The Norwegian College of Fishery Science, Faculty of Biosciences, Fisheries and economics, UiT – The Arctic University of Norway

Exploring the Antibacterial and Anticancer potential of Five Marine Fungi

With the use of OSMAC-approach

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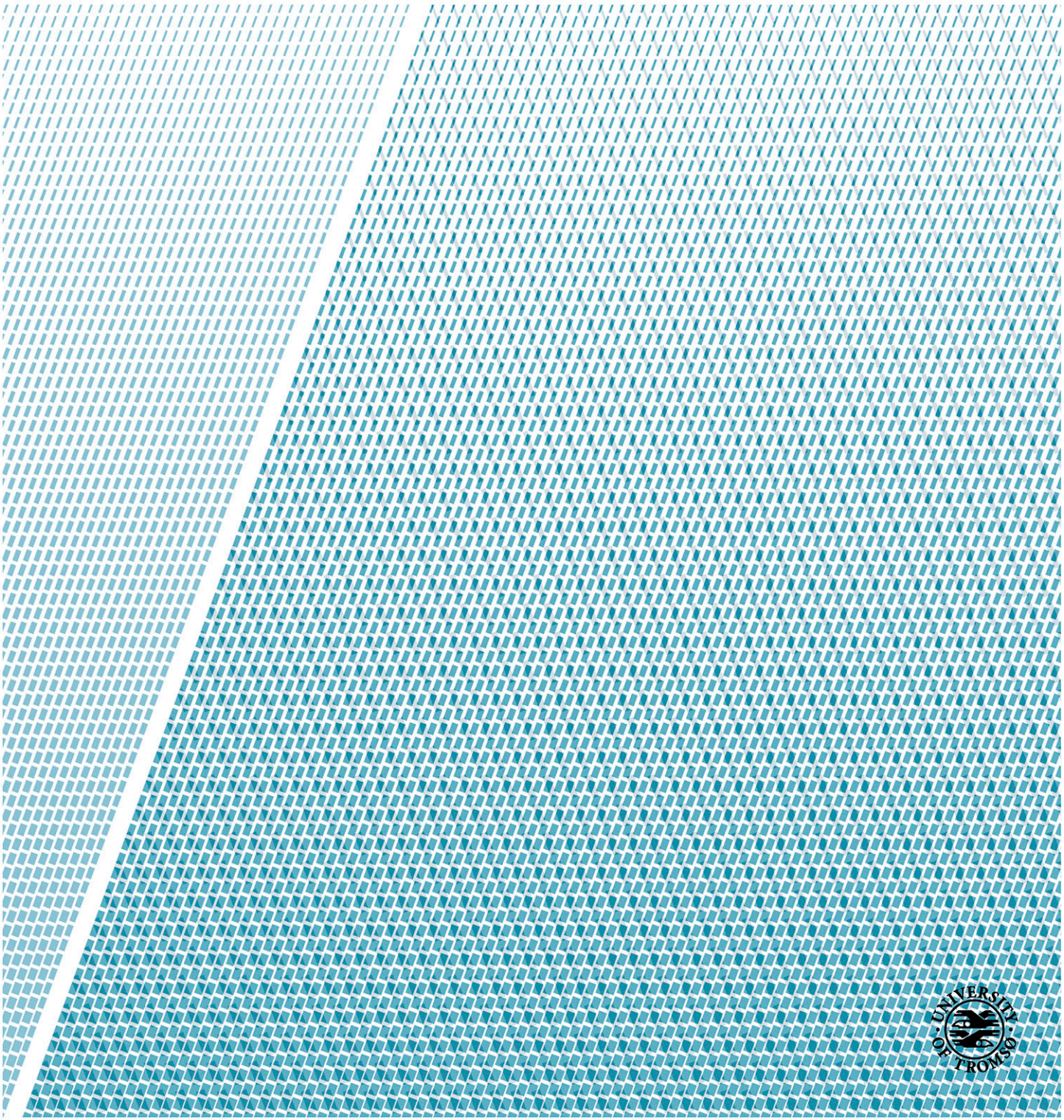
Hanne Bragmo

Master thesis in Marine Biotechnology (60 STP) May 2017



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Tromsø, May 2017
Hanne Bragmo

Abstract

The marine environment is an untapped source for biodiversity and has a great potential to provide the drugs of the future. Antibiotic resistance is an increasing threat worldwide and the need for discovering new antibacterial compounds is urgent. Marine microorganisms produce a wide range of bioactive compounds, and marine fungi have only been exploited to a small extent. This creates a great potential for finding novel antibacterial compounds in marine fungi.

In this study, the antibacterial and anticancer potential for five marine fungi *Acremonium* sp. TS7**,** *Typhula* sp**.,** *Amylocarpus encephaloides, Pseudogymnoascus* sp. TS12 and *Digitatispora marina* have been investigated for antibacterial and anticancer activity. The One-strain-many-compounds (OSMAC)-approach was to try to induce the production of secondary metabolites by applying stress to the marine fungi. These five marine fungi were fermented on four different media and at two different temperatures. Half of the fermentations were co-cultivated with the marine bacteria *Leeuwenhoekiella* sp. The active fractions were dereplicated with UPLC-QToF-MS. The antibacterial compounds were identified as rhamnolipids and were found in all active samples. Rhamnolipids were also identified in the bacteria controls with *Leeuwenhoekiella* sp., suggesting that *Leeuwenhoekiella* sp. is the producer of rhamnolipids. The bioactivity effects of our OSMAC-approach were not as expected, this is probably due to that the culture conditions selected for this study did not trigger the production of secondary metabolites. The full bioactivity potential for *Acremonium* sp. TS7**,** *Typhula* sp**.,** *Amylocarpus encephaloides, Pseudogymnoascus* sp. TS12 and *Digitatispora marina* has not been fully investigated in this study, but should be further explored.

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Abbreviations

ASCMA	Artificial Seawater Corn Meal Agar
0.2 ASMEA	0.2 Artificial Seawater Malt Extract Agar
BHI	Brain Heart Infusion broth
CFM	Corn Flour Medium
DCM	Dichloromethane
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacetic Acid
EtAOc	Ethyl acetate
HPLC	High Pressure Liquid Chromatography
LC	Liquid Chromatography
LPLC	Low Pressure Liquid Chromatography
MEM	Malt Extract Medium
MeOH	Methanol
MH	Mueller Hinton broth
MS	Mass Spectrometer
MQ-H_2O	Milli-Q water
NP	Natural Products
OD	Optical Density
PCR	Polymerase Chain Reaction
SM	Seaweed Medium
ToF	Time-of-flight
UPLC	Ultra Pressure Liquid Chromatography
WM	Wood Medium

1. Introduction

1.1 Marine bioprospecting

Marine bioprospecting is the process of discovery and commercialization of new products based on marine organisms. The most explored resources in the marine environment include seafood, fish oil and food additives. There is a huge potential to increase the discovery of new high-end products, such as pharmaceuticals. The oceans represent a highly competitive environment with a longer evolutionary history and greater less-exploited biodiversity, compared to the terrestrial habitat (Bolhuis & Cretoiu, 2016). It can be assumed that the potential for discovery of new bioactive molecules in marine fungi could exceed that of fungi from other ecosystems (Silber, Kramer, Labes, & Tasdemir, 2016).

Compounds originating from living organisms can be called natural products. What these natural products are differs greatly and includes entire organisms (plant, animal or a microorganism), parts of an organism (e.g. leafs or flowers), an extract of an organism, or pure compounds of microorganisms (Sarker & Nahar, 2012b). In most cases the term "natural products" refers to secondary metabolites produced by a living organism. Secondary metabolites molecules that are not strictly necessary for survival, growth, development or reproduction for the organism that produces them (Martins, Vieira, Gaspar, & Santos, 2014). In the field of marine bioprospecting, there have over the years been developed strategies in order to isolate bioactive compounds as efficiently as possible. Initial approaches were primarily focused on the characterization of chemical compounds in natural products, before collecting bioactivity data. This strategy is labor-intensive, and may result in finding new chemistry, but with no guarantee for bioactive molecules. Today it is more common to use bioassay-guided isolation strategy and to only isolate bioactive compounds.

1.2 Marine fungi

Marine fungus is a group of eukaryotic organisms in the kingdom Fungi. They can be divided in two major groups: unicellular (yeasts) and filamentous (molds) fungi. Filamentous fungi grow as tread-like structures called hyphae. Hyphae can be from 2-10 μm in diameter to several centimeters and the structures typically grow in a network

called mycelium (Madigan, Martinko, Stahl, & Clark, 2012a). Marine fungus is an ecological group of organisms estimated to consist of 1500 species, excluding those that form lichens (Hyde et al., 1998). They inhabit most of the marine habitats and are distributed in tropical, temperate and Arctic waters (Redou et al., 2016). Marine fungi can grow on a variety of substrata, such as wood, algae, sediments, corals and decaying leafs of mangroves (Kohlmeyer & Kohlmeyer, 1979; Redou et al., 2016). The major role of marine fungi in their ecosystem is to function as decomposers, the same role terrestrial fungi have in the terrestrial environment, but parasitic, pathogenic and symbiotic fungi occur also commonly. Marine fungi are one of the main decomposers of wood and marine plants in the marine environment (Hyde et al., 1998). Many marine fungi appear to be able to tolerate low oxygen tension and are found to be the dominant decomposer of wood in marine ecosystems with low oxygen tension (Hyde et al., 1998).

Several scientists have tried to define a marine fungus. Jones and Jennings (1964) determined that a marine fungus is a fungus with physiological requirements for sodium chloride to sustain growth. Kohlmeyer and Kohlmeyer (1979) had the most supported definition as of late:

> *Obligate marine fungi are those that grow and sporulate exclusively in a marine or estuarine habitat; facultative marine fungi are those from freshwater and terrestrial milieus able to grow and possibly also sporulate in the marine environment* (Kohlmeyer & Kohlmeyer, 1979, p. 3).

There are, from the definition from Kohlmeyer and Kohlmeyer (1979), two major groups of marine fungi: obligate and facultative. Facultative marine fungi are the most studied group (Ebel, 2012). The obligate marine fungi have been less studied (Sithranga Boopathy & Kathiresan, 2010) and have very few reported secondary metabolites (Ebel, 2012). According to Jones et al. (2009), classification of marine fungi has become difficult and unclear, much due to the Kohlmeyers' definition. This definition, which defines what constituents a marine fungus has, has been suggested to be too strict by some researchers (E. Jones, Sakayaroj, Suetrong, Somrithipol, & Pang, 2009). The term "marine-derived fungi" has been used broadly to describe isolated fungi with unknown ecology from marine or marine-related habitats. (Pang et al., 2016).

1.3 Antibacterial resistance and discovery of antibacterial compounds

The discovery of the antibiotic compound penicillin by Alexander Fleming in 1928 marked a new era (Raper, Alexander, & Coghill, 1944). Since then, antibiotic agents have been incorrectly used for many years. Excessive use of antibiotic agents against human pathogens, as well as the use of antibiotic compounds in animal feed, has accelerated the development of antibiotic resistance (Bartlett, Gilbert, & Spellberg, 2013). This has inevitably contributed to the evolution of resistant bacterial strains against most of antibiotic drugs on the market (Davies & Davies, 2010). T carbapenem-resistant Enterobacteriaceae (CRE) *Klebsiella pneumonia* was discovered in a patient in 2016 and was resistant against all antibacterial drugs on the market (Chen, Todd, Kiehlbauch, Walters, & Kallen, 2017). With the rise of these multi-drug resistant pathogens, the number of effective antibiotics has dropped dramatically. Infectious diseases are an increasing public health threat and are regarded as one of the major challenges in this century (WHO, 2014).

Microbial sources have been the main contributor for antibacterial discoveries over the years. Most of these organisms come from the terrestrial environment, so these microorganisms represent only a small portion of the microbial diversity (Imhoff, 2016). The marine ecosystems are less explored, even though marine microorganisms have the potential to produce structurally unique bioactive compounds that cannot be found in the terrestrial ecosystems (Bolhuis & Cretoiu, 2016). The number of novel natural products isolated from marine-deprived fungi reached 1100 in 2010 (Rateb & Ebel, 2011). In 2002, only 272 novel natural products had been reported. It is evident that the focus on marine-derived fungi has increased (Ebel, 2012), however the majority of the sampled compounds are from fungal genera *Penicillium* and *Aspergillus* (Silber et al., 2016). Even though marine fungi are starting to get more attention, they are still underrepresented (Imhoff, 2016). The lack of research in the field of marine fungi creates opportunities to discover novel antibacterial and anticancer compounds that can be developed for future drug treatments.

1.4 Bioprospecting pipeline

A pipeline is commonly used to describe the process of marine bioprospecting. It includes the main steps of the workflow from sample collection or marine organisms to structure elucidation and bioactivity profiling of pure compounds. The pipeline is constructed following a bioassay-guided purification strategy and is designed to identify novel bioactive compounds. At Marbio, the major steps in the pipeline include sample collection, fermentation, extraction, prefractionation, bioassays, dereplication, compound purification, structure elucidation and bioactivity profiling. The pipeline steps from fermentation dereplication are included in this study (Fig. 1). Marbio applies High Throughput Screening (HTS), a process that analyzes many samples (crude extracts or fractions) in one set against a selected target (e.g. antibacterial and anticancer assays)(Carnero, 2006). In order to have a successful HTS, a platform with automated instruments and standardized assay protocols is implemented (Mishra, Ganju, Sairam, Banerjee, & Sawhney, 2008)

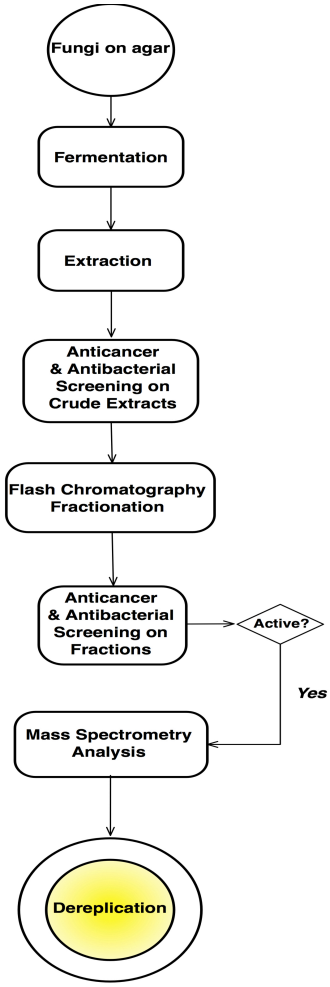

Figure 1: General workflow from raw biomaterial to dereplication of active compounds.

1.5 Cultivation strategies

The cultivation of microorganisms is important to be able to get enough biomass for every step in the bioprospecting pipeline. It is estimated that only 0.1-1% of marine microorganisms can be cultivated under laboratory conditions (Chai et al., 2012). Standard culturing methods are limited and fail to activate every metabolic pathway. Many biosynthetic gene clusters remain inactive or under-expressed in artificial laboratory culture conditions (Chiang, Lee, Sanchez, Keller, & Wang, 2009). Efforts to activate these silent gene clusters and trigger production of unknown compounds have been researched by adding external stimuli. Modifications of culture parameters, such as media, temperature and micronutrients, have proved to be very effective to activate

silent gene clusters that are often inactive in standard culture conditions (Bode, Bethe, Höfs, & Zeeck, 2002).

Addition of trace minerals and other micronutrients have shown to be effective for increased growth and sporulation (Vishniac & Santer, 1957). This approach, which uses modified traditional culture conditions, is commonly known as OSMAC (One Strain, Many Compounds) (Bode et al., 2002). The OSMAC-approach is not the only approach that differs from the traditional methods. Modifying simple parameters (e.g. temperature, aeration and salinity) can trigger production of several unknown secondary (Rateb & Ebel, 2011). Mimicking environmental conditions and co-cultivation with other microorganisms has become a common approach to activate secondary metabolite production in microorganisms (Schroeckh et al., 2009). One of the major challenges with co-cultivation with slow-growing marine fungi is that bacteria can take over the culture. Another problem is that the bacteria itself can get triggered and start producing bioactive secondary metabolites. A study found increased production of secondary metabolites by adding lipopolysaccharides (LPS) instead of co-cultivation (Khalil, Kalansuriya, & Capon, 2014). This could be alternative method for co-cultivation.

1.6 Extraction

Extraction is a necessary step in order to harvest the produced target compounds from the cultivated organism. The target compounds can be extracellular compounds, intracellular proteins or both. The choice of extraction method depends heavily on target compound and what the source material is (Sarker & Nahar, 2012b). To have a successful extraction, several parameters have to be considered. The first parameter is deciding what the target is. The target may be an unknown bioactive compound, a known compound, all secondary metabolites produced from same organism under different condition or all secondary metabolites present in an organism (Sarker & Nahar, 2012b).

Solvents are necessary in most extraction methods to release target compounds and separate them. The three major groups of solvents used in extractions are polar, medium polar and nonpolar solvents. Fermentation media and other liquid culture media contain mainly water, which is a polar solvent. Since it is vital to have a solvent

that is soluble with the liquid culture media, polar solvents are the preferred solvents for the extractions of the fungi fermentations (Houssen & Jaspars, 2012).

There are many extraction methods used for fungi (Houssen & Jaspars, 2012; Wiese, Ohlendorf, Blümel, Schmaljohann, & Imhoff, 2011). Two of the extraction methods are efficient, but have different focus points. Secondary metabolites are both found extracellular and intracellular. Different techniques can be applied depending on whether the focus is on extracellular secondary metabolites or both intra- and extracellular secondary metabolites. In the latter, extractions consists of three parts (Wu, Oesker, Wiese, Schmaljohann, & Imhoff, 2014). The mycelium is separated from the fermentation media and homogenized. Ethanol is then added and centrifuged, and the supernatant collected. The remaining aquatic phase and the fermentation media is extracted with EtAOc. The remaining residue from the aquatic phase and fermentation media is extracted with methanol (Wu et al., 2014). This method is more labor-intensive, but have a higher probability to uncover novel compounds. An alternative method extracts only extracellular secondary metabolites, using macroporous adsorptive resin (Houssen & Jaspars, 2012). This method was developed specifically to extract secondary metabolites produced by marine microorganisms in liquid cultures. The resin is added directly to the liquid medium absorbing the produced and excreted secondary metabolites. This method removes inorganic salts, which is abundant in marine liquid culture medias. The secondary metabolites are eluted from the resin with methanol, which is evaporated easily under vacuum. This method is the preferred at Marbio, and is used as extraction method in this study.

