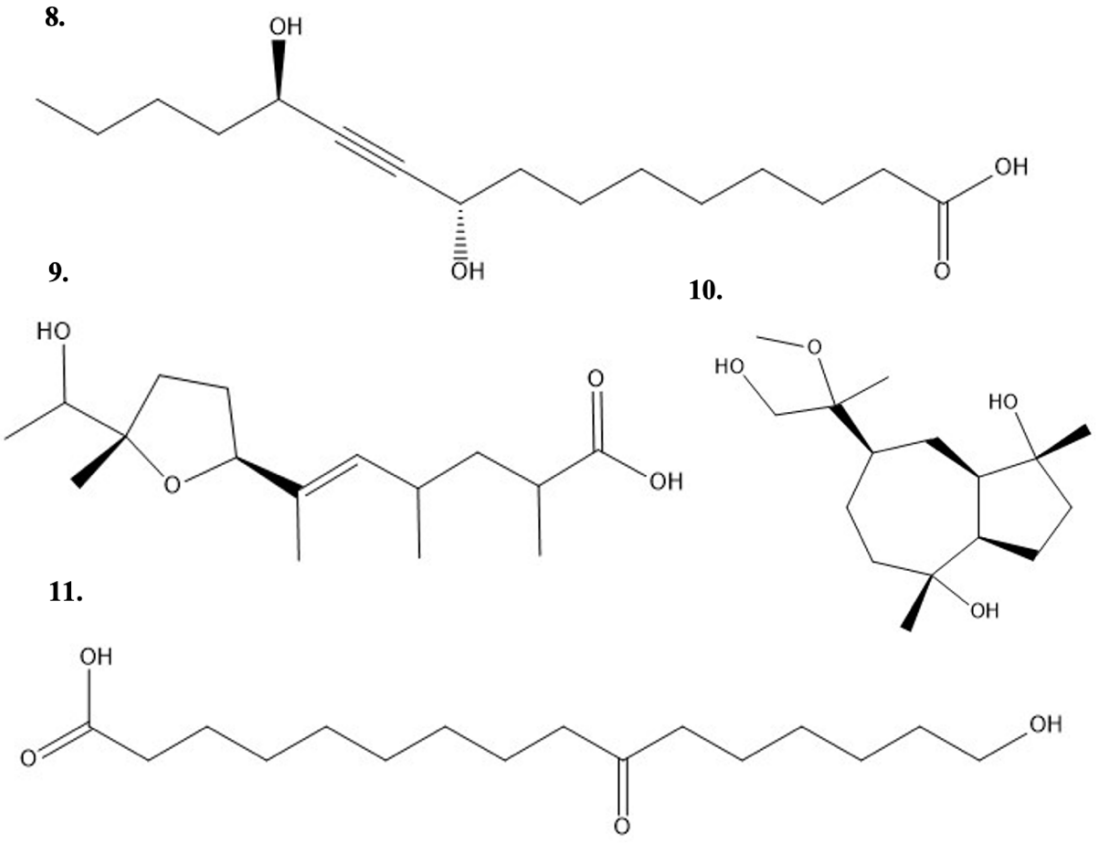

*Figure 5.6: The chemical structure of **8.** (9S,12R)-9,12-Dihydroxy-10-hexadecynoic acid (ChemSpider ID: 78440967), **9.** (5E)-6-[(2S,5S)-5-(1-Hydroxyethyl)-5-methyltetrahydro-2-furanyl]-2,4-dimethyl-5-heptenoic acid (ChemSpider ID: 8788439), **10.** (1R,3aR,4R,7R,8aR)-7-[(2R)-1-Hydroxy-2-methoxy-2-propanyl]-1,4-dimethyldecahydro-1,4-azulenediol (ChemSpider ID: 78441131) and **11.** 16-Hydroxy-10-oxohexadecanoic acid (ChemSpider ID: 30777548).*

Molecule **8.** and **9.** were assigned the signal 285,20 m/z in ESI^+ (figure 5.5). Molecule **8.** was identified as an acetylenic acid, and marine organisms (microbes, algae and sponges) have been known to produce these types of compounds with biologic activities, such as antibacterial and anticancer [55]. Molecule **9.** was identified as an asperic acid previously isolated for a marine fungi, but with no recorded bioactivity [56].

For signal 285,20 m/z in ESI^- the molecules **10.** and **11.** were identified (figure 5.5). The sesquiterpene (molecules **10.**) has been previously isolated by Huang *et al.* from a fungi, and exhibit antifungal activity [57]. Molecule **11.** was identified as an fatty acid with a role as a plant metabolite [58], and if the signal 285,20 m/z is a result of this compound, it is with high probability a media component.

6 Conclusion

The seven isolates were successfully cultivated with the ArtM growth media at 10°C, and also for the selected isolates grown under different cultivation conditions, DVR2, ArtG and ArtM at room temperature, utilizing the OSMAC approach. The cell pellet mass and extraction yields indicated that the different growth media (ArtG and DVR2) were preferable to ArtM, and more suitable for the cultivation of the selected *Arthrobacter* sp.. Higher temperature under cultivation was shown to have a negative effect on the biomass and metabolite production yield, indicating that the isolates were adapted to colder growth temperatures. It was shown that the different cultivation conditions and growth media influenced the metabolites produced by the different isolates, and the application of the OSMAC approach was deemed successful in this project. The different extraction methods utilized, cell pellet and resin extraction, both gave approximately the same metabolite yield, but resin extraction was concluded to be the preferred method, due to it being easier to perform and giving the same results.

The 48 fractionated resin extracts and eight unfractionated pellet extracts yielded 26 hits deemed active or questionably active in the primary screenings of anticancer, antibacterial and biofilm formation inhibiting activity. Several fractions resulted in activity against the human melanoma cell-line (A2058) and against the bacteria *S. agalactiae*, and were included in the secondary screening. For the cell-line MRC5 one fraction was deemed questionably active and, for the four remaining test-bacteria in the MIC assay there was no activity observed, thus there was not conducted a secondary screening for these. For the biofilm formation inhibition activity assay there were some that showed activity, but were due to time limitation and prioritization not included in the secondary screening. The three fractions X0870A-05, X0871A-05 and X0872A-05, originating from the strains T009, T011 and T040 respectively, exhibited activity at the lowest concentrations in the secondary screening for both assays. These were selected as the most promising fractions and included in dereplication.

The dereplication showed a high quantity of media and modified media components in the fractions originating the cultures cultivated in ArtM or modified ArtM (ArtG). The identification of flavonoids, isoflavonoids and phytosterols, as well as many lipids, suggested that some of the bioactivity of these fractions could be due to these compounds. This assumption was strengthened by the activity shown for the ArtM in a MIC assay. ArtM and media containing soybean meal are thus not recommended as cultivation media in bioassay-guided identification.

The dereplication of the pellet and resin extract did result in some peaks with corresponding m/z as discovered for arthrobactilins by Ohtsuka *et al.*, but were found to be caused by modified media components. Thus, it was concluded that the selected *Arthrobacter* sp., with high probability, did not produce the antibiotic arthrobactilins.

The molecules deemed promising after the exclusion of media components resulted in 11 potentially interesting molecules, identified by elemental composition and common fragments. In fraction X0870A-05 the molecules **1.**, **2.**, **3.** and **4.** were pointed out, and for fraction X0871A-05 the molecules **5.**, **6.** and **7.** were selected as potentially bioactive and new molecules. The molecules **6.-11.** were pointed out for the fraction X0872A-05. These resulted in several suggested molecules with identified bioactivity, and some originated from marine organisms. These were selected as the most promising candidates for further purification and isolation.

6.1 Future perspectives

Only the resin extracts were fractionated due to time constraints, but to compare the efficiency of capturing secreted metabolites for the two extraction methods, the extracts from both methods should be fractionated and tested for bioactivity in the same assays. The fractions that showed activity and not included in the dereplication could also contain molecules with potential bioactivity and should in future work be dereplicated.

Future work based on the results achieved in this thesis could be to divide the fractions containing the potential bioactive molecules so that these molecules are separated from one another. These should then be tested for antibacterial and anticancer activity again, to exclude the ones not containing the active compound or compounds, before further purification and isolation. The isolated compounds should then be screened for bioactivity again, to determine if it is active and its potency.

The OSMAC approach led to the bioactive fractions with the most promising candidate compounds, even using only three additional conditions with three of the five strains of *Arthrobacter*. This shows that there is a potential to further explore the other strains for their metabolite production using various growth conditions. Future work could therefore include additional growth conditions and additional bacterial strains. The results from this thesis give a valuable starting point for further research on cultivation of Arctic marine *Arthrobacter* and *Actinobacteri*a in general, with the purpose of producing bioactive secondary metabolites.

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