UiT The Arctic University of Norway

Norwegian College of Fishery Science

Manipulating the Epigenetic Machinery of Marine Fungi Through the Application of Epigenetic Modifiers to Induce the Biosynthesis of Novel Secondary Metabolites

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60 credits

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Cathrine Eiken Marø

Abstract

Fungi are among the most prolific producers of secondary metabolites, many of which have huge utility for society as various anti-infective and anticancer drugs. The genes encoding these secondary metabolites are arranged in continuous biosynthetic gene clusters (BGCs). Each fungus can have tens of different BGCs in its genome, possessing the ability to produce a multitude of secondary metabolites with potential for medical utility. However, most of the BGCs are silent under standard laboratory conditions, packaged in heterochromatin, inaccessible for transcription. To activate these clusters the heterochromatin must be converted to euchromatin, the transcription accessible form. The addition of small molecule epigenetic modifiers, inhibiting the activity of transcription repressive epigenetic enzymes, have been proven to effectively induce secondary metabolism in many fungi. As this approach does not require genetic manipulation, it is potentially applicable to a broad range of fungi, recalcitrant to genetic manipulation. This method could therefore have the potential for implementation. the high-throughput screening of microorganisms at Marbio.

In this thesis, the method of manipulating the epigenetic machinery of marine filamentous fungi by the application of epigenetic modifiers 5-azacytidine, SBHA, and nicotinamide were employed as a means for inducing activation of biosynthetic gene clusters. This is the first time epigenetic modifiers have been applied to any microbial cultures at Marbio. The fungi investigated were cultivated in solid and liquid media with added epigenetic inhibitors in different combinations to study the effects of the modifiers on the fungi. The fungal cultures were subsequently extracted and subjected to bioactivity testing. The bioactivity screening revealed that epigenetic modifiers did not induce the production of bioactive secondary metabolites in detectable amounts. However, SBHA was found to alter colony morphology and pigmentation in some cultures, indicating that these fungi are accessible for epigenetic manipulation by HDAC inhibitors. Increased concentrations or applications of more potent HDAC inhibitors such as SAHA, might be necessary to observe more evident changes in the fungal metabolomes.

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Abbreviations

A	5-azacytidine
Abs	Absorbance
ANA	*Ascophyllum nodosum* agar
BGC	Biosynthetic gene cluster
BHI	Brain heart infusion
CMA	Corn meal agar
D-MEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxid
DNMT	DNA methyltransferase
DWP	Deep well plate
E-MEM	MEM Earle's
ESI	Electrospray ionization
ITS	Internal Transcribed Spacer
HDAC	Histone deacetylase
LLE	Liquid-liquid extraction
LPS	Lipopolysaccharide
MeOH	Methanol
MH	Mueller Hinton
MIC	Minimum inhibitory concentration
MNP	Marine natural product
MS	Mass spectrometry
MTS	[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS]
m/z	Mass per charge
N	Nicotinamide
OD	Optical density
PDA	Potato dextrose agar
RT	Retention time
S	SBHA/ Suberoyl bis-hydroxamic acid
SBHA	Suberoyl bis-hydroxamic acid
SAHA	Suberoylanilide hydroxamic acid
SPE	Solid phase extraction
SM	Secondary metabolite
TOF	Time of flight
TF	Transcription factor
TSA	Trichostatin A

1 Introduction

1.1 Secondary metabolites for drug discovery

Secondary metabolites are compounds of low molecular weight with restricted taxonomic distribution. The term natural product (NP) is often used as a synonym for secondary metabolites of natural origin. The most prominent producers of secondary metabolites are microorganisms such as bacteria and fungi(Hutchings et al., 2019), where their primary function is to enhance the producing organism's fitness and chance of survival by repelling or attracting other organisms (Hanson, 2003; McMurry, 2009). These compounds are also widely recognized to frequently exhibit activities of high medical utility such as anti-infective, immunosuppressive and anticancer properties. (Keller et al., 2005; Winter et al., 2011; Zerikly & Challis, 2009). Therefore over half of all drugs are secondary metabolites of natural origin or based on such compounds (Montaser & Lunesch, 2011). Despite this, the research into secondary metabolites has diminished during the last two decades due to technical barriers and high rediscovery rate. However, in the era of genomics secondary metabolites are regaining attention. New genomics approaches have revealed that the potential of secondary metabolite production, in fungi especially, is much larger than formerly believed and technical advancements are making secondary metabolites increasingly accessible (Harvey et al., 2015; Koehn & Carter, 2005).

1.1.1 Potential of marine fungi

There is a high abundance of species within the fungal kingdom with unique and unusual biochemical pathways producing secondary metabolites and enzymes of high pharmaceutical utility such as penicillin, cyclosporin and aflatoxins (Keller et al., 2005). The first systematic study of fungal secondary metabolites started in 1922, and the following discovery of penicillin in 1928 led to a widespread interest in fungal metabolites (Fleming, 1929). Since then the screening for fungal secondary metabolites has continued unabated and thousands of compounds possessing antibacterial, antifungal, antiprotozoal, antiparasitic anti-insect, antivirus and anticancer activity have been identified (Keller et al., 2005).

Terrestrial fungi have been studied and utilized thoroughly for the last 50 years yielding thousands of fungal secondary metabolites with enormous therapeutic value (Aly et al., 2011), while marine fungi have just recently started to gain the same attention in the wake of the increasing rediscovery rate of already known compounds from terrestrial sources. Due to their harsh environment, marine species have had to evolve specialized mechanisms to survive in terms of light, salinity and pressure. This is reflected by the multitude of compounds produced by marine species to protect themselves from biotic and abiotic stresses, multiple compounds which has no terrestrial analogues (Jaspars et al., 2015).

Marine fungi are a highly diverse taxonomic group and inhabits a huge variety of marine habitats, such as sea water, corals, algae and marine sediment. They have numerus ecological roles in their habitats, including degradation of organic material, parasitic and mutualistic interactions with other marine organisms such as algae and animals, and some are involved in denitrifications processes in marine sediments (Zhang et al., 2015). Marine fungi are often divided into two groups, obligate marine fungi and facultative marine fungi. While facultative

marine fungi are able to grow and sporulate in both marine, freshwater and terrestrial environments, obligate marine fungi grow and sporulate exclusively in marine habitats (Pang et al., 2016).

The number of new compounds identified from marine fungi has increased significantly recent years with an increase of 38% from 2017 to 2018 (figure 1). The increase in overall discovery rate during the last three years is 85%. If this discovery rate continues, fungi are predicted to exceed cnidarians as second most prolific source of marine natural products in 2021 and overtake the status as most prolific source of new marine natural products from sponges in 2024 (Carroll et al., 2020).

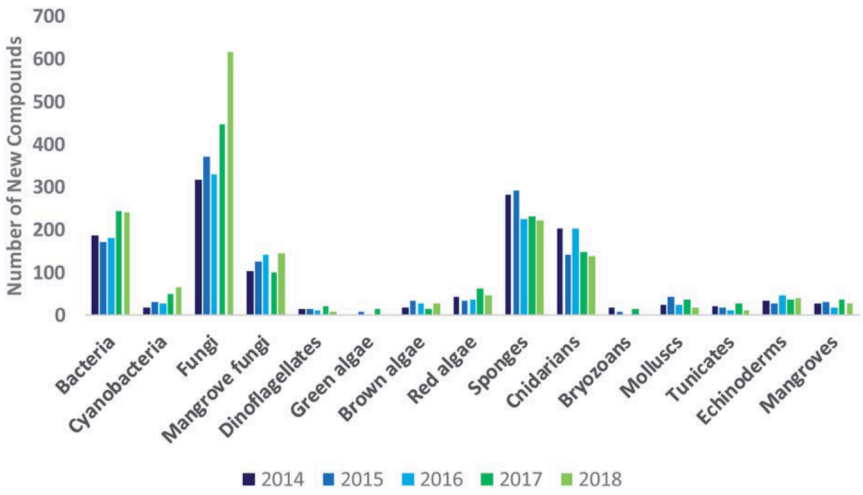

Figure 1 - Trends in discovery of new marine secondary metabolites from 2014 to 2018 (Carroll et al., 2020).

1.1.2 Types of secondary metabolites

There is no rigid classification of secondary metabolites as their great diversity in terms of structure, function and biosynthetic makes them hard to put in confined categories. However, most can be grouped into the three chemical categories: polyketides (derived from acyl-CoA), terpenes (derived from acyl-CoA) and small peptides (derived from amino acids) (McMurry, 2009). In addition there are also hybrid molecules such as polyketide-terpenes, non-ribosomal peptide-polyketides and peptide-fatty acids (Keller, 2019).

1) Terpenes are the largest and most diverse group of secondary metabolites. They are made up of isoprene units that are combined in a linear or cyclic structure that are saturated or unsaturated. Terpenes vary greatly in structure, with many structures seemingly unrelated. This great diversity is likely a result of the many enzymatic modifications employed such as alkylation, cyclization, decarboxylation, glycosylation and redox reactions (Daley et al., 2017; Gershenzon & Dudareva, 2007). **2)** Polyketides are built by a stepwise condensation of acyl units using carbon atoms from methyl and carboxyl groups as acetate building blocks. (McMurry, 2009). The great number of possible reductions, iterations, type of extender units and possible cyclization of the polyketide chain make them a highly diverse group of compounds (Daley et al., 2017). **3)** Peptides functioning as secondary metabolites can be divided into two classes, the (natural) ribosomal synthesized peptides, and the non-ribosomally synthesized peptides formed by both proteinogenic and nonproteinogenic amino acids by non-ribosomal peptide synthases. While the peptides directly translated from RNA are present in all organisms, non-ribosomal peptides are more restricted to bacteria and fungi (Daley et al., 2017; Jenssen et al., 2006; McMurry, 2009).

1.1.3 Biosynthesis of secondary metabolites

In fungi the genes involved in the production of secondary metabolites are grouped together in a continuous manner as biosynthetic gene clusters (BGCs) (figure 2) in the sub-telomeric regions of the chromosomes (Pfannenstiel & Keller, 2019). The synthesis of a secondary metabolite is a multistep process and involves **1)** one or more *core enzymes*, these are the chemical class-defining synthase and/or synthetase (e.g. terpene synthase (TS), terpene cyclase (TC), polyketide synthase (PKS), and/or non-ribosomal peptide synthetase (NRPS) that utilizes a primary metabolite to form the backbone of the secondary metabolite, also known as the chemical scaffold. **2)** Next *tailoring enzymes* can modify the carbon backbone further (e.g. methyltransferase, p450 monooxygenase, hydroxylase and/or epimerase). **3)** Some BGCs also encode a cluster-specific transcription factor that upregulate the transcription of the other genes in the cluster. **4)** If the BGC generate a compound that can be harmful to the producing organism the cluster might contain protective genes to minimize the damages. The four main types of in-cluster self-protection genes are *efflux transporters* (e.g. GliA, an efflux pump important for the resistance to gliotoxin (Dolan et al., 2015)); *cellular BGC intermediate transporters* (e.g. CefM which translocates the cephalosporin C intermediate penicillin N from a microbody to the cytosol, where it is transformed to the end product (Teijeira et al., 2009)); *detoxifying enzymes* that modifies the chemical structure of the finished secondary metabolite to reduce target-binding properties and lower affinity to the target protein (e.g. the oxidoreductase GliT modifies gliotoxin structure to less-toxic molecule (Dolan et al., 2015; Scharf et al., 2010)); lastly the cluster can contain duplicated and resistant copies of the target protein (e.g. the lovastatin BGC contains an extra copy of its target protein 3-hydroxy-3-methylglutaryl-CoA (Abe et al., 2002)). **5)** BGCs can also have several *incongruous genes with hypothetical functions* that are not obviously involved in the synthesis of the secondary metabolite nor the protection from it, which functions are not fully understood (Keller, 2015, 2019).

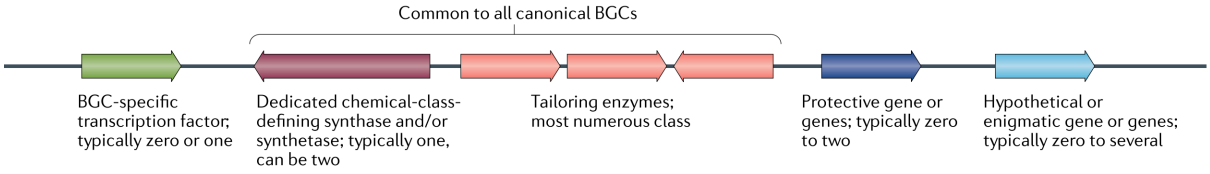

Figure 2 - Schematic overview of the general organization of a biosynthetic gene cluster (Keller, 2019).

1.2 Connecting biosynthetic gene clusters and secondary metabolites

Despite the great diversity of secondary metabolite chemical scaffolds, the biosynthetic principles for most secondary metabolites are highly conserved. Certain collections of enzyme families are often and very specifically associated with the biosynthesis of specific categories of secondary metabolites. Thus, the sequence information of these biosynthetic gene families can be utilized to screen genomes of the biosynthetic gene clusters they are associated with (Dimova et al., 2018; Weber & Kim, 2016).The clustering of the genes combined with the conserved sequences of synthases such as PKS (polyketide synthase) and synthetases such as NRPS (non-ribosomal peptide synthetase) has enabled the development of algorithms able to predict the number of BGCs in any sequenced genome. This innovation led to the realization that many of the acknowledged drug producing microorganisms actually have the genetic

predisposition for producing dozens of more secondary metabolites than they are already known to produce (Keller, 2019; Keller et al., 2005; Pfannenstiel & Keller, 2019).

The recognition that microorganisms has much higher genetic predisposition for synthesis of secondary metabolites than first assumed came with the complete sequencing of the model organism *Streptomyces coelicolor* by Bentley et al. in 2002. *S. coelicolor* had already been found to possess five BGCs, but the whole genome analysis revealed 18 additionally clusters (Bentley et al., 2002; Rigali et al., 2018). This marked as a landmark event that influenced the scientific community on how immense the reservoir of still unknown microbial secondary metabolites might be. Several other actinobacteria have now been whole genome sequenced and screened for BGCs, and *Streptomyces bingchenggensis* and *Streptomyces rapamycinicus* with 54 and 51 clusters respectively, are the current most "gifted" actinobacteria. However, an even higher potential have been found in filamentous fungi (Baranasic et al., 2013; Rigali et al., 2018; X. J. Wang et al., 2010).

An analysis of 19 *Aspergillus* genomes by de Vries et al. found a range of 21-66 BGCs in each species (de Vries et al., 2017). Some species, such as *Aspergillus westerdijkiae*, have been predicted by antiSMASH to contain 88 BGCs (Han et al., 2016). Considering it is estimated that there are 2.2 to 3.8 million fungal species in the world and only about 120 000 species are considered well described, the potential for new discoveries is immense (Hawksworth & Lücking, 2017). Though this is a huge number, the majority of BGCs are either cryptic or expressed at a level too low to detect in standard laboratory culture conditions. This is also the case for most other gifted microorganisms as well (Pfannenstiel & Keller, 2019).

Secondary metabolite production is tightly regulated in microbes due to the high energy requirement for the production of these complex molecules due to the many enzymatic steps involved. This often makes it very hard to induce the activation of silent BGCs as the complexity of the natural induction is hard to mimic in the laboratory (Chianga et al., 2009). However, multiple strategies have been established for inducing the expression of BGCs and identifying the produced secondary metabolites. The increasing number of whole genome sequenced organisms is leading to an emerging number of BGCs identified. In the next sections follows a short introduction to some approaches for studying secondary metabolites from active BGCs, approaches for identifying associated BGCs of characterized secondary metabolites and strategies for activating silent BGCs.

1.2.1 Strategies for identifying the secondary metabolites produced by active BGCs

1.2.1.1 Mass Spectrometry

Mass spectrometry (MS) is an analytical technique that can be applied to study the molecular composition of biological samples, determine elemental composition, and predict some molecular structures (Watson & Sparkman, 2007). It works by ionizing a net charge on the analyze injected and subsequently sorting the ions based on their mass-to-charge ratio (m/z). A mass spectrometer consists of an ion source, a mass analyzer that measures the m/z of the ionized analytes, and a detector that records the number of ions at each m/z value. Electrospray ionization (ESI) is one of the techniques for volatizing and ionizing analytes, and the best suited for analysis of complex samples as it covers a broad area of compouds in terms

of size and polarity. ESI ionizes analytes out of a solution and is therefore readily applicable for analysis of liquid samples, and thus will be applied in this thesis. The quadrupole time-of-flight (qTOF) will be applied as the mass analyzer. The TOF analyzer has high sensitivity, resolution and mass accuracy. In the analyzer, the ions are accelerated to a high kinetic energy and separated along the flight tube based on their different velocities. A collision cell is placed between a quadrupole mass filter and a TOF mass analyzer. Ions of specific m/z are selected in the TOF mass analyzer, then fragmented in the collision cell. The ions are reversed in a reflector before impinging on a detector, that amplifies and records the arriving ions (Aebersold & Mann, 2003; Covington et al., 2017).

MS predictions are made on the compounds ability to ionize. Neutral or poorly ionizing compounds are therefore invisible in MS, and the number of detectable compounds thus depend the ionization source and conditions applied (Covington et al., 2017).

1.2.1.2 Gene deletion/disruption

Once a secondary metabolite has been identified by mass spectrometry it can be linked to its producing BGC by gene deletion or disruption of core genes and a following metabolite profiling. The comparison of the chemical spectra of the reference strain to the deletion mutant should reveal MS signals absent in the deletion mutant, indicating the missing signal represent the relevant secondary metabolite (Kjærbølling et al., 2019; Sanchez et al., 2011).

The gene deletion strategy can be further sophisticated by the generation of deletion libraries focusing on one BGC. The libraries can then be screened for the absence of the compound of interest, and the genes involved in the synthesis of the secondary metabolite can thereby be identified (Kjærbølling et al., 2018).

1.2.2 *In silico* strategies for identifying the associated BGCs of identified secondary metabolites by whole genome sequences

One can also connect secondary metabolite and BGCs in the opposite direction by identifying a BGC starting from a known secondary metabolite. There are several methods for making this connection and the most suiting approach depends on the acquired information/knowledge of the specific secondary metabolite. The three main approaches are **1)** Homology search; identification of the BGC producing a characterized compound by using the BGC of a homologous compound as query for a genome search of the homologues BGC. **2)** Retro biosynthesis; prediction of the enzymatic activities needed to form a compound based on its composition and side chains, and subsequent genome search for enzymes of fairly close proximity that possess these activities. And **3)** Comparative genomics; the BGC for a secondary metabolite can be identified by comparing the genomes of organisms producing that compound looking for common clusters and checking close related non-producing species for the lack of those clusters (Kjærbølling et al., 2019).

1.2.3 Bioinformatic tools for studying secondary metabolites and BGCs

The pipeline antibiotics and Secondary Metabolite Analysis SHell (antiSMASH) is currently the most comprehensive software for screening of microbial genomes for BGCs (Blin et al., 2019). In addition to providing BGC identification antiSMASH also includes detailed annotation of domain structures of modular NRPS and PKS, substrate prediction, pathways, whole genome

metabolic modelling and comparative genomics tools for identifying conserved sub-clusters that synthesize building-blocks, homologues BGCs in other sequenced genomes and the Minimum Information about a Biosynthetic Gene cluster (MIBiG) data standard (Weber & Kim, 2016).

1.2.4 Strategies for activating silent BGCs

1.2.4.1 OSMAC

The OSMAC (One Strain Many Compounds) approach was introduced by (Bode et al., 2002) as a method for inducing the activation of secondary metabolisms in microorganisms. The method aims at inducing activation by systematically altering cultivation parameters such as medium composition, pH, temperature, oxygen availability, culture vessel, and enzymatic inhibitors present, and can also include co-cultivation with other microorganisms as an attempt to try and simulate the natural variations in the fungal environment and mimic the natural inducers of the secondary metabolism (Bode et al., 2002; VanderMolen et al., 2013). With this approach researchers have managed to uncover some of the hidden metabolic potential of fungi and increase the number of expressed BGCs. However, the efficiency of OSMAC is low and this approach is simply not sufficient in activating all clusters alone, but the combination of OSMAC with e.g. epigenetic modification have showed increased effectivity in inducing BGCs (Chianga et al., 2009; Pfannenstiel & Keller, 2019).

1.2.4.2 Epigenetic modification

Some BGCs are physically unavailable for transcription as they are "silenced" and highly compressed in heterochromatin and hidden away from transcription enzymes. This is due to the epigenetic regulation of the fungal genome performed by the epigenetic machinery (Pfannenstiel & Keller, 2019). The enzymes constituting this machinery can regulate the tightness of the DNA packaging, making the encoded genes more or less available for the transcription apparatus. This can be done by either deleting the genes for the epigenetic enzymes, responsible for making the DNA packaging denser, or inhibiting their activity by supplementing chemical small molecule inhibitors that blocks their enzymatic activity, and thus inhibit the formation of heterochromatin. Multiple of the enzymes of the epigenetic machinery have been found to be involved in the regulation of secondary metabolism (Seto & Yoshida, 2014). The strategy of this approach is therefore to delete or inhibit the action of the enzymes that repress transcription of BGCs and thereby have more BGCs accessible for transcription (Pfannenstiel & Keller, 2019).

1.2.4.3 Other approaches for BGC activation

The deletion or overexpression of the genes for global regulators of secondary metabolism have been found to result in the silencing or increased production, respectively, of multiple secondary metabolites and theirs BGCs in various *Aspergillus* spp. (Bayram et al., 2008). Some BGCs also have cluster-specific transcription factors, that can be utilized for targeted activation of BGCs. Clusters lacking such activators can alternatively be induced by replacing all promoters in the BGC to force the expression of the in-cluster genes (Bayram et al., 2008; Keller et al., 2005; Kjærbølling et al., 2019; Perrin et al., 2007).

Sometimes the BGC of interest is located in a species which is uncultivable, hard to cultivate or not easily genetically manipulated. In these cases, heterologous expression strategies can be applied to try and study the BGCs of interest in a host organism more responsive and adapted for genetic manipulation (Kjærbølling et al., 2019).