1.7 Prefractionation

Prefractionation of crude extracts reduces the complexity of crude extracts by separating the molecules after certain parameters. Prefractionation can increase the probability of finding activity (Pham, Toms, Camp, & Quinn, 2015). Chromatography is usually used for this purpose and exploits how a compound distributes between the mobile phase and the solid phase. Compounds in the mixture interact with the stationary phase based on charge, relative solubility or adsorption. The retention time is the time a compound uses to travel through the column in a chromatographic system. A wide range of chromatographic separation methods exists. When choosing a method,

the sample material that is to be fractionated and the desired outcome of the fractionation is taken into consideration. Chromatographic methods like liquid chromatography (LC) and low-pressure liquid chromatography (LPLC) are more commonly used to purify larger samples (1-10 grams) for further analysis.

Flash chromatography is a LPLC method, which is a preparative method that separates compounds. Depending on column material and solvents, it can be used to separate compounds by size, polarity, charge or affinity. It is a an efficient method for separating complex mixture of compounds. The principle of flash chromatography is the same as in all liquid chromatography methods. There are two parts: a solid phase and a mobile phase. The different phases are chosen to best separate the components in the sample. Choosing a solvent system can be challenging, especially when the goal compounds are unknown. Flash chromatography is usually carried out with a mixture of two solvents as the mobile phase, where one solvent is polar and the other is non-polar. In some cases one solvent can be enough or a mixture of three solvents can be applied. The application of the solvent mixture can be either of the same concentration throughout the run or as a gradient were the concentration of the different solvents are changed during the run. A column material, solvent system and applications has to work for most compounds, making it possible to prefractionate as many compounds as possible at a low cost and over a short time in a HTS platform.

1.8 Bioassays

A bioassay is a standardized experiment that, in either an in vivo or in vitro system, determines the biological activity of a compound in a sample. There are two groups of bioassays: target-based and phenotypic assays. A target-based assay measures the compound's effect on a specific target that usually is a protein with a key role (Vasaikar, Bhatia, Bhatia, & Chu Yaiw, 2016). The phenotypic assay is used to detect an activity using of cells, tissue or whole organisms in a sample. Knowledge of potential targets is not needed (Swinney, 2013). The main purpose with phenotypic assay is to discover a desired effect on the test subject, e.g. the bacteriosidal or bacteriostatic effect on test bacteria.

1.8.1 Antibacterial screening

A Minimal Inhibitory Concentration (MIC) assay is a commonly used method to screen for antibacterial activity. MIC is the lowest concentration of an antibacterial compound that can inhibit visible growth of a microorganism after overnight incubation (or 20-24 hours) (Madigan, Martinko, Stahl, & Clark, 2012b). In addition, the MIC assay can be used as a diagnostic tool as well as evaluating the efficacy of an antimicrobial agent. The MIC can be used in discovering antibacterial secondary metabolites in crude extracts or fractions (Valgas, Souza, Smânia, & Smânia Jr, 2007). At Marbio the following common human pathogenic bacteria are used: *Escherichia coli* (Gram neg.), *Staphylocuccus aureus* (Gram pos.), *Streptococcus agalactiae* (Gram pos.), *Pseudomonas aeruginosa* (Gram neg.) and *Enterococcus faecalis* (Gram pos.).

MIC is used to determine the lowest concentration of different antibacterial agents exerting bacteriosidal or bacteriostatic effect against the exposed bacteria. With regards to antibacterial drug discovery, these agents can be crude extracts, fractions, purified natural products or synthetically produced compounds. If an antibacterial agent does not inhibit or halt bacterial growth, the bacteria will grow and cloud the growth media. Inhibition of bacterial growth by the tested agents will on the other hand result in a clear assay well. MIC is a visible assay, were growth and clouding of the growth media differs from wells without growth. In addition to visual well inspection to detect activity, a supplementary optical density measurement is performed. This measurement is usually conducted by measuring the optical density or absorbance at 600 nm (OD_{600}). This method is common to use to indicate the concentration of bacteria cells in a liquid. There are several parameters that can affect the growth of the bacteria and some media components in crude extracts or fractions can affect the growth in some degree. Gentamycin control is used to evaluate the assay in general.

1.8.2 Anticancer screening

Anticancer screening can be accomplished in several ways, but a common assay is cell proliferation evaluation using various cancer cell lines. A cell proliferation assay can be applied to find anticancer activity in crude extracts and fractionated samples. The Promega CellTiter 96® Aqueous One solution Cell Proliferation Assay is a colorimetric method to determine the number of living cells in proliferation, cytotoxicity or

chemosensitivity assays (Promega, 2012). In this assay, a yellow tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; short MTS] is added at the end of the incubation time to each well. The tetrazolium salt enters the cytocol and is reduced to a purple end product by the mitochondrion in metabolically active cells. The formazan product is soluble over the cell membrane. The reduction of MTS to formazan will only take place in living cells, whereas dead cells will not reduce MTS. The number of living cells is directly proportional with the level of formazan product produced. The color concentration is measured spectrophotometric at 490 nm (Promega, 2012). Negative and positive controls are used to calculate percent survival.

1.9 Dereplication

Dereplication is a process used in screening for natural products with bioactivity screening, mass spectrometric techniques and mass spectrometric databases (Gaudencio & Pereira, 2015). It is a rapid process that is a key element in the discovery of novel natural products (Nielsen, Månsson, Rank, Frisvad, & Larsen, 2011). Compounds in active crude extracts or fractions are identified with molecular mass and calculated molecular formula with mass spectrometry. The analyses distinguish previously reported bioactive secondary compounds, from those that contain secondary metabolites with novel chemistry and/or novel bioactivity. Isolation of compounds is a labor-intensive task, thus dereplication is done to eliminate known compounds before an isolation process is initiated (Sarker & Nahar, 2012a).

1.9.1 UPLC-QToF-MS

The bioactive samples are analyzed in this study for with UPLC-QToF-MS. However, a wide range of options exist when it comes to inlet systems, isolation techniques and mass filters (Lampman, Pavia, Kriz, & Vyvyan, 2010; Sarker & Nahar, 2012a). The components in Marbio's mass spectrometry system include Ultra Pressure Liquid Chromatography (UPLC), electrospray ionization (ESI), Quadrupole and Time-of-Flight (ToF .

The sample is injected to a column in a UPLC system, which separates the compounds in the sample. The eluted compounds are then pumped through a thin capillary tube and sprayed out in a fine aerosol with the help of nitrogen gas. Exposure to a very high

voltage electrically charges the droplets that rapidly shrink in size as mobile phase molecules evaporate from their surface, which ionizes the compounds. This method is commonly known as electrospray ionization (ESI) and can be performed in positive (ESI +) or negative (ESI-) mode (Lampman et al., 2010). The Quadople and Time-of-Flight (ToF) are both mass analyzers. They work together to separate the ionized compounds according to mass-to-charge (*m/z*) ratios (Chernushevich, Loboda, & Thomson, 2001). When the ions have been separated according to *m/z*, a detector detects them at the end of the mass analyzer. The detector consists of a counter that produces a current that is proportional to the number of ions that strike it. The signal created is fed to a recorder, which produces the chromatogram. The output of the detector is fed through an interface to a computer. The computer stores the data; provides output in graphic and tabular form, and compares data libraries (Lampman et al., 2010).

Aim of the study

The aims of this study are:

1) To study the bioactivity potential of the selected marine fungi using different fermentation conditions (OSMAC-approach)
2) To identify extracts, fractions or potential compounds with anticancer or antibacterial activity
3) To study whether co-cultivation increases production of secondary metabolites

In order to reach the set of goals the following steps in Marbio's biodiscovery pipeline (Fig.1) Needs to be completed: fermentation extraction and pre-fractionation, bioassays and MS analysis for metabolite profiling (dereplication in Fig. 1)

2. Materials and methods

2.1 Biological material

Five marine fungi isolates were selected for cultivation (Table 1-2). These five marine fungi were fermented based on the OSMAC-approach on four different media and at two different temperatures. Half of the fermentations were co-cultivated with the marine bacteria *Leeuwenhoekiella* sp. (strain M09W024). This resulted in 120 fermentations with three different parameters. These 120 fermentations were extracted and tested in a bioactivity screening (Fig. 2).

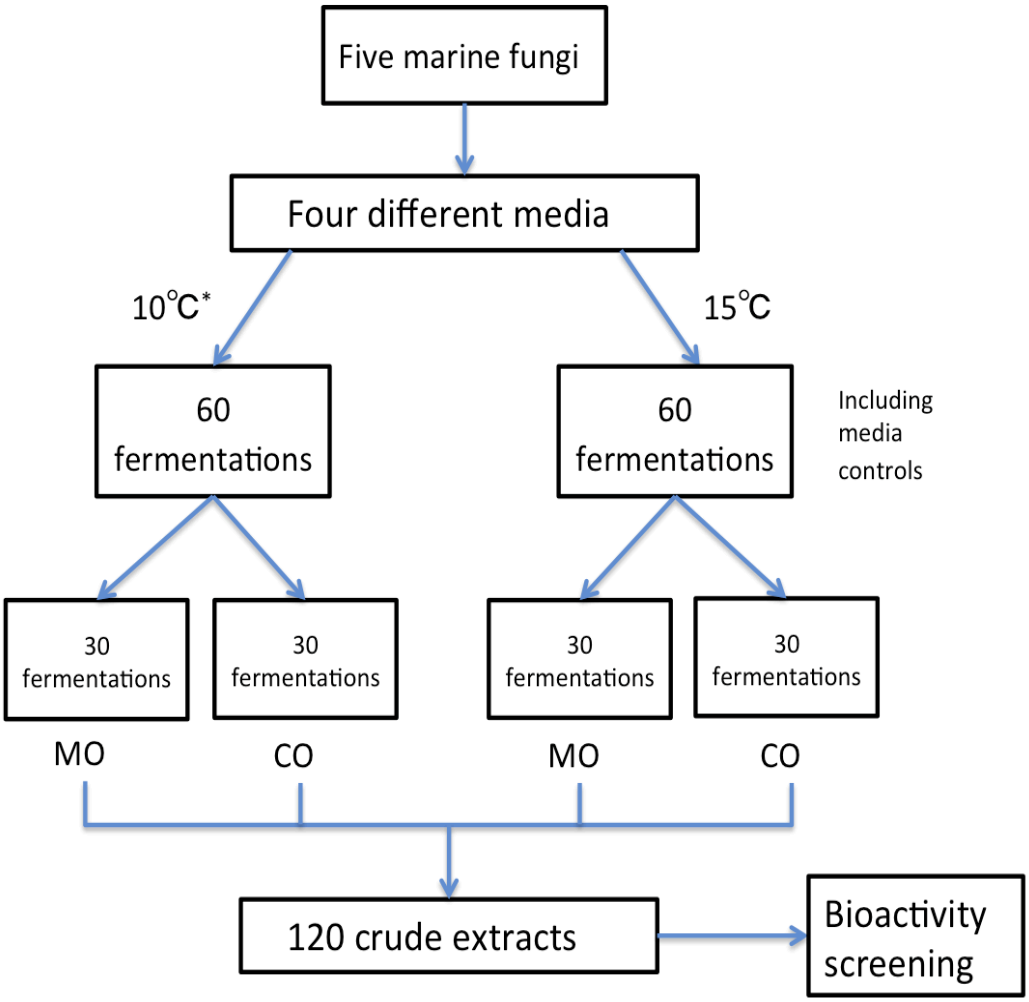

Figure 2: Five marine fungi were fermented on four different media. 60 fermentations were incubated at 10℃ and 100 rpm shaking, while 60 other fermentations were incubated at 15℃. The 10℃ and 15℃ fermentations were split in half, were 30 fermentations were co-cultivated (CO) and 30 fermentations were monocultivated (MO). This resulted in 120 crude extracts that were tested for antibacterial and anticancer activity.

The five marine fungi fermented were assigned fermentation number. These numbers are used throughout the study (Table 1).

Table 1: Notation system with fermentation number for each species

Species	Isolate Number	Fermentation Number
Acremonium sp. TS7	1	701
Typhula sp.	2	702
Amylocarpus encephaloides	3	703
Pseudogymnoascus sp. TS12	4	704
Digitatispora marina	5	705

Three of the species belonged to the fungal phylum Ascomycota (*Acremonium* sp. TS7, *Amylocarpus encephaloides* and *Pseudogymnoascus* sp. TS12). The last two belonged to the Basidiomycota phylum (*Typhula* sp. and *Digitatispora marina*). These strains were sampled from different parts of the Atlantic Ocean from deep-sea sponges, wood and algae (Table 2). The wood and seaweed-associated fungal species are obligate marine fungi, whereas the ecology of the sponge-associated strains remains unsolved. These five marine fungi have not been investigated for bioactive secondary metabolites. The human pathogenic bacteria used in antibacterial assay are *S. aureus* (ATCC 25923), *E. coli* (ATCC 25922), *E. faecalis* (ATCC 29212), *P. aeruginosa* (ATCC 27853) and *S. agalactiae* (ATCC 12386). The human pathogenic bacteria were bought at The University Hospital in North Norway (UNN). The adherent A2058 melanoma cancer cell line (ATCC CRL-11147, LGC Standards, UK) was used in anticancer screening.

Table 2: Marine fungi strains used

Species	Strain	Substrate/host	Habitat	Location
Acremonium sp. TS7 (Hypocreales incertae sedis)	TS7	The marine sponge *Stelletta normani*	Seafloor 1.350 m depth.	Atlantic Ocean, west coast of Ireland.
Typhula sp.	TRa3160C	Brown seaweeds	Dead, decaying algae in slash zone onshore.	Tromsø, Håkøya (Station M14TMU0001)
Amylocarpus	TRa018bII	Driftwood	Submerged and	Tromsø,

encephaloides			intertidal wood in temperate and arctic waters.	Ringvassøya, Skarsfjord (Station M10TMU0008)
Pseudogymnoascus sp. TS12 (Leotiomycetes incertae sedis)	TS12	The marine sponge *Stelletta normani*	Seafloor 1.350 m depth.	Atlantic Ocean, west coast of Ireland.
Digitatispora marina	008cD1.1	Driftwood	Submerged and intertidal wood in temperate and arctic waters.	Karlsøy, Vannøya. Sandefjorden (Station M10TMU0003)

2.2 Media

All media used, both liquid and solid, are listed in table 3. The list includes agar plate media, fermentation media, media used in bioassays and solutions. Details of the media and solution constituents are given in table 3.

Table 3: List of media and solutions used in this thesis

Media/solution	Content	Purpose
0.2 Artificial Sea Water Malt Extract Agar (0.2 ASMEA)	4 g/L Malt Extract, 15 g/L Agar, 40 g/L Sea Salts	Precultivation of marine fungi
Artificial Sea Water Corn Meal Agar (ASCMA)	17 g/L Corn Meal Agar (2 g/L Corn Meal, 15 g/L Agar), 40 g/L Sea salts	Precultivation of marine fungi
Vishniac Spore Solution (VSS)	10 g/L EDTA, 4.4 g/L $ZnSO_4$* $7H_2O$, 1.01 g/L $MnCl_2$*$4H_2O$, 0.32 g/L $CoCl_2$*$6H_2O$, 0.315 g/L $CuSO_4$* $5H_2O$, 22 g/L $(NH_4)_6Mo_7O_{24}$*$4H_2O$, 1.47 g/L $CaCl_2$*$2H_2O$, 1.0 g/L $FeSO_4$* $7H_2O$ and MQ- H_2O.	Fermentation, trace metal solution

Two times Minimal Medium (2xMM)	12 g/L $NaNO_3$, 3 g/L KH_2PO_4, 1 g/L KCl, 1 g/L $MgSO_4*7H_2O$, MQ- H_2O and 400 µL Vishniac Spore Solution. pH= 6.00	Fermentation, mineral solution
Seaweed Medium (SM)	10 g/L *Ascophyllum nodosum*, 40 g/L Sea Salts (Sigma Aldrich)	Fermentation
Wood Medium (WM)	10 g/L grinded wood chips, 40 g/L Sea Salts (Sigma Aldrich)	Fermentation
Corn Flour Medium (CFM)	2 g/L Corn Flour (Risenta®), 40 g/L Sea Salts (Sigma Aldrich)	Fermentation
Malt Extract Medium (MEM)	4 g/L Malt Extract (MOSS), 40 g/L Sea Salts (Sigma Aldrich)	Fermentation
M19 medium	20 g/L peptone, 20 g/L D-mannitol	Inoculation
Mueller Hinton Broth	0.2 % Beef Extract Powder, 1.75 % Acid Digest of Casein, 0.15 % Soluble Starch	MIC assay
Brain Hearth Infusion	1.25% Calf brains, 0.5% Beef Heart, 1.0% Peptone, 0.5% Sodium Chloride, 0.2% D(+)-Glucose, 0.25% Disodium hydrogen phosphate	MIC assay
RPMI-1640	10% FBS, 1% L-Alanyl-L-Glutamine, 0.1% Gentamycin	Cancer assay
D-MEM	10% FBS, 1% L-Alanyl-L-Glutamine, 0.1% Gentamycin	Cancer assay
Phosphate Buffer Saline (PBS)	0.80 g/L NaCl, 2.16 g/L $Na_2HPO_4*2H_2O$, 0.2 g/L KH_2PO_4, 0.2 g/L KCl	Cancer assay
Trypsin solution	25 g/L Trypsin, 5 g/L EDTA	Cancer assay

2.3 Chemicals and reagents

The chemicals, reagents and other products used in this study are found in table 4.

