INVESTIGATIONS ON THE PRIMARY AND SECONDARY METABOLISM OF MARINE AND TERRESTRIAL ENDO SYMBIONTS

I n a u g u r a l d i s s e r t a t i o n

zur

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7th frame network Initial training program within the symbiomics project.

Dedication

I dedicate this thesis work

to my husband Mr. Nandakumar Sundaramoorthy

who stood for me throughout.

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List of Abbreviations and Symbols

1-D - one-dimensional

2-D - two-dimensional

ATCC - American type culture collection

AS - Anisaldehyde-sulphuric acid

ABC- ATP binding cassette

ATP- Adenosinetriphosphate

B. subtilis - *Bacillus subtilis*

Br - Broad

C18 - Octadecyl

calc. - Calculated

CC - Column chromatography

CCDC - Cambridge crystallographic data centre

CD_3OD – Deuterated methanol

COSY - Correlation spectroscopy

C-source - carbon source

δ - chemical shift

D - Doublet

Dd -Doublet of doublets

Ddd - *D*oublet of doublet of doublets

DAD - Diode array detector

DCM - Dichloromethane

DEPT - Distortion less excitation by polarization transfer

diam.- Diameter

DMSO - Dimethylsulfoxide

DNA - Deoxyribonucleic acid

DPPH - 2,2-Diphenyl-1-picrylhydrazyl

Dq - Doublet of quartets

DSMZ - Deutsche Sammlung für Mikroorganismen und Zellkulturen

Dt - Doublet of triplets

EDTA - Ethylene diamine tetra acetic acid

e.g. - *exempli gratia* (for example)

ELISA - Enzyme linked immunosorbent assay

E. coli – Escherichia coli

ESI - Electrospray ionization

et. al. - et altera (and others)

EU - European Union

EtOAc/EA - Ethyl acetate

EtOH - Ethanol

Ev - Electron volt

FASEB - Federation of American Societies for Experimental Biology

FCS - Fetal calf serum

FDA - Food and Drug Administration

FTICR - Fourier transform ion cyclotron resonance

Fig (s). - Figure (s)

GC-MS – Gas Chromatography Mass spectrometry

H - Hour

HBSS - Hank’s balanced salt solution

HMBC - Heteronuclear multiple bond correlation

HMQC - Heteronuclear multiple quantum coherence

HSQC - Heteronuclear single quantum correlation

HPLC - High performance liquid chromatography

HR-ESI-MS - High resolution-ESI-MS

Hz - Hertz

H2O - water

IC_{50} – 50% Inhibitory concentration

i.e. - *id est* (that is)

INT - Iodonitrotetrazolium chloride

IR - Infrared

IZ - Inhibition zone

J - Coupling constant (in Hz)

K_2HPO_4 - dipotassium hydrogen phosphate

LC - liquid chromatography

M - Multiplet

m/z - Mass/charge

M^+ - Molecular ion

Me - Methyl

MEA - Malt extract agar

MeCN - Acetonitrile

MeOH - Methanol

Mg - Microgram

Mg - Milligram

MHz - Megahertz

Min - Minute

Ml - Milliliter

µl - Microlitre

MNP - Marine Natural Product

MRSA - Methicillin-resistant *Staphylococcus aureus*

MS - Mass spectrometry

MW - Molecular weight

NMR - Nuclear magnetic resonance

NOESY - Nuclear overhauled effect spectroscopy

NRU - Neutral red uptake

NaH_2PO_4 - sodium dihydrogen phosphate

Na_2HPO_4 - disodium hydrogen phosphate

OD - Optical density

PBS - Phosphate buffered saline

PDA - Potatoes dextrose agar

Ppm - Part per million

PTLC - Preparative-TLC

PTS - phosphotransferase system

Q - *Q*uartet

qd - quartet of doublets

R_f - Retention factor

ROESY - Rotating frame nuclear Overhauled effect spectroscopy

RP-18 - Reversed phase C18

Rpm - Rotation per minute

RPMI - Roswell Park Memorial Institute

rRNA - ribosomal ribonucleic acid

s - Singlet

SBUG - Stammsamlung Biologie der Universität Greifswald

SCUBA - Self-contained underwater breathing apparatus

SD - Standard deviation

sp. - Species (singular)

SS - Solvent system

S. aureus - *Staphylococcus aureus*

δ - Chemical shift (in ppm)

t - Triplet

t_R - Retention time

T - Tesla

TLC - Thin layer chromatography

TOCSY - Total correlation spectroscopy

TSA - Tryptic soy-bean agar

TSP - trimethylsilylpropionic acid

V. tuberculata - *Verminephrobacter eiseniae*

V. eiseniae - *Verminephrobacter tuberculatae*

UV/Vis - Ultraviolet/visible

Amino acids were denoted following the international three letter abbreviation code.

1 MOTIVATION OF RESEARCH

1.1 Microbial communication:

Communication is the most omnipresent social phenomenon and a primary mean through which social interaction occurs. Millions of languages have been discovered and adopted in human evolution. Besides human, communication among the microbial world is a noteworthy aspect in the social ecology. Microbial conversation taking place through chemicals produced by themselves and terpenoids found to be the world oldest language spoken ever by the bacterias and fungus (Schmidt et al. 2017). Moreover, human-microbial interactions also occur while bacteria harbored in human habitat and plays essential role in disease and heath. It is a mammoth task to understand the molecular responses of such ineractions. However, chemical compounds with potential pharmacological activity produced by micro organisms are isolated and used as natural products (therapeutics) to communicate to microbes causing life threatening diseases in human.

Biosynthesis of bioactive chemicals in the microorganisms is the natural process of metabolism of up taken nutrients, called as metabolites and further can be categorized as “primary metabolites” and “secondary metabolites”. Metabolites are spoken language in broadcasting signals from the genetic architecture and the environment (Jewett et al. 2006).

The current Ph.D thesis has a focus on natural product science and microbial symbiosis through metabolomics approach. Metabolomics is the emerging bioanalytical platform affords to investigate both primary and secondary metabolites under various research areas with specific objectives. Natural product science has a focus on secondary metabolites with potential pharmacological activity for human use. Microbial symbiosis deals with primary metabolites and their key role in the symbiotic communication. Findings from this research would be a partial contribution to individual research areas.