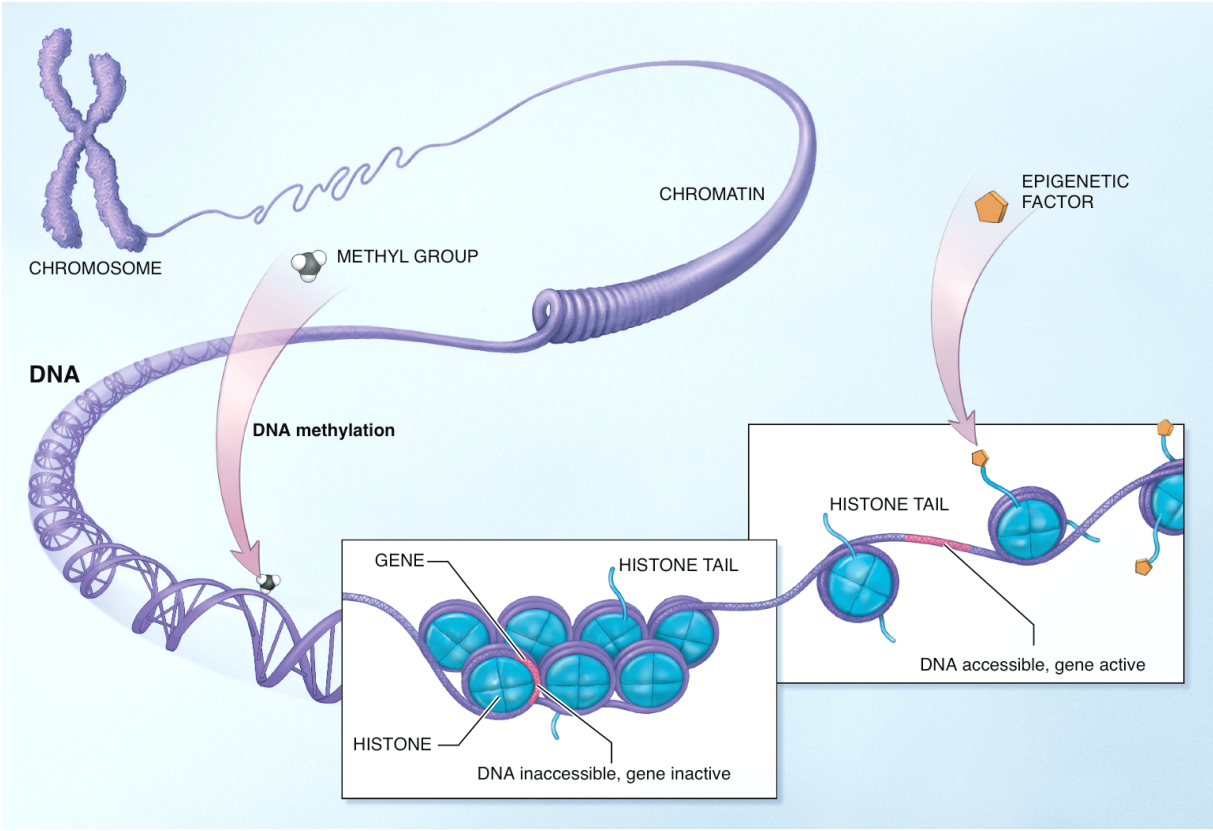

Figure 3 - Epigenetic mechanisms (National Institutes of Health, 2018).

1.3 Epigenetic manipulation

Epigenetics is the study of heritable phenotypes, such as secondary metabolite production, and do not involve modifications to the DNA sequence. Instead of modifying the DNA sequence, the proteins constituting the epigenetic machinery alter gene expression by manipulating chromatin (Pfannenstiel & Keller, 2019). Epigenetic means "in addition to changes in genetic sequence" and the term covers all processes that lead to changes in gene activity without changing the DNA sequence. These modifications of gene activity that can be transferred to the next generation of cells (Weinhold, 2006).

Chromatin is a complex of DNA and proteins in the nucleus. It is made up by DNA wounded around small proteins called histones to form structural units named nucleosomes (figure 3). There are two main types of chromatin – euchromatin, which is loosely packaged, accessible DNA that is available for transcription, and heterochromatin, which is densely packaged and inaccessible for transcription. The strength of the interaction between the histones and the DNA and the density of the DNA packaging can be controlled by modifying the chromatin by the placement or removal of epigenetic factors (figure 3) (Pfannenstiel & Keller, 2019).

1.3.1 Chromatin modulation

There is a wide array of epigenetic processes that can be applied to modify chromatin, these can either be applied directly to the DNA, e.g. by adding methyl groups to cytosines, or to the histone tails (figure 3

). Histone tails are the main target for chromatin regulation of secondary metabolism. There are multiple types of highly dynamic, reversable modifications, known as epigenetic factors or post translational modifications (PTMs) that can be placed on the many residues of the histone tail and multiple PTMs that can be placed on the same residue. These are methylation, acetylation, ubiquitination, deamination, phosphorylation, ADP ribosylation, sumolyation, and several others. Methylation and acetylation are the best studied in relation to secondary metabolism (Bannister & Kouzarides, 2011; Pfannenstiel & Keller, 2019; Weinhold, 2006). The current hypothesis is that histone PTMs either act as signals or binding sites for downstream transcriptional processes or act by influencing the structure of the local chromatin (Pfannenstiel & Keller, 2019).

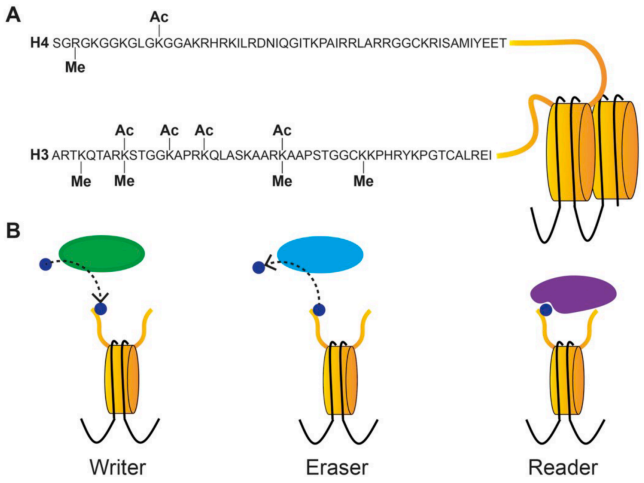

Figure 4 – Histone tail PTMs and the enzymes regulating them. A) Illustration of the histone tails and the many amino acid residue that can be targeted for PTMs. B) The proteins constituting the epigenetic machinery; writers (green), erasers (light blue) and readers (purple), the dark blue dots represents PTMs placed on the histone tails (Pfannenstiel & Keller, 2019).

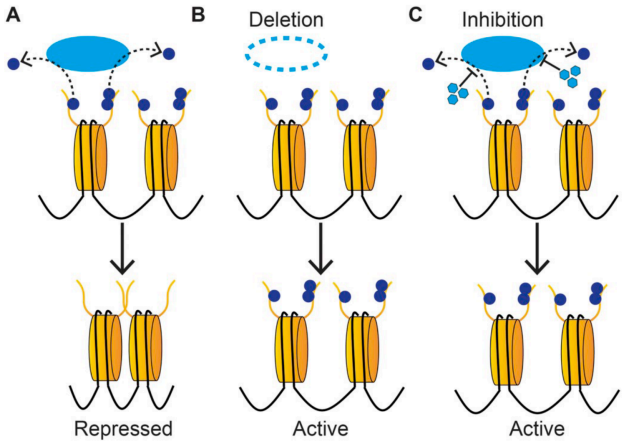

Figure 5 – The two main strategies for interfering with chromatin regulation. A) the action of an eraser under wild type conditions leading to the formation of heterochromatin and the repression of transcription. B) eraser gene deletion resulting in no erasing of PTMs and genes remaining active in euchromatin conformation, accessible for transcription. C) inhibition of eraser by small molecule chemical inhibitors resulting in no PTMs removed and persistently active genes (Pfannenstiel & Keller, 2019).

The histone modifications are controlled and decoded by three types of proteins constituting the epigenetic machinery. *Writers* place modifications on histone tails, *erasers* remove modifications, and *readers* recognizes the modifications on histone tails and brings writers and/or erasers to the correct genomic loci to act (figure 4). Acetyltransferases (HATs) are the *writer* enzymes that transfer acyl group to specific lysine residues of the histone tails from acetyl-CoA. The acetylation of histones is hypothesized to neutralize the positive charge of the histone, making the interaction with the negatively charged DNA weaker, thus loosen the DNA packaging, making the DNA accessible for transcription (Pfannenstiel & Keller, 2019). Deacetylation of histone tails is performed by the *eraser* enzymes, histone deacetylases (HDACs). They increase the positive charge of the

histones and thereby strengthen the interaction to the negatively charged DNA and repress transcription. Histone deacetylases are divided into two families, the zinc depended histone deacetylase (HDAC) family and the NAD^+ dependent Sir2 regulatory family. There are four classes of HDACs. Class; I, II and IV belong to the HDAC family and Class III belongs to the Sir2 regulatory family (Seto & Yoshida, 2014).

Methyltransferases are the *writer* enzymes responsible for methylation of histone tails. In contrary to acetylation, which is mostly associated with transcription activation, the effects of methylation are more complex and depends on which specific histone tail residue. In addition, residues can be methylated in multiple levels (mono- di- or tri-) with each having specific biological effects. These histone methylations can be removed by the *erasers* histone demethylases (Pfannenstiel & Keller, 2019).

Currently there are two main strategies for interfering with chromatin regulation to activate silent BGCs. These are deletion or overexpression of chromatin modifying enzymes or addition of small molecule chemical inhibitors to prevent the formation of heterochromatin (figure 5) (Pfannenstiel & Keller, 2019).

1.4 Epigenetic modifiers

The two major groups of small molecule epigenetic modifiers are the DNA methyltransferase (DNMT) inhibitors and the histone deacetylase (HDAC) inhibitors.

1.4.1 DNA methyltransferase inhibitors

In contrary to histone methyltransferases, DNA methyltransferases (DNMT) targets DNA directly. DNMTs methylate cytosine bases by binding the cytosine, transferring a methyl group to C5 from the cofactor S-adenosyl-L-methionine (SAM), producing 5-methylcytosine. the DNMT is then released by β-elimination. 5-azacytidine is the most widely used DNMT inhibitor. It inhibits DNMT activity by a trapping mechanism. DNMTs bind 5-azacytidine in the same manner as cytidine. However, because 5-azacytidine has a nitrogen located at the 5-position the DNMT is not able to release itself by β -elimination and becomes irreversibly covalent bound (figure 6) (Gnyszka et al., 2013; Stresemann & Lyko, 2008). 5-azacytidine has been used to identify novel secondary metabolites in several fungal species including *Cladosporium cladosporioides, Diatrype disciformis* (Williams et al., 2008) and *Beauveria bassiana* (Yakasai et al., 2011).

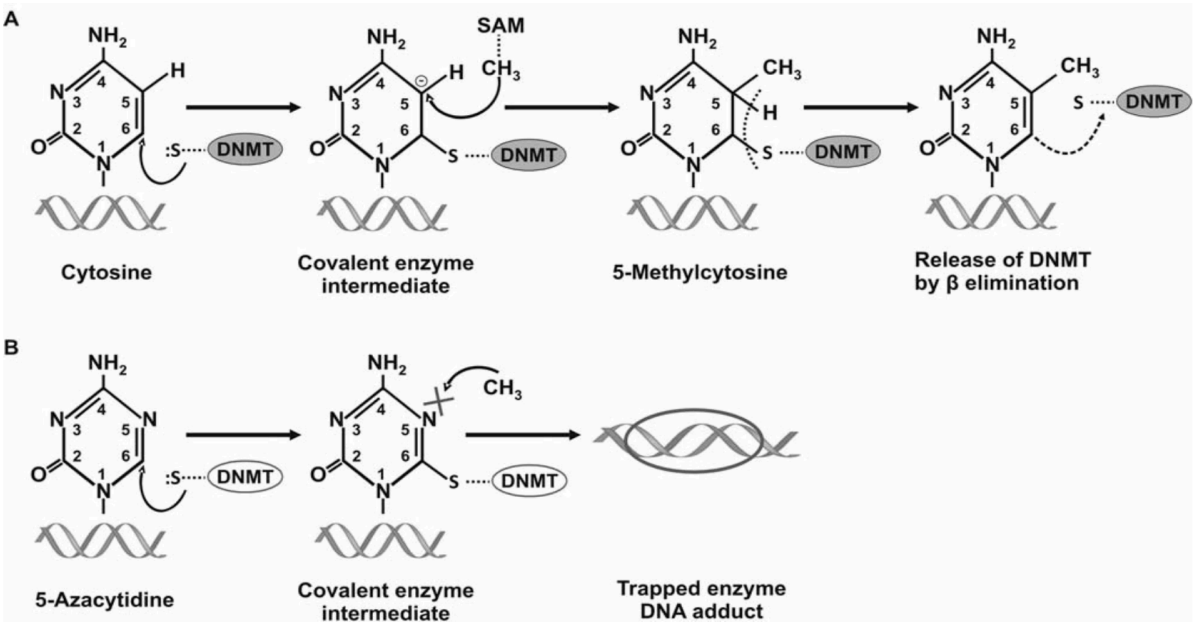

Figure 6 - Mechanism of action for DNMT inhibitor 5-azacytidine. A) Mechanism of DNMT for methylation of cytosine bases. B) Inhibition of DNMT by 5-azacytidine resulting in trapped enzyme irreversible bound to 5-azacytidine (Gnyszka et al., 2013).

1.4.2 Histone deacetylase inhibitors

Histone acetylation is associated with transcription activation. Histone deacetylases are divided into two families, the zinc depended histone deacetylase (HDAC) family and the NAD^+ dependent Sir2 regulatory family. There are four classes of HDACs. Class; I, II and IV belong to the HDAC family and Class III belongs to the Sir2 regulatory family (Pfannenstiel & Keller, 2019; Seto & Yoshida, 2014).

1.4.2.1 HDAC class I, II and IV – inhibitors

HDAC inhibitors can be divided into four groups based on their chemical structures: hydroxamate, benzamide, cyclic peptide and short-chain fatty acid. The two hydroxamates Trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA) are the best studied. They bind the HDAC by inserting their long aliphatic chain into the catalytical core of the HDAC, (consisting of a tubular pocket, the zinc-binding site and the active-site residues) with multiple contact points to the tubular pocket. There they make hydrogen bonds with histidine and tyrosine residues located around the zinc ion. At the other end of the TSA and SAHA molecules, their aromatic ring group interacts with the residues at the lining of the rim of the pocket, thereby locking the inhibitor in the pocket. TSA and SAHA (figure 7) are considered pan-HDAC inhibitors capable of inhibiting the activity of both class I, II and IV HDACs due to this strong binding (Seto & Yoshida, 2014).

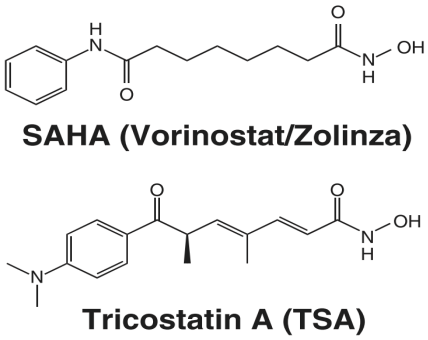

Figure 7 - Chemical structure of the two HDAC inhibitors TSA and SAHA.

1.4.2.2 HDAC class III – inhibitors

Sirtuins are known to perform two enzymatic activities: histone deacetylase and ADP-ribosyltransferase, and they require an NAD^+ cofactor for their enzymatic activity. The catalytic site of sirtuins is located in a cleft between the large and small domain of the protein. This cleft form a protein-tunnel in which the substrate can interact with NAD^+. Sirtuins deacetylate lysine residues of histone tails by binding NAD+ to the acetylated lysine and cleaving off nicotinamide and thereafter removal of O-acetyl-ADP-ribose by water resulting in the deacetylated lysine residue and 2'-O-acetyl-ADP-ribose (figure 8) (Seto & Yoshida, 2014).

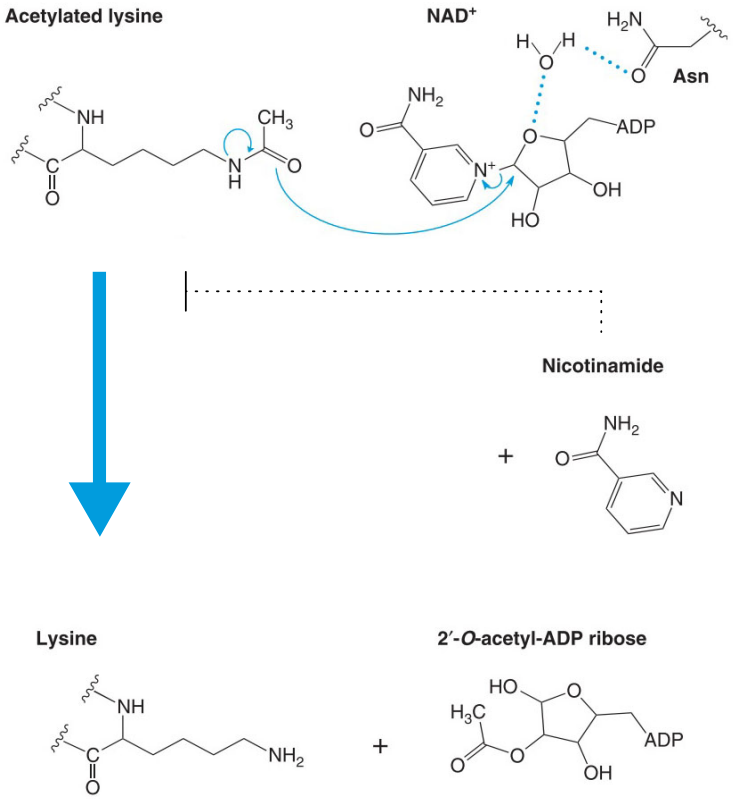

Figure 8 - Mechanism of deacetylation by sirtuin and nicotinamides physiological inhibiting action (Seto & Yoshida, 2014).

Nicotinamide is widely used as a sirtuins inhibitor. Because it is a by-product of sirtuin deacetylase activity, and thus a physiological inhibitor it requires high concentrations to shift the direction of the reaction to decease the gene silencing. Some sirtuin specific inhibitors have also been discovered, such as sirtinol, splitomicin and sodium butyrate, however, their specific mechanisms for inhibiting sirtuin activity are not yet characterized (Seto & Yoshida, 2014).

1.4.3 Studies of BGC activation by the application of epigenetic modifiers

The first study of awakening silent BGCs by the supplementation of epigenetic modifiers was the treatment of Aspergillus alternata and Pencillium expansum with the histone deacetylase (HDAC) inhibitor and antifungal drug, trichostatin A (TSA) (Shwab et al., 2007). It demonstrated HdaA (histone deacetylase) only regulated genes in the telomere-proximal regions, in contrary to LaeA which regulates clusters in both telomere proximal regions and the internal regions of the chromosomal arms. (Shwab et al., 2007).

Since this initial study there have been many supporting reports of the successful awakening of BGCs in a diverse array of filamentous fungi (Akone et al., 2016; Asai et al., 2011, 2013, 2016; Asai, Luo, Obara, Taniguchi, et al., 2012; Asai, Yamamoto, & Oshima, 2012; Asai, Yamamoto, Chung, et al., 2012; J. Sun et al., 2012; Williams et al., 2008; Yakasai et al., 2011; Yang et al., 2014). There are also some studies applying epigenetic modifiers to marine and marine-derived fungi with successful induction of BGCs. The cultivation of the marine-derived

fungus Penicillium brevicompactum treated with class III HDAC inhibitors nicotinamide and sodium butyrate, were found to induce the production of several phenolic compounds. Nine new phenolic compounds were isolated from the nicotinamide treatment, three of which were found to show antiproliferative activity against HepG2. Two new compounds were also isolated from the sodium butyrate treatment (El-Hawary et al., 2018). Two new polyketides were isolated from marine fungus Asteromyces cruciatus exposed to SAHA and osmotic stress (Igboeli et al., 2019). These studies demonstrate the potential of applying epigenetic modifiers to marine and marine-derived fungi.

1.5 Extraction of secondary metabolites from cultures

1.5.1 Liquid-liquid extraction using ethyl acetate

In liquid-liquid extraction (LLE) hydrophobic sample constituents are extracted from aqueous samples with a water-immersible organic phase. Volatile organic solvents that are usually used include methyl chloride, diethyl ether, pentane, hexane, chloroform and ethyl acetate. Liquid-liquid extraction works by extracting hydrophobic compounds from the sample to the organic phase of the organic solvent. For a successful LLE the extraction efficiency or the recovery (extracted amount of compound/original amount of compound) should be close to 100%. The extraction efficiency is closely related to the volume of organic solvent and the partition coefficient of the analyte of interest. Partition coefficients are defined as the concentration ratio of a chemical compound between to immersible solutions at equilibrium (Johanson, 2010; Pedersen-Bjergaard et al., 2000).

Extraction efficiency might be increased by increasing the volume of organic solvent relative to the sample volume or extracting several times. For extraction of basic or acidic compound, pH adjustments to alkaline or acidic range, respectively can ensure high extraction efficiency. In addition to analyte enrichment, LLE provides a clean-up of the sample as inorganic salts are usually insoluble in the organic solvents used and consequently remain in the sample (Pedersen-Bjergaard et al., 2000).

1.5.2 Solid phase extraction using Diaion® HP-20

Solid-phase extraction (SPE) using Diaion® HP-20 resin is a more efficient extraction method than LLE. It yields quantitative extracts and the process can be automated. By applying this technique, many of the disadvantages of liquid-liquid extraction such as incomplete phase separation, less than quantitative recoveries and disposal of large quantities of organic solvents are avoided (Supelco Analytical, 2004). It is applied as a preconcentration and clean-up method for analytical samples, purification of chemicals and the extraction of toxic of valuable compounds from aqueous samples (J. J. Sun & Fritz, 1992).

There are a wide range of adsorbents to choose from when performing SPE and the choice of solid sorbent is the most crucial factor, which controls the selectivity, affinity, capacity, recovery and enrichment (Yavuz et al., 2017). Diaion HP-20 is a non-polar copolymer styrene-divynibenzene adsorbent resin. It constitutes porous spherical particles of 0.5 mm diameter in which compounds in the sample can be attracted into. Diaion HP-20 is considered the most effective adsorbent for preconcentration and eliminating toxic compounds in fermentations (Sigma-Aldrich, 2020a).

1.6 Screening of bioactivity

The emergence of genomics-based methods in drug discovery has led to an enormous amount of analytical data of microbial genomics, transcriptomics and proteomics. To figure out if the compounds constituting this data has potential to be utilized as drugs or other agents in a biological system, it is essential to identify if they have biological targets. If a compound has no biological target it has no drug potential. To study this, bioassays must be performed (Malviya & Malviya, 2017; Weller, 2012). The focus was in antibacterial assays that are shortly introduced in the following section.