Table 4: Chemicals, reagents and products

Chemical	Supplier	Distributor	Product number
Acetone	Sigma Aldrich	MO, USA	
DMSO	Sigma Aldrich	MO, USA	W387520
EDTA	Sigma Aldrich	MO, USA	E6758-100G
$ZnSO_4*7H_2O$	Sigma Aldrich	MO, USA	24750-100G
$MnCl_2*4H_2O$	Sigma Aldrich	MO, USA	221279-100G
$CoCl_2*6H_2O$	Sigma Aldrich	MO, USA	C8661-25G
$CuSO_4*5H_2O$	Sigma Aldrich	MO, USA	209198-100G
$(NH_4)_6Mo_7O_{24}*4H_2O$	Sigma Aldrich	MO, USA	09878
$CaCl_2*2H_2O$	Sigma Aldrich	MO, USA	C3881
$FeSO_4*7H_2O$	Sigma Aldrich	MO, USA	215422
$NaNO_3$	Sigma Aldrich	MO, USA	S5506
KH_2PO_4	Merck KGaA	Germany	1.04871.
KCl	Merck KGaA	Germany	1.04935.5000
$MgSO_4*7H_2O$	Sigma Aldrich	MO, USA	63138-250G
NaCl	Sigma Aldrich	MO, USA	S5886
$Na_2HPO_4*2H_2O$	Sigma Aldrich	MO, USA	30412
Diaion® HP-20 (Supelco)	Sigma Aldrich	MO, USA	13607
Diaion® HP-20SS	Sigma Aldrich	MO, USA	13613-U
Sea Salts	Sigma Aldrich	MO, USA	S9883-1KG
UtraPure™ Low Melting Point Agarose	Invitrogen	CA, USA	15510-027
Gel red (10.000x)			41003
UtraPure™ TBE Buffer 10X	Thermo Fisher Scientific	MA, USA	15581-044
DreamTaq Green PCR Master Mix (2X)	Thermo Fisher Scientific	MA, USA	K1081
1 kb Plus DNA Ladder	Thermo Fisher Scientific	MA, USA	10787-018
Agarose gel loading dye (6x)	Amresco®	OH, USA	E190-5ml
BigDye® Terminator v1.1 & v.3.1 5X Sequence buffer	Thermo Fisher Scientific	MA, USA	4226697
Brain Hearth Infusion Broth	Sigma Aldrich	MO, USA	53286-500G
Mueller Hinton Broth	Becton, Dickinson and Company	NJ, USA	275730

Gentamycin (10 mM)	Merck KGaA	Germany	A2712
DMEM, high glucose, GlutaMAX™ Supplement, HEPES	Thermo Fisher Scientific	MA, USA	32430-027
RPMI-1640	Merck KGaA	Germany	FG 1383
Fetal Bovine Serum (FBS)	Merck KGaA	Germany	S0115
Trypsin (1:250)	Thermo Fisher Scientific	MA, USA	27250018
L-Alanyl-L-glutamine (200 mM)	Merck KGaA	Germany	K0302
Triton x100	Sigma Aldrich	MO, USA	T8787
CellTiter 96® AQueous One Solution Reagent	Promega	WI, USA	G358B
Methanol LC-MS Ultra CHROMASOLV®	Thermo Fisher Scientific	MA, USA	14262
Acetonitrile LiChrosolv® (Hypergrade for LC-MS)	Merck KGaA	Germany	1.00029
Formic acid 99% UMC-MS	BioSolve BV	Nederland	069141
MOSS Malt Extract	Jensen & Co	Norway	n/a
Filtered Seawater	Norwegian College of Fishery Sciences	Norway	
D-Mannitol	Sigma Aldrich	MO, USA	63560
Peptone from casein	Sigma Aldrich	MO, USA	82303
Corn Meal Agar	Sigma Aldrich	MO, USA	42347
Agar-agar	Sigma Aldrich	MO, USA	A1296-1KG
Majsmjöl	Risenta AB	Sweden	
Wood briquettes	Biltema	Norway	879110
Dried *Ascophyllum nodosum*	Self-made. Collected in the tidal zone.	Norway	n/a

2.4 Preculture

The isolated marine fungi were either kept in a glycerol solution in -80°C or on agar plates at 10°C. The marine fungi were subcultivatied to grow enough biomass for fermentation. The cryopreserved fungi isolates were taken out of the -80 freezer and subcultivated on 0.2 ASMEA and ASCMA plates and grew 3-4 weeks. The subcultivated fungi were subcultivated on multiple 0.2 ASMEA and ASCMA plates achieve enough biomass for fermentation. The subcultivated fungi grew between 4-8 weeks, until their biomass was sufficient for fermentation in liquid media.

2.5 Fermentation

The marine fungi were fermented as monoculture or co-culture with four different media, with 10°C and 15°C incubation (Fig.1). The fermentation media were constructed with a carbon source, artificial seawater, two times minimal medium, Vishniac Spore solution (a trace metal solution) and MQ-H_2O (Table 3). The Vishniac Spore Solution was made accordingly to Vishniac and Santer (1957). Four different carbon sources were selected, giving a total of four different liquid fermentation media. The dried *Ascophyllum nodosum* and the wood chips were pulverized with IKA® A11 basic (IKA Werke GmbH & Co. KG, Germany) before they were added to the medium. The seaweed medium (SM), the wood medium (WM), the corn flour medium (CFM) and the malt extract medium (MEM) were made according to table 3. They were autoclaved at 121°C for 60 minutes with absorbent cotton (Macdonald & Taylor Limited, UK) in the flask opening and covered with commercial aluminum foil. For each fungus, 1.5 L of each fermentation medium 1.5 L was made, of which 0.250 L liquid media was used per fermentation flask. Six fermentation flasks were used per medium, giving a total of 24 fermentations per isolate. The total number of fermentations were120 flasks.

Two flasks with monoculture, two with co-cultivation treatment with a marine bacterium and two media controls (were one had co-cultivation) were used (Fig. 3). These were divided in two treatments, were the first group had 10°C incubation temperature with 100 rpm shaking and the other group were incubated at 15°C with no shaking. They grew for minimum 116 days and maximum 140 days.

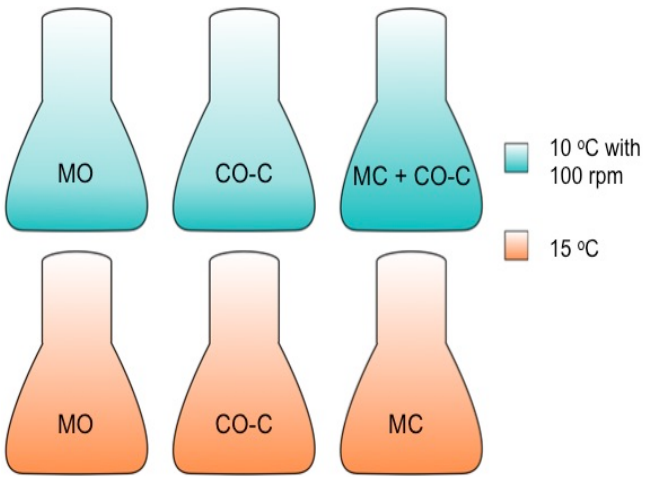

Figure 3: Shows setup of fermentation per media used. MO = monoculture, CO-C = co-cultivation, MC= media control and MC +CO-C = bacteria control. The blue flasks are incubated at 10℃ with 100 rpm shaking and the orange flasks are incubated at 15 ℃ without shaking.

To simplify the notation of the different media and treatments of each flask, a note system was created. The marine fungi species were annotated with fermentation number (Table 1) and parameter codes (Table 5). For example: species *Acremonium* sp.

TS7 fermented with MEM, co-cultivation and incubation at 10°C and 100 rpm shaking, the fermentation ID will be XY701A.2 (Fig. 4)

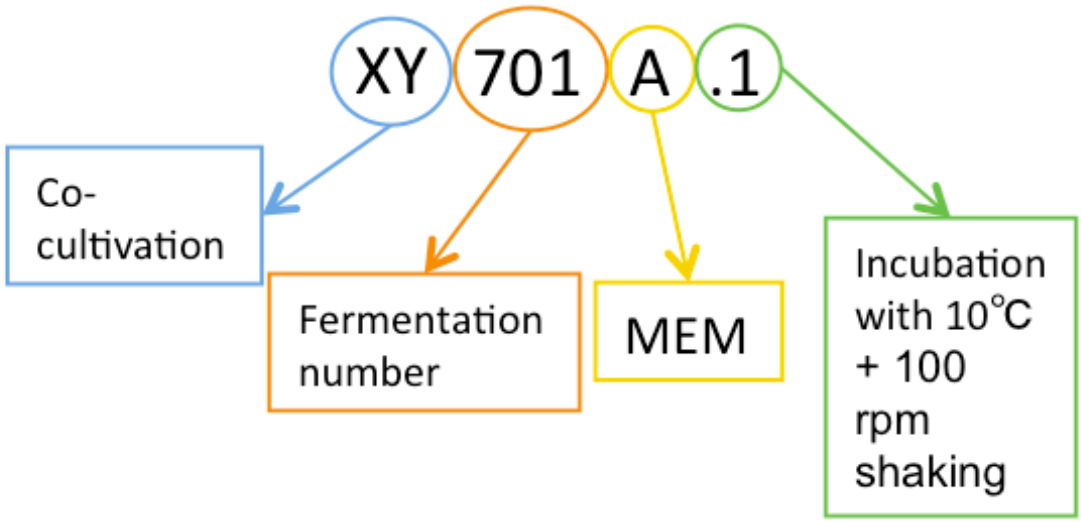

Figure 4: Shows an example of the notation for the fermentation ID of *Acremonium* sp. TS7 fermented with MEM, co-cultivation and incubation at 10°C and 100 rpm shaking. It is based on cultivation method (co-cultivation or monoculture), species (fermentation number), fermentation medium (MEM, CFM, SM or WM) and incubation conditions (incubation at 15°C or at 10°C with 100 rpm shaking.

Table 5: Notation system for the different fermentation treatments

Treatment	Notation
Malt Extract Medium (MEM)	A
Corn Flour Medium (CFM)	B
Wood Medium (WM)	C
Seaweed Medium (SM)	D
Co-Cultivation	XY
Monoculture	X
Media control	K
Incubation with 10°C and 100 rpm	.1
Incubation with 15°C	.2

2.5.1 Inoculation of co-cultivation fermentations

The 60 co-cultivated fermentations were inoculated at least two weeks before extraction. The bacteria *Leeuwenhoekiella* sp. (strain M09W024) was kept on agar plate at 10°C incubation. A scoop of the bacteria were transferred to a 15 mL Falcon tube along with 5 mL M19 medium (autoclaved at 121°C for 60 min beforehand). The Falcon tube was incubated at 10°C with 150 rpm until growth was visible, roughly two to three days. The bacteria solution was transferred over to a 400 mL Erlenmeyer flask with 200 mL M19 medium. The Erlenmeyer flask was incubated at 10°C with 100-150 rpm for

three days, until growth was visible. The 60 co-culturing fermentations were inoculated by adding 2.5 mL of the bacteria solution to each fermentation of marine fungi. The inoculation was done under sterile conditions (Class II hood) to prevent contamination. The 60 inoculated co-cultivated fermentations were incubated at either 10 °C or 15°C depending on treatment until they were extracted.

2.6 Extraction

2.6.1 Analysis for contamination with PCR

Before extraction samples from the fermentations were taken out in order to conduct DNA analysis to identify possible contaminates. From each fermentation, 400 µL sample was transferred over to a eppedorftube. The sampling was conducted for all 120 fermentations to identify possible fungal contaminations in the fermentations. The samples were stored at -20°C until they were used.

The internal transcribed spacer (ITS) regions 4 and 5 were used to identify fungal contamination (Schoch et al., 2012). To identify contaminants from other fungal species, some samples from the fermentations were analyzed with PCR and sequencing. The samples were taken out from the freezer and thawed on ice. The samples were diluted 10 times to avoid too much template in the PCR reaction. Each PCR mix was made per sample containing 1 µL fungal template, 12.5 µL 2x DreamTaq PCR, 1 µL ITS4 primer (10 µM), 1 µL ITS5 primer (10 µM) and 9.5 µL ddH_2O. The PCR reaction followed the following PCR cycle:

95°C	5 min	
95°C	30 sec	} 35 cycles
55°C	30 sec	
72°C	1 min	
72°C	10 min	
4°C	∞	

The PCR products were determined by gel electrophoresis. The 1% agarose gel was made by melting 1 g agarose in 100 mL 1xTBE Buffer. The solution was cooled down before 2.5 µL 100x gel red was added. The solution was added to Owl™ EasyCast™ B2 Mini Gel Electrophoresis System (Thermo Fisher Scientific™, MA, USA). When the gel had completely cooled down, the following was added to the wells; 6 µL 1kb ladder and 6 µL sample mix (5 µL PCR sample and 1 µL 6x loading dye). The gel was run for 15 min

at 150-200 V. Picture of the gel was executed by GeneFlash® (SYNGENE, Great Britain). When the PCR products had been confirmed with the correct size, the PCR products were purified for sequencing. The QIAquick PCR purification kit was used. The manufactures instructions were followed. The concentration and quality of the purified PCR products was investigated with NanoVue Plus™ (GE Healthcare, Great Britain).

The master mix for PCR sequencing was made with 2 µL template (purified PCR product), 2 µL BigDye 3.1, 2 µL 5x sequencing buffer, 1 µL ITS4 (or ITS5) primer (1 µM) and 3 µL ddH_2O. The PCR products were amplified for sequencing with the following PCR cycle:

96°C	1 min		
96°C	10 sec	}	30 cycles
50°C	5 sec		
60°C	4 min		
4°C	∞		

The amplified PCR products were sequenced at MH. Sequence similarity searches were preformed with BLAST software on the NCBI.

2.6.2 Preparation for extraction

The 120 fermentations were extracted to create crude extracts that could be tested for antibacterial and anticancer activity. To extract the produced secondary metabolites from the fermentationse, the Resin Diaon® HP-20 was used. This has a high affinity for medium to non-polar compounds. The larger compounds will not be able to bind the resin beads. The resin beads were pretreated with 100% methanol to activate the resin beads. For this, 10 g Diaon® HP-20 was added to a 100 mL Erlenmeyer flask with 100 mL methanol per fermentation (250 mL) and this mixture was incubated for 30 minutes. The methanol was poured gently off while keeping the resin beads in the Erlenmeyer flask. The resin beads were washed with 100 mL MQ-H_2O with 20 minutes incubation. Most of the water was poured gently off with some water remaining in the Erlenmeyer flask. The remaining water enables the transfer of resin beads, without losing too much resin. The transfer of resin was conducted in sterile conditions to prevent contamination

of the fermentations. The fermentations were incubated at the initial fermentation conditions for two days before they were extracted.

2.6.3 Extraction of metabolites in the fermentation media

The metabolites secreted in the fermentation media were extracted after two days of incubation. The fermentation medium was first separated by using cheesecloths (Osteklede, finmasket, Dansk hjemmeproduktion, Denmark), 1L filter flask and a Buchner funnel with vacuum filtration. The cheesecloth restrained the resin, mycelia and other large particles from escaping with the water phase. The resin and cheesecloth were washed with 200 mL MQ-H_2O and transferred back to the fermentation flask without losing the contents on the filter. Then 100 mL methanol was added and incubated for 1-1.5 hours. The water phase collected was discarded. After the incubation, the methanol was poured gently over a Whatman grade 3 90 mm filter (Sigma Aldrich, MO, USA) connected to a new Buchner funnel and filtration flask under vacuum. The resin was kept in the bottle during the first filtration. The filter was discarded and 100 mL methanol was added to the bottle for a second extraction. The flask was incubated for at least 30 minutes and was then filtrated with a new Whatman grade 3 90mm filter (Sigma Aldrich, MO, USA). The methanol phases were combined and transferred over to a 100 mL round bottom flask and dried under vacuum (Laborota, Heidolph Instruments GmbH and Co, Germany). This extraction method was repeated for all 120 fermentations. Software R was used for creating boxplots from the crude extracts yields.

2.6.4 Preservation of extracts

The 120 crude extracts were preserved in DMSO. A standard concentration of 40 mg/mL DMSO was first used, but the extracts did not dissolve properly. The DMSO volume was doubled, making the final concentration 20 mg/mL DMSO. The DMSO solutions were transferred to cryotubes and stored at -20°C until further use.

2.7 Bioactivity screening – crude extracts

2.7.1 Preparation of crude extracts for bioactivity screening

The DMSO in the 120 crude extracts had to be removed before they could be used in the bioactivity screening. The crude extracts were taken out of storage and thawed completely in room temperature. Stock plates for bioactivity testing were made by

transferring 250 µg crude extracts into deep well plates. The plates were frozen solid and freeze-dried (Heto Powerdry PL9000, Thermo Fisher Scientific, MA, USA). The freeze-drying process took approximately three days. The freeze-dried crude extracts were redissolved with MQ-H_2O and 2.5% DMSO. Final concentration for the deep well stock was 1 mg/mL.

2.7.2 Antibacterial screening

The 120 redissoved crude extracts were tested for antibacterial activity against *E. coli, S. aureus, S. agalactiae, P. aeruginosa and E. faecalis*. The bacteria were taken out from the -80°C freezer and kept on ice. The bacteria strains were plated on blood agar plates and incubated at 37°C for 24 hours. Overnight cultures were made by adding one scoop of bacteria from the blood agar plates into 8 mL of fresh growth medium in falcon tubes with all five bacteria strains. Growth media used were specific for each bacteria strain (Table 6). The overnight cultures were incubated at 37°C for 24 hours.

On day three, the crude extracts were prepared for antibacterial screening by making a test solution with the crude extracts and MQ-H_2O. The antibacterial screening requires 100 µL crude extract solutions per bacteria and the crude extract solutions were diluted 1:2 in the assay plate. The crude extract solution were made 100µg/mL concentration, making the final test concentration to 50 µg/mL in the assay plate.

The five overnight cultures were taken out of the incubator and 2 mL of the bacteria suspensions were transferred over to five 100 mL Erlenmeyer flask containing 25 mL fresh growth media (Table 6). The bacteria were incubated for 1.5-2.5 hours to reach the exponential phase (Table 6). Varying incubation times ensures that each bacteria strain reaches the turbidity of 0.5 McForland Standard ($1,0*10^8$ bacteria per ml). The bacteria cultures were incubated at 37°C and 150 rpm for either 1.5 or 2.5 hours depending on the strain (Table 6).

Table 6: Information on the specifications for each bacteria strain

Bacteria strain	Growth media	Incubation time	Bacterial density
S. aureus	MH-broth	2.5 hours	0,5-3*10^5 CFU[1] (2500-15000 CFU/well)
E. coli	MH-broth	1.5 hours	0,5-3*10^5 CFU[1] (2500-15000 CFU/well)
E. faecalis	BHI-broth	1.5 hours	0,5-3*10^5 CFU[1] (2500-15000 CFU/well)
P.aeruginosa	MH-broth	2.5 hours	3-7*10^4 CFU[1] (1500-3500 CFU/well)
S. agalactiae	BHI-broth	1.5 hours	0,5-3*10^5 CFU[1] (2500-15000 CFU/well)

[1]Colony-forming Units (CFU), a measure of the number of viable microorganisms present on a surface.