2 AIM AND SCOPE OF THE CURRENT STUDY

2.1 Part I:

Natural product science project as part-I focus on investigation pharmacologically active secondary metabolites from marine fungi. This project aimed at fungal strains being able to produce bioactive secondary metabolites for the pharmaceutical purpose and may be structurally novel in chemical library. The primary objective is to make batch cultures of one free living fungi isolated from draft wood and two marine derived fungus isolated from unknown sponges, extraction of culture medium and the mycelium, compound separation by bioassay-guided fractions, isolation of bioactive compounds and purification of compounds of interest, performing high throughput analysis for chemical characterization and compound identification.

2.2 Part II:

Microbial symbiosis project in collaboration with Aarhus University, Denmark as part II partially contributing with metabolomics part. The main objective is to explore the time resolved metabolom of *V. eiseniae* and *V. tuberculata* in In-vitro batch culture, aiming not only to comprehensively analyze the true metabolism of the symbionts, but also trying to identify expressed traits valuable for its symbiotic lifestyle by understanding which metabolic pathways are active under the mimicking conditions. The cultivation temperature and oxygen supply were selected and set to conditions to closely resemble the conditions in the nephridia of the earthworms to make sure that during the cultivation *V. eiseniae* and *V. tuberculata* grow and behave as inside their host, knowing more about the metabolic pattern may help gaining insight into the reasons for the endosymbiosis since this is yet to clarify by scientists. Different potential functions of the bacteria for their host have been mentioned in recent research articles in this field. However, so far researchers have only been able to show that the symbionts have a positive effect on the fitness of the earthworms and increase the hatching success but how is still a question to be answered (Lund et al. 2010).

3 INTRODUCTION

3.1 PART - I Secondary metabolites in marine drug discovery

3.1.1 Marine Bioprospecting

All forms of life first appeared in the oceans and are still there from archaea to mammals which explain why marine biodiversity is much richer than terrestrial and freshwater biodiversity. The ocean is the largest habitat on earth and the biodiversity of the sea is extraordinary with 34 of the 37 phyla of life represented in the ocean. In contrast, there are only 17 phyla that occur on land. Thus it is unsurprising that the relentless quest for new scientific knowledge coupled with the rapid development of new ocean exploration technology has placed the spotlight on the legal regime governing the conduct of scientific research in the marine environment (R.Long 2009).

3.1.2 Marine resources studied so far:

For the first time, In 1951 Werner Bergmann published three reports of unusual arabino- and ribo-pentosyl nucleosides obtained from the marine sponge, Tethya crypta collected in Florida, USA (Bergmann and Feeney, 1951; Bergmann and Burke, 1956; Bergmann and Stempien, 1957). Later, from 1963, 9220 papers have reported the isolation of 24 662 new compounds. Figure 1 shows the mostly preferred phyla called Porifera. These 9220 papers constitute 37% of the total papers in Marine literatures. In fact, the 9220 papers describe compounds elicited from just 2657 named species with another 2485 occurrences across 484 genera that are only described as ‘‘species’’. So, the number of distinct species studied is a long way short of 9220. This emphasizes the point that there is still an enormous MNP resource to be explored; probably in excess of 200 000 species still to be evaluated (Blunt et al. 2015).

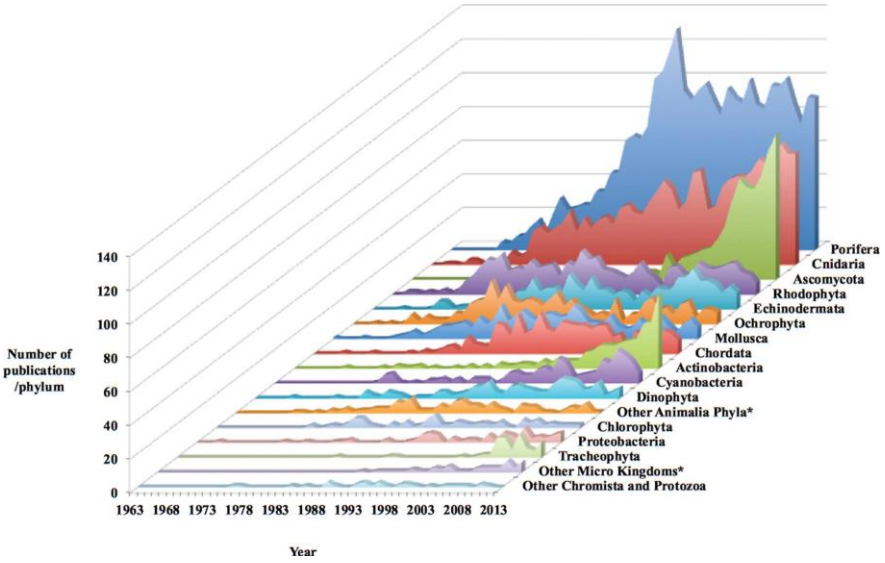


Figure 1 : The phylum-preferences of the marine natural product research community across a 50-year period from 1963. Picture courtesy marine natural products nat. Prod. Rep., 2015, 32, 116–211 | 189

3.1.3 Marine natural products – Primary and secondary metabolites:

All organisms metabolize the nutrients into functional end products in order to live, grow and reproduce. Marine natural products are any bioactive chemical substances produced by exclusively aqueous living organisms, i.e. Marine invertebrates, Cyanobacteria, Actinomycetes and Marine fungi.

A primary metabolite is a chemical substance produced by the organism that is directly involved in normal growth, development, and reproduction. These include common carbohydrates, proteins, fats, and nucleic acids. It usually performs a physiological function in the organism (i.e. an intrinsic function) For example, Ergosterol is a well known primary metabolite from micro organism also used as a natural products.