1.6.1 Antibacterial activity

The current spread of antimicrobial resistance is one of the greatest global threats to human health world-wide causing over 700 000 deaths yearly, and the number is estimated to increase to 10 million yearly deaths by 2050 if measures are not put in place. The current rate of antibiotic development has not hold up with the rapid development of resistance among human pathogenic bacteria. (Genilloud, 2019; Trotter et al., 2019; WHO, 2017)

The World Health Organization (WHO) have made multi drug resistant pathogens one of their top priorities and released a global priority list of antibiotic resistant bacterial to guide research and development (table 1). Carbapenem-resistant *Pseudomonas aeruginosa* and carbapenem and 3rd generation cephalosporin resistant *Escherichia coli* are ranked as some of the most critical pathogens on this list. Vancomycin resistant *Enterococcus faecalis* and vancomycin and methicillin resistant *Staphylococcus aureus* are also of high priority (WHO, 2017). These four bacteria; *E. coli, P. aeruginosa, S. aureus* and *E. faecalis* together with *Streptococcus agalactiae* (not on WHO's list) were tested against in the screenings for antibacterial activity.

Table 1 - The global priority list of antibiotic resistant bacteria presented by WHO (WHO, 2017).

Priority	Pathogens	Resistance
Critical	*Acinetobacter baumannii*	Carbapenem
	Pseudomonas aeruginosa	Carbapenem
	Enterobacteriaceae: *Klebsiella pneumonia, Escherichia coli, Enterobacter spp., Serratia spp., Proteus spp., Providencia spp., Morganella spp.*	Carbapenem, 3rd generation cephalosporin
High	*Enterococcus faecalis*	Vancomycin
	Staphylococcus aureus	Methicillin Vancomycin
	Helicobacter pylori	Clarithromycin
	Campylobacter	Fluoroquinolone
	Salmonella spp.	Fluoroquinolone
	Neisseria gonorrhoeae	3rd generation cephalosporin Fluoroquinolone

Due to the different cultivation approaches applied in this thesis, antibacterial activity had to be tested in several different assays. The solid cultures were tested by an agar plug diffusion

method, the liquid micro cultures were tested in an agar disc-diffusion method and the extracts were tested by a broth dilution method.

1.6.1.1 Agar plug diffusion method

In the agar plug diffusion method the microbial strains of interest are first cultivated on agar medium. During this growth it is assumed the microbe secretes molecules in the agar medium. After cultivation plugs of the microbes are cut out using sterile cork borer and placed either with aerial side down on agar inoculated with the microbe tested against, or with the aerial side up an empty petri dish which then is filled with liquid agar inoculated with the microbe tested against. I will apply the latter method. The activity of the microbial strain tested can be evaluated by the size of the inhibition zone around the plug. (Balouiri et al., 2016)

1.6.1.2 Agar disc-diffusion method

The agar disk disc-diffusion method is an official method for routine antimicrobial susceptibility testing in many clinical laboratories. In this procedure the surfaces of agar plates are inoculated by spreading a volume of a standardized inoculum of the test microbe. Filter paper discs with samples for testing are evenly dispersed on the solidified surfaces (Balouiri et al., 2016; Hudzicki, 2009). The bacteria tested against can be classified as susceptible, intermediate or resistant to the extracts based on the size of the inhibition zone or the lack of one. This method is not able to distinguish between bactericidal and bacteriostatic effects as inhibition of bacterial growth does not necessarily mean bacterial death. However, the advantages of this assay include simple design, low cost, and ability to test a high number of microbes and antimicrobial agents. The minimum inhibitory concentration (MIC) cannot be determined by dilution methods as it is not possible to quantify the amount of compounds diffused into the agar (Balouiri et al., 2016).

1.6.1.3 Broth dilution method

Broth dilution testing is commonly performed in 96-well microtitration plate format. Generally, each well is added the microbial agents for testing diluted in a culture medium. Then each well the microbial strain tested against, inoculated in the same medium to a standardized concentration, mixed and incubated. The activity of the microbial agents tested can be determined by measuring the optical density (OD), the amount of light absorbed by the samples, in relation to a blank sample only containing assay medium. Dense microbial growth will give a high OD value and indicate poor antimicrobial inhibition, while a low OD value indicates no or sparse microbial growth and successful microbial inhibition (Balouiri et al., 2016).

The MIC value recorded is the lowest concentration of the tested agent able to inhibit visible/detectable growth of the microbe screened against. it is usually expressed as µg/mL or mg/L. Dilution methods are the most suitable for determination of MIC values as they can estimate the concentration of the tested antimicrobial agent (sample) in the medium.

1.6.2 Antibiofilm activity

In the antibiofilm assay the samples ability to inhibit the formation of biofilm by *Staphylococcus epidermidis* is investigated, with non-biofilm forming bacterium *Staphylococcus haemolyticus* as planktonic growth control. In the assay, *S. epidermidis* biofilm is grown on the surface of polystyrene wells of 96-well plates filled with liquid media. Extracts are then added to test their ability to inhibit biofilm formation. After incubation the biomass build-up is quantified by crystal violet staining. After staining the crystal violet is removed and washed and dried before the biomass is dissolved in ethanol. The extracts are then evaluated on their inhibition of biofilm formation by their optical density as wells with biofilm will be stronger colored than wells without or small amounts of biofilm (Kragh et al., 2019; O'Toole et al., 1999).

S. epidermidis is a Gram-positive bacterium of the Staphylococcaceae family. It is a commensal bacterium of the microbiota and inhabits multiple skin surfaces. *S. epidermis* is one of the most frequent causes of medical device-related infections resulting from its biofilm-forming abilities (Rupp & Fey, 2014; Toltzis, 2018; X. Wang et al., 2009).

2 Background

Marbio is a high-throughput analytical laboratory with a broad range of chromatographic and spectrometric tools for extracting and fractionating biological samples and biochemical and cellular assays for screening of samples for bioactivity (Svenson, 2013). Collected microorganisms are cultivated and applied the OSMAC approach for inducing secondary metabolism. However, identifying the exact culture conditions that induces secondary metabolite production might be a labor-intensive approach as the biosynthetic gene clusters producing secondary metabolites are repressed by many levels of transcription regulation (Frisvad, 2012).

Silent BGCs are physically unavailable for transcription because their DNA are highly compressed in heterochromatin and hidden away from transcription enzymes. This is due to the epigenetic regulation of the fungal genomes performed by the epigenetic machinery (Pfannenstiel & Keller, 2019). In this thesis I will be applying the strategy of inducing the activation of biosynthetic gene clusters in marine filamentous fungi by the supplementation of small molecule chemical inhibitors as a way of manipulating the epigenetic machinery of the fungi to keep the BGCs transcriptionally accessible. This method has been acknowledged to be more effective in inducing the activation of silent BGCs than the OSMAC approach and is a simpler and less labor intensive than the other strategies previously mentions. As many fungi still are recalcitrant to genetic transformation, the supplementation of small molecule inhibitors might be more applicable to the many species of filamentous fungi less responsive to genetic manipulation (Pfannenstiel & Keller, 2019).

2.1 Epigenetic modifiers applied to Marbio's marine fungi

Epigeneitc modifiers have not previously been applied to the selection of marine filamentous fungi studied in this thesis. Consequently, there were no data available on which modifiers are effective and not in these strains. It was therefore chosen to implement one inhibitor of each of the three major groups: the DNMT inhibitor 5-azacytidine, the HDAC inhibitor suberoyl bis-hydroxamic acid (SBHA) (also known as suberohydroxamic acid) and the sirtuin inhibitor nicotinamide (figure 9). These inhibitors were selected because of the many report of successful induction of BGCs by their application, they constituted the best economic alternatives and were some of the least toxic compounds compared to other inhibitors.

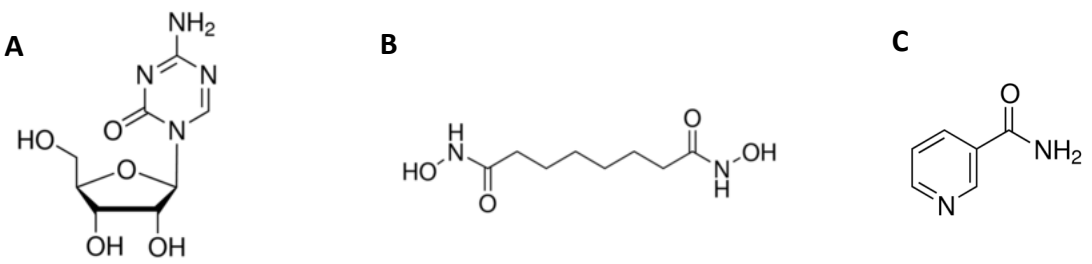

Figure 9 - Chemical structure of A) 5-azacytidine, B) SBHA and C) nicotinamide

Although RG-108 is less hazardous (acute toxic if swallowed and acute aquatic toxic) compared to 5-azacytidine (acute toxic if swallowed and carcinogenic), 5-azacytidine was selected as the DNMT inhibitor. This is because it has the most reported cases of inducing secondary

metabolism and a significantly lower price than the alternative RG-108 (Appendix 3). SBHA was selected as the HDAC inhibitor as it is much safer to apply than SAHA and VPA (both posing reproductive toxicity) and substantially cheaper than the widely used SAHA. Lastly nicotinamide was the selected sirtuin inhibitor because of the significantly lower price compared to the two alternative inhibitors, sirtinol and splitomicin.

2.2 Selection of fungal strains

I started the MSc work with eight fungal isolates listed in table 2. These fungal isolates were selected for investigation because they were all poorly studied in terms of bioactivity and were all already whole genome sequenced or planned to be sequenced. So I had the possibility to link metabolites with BGCs. These were subjected to two liquid and solid cultivation in four different growth media: M6, M6 LPS (lipopolysaccharide), FMAP, and FMAP LPS, with agarose in the solid media.

Table 2 - Fungal isolates investigated

Taxon	Isolate name	Source of isolation	Reference
Digitatispora marina	008cD1.1	Driftwood	(Rämä et al., 2014)
Amylocarpus encephaloides	018bII1.1	Driftwood	(Rämä et al., 2014)
Tolypocladium inflatum	010cU1.3	Driftwood	(Rämä et al., 2014)
Calycina marina	TRa3180AII.4	Alga	(Baral & Rämä, 2015)
***Typhula* sp.**	TRa3160C	Alga	(Rämä & Spatafora, 2019b)
***Mytilinidion* sp.**	M16HEL1360D1-10.1	Driftwood	(Hagestad et al., 2019)
***Lulworthia* sp.**	TRa3202.III.1	Fruiting body (on driftwood)	(Hagestad et al., 2019)
***Acremonium* sp.**	TS7	Deep-sea sponge	(Rämä & Spatafora, 2019a)

2.3 Metabolic potential of *Acremonium* sp. (TS7)

Prior to thesis, TS7 had been genome sequenced and draft genome assembled. Following genome annotation and BGC prediction done by Ph.D. student Ole Christian Hagestad using antiSMASH revealed 16 NRPS, 9 terpene, 6 PKS, 1 indole, 1 phosphonate, and 3 NRPS-PKS hybrid BGCs to a total of 36 BGCs. Homology search of TS7 BGCs revealed clusters likely to produce homologous compounds of botrydial, helvolic acid, cephalosporin C, ascochlorin and leucinostatin A.

3 Workflow

This thesis was performed at Marbio – an analytical platform for natural products. This was the first study into supplying epigenetic modifying compounds to any microbial culture at the Marbio research laboratory, and because of the time constraints of conducting a master's thesis not all aspects of optimising the use of epigenetic modifiers as a way of inducing biosynthetic gene clusters were studied. Starting out with cultivating the 8 fungal isolates *Digitatispora marina* (008cD1.1), *Amylocarpus encephaloides* (018bII1.1), *Tolypocladium inflatum* (010cU1.3), *Calycina marina* (TRa3180AII.4), *Typhula* sp. (TRa3160C), *Mytilinidion* sp. (M16HEL1360D1-10.1), *Lulworthia* sp. (TRa3202.III.1), and *Acremonium* sp. (TS7) in liquid and solid media for 30 days and subsequently subjecting the cultures to antibacterial and protease activity testing. Based on these preliminary results, the 3 fungal isolates 008cD1.1, TRa3160C, and TS7 were selected for further cultivation for 30 days on solid media with epigenetic modifiers 5-azacytidine, SBHA, and nicotinamide. After cultivation these cultures were screened for antibacterial and antifungal activity. Because of the uniform activity of TS7, and lack of activity by 008cD1.1 and TRa3160C, all solid TS7 cultures were combined in one liquid-liquid extraction and 008cD1.1 and TRa3160C were not further studied. TS7 were subjected for a final cultivation in liquid media to investigate if the epigenetic modifiers might work better in a liquid environment and extracted by solid phase extraction after 16 days of cultivation. All extracts were screened for antibacterial, antibiofilm, antifungal and anticancer activity and analyzed by MS (figure 10).

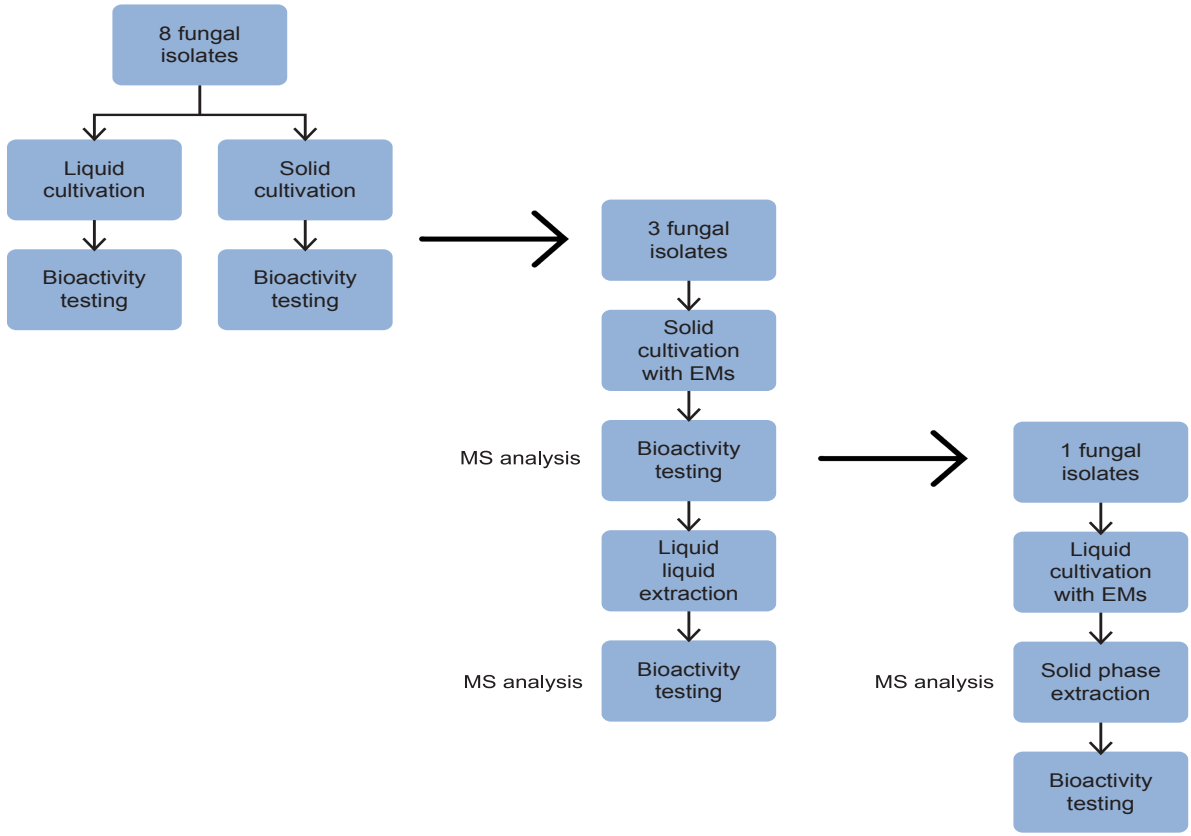

Figure 10 - Workflow of the thesis. The original selection of isolates was first cultivated in liquid and solid media and subsequently tested for bioactivity. Based on these results the three most promising fungi were subjected for new rounds of cultivation on solid media with added epigenetic modifiers and thereafter tested for bioactivity and analyzed using mass spectrometry. On the basis of these results one fungal isolate was selected for further research and cultivation in liquid media. EMs: epigenetic modifiers, MS: mass spectrometry.

4 Aim of the Thesis

The main aim of this thesis was to identify if applying epigenetic modifiers would have an effect on secondary metabolites produced by the studied marine filamentous fungi.

Other aims were to:

- Identify bioactive secondary metabolites, and study which epigenetic modifiers yielded their production
- Develop a method for implementing the application of epigenetic modifiers as part of the biodiscovery pipeline at Marbio

5 Materials and methods

5.1 Initial cultivation of fungi on solid and liquid media

Table 3 - Products and equipment used in the initial cultivation.

Product/Equipment	Product ID/Equipment specifications	Distributor (Country)
Herasafe biological safety cabinet	Class II	Thermo Fisher Scientific (Massachusetts, USA)
Termaks Cooling Incubator	KB8182	Termaks AS, (Norway)
Petri dishes, 9 cm diameter	391-0605	VWR International (Pennsylvania, USA)
Deep well storage plates, 96-well, 2,2 mL	732-4843	VWR International (Pennsylvania, USA)

Initial cultivations of the eight fungal isolates (table 2) was first performed to select which isolates to go forward with and cultivate with added epigenetic modifiers. In this initial cultivation all fungal isolates were cultivated in both liquid and solid M6, M6 LPS, FMAP, and FMAP LPS media (Appendix 1).

Solid cultures were grown in petri dishes containing 20 mL medium and liquid micro-cultures were grown in deep well plates (DWPs) in volumes of 1.5 mL. Fungi were inoculated on solid cultures by cutting out plugs of mycelia from established plates and transferring three plugs with the aerial side down to each agar plate. In liquid cultures, fungi were inoculated by taking plugs from established plates with a syringe and inserting the plugs into the media containing wells of the DWPs.

Both solid and liquid cultures were incubated at 10°C for 30 days before screening for bioactivity. Solid cultures were only tested for antibacterial activity by the agar plug diffusion method (described in section 5.2.1) and the liquid cultures were tested for antibacterial activity by the agar disc diffusion method, so that the two antibacterial assays could be easily compared. In addition, the liquid micro-cultures were tested for protease activity. After cultivation the liquid micro-cultures were centrifuged for 30 minutes at 3 300 G and supernatants collected. Only supernatants were investigated for bioactivity.

5.1.1 Characterization of fungal strains

Table 4 - Products and equipment used in the characterization of fungal strains.

Product/Equipment	Product ID/Equipment specifications	Distributor (Country)
DreamTaq Green PCR Master Mix (2X)	K108/82	Thermo Fisher Scientific (Massachusetts, USA)
Forward primer ITS5	OLIGO, sequence: 5'-GGAAGTAAAAGTCGTAACAAGG-3'	Sigma-Aldrich (Missouri, USA)
Reverse primer ITS4	OLIGO, sequence: 5'-TCCTCCGCTTATTGATATGC-3'	Sigma-Aldrich (Missouri, USA)
Reverse primer LR0Ri	OLIGO, sequence: 5'-GCTTAAGTTCAGCGGGT-3'	Sigma-Aldrich (Missouri, USA)
Gel Red (10,000 x)	41003	BioTium, (California, USA)
Agarose Ultrapure™	15510-027	Life Technologies, (California, USA)
10 x TBE	15581-044	Life Technologies, (California, USA)
DNA ladder	10787-018	Life Technologies, (California, USA)
Agarose gel loading dye (6x)	E190-5ml	Amresco®, (Ohio, USA)
A-SAP Kit	Shrimp Alkaline Phosphatase (70700-201) and Exonuclease 1	ArcticZymes, (Norway)
BigDye 3.1		MH sequencing lab at UiT, Norway
5x sequencing buffer		MH sequencing lab at UiT, (Norway)
Agarose Gel electrophorese system	Owl separation system, B2 model	Thermo Fisher Scientific (Massachusetts, USA)
Gel image system	GeneFlash	SYNGENE Bio imaging, (UK)
PCR machine		
Ultra Pure water	L0015	Biochrom Gmbh, (Germany)

To verify the identity of the cultivated fungi and check for contamination of other microbes, the internal transcribed spacer (ITS) was sequenced This spacer DNA located between the small and large ribosomal subunit rRNA genes, which is used as DNA barcoding of fungal species (Fajarningsih, 2016; Lafontaine & Tollervey, 2001).

DNA samples of fungal mycelia were taken from established solid fungal cultures with an inoculation loop and inoculated in 100 µL Ultra Pure water in Eppendorf tubes. The samples were vortexed, spun down on table centrifuge and incubated for 30 minutes at -80°C to lyse the cells and release their DNA into the water. The amplification PCR (polymerase chain reaction) reaction mix was prepared for each fungal sample by mixing the following: 1 µL fungal template, 12.5 µL2x Dream Taq, 1 µL (10 µM) forward primer, 1 µL (10 µM) reverse primer, and 9.5 µL Ultra Pure water to a volume of 25 µL for each PCR sample. Fungal isolate M16HEL1360D1-10.1 was amplified using forward primer ITS5 and reverse primer LR0Ri. The remaining fungal isolates were amplified using the ITS5/ITS4 primer pair. The amplification PCR was run as specified in table 5.

Table 5 - Cycle scheme for amplification PCR

Initial Denaturation		**95°C**	**5 min**
Cycle x35	Denature	95°C	30 sec
	Annealing	47°C	30 sec
	Elongation	72°C	1 min
Final Extension		72°C	10 min
Hold		4°C	∞

The PCR products were analysed by gel electrophoresis in 1% agarose gel (1 g agarose in 100 mL 1x TBE buffer) added 10 µL 10,000x GelRed. Loaded 5 µL of each PCR product and 5 µL 1kb ladder to the outer wells and ran the gel for 20 min with 180 V. Exposed the gel with UV light.

The PCR products were purified using the A-SAP Kit by mixing 5 µL of each sample with 1 µL Shrimp Alkaline Phosphatase and 1 µL Exonuclease 1. The mixtures were run by PCR machine for 15 min at 37°C and 15 min at 80°C to deactivate the enzymes.

Lastly the sequencing PCR was performed. Prepared the sequencing PCR mix by mixing the following: 1 µL fungal template, 1 µL BigDye 3.1, 2 µL 5x sequencing buffer, 1 µL (1 µM) forward or reverse primer (in separate samples), and 5 µL Ultra Pure water to a total volume of 10 µL. The sequencing PCR reaction was run as specified in table 6.

Table 6 - Cycle scheme for sequencing PCR

Initial Denaturation		**96°C**	**5 min**
Cycle x30	Denature	96°C	10 sec
	Annealing	47°C	5 sec
	Elongation	60°C	2 min
Hold		4°C	∞

After sequencing PCR, the samples were delivered to the sequencing lab at UiT/UNN for Sanger sequencing. The sequencing outputs were analyzed in Geneious R10.2 (2017 version) and the fungi were characterized using BLAST search of the ITS sequences.

5.1.2 Antibacterial agar disc diffusion assay

Table 7 - Products and equipment used in the antibacterial agar disc diffusion assay.

Product/Equipment	Product ID/Equipment specifications	Distributor (Country)
Mueller Hinton broth (MH)	275730	Becton, Dickinson and Company (New Jersey, USA)
Brain heart infusion broth (BHI)	53286	Sigma-Aldrich (Missouri, USA)
Agarose	A1296	Sigma-Aldrich (Missouri, USA)
Blood agar plates		University hospital of North Norway (UNN), (Norway)
Gentamycin (10 mg/mL)	A 2712	Merck KGaA (Germany)
NaCl	S3014	Sigma-Aldrich (Missouri, USA)
MilliQ H_2O	MilliQ Gradient A10	Merck Millipore KGaA, Germany
Whatman® Antibiotic Assay Discs	WHA2017006	Sigma-Aldrich (Missouri, USA)
Heated Incubator MIR-262		Panasonic Healthcare (Japan)
Incubator Unimax 1010		Heidolph Instruments GmbH & Co (Germany)
Herasafe biological safety cabinet	Class II	Thermo Fisher Scientific (Massachusetts, USA)
Staphylococcus aureus	ATCC® 25923	LGC Standards (UK)
Escherichia coli	ATCC® 25922	LGC Standards (UK)
Enterococcus faecalis	ATCC® 29212	LGC Standards (UK)
Pseudomonas aeruginosa	ATCC® 27853	LGC Standards (UK)
Streptococcus agalactiae	ATCC® 12386	LGC Standards (UK)

Blood plates of *E. coli*, *P. aeruginoa*, *S. aureus*, *E. faecalis* and *S. agalactiae* were prepared and incubated at 37°C over-night. A scoop from each bacterial blood plate were inoculated in 2 mL sterile saline (0.9% NaCl) and vortexed to create a smooth suspension. Plated out 50 µL *E. coli*, *P. aeruginoa* and *S. aureus* suspension on MH agar plates and 50 µL *E. faecalis* and *S. agalactiae* suspension on BHI agar plates. Distributed 5 disc evenly on each plate. Each disc was added 5 µL supernatant to each disc and 5 µL gentamycin (10 mg/mL) as negative control. The plates were incubated at 37°C for 24 hours before reading. The activity was measured by the size of the inhibition zone around the discs.

5.1.3 Protease activity assay

Table 8 - Products and equipment used in the protease activity assay.

Product/Equipment	Product ID/Equipment specifications	Distributor (Country)
Tris-HCl	T5941	Sigma-Aldrich (Missouri, USA)
HEPES	H4034	Sigma-Aldrich (Missouri, USA)
NaCl	S3014	Sigma-Aldrich (Missouri, USA)
Suc-Ala-Ala-Pro-Phe-pNA	S7388	Sigma-Aldrich (Missouri, USA)
Protease (reference sample)		ArcticZymes, (Norway)
$CaCl_2$	72340	Sigma-Aldrich (Missouri, USA)
96 MicroWell™ plates, Nuclon™Δ	734-2073	VWR International AS, Pennsylvania, USA
MilliQ H_2O	MilliQ Gradient A10	Merck Millipore KGaA, Germany

90 µL assay mix (3 mL 1M Tris-HCl pH 8.0 at 25°C, 3 mL 0.1 M $CaCl_2$, 0.3 mL 100 mM substrate stock and 23.7 mL MilliQ H_2O) was transferred to all wells of the micro titer plates and 10 µL enzyme dilution buffer (25 mM HEPES, pH 8 and 0.3 M NaCl) to well C-H 11-12 and 10 µL reference sample (1.2 U/mL) to well A and B 11-12 (final concentration 0.120 U/mL). 10 µL of the supernatants were added in parallels to the remaining wells and read the plates immediately. The maximum slope was of the enzyme reactions were calculated, and samples with maximum slope above 0.0005 were classified as active.

5.2 Cultivation of fungi on solid media with epigenetic modifiers

Table 9 - Products and equipment used in the cultivation of fungi on solid media.

Product/Equipment	Product ID/Equipment specifications	Distributor (Country)
Herasafe biological safety cabinet	Class II	Thermo Fisher Scientific (Massachusetts, USA)
Termaks Cooling Incubator	KB8182	Termaks AS, (Norway)
Petri dishes, 9 cm diameter	391-0605	VWR International (Pennsylvania, USA)
5-azacytidine	10003160	Fisher Scientific, (Norway)
SBHA	390585	Sigma-Aldrich (Missouri, USA)
Nicotinamide	72340	Sigma-Aldrich (Missouri, USA)

Based on preliminary bioassay results, *Digitatispora marina* (008cD1.1), *Typhula* sp. (TRa3180AII.4) and *Acremonium* sp. (TS7) were selected for further investigation and cultivation with the epigenetic modifiers SBHA, 5-azacytidine, and nicotinamide. 008cD1.1 and TS7 were cultivated on M6 agar and M6 LPS agar, and Tra3180AII.4 was cultivated on *Ascophyllum nodosum* agar and corn meal agar (Appendix 1). Epigenetic modifiers were added to lukewarm solid medium before 20 mL were pipetted in each petri dish. SBHA, 5-azacytidine, and nicotinamide were added to concentrations of 500 µM, 50 µM and 50 µM, respectively in seven combinations. Plugs from established plates were inoculated on the solidified medium, placed with the aerial side against the agar. Three plugs on each plate. Plates were incubated at 10°C for 30 days. The cultures established and the combinations of epigenetic modifiers are listed in table 10. All combinations were prepared in triplets making a total of 144 plates.

Table 10 - List of fungal solid cultures with epigenetic modifiers. 5-aza: 5-azacytidine, Nico: nicotinamide, null: no epigenetic modifiers added, S: SBHA, A: 5-azacytidine, N: nicotinamide, ANA: Ascophyllum nodosum *agar, CMA: corn meal agar.*

Fungal strain	Culture name	5-aza (µM)	SBHA (µM)	Nico (µM)
***Acremonium* sp.**	TS7 M6 null	-	-	-
***Acremonium* sp.**	TS7 M6 S	-	500	-
***Acremonium* sp.**	TS7 M6 A	50	-	-
***Acremonium* sp.**	TS7 M6 N	-	-	50
***Acremonium* sp.**	TS7 M6 SN	-	500	50
***Acremonium* sp.**	TS7 M6 AS	50	500	-
***Acremonium* sp.**	TS7 M6 AN	50	-	50
***Acremonium* sp.**	TS7 M6 ASN	50	500	50
***Acremonium* sp.**	TS7 M6 LPS null	-	-	-
***Acremonium* sp.**	TS7 M6 LPS S	-	500	-
***Acremonium* sp.**	TS7 M6 LPS A	50	-	-
***Acremonium* sp.**	TS7 M6 LPS N	-	-	50
***Acremonium* sp.**	TS7 M6 LPS SN	-	500	50
***Acremonium* sp.**	TS7 M6 LPS AS	50	500	-
***Acremonium* sp.**	TS7 M6 LPS AN	50	-	50
***Acremonium* sp.**	TS7 M6 LPS ASN	50	500	50
Digitatispora marina	008 M6 null	-	-	-
Digitatispora marina	008 M6 S	-	500	-
Digitatispora marina	008 M6 A	50	-	-
Digitatispora marina	008 M6 N	-	-	50
Digitatispora marina	008 M6 SN	-	500	50
Digitatispora marina	008 M6 AS	50	500	-
Digitatispora marina	008 M6 AN	50	-	50
Digitatispora marina	008 M6 ASN	50	500	50
Digitatispora marina	008 M6 LPS null	-	-	-
Digitatispora marina	008 M6 LPS S	-	500	-
Digitatispora marina	008 M6 LPS A	50	-	-
Digitatispora marina	008 M6 LPS N	-	-	50
Digitatispora marina	008 M6 LPS SN	-	500	50
Digitatispora marina	008 M6 LPS AS	50	500	-
Digitatispora marina	008 M6 LPS AN	50	-	50
Digitatispora marina	008 M6 LPS ASN	50	500	50
***Typhula* sp.**	3160 ANA null	-	-	-
***Typhula* sp.**	3160 ANA S	-	500	-
***Typhula* sp.**	3160 ANA A	50	-	-
***Typhula* sp.**	3160 ANA N	-	-	50
***Typhula* sp.**	3160 ANA SN	-	500	50
***Typhula* sp.**	3160 ANA AS	50	500	-
***Typhula* sp.**	3160 ANA AN	50	-	50
***Typhula* sp.**	3160 ANA ASN	50	500	50
***Typhula* sp.**	3160 CMA null	-	-	-
***Typhula* sp.**	3160 CMA S	-	500	-
***Typhula* sp.**	3160 CMA A	50	-	-
***Typhula* sp.**	3160 CMA N	-	-	50
***Typhula* sp.**	3160 CMA SN	-	500	50
***Typhula* sp.**	3160 CMA AS	50	500	-
***Typhula* sp.**	3160 CMA AN	50	-	50
***Typhula* sp.**	3160 CMA ASN	50	500	50

5.2.1 Antibacterial agar plug diffusion assay

Table 11 - Products and equipment used in the antibacterial plug assay.

Product/Equipment	Product ID/Equipment specifications	Distributor (Country)
Mueller Hinton broth (MH)	275730	Becton, Dickinson and Company (New Jersey, USA)
Brain heart infusion broth (BHI)	53286	Sigma-Aldrich (Missouri, USA)
Blood agar plates		University hospital of North Norway (UNN), (Norway)
Gentamycin (10 mg/mL)	A 2712	Merck KGaA (Germany)
MilliQ H_2O	MilliQ Gradient A10	Merck Millipore KGaA, Germany
Heated Incubator MIR-262		Panasonic Healthcare (Japan)
Incubator Unimax 1010		Heidolph Instruments GmbH & Co (Germany)
Herasafe biological safety cabinet	Class II	Thermo Fisher Scientific (Massachusetts, USA)
Staphylococcus aureus	ATCC® 25923	LGC Standards (UK)
Escherichia coli	ATCC® 25922	LGC Standards (UK)
Enterococcus faecalis	ATCC® 29212	LGC Standards (UK)
Pseudomonas aeruginosa	ATCC® 27853	LGC Standards (UK)
Streptococcus agalactiae	ATCC® 12386	LGC Standards (UK)

Solid fungal cultures were tested for antibacterial activity in parallels directly by incubating plugs of full-grown fungal cultures in solid medium containing bacteria to study the fungi for bacterial growth inhibiting abilities.

The fungi were screened against the five pathogenic bacteria *Staphylococcus aureus, Escherichia coli, Enterococcus faecalis, Pseudomonas aeruginosa* and *Streptococcus agalactiae.* These were plated out from freeze stock on blood agar plates. The plates were incubated over night at 37°C and thereafter stored at 4°C. Overnight cultures of the bacteria were prepared by inoculating a scoop of each bacteria from blood plates in 5 mL growth media and incubated over night at 37°C with shaking.

Plugs with mycelia were cut out of fungal plates using a cork borer with diameter of 6 mm and placed in empty petri dishes with the aerial side up. 5-6 plugs were placed in each dish, evenly dispersed. Inoculated 100 µL overnight cultures of bacteria in 100 mL lukewarm MH (*S. aureus, E. coli* and *P. aeruginosa*) or BHI (*E. faecalis* and *S. agalactiae*) medium with 0.8% agarose. 20 mL bacterial cultures were then added the petri dishes containing fungal plugs without covering the top of the plugs. After the agar had solidified the plates were incubated for 24 hours at 37°C. After 24 hours the inhibition of bacterial growth was determined by measuring the diameter of the inhibition zones around the plugs.

5.2.2 Antifungal agar plug diffusion assay

Candida albicans it is one of the most prevalent fungi of the human microbiota and the predominating infection causing fungus. It can act as both a commensal and opportunistic pathogen and hold the ability to cause both superficial mucosal and dermal infections as well as life-threatening often fatal, bloodstream infections (Calderone & Fonzi, 2001).

The screening for antifungal activity, was performed in multiple formats like the antibacterial screening. The solid cultures were tested for antifungal activity by the agar plug diffusion method, while the extracts were tested by the broth dilution method. In both assays the activity were tested against *Candida albicans* with the antifungal agent Amphotericin B as negative control.

Table 12 - Products and equipment used in the antifungal plug assay.

Product/Equipment	Product ID/Equipment specifications	Distributor (Country)
Potato dextrose agar	70139	Sigma-Aldrich (Missouri, USA)
RPMI	R7755	Sigma-Aldrich (Missouri, USA)
MOPS	M3183	Sigma-Aldrich (Missouri, USA)
L-glutamine	K0302	VWR International (Pennsylvania, USA)
NaCl	S5886	Sigma-Aldrich (Missouri, USA)
Amphotericin B (AMP-B)	A 2942	Sigma-Aldrich (Missouri, USA)
MilliQ H_2O	MilliQ Gradient A10	Merck Millipore KGaA, Germany
Remel 0.5 McFarland Equivalence Turbidity Standard	10026732	Thermo Fisher Scientific (Massachusetts, USA)
Heated Incubator MIR-262		Panasonic Healthcare (Japan)
Herasafe biological safety cabinet	Class II	Thermo Fisher Scientific (Massachusetts, USA)
Candida albicans	ATCC® 90028	LGC Standards (UK)

There was no established protocol for testing of solid cultures activity against *Candida albicans* at Marbio, so modifications were made to the established protocol for the testing of liquid cultures' against *Candida albicans* at Marbio, so modifactions were made to the established protocol for testing of liquid cultures against *Candida albicans* and three alternative methods were tested out.

A scoop of *Candida albicans* cells from the freeze stock were plated out on potato dextrose agar (PDA) and incubated over night at 37°C. 5-8 colonies from the overnight plate were resuspended in 5 mL sterile 0.9% NaCl. The suspensions were vortexed for 15 seconds and adjusted the cell density to the McFarland standard (1-5 x 10^6 cells/mL).

Alternative method 1 - *Candida albicans* and fungal plugs in the agar

Plugs were cut out using cork borer of the fully grown fungal solid cultures and placed in empty petri dishes with the aerial side up. 100 µL *Candida albicans* suspension adjusted to the McFarland standard, were inoculated and in 100 mL lukewarm PDA. 20 mL *Candida albicans*

inoculated in PDA were added the petri dishes containing the fungal plugs without coving the top of the plugs.

Alternative method 2 - *Candida albicans* and fungal plugs on top of the agar

20 µL *Candida albicans* suspension adjusted to the McFarland standard, were plated out on PDA plates. Plugs of the fully grown fungal solid cultures were placed on top with the aerial side against the agar.

Alternative method 3 - *Candida albicans* inside and fungal plug on top of the agar

100 µL *Candida albicans* suspension adjusted to the McFarland standard were inoculated in 100 mL lukewarm PDA. Further, 20 mL suspension were pipetted to empty petri dishes. Plugs of the fully grown fungal solid cultures were placed on top with the aerial side against the agar. All plates were incubated at 37°C for 48 hours and were examined after 24 and 48 hours.

5.2.3 Ethyl acetate extraction of solid cultures

Table 13 - Products and equipment used in the extraction of fungal solid cultures.

Product/Equipment	Product ID/Equipment specifications	Distributor (Country)
Ethyl acetate	33211N	Sigma-Aldrich (Missouri, USA)
Whatman® qualitative filter paper, grade 3	1003-090	Sigma-Aldrich (Missouri, USA)
Rotary Evaporator (Rotavapor)	Laborota	Heidolph Instruments GmbH & Co. (Germany)
Heidolph Unimax 1010		Heidolph Instruments GmbH & Co (Germany)
Herasafe biological safety cabinet	Class II	Thermo Fisher Scientific (Massachusetts, USA)

One of the three triplicate plates of each fungal solid culture with epigenetic modifiers was used for bioactivity testing. The two remaining plates for each culture condition were used for metabolite extraction. Based on the results from the bioactivity screening it was decided to combine all remaining plates of TS7 for one extraction. 32 plates of 20 mL each were combined, making a total of 640 mL solid cultures. The cultures were cut into pieces of approximately 3mm x 3mm using sterile plastic knife and transferred to 1 L glass Erlenmeyer flasks.

A 1:2 extraction of the solid cultures in the extraction solvent (ethyl acetate) was performed. The cultures were extracted three times while shaking under fume hood, first time for 12 hours and second and third for 3 hours each. The solvent extract was then filtrated through Whatman® filter into vacuum flask in fume hood after terminated extraction. Lastly, the extract was dried under reduced pressure using rotary evaporator.

Before the combined extraction of all solid cultures of TS7, plugs of the cultures were analyzed by MS separately. Three plugs of 7 mm were cut out of each full-grown solid culture using a 1000 µL pipette tip. The plugs were transferred to Eppendorf tubes and inoculated in 1 mL extraction solvent (isopropanol:ethyl acetate 1:3) with 1 % formic acid and sonicated for one hour. The samples derived from the same plates were combined and evaporated to dryness. The dry samples were re-dissolved in 100 µL MeOH, spun down and transferred to MS vials for analysis.

5.3 Cultivation of fungi in liquid media

Table 14 - Products and equipment used in the cultivation of fungi in liquid media.

Product/Equipment	Product ID/Equipment specifications	Distributor (Country)
Herasafe biological safety cabinet	Class II	Thermo Fisher Scientific (Massachusetts, USA)
5-azacytidine	10003160	Fisher Scientific, (Norway)
SBHA	390585	Sigma-Aldrich (Missouri, USA)
Nicotinamide	72340	Sigma-Aldrich (Missouri, USA)

It is well known that cultivation parameters greatly affects the metabolome of the cultured microbes (Bode et al., 2002; VanderMolen et al., 2013), epigenetic modifiers were also supplemented to liquid fungal cultures to study if their effects on the liquid cultures compared to the solid cultures. It appears that most successful studies of usage of epigenetic modifiers to induce fungal secondary metabolism have been cultivating fungi in liquid cultures (Akone et al., 2016; Asai et al., 2011; Asai, Yamamoto, & Oshima, 2012; Asai, Luo, Obara, Taniguchi, et al., 2012; Asai, Morita, Shirata, Taniguchi, et al., 2012; Asai et al., 2013; El-Hawary et al., 2018; Igboeli et al., 2019; J. Sun et al., 2012; Yakasai et al., 2011; Yang et al., 2014). The epigenetic modifiers were therefore also applied to liquid cultures.

As antibacterial activity was recorded from TS7 grown on solid M6, M6 LPS and FMAP LPS media in the preliminary antibacterial plug assay with all eight fungal isolates, these media were used for the liquid culturing as well. In addition, TS7 were cultivated in ASME medium, to have a simpler medium to compare the more complex M6 and FMAP media to. The number of combinations of epigenetic modifiers were reduced from 7 in the solid cultivation to 5 in the liquid cultivation. The liquid cultures are listed in table 15.

The liquid cultivation was performed based on the experimental procedure of culturing fungi with epigenetic modifiers (Asai, Chung, Sakurai, Ozeki, et al., 2012; Asai et al., 2016; El-Hawary et al., 2018; Igboeli et al., 2019; Williams et al., 2008; Yakasai et al., 2011).

Table 15 - List of liquid fungal cultures with added epigenetic modifiers.

Fungal strain	Culture name	5-aza (µM)	SBHA (µM)	Nico (µM)
***Acremonium* sp.**	TS7 M6 null	-	-	-
***Acremonium* sp.**	TS7 M6 S	-	500	-
***Acremonium* sp.**	TS7 M6 SN	-	500	50
***Acremonium* sp.**	TS7 M6 AS	50	500	-
***Acremonium* sp.**	TS7 M6 AN	50	-	50
***Acremonium* sp.**	TS7 M6 ASN	50	500	50
***Acremonium* sp.**	TS7 M6 LPS null	-	-	-
***Acremonium* sp.**	TS7 M6 LPS S	-	500	-
***Acremonium* sp.**	TS7 M6 LPS SN	-	500	50
***Acremonium* sp.**	TS7 M6 LPS AS	50	500	-
***Acremonium* sp.**	TS7 M6 LPS AN	50	-	50
***Acremonium* sp.**	TS7 M6 LPS ASN	50	500	50
***Acremonium* sp.**	TS7 FMAP null	-	-	-
***Acremonium* sp.**	TS7 FMAP S	-	500	-
***Acremonium* sp.**	TS7 FMAP SN	-	500	50
***Acremonium* sp.**	TS7 FMAP AS	50	500	-
***Acremonium* sp.**	TS7 FMAP AN	50	-	50
***Acremonium* sp.**	TS7 FMAP ASN	50	500	50
***Acremonium* sp.**	TS7 FMAP LPS null	-	-	-
***Acremonium* sp.**	TS7 FMAP LPS S	-	500	-
***Acremonium* sp.**	TS7 FMAP LPS SN	-	500	50
***Acremonium* sp.**	TS7 FMAP LPS AS	50	500	-
***Acremonium* sp.**	TS7 FMAP LPS AN	50	-	50
***Acremonium* sp.**	TS7 FMAP LPS ASN	50	500	50
***Acremonium* sp.**	TS7 ASME null	-	-	-
***Acremonium* sp.**	TS7 ASME S	-	500	-
***Acremonium* sp.**	TS7 ASME SN	-	500	50
***Acremonium* sp.**	TS7 ASME AS	50	500	-
***Acremonium* sp.**	TS7 ASME AN	50	-	50
***Acremonium* sp.**	TS7 ASME ASN	50	500	50

Full-grown agar plates with TS7 were cut into pieces of approximately 3 mm x 3 mm using sterile plastic knife. One fourth of each plate were transferred into sterile 100 mL Erlenmeyer flasks and inoculated in 75 mL M6 medium to prepare seed cultures. These were incubated at 13°C with shaking at 200 rpm for 3 days, when sufficient growth was observed. 25 mL seed cultures were inoculated in 250 mL medium in 1 L Erlenmeyer flasks and incubated static for 16 days at 13°C. Epigenetic modifiers, 5-azacytidine, SBHA and nicotinamide were added after 9 days of incubation at volumes of 50 µL, 500 µL and 50 µL, respectively. Cultures were placed on shaker for 4 hours after addition of epigenetic modifiers to evenly distribute the modifiers in the cultures.

5.3.1 Extraction of liquid fungal culture

Table 16 - Products and equipment used in the extraction of the liquid fungal cultures.

Product/Equipment	Product ID/Equipment specifications	Distributor (Country)
Methanol	34860	Sigma-Aldrich (Missouri, USA)
Diaion® HP-20	13607	Sigma-Aldrich (Missouri, USA)
MilliQ H_2O	MilliQ Gradient A10	Merck Millipore KGaA, Germany
Cheesecloth filter, fine mesh		Dansk Hjemmeproduksjon (Denmark)
Whatman® qualitative filter paper, grade 3	1003-090	Sigma-Aldrich (Missouri, USA)
Rotary Evaporator (Rotavapor)	Laborota	Heidolph Instruments GmbH & Co. (Germany)

The liquid fungal cultures were extracted using resin Diaion HP-20 with affinity for non-polar compounds.

Resin was added to the cultures 3 days before extraction at 40 g/L culture. To activate the resin, 75 mL MeOH was added to the 10 grams of resin for each 250 mL fungal culture in 100 mL Erlenmeyer flasks. The flasks were shaken to make sure all resin beads were in contact with the MeOH and left for 1 hour. All MeOH was carefully removed and replaced with 75 mL MilliQ H_2O, shaken briefly to make sure all resin beads were in contact with the MilliQ H_2O and left for 30 minutes. MilliQ H_2O was removed from the resin, before the resin was added to the culture flasks. After addition of resin the incubation was continued with shaking at 100 rpm at 13°C.

The cultures were filtrated through a cheesecloth using vacuum. To make the hydrophobic cheesecloth stick to the funnel it was first moistened with MeOH and washed with MilliQ H_2O to remove traces of MeOH. The cultures were then carefully filtered to remove the growth media. The culture flasks were rinsed with MilliQ H_2O and transferred the cheesecloth and resin back into the now empty culture flasks. 200 mL MeOH was added and extracted for 1 hour while shaking.

The second filtration was through a Whatman no. 3 filter. The filter was moisten with MeOH to make it stick to the funnel and the extract carefully filtrated with vacuum, making sure the resin stayed in the culture flasks. Added 200 mL MeOH to the culture flasks and extracted once more for 30 minutes. Filtrated again through the Whatman no. 3 filter. The filtrates were collected and transferred to round flasks before drying under reduced pressure using rotavapor. The extraction of each liquid fungal culture was performed separately.

Based on the number of extracts it was decided to skip the time-consuming fractionation step and test the crude extracts directly in bioassays. To prepare the extracts for bioassay testing they were, after drying, weighted and dissolved in MeOH to 40 mg/mL. 1 mL were then transferred to deep well plate and freeze dried. The freeze-dried extracts were then dissolved in 2.5% DMSO and MilliQ H_2O to stock solution of 1 mg/mL.

5.3.2 Antifungal broth dilution assay

Table 17 - Products and equipment used in the antifungal broth dilution assay.

Product/Equipment	Product ID/Equipment specifications	Distributor (Country)
Potato dextrose agar	70139	Sigma-Aldrich (Missouri, USA)
RPMI	R7755	Sigma-Aldrich (Missouri, USA)
MOPS	M3183	Sigma-Aldrich (Missouri, USA)
L-glutamine	K0302	VWR International (Pennsylvania, USA)
NaCl	S5886	Sigma-Aldrich (Missouri, USA)
Amphotericin B (AMP-B)	A2942	Sigma-Aldrich (Missouri, USA)
MilliQ H_2O	MilliQ Gradient A10	Merck Millipore KGaA, Germany
Remel 0.5 McFarland Equivalence Turbidity Standard	10026723	Thermo Fisher Scientific (Massachusetts, USA)
96 MicroWell™, Nunclon™Δ, U bottom	734-1192	VWR International (Pennsylvania, USA)
Sanyo Incubator MIP-262		Sanyo Electric Biochemical (Japan)
Heidolph Unimax 1010		Heidolph Instruments GmbH & Co (Germany)
Victor Multilabel Counter	1420 Multilabel Counter	Perkin Elmer (Massachusetts, USA)
Herasafe biological safety cabinet	Class II	Thermo Fisher Scientific (Massachusetts, USA)
Candida albicans	ATCC® 90028	

Candida albicans was collected from freeze stock and plated out on PDA plate. The plate was incubated over night at 37°C. 5-8 colonies from the overnight plate were inoculated in 5 mL sterile 0.9% NaCl. Corrected the cell density to 1-5 x 10^6 cells/mL in 0.9% NaCl using the 0.5 McFarland standard as guide. The *Candida* suspension were then diluted 1:50 in 0.9% NaCl and thereafter 1:20 in assay medium (10.4 g RPMI, 34.53 g MOPS, 1 L MilliQ H_2O, and 10.25 g L-glutamine (added after autoclavation))

The assay was performed in 96 MicroWell™ plates with rounded wells. Stock solutions of the extracts (1 mg/ml) were diluted to 500 µg/mL in assay medium and 100 µg/mL were added in two parallels as illustrated in figure 11. Further 100 µL diluted *Candida* suspension were then added to the same wells to a total of 200 µL in each well. As antifungal control, 100 µL 16 µg/mL Amphotericin B (AMP-B) diluted in sterile MilliQ H_2O + 100 µL diluted *Candida* suspension was added to wells B10-G10. As growth control 100 µL sterile MilliQ H_2O + 100 µL diluted *Candida* suspension were added wells B11-G11. As medium control 100 µL assay medium + 100 µL sterile MilliQ H_2O were added wells B12-G12. Because of evaporation all outermost wells were also only added 100 µL assay medium + 100 µL sterile MilliQ H_2O.

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

Figure 11 - Plate set up for Candida *assay. Extracts were added to blue colored wells in parallels as illustrated. Ampotericin B controls were added wells marked "A", growth controls were added wells marked "G", and media controls were added wells marked "M".*

Optical density (OD) at 600 nm was measured directly after addition of *Candida* suspension before incubation start. OD was measured again after 24 and 48 hours of incubation at 37°C.

5.3.3 Antibacterial broth dilution assay

Table 18 - Products and equipment for the antibacterial broth dilution assay.

Product/Equipment	**Product ID/Equipment specifications**	**Distributor (Country)**
Mueller Hinton broth (MH)	275730	Becton, Dickinson and Company (New Jersey, USA)
Brain heart infusion broth (BHI)	53286	Sigma-Aldrich (Missouri, USA)
Luria-Bertoni plates		University hospital of North Norway (UNN), (Norway)
Blood agar plates		University hospital of North Norway (UNN), (Norway)
96 MicroWell™ plates, Nuclon™Δ	734-2073	VWR International AS, Pennsylvania, USA
Gentamycin (10 mg/mL)	A 2712	Merck KGaA (Germany)
MilliQ H_2O	MilliQ Gradient A10	Merck Millipore KGaA, Germany
Sanyo Incubator MIP-262		Sanyo Electric Biochemical (Japan)
Heidolph Unimax 1010		Heidolph Instruments GmbH & Co (Germany)
Victor Multilabel Counter	1420 Multilabel Counter	Perkin Elmer (Massachusetts, USA)
Herasafe biological safety cabinet	Class II	Thermo Fisher Scientific (Massachusetts, USA)
Staphylococcus aureus	ATCC® 25923	LGC Standards (UK)
Escherichia coli	ATCC® 25922	LGC Standards (UK)
Enterococcus faecalis	ATCC® 29212	LGC Standards (UK)
Pseudomonas aeruginosa	ATCC® 27853	LGC Standards (UK)
Streptococcus agalactiae	ATCC® 12386	LGC Standards (UK)

Freeze stock of the bacteria were collected and transferred to blood agar plates. The plates were incubated overnight at 37°C and stored at 4°C. Overnight cultures were prepared by inoculating a scoop of the bacteria in 8 mL growth medium. 2 mL overnight culture was inoculated in 25 mL fresh medium in sterile Erlenmeyer flasks and incubated each bacterium as specified in table 19 at 37°C with shaking.

Table 19 – Incubation parameters for bacteria used in MIC assay.

Bacterial strain	Medium	Incubation time	Bacterial density
S. aureus	MH-broth	2.5 hours	0,5-3x10^5 CFU/mL (2500-15000 CFU/well)
E. coli	MH-broth	1.5 hours	0,5-3x10^5 CFU/mL (2500-15000 CFU/well)
E. faecalis	BHI-broth	1.5 hours	0,5-3x10^5 CFU/mL (2500-15000 CFU/well)
P. aeruginosa	MH-broth	2.5 hours	0,5-3x10^4 CFU/mL (2500-15000 CFU/well)
S. agalactiae	BHI-broth	1.5 hours	0,5-3x10^5 CFU/mL (2500-15000 CFU/well)

After completed incubation, the bacterial cultures were diluted 1:1000 in their appropriate growth medium. Stock solutions of extracts were diluted to 100 µg/mL in sterile MilliQ H_2O. In the primary screening for antibacterial activity all extracts were tested at a final concentration of 50 µg/mL. In the secondary screening the extracts were tested at 50, 25, 10, and 1 µg/mL, both rounds of screening in duplicates.

50 µL diluted extracts were added to column 2-11, as illustrated in figure 12, to five microtiter plates, one for each bacterial strain. Thereafter 50 µL diluted bacterial suspension was added to the same wells. Medium control constituting 50 µL growth medium + 50 µL sterile MilliQ H_2O were added column 1. And growth control, 50 µL bacterial suspension + 50 µL sterile MilliQ H_2O were added column 12.

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

Figure 12 - Plate set up for the antibacterial MIC assay. Extracts were added blue colored wells added numbered wells. G: growth control (MilliQ H_2O + bacterial suspension), M: medium control (growth medium + MilliQ H_2O).

The plates were incubated overnight at 37°C. Before reading, the plates were inspected for observable inhibition and thereafter read using Victor plate reader by measuring the absorbance (Abs) at 600 nm. The extracts were then classified as active, questionable or inactive by the threshold Abs_{600} values:

Active ≤ 0.05

Questionable 0.05 – 0.09

Inactive ≥ 0.09

In the assay design used for MIC screening the antibacterial activity is tested in two rounds. The first round all samples are tested at 50 µM and in the subsequent round only the samples that exhibited activity are retested in a dilution series to identify the MIC value. However, as no actives were observed at 50 µM, the second round of dilution series testing was not performed.

5.3.4 Antibiofilm assay

In the antibiofilm assay the extracts ability to inhibit the formation of biofilm by the biofilm producing bacteria *Staphylococcus epidermidis* was evaluated. The non-biofilm producing bacteria *Staphylococcus haemolyticus* was used as negative control and the extracts were tested in triplets.

Table 20 - Products and equipment used in the biofilm assay.

Product/Equipment	**Product ID/Equipment specifications**	**Distributor (Country)**
Tryptic soy broth (TBS)	1.05459	Merck KgaA (Germany)
Dextrose (Glucose)	D9434	Sigma-Aldrich (Missouri, USA)
Ethanol	24106	Sigma-Aldrich (Missouri, USA)
Crystal violet	1.15940	Merck KgaA (Germany)
MilliQ H_2O	MilliQ Gradient A10	Merck Millipore KGaA, Germany
Blood agar plates		University hospital of North Norway (UNN), (Norway)
96 MicroWell™ plates, Nuclon™Δ	734-2073	VWR International, Pennsylvania, USA
Sanyo Incubator MIP-262		Sanyo Electric Biochemical (Japan)
Heidolph Unimax 1010		Heidolph Instruments GmbH & Co (Germany)
Victor Multilabel Counter	1420 Multilabel Counter	Perkin Elmer (Massachusetts, USA)
Herasafe biological safety cabinet	Class II	Thermo Fisher Scientific (Massachusetts, USA)
Staphylococcus epidermis	ATCC® 35984	LGC Standards (UK)
Staphylococcus haemolyticus	Clinical isolate 8-7 A	University hospital of North Norway (UNN), (Norway)

Freeze stock of *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* was collected and transferred to blood agar plates. The plates were incubated overnight at 37°C and stored at 4°C.

Overnight cultures were prepared by inoculating a scoop of each bacterial strain in 5 mL TBS medium and incubated with shaking overnight at 37°C. The overnight cultures were diluted 1:100 in fresh TBS medium with 1% glucose. The stock solution of the extracts were diluted to 100 µg/mL in MilliQ H_2O. 50 µL of the extracts were transferred to the numbed wells, and 50 µL MilliQ H_2O to the remaining wells as illustrated in figure 13. 50 µL *S. epidermidis* suspension with 1% glucose were transferred to column 1-10, and 50 µL *S. haemolyticus* suspension with 1% glucose were transferred to column 11 and 50 µL TBS medium with 1% glucose to column 12.

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

*Figure 13 - Plate set up for the biofilm assay. Extracts were added the blue colored wells, dispersed as illustrated in triplets. G: growth control (*S. epidermidis *+ MilliQ H_2O), N: negative control (*S. haemolyticus *+ MilliQ H_2O), and M: medium control (TBS medium + MilliQ H_2O).*

Incubated the plates over night at 37°C. Examined the plates for lethal effects on the bacteria. Removed the bacterial suspensions from all wells by tapping the plates against cell paper. Rinsed the plates with water and tapped the plates dry against the cell paper. Fixated the biofilm by incubating at 55°C for 1 hour. After fixation, 70 µL 0.1% crystal violet were added to the plates for 5 minutes to color the biofilm. The crystal violet was removed by tapping against cell paper. Cleansed the plates twice with water and dried at 55°C for 1 hour. Then 70 µL 70% ethanol were added all wells and incubated for 10 minutes with shaking at room temperature. Absorbance at 600 nm were measured using Victor plate reader. The extracts were classified as active (A), inactive (I) or questionable (Q) by the threshold Abs_{600} values:

Active: OD < 30 % of growth control

Questionable: OD = 30-40% of growth control

Inactive: OD > 40% of growth control

5.3.5 Anticancer assay

In the anticancer assay the extracts were investigated for toxic effects on the human cancer cell lines A2058 (human melanoma), HepG2 (human hepatocellular carcinoma) and MCF7 (human breast adenocarcinoma), and the non-cancerous lung fibroblast cell line MRC5 as control for normal human cells and toxicity.

The anticancer activity of the extracts were investigated by performing the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay. This is a colorimetric method that can be applied

to find the number of viable cells in proliferation or cytotoxicity assays. the CellTiter 96® AQ_{ueous} One Solution Reagent contains the yellow colored novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS]. This reagent is added to the micro titer plate after incubation 1-4 hours before reading of assay results. In living cells, the MTS tetrazolium compound is bioreduced to a red colored formazan product that is soluble in the culture medium. Measured at 490nm, the amount of formazan is directly proportional to the number of living cells in the culture (Promega Corporation, 2012).

Table 21 - Products and equipment used in the cancer assay.

Product/Equipment	Product ID/Equipment specifications	Distributor (Country)
Earle's Minimal Essential Medium (E-MEM) with 20 mM HEPES	F4315	Merck KGaA (Germany)
Roswell Park Memorial Institute medium (RPMI) 1640	FG 1383	Merck KGaA (Germany)
Dulbecco's Modified Eagle Meduim (D-MEM), high glucose, GlutaMAX™ Supplement, HEPES	32430027	Thermo Fisher Scientific (Massachusetts, USA)
Non-essential amino acids (NEA) (100x)	K0302	Merck KGaA (Germany)
L-Alanyl-L-Glutamine (200 mM)	K 0302	Merck KGaA (Germany)
CellTiter 96® Aqueous One Solution Reagent	G358B	Promega (Wisconsin, USA)
Gentamycin (10 mg/mL)	A 2712	Merck KGaA (Germany)
Trypsin (1:250)	27250018	Thermo Fisher Scientific (Massachusetts, USA)
Triton™ X-100	T8787	Sigma-Aldrich (Missouri, USA)
Trypan blue	T 6146	Sigma-Aldrich (Missouri, USA)
96 MicroWell™ plates, Nuclon™Δ	734-2073	VWR International AS, Pennsylvania, USA
DTX 880 Multimode Detector		Beckman Coulter
HeraCell Vios 160L CO2 Incubator, Thermo Scientific		Thermo Fisher Scientific (Massachusetts, USA)
Herasafe biological safety cabinet	Class II	Thermo Fisher Scientific (Massachusetts, USA)
A2058	ATCC® CRL-11147™	LGC Standards (UK)
MCF7	ATCC® HTB-22	LGC Standards (UK)
MRC5	ATCC® CCL-171	LGC Standards (UK)
HepG2	ATCC® HB-8065	LGC Standards (UK)

RPMI, D-MEM, PBS and Trypsin were preheated to 37°C. Cell culture flasks were collected from incubator and media were removed from the cells. 10 mL PBS were transferred to culture flasks to wash the cells for 1 minute before removal. Thereafter, 5 mL trypsin were transferred

to the culture flasks until the cells detached from the flask wall. The cultures were inspected under microscope to conform that the cells had detached from the flask. The cells were resuspended in 10 mL growth medium (D-MEM for A2058, E-MEM for HepG2, MCF7 and MRC5). 100 µL cell suspension were mixed with 100 µL Trypan Blue in an Eppendorf tube. 10 µL of the mixture were transferred to Bürker-Türk counting chamber and the cells were counted under the microscope. The cell density was adjusted to 2 x 10^4 cells/mL, before 100 µL cell suspension were added to each well achieving a final concentration of 2000 cells per well for A2058 and MCF7, 4000 for MRC5 and 20 000 cells per well for HepG2. The plates were incubated overnight at 37°C, 5% CO_2.

Growth media were removed from all wells and replaced with 5 µL of the extracts in triples and 95 µL fresh media in the numbered wells as illustrated in figure 14. Positive control (90 µL growth media + 10 µL DMSO) were added wells marked "P". The remaining wells were added 100 µL growth media, including the wells marked N (negative control). Incubated the HepG2 plates for 24 hours and the A2058, MCF7 and MRC5 plates for 72 hours at 37°C, 5% CO_2.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A												
B		1	2	3	4	5	6	7	8	N		
C		1	2	3	4	5	6	7	8	N		
D		1	2	3	4	5	6	7	8	N		
E		9	10	11	12	13	14	15	16	N		
F		9	10	11	12	13	14	15	16			G
G		9	10	11	12	13	14	15	16			G
H												G

Figure 14 - Plate set up for the cancer assay. Extracts were added blue colored, numbered wells in triplets, P: positive control (growth media + DMSO), N: negative control (growth media). All remaining wells were only added growth media.

Before reading, 10 µL Aqueous One Solution (AQOS) were added to each well, the plates were then incubated for 1 hour at 37°C, 5% CO_2. Thereafter the plates were read using DTX 880 Multimode Detector by measuring the absorbance at 485 nm. The percentage survival of cancer cells were calculated, and based on this estimate the extracts were classified as active (A), inactive (I) or questionable (Q) according to the threshold values:

Active < 50% survival

Questionable = 50-60% survival

Inactive > 60% survival

5.4 Mass spectrometry

Mass spectrometry (MS) was used to analyze all the solid cultures with epigenetic modifiers, the ethyl acetate extract of the solid cultures as well as the solid phase extracts of the liquid cultures with epigenetic modifiers.

To prepare samples for the MS run, 20 µL of each extract were added 100 µL MS grade MeOH to dilute the samples in Eppendorf tubes. All MS samples were then centrifuged for 10 min at 13 000 rpm to spin down particles that can clog the MS machine. Transferred 100 µL of each sample to MS vials. The Waters Acquity I-class UPLC system interfaced with a PDA Detector and VION IMS-qTOF was used for the UPLC-HR-MS analysis. Electrospray ionization (ESI) in positive mode and detected wavelengths of 190-500nm was applied. The following VION IMS qTOF settings were applied for the UPLC-HR_MS analysis: 0.80 kV capillary voltage, 50 l/h cone gas, desolvation temperature of 350°C, 800 L/h desolvation gas, source temperature of 120°C, acquisition range of 50-2000 m/z. The system was controlled by UNIFY 1.8.2 (Waters), which also were used to process the data. BFH C18 1.7 µm (2.1 x 100 mm) column (Waters) was used for the chromatographic separation and maintained at 40°C. The peaks of interest were dereplicated using Bovine Metabolome Database, Bovine Rumen Metabolome Database, Carotenoids Database, CSF Metabolome Database, DrugBank, E. coli Metabolome Database, Fecal Metabolome Database, Golm Metabolome Database, Human Metabolome Database, LipidMAPS, Marine Drugs, Nature Chemical Biology, Nature Chemistry, Nature Communications, Peptides, Saliva Metabolome Database, Serum Metabolome Database, Springer Nature, Urine Metabolome Database and Yeast Metabolome Database.

6 Results

6.1 Bioactivity of initial cultures

The initial solid cultures and liquid micro-cultures of the eight fungal isolates without epigenetic modifiers were tested for antibacterial activity against the five pathogenic bacteria *E. coli*, *P. aeruginosa*, *S. aureus, E. faecalis* and *S. agalactiae*. Of the solid cultures only TS7 were found active in the antibacterial agar plug assay, exhibiting activity against *E. faecalis* and *S. agalactiae* (Appendix 2). In the antibacterial screening of the liquid micro-culture supernatants and in the antibacterial agar disc diffusion assay none of the fungal isolates exhibited any activity. The liquid micro-culture supernatants were also tested for protease activity, in which 008cD1.1 in M6 and M6 LPS medium exhibited activity. On the basis of these results TS7 and 008cD1.1 were selected for further cultivation together with TRa3160C, which were of special interest for my advisors.

6.2 Effects of epigenetic modifiers on fungal growth pattern

The growth of *Digitatispora marina* (008cD1.1), *Typhula* sp. (TRa3160C) and *Acremonium* sp. (TS7) were inspected after 22 and 25 days of incubation on solid medium with the epigenetic modifiers 5-azacytidine, SBHA and nicotinamide.

6.2.1 Solid cultures of *Digitatispora marina* (008cD1.1)

Digitatispora marina plates were not full-grown after 3 weeks of cultivation (figure 15) and grew slower than the two other fungi. No clear difference in growth pattern were observed by the application of different epigenetic modifiers on neither M6 or M6 LPS agar, and the growth did not seem to be much affected by their application compared to control cultures, not added epigenetic modifiers.

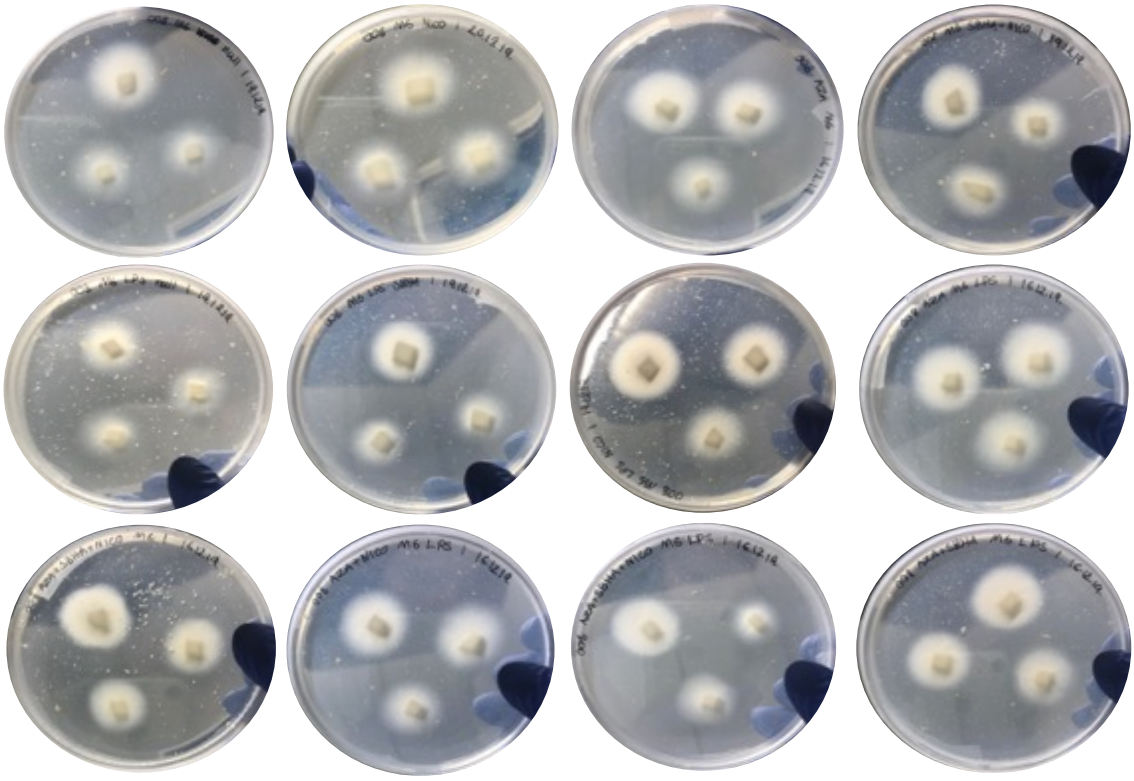

Figure 15 - Growth of Digitatispora marina spotted at three locations on each plate *of M6 and M6 LPS agar with epigenetic modifiers 5-azacytidine, SBHA and nicotinamide after 22 days of incubation.*

6.2.2 Solid cultures of *Typhula* sp. (TRa3160C)

Typhula sp. grew significantly better on corn meal agar (CMA) compared to *Ascophyllum nodosum* agar (ANA) and were full-grown on CMA plates after 3 weeks of cultivation (figure 16), while only forming thin treads of mycelia on ANA at the same point of cultivation (figure 17). *Typhula* sp. exhibited visible changes of growth pattern on CMA in response to different epigenetic modifiers.

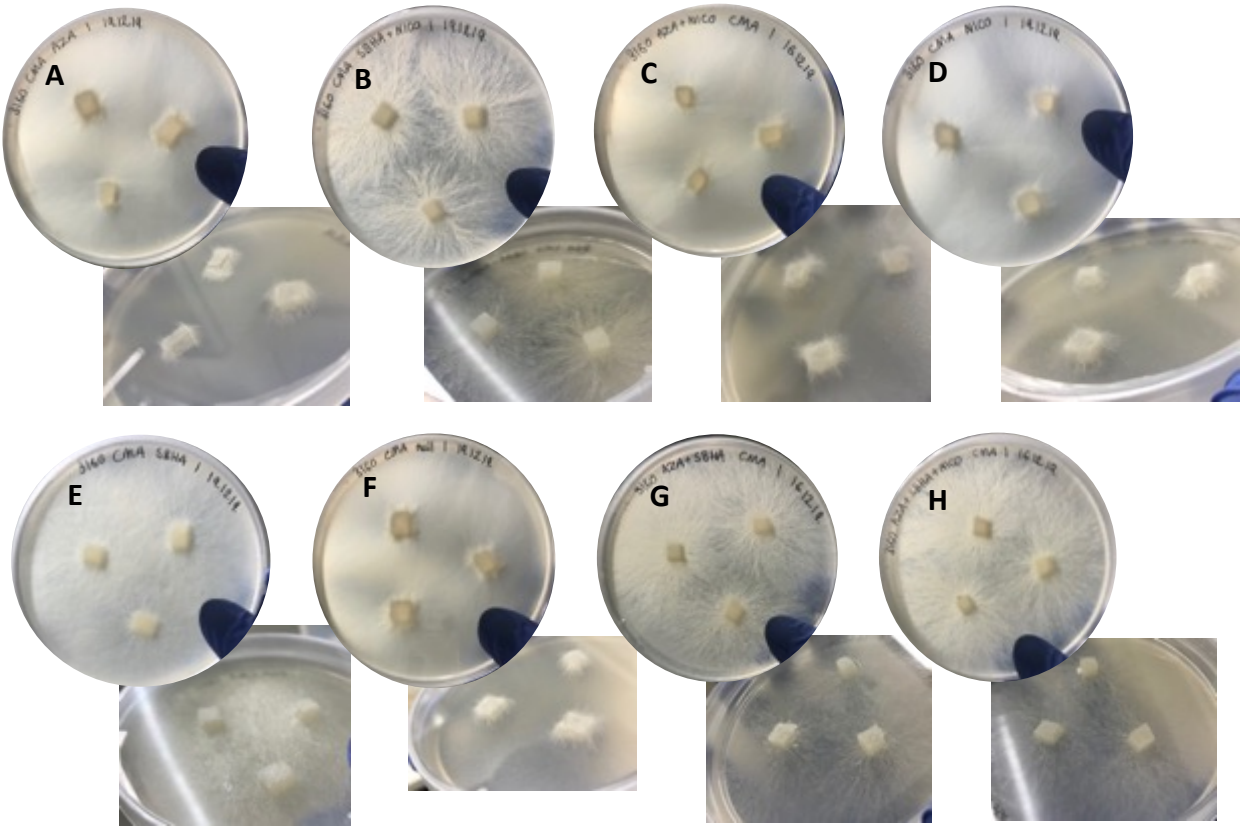

Figure 16 - Growth of Typhula *sp.* spotted at three locations on each plate *of corn meal agar with epigenetic modifiers 5-azacytidine, SBHA and nicotinamide after 22 days of incubation. A) 5-azacytidine, B) SBHA + nicotinamide, C) 5-azacytidine + nicotinamide, D) nicotinamide, E) SBHA, F) control, G) 5-azacytidine + SBHA, H) 5-azacytidine + SBHA + nicotinamide.*

Addition of 5-azacytidine (figure 16 A) and nicotinamide (figure 16 D) both separately and conjointly (figure 16 C) seemed to have the most similar growth pattern to the control cultures (figure 16 F) with dense, thin mycelia evenly dispersed all over the plate with some growth on top of the plugs as well. Cultures only added SBHA (figure 16 E) also formed dense, thin mycelia but exhibited in addition more growth on top of the agar and had denser growth near the plugs. Culture added SBHA in addition to 5-azacytide and nicotinamide, both separately and combined (figure 16 B, G and H), exhibited similar growth pattern differing both from cultures only added SBHA and cultures with 5-azacytidine and nicotinamide without SBHA. In these cultures *Typhula* sp. grew thicker mycelia with denser growth near plugs and not as evenly dispersed growth over the whole plates as seen in figure 16 A, C, D and F, and with less growth on the surface of the agar.

Growth of *Typhula* sp. on ANA (figure 17) were quite frail, forming really thin mycelia only visible by close examination.

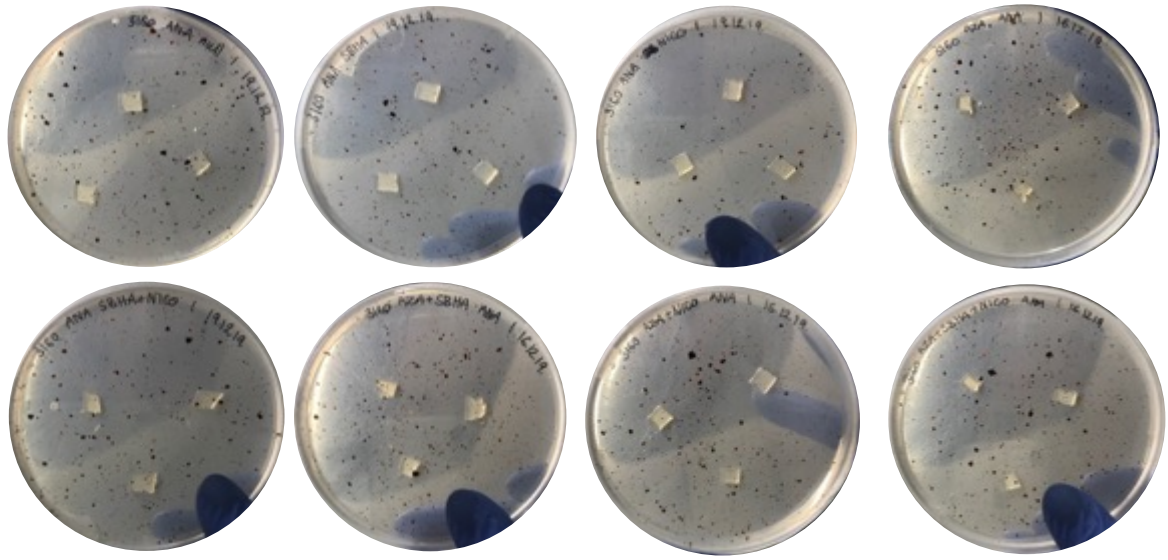

Figure 17 - Growth of Typhula *sp. on* Ascophyllum nodosum agar *with epigenetic modifiers 5-azacytidine, SBHA and nicotinamide after 22 days of incubation.*

6.2.3 Solid cultures of *Acremonium* sp. (TS7)

Acremonium sp. grew well on both M6 and M6 LPS agar in the presence of epigenetic modifiers (figure 18) and were almost full-grown after 3 weeks of cultivation. However, visible differences in growth pattern in response to different epigenetic modifiers were not observed.

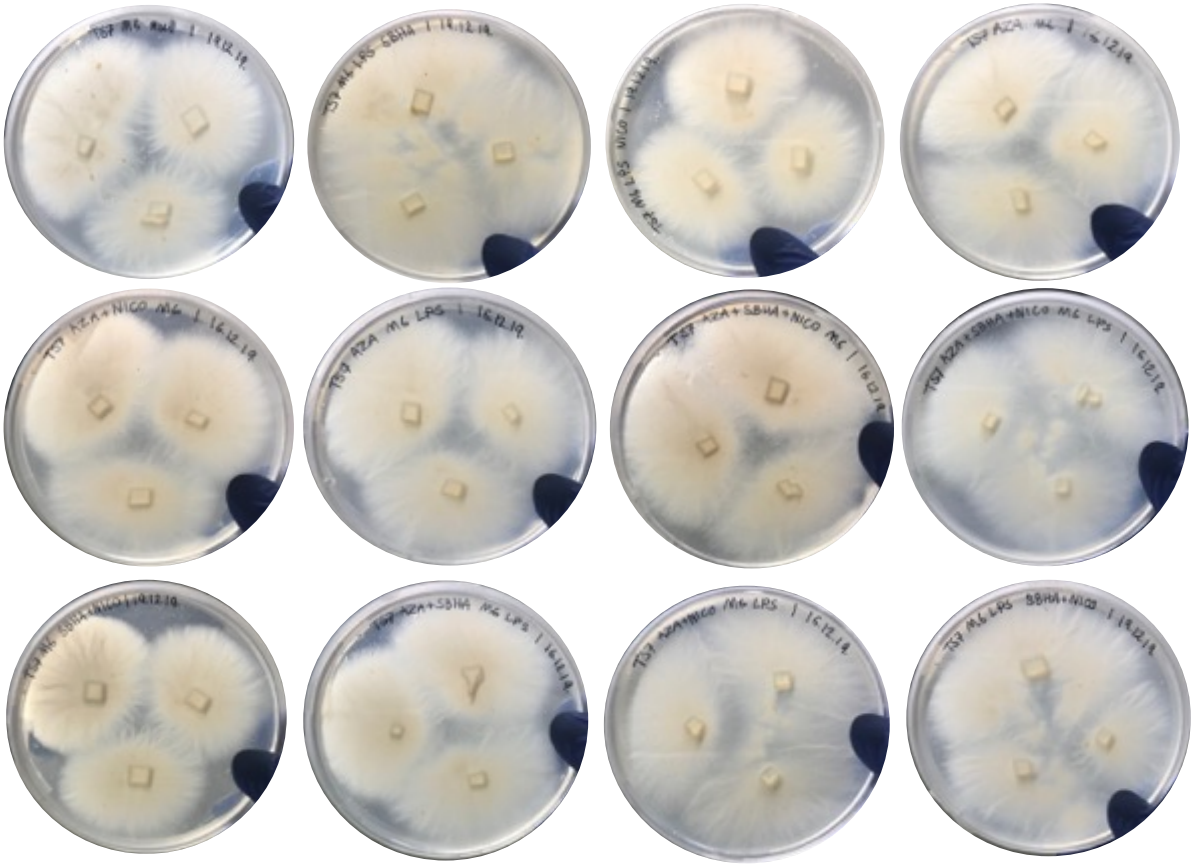

Figure 18 - Growth of Acremonium *sp. on M6 and M6 agar with epigenetic modifiers 5-azacytidine, SBHA and nicotinamide after 22 days of incubation.*

6.2.4 Liquid cultures of *Acremonium* sp. (TS7)

Acremonium sp. cultivated in liquid M6, M6 LPS and ASME media exhibited similar growth growing in evenly dispersed threads and lumps out of the agar plugs used for inoculation of the cultures. It seemed to grow a bit faster and denser in M6 and M6 LPS compared to ASME but displayed the same pigmentation and growth pattern.

Liquid FMAP and FMAP LPS cultures initially displayed the same growth pattern and culture pigmentation but after a week of cultivation started growing in thin biofilm looking layers on top of the media in some cultures (figure 19). These layers were disrupted to assure all fungal material were in contact with the media and had access to nutrients. DNA samples were taken and analysed of all cultures and were pure.

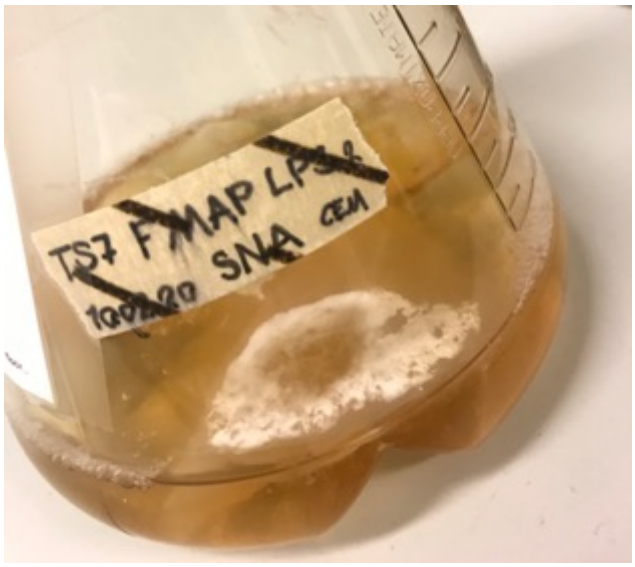

Figure 19 - Biofilm looking growth observed in TS7 FMAP LPS ASN culture. Pictures taken a few days after attempting to disrupt the growth structure.

After addition of epigenetic modifiers cultures added SBHA were observed becoming more pigmented than cultures not added SBHA (TS7 FMAP null, TS7 FMAP AN, TS7 FMAP LPS null and TS7 FMAP LPS AN) (figure 20 A). This pigment seemed to also be present in the extracts of the cultures as the extracts had the same pigmentation as the cultures (figure 20 B). This pigmentation was not observed in SBHA containing cultures in different media.

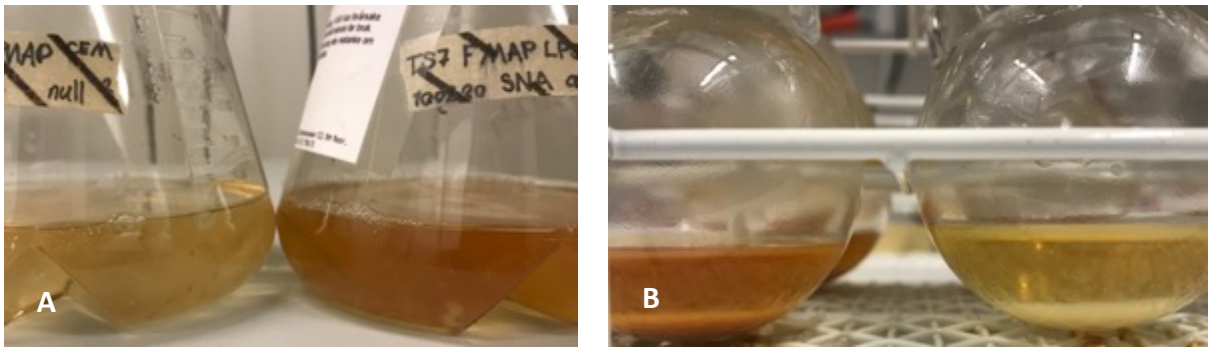

Figure 20 - Photographs of the evident pigmentation difference between various TS7 FMAP liquid cultures and extracts. A) TS7 FMAP null culture to the left and TS7 FMAP LPS ASN culture to the right. B) TS7 FMAP S extract to the left and TS7 FMAP AN extract to the right.

6.3 Bioactivity of solid cultures

Solid cultures of TS7, 008cD1.1 and TRa3160C with epigenetic modifiers 5-azacytidine, SBHA and nicotinamide were tested for antibacterial activity against *E. coli*, *P. aeruginosa*, *S. aureus, E. faecalis* and *S. agalactiae* and antifungal activity against *Candida albicans*. Only TS7 showed activity in the antibacterial assay, results of 008cD1.1 and TRa3160C are therefore not shown. TS7 exhibited activity against the three Gram-positive bacteria *S. aureus, E. faecalis* and *S. agalactiae*, with the strongest activity against *S. agalactiae* (table 22). The activity against *S. agalactiae* were mostly consistent for all TS7 cultures, regardless of supplemented epigenetic modifiers, with the small exception of TS7 M6 LPS AN that also were active against *S. agalactiae*, but were slightly less potent, making an inhibition zone of 17 mm compared to the average inhibition zone of 23 mm of the remaining cultures.

Table 22 – Bioactivity results of solid cultures of TS7 *from antibacterial plug assay.* E. c: E. coli., P. a: P. aeruginosa, S. a: S. aureus, E. f: E. faecalis, S. B: S. agalactiae (Streptococcus type B). (-): Gram-negative bacteria, (+): Gram-positive bacteria. Values presented represent diameter of inhibition zone around the fungal plug (including the plug of 6 mm) in millimeters.

Fungus	**Culture**	***E. c*** (-)	***P. a*** (-)	***S. a*** (+)	***E. f*** (+)	***S.* B** (+)
***Acremonium* sp.**	TS7 M6 null	-	-	13	11	23
***Acremonium* sp.**	TS7 M6 S	-	-	13	11	23
***Acremonium* sp.**	TS7 M6 A	-	-	7	6	24
***Acremonium* sp.**	TS7 M6 N	-	-	12	10	22
***Acremonium* sp.**	TS7 M6 SN	-	-	13	11	23
***Acremonium* sp.**	TS7 M6 AS	-	-	7	6	25
***Acremonium* sp.**	TS7 M6 AN	-	-	6	6	24
***Acremonium* sp.**	TS7 M6 ASN	-	-	7	6	24
***Acremonium* sp.**	TS7 M6 LPS null	-	-	11	11	22
***Acremonium* sp.**	TS7 M6 LPS S	-	-	10	10	20
***Acremonium* sp.**	TS7 M6 LPS A	-	-	6	6	23
***Acremonium* sp.**	TS7 M6 LPS N	-	-	11	10	23
***Acremonium* sp.**	TS7 M6 LPS SN	-	-	12	11	23
***Acremonium* sp.**	TS7 M6 LPS AS	-	-	6	6	23
***Acremonium* sp.**	TS7 M6 LPS AN	-	-	6	-	17
***Acremonium* sp.**	TS7 M6 LPS ASN	-	-	6	-	22

No fungal cultures showed any antifungal activity toward *Candida albicans*. Despite several attempts at modifying the assay for testing antifungal activity of liquid cultures to a solid culture format, there were no observed inhibition of *Candida albicans* growth by the antifungal agent Amphotericin B. The antifungal assays of the solid cultures were therefore invalidated.

6.4 Extraction yields

Due to the high similarity in bioactivity between solid TS7 cultures, and the low yield normally obtained from extraction of solid cultures, it was decided to combine all 32 remaining solid TS7 cultures after bioassays (16 culture conditions in parallels) to perform one extraction. The ethyl acetate extraction of these cultures yielded an extract of 90.8 mg dry weight.

The liquid cultures of TS7 with epigenetic modifiers were extracted by solid phase extraction using Diaion® HP-20, which is the standard extraction protocol used at the Marbio lab. Each liquid culture of 250 mL was extracted separately and yielded significantly bigger extracts than the ethyl acetate extraction of the solid cultures, with an average extract weight of 589.4 mg.

Table 23 – Extract yield in mg for liquid cultures of TS7 with added epigenetic modifiers and media controls (Ctr).

	Ctr	null	S	SN	SA	NA	SNA
M6	326.9	301.1	751.9	362.4	526.2	586.5	454.3
M6 LPS	663.8	221.3	286.9	395.3	510.0	1255.3	598.0
FMAP	781.6	607.8	527.2	892.8	612.0	1064.2	1060.1
FMAP LPS	799.7	545.3	963.7	693.2	706.6	1216.1	989.8
ASME	313.3	255.4	310.4	169.8	224.2	344.4	310.6

FMAP and FMAP LPS cultures yielded on average the biggest extracts with mean weights of 792.3 and 844.9, respectively compared to 472.8, 561.5 and 275,4 for M6, M6 LPS and ASME, respectively.

6.5 Bioactivity of extracts

The extracts of both liquid and solid cultures were tested for antibacterial activity against *E. coli*, *P. aeruginosa*, *S. aureus, E. faecalis* and *S. agalactiae*, antibiofilm activity against *S. epidermidis,* antifungal activity against *Candida albicans* and anticancer activity against the human cancer cell lines A2058 (human melanoma), HepG2 (human hepatocellular carcinoma) and MCF7 (human breast adenocarcinoma) with the non-cancerous lung fibroblast cell line MRC5 as control for normal human cells and toxicity. All extracts were first tested in concentration of 50 µg/mL in all assays, which is the standard concentration for screening of flash fractions at Marbio. At this concentration all extracts were inactive in all assays, except for the solid culture ethyl acetate extract, which were classified as *questionable* with OD=0.06 (threshold for activity: <0.05) exhibiting activity against *S. agalactiae* (*Strep .B*) (table 24).

Table 24 - Results of antibacterial screening of ethyl acetate extract of solid TS7 M6 and M6 LPS cultures. Number represent measured OD. Threshold for activity: 0.05, questionable: 0.09-0.05. E. c: E. coli, P. a: P. aeruginosa, S. a: S aureus, E. f: E. faecalis, S.B: S. agalactiae *(staphylococci group B).*

	E. c	***P. a***	***S. a***	***E. f***	***S. B***
50 µM	0.43	0.47	0.30	0.29	0.06
150 µM	0.36	0.49	0.13	0.10	0.03

It was decided to retest the extract at a higher concentration due to the lack of activity in most extracts. This may be due to the complexity of crude extracts that possibly contain contradictory compounds inhibiting bioactive compounds, compared to purified fractionated samples. The antibacterial and anticancer assays were repeated at 150 µg/mL. At this concentration the solid culture ethyl acetate extract was active against *Strep. B* with OD=0.03 and exhibited some tendency to activity against *S. aureus* and *E. faecalis* as well (table 24),

with OD of 0.13 and 0.10, respectively (threshold for classification as questionable: 0.06-0.09). The remaining extract did not exhibit activity at increased concentration, therefore only the results of the solid culture ethyl acetate extract are presented here. The results of the liquid culture extract are in Appendix 2.

No anticancer activity was observed at increased concentrations in any of the extracts. Additional assays were not possible to perform due to the occurrence of the COVID-19 pandemic resulting in the closing off of university locations, including laboratories, for all students and restricted access for most employees.

6.6 Mass spectrometry analysis

Bioactivity was only observed in the solid cultures and the ethyl acetate extract produced from them. The identification of the compound(s) accountable for this activity in these samples was the main focus of the MS analysis. Generally, most of the cultures seemed to have the same peaks in the MS chromatograms, with some variation in the height of the peaks and the strength of the signals of various candidate masses. A common feature in these samples was strong responses for the candidate mass 1050.75 m/z RT: 8.72 (predicted mainly to $C_{57}H_{105}N_5O_8P_2$ or $C_{54}H_{99}N_9O_{11}$) (figure 21). The signal of 1050.75 m/z occurred in all solid TS7 cultures as well as the solid culture extract (table 25). This signal represented the strongest or second strongest signal in 12 out of 16 solid cultures and was the third, fourth and seventh highest in the remaining 4 cultures, with TS7 M6 LPS AN having the weakest signal with 1050.75 m/z as the seventh strongest signal. The solid culture extract had 1050.75 m/z as the second highest signal.

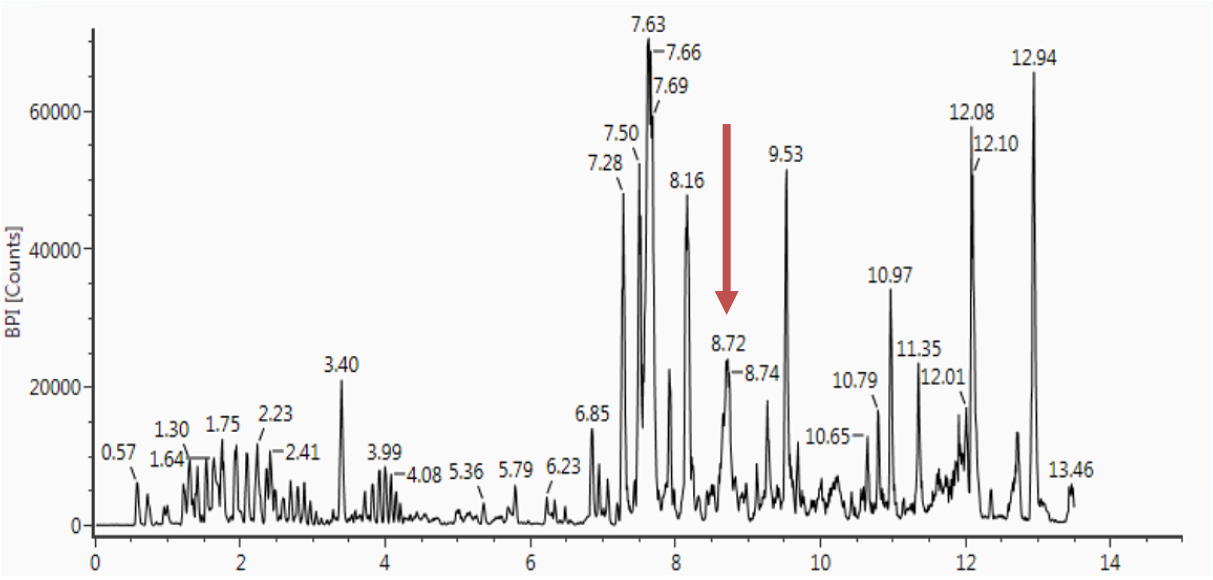

Figure 21 - Mass spectrometry chromatogram of ethyl acetate extract of all solid TS7 M6 and M6 LPS cultures. The red arrow indicated to the peak of 1050.7514 m/z, which represents the second strongest response after 511.3740 m/z at RT: 7.63. Height of peaks in this chromatogram represents their base peak counts, (not the same as response).

Table 25 - Mass to charge (m/z), retention time (RT), response, predicted elementary compositions (with elements: C, H, N, O, (P) and adduct: +H) and iFIT confidence of prediction, and intensity of response in sample (1st: strongest response in sample etc.) of TS7 ethyl acetate extract (EtoAcE) of solid cultures and solid TS7 cultures with epigenetic modifiers before extraction.

	m/z	RT	Response	Prediction	Response rank
EtoAcE	1050.7514	8.72	1 039 529	$C_{57}H_{105}N_5O_8P_2$ (97.51%)	2nd
M6 null	1050.7515	8.54	1 964 987	$C_{57}H_{105}N_5O_8P_2$ (97.29%)	1st
M6 S	1050.7516	8.57	1 273 352	$C_{57}H_{105}N_5O_8P_2$ (99.89%)	1st
M6 A	1050.7521	8.57	564 278	$C_{57}H_{105}N_5O_8P_2$ (77.64%) $C_{54}H_{99}N_9O_{11}$ (91.96%)	3rd
M6 N	1050.7512	8.56	823 205	$C_{57}H_{105}N_5O_8P_2$ (97.15%)	1st
M6 SN	1050.7515	8.57	688 899	$C_{57}H_{105}N_5O_8P_2$ (91.64%)	2nd
M6 AS	1050.7516	8.58	852 158	$C_{57}H_{105}N_5O_8P_2$ (84.44%)	2nd
M6 AN	1050.7527	8.57	1 262 934	$C_{57}H_{105}N_5O_8P_2$ (59.74%) $C_{54}H_{99}N_9O_{11}$ (92.14%)	1st
M6 ASN	1050.7521	8.56	1 145 116	$C_{57}H_{105}N_5O_8P_2$ (24.14%) $C_{54}H_{99}N_9O_{11}$ (96.90%)	1st
M6 LPS null	1050.7524	8.60	1 336 598	$C_{57}H_{105}N_5O_8P_2$ (32.08%) $C_{54}H_{99}N_9O_{11}$ (96.04%)	1st
M6 LPS S	1050.7518	8.62	621 794	$C_{57}H_{105}N_5O_8P_2$ (81.95%) $C_{54}H_{99}N_9O_{11}$ (89.53%)	3rd
M6 LPS A	1050.7522	8.63	807 505	$C_{57}H_{105}N_5O_8P_2$ (37.47%) $C_{58}H_{101}N_9O_4P_2$ (42.77%) $C_{59}H_{112}N_3O_2P_5$ (16.54%)	2nd
M6 LPS N	1050.7518	8.62	769 632	$C_{57}H_{105}N_5O_8P_2$ (89.92%)	2nd
M6 LPS SN	1050.7516	8.60	865 759	$C_{57}H_{105}N_5O_8P_2$ (85.02%)	2nd
M6 LPS AS	1050.7511	8.58	480 776	$C_{57}H_{105}N_5O_8P_2$ (69.32%) $C_{58}H_{101}N_9O_4P_2$ (10.80%)	4th
M6 LPS AN	1050.7523	8.60	421 714	$C_{57}H_{105}N_5O_8P_2$ (68.05%) $C_{54}H_{99}N_9O_{11}$ (78.05%)	7th
M6 LPS ASN	1050.7519	8.59	959 513	$C_{57}H_{105}N_5O_8P_2$ (81.55%) $C_{54}H_{99}N_9O_{11}$ (89.27%)	1st

In 7 out of the 16 solid cultures the elementary composition of $C_{54}H_{99}N_9O_{11}$ were predicted more likely than $C_{57}H_{105}N_5O_8P_2$ (which was the most likely elementary composition of 8 out of the 16 solid cultures and the solid culture extract). One culture, M6 LPS A, did not have high iFIT confidence for neither $C_{57}H_{105}N_5O_8P_2$ nor $C_{54}H_{99}N_9O_{11}$. In contrary to $C_{57}H_{105}N_5O_8P_2$, $C_{54}H_{99}N_9O_{11}$ had three hits for molecular structure in PubChem as well as a structure named, Emericellipsin A (figure 22), isolated from a closely related fungus to TS7, *Emericellopsis alkalina* (sexual form of *Acremonium alkalina*), which has been found to exhibit bactericidal activity against methicillin resistant *S. aureus* and vancomycin resistant *E. faecalis* (Rogozhin et al., 2018).

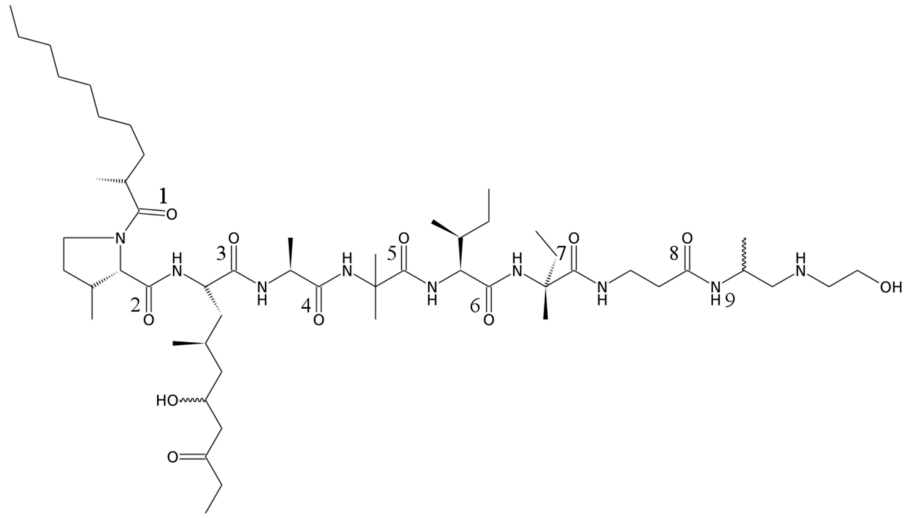

Figure 22 - Structure of Emericellipsin A ($C_{54}H_{99}N_9O_{11}$, 1049.76 Da.) (Rogozhin et al., 2018).

Table 26 - Mass to charge (m/z), retention time (RT), response, and intensity of response in sample (1st: strongest response in sample etc.) of the extracts of liquid TS7 cultures which contained 1050.75 m/z.

	m/z	RT	Response	Response rank
M6 null	1050.7525	8.64	34 567	571st
M6 S	1050.7516	8.65	19 064	868th
M6 SN	1050.7533	8.65	19 027	768th
M6 AS	1050.7526	8.64	10 872	1225th
M6 AN	1050.7527	8.65	14 664	993rd
M6 ASN	1050.7520	8.64	15 592	1388th
M6 LPS null	1050.7526	8.64	63 694	342nd
M6 LPS S	1050.7521	8.64	67 918	249th
M6 LPS SN	1050.7532	8,65	28 962	551st
M6 LPS AS	1050.7520	8.64	7 067	981st
M6 LPS AN	1050.7533	8.64	4 927	1306th
M6 LPS ASN	1050.7526	8.63	3 686	1624th
ASME null	1050.7524	8.62	20 659	240th
ASME S	1050.7521	8.63	20 788	271st
ASME SN	1050.7533	8.62	4 804	454th
ASME AS	1050.7515	8.61	12 466	338th
ASME AN	1050.7508	8.62	9 968	330th
ASME ASN	1050.7530	8.62	4 203	550th
FMAP LPS AS	1050.7555	8.64	2 904	4621st

The signal of 1050.75 m/z with RT of approximately 8.60 were also observed in all liquid culture extracts of TS7 M6, M6 LPS and ASME and one TS7 FMAP LPS extract; TS7 FMAP LPS AS, however with significantly weaker responses than in the solid cultures (table 26). Responses of 1050.75 m/z varied from 2 904 to 67 918 in the liquid culture extracts compared to 421 714 to 1 964 987 in the solid culture extracts.

7 Discussion

Marine filamentous fungi were investigated in three rounds of cultivation and bioactivity testing. The initial and preliminary cultivation included the eight fungal isolates *Digitatispora marina* (008cD1.1), *Amylocarpus encephaloides* (018bII1.1), *Tolypocladium inflatum* (010cU1.3), *Calycina marina* (TRa3180AII.4), *Typhula* sp. (TRa3160C), *Mytilinidion* sp. (M16HEL1360D1-10.1), *Lulworthia* sp. (TRa3202.III.1), and *Acremonium* sp. (TS7). These were all cultivated in both solid and liquid M6, M6 LPS, FMAP, and FMAP LPS media and tested for antibacterial and protease activity. The three most interesting fungi from these screening, *Digitatispora marina* (008cD1.1), *Typhula* sp. (TRa3160C), and *Acremonium* sp. (TS7), were subjected to another round of cultivation on solid media supplemented with epigenetic modifiers: DNA methyltransferase inhibitor (DNMT) 5-azacytidine, histone deacetylase inhibitor (HDAC) SBHA and sirtuin inhibitor nicotinamide. These cultures were screened for antibacterial and antifungal activity, in which only TS7 exhibited activity. All TS7 cultures were then combined for a liquid-liquid extraction using ethyl acetate. Lastly, TS7 was cultivated in liquid M6, M6 LPS, FMAP, FMAP LPS and ASME media with a reduced number of combinations of epigenetic modifiers (S, AS, SN, AN and ASN) to ease the workload. The cultures with 5-azacytidine (A) and nicotinamide (N) added singly were excluded because the MS analysis of the solid cultures added these epigenetic modifiers showed the flattest peaks and seemingly lowest production of compounds. The liquid cultures were extracted by solid phase extraction using Diaion® HP-20 and screened for antibacterial, antibiofilm, antifungal and anticancer activity. Only antibacterial activity was observed, exhibited by the ethyl acetate extract of the solid cultures. All extracts and solid cultures with epigenetic modifiers were analyzed using mass spectrometry (MS).

The aim of this thesis was to identify if the application of small molecule inhibitors would have an impact on the secondary metabolites produced by the selected fungi, and if bioactive secondary metabolites could be identified through this approach. If so, develop a method for the implementing the of supplementation of these inhibitors to fungal cultures as part of the biodiscovery pipeline at Marbio.

These small molecule inhibitors serve as epigenetic modifiers, inhibiting the transcription repressive activity of epigenetic enzymes DNMTs, HDACs and sirtuins. These enzymes repress transcription by adding or removing epigenetic markers on DNA or histone resulting in denser packing of DNA to the inaccessible conformation, heterochromatin. BGCs in this conformation are so called silent. By inhibiting this activity more BGCs remain in the euchromatin conformation, accessible for transcription. Epigenetic modifiers were employed to fungal cultures to investigate if their application could be implied as an extension of the OSMAC approach. And by this establish a method for studying the metabolic potential of fungi at Marbio that is more efficient in awakening silent BGCs than OSMAC alone. As the application of epigenetic modifiers is a fairly straight forward strategy and does not require genetic manipulation of fungi, I imagine this could be applied as a method for early investigation of fungal secondary metabolite production.

7.1 Bioactivity observed from the fungal cultures

The results of the first application of epigenetic modifiers to solid cultures of TS7, which had already been found active to a selection of bacterial strains, showed rather unsatisfactory results. All TS7 cultures, regardless of epigenetic modifiers supplemented had similar bioactivities and MS chromatograms to control cultures, and only minor differences in the intensity of MS signals were observed. This indicated that the same biosynthetic gene clusters might have been active in all cultures, producing the same metabolomes and secondary metabolites, but in different quantities. The same were observed for 008cD1.1 and TRa3160C which were uniformly inactive in all assays and had rather indistinguishable MS chromatograms.

The cultivation of fungi with epigenetic modifiers on solid media, were based on the initial cultivations without epigenetic modifiers. Fungi were cultured for 30 days at 10°C on solid media added 50 µM 5-azacytidine, 500 µM SBHA and/or 50 µM nicotinamide. These concentrations were used based on the reporting of 5-azacytidine being applied at 0.1 µM to 10 mM to fungal cultures and showing effective induction of BGCs from 10 µM (Asai, Yamamoto, Chung, et al., 2012; Chen et al., 2016; González-Menéndez et al., 2014; Igboeli et al., 2019; J. Sun et al., 2012; Williams et al., 2008; Yakasai et al., 2011). SBHA have also been applied in the range of 0.1 µM to 10 mM but have mostly been reported effective at 500 µM to 1 mM (Asai et al., 2011; Asai, Chung, Sakurai, Ozeki, et al., 2012; Asai, Luo, Obara, Taniguchi, et al., 2012; Asai, Yamamoto, & Oshima, 2012; J. Sun et al., 2012; Yakasai et al., 2011). Lastly, nicotinamide has been reported applied at 10-500 µM with the most significantly effects on secondary metabolism at 50 and 100 µM (Asai et al., 2016; Asai, Morita, Shirata, Taniguchi, et al., 2012; El-Hawary et al., 2018).

Based on the results from the analysis of the solid cultures, it was decided to attempt to do further cultivating with epigenetic modifiers. As the majority of studies reporting successful induction of secondary metabolism and identification of novel secondary metabolites have cultivated fungi in liquid media, it was decided to try and cultivate TS7 in this way as well. Due to time constrains and TS7 growing notably faster than the other fungi in this study in the previous cultivations it was decided to shorten the incubation time. Several studies have reported successful induction of fungal secondary metabolism after one to two weeks of cultivation, including one study of cultivating the marine fungus *Asteromyces cruciatus* for 14 days (Igboeli et al., 2019). It was therefore decided to test if cultivation of TS7 for 16 days would be sufficient.

Epigenetic modifiers SBHA, 5-azacytidine and nicotinamide were added at day 9 of cultivation at concentrations of 500 µM, 50 µM and 50 µM, respectively. Secondary metabolism is generally repressed during the initial, logarithmic growth and gets derepressed during the stationary growth phase (Malik, 1980). As fungi had been observed to grow slower in the presence of epigenetic modifiers in the solid cultures, epigenetic modifiers were therefore tried added after the logarithmic growth had ceased at day 13 of incubation. Cultures were extracted by solid-phase extraction using Diaion® HP-20 after 16 days of cultivation. However, MS chromatograms showed only minor differences in peak height and bioassay results showed no activity.

7.2 Investigation of active cultures

Because only solid cultures and the ethyl acetate extract produced from them exhibited activity in the bioassays, these samples and the potential compound(s) causing this activity were the main focus of the mass spectrometry analysis. Just as the bioactivity results, the MS chromatograms for each culture were very similar, with generally the same MS peaks at the same retentions times with minor differences in peak intensity. As solid cultures and the ethyl extract exhibited the same activity and were fairly potent in its activity against *S. agalactiae* the peak(s) for the active compound(s) should represent a relatively big peak in all samples. A common feature in all samples were high responses for the signal of 1050.75 m/z RT: 8.72 (predicted to $C_{57}H_{105}N_5O_8P_2$ or $C_{54}H_{99}N_9O_{11}$). The elementary composition of $C_{57}H_{105}N_5O_8P_2$ gave no hits for molecular structure in any of the available databases. However, $C_{54}H_{99}N_9O_{11}$ had three hits in PubChem of potential structures in addition to a fourth potential molecular structure (Rogozhin et al., 2018). In this study they found a novel lipopeptide, *Emericellipsin A* ($C_{54}H_{99}N_9O_{11}$) isolated from *Emericellopsis alkalina* (sexual form of *Acremonium alkalina*) to exhibit bactericidal activity against methicillin resistant *S. aureus* and vancomycin resistant *E. faecalis* which both are classified as pathogens of high priority for antibiotic research by WHO (Rogozhin et al., 2018; WHO, 2017). This could correspond with my finding of the solid TS7 cultures to inhibit growth of *S. aureus* and *E. faecalis* in the agar plug diffusion assay, although I did not screen for activity against the resistant strains of these bacteria.

In the ethyl acetate extract and eight out of the 16 solid cultures the signal of 1050.75 m/z were predicted to $C_{57}H_{105}N_5O_8P_2$ with highest iFIT confidence, and in seven other cultures it was predicted to $C_{54}H_{99}N_9O_{11}$ with the highest iFIT confidence. However, the presence of two phosphor atoms in one compound is quite rare, making the elementary composition of $C_{54}H_{99}N_9O_{11}$ more probable.

The number of amino acids of 1050.75 m/z predicted based of the fragmentation pattern observed in the MS/MS spectra of the ethyl acetate extract, exceeded the maximum number of amino acids of the elementary composition $C_{57}H_{105}N_5O_8P_2$. An additional indication that $C_{54}H_{99}N_9O_{11}$ is a more likely elementary composition. However, the fragmentation pattern did not match the amino acids constituting Emericellipsin A. This indicates that the observed compound might not be Emericellipsin A, but perhaps a compound of similar molecular weight.

TS7 was previous to this thesis whole genome sequenced and annotated by Ph.D. student Ole Christian Hagestad and identified BGCs were subject to homology search to identify similar BGCs and possible products for the BGCs of TS7. Among the identified homologues were the leucinostatin A ($C_{62}H_{111}N_{11}O_{13}$, 1218.6 Da) BGC from *Purpureocillium lilacinum* (G. Wang et al., 2016). This BGC were found to be 15% similar to a PKS BGC and 35% similar to a NRPS BGC in TS7. Both these clusters are located on contig ends, and as the compound of 1050.75 m/z is probably a lipopeptide (hybrid compound), such as Emericellipsin A, these two BGCs might be the two halves of the one BGC producing the compound of 1050.75 m/z. This cluster could probably be identified using real time PCR to sequence the transcriptome of the TS7 samples with strong responses for 1050.75 m/z. If *Emericellopsis alkalina* were whole genome sequenced, the genomes of *Acremonium* sp. TS7 and *Emericellopsis alkalina* could be analyzed by a homology search to identify the BGC producing Emericellipsin A in *Emericellopsis alkalina*

and the homologous cluster in TS7. This analysis would also confirm if the compound observed in this thesis is Emericellipsin A or a similar compound of the same molecular weight, by comparison of the genes constituting the two clusters.

7.3 Induced colony morphological changes and pigmentation

It seems that the solid TS7 M6 and TS7 M6 LPS cultures, and their extract, which were the only cultures showing activity, did not exhibit this activity because of the addition of epigenetic modifiers. The antibacterial activity observed in solid cultures were also present in samples without added epigenetic inhibitors, which showed the same potency as the other cultures. Highlighted by the fact that all cultures of TS7 in M6 media with and without LPS exhibited activity in the antibacterial plug assay with no significant difference in the inhibition between the different combinations of epigenetic modifiers and the control cultures, these results lead me to believe the BGCs responsible of producing the secondary metabolites possessing this activity are not silent. The attempt of activating silent BGCs for the purpose of identifying novel compound was not successful. However, the epigenetic modifiers had some visible effects on the growth of both TS7 and TRa3160C, resulting in increased pigmentation of liquid TS7 cultures in FMAP and FMAP LPS media added SBHA and altered colony morphology of TRa3160C on corn meal agar added SBHA. Although the pigment in FMAP and FMAP LPS cultures was not found in the MS chromatogram, and TRa3160C was not found to exhibit activity in the performed bioassays. These changes to the culture appearances indicate that the epigenetic modifiers still had some effects on the metabolome of these fungi.

7.4 Cultivation optimization measures for increased efficacy of epigenetic modifiers

7.4.1 Amount and time of addition of epigenetic modifiers

Solid cultivation was preformed first because fungi are known to generally produce more secondary metabolites on solid media compared to liquid media (VanderMolen et al., 2013). However, solid cultivation introduces some limitations to the application of epigenetic modifiers as these must be added while the medium is still liquid and must therefore be added at the start of incubation and cannot be replenished at a later time during the cultivation. In addition, compounds are generally harder to extract from solid cultures and the extraction yield are thus often significantly lower (VanderMolen et al., 2013). These limitation might be the reasons most researches choose liquid cultivation when applying epigenetic modifiers to fungal cultures (Akone et al., 2016; Asai et al., 2011; Asai, Luo, Obara, Taniguchi, et al., 2012; Asai, Yamamoto, & Oshima, 2012; Asai et al., 2013, 2016; El-Hawary et al., 2018; Igboeli et al., 2019; J. Sun et al., 2012; Williams et al., 2008; Yakasai et al., 2011; Yang et al., 2014). Only one study of the application of epigenetic modifiers to solid cultures were found, in which the marine derived fungus *Cochliobolus lunatus* was cultivated with 5-azacytidine (Chen et al., 2016). Liquid cultivation was therefore also applied to utilize the possibility to add the epigenetic modifiers at a later time, and let the fungi grow undisturbed for the initial part of cultivation.

The observed indistinguishableness of both solid and liquid cultures might be due to addition of too low concentration of epigenetic modifiers, the modifiers being degraded during the cultivation or the epigenetic modifiers having no or small effect on the fungi tested in this thesis. Although all three epigenetic modifiers were applied at concentrations found effective in previous studies (Asai et al., 2016; Asai, Yamamoto, Chung, et al., 2012; Chen et al., 2016), these might be too low for these specific fungi. 5-azacytidine is an irreversible inhibitor of its target, DNA methyltransferase (Gnyszka et al., 2013). However, SBHA and nicotinamide are not. Nicotinamide is a physiological inhibitor of sirtuins and must be maintained at high concentrations to block the effect of sirtuins. The activity of histone deacetyl inhibitor SBHA is also reversible, as SBHA lack the aromatic ring the irreversible HDACs SAHA and TSA uses to lock themselves in the catalytic pocket of HDACs (Seto & Yoshida, 2014). It might therefore have been advantageable, at least for the liquid cultures, to apply the modifiers several times during the cultivation to sustain the concentration of the modifiers at an effective level, and possibly increase the amounts added.

Although epigenetic modifiers were found to modify the pigmentation of liquid TS7 cultures and colony morphology of solid TRa3160C, I did expect more evident changes to the metabolome of the cultures. It could be the case that the epigenetic modifiers applied in this thesis are suboptimal for the fungi investigated. Despite SBHA seemingly being the inducer of the changes observed in the liquid TS7 cultures and solid TRa3160C cultures, it could possibly yield even more potent effects. SAHA which is also a class I and II HDAC inhibitor like SBHA, has more reported cases of successful activation of silent BGCs. Still, SBHA were selected for application over SAHA in this thesis, as SAHA costs about four times as much as SBHA and is significantly more toxic. SBHA and SAHA inhibit HDAC by the same mechanism, except for the lock in place ability of SAHA due to its aromatic ring. However, SAHA is classified as causing germ cell mutagenicity and reproductive toxicity while SBHA is considered non-hazardous (Sigma-Aldrich, 2019c, 2019f). As the toxic effects of SAHA are directly related to its ability, the classification of SAHA as more hazardous could indicate the higher potency and efficacy of SAHA.

7.4.2 Cultivation length

The liquid cultures not showing any activity when exposed to epigenetic modifiers might also be due to too short cultivation time. Although several studies reported isolating novel compounds after only one or two weeks, some studies reported cultivating the fungal strains for four weeks before extracting (Asai, Luo, Obara, Taniguchi, et al., 2012; Chen et al., 2016; El-Hawary et al., 2018). Despite the rapid growth observed by TS7 in the liquid cultures, it might have been wise to continue the cultivation for two additional weeks to obtain a higher yield of secondary metabolites as the secondary metabolism is most active in the stationary growth phase after the initial growth has ceased (Malik, 1980).

7.4.3 Time of extraction

Solid cultures with 5-azazcytidine (A, AS, AN and ASN) were started three days before the cultures only added SBHA and nicotinamide (null, S, N, SN). Still, all cultures were screened for antibacterial activity precisely 30 days after incubation start. In the ethyl acetate extraction, all solid cultures were combined and extracted simultaneously at 46 days after cultivation start of the culture null, S, N, and SN and 49 days after cultivation start of the cultures A, AS, AN and ASN. The extraction was not performed immediately after activity testing because the workflow of the thesis was not predefined and the methods and analyzes performed were determined based on the retrieved results. However, the delay before extracting the cultures might have affected the extraction yield of the solid cultures as reported by (Chen et al., 2016) after cultivating the marine-derived fungus *C. lunatus* on solid medium with 5-azacytidine. They studied the optimal extraction time for obtaining the highest extract yield by extracting the cultures after 20, 22, 24, 26, 28, 30, 32 and 35 days and found the optimal days were 26, 28 and 30 days, with yields decreasing after 32 days (Chen et al., 2016). As the solid cultures of TS7 were first extracted after 46 and 49 days, the obtained extract might therefore not represent the actual activity of the cultures, as observed in the antibacterial plug assay performed after 30 days of cultivation, as active compounds might have been degraded in the time after activity testing before extraction. The MS analysis of the solid cultures was performed one week before the extraction (40 and 43 days after incubation start).

The solid cultures were extracted using a micro ethyl acetate plug extraction method before MS analysis which should have led to the extraction of same type of compounds as extracted from all the solid cultures combined. The delay before extracting the cultures may have led to partial degradation of the potential lipopeptide representing 1050.75 m/z. Amino acids in peptide are targeted by numerous degradation pathways including deamination, hydrolysis and oxidation (Bell, 1997). If this potential lipopeptide have been exposed to degradation, the observed activity in the ethyl acetate extract might therefore be weaker that its true potential.

7.4.4 Extraction procedure

Ethyl acetate extraction was selected as the extraction method for the solid cultures as the standard extraction method at Marbio, solid phase extraction using Diaion® HP-20, was not applicable to solid cultures. Ethyl acetate extraction is routinely used for extraction of plugs from solid cultures at Marbio, and seems to be the preferred organic solvent for liquid-liquid extraction as the great majority of studies of epigenetic modifiers applied to fungal cultures to induce secondary metabolite production reported extracting the cultures using ethyl acetate (Akone et al., 2016; Asai et al., 2011, 2013, 2016; Asai, Luo, Obara, Taniguchi, et al., 2012; Asai, Yamamoto, & Oshima, 2012; Chen et al., 2016; El-Hawary et al., 2018; Igboeli et al., 2019; Yakasai et al., 2011; Yang et al., 2014).

Only cultures extracted by liquid-liquid extraction (LLE) using ethyl acetate exhibited activity while all extracts prepared by solid phase extraction (SPE) using Diaion HP-20 resin beads were inactive. Although the liquid cultures extracted by SPE yielded significantly bigger extracts, the media controls, which were also extracted by SPE, yielded as big extracts. Indicating that although SPE produced significantly bigger extract, a large portion of the weight seems to be medium components. Regardless of the extraction method, solid cultures are known to generally have lower extraction yields than liquid cultures (VanderMolen et al., 2013).

Although extraction using Diaion HP-20 is known to be effective, this method might be inferior to ethyl acetate extraction in this case. The liquid cultures that were extracted were fairly small (only 250 mL) and considering the "target compound" often constitutes less than 1% of the crude extract weight (Koehn & Carter, 2005), one could argue that the chances of detecting more compounds would have been greater if bigger cultures were extracted. Nevertheless, I believe the extraction methods applied had huge impact on the compound composition in the crude extracts. The SPE seems to extract a lot of unnecessary compounds producing noisy extracts while the ethyl acetate extract seemed purer. To properly identify which of LLE and SPE is best for extraction of liquid cultures for the purpose of extracting and identifying secondary metabolites, parallel liquid cultures should be cultivated and subjected to the two extraction procedures.

8 Conclusion

By the observed change in pigmentation of liquid TS7 cultures and altered colony morphology of solid TRa3160c cultures, both by HDAC inhibitor SBHA, it appears this type of epigenetic modifies are able to alter gene expression in these fungi. Still, the treatment of marine fungi with DNMT inhibitor 5-azacytidine, HDAC inhibitor SBHA and sirtuin inhibitor did not result in evident changes in secondary metabolite production. Antibacterial activity against *S. agalactiae* and partial activity against *S. aureus* and *E. faecalis* were still observed in TS7 cultures, but seemingly regardless of addition of epigenetic modifiers. As SBHA were able to induce visible changes in the fungal cultures, the lack of effects to secondary metabolism could be due to too low concentration of the applied epigenetic modifiers, or in the case of the liquid cultures, too short cultivation time.

For further studies of application of epigenetic modifiers to TS7 or other filamentous fungi I believe that the application of SAHA instead of SBHA should be attempted as SAHA is acknowledged as a more potent HDAC inhibitor due to its irreversible inhibiting activity. Because SBHA was here proven to affect the appearance of TS7 and TRa3160c cultures, indicating changes in gene expression, SAHA could be effective as well. A number of different concentrations of the applied epigenetic modifiers should also be applied as it seems that the necessary concentration for effective DNMT and HDAC inhibition differs between fungi. And lastly, different cultivation lengths and extraction timings should be tested out to identify the optimal timing for the specific fungus. The transcriptomes of the cultures should also be analyzed by RT-PCR to identify which BGCs are active and quantify the rate of their transcription.

If these measure does not yield an increased number of identified secondary metabolites from the BGCs of the fungi investigated other strategies such as promoter exchange for all genes in a cluster of interest or transformation of clusters into a heterologous host could be applied.

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Appendix

Appendix 1 – Culture media

Table 27 – Products and equipment's used for the making of fungal culture media

Glucose	D9434	Sigma-Aldrich (Missouri, USA)
Fish filet powder	Polarsnacks Tørrfiskfilet	Arctic Taste AS
Peptone from casein, enzymatic digest	82303	Sigma-Aldrich (Missouri, USA)
Sea salts	S9883	Sigma-Aldrich (Missouri, USA)
MilliQ H_2O	MilliQ Gradient A10	Merck Millipore KGaA, Germany
Agarose	A1296	Sigma-Aldrich (Missouri, USA)
Difco Marine Broth	279110	Becton, Dickinson and Company, New Jersey, USA
Filtrated sea water	5 µm pore size, ceramic membrane filter 0.2 µm, UV filter	Norwegian College of Fishery Science, UiT (Norway)
Corn meal agar	42347	Sigma-Aldrich (Missouri, USA)
***Ascophyllum nodosum* powder**		
Malt Extract	70167	Sigma-Aldrich (Missouri, USA)

M6 medium
5 g glucose, 0.5 g fish filet powder, 1 g peptone, 40 g sea salts, and 1 L MilliQ H_2O (15 g agar) (0.6 ng/mL LPS)

FMAP medium
15 g Difco Marine Broth, 5 g peptone, 300 mL filtrated sea water, and 700 mL MilliQ H_2O (15 g agar) (0.6 ng/mL LPS)

Ascophyllum nodosum agar
10 g *Ascophyllum nodosum* powder, 15 g agar, 500 mL filtrated sea water and 500 mL MilliQ H_2O

Corn meal agar
17 g corn meal agar, 500 filtrated sea water and 500 mL MilliQ H_2O

ASME medium
4 g malt extract, 40 g sea salts, and 1 L MilliQ H_2O

Appendix 2 – Bioassay results

Table 28 - Results of antibacterial agar plug diffusion assay of initial solid fungal culture without added epigenetic modifiers. Numbers represent size of inhibition zones in millimeters.

Fungus	Medium	*E. c* (-)	*P. a* (-)	*S. a* (+)	*E. f* (+)	*S.* B (+)
Digitatispora marina	FMAP	-	-	-	-	-
	FMAP LPS	-	-	-	-	-
	M6	-	-	-	-	-
	M6 LPS	-	-	-	-	-
Amylocarpus encephaloides	FMAP	-	-	-	-	-
	FMAP LPS	-	-	-	-	-
	M6	-	-	-	-	-
	M6 LPS	-	-	-	-	-
Tolypocladium inflatum	FMAP	-	-	-	-	-
	FMAP LPS	-	-	-	-	-
	M6	-	-	-	-	-
	M6 LPS	-	-	-	-	-
Calycina marina	FMAP	-	-	-	-	-
	FMAP LPS	-	-	-	-	-
	M6	-	-	-	-	-
	M6 LPS	-	-	-	-	-
***Typhula* sp.**	FMAP	-	-	-	-	-
	FMAP LPS	-	-	-	-	-
	M6	-	-	-	-	-
	M6 LPS	-	-	-	-	-
***Mytilinidion* sp.**	FMAP	-	-	-	-	-
	FMAP LPS	-	-	-	-	-
	M6	-	-	-	-	-
	M6 LPS	-	-	-	-	-
***Lulworthia* sp.**	FMAP	-	-	-	-	-
	FMAP LPS	-	-	-	-	-
	M6	-	-	-	-	-
	M6 LPS	-	-	-	-	-
***Acremonium* sp.**	FMAP	-	-	-	-	-
	FMAP LPS	-	-	-	14	15
	M6	-	-	-	11	23
	M6 LPS	-	-	-	11	23

Table 29 - Protease activity results. Numbers represent maximum slope of the enzymatic reaction for each sample. Threshold for activity: > 0.0005

	FMAP	FMAP LPS	M6	M6 LPS
008cD1.1	0.00001	0.00001	0.00059	0.00073
018bII1.1	-	-	-	-
010cU1.3	-	0.00001	0.00001	0.00005
TRa3180AII.4	0.00001	0.00001	0.00001	-
TRa3160C	000001	-	-	-
M16HEL1360D1-10.1	-	-	-	-
TRa3202.III.1	0.00001	0.00001	-	-
TS7	0.00003	0.00002	0.00005	0.00008

Table 30 – Results of antifungal activity against Candida albicans *of TS7 extracts, EtoAcE: ethyl acetate extract. Numbers represent measured OD. Threshold for activity: < 0.05, questionable: 0.05 – 0.09.*

	Ctr	null	S	SN	SA	NA	SNA
M6	0.268	0.255	0.229	0.234	0.206	0.228	0.192
M6 LPS	0.259	0.261	0.257	0.229	0.221	0.228	0.240
FMAP	0.288	0.205	0.272	0.229	0.252	0.218	0.211
FMAP LPS	0.270	0.247	0.237	0.235	0.222	0.202	0.201
ASME	0.259	0.241	0.239	0.248	0.249	0.219	0.210
EtoAcE							0.174

Table 31 - Results of antibiofilm activity of extracts, EtoAcE: ethyl acetate extract. Number represent percentage survival of cells compared to growth control. Threshold for activity: < 30 % of growth control, questionable 30-40% of growth control.

	Ctr	null	S	SN	SA	NA	SNA
M6	135.3	134.9	130.6	115.9	113.5	112.6	111.3
M6 LPS	119.3	131.3	90.6	118.7	110.3	101.0	109.3
FMAP	116.6	121.2	109.3	105.5	103.6	94.2	97.2
FMAP LPS	120.2	118.5	111.5	115.5	109.8	115.3	110.9
ASME	124.0	118.6	104.0	109.6	111.6	110.4	110.8
EtoAcE							107.3

Table 32 - Results of antibacterial screening of liquid culture extracts at 50 μM, EtoAcE: ethyl acetate extract. Numbers represent measured OD. Threshold for activity: < 0.05, questionable: 0.05-0.09

		Ctr	**null**	**S**	**SN**	**SA**	**NA**	**SNA**
E. coli	M6	0.641	0.572	0.579	0.545	0.559	0.563	0.537
E. coli	M6 LPS	0.652	0.546	0.620	0.451	0.515	0.532	0.443
E. coli	FMAP	0.567	0.549	0.551	0.564	0.560	0.533	0.532
E. coli	FMAP LPS	0.560	0.509	0.489	0.476	0.511	0.555	0.586
E. coli	ASME	0.469	0.548	0.498	0.454	0.487	0.514	0.493
P. aeruginosa	M6	0.550	0.547	0.651	0.669	0.595	0.652	0.606
P. aeruginosa	M6 LPS	0.570	0.566	0.576	0.640	0.628	0.787	0.660
P. aeruginosa	FMAP	0.559	0.620	0.565	0.645	0.599	0.633	0.504
P. aeruginosa	FMAP LPS	0.537	0.615	0.623	0.700	0.647	0.606	0.634
P. aeruginosa	ASME	0.569	0.621	0.689	0.726	0.633	0.633	0.613
S. aureus	M6	0.248	0.315	0.344	0.337	0.344	0.346	0.363
S. aureus	M6 LPS	0.315	0.258	0.343	0.376	0.363	0.375	0.395
S. aureus	FMAP	0.279	0.330	0.397	0.355	0.330	0.386	0.387
S. aureus	FMAP LPS	0.366	0.389	0.415	0.371	0.369	0.349	0.368
S. aureus	ASME	0.354	0.388	0.368	0.374	0.337	0.336	0.397
E. faecalis	M6	0.397	0.367	0.376	0.355	0.383	0.365	0.395
E. faecalis	M6 LPS	0.363	0.337	0.381	0.370	0.368	0.391	0.334
E. faecalis	FMAP	0.376	0.359	0.358	0.360	0.365	0.359	0.380
E. faecalis	FMAP LPS	0.317	0.353	0.381	0.349	0.352	0.368	0.365
E. faecalis	ASME	0.346	0.350	0.364	0.350	0.408	0.388	0.372
Strep. B	M6	0.600	0.507	0.566	0.348	0.578	0.317	0.329
Strep. B	M6 LPS	0.560	0.446	0.578	0.577	0.283	0.410	0.308
Strep. B	FMAP	0.519	0.572	0.378	0.530	0.348	0.277	0.343
Strep. B	FMAP LPS	0.501	0.540	0.485	0.475	0.385	0.315	0.328
Strep. B	ASME	0.511	0.577	0.516	0.428	0.270	0.347	0.308

Table 33 - Results of antibacterial screening of extracts of 150 µM, EtoAcE: ethyl acetate extract. Numbers represent measured OD. Threshold for activity: < 0.05, questionable: 0.05-0.09

		Ctr	**null**	**S**	**SN**	**SA**	**NA**	**SNA**
E. coli	M6	0.499	0.452	0.481	0.463	0.491	0.488	0.441
E. coli	M6 LPS	0.441	0.415	0.432	0.457	0.437	0.468	0.458
E. coli	FMAP	0.398	0.411	0.435	0.452	0.439	0.443	0.456
E. coli	FMAP LPS	0.404	0.406	0.424	0.418	0.444	0.435	0.441
E. coli	ASME	0.368	0.384	0.412	0.396	0.411	0.426	0.425
P. aeruginosa	M6	0.719	0.745	0.783	0.840	0.839	0.845	0.768
P. aeruginosa	M6 LPS	0.719	0.759	0.813	0.779	0.801	0.725	0.842
P. aeruginosa	FMAP	0.618	0.636	0.628	0.774	0.705	0.788	0.793
P. aeruginosa	FMAP LPS	0.595	0.693	0.710	0.724	0.742	0.855	0.750
P. aeruginosa	ASME	0.607	0.571	0.675	0.699	0.641	0.756	0.671
S. aureus	M6	0.286	0.319	0.309	0.325	0.303	0.325	0.286
S. aureus	M6 LPS	0.312	0.319	0.296	0.331	0.271	0.302	0.298
S. aureus	FMAP	0.305	0.269	0.236	0.298	0.258	0.297	0.293
S. aureus	FMAP LPS	0.292	0.288	0.271	0.285	0.266	0.287	0.306
S. aureus	ASME	0.264	0.295	0.294	0.358	0.281	0.328	0.311
E. faecalis	M6	0.365	0.386	0.367	0.398	0.375	0.351	0.389
E. faecalis	M6 LPS	0.329	0.357	0.358	0.350	0.340	0.345	0.351
E. faecalis	FMAP	0.287	0.337	0.348	0.322	0.342	0.339	0.371
E. faecalis	FMAP LPS	0.289	0.341	0.356	0.342	0.341	0.346	0.375
E. faecalis	ASME	0.292	0.353	0.340	0.360	0.334	0.358	0.359
Strep. B	M6	0.277	0.289	0.303	0.287	0.285	0.289	0.265
Strep. B	M6 LPS	0.272	0.282	0.288	0.293	0.252	0.245	0.281
Strep. B	FMAP	0.267	0.229	0.247	0.240	0.258	0.270	0.232
Strep. B	FMAP LPS	0.286	0.268	0.323	0.224	0.267	0.264	0.267
Strep. B	ASME	0.266	0.268	0.282	0.239	0.252	0.246	0.255

Appendix 3 – Hazards and prices of epigenetic modifiers

Table 34 – Hazards and prices of epigenetic modifiers considered for application.

	Name	Hazards	Price	Distributor
DNMT inhibitors	**5-azacytidine**	Acute toxicity, oral Carcinogenicity	€ 70.20 for 100 mg (702.00 €/g)	(Sigma-Aldrich, 2019a)
	RG-108	Acute toxicity, oral Acute aquatic toxicity	€ 953.00 for 200 mg (4 765.00 €/g)	(Selleck Chemicals, 2019)
HDAC inhibitors	**SAHA**	Germ cell mutagenicity Reproductive toxicity	€ 282.00 for 25 mg (11 280 €/g)	(Sigma-Aldrich, 2019c)
	SBHA	-	€ 54.20 for 1 g (54.20 €/g)	(Sigma-Aldrich, 2019f)
	VPA	Acute toxicity, oral Skin irritation Eye irritation Reproductive toxicity	€ 51.60 for 1 g (51.60 €/g)	(Sigma-Aldrich, 2020b)
Sirtuin inhibitors	**Nicotinamide**	Eye irritation	€ 27.50 for 100 g (275.00 €/g)	(Sigma-Aldrich, 2019b)
	Sirtinol	-	€ 354.00 for 5 mg (70 800 €/g)	(Sigma-Aldrich, 2019d)
	Splitomicin	-	€ 88.20 for 5 mg (17 640 €/g)	(Sigma-Aldrich, 2019e)