The microtiter plates were prepared under the incubation period for the bacteria by adding 50 µL of each crude extract test solution in microtiter plates. Each crude extract was tested in parallels and against the five bacteria strains (Table 7).

Table 7: Setup for MIC assay

	1	2	3	4	5	6	7	8	9	10	11	12
A	**N**	1	1	9	9	17	17	25	25	33	33	**P**
B	**N**	2	2	10	10	18	18	26	26	34	34	**P**
C	**N**	3	3	11	11	19	19	27	27	35	35	**P**
D	**N**	4	4	12	12	20	20	28	28	36	36	**P**
E	**N**	5	5	13	13	21	21	29	29	37	37	**P**
F	**N**	6	6	14	14	22	22	30	30	38	38	**P**
G	**N**	7	7	15	15	23	23	31	31	39	39	**P**
H	**N**	8	8	16	16	24	24	32	32	40	40	**P**

The bacteria suspensions were diluted 1:100 before adding 50 µL of bacteria suspension in each well of the microtiter plates after incubation.

Negative control was added to column 1 on the microtiter plates (Table 7) consisting of 50 µL growth media and 50 µL MQ-H_2O. Positive controls were added to column 12 on the microtiter plates (Table 7) consisting of 50 µL bacteria suspenstion and 50 µL MQ-H_2O. A gentamicin control of the setup was used to control and validate the stability of

the bacteria strains used. The gentamicin was tested with the five bacteria strains on the following concentrations: 8 µg/mL, 4 µg/mL, 2 µg/mL, 1 µg/mL, 0.5 µg/mL, 0.25 µg/mL, 0.12 µg/mL, 0.06 µg/mL and 0.03 µg/mL. The dilution series of gentamicin was added to five microtiter plates with 50 µL bacteria suspention, one microtiter plate per bacteria strain. All the microtiter plates were incubated at 37°C for 20-24 hours.

On the forth day of the antibacterial screening, the microtiter plates were visually inspected for inhibition. The photometric instrument 1420 Multilabel Counter VICTOR^3 ™ (PerkinElmer, MA, USA) was used to measure OD. Cut-off values have been established as guidelines to define the antibacterial activity the sample has with OD_{600} values.

Active $<$ 0.05
Questionable 0.05 – 0.09
Inactive $>$ 0.09

An additional secondary screening is conducted on active and questionable samples with lower concentrations to find the lowest concentration for inhibition and to confirm the activity of the fractions.

2.7.3 Anticancer screening

The 120 redissoved crude extracts were tested for anticancer activity against the adherent A2058 melanoma cancer cell line. The A2058 were detached from the growth flask with PBS-wash and trypsin treatment, and added to microtiter plates with 2000 cells/well. The plates were incubated for 24 hours at 37°C and 5% CO_2 (Panasonic Biomedical, Japan) to allow the cells to attach. The crude extract test solutions were prepared by adding crude extracts from stock plate and RPMI-1640 cell medium in a new deep well plate with 50 µg/ml test concentration. The incubated microtiter plates were inspected microscopically to check for good growth of the cancer cells. The cell media were then removed from each well with a multichannel pipette and 100 µl crude extracts test solutions and cell medium were added to the wells. Two parallels were used. Cancer cells with cell medium were used as negative controls and positive control was cell media and 0.5% Triton. Triton and A2058 cancer cells were tested vigorously and a standard OD measurement has been set. Triton has previously been added to a separate test plate and the average 0.13 OD_{485} is used as positive control. The microtiter

plates were incubated for 72 hours at 37°C and 5% CO_2 (Panasonic Biomedical, Japan). All cancer cell work was conducted under sterile condition in a Class II hood.

Table 8: Setup for cancer assay

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		1	1	7	7	13	13	19	19	Neg		
C		2	2	8	8	14	14	20	20	Neg		
D		3	3	9	9	15	15	21	21	Neg		
E		4	4	10	10	16	16	22	22			
F		5	5	11	11	17	17	23	23			
G		6	6	12	12	18	18	24	24			
H												

After the 72 hour incubating, 10 µL CellTiter 96® Aqueous One Solution were added to wells. The microtiter plates were incubated for 1 –1.5 hours at 37°C and 5% CO_2 (Panasonic Healthcare, Osaka, Japan). The absorbance was measured at 485 nm with DTX 880 Multimode Detector (Beckman Coulter, California, USA). The absorbance is used to determine if crude extracts are active, questionable or inactive with the equation (1):

$$(1) \quad \frac{(\text{average parallels} - \text{average postive controll})}{(\text{average negative controll} - \text{average positive control})} \text{X } 100$$

This percent is used to determine if the crude extracts are active, questionable or inactive:

$$\text{Active} < 50\ \% \text{ cell survival}$$
$$\text{Questionable} = 50 - 60\ \% \text{ cell survival}$$
$$\text{Inactive} > 60\ \% \text{ cell survival}$$

2.8 Prefractionation

There was not found any anticancer or anticancer activity in the bioactivity screening, therefore were eight crude extracts selected for prefractionation based the crude extracts yields. One monoculture and one co-cultivated crude extracts were selected per isolate. The selected crude extracts were X701A.2, XY701A.2, X702D.2, XY702D.2, X703C.2, XY703C.2, X705B.2 and XY702B.2.

The prefractionation was conducted with flash chromatography. The crude extract stock was freeze-dried (Heto Powerdry PL9000, Thermo Fisher Scientific, MA, USA) to remove the DMSO and redissolved with methanol. The crude extract solutions were transferred to 100 mL round bottom flasks and dried under vacuum with a rotavapor (Laborota, Instruments GmbH and Co, Germany). The dried solutions were then resolved in 8 mL 90% methanol and 2 g Diaion® HPSS-20 was added to each sample. The dried under vacuum with rotavapor until it was completely dry. The dry crude extract and column material was added on top of the flash column (Biotage® SNAP Cartridge KP-Sil 10g, Uppsala Sweden) and attached to the Biotage® HPFC SP4 Flash Purification system (Uppsala, Sweden). The column was packed with 6.5 g of HPSS-20 column material. The column material was equilibrated beforehand with 1:1 methanol:MQ-H_2O. The following step gradient was applied: Methanol/H_2O to methanol in five steps (5:95, 25:75, 50:50, 75:25 and 100:0) followed by methanol:acetone to acetone in two steps (50:50 and 0:100). The fractionations were done in a two-step flash chromatography program. The first step fractionated and eluted the crude extract with an increasing gradient of methanol and decreasing the concentration of MQ-H_2O. The second step eluted compounds by increasing the acetone concentration in the column. This approach gave in total six fractions, were the first fraction contained the most hydrophilic compounds (polar) and fraction six are the most lipophilic (nonpolar). This prefractionation created 48 fractions in total.

The solvents of the six fractions per crude extract sample were evaporated and removed (Syncore® Polyvap, Butchi Corporation, Switzerland). A DMSO stock of the dried fractions were made and stored in cryrotubes at -20°C until bioactivity screening.

2.9 Bioactivity screening – fractions

2.9.1 Preparation of fractions for bioassays

DMSO in the 48 fractions had to be removed in order to use them in activity screening. The fractions were taken out of storage freezer and thawed completely in room temperature. A stock plate for bioactivity testing was made by transferring 500 µg fractions in a deep well plate. The plate was frozen solid and freeze-dried (Heto Powerdry PL9000, Thermo Fisher Scientific, MA, USA). The freeze-drying process took approximately 3 days. The freeze-dried fractions were redissolved with MQ-H_2O and 2.5% DMSO. Final concentration for the deep well stock was 1 mg/mL.

2.9.2 Antibacterial screening

The total of 48 fractions from the eight fermentations were tested for antibacterial activity. The same procedure for antibacterial screening with crude extracts (2.7.2) was conducted on the 48 fractions. The test concentration was 50 µg/mL with two parallels (Table 7). The active or questionable fractions were taken forward for MIC retest.

2.9.3 Anticancer activity

The total of 48 fractions from the eight fermentations were tested for anticancer activity. The same procedure for anticancer screening as with crude extracts (2.7.3) was conducted on the 48 fractions. The test concentration was 50 µg/mL with two parallels (Table 8).

2.9.4 Antibacterial screening – retest of active fractions

The five active or questionable fractions from the antibacterial screening (2.9.2) were retested to find minimal inhibition concentration (MIC). The same procedure for antibacterial screening for crude extracts (2.9.1) were used. Test concentrations were 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL and 3.125 µg/mL. The retest was conducted two times. In the first retest, none of the five fractions were active. To investigate if this was a solubility issue, a new deep well plate was made with the active fractions from the antibacterial screening. The plate was freeze-dried and dissolved with the same procedure as in 2.5.3. The five active fractions were rested again with the same test concentrations as above.

2.10 Dereplication

2.10.1 MS-analysis of bioactive fractions

The five active fractions were investigated with UPLC-QToF-MS. The inactive fractions X701A.2-5, X702D.2-5 and X705B.2-5 were also included in the analysis to be able to identify peaks that were responsible for the bioactivity. The samples were analyzed on a Vion® IMS QToF with a C18 1.7µM reverse column in the ESI+ mode (Table 9). The samples were prepared by adding 10 µL sample in a vial with 90 µL 90% methanol. The sample injection volume was 1µL. The UPLC used two solvents. Solvent A was water with 0.1% formic acid. Solvent B was acetonitrile with 0.1% formic acid. Both solvents had adjusted pH = 3.75. The UPLC gradient conditions are listed in table 10. The source parameters used are listed in table 11.

Table 9: Instrument list for the UPLC-QToF-MS.

Instrument	Producer
Vion® IMS QToF	Waters Corporation (Massachusetts, USA)
Acquity PDA Detector	Waters Corporation (Massachusetts, USA)
Acquity Column Manager	Waters Corporation (Massachusetts, USA)
Acquity Sample Manager - FTN	Waters Corporation (Massachusetts, USA)
Acquity Binary Solvent Manager	Waters Corporation (Massachusetts, USA)
Acquity UPLC® BEH C18 1.7µM Column	Waters Corporation (Massachusetts, USA)

Table 10: UPLC gradient conditions.

Time (min)	Flow rate (mL/min)	Solvent A gradient (%)	Solvent B gradient (%)
0.00	0.450	90.0	10.0
12.00	0.450	0.0	100.0
13.50	0.450	0.0	100.0

Table 11: Source parameters for MS analysis of flash fractions.

Source parameters	ESI+
Capillary voltage (kV)	0.80
Cone voltage (V)	30
Cone gas flow (L/h)	50
Desolvation gas flow (L/h)	800
Temperature desolvation (°C)	450
Temperature source (°C)	120
Low mass (m/z)	50
High mass (m/z)	2000
Low collision energy (eV)	6.0
High collision energy (eV)	15-45

2.10.2 MS-analysis of bacteria controls

Four bacteria controls containing only medium and *Leeuwenhoekiella* sp were investigated with UPLC-QToF-MS to be able to identify possible production of bioactive compounds from *Leeuwenhoekiella* sp. The samples was prepared by adding 15 µL sample in a vial with 85 µL 90% methanol. Sample injection volume was 1 µL. The same procedure as in 2.10.1 was used.

3. Results

3.1 Contamination analysis of the fermentations

A selection of DNA samples from the fermentations was used to check for fungal contamination. The ITS4 and ITS5 primer sequences were used to amplify the ITS region in the DNA samples. The amplified PCR products were sequenced and generated sequences were used in BLAST searches against the National Center for Biotechnology Information's nucleotide database. Comparison of BLAST hits with initial strain identity showed contamination in the *Pseudogumnoacus* sp. TS12 (704). The contaminant was the *Acremonium* sp. TS7 (701). The *Pseudogumnoacus* sp. TS12 (704) was probably contaminated on solid media when the precultures were made. The 704 fermentations were terminated for further work due to the contamination.

3.2 Crude extract yield

The yield of an extraction is important because a high yield gives more material for the subsequent studies. The medians of the extraction yields were quite similar between the isolates (Fig. 5). The fermentations did not get extracted at the same day, so the incubation periods varied somewhat across the isolates. *Typhula* sp. (702) had the longest incubation time with an average of 135 days followed with the *Acremonium* sp. TS7 (701) with 130 days, the *A encephaloides* (703) with 122 days and *D. marina* (705) with 121.5 incubation days.

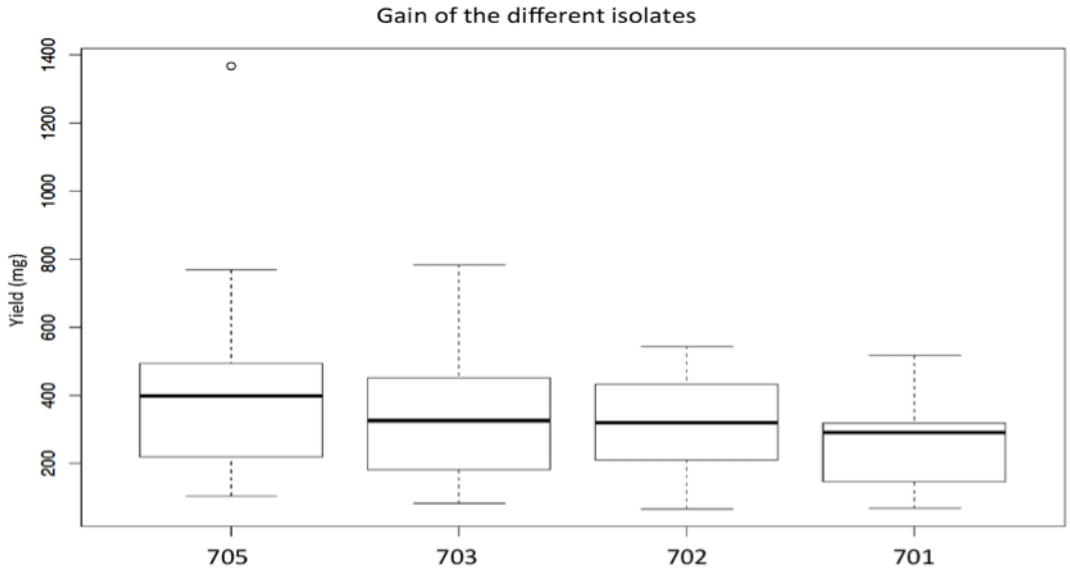

Figure 5: Boxplot for the yield of the different isolates, which includes 701 (*Acremonium* sp. TS7), 702 (*Typhula* sp.), 703 (*A. encephaloides*) and 705 (*D. marina*). All of the fermentations (with all the different parameters) are included. Fermentation media controls are excluded. The black line in the four boxplots is the median.

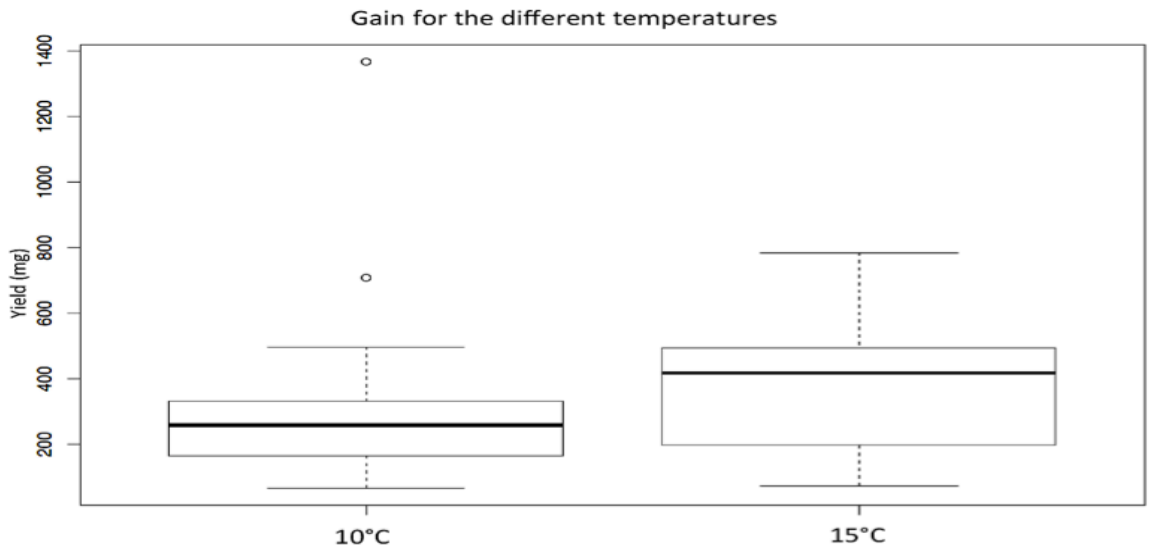

Figure 6: Boxplot of the yield for the different temperature conditions. The 15℃ incubation had significant higher yield than 10℃ incubation. The fermentation media controls are not included. The black line in each boxplot is the median.

The fermentations with 10℃ incubation and 100 rpm shaking had generally lower yields compared to fermentations with 15℃ incubation without shaking (Fig. 6). The trend is evident on isolate level as well. All four isolates had significantly higher crude extract yields with 15℃ incubation compared to fermentations with 10℃ incubation (Fig.7).

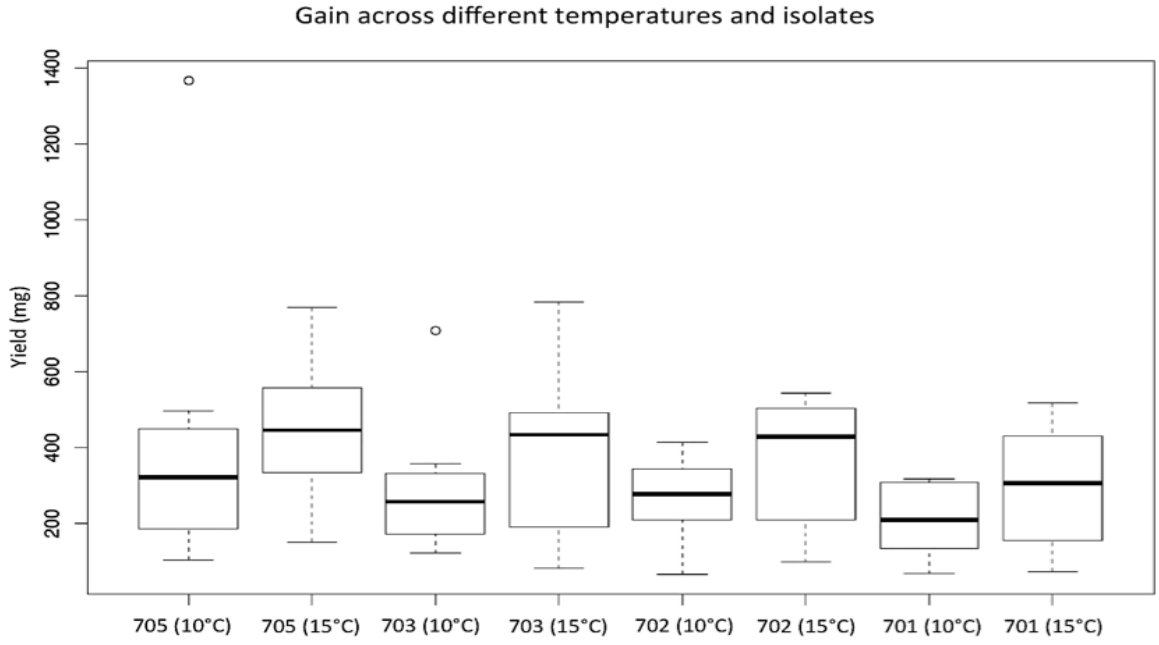

Figure 7: Boxplot of all crude extracts from each isolate separated after the incubation temperature 10℃ and 15℃. Overall higher yield are found with 15℃ incubation in all four isolates. Media controls are not included. The black line in each boxplot is the median.

The four different media used for these fermentations had overall no major advantages over the other when the crude extracts yields are considered (Fig. 8). The SM, WM and CFM were all media that contained particles and were heterogenic. The residues from the particles in the media did end up in the extracts in some degree, thus can have increased the yield in total. MEM was the only media that was homogeneous. The different isolates were collected from different habitats, but no media specificity was found amongst them.

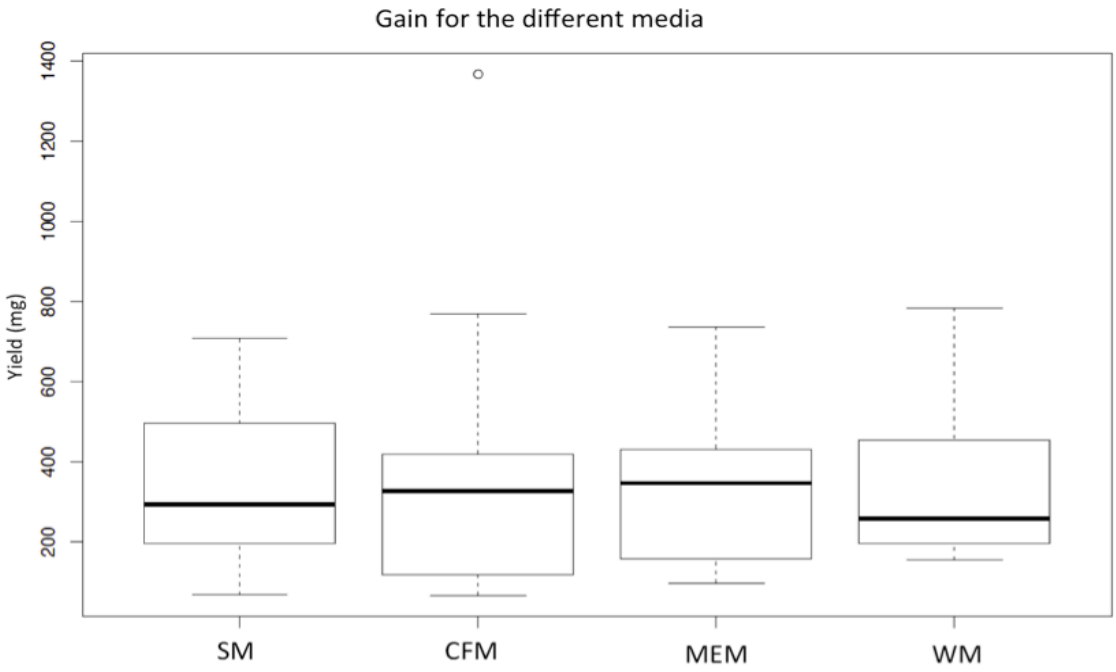

Figure 8: Gain for the different media. Four media was used: Seaweed Medium (SM), Corn Flour Medium (CFM), Malt Extract Medium (MEM) and Wood Medium (WM). The black line in each boxplot is the median.

3.3 Bioassays on crude extracts

Prefractionations of crude extracts are normally executed before bioactivity screening. With 96 fermentations in this study, that would have been an enormous task. Instead, the crude extracts were tested for antibacterial –and anticancer activities to identify crude extracts with bioactivity. The active crude extracts would subsequently be prefractonated. This would save time and labor by removing inactive samples.

3.3.1 Antibacterial screening

In the antibacterial screening of the 96 crude extracts, no activity was found against *E. coli*, *S. agalactiae*, *P. aeruginosa*, *S. aureus* or *E. faecalis* at 50 and 100 µg/ml test concentrations. As illustratied with 8 different crude extracts against *S. agalactiae* in figure 9. The results from a selection crude extracts tested against *S. agalactiae* show no inhibition (Fig.9).

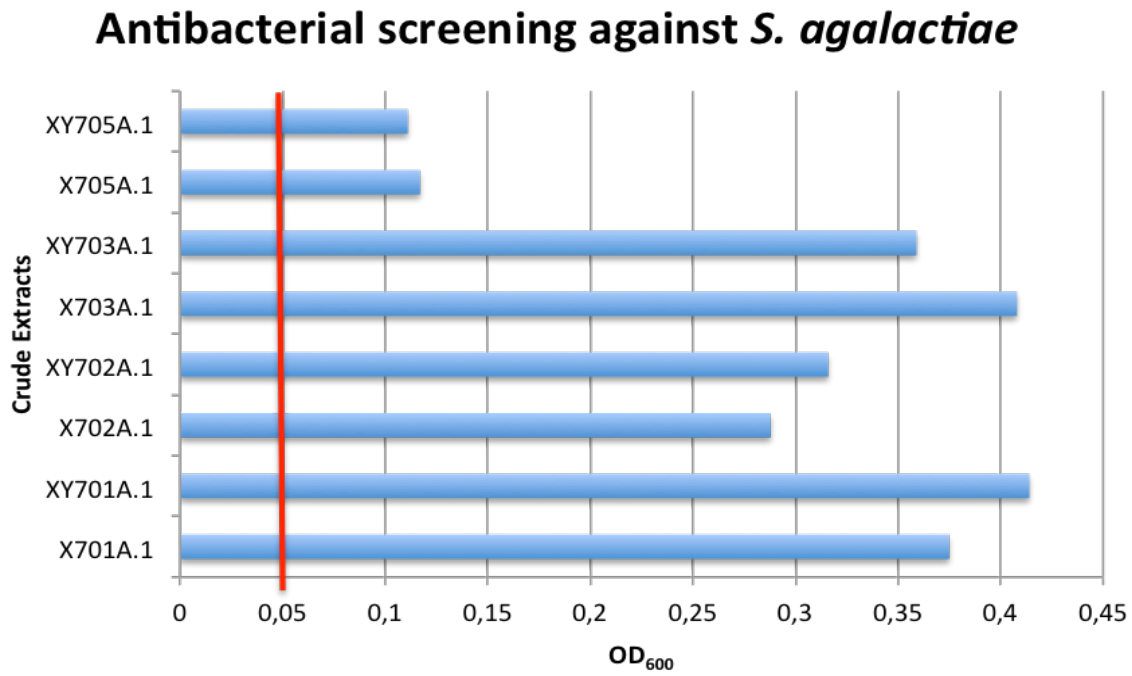

Figure 9: The results from the antibacterial screening of 8 crude extracts against *S. agalactiae*. None of the crude extracts were under the cut-off value 0.05 (red line). The test concentrations for these crude extracts were 100 µg/ml.

3.3.2 Anticancer screening

In the anticancer screening of the 96 crude extracts, no anticancer activity against the cancer cell line A2058 were found with the cut-off value (<50% cell survival). However, some of the extracts did reduce the survival of the cancer cells (Fig. 10). The average cell survival was between 100-110 %. The samples that affected the cancer cell growth were all from *D. marina* (705) and included samples X705A.1, XY705A.1, X705B.1, XY705B.1 and X705A.2 (Table 12). All these extracts gave cell survival between 72-75 % at

50 µg/ml test concentration. Crude extracts X701A.2 and XY701A.2 were not screened for antibacterial activity and are therefore not included.

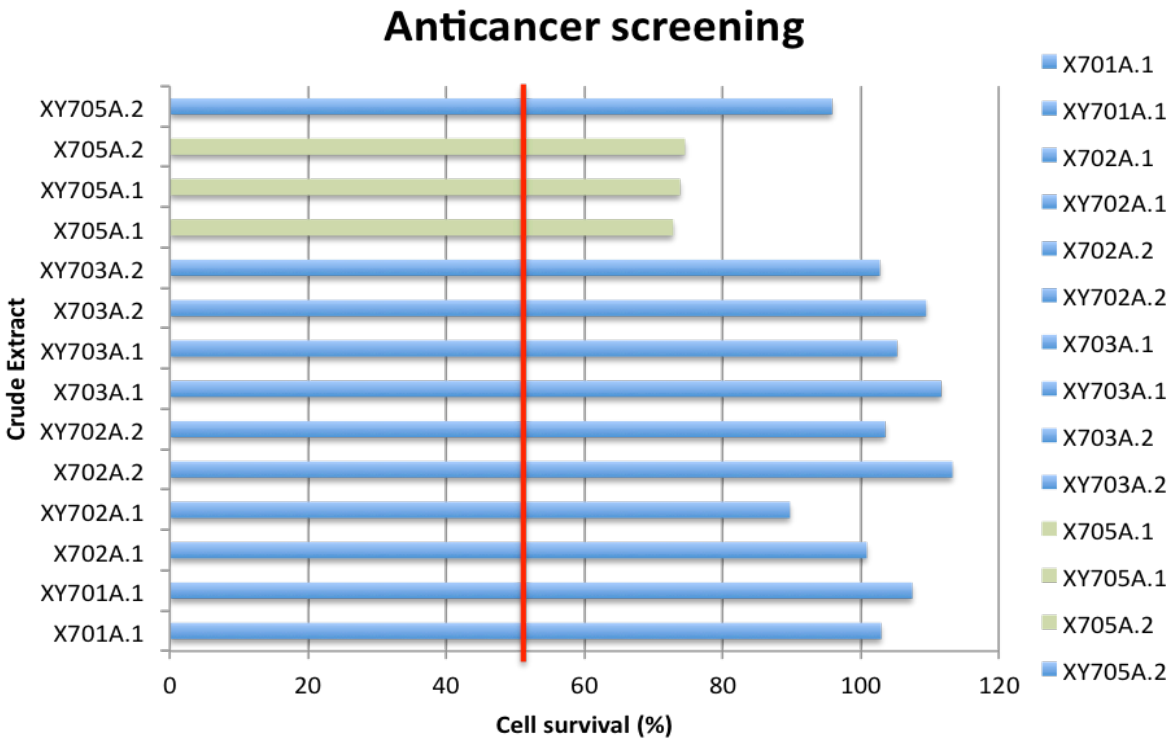

Figure 10: The figure shows results from the anticancer screening of the 14 different extracts, two from isolate 701, four extracts from each of the three isolates 702, 703 and 705. The screening concentration was 50 µg/ml. The crude extracts from 705 (*D. marina*) were the only isolate that showed any effect on the cell survival (in green). The red represents the cut-off value for active samples (>50% cell survival).

Table 11: Crude extracts with reduced cell survival in the anticancer screening

Sample	Cell survival (%)
X705A.1	72,8
XY705A.1	73,8
X705B.1	72,0
XY705B.1	75,1
X705A.2	74,5

3.4 Prefractionation

Due to the lack of hits from the bioassays done on the crude extracts, several fermentations were selected for prefractionation based on other criteria. Pairs of monoculture – and co-culture fermentation from the four different isolates were selected. The pairs had the same fermentation medium. The following samples were prefractionated: X701A.2,
XY701A.2, X702D.2, XY702D.2, X703C.2, XY703C.2, X705B.2 and XY705B.2. These crude extracts had overall good yield. The distribution of the material between the fractions for each crude extract is represented in figure 11.

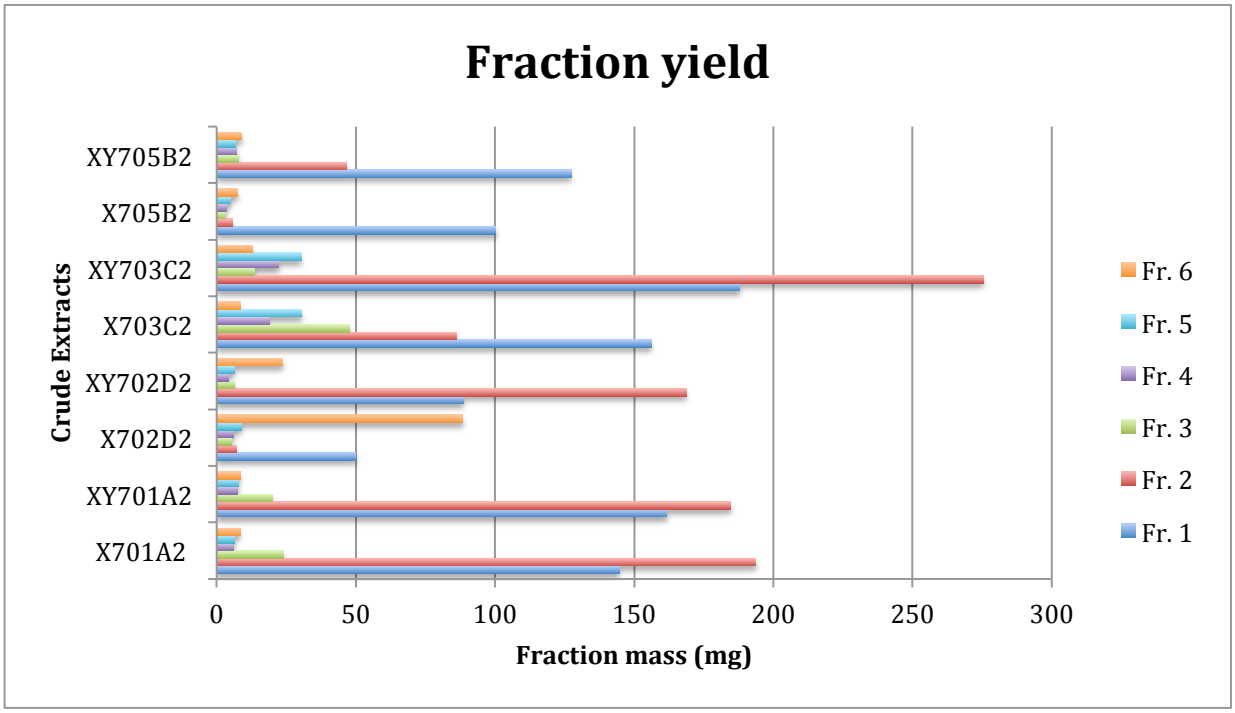

Figure 11: Illustrated the crude extracts yield for the prefractionated X701A.2, XY701A.2, X702D.2, XY702D.2, X703C.2, XY703C.2, X705B.2 and XY705B.2. The figures illustrate how much material in each fraction was eluted from the prefractionation. Each crude extract were prefractionated into six fractions. The most polar compounds were eluted in fraction 1 (blue) and 2 (red), less polar compounds were eluted in fractions 3 (green) and 4 (purple) and the more non-polar compounds were eluted in fraction 5 (turquois) and 6 (orange).

The amounts of material in the six different fractions from the extracts X701A.2 and XY701A.2 were very similar, and fractions 1 and 2 contained most of the material in both of them (Fig. 11). Fraction 1 contained 50% of the total mass, wheras fraction 2 contained 30-40%. Fraction 3, 4, 5 and 6 held the rest of the mass, around 10-20%.

The prefractionation of X702D.2 and XY702D.2 gave very different distributuion of material between the fractions (Fig. 11). Fraction 1 represented roughly 30% of the total

mass in both X702D.2 and XY702D.2. However, fraction 2 contained about 55% of the XY702D.2, but only 5% for X702D.2 whilst the fraction 6 is the largest. The X703C.2 and XY703C.2, the fraction 1 and 2 stands for the majority of the total mass. The rest is distributed between the rest of the fractions. The X705B.2 and XY705B.2 have the same trend, fraction 1 and 2 are the largest and the rest is more or less distributated evenly between the remaining fractions.

3.5 Bioassays on flash fractions

3.5.1 Antibacterial assay

The 48 fractions from the eight prefractionated fermentations were tested for antibacterial activity. Only activity against *S. agalactiae* was found of the five bacteria tested against (Table 12). Five fractions were active or questionable and included XY701A.2-5, XY705D.2-5, X703C.2-5, XY703C.2-5 and XY705B.2-5 (Fig. 12). All of the active fractions were from fraction 5. The active fractions (OD_{600} < 0.05) were XY702D.2-5, X703C.2-5 and XY705B.2-5. The questionable fractions (OD_{600} = 0.05-0.09) were XY701A.2-5 and XY703C.2-5. The rest of the tested fractions were above the cut-off value (OD_{600} > 0.09).

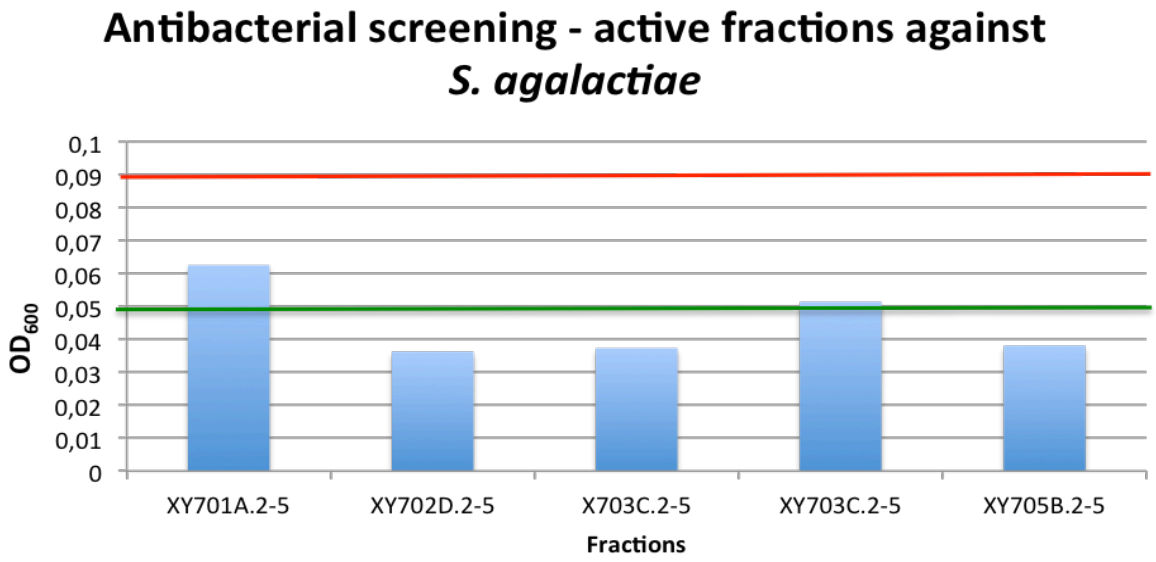

Figure 12: The figure shows the antibacterial screening results from five fractions from four isolates against *S. agalactiae*. The active fractions against *S. agalactiae* were the following XY702D.2-5, X703C.2-5 and XY705B.2-5 with OD_{600} under 0.05. The questionable fractions against *S. agalactiae* were XY701A.2-5 and XY703C.2-5. Fractions below the green line are active, the fractions between the green line and the red line are questionable and fractions over the red line would be considered inactive.

Table 12: Antibacterial assay: Active fractions[1]

Sample	*E. coli*	*S. agalactiae*	*P. aeruginosa*	*S. aureus*	*E. faecalis*
X701A.2-5	÷	÷	÷	÷	÷
XY701A.2-5	÷	+	÷	÷	÷
X702D.2-5	÷	÷	÷	÷	÷
XY702D.2-5	÷	++	÷	÷	÷
X703C.2-5	÷	++	÷	÷	÷
XY703C.2-5	÷	+	÷	÷	÷
X705B.2-5	÷	÷	÷	÷	÷
XY705B.2-5	÷	++	÷	÷	÷

1: The active fractions (OD_{600} < 0.05) are marked with (++). Questionable fractions (OD_{600} = 0.05-0.09) are marked with (+). Inactive fractions are marked with (÷).

3.5.2 Cancer assay

None of the 48 fractions from the eight prefractionated crude extracts showed any activity against the cancer cell line A2058.

3.5.3 Antibacterial assay – Retest on active fractions

The five active or questionable fractions from the antibacterial screening were retested on *S. agalactiae* for determining the lowest inhibitory concentration. The highest concentration tested (50 µg/mL) was the same as the primary screening. The first retest gave no inhibition in any of the test concentrations. The second retest was conducted to see if there was a solubility issue with the fractions and only fraction XY702D.2-5 was found active (Table 13). The results from the primary antibacterial screening and antibacterial retest were not coherent. The activity against *S. agalactiae* were lost for four out of five fractions.

Table 13: MIC assay retest with different concentrations[1].

Concentration	XY701A.2-5	XY702D.2-5	X703C.2-5	XY703C.2-5	XY705B.2-5
50 µg/mL	÷	++	÷	÷	÷
25 µg/mL	÷	÷	÷	÷	÷
12,5 µg/mL	÷	÷	÷	÷	÷
6,25 µg/mL	÷	÷	÷	÷	÷

1: The active fractions (OD_{600} < 0.05) are marked with (++). Questionable fractions (OD_{600} = 0.05-0.09) are marked with (+). Inactive fractions are marked with (÷)

3.6 Dereplication

Of the 48 fractions, five were active against *S. agalactiae* in the primary antibacterial screening. The active fractions XY701A.2-5, XY702D.2-5, X703C.2-5, XY703C.2-5 and XY705B.2-5 were dereplicated with UPLC-QToF-MS with ESI+. In addition, the inactive monocultured fractions X701A.2-5, X702D.2-5 and X705B.2-5 were analyzed to compare against the active fractions.

3.6.1 Dereplication of active fractions

In the active fraction XY701A.2-5, peaks were found that were not present in the inactive fraction X701A.2 from a monoculture fermentation (Fig. 13). Peaks at *m/z* 527, 553, 555 and 581 were not present in X701A.2. The same peaks were also found in XY702D.2-5 at identical retention times (Fig. 14). X702D.2-5 did not contain compounds at *m/z* 527, 553, 555 and 581 (Fig. 14).

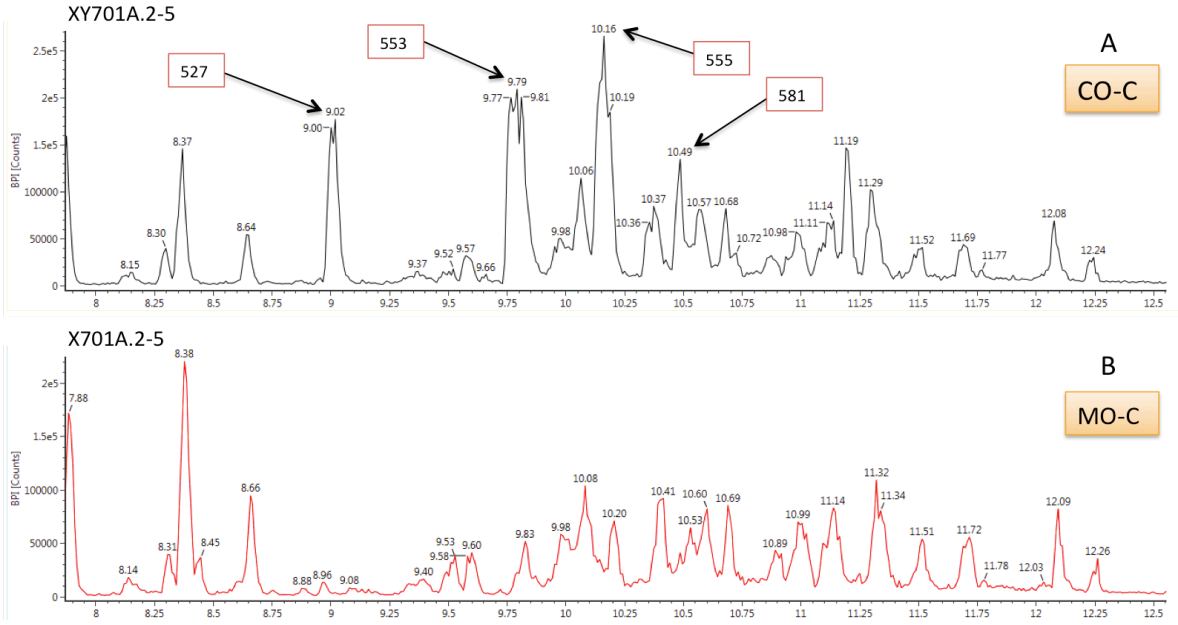

Figure 13: Chromatograms of the MS analysis of XY701A.2-5 (A) and X701A.2-5 (B) with ESI+. All compounds are sodium adducts. There was found four peaks (527, 553, 555 and 581) in chromatogram B, which was not present in chromatogram A.

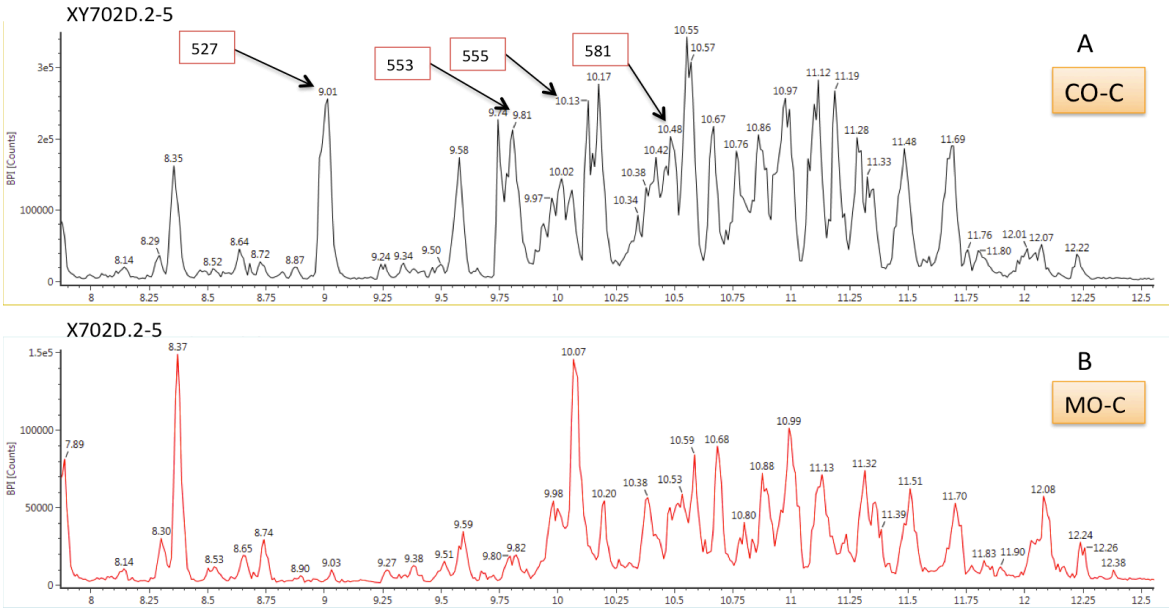

Figure 14: Chromatograms of the MS analysis of XY702D.2-5 (A) and X702D.2-5 (B) with ESI+. All compounds are sodium adducts. There was found four peaks (527, 553, 555 581) in chromatogram B, which was not present in chromatogram A.

In fraction XY703C.3-5 the compounds at *m/z* 527, 553 and 555 were found, but not compound *m/z* 581 as previously described in XY701A.2-5 and XY702D.2-5 (Fig. 15). Again, as for the other monocultures X701A.2-5 and X702D.2-5, none of these peaks were found in X703C.2-5. The fraction X703C.2-5 had antibacterial activity, but no specific peaks were found that could be responsible for that activity. The fraction XY705B.2-5 had the same peaks as the other co-culture fractions, namely *m/z* 527, 553, 555 and 581 (Fig. 16). No peaks with corresponding retention times and *m/z* values were found in X705B.2-5.

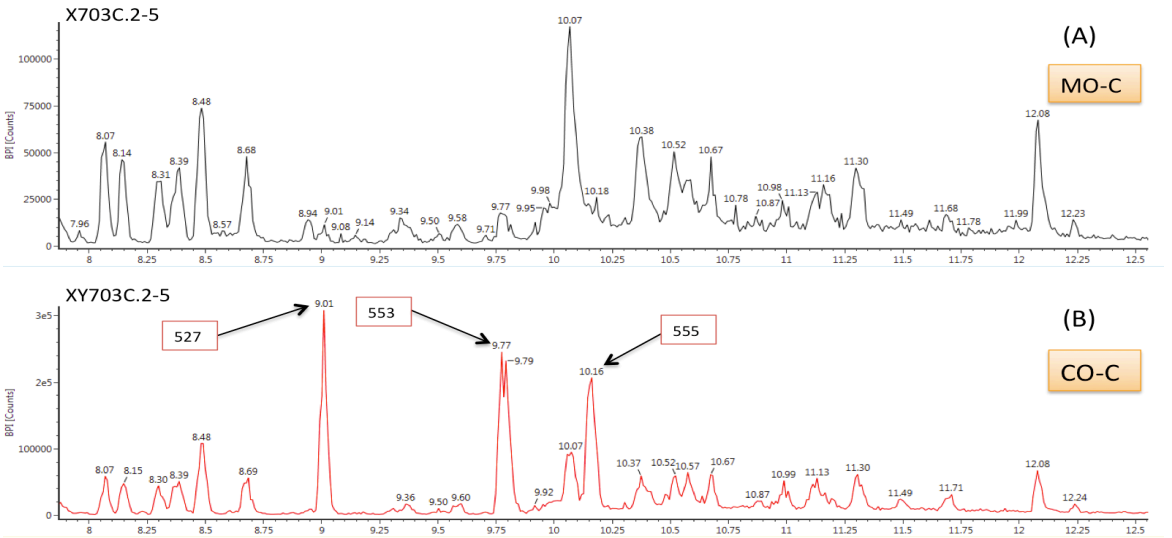

Figure 15: Chromatograms of the MS analysis of X703C.2-5 (A) and XY703C.2-5 (B) with ESI+. All compounds are sodium adducts. There was found three peaks (527, 553 and 555) in chromatogram B, which was not present in chromatogram A.

Peak 527 had a *m/z* 527.3192 and a retention time of 9.01 min. The mass represented the sodium adduct of the molecule ($[M+Na]^+$) and the elemental composition was calculated to be $C_{26}H_{48}O_9$. Peak 553 had a *m/z* 553.3348 and a retention time of 9.79 min. The mass represented the sodium adduct of the molecule ($[M+Na]^+$) and the elemental composition was calculated to be $C_{28}H_{50}O_9$. Peak 555 had a *m/z* 555.3504 and a retention time of 10.15 min. The mass represented the sodium adduct of the molecule ($[M+Na]^+$) and the elemental composition was calculated to be $C_{28}H_{52}O_9$. Peak 581 had a *m/z* 581.3671 and a retention time of 10.48 min. The mass represented the sodium adduct of the molecule ($[M+Na]^+$) and the elemental composition was calculated to be $C_{30}H_{54}O_9$ (Table 15).

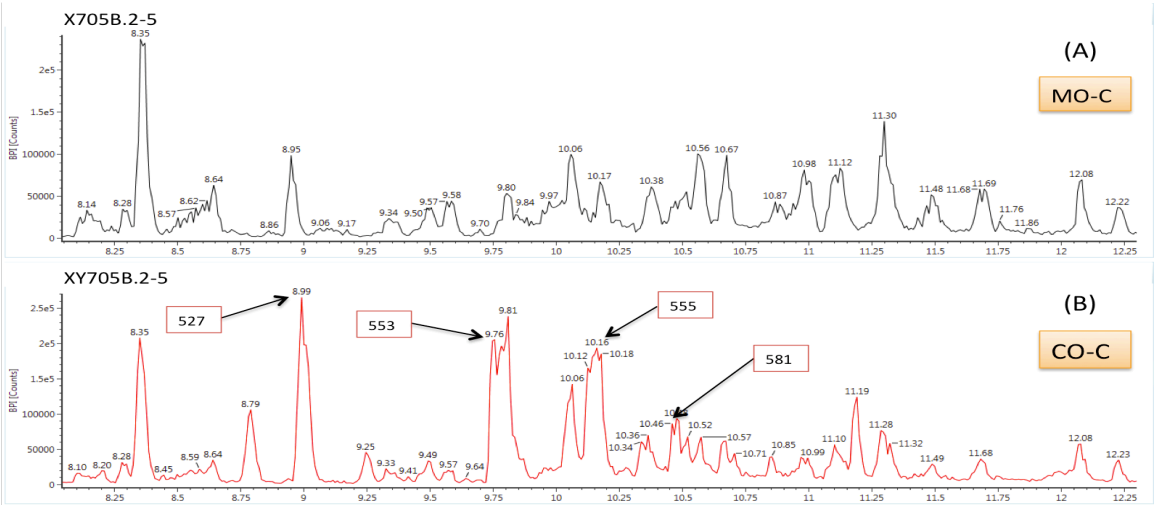

Figure 16: Chromatograms of the MS analysis of X705B.2-5 (A) and XY705B.2-5 (B) with ESI+. All compounds are sodium adducts. There was found four peaks (527, 553, 555 and 581) in chromatogram B, which was not present in chromatogram A.

Table 15: List of identified compounds

Peak	Retention time	*m/z* [M+Na$^+$]	Elemental composition
527	9.01	527.3192	$C_{26}H_{48}O_9$
553	9.79	553.3348	$C_{28}H_{50}O_9$
555	10.15	555.3504	$C_{28}H_{52}O_9$
581	10.48	581.3671	$C_{30}H_{54}O_9$

From the *m/z*, the retention time and fragment patterns, it was quite clear that these compounds have been identified in previous projects at Marbio. The $C_{26}H_{48}O_9$, $C_{28}H_{50}O_9$, $C_{28}H_{52}O_9$ and $C_{30}H_{54}O_9$ are most likely Rhamnolipids.

3.6.2 Dereplication of bacteria controls

The fractions of the co-cultivated fermentations contained the same compounds, independent of isolate and fermentation media used. The only common parameters these have are co-cultivation with *Leeuwenhoekiella* sp. After the discoveries in 3.8.1, the bacteria controls were analyzed with UPLC-QToF-MS with ESI+. The bacteria controls contained only *Leeuwenhoekiella* sp. and medium. The following bacteria controls were analyzed: XY701KA, XY702KD, XY703KC and XY705KB. Peak at *m/z* 527, 553 and 555 were found in three out of the four bacteria controls (Fig. 13). Peak 581 were absent in all bacteria controls. XY702KD did not have any of the known peaks with the same *m/z* and retention time as previously (Fig. 17). The calculated elemental composition for the peaks at *m/z* 527, 553 and 555 are the same found in 3.8.2.

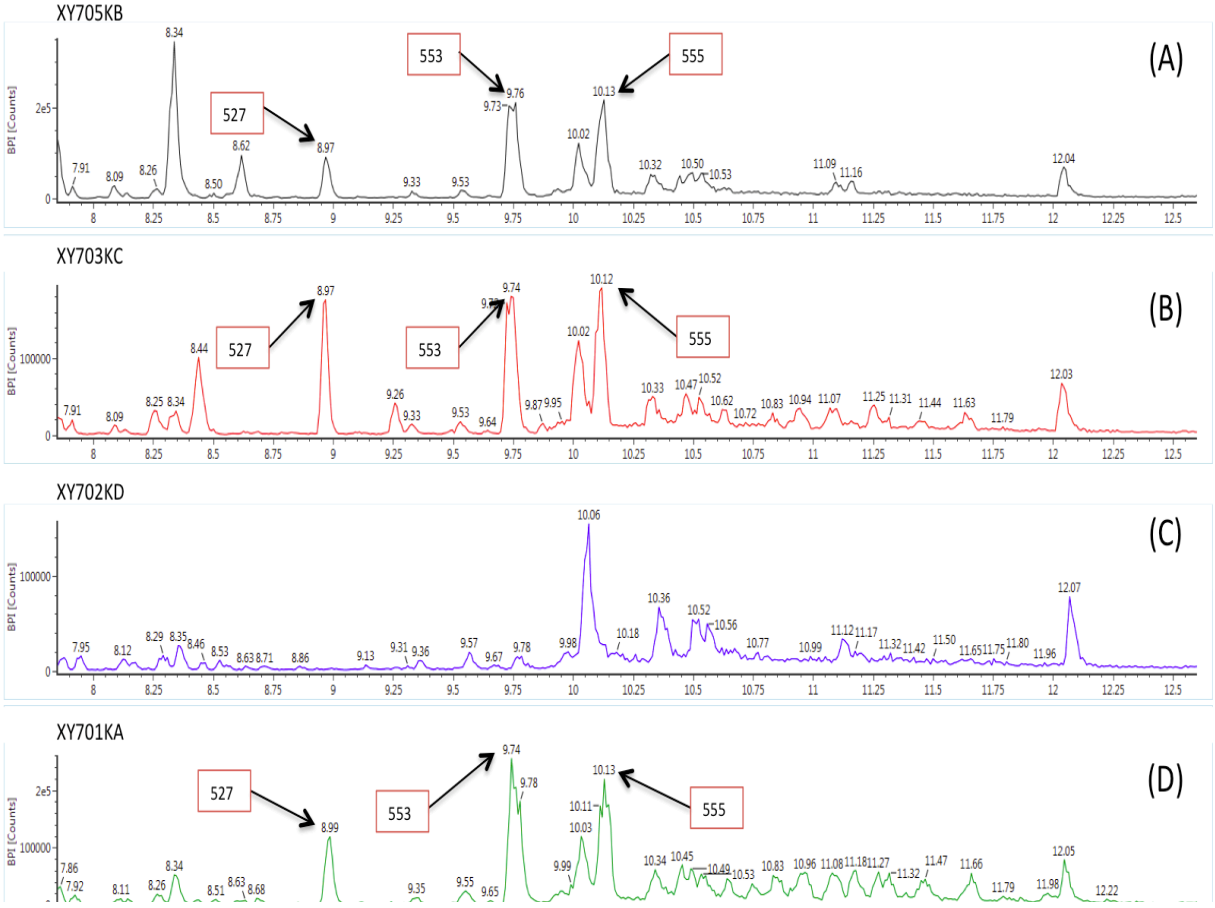

Figure 17: Chromatograms of the MS analysis of media controls with *Leeuwenhoekiella* sp. with ESI+. All compounds are sodium adducts. The media controls included are XY701KA (D), XY702KD (C), XY703KC (B) and XY705KB (A). Peak 527, 553 and 555 were found in chromatogram A, B and D. Peak 581 were absent in all samples. All peaks (527, 553, 555) were absent in chromatogram C.

4. Discussion

The marine environment provides a unique diversity and is a good source for discovering novel bioactive compounds. Lately, there has been an increasing interest towards marine fungi, which have proven to be an excellent source for novel bioactive molecules with antibacterial and anticancer properties (Ebel, 2012). Yet, prior to this study, the five marine fungi selected (*Acremonium* sp. TS7, *Typhula* sp., *A. encephaloides*, *Pseudogumnoacus* sp. TS12 and *D. marina*) have only been investigated to a small extent. The three wood- and algae-associated obligate species have not been studied for bioactivity before, whereas there are some studies on the two remaining species (e.g. Khamthong et al., 2014). The marine fungi were fermented using the OSMAC-approach with four different media and with two different temperatures, to investigate their antibacterial and anticancer potential. Half of the fermentations were co-cultivated with the marine bacteria *Leeuwenhoekiella* sp. The total number of fermentations was 120. The aim with the OSMAC-approach was to activate possible silent gene clusters that could activate production of bioactive secondary metabolites.

4.1 Crude extract yield

The 120 crude extracts varied in the terms of yields. *D. marina* (705) had overall a higher extraction yield from fermentations, but its incubation period was almost two weeks shorter than *Acremonium* sp. TS7 (701) which had the smallest yield of the four marine fungi (Fig. 5). This indicates that the crude extract yields are dependent on the fungal species. The parameter that showed significant increase in the mass of the crude extracts was incubation temperature. *Acremonium* sp. TS7 (701), *Typhula* sp. (702), *A. encephaloides* (703) and *D. marina* (705) all had higher crude extracts yields from the fermentations with 15°C incubations (Fig. 6). Although the natural habitats of the four marine fungi are in cold water, they seemingly grow better at higher temperatures. It is therefore possible that using incubations at higher temperature would lead to better growth (e.g. 20°C or 30°C). Contrary to the fermentations with 15°C incubation, the 10°C incubation also had 100 rpm shaking. It was observed that the mycelia in the 15°C incubations were fluffy-like, while the mycelia in the fermentations with 10 °C incubations were more compact. The constant movement may have reduced the growth by constantly disturbing the mycelia growth, resulting in compact mycelia. The setup of

the fermentation did not include 15°C with 100 rpm shaking, therefore it is not possible to extract an explanation, besides the temperature, for the higher yield overall in the 15°C incubation. The other parameters selected for this study, i.e. four different media and co-cultivation (with *Leeuwenhoekiella* sp.) did not show any effect on the mass of the crude extracts.

The marine fungi *A. encephaloides* (703) and *D. marina* (705) were sampled from driftwood, but did not show any improved growth in the wood medium compared with the other three media. *Typhula* sp. (702) was sampled from brown seaweeds and did not grow any better on seaweed medium compared with the other three media. *Acremonium* sp. TS7 (701) was isolated from a marine sponge and did not favor any of the four media. Which media type used did not have a great effect on extraction yield (Fig. 8). The complex carbohydrate media (SM and WM) had a little higher crude extracts yields compared to MEM and CFM. The complex carbohydrates media (SW and VM) were not homogeneous and contained both small and large particles. Some of the medium particles were transferred during the extraction process over to the crude extracts. The mass of the crude extracts yields may have measured higher than the true mass of the crude extracts. This may have inflicted on the crude extracts stock solution, which gave lower crude extracts concentrations. It was not found any significant difference in crude extracts yields between monoculture and co-culture. The reason for this is unknown.

4.2 Bioactivity – Crude extracts

There were 120 fermentations that were extracted. The fermentations with *Pseudogumnoacus* sp. TS12 (704) were contaminated and excluded from further work. The remaining 96 crude extracts remaining would have taken up to four months to prefractionate with flash chromatography. This had been a too laborious work to be included in the project, and for this reason a prioritizing of the extracts for prefractionation was done based on their potential antibacterial and anticancer activity. The crude extracts were tested with 50 µg/mL and 100 µg/mL concentrations for antibacterial activity. None of the crude extracts showed any activity against the five human pathogenic bacteria used. The lack of activity was surprising. The cancer screening showed no anticancer activity for the 96 crude extracts tested. Five of the samples did, however, give a reduction of cell survival (~75% cell survival) (Fig. 10).

The reductions in these five crude extracts did not go under the cut-off value (< 50% cell survival), therefore they cannot be classified as active. The cut-off value is set for guidance and is mainly used for fractions tested. Fractions are more concentrated and would contain more of the bioactive compounds. Crude extract is a solution that contains every extracted metabolite and is therefore very complex (Reid & Sarker, 2012). The complexity of the crude extracts may be one reason why all tested crude extracts were inactive in the antibacterial and anticancer screening. Secondary metabolites are usually produced in small quantities and the other components in the crude extracts can overshadow these secondary metabolites (Pham et al., 2015). The complexity of the crude extracts and the fact that every compound extracted is theoretically added to each well in small amounts makes the test concentration contain very small quantities of the active compound.

4.3 Prefractionation

No hits were generated from antibacterial and anticancer screening with the crude extracts. Prefractionating every crude extract was excluded, as it would have been too time consuming. A selection of crude extracts for prefractionation was selected based on crude extract yield. All crude extracts selected were incubated at 15°C, where the yield was the highest. Two crude extracts from each isolate were selected, one monoculture and one co-culture, both with same media within the isolate. The following samples were prefractionated: *Acremonium* sp. TS7 X701A.2 and XY701A.2, *Typhula* sp. X702D.2 and XY702D.2, *A. encephaloides* X703C.2 and XY703C.2, *D. marina* X705B.2 and XY705B.2. The prefractionation of the eight crude extracts gave in total 48 fractions. The crude extracts are from different marine fungi, therefore the amounts of material eluted in the different fractions were expected to be different when all fractions are compared. Fractions 1 and 2 contained the most material eluted in majority of the prefractionated samples (Fig. 11). These fractions contained the most polar compounds in the crude extracts. Carbohydrates and salts are usually the main contributors to these fractions. The rest of the fractions vary in yield. The media differs in composition and can contribute to the variations. The amount of material in each fraction does not give any information of what the fractions contain. Fraction 6 in X702D.2 is unusually large (Fig. 11). The overall yield in each fraction was very small (fraction 2-5 <10 mg). When

comparing them with fraction 6 (80 mg), they would seem abnormally small. The X702D.2 was fermented on SM, which had media components (particles) transferred during the extraction. A possible explanation for the low yields in the fractions may be that these impurities contributed to large portion of the crude extract mass. The prefractionation column used would naturally remove these impurities.

4.4 Bioassays – Flash fractions

The prefractionation of the eight crude extracts gave in total 48 fractions. These were tested for antibacterial activity. From the antibacterial screening three active and two questionable fractions were found against *S. agalactiae* (Table 14). All four marine fungi had at leaset one active fraction. No activity was found against the other pathogenic bacteria. The cut-off value is a predetermined value for guidance. Four out of five active fractions were co-cultured with *Leeuwenhoekiella* sp. and were all from fraction 5. Fraction 5 from prefractionation is quite nonpolar, while fraction 6 is the most nonpolar. Polarity is an important factor for finding activity and very polar compounds are usually incapable to enter cells through the cell membrane. Compounds from fractions 4 and 5 are usually polar enough to penetrate the cell membrane without precipitating in an aqueous environment. The five active fractions (XY701A.2-5, XY702D.2-5, X703C.2-5, XY703C.2-5 and X705D.2-5) were retested in an antibacterial assay to find minimal inhibition concentration (MIC). The first round of retesting showed no activity for any of the fractions tested and it was suspected that the fractions had precipitated in the stock plate. To investigate a possible solubility issue, a new deep well plate with the active fractions was made. Even though all five fractions should have given activity, only the second retest produced a hit with fraction XY705D.2-5 at only 50 µg/mL. The five fractions tested should all have been active (or questionable) at 50 µg/mL. Thus it appeared that the activities against *S. agalactiae* were lost. The absence of bioactivity is a problem and the solubility may be an issue. The fractions were dissolved in 2.5% DMSO and MQ-H_2O. DMSO was added to dissolve the dry fraction and increase the solubility of nonpolar compounds with water. The concentration may have been too low to make a stable homogenous solution, which in turn led to the inconsistent results.

The 48 fractions were tested for anticancer activity. None of the fractions had any activity against the A2058 cancer cell line. It was expected to find more activity, especially in fractions from *D. marina* (705) that showed reduction in cell survival when tested on crude extracts. The *D. marina* (705) crude extracts that were found to reduce cell survival were not the same crude extracts that were prefractionated. They were either fermented on different media or incubated at different temperatures. The crude extracts that reduced cell survival should be prefractionated and screened for anticancer activity to be certain that *D. marina* (705) did not produce any anticancer active compounds.

4.5 Dereplication

The following samples were dereplicated: *Acremonium* sp. TS7 X701A.2-5 and XY701A.2-5, *Typhula* sp. X702D.2-5 and XY702D.2-5, *A. encephaloides* X703C.2-5 and XY703C.2-5, *D. marina* X705B.2-5 and XY705B.2-5. The chromatograms revealed that the active fractions, excluding X703C.2-5, had the same compounds that were missing from the inactive fractions. Compounds *m/z* 527, 553, 555 and 581 were eluted with identical retention time had similar fragment patterns. The compounds were found in all four species (*Acremonium* sp. TS7, *Typhula* sp., *A. encephaloides* and *D. marina*) and only in the co-cultured fractions. The only commonality between the active fractions was co-cultivation with *Leeuwenhoekiella* sp. The chromatograms of the active and inactive fractions indicate that the fungi are not responsible for the antibacterial activity found, but *Leeuwenhoekiella* sp. may be the responsible species. The chromatograms of the media controls with *Leeuwenhoekiella* sp. added, revealed three out of the four compounds found in the active fractions. Only compound *m/z* 581 is absent in the media controls, the reason for this is unknown. The most likely explanation for the antibacterial activity found is that *Leeuwenhoekiella* sp. is the responsible producer of these compounds. The marine fungi may have induced production of compound *m/z* 581 in the active fractions, explaining the absence of peak 581 in the media controls.

From the *m/z*, the retention time and the fragment patterns, it was quite clear at Marbio that these compounds have been seen before and that the compounds $C_{26}H_{48}O_9$, $C_{28}H_{50}O_9$, $C_{28}H_{52}O_9$ and $C_{30}H_{54}O_9$ are most likely Rhamnolipids. These four compounds, with the same molecular weight and formula, are among known mono-rhamno-di-lipidic congeners (Abdel-Mawgoud et al., 2010). Rhamnolipids are a diverse group of glycolipids that have been extensively investigated. Rhamnolipids typically contain 3-hydroxyfatty acids linked through a beta-glycosidic bond to a mono- or di-rhamnoses (Vatsa, Sanchez, Clement, Baillieul, & Dorey, 2010). Rhamnolipids are produced by bacteria were *P. aeruginosa* being the most known producer, but several other rhamnolipid-producing bacteria have been reported. The functions of rhamnolipids are unclear (Abdel-Mawgoud et al., 2010). Most rhamnolipids have been reported as powerful biosurfactants and antimicrobial properties of rhamnolipids were found as early as 1971 (Itoh, Honda, Tomita, & Suzuki, 1971). They have shown antibacterial activity against both gram-positive and gram-negative bacteria (Lang et al 1989). Antifungal activities have also been found (Benincasa, Abalos, Oliveira, & Manresa, 2004; Haba et al., 2003). Benincasa et al. (2004) found a varying activity against *E. coli, S. aureus, P. aeruginosa and S. faecalis*, but no activity was found against these bacteria strains in 2.9.2. Not necessary unexpected, since the rhamnolipids used by Benincasa et al. (2004) were far more pure than fractions used on the antibacteria screening (2.9.2). Even though the fractions were separated by polarity, the mixture still contained a mixture of compounds. All of the bacteria controls with *Leeuwenhoekiella* sp. contained rhamnolipids, which indicates that these compounds may be produced independently of other microorganisms present. Compound *m/z* 581 were not present in the bacteria controls, suggesting that the marine fungi may induce this compound.

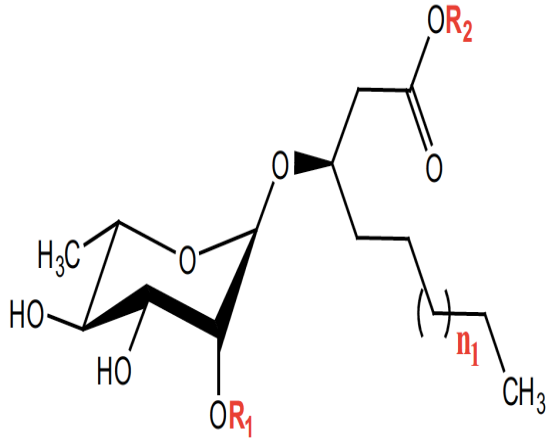

Figure 1: The general structure for a mono-rhamno-di-lipidic compound. The molecule contains one rhamnose and two lipid parts. The R_1, R_2 and n_1 vary depending on molecular formula (Abdel-Mawgoud, Lépine, & Déziel, 2010).

4.6 The OSMAC-approach

The OSMAC-approach does have documented effects on the production of bioactive secondary metabolites, either as inducing or increasing production (Bode et al., 2002; Schroeckh et al., 2009). Despite of this, the bioactivity found in the 120 crude extracts and 48 fractions was far less than expected. The antibacterial retest did not give consistent results, as four out of five fractions had lost the activity. The fermentation parameters selected did not affect the production of bioactive compounds as expected. The media that were used contained either small amounts of complex carbohydrates or simple carbohydrates. This makes them nutrient-poor, which is what marine fungi generally prefer. One of the purposes of using the OSMAC-approach is to stress the marine fungi. The nutrient-poor media may have not stressed the marine fungi enough and perhaps a media containing more nutrients would be more stressful for the marine fungi, than lowering the nutrients even more. The OSMAC-approach is a try-and-fail method; not every altered parameter is going to give good results. Warmer incubation gave higher crude extract yield. Starting new cultures may not guarantee that the same compounds are produced, but using simple modifications such as temperature alterations can beneficial in discovering novel compounds. Modifications of fermentation medium based on the fungal habitat did not give beneficial results as expected.

Co-cultivation has been proven to increase production of secondary metabolites and can activate silent gene clusters (Rateb & Ebel, 2011; Rateb et al., 2013). This was not seen in the 60 fermentations that were co-cultivated with *Leeuwenhoekiella* sp. The major problems of co-cultivations are that bacteria can produce bioactive molecules and take over the culture, especially when cultured with generally slow-growing marine fungi. The inoculations with *Leeuwenhoekiella* sp. were executed for all fermentations at the same day and many of the fermentations were extracted between 4-8 weeks after the inoculation. There is a great possibility that *Leeuwenhoekiella* sp. overtook several of the fermentations during incubation. The timing of inoculation may be very crucial for the ability of the marine fungi to respond with production of secondary metabolites that inhibit bacteriocidal or bacteriostatic effect. Too long incubation time and the bacteria may overtake the culture. Too short and the fungi may not be able to respond to the invasion of bacteria and produce antibacterial compounds. The dereplication of the five

active fractions identified that *Leeuwenhoekiella* sp. are the likely producer of the bioactive rhamnolipids. There will always be a probability that the inoculated bacteria can produce bioactive compounds. A possible way to reduce this risk is by adding the bacteria wall component lipopolysaccharides (LPS) to the fermentations. A preliminary study found that 15% of the tested fungi reacted to inoculation of LPS by activating or increasing productions of secondary metabolites (Khalil et al., 2014), This method may be an alternative to the more traditional co-cultivation strategy, but will need more investigation. Very few of the fermentations were prefractionated, thus a full investigation of the OSMAC effect on the fungi on the metabolomic level cannot be done. This can however be executed in the future.

There is a lot of time invested in the fermentation process and it is a question whether the time invested gave the maximal yield in the end. The extraction method with Diaon® HP-20 and methanol is used with marine bacteria as well. This method extracts, for the most part, only extracellular secondary metabolites. The full potential of the four marine fungi would have been explored if the intracellular secondary metabolites had also been extracted. With the time used to ferment these four marine fungi, it would not require that much more work to extract both extracellular and intracellular secondary compounds. It is perhaps beneficial to investigate if extraction of extracellular and intracellular secondary metabolites increases the change to discover novel compounds with bioactivity in the future.

5. Conclusions

The four marine fungi *Acremonium* sp. TS7, *Typhula* sp., *A. encephaloides* and *D. marina* were studied for discovery of antibacterial and anticancer secondary metabolites with the OSMAC-approach. These four marine fungi were fermented on four different media and at two different temperatures. Half of the fermentations were co-cultivated with the marine bacteria *Leeuwenhoekiella* sp. In total, 96 fermentations were made and extracted with Diaon® HP-20 and methanol. The only parameter that increased crude extracts yields was 15°C incubation. No antibacterial or anticancer activity was found in the 96 crude extracts tested. Eight crude extracts were prefractionated based on crude extracts yields, while five of 48 fractions were found active against *S. agalactiae*. Mass spectrometric analysis of the five active fractions and bacteria controls revealed the same compounds responsible for the antibacterial activity. These compounds were identified as Rhamnolipids and have known bioactivity. The producer of Rhamnolipids is most likely the marine bacterium *Leeuwenhoekiella* sp., which was used in the co-cultivated fermentations. The OSMAC-approach is a method for inducing the activation of silent gene clusters and the production of secondary metabolites by applying stress to the fungi. The observed effects of this approach were not as expected, which might due to: not enough stress; wrong microorganism used in co-cultivation; the four marine fungi cannot produce antibacterial and anticancer secondary metabolites, though this is unlikely. The potential of the four marine fungi *Acremonium* sp. TS7, *Typhula* sp., *A. encephaloides* and *D. marina* have not been fully explored with the parameters used. More stressful growth conditions should be tested in the future or a genome analysis that could find hidden gene clusters should be explored. Alternative extractions methods could also be explored. This study alone cannot exclude the possibility that *Acremonium* sp. TS7, *Typhula* sp., *A. encephaloides* and *D. marina* can produce antibacterial and anticancer secondary metabolites. This study has given useful information that could assist future work in the field.

6. References

Abdel-Mawgoud, A. M., Lépine, F., & Déziel, E. (2010). Rhamnolipids: diversity of structures, microbial origins and roles. *Applied microbiology and biotechnology, 86*(5), 1323-1336.

Bartlett, J. G., Gilbert, D. N., & Spellberg, B. (2013). Seven Ways to Preserve the Miracle of Antibiotics. *Clinical Infectious Diseases, 56*(10), 1445-1450.

Benincasa, M., Abalos, A., Oliveira, I., & Manresa, A. (2004). Chemical structure, surface properties and biological activities of the biosurfactant produced by Pseudomonas aeruginosa LBI from soapstock. *Antonie Van Leeuwenhoek, 85*(1), 1-8.

Bode, H. B., Bethe, B., Höfs, R., & Zeeck, A. (2002). Big effects from small changes: possible ways to explore nature's chemical diversity. *ChemBioChem, 3*(7), 619-627.

Bolhuis, H., & Cretoiu, M. S. (2016). What is so Special About Marine Microorganisms? Introduction to the Marine Microbiome - From Diversity to Biotechnological Potential. In L. J. Stal & M. S. Cretoiu (Eds.), *The Marine Microbiome: An Untapped Source of Biodiversity and Biotechnological Potential* (p. 3-17). Switzerland: Springer International Publishing

Carnero, A. (2006). High throughput screening in drug discovery. *Clinical and Translational Oncology, 8*(7), 482-490.

Chai, Y.-J., Cui, C.-B., Li, C.-W., Wu, C.-J., Tian, C.-K., & Hua, W. (2012). Activation of the dormant secondary metabolite production by introducing gentamicin-resistance in a marine-derived Penicillium purpurogenum G59. *Marine drugs, 10*(3), 559-582.

Chen, L., Todd, R., Kiehlbauch, J., Walters, M., & Kallen, A. (2017). Notes from the Field: Pan-Resistant New Delhi Metallo-Beta-Lactamase-Producing Klebsiella pneumoniae - Washoe County, Nevada, 2016. *MMWR Morb Mortal Wkly Rep, 66*(1), 33.

Chernushevich, I. V., Loboda, A. V., & Thomson, B. A. (2001). An introduction to quadrupole‐time‐of‐flight mass spectrometry. *Journal of Mass Spectrometry, 36*(8), 849-865.

Chiang, Y.-M., Lee, K.-H., Sanchez, J. F., Keller, N. P., & Wang, C. C. (2009). Unlocking fungal cryptic natural products. *Natural product communications, 4*(11), 1505.

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

Ebel, R. (2012). Natural products from marine-derived fungi. In E. G. Jones & K.-L. Pang (Eds.), *Marine Fungi: and Fungal-like Organisms*. Germany: Walter de Gruyter.

Gaudencio, S. P., & Pereira, F. (2015). Dereplication: racing to speed up the natural products discovery process. *Natural Product Reports, 32*(6), 779-810.

Haba, E., Pinazo, A., Jauregui, O., Espuny, M., Infante, M. R., & Manresa, A. (2003). Physicochemical characterization and antimicrobial properties of rhamnolipids produced by Pseudomonas aeruginosa 47T2 NCBIM 40044. *Biotechnology and bioengineering, 81*(3), 316-322.

Houssen, W. E., & Jaspars, M. (2012). Isolation of Marine Natural Products. In S. D. Sarker & L. Nahar (Eds.), *Natural products isolation* (p. 367-390). New York City: Humana Press Inc. .

Hyde, K. D., Jones, E. B. G., Leaño, E., Pointing, S. B., Poonyth, A. D., & Vrijmoed, L. L. P. (1998). Role of fungi in marine ecosystems. *Biodiversity & Conservation, 7*(9),

Imhoff, J. F. (2016). Natural products from marine fungi—Still an underrepresented resource. *Marine drugs, 14*(1), 19.

Itoh, S., Honda, H., Tomita, F., & Suzuki, T. (1971). Rhamnolipids produced by Pseudomonas aeruginosa grown on n-paraffin (mixture of C12, C13 and C14 fractions). *The Journal of antibiotics, 24*(12), 855-859.

Jones, E., Sakayaroj, J., Suetrong, S., Somrithipol, S., & Pang, K. (2009). Classification of marine Ascomycota, anamorphic taxa and Basidiomycota. *Fungal Diversity, 35*(1), 187.

Jones, E. G., & Jennings, D. (1964). The effect of salinity on the growth of marine fungi in comparison with non-marine species. *Transactions of the British Mycological Society, 47*(4), 619-625.

Khalil, Z. G., Kalansuriya, P., & Capon, R. J. (2014). Lipopolysaccharide (LPS) stimulation of fungal secondary metabolism. *Mycology, 5*(3), 168-178.

Khamthong, N., Rukachaisirikul, V., Pakawatchai, C., Saithong, S., Phongpaichit, S., Preedanon, S., & Sakayaroj, J. (2014). Acremonoside, a phenolic glucoside from the sea fan-derived fungus Acremonium polychromum PSU-F125. *Phytochemistry Letters, 10*, 50-54.

Kohlmeyer, J., & Kohlmeyer, E. (1979). Marine mycology: the higher fungi (p. 1-5): Academic Press.

Lampman, G. M., Pavia, D. L., Kriz, G. S., & Vyvyan, J. R. (2010). Mass Spectrometry *Spectroscopy* (p. 418-439). Canada: Brooks/Cole, Cengage Learning

Madigan, M. T., Martinko, J. M., Stahl, D. A., & Clark, D. P. (2012a). Eukaryotic Cell Biology and Eukaryotic Microorganisms In D. Espinoza (Ed.), *Brock Biology of Microorganisms* (13 ed., p. 612-636). USA: Pearson Education

Madigan, M. T., Martinko, J. M., Stahl, D. A., & Clark, D. P. (2012b). Microbial Growth Control In D. Espinoza (Ed.), *Brock Biology of Microorganisms* (13 ed., p. 790-791). USA: Pearson Education

Martins, A., Vieira, H., Gaspar, H., & Santos, S. (2014). Marketed Marine Natural Products in the Pharmaceutical and Cosmeceutical Industries: Tips for Success. *Marine drugs, 12*(2), 1066.

Mishra, K., Ganju, L., Sairam, M., Banerjee, P., & Sawhney, R. (2008). A review of high throughput technology for the screening of natural products. *Biomedicine & Pharmacotherapy, 62*(2), 94-98.

Nielsen, K. F., Månsson, M., Rank, C., Frisvad, J. C., & Larsen, T. O. (2011). Dereplication of Microbial Natural Products by LC-DAD-TOFMS. *Journal of Natural Products, 74*(11), 2338-2348.

Pang, K.-L., Overy, D. P., Jones, E. B. G., Calado, M. d. L., Burgaud, G., Walker, A. K., Bills, G. F. (2016). 'Marine fungi' and 'marine-derived fungi' in natural product chemistry research: Toward a new consensual definition. *Fungal Biology Reviews, 30*(4), 163-175.

Pham, N. B., Toms, S., Camp, D., & Quinn, R. J. (2015). Logistic Considerations to Deliver Natural Product Libraries for High-Throughput Screening. In B. J. Baker (Ed.), *Marine biomedicine: from beach to bedside* (pp. 90-92). Boca Raton, Florida CRC Press.

Promega. (2012). *CellTiter 96 AQueous One Solution Cell Proliferation Assay*. Retrieved from USA:

Raper, K. B., Alexander, D. F., & Coghill, R. D. (1944). Penicillin: II. Natural Variation and Penicillin Production in Penicillium notatum and Allied Species1, 2. *Journal of bacteriology, 48*(6), 639.

Rateb, M. E., & Ebel, R. (2011). Secondary metabolites of fungi from marine habitats. *Natural Product Reports, 28*(2), 290-344.

Rateb, M. E., Hallyburton, I., Houssen, W. E., Bull, A. T., Goodfellow, M., Santhanam, R., Ebel, R. (2013). Induction of diverse secondary metabolites in Aspergillus fumigatus by microbial co-culture. *RSC Advances, 3*(34), 14444-14450.

Redou, V., Vallet, M., Meslet-Cladiere, L., Kumar, A., Pang, K.-L., Pouchus, Y.-F., . . . Burgaud, G. (2016). Marine Fungi. In L. J. Stal & M. S. Cretoiu (Eds.), *The Marine Microbiome: An Untapped Source of Biodiversity and Biotechnological Potential* (p. 99-154). Switzerland: Springer International Publishing

Reid, R. G., & Sarker, S. D. (2012). Isolation of Natural Products by Low-Pressure Column Chromatography. In S. D. Sarker & L. Nahar (Eds.), *Natural products isolation* (3 ed., p. 155-183). USA: Humana Press Inc.

Sarker, S. D., & Nahar, L. (2012a). Hyphenated Techniques and Their Applications in Natural Products. In S. D. Sarker & L. Nahar (Eds.), *An introduction to natural products isolation* (3 ed., Vol. 864, p. 307-309). USA: Humana Press Inc. .

Sarker, S. D., & Nahar, L. (2012b). Natural products isolation. In S. D. Sarker & L. Nahar (Eds.), *An introduction to natural products isolation* (3 ed., Vol. 864, p. 1-22). USA: Humana Press Inc.

Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., . . . Crous, P. W. (2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences, 109*(16), 6241-6246.

Schroeckh, V., Scherlach, K., Nützmann, H.-W., Shelest, E., Schmidt-Heck, W., Schuemann, J., Brakhage, A. A. (2009). Intimate bacterial–fungal interaction triggers biosynthesis of archetypal polyketides in Aspergillus nidulans. *Proceedings of the National Academy of Sciences of the United States of America, 106*(34), 14558-14563.

Silber, J., Kramer, A., Labes, A., & Tasdemir, D. (2016). From Discovery to Production: Biotechnology of Marine Fungi for the Production of New Antibiotics. *Marine drugs, 14*(7), 137.

Sithranga Boopathy, N., & Kathiresan, K. (2010). *Anticancer Drugs from Marine Flora: An Overview* (Vol. 2010).

Swinney, D. (2013). Phenotypic vs. target-based drug discovery for first-in-class medicines. *Clin Pharmacol Ther, 93*(4), 299-301.

Valgas, C., Souza, S. M. d., Smânia, E. F., & Smânia Jr, A. (2007). Screening methods to determine antibacterial activity of natural products. *Brazilian Journal of Microbiology, 38*(2), 369-380.

Vasaikar, S., Bhatia, P., Bhatia, P. G., & Chu Yaiw, K. (2016). Complementary Approaches to Existing Target Based Drug Discovery for Identifying Novel Drug Targets. *Biomedicines, 4*(4), 27.

Vatsa, P., Sanchez, L., Clement, C., Baillieul, F., & Dorey, S. (2010). Rhamnolipid biosurfactants as new players in animal and plant defense against microbes. *International journal of molecular sciences, 11*(12), 5095-5108.

Vishniac, W., & Santer, M. (1957). The thiobacilli. *Bacteriological Reviews, 21*(3), 195.

WHO. (2014). *Antimicrobial resistance: global report on surveillance*. Switzerland: World Health Organization.

Wiese, J., Ohlendorf, B., Blümel, M., Schmaljohann, R., & Imhoff, J. F. (2011). Phylogenetic identification of fungi isolated from the marine sponge Tethya aurantium and identification of their secondary metabolites. *Marine drugs, 9*(4), 561-585.

Wu, B., Oesker, V., Wiese, J., Schmaljohann, R., & Imhoff, J. F. (2014). Two new antibiotic pyridones produced by a marine fungus, Trichoderma sp. strain MF106. *Marine drugs, 12*(3), 1208-1219.

Appendix 1

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Exploring the Antibacterial and Anticancer Potential of Five Marine Fungi

Hanne Bragmo, Jeanette Hammer Andersen, Espen Hansen, Teppo Rämä
Marbio, The Norwegian College of Fishery Science, Faculty of Biosciences, Fisheries and Economics, UiT – The Arctic University of Norway, Norway

/ INTRODUCTION

The marine environment has in little degree been exploited for natural products compared to the terrestrial parts of the world. Even less research has been done on marine microorganisms. The marine environment offers a huge diversity that has through evolution given highly adapted and specialized organisms. The traditional methods of fermentation using media for optimal growth can restrict the activation of metabolic pathways and as a result various metabolites will not be formed. The OSMAC-approach (One Strain, Many Compounds) include stressing the organism by multiple factors like temperature, competition from other species and limitation of nutrients. The theory with the OSMAC-approach is that this stress will activate more metabolic pathways, resulting in a higher diversity in the secondary metabolites produced.

/ AIMS OF THE STUDY

- Study whether fungal isolates produce different secondary metabolites under different fermentation conditions.
- Identify crude extracts and Flash fractions with anticancer or antibacterial activity.
- Study whether co-cultivation increases production of secondary metabolites.

/ MATERIALS AND METHODS

Four marine fungi isolated from different habitats where studied for anticancer and antibacterial activity. *Amylocarpus encephaloides* and *Digitatispora marina* were both isolated from driftwood in arctic waters. *Typhula* sp. was isolated from seaweeds in Northern Norway. The last studied marine fungus was *Acremonium* sp. that was isolated from a deep-sea marine sponge. Four different media were prepared using artificial seawater and minimal medium (Table 1, fig. 2). The marine bacteria *Leeuwenhoekiella* sp.(strain M09W024) was used for co-cultivation. Media controls with and without co-cultivation for each media where made. The marine fungi where extracted using the resin Diaon® HP-20 as an absorbent. The resin was added two days before extraction. The small compounds absorbed by the resin where eluted with 100% methanol and vacuum filtration.

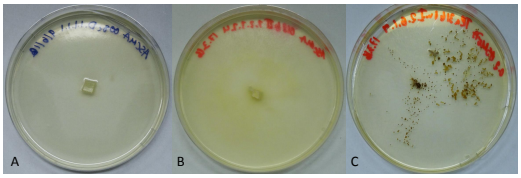

Figure 1. *Digitatispora marina* (A), *Amylocarpus encephaloides* (B) and *Typhula* sp. on agar before fermentation.

Table 1. Media used

Media	Carbohydrate source
Simple Carbohydrates Media 1	Malt Extract
Simple Carbohydrates Media 2	Corn Flour
Complex Carbohydrates Media 1	Woodchips
Complex Carbohydrates Media 2	Seaweed

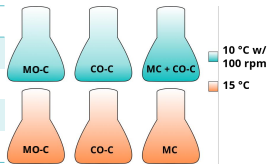

Figure 2. Illustrates setup for fermentation per isolate per media. MO-C = Monoculture, CO-C = Co-cultivation, MC = Media Control and MC + CO-C = Media control + Co-Cultivation.

/ RESULTS

Preliminary results show a trend in the following:

- Simple carbohydrate sources and higher temperature resulted in higher biomass production.
- Anticancer activity against human melanoma cell line A2058 was observed. The majority of active crude extracts came from low temperature with shaking.
- No antibacterial activity was observed on any of the crude extracts.
- Antibacterial activity was observed in all prefractionated extracts. Three out of four of the isolates had only activity in the co-cultivations.

/ DISCUSSION

- All species grew better on simple than complex carbohydrates sources, although three of the fungi studied were either seaweed- or driftwood-associated. This indicates that specialized growth media may not be as beneficial for growth as expected.
- The lack of antibacterial activity in the crude extracts was unexpected and the concentrations may be too low to be detected in bioassays. Furthermore, active compounds may be masked by media components and other compounds in the crude extracts.
- Co-cultivation induced antibacterial activity which suggests co-cultivation to be an effective tool for triggering production of antibacterial compounds.

/ FUTURE WORK

- Use Flash Chromatography to prefractionate all 96 crude extracts by polarity.
- Test fractions for anticancer and antibacterial activity.
- Dereplicate active fractions.

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