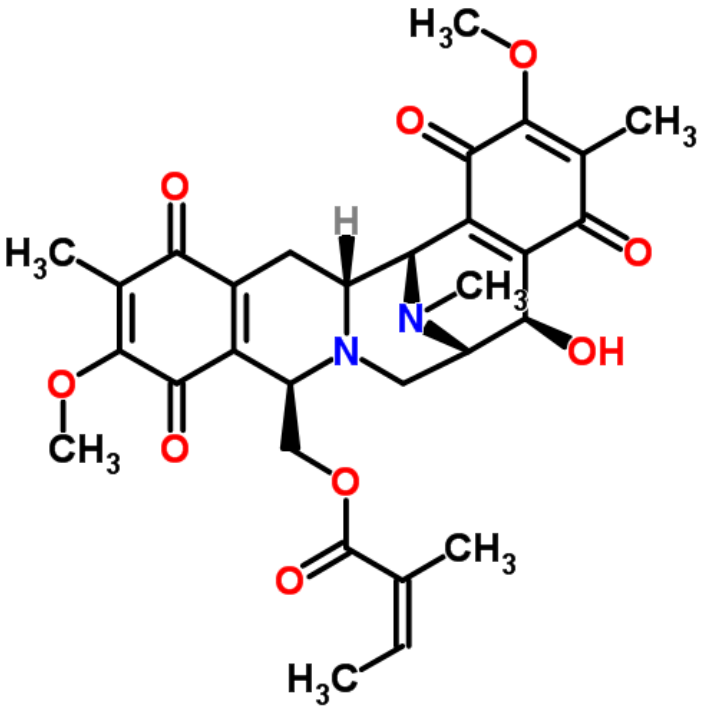
A “secondary metabolite” is the end product of metabolic pathway which may not be essential for the physiological function of an organism. But, it plays a vital role for the eco system. It is their chemical properties which makes them interesting for the Natural product scientists.

3.1.4 Secondary metabolites discovery from marine organisms:

At a global level, the first flavor of marine natural products originated in 1967 during a small symposium held in Rhode Island, USA with an ambitious title “Drugs from the Sea”. Although the tone and theme of the meeting proved to be somewhat hesitant, it marked the birth of marine natural product chemistry by Freudenthal in 1967 (Beedessee et al. 2015) With time, several drug discovery programs have been initiated to investigate the potential of the marine environment as a source of natural products. There are enough literatures have covered 50 years of secondary metabolite discovery from marine sources since year 1951 to year 2000. D.J. Faulkner and R. J Anderson (In press) have provided a first review of the chemistry of the “natural products” of marine organisms in 1973. Faulkner and his group had special reviews on marine natural products including ‘metabolites from marine fungi’ annually since 1974 to 2002 in natural product reports. After him, this annual review has been continued since 2002 by J.W. Blunt and his group.

According to the statistical survey by Jie Yuan et.al report in Marine drugs 2015, the number of bioactive compounds dramatically changed every year. For example, more than 250 bioactive compounds were reported in 1997, 2000, 2003, 2008, and 2011, but less than 100 in 1990, 1994, 1998, and 2006. Particularly in 2007, around 900 secondary metabolites got reported (D J Faulkner until 2002 and J.W Blunt 2003, 2004, 2005, 2006, 2007). Since 2008, more than 1000 new compounds are discovered each year (Hu et al. 2015) Recently, J W Blunt has mentioned in his review for 2013 that is of the marine natural product (MNP) literature for 2012 and describes 1241 new MNPs from 382 articles, an 8% increase in the number of compounds reported for 2011. Now recently, the same author has reviewed again in natural products report for 2013, and describes 1163 new compounds from 379 articles, a 6% decrease in the number of compounds reported in 2012 (J W Blunt 2015). From the longitudinal study by Jie Yuan et.al reported in marine drugs 2015, out of 16617 new compounds, the number of bioactive compounds is 4196, or 25.25%. However, this does not mean that the other 74.75% are inactive. Indeed, their bioactivity may be discovered in future studies. For

example: Accidental discovery of cephalosporin C in 1949, which was produced by a culture of a *Cephalosporium* species obtained off the ,Sardinian coast, it took another thirty years for marine-derived fungi to be more systematically evaluated for their chemical potential in 1979 (Rateb & Ebel 2011). And, perhaps the earliest example was the compound known as Renieramycin A first reported by Faulkner's group in 1982 from an eastern Pacific sponge of the genus Reniera (Newman & Giddings 2014). Twenty years later, in 2004 the same compound was isolated from another marine sponge, but this time a *Neopetrosia* species, was found to be a potent antileishmanial agent by a group led by Fusetani in Tokyo, (Newman & Giddings 2014).



Renieramycin A

A number of biologically active compounds with various pharmacological actions, such as anti-tumor, photo protective, anti-cancer, anti-proliferative, anti-microtubule, cytotoxic, as well as antibiotic and antifouling properties, have been isolated to date from marine sources. In general, The bioactive compounds are classified chemically into eight classes according to different types of chemical structure: Terpenoids, steroids (including steroidal saponins), alkaloids, ethers (including ketals), phenols (including

quinones), strigolactones, peptides, and others those that cannot be classified into the above seven classes (Hu et al. 2015). Several review papers strongly mentioned that polyketides are the main output of marine natural products of fungal origin. Some of these bioactive secondary metabolites of microbial origin with strong antibacterial and antifungal activities are being intensely used as antibiotics and may be effective against infectious diseases such as HIV, conditions of multiple bacterial infections (penicillin 1928, cephalosporin 1945, streptomycin 1943, and vancomycin 1950-1980) or neuropsychiatric sequelae (Bhatnagar & Kim 2010). Some drugs have also been found to be useful against carcinomas (Beomycin 1962, Dactinomycin 1940, Doxorubicin 1969 and Staurosporin 1977), risk of coronary heart disease, or may act as immune-suppressants (cyclosporine) to aid in organ transplantation (Ruiz et al. 2010) thus making the microbial secondary metabolites an enormous source of pharmaceutical importance (Bhatnagar & Kim 2010). The FDA granted orphan drug designation for a developmental agent known as GPX-150, an analog of doxorubicin currently being tested for use in advanced or metastatic soft-tissue sarcoma (journal of Oncology 2016). There are some novel secondary metabolites isolated in the last five years with various bioactivities from various marine derived fungal sources are listed below in Table 1.

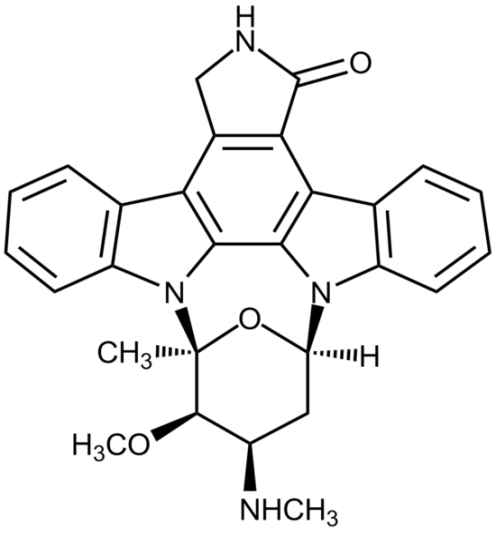
Table 1: Some novel secondary metabolites isolated from various marine sources.

Novel secondary metabolite Isolated	Bioactivity	Marine organisms	Actual source	Reference
Anthracyclines: Aranciamycin I & J	Antibacterial Cytotoxic	*Streptomyces marinus*	Marine sediment	(Khalil et al. 2015)
Thiasporines: A,B,C	Antibacterial Antifungal Cytotoxic	*Actinomycetospora chlora*	Marine sediment	(Fu& MacMillan 2015)
carbamate macrolide: Callyspongiolide	Cytotoxic	*Callyspongia sp.*	Sponge (Ambon, Indonesia)	(Blunt et al. 2016)
scalarane sesterterpenes: 24-methoxy-	Cytotoxic	*Hyrtios erectus*	Red Sea sponge	(Elhady & El-Halawany 2016)

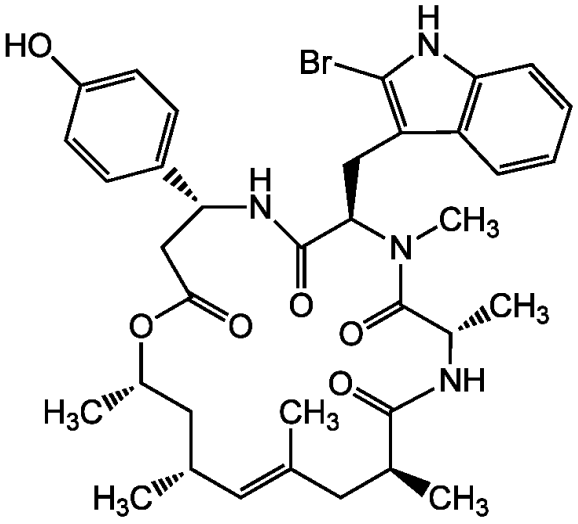
petrosaspongia C				
cyclodepsipeptide, Jaspamide	Cytotoxic	*Jaspis diastral*	Sponge	(Beedessee et al. 2015)
Petrosynol	Cytotoxic	*Petrosia tuberose*	Sponge	(Beedessee et al. 2015)
Angucycline-like compound actinosporin	Antitrypanosomal	*Actinokineospora sp.* strain EG49	Sponge-Spheciospongia vagabunda	Abdelmohsen et al. (2014b; Harjes et al. 2014)
Valinomycin, Staurosporine and butenolide	Antiparasitic	*Streptomyces. sp*	Mediterranean sponges	Pimentel-Elardo et al. (2010
Bisucaberin B	Siderophore	*Tenacibaculum mesophilum*	Unidentified sponge (from Palau)	Fujita et al. (2013
Thiocoralines	Cytotoxic activity against human cancer cell lines	*Verrucosispora sp.*	Sponge-Chondrilla caribensis f. caribensis	Wyche et al. (2011
Mevinolin	Cholesterol lowering	*Salinispora arenicola*	Sponge-Cinachyrella australiensis, Hyatella intestinalis	Bose et al. (2014b)
Aranciamycin, tetracenoquinocin, 5-iminoaranciamycin, novel compound JBIR-43, novel peptides, chlorinated peptide, fumaquinone, fredericamycin A, B	Anticancer cell line cytotoxic activity (for JBIR-43) including antimesothelioma cell line activity	*Streptomyces*	Sponge-Haliclona sp.	Khan et al. (2011)
Rifamycin O, rifamycin W, cycloaspeptide A, nazumamide A	Antibiotic (antimycobacterial	*Salinispora arenicola, Salinispora pacifica*	Great Barrier Reef sponge Species	Bose et al. (2014a, c)
Indoloditerpenes Sterols	Alzheimer dementia	*Dichotomomyces Cejpii*	Marine derived fungus	Henrik Harms (2015)
Phenolic, triterpenoid and Flavanoids	Inhibition of pathogenic fungal growth	*Aspergillus unguis*	Soft coral *Sinularia* sp.	Andidni Putria (2015)

	in host			
New Alkaloids	Cytotoxicity	Closely related to *Aspergillus sp.*	Mediterranean sponge *Tethya aurantium*	Virupakshaiah (2014)
Unknown metabolites	Free radical scavenging activity α - glucosidase and acetyl cholinesterase inhibition,	*Aspergillus unguis*	Marine Sponge (*Agelas sp.,* Red Sea , Egypt)	El- Hady *et al.,* 2015
Phenalenone derivatives	Cytotoxic	*Coniothyrium cereal*	Marine-Derived Fungus	(Elsebai et al. 2016)
Sesquiterpenois	Antibacterial	*Aspergillus sp.*	Marine fungus	Mei Yan (2011)
Alkaloids	anticancer activity	*Penicillium citrinum*, *Fusarium* sp., *Apiospora montagnei*	Marine fungus	Joel Q *et al. Adv Marine Genomics*. 2010
Polyketide asco-sali pyrrolidinone-A40	Antimalarial	*Ascochyta Sali-corniae*	Marine fungus	Joel Q *et al. Adv Marine Genomics*. 2010
new indoloditerpene derivatives	Antimicrobial	*Aspergilluso oryzae*	Marine red alga Heterosiphonia japonica (Dasyaceae)	Qiao et al. 2010
New cytochalasin derivatives	Cytotoxicity	*Chaetomium globosum*	Marine green alga Ulva pertusa (Ulvaceae)	Cui et al. 2010a

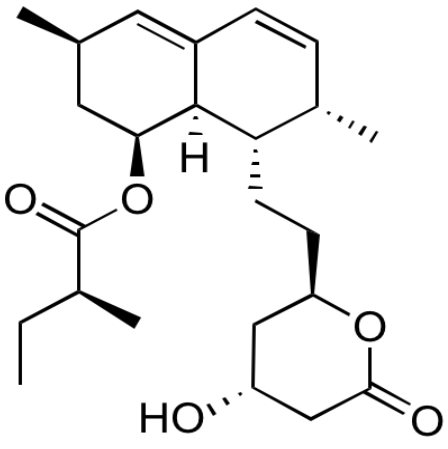
3.1.5 Chemical structure for few novel secondary metabolites listed in the Table 1 obtained from literatures:



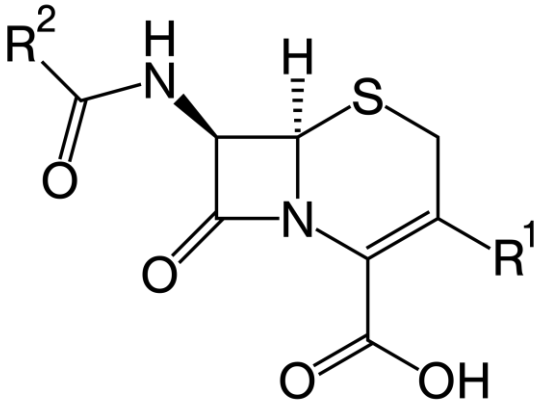
Staurosporine



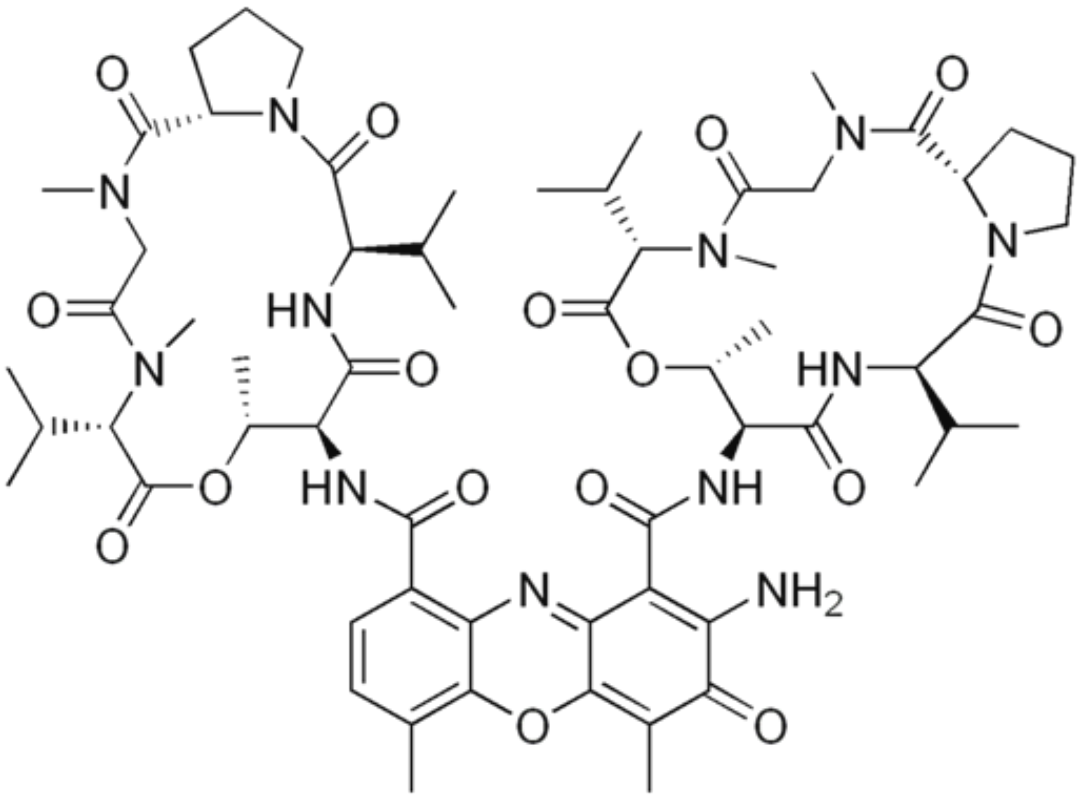
Jaspamide



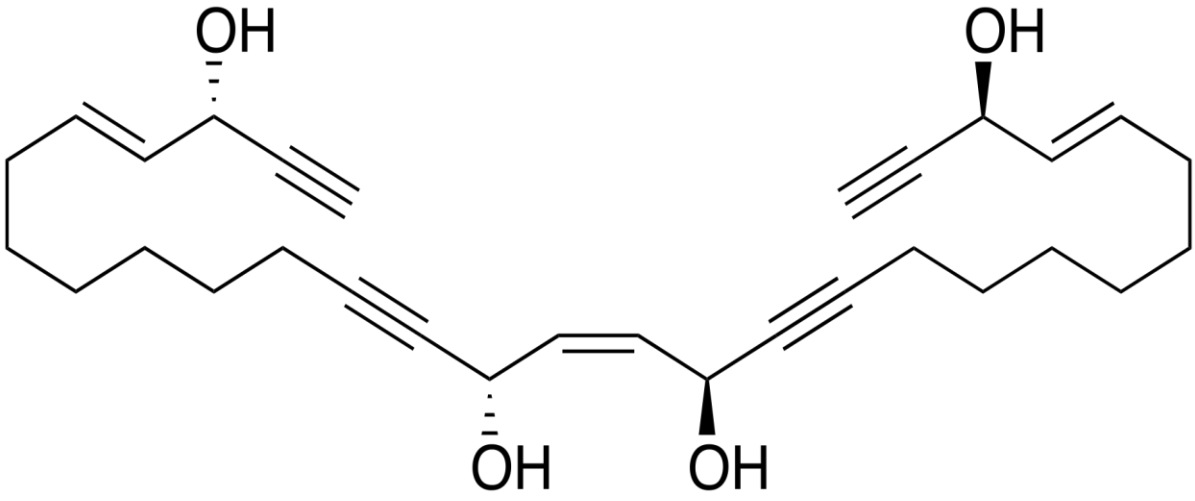
Mevinolin



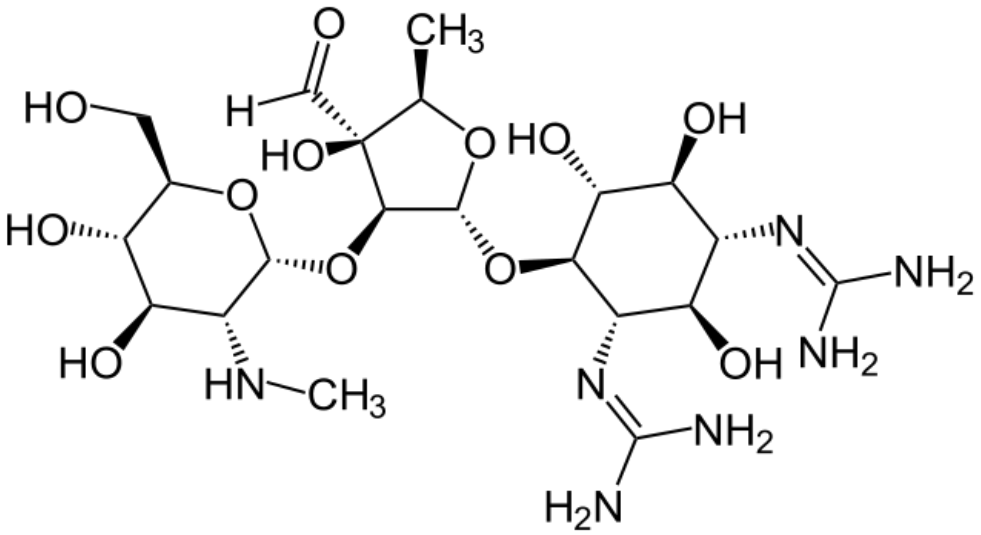
Cephalosporin



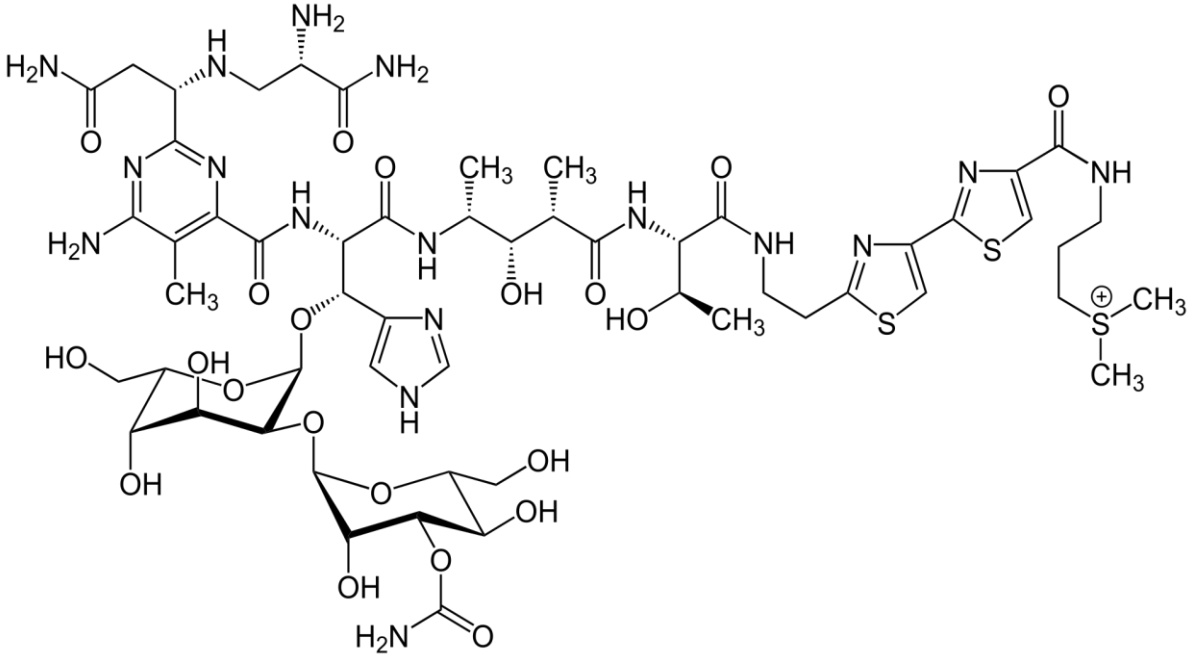
Actinomycin_D



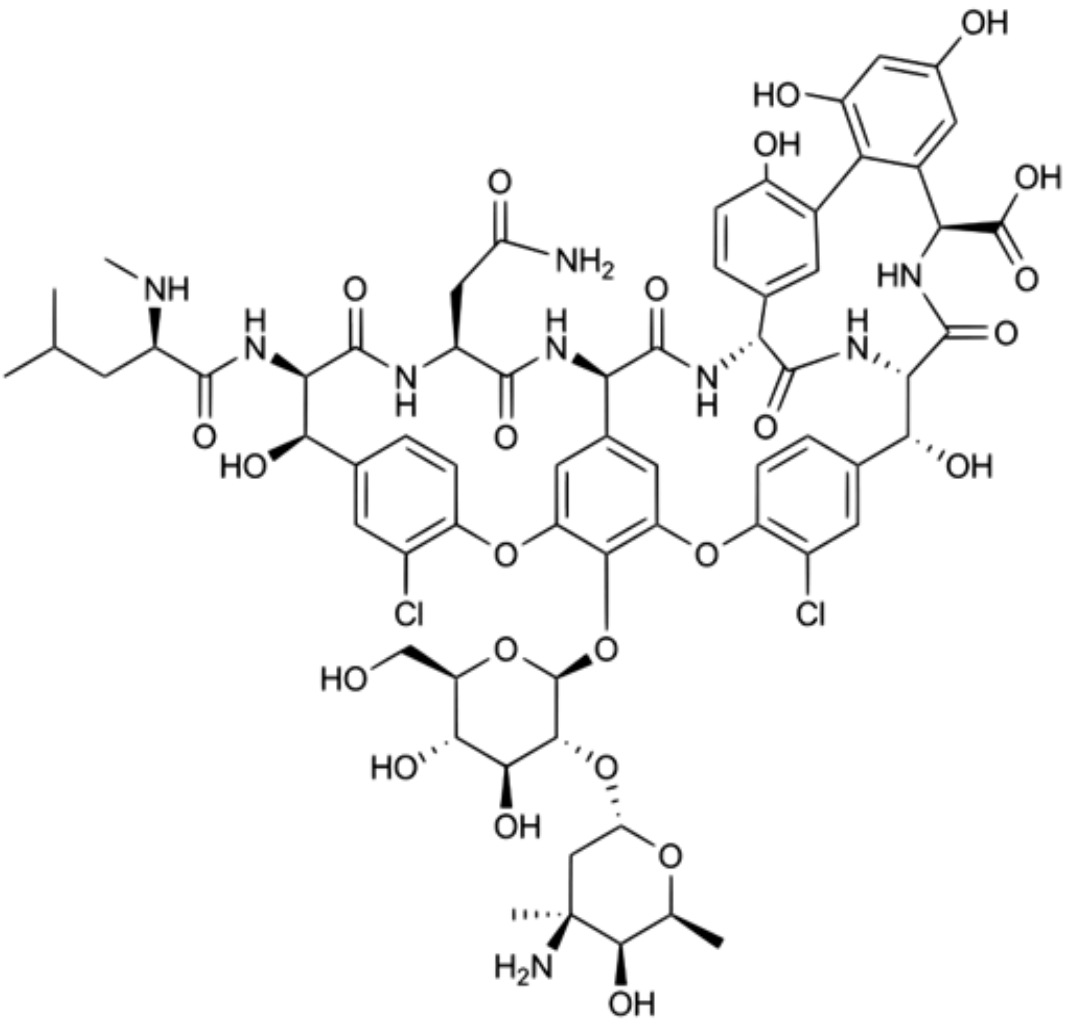
Petrosynol



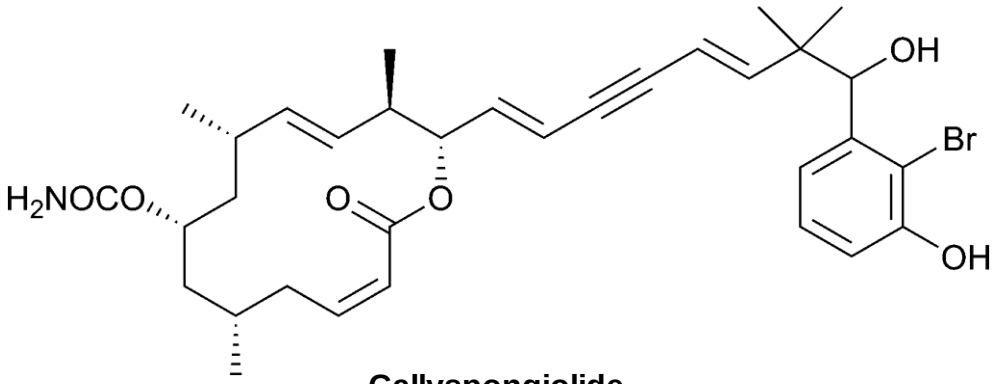
Streptomycin



Bleomycin_A2



Vancomycin



Callyspongiolide

3.1.6 Marine Secondary metabolites accepted as pharmaceuticals:

Werner Bergmann and group discovered unusual arabino- and ribo-pentosyl nucleosides obtained from the marine sponge, *Tethya crypta* collected in Florida, USA (Bergmann and Feeney, 1951; Bergmann and Burke, 1956; Bergmann and Stempien, 1957).These compounds eventually led to the development of Ara-C (cytarabine, an antileukemia agent) and Ara-A (vidarabine, an anti-viral agent) that received Food and Drug Administration (FDA) approval in 1969 and 1976 respectively and which have been in clinical use for decades (Beedessee et al. 2015). Following that all the progressive improvements in the past 50 years of exploration of the marine environment, have resulted in the isolation of approximately 20,000 structurally unique bioactive MNP (Mayer et al. 2013). Nevertheless, despite this enormous number of structurally unique bioactive MNP, to the date the global marine pharmaceutical pipeline includes only eight approved drugs, twelve NP (or derivatives thereof) in different clinical phases and a large number of marine chemicals in the preclinical phase (Mayer et al. 2010) Glaser and Mayer have assessed the status of the clinical marine pharmaceuticals pipeline (Mayer et al. 2016). Results were the following: there were five FDA-approved marine-derived drugs in the US Market, namely cytarabine for cancer (Cytosar-U®, Depocyt®, FDA-approved 1969), ziconotide for pain (Prialt®, FDA-approved 2004), omega-3-acid ethyl esters for hypertriglyceridemia (Lovaza®, FDA-approved 2004), eribulin mesylate for cancer (Halaven®, FDA-approved 2010), brentuximab vedotin for cancer (Adcertis®, FDA-approved 2011), while vidarabine as an antiviral (Vira-A®, FDA-approved 1976) was no longer available, and trabectedin for cancer (Yondelis®, FDA-orphan drug approval 2005) being EU-registered. The clinical marine pharmaceutical pipeline recently reviewed that as of October 2012 consisted of 11 marine-derived compounds in clinical development. These included three new monoclonal antibodies conjugated to synthetic dolastatin derivatives that were in either Phase I, Phase II or Phase III clinical trials. Finally, the preclinical marine pharmacology pipeline remained a global enterprise with researchers from several countries reporting novel mechanisms of action for multiple marine chemicals (Mayer et al. 2011). It was concluded that both marine pharmacology preclinical research as well as the clinical pharmaceutical pipeline

remained very active in 2012. The recent approval of Eribulin mesylate, a synthetic derivative of halichondrin B originally isolated from the marine sponge *Halichondria okadai*, for metastatic breast cancer by the US FDA and European Medicines Agency (EMEA) is proof of how marine organisms can provide new avenues for the treatment of diseases (Twelves et al. 2010). The global market for marine-derived drugs is forecasted to reach $8.6 B by 2016 (BBC report 2011) . In conclusion, marine natural products constitute a strategic research area with enormous economic and social revenues, where scientists all over the world are interested. According to Euro Ocean there are over 590 European marine projects funded by both FP6 and FP7 action which shows the engagement of academia and industry in bringing more marine bioactives into the market (Martins et al. 2014) .

3.1.7 Technologies used in secondary metabolite discovery:

Marine natural products chemistry has passed through several phases of development. The scuba diving made the collection of materials from deep seas easy (Kornprobst & Barre 2014). A growing number of American, European and Japanese scientific teams public and private have systematically explored and exploited a now easily accessible resource taxa by taxa, using safer SCUBA diving equipment and trawling along extensive transects (D.S.Bhakuni & D.S. Rawat n.d.; Martins et al. 2014; Blunt et al. 2015; Micco et al. 2012; Kornprobst & Barre 2014). This technique favored the discovery of completely novel series of molecules with hitherto unknown terrestrial equivalents. For example, cyclic polyether produced by microalgae, volatile halocarbons and tissue-bound terpenes of red algae, linear diterpenes and meroditerpenes of brown algae, chlorinated diterpenes of cnidarians, bryostatins of bryozoans, saponins of echinoderms, non ribosomal cyclic peptides of ascidians, sulfated polysaccharides from red and brown algae, and dozens of examples with original structures isolated from multiple source (Kornprobst & Barre 2014). Traditional extraction techniques such as soxhlet, solid-liquid extraction (SLE), or liquid-liquid extraction (LLE) are characterized by using high volumes of solvents and long extraction times. These techniques often produce low extraction yields of bioactives and present low selectivity (Ibañez et al. 2012).

Furthermore, traditional extraction techniques are usually not automated procedures and their reproducibility can therefore be compromised. The use of new state-of-the art extraction techniques, such as supercritical fluid extraction (SFE), pressurized liquid extraction (PLE), accelerated solvent extraction (ASE®), pressurized hot water extraction (PHWE), ultrasound-assisted extraction (UAE), and microwave assisted extraction (MAE) techniques, among others, may provide an effective alternative to the problems encountered with the use of traditional extraction procedures (Ibañez et al. 2012). Bioactivity-guided separation and purification of marine secondary metabolites afforded the discovery of new compounds with bioactivity assays. Effective methods of separation and isolation such as glass column chromatography, Vacuum liquid chromatography, Flash column chromatography, Preparative Thin layer chromatography and further purification by high performance liquid chromatography both analytical and preparative, capillary electrophoresis methods provided many potent compounds in pure form. The development of refined analytical techniques, particularly high resolution nuclear magnetic resonance (NMR), mass spectrometry (MS) and X-ray diffraction has also contributed tremendously. They have helped to solve many intricate structural and stereo chemical problems. In fact, many advances in spectroscopic methods, allowed the *de novo* structure determination of new chemical entities (NCEs) in very small concentrations even in complex mixtures as crude extracts (Martins et al. 2014).

3.1.8 Why to discover new drugs further?

The need for new biologically active compounds with application as pharmaceuticals, nutraceuticals and cosmeceuticals is undeniable. In the very near future, there is a serious risk that a significant number of infections, particularly nosocomial infections, will be untreatable. There are no effective synthetic drugs for many forms of cancer. The obesity epidemic has created a need for “fat blockers.” New, safe and effective sunscreens are needed to protect the public from damaging UV radiation.

Natural products have fulfilled a critical role in the discovery of pharmaceuticals, not to mention their use in nutritional supplements and cosmetics. The marine environment has proven to be an exceptional resource for the discovery of bioactive natural products

as is evident from the observation that more than 30,000 new metabolites have been isolated from marine biota in the past 30 years. Notably, the marine natural products Cytarabine, Yondelis and Eribulin (a derivative of a sponge metabolite) and monomethyl auristatin E (MMAE, a dolastatin derivative) have been approved for use in human chemotherapy. According to a 2014 review by David Newman and Gordon Cragg of the Natural Products Branch of the National Cancer Institute in Maryland, an additional 39 marine natural products or derivatives thereof are currently in clinical trials as anti-cancer and cancer pain control agents.

Our oceans, much like the rain forests, are suffering from a loss of biodiversity. Until recently, only two corals were listed in the Endangered Species Act, however, earlier this year, the US National Oceanic and Atmospheric Administration (NOAA) added an additional 20 species of coral to this list and a further 83 species are being considered for future listing. Clearly, the rate of loss of our marine biodiversity is occurring at an alarming rate. Given that each sponge or coral will contain thousands of microbial symbionts, loss of dozens of coral species could result in the loss of substantial biodiversity and, possibly, the loss of a life-saving cancer drug, a new class of antibiotic or a new sunscreen. The public is likely aware of the value of coral reefs in terms of the impact on tourism and fishing, however, the potential of microbes living within coral reef invertebrates to produce life-saving medicines and other bio-products is likely not well understood. The world ocean assessment report has been released at end of 2014 and we would be well served to consider the impact of environmental damage on our access to yet-to-be discovered marine natural products. According to the review of marine natural products 2015 by Blunt et al. “Fifty years ago around 1963 just four papers were published on MNPs with only one paper containing new compounds (Blunt et al. 2015) . To date, chemists published 9220 papers over the past 50 years describing new compounds.” This emphasize the point that there is still an enormous MNP resource to be explored; probably in excess of 200 000 species still to be evaluated (Blunt et al. 2015) .

3.1.9 Marine fungal candidates taken for the current thesis

3.1.9.1 Definitions:

Marine fungi are an ecologically rather than physiologically or taxonomically defined group of organisms. According to the ‘‘classic’’ definition that appears to be universally accepted in the scientific community, marine fungi are divided into two groups, obligate and facultative marine fungi obligate marine fungi are those that grow and sporulate exclusively in a marine or estuarine habitat, while facultative marine fungi are those from freshwater or terrestrial milieus able to grow (and possibly also to sporulate) in the marine environment (Kohlmeyer & kohlmeyer 1979) It has been suggested that indigenous marine species (obligate and facultative) should be separated from non-indigenous species (sometimes referred to as ‘‘contaminants’’ or ‘‘transients’’, i.e. terrestrial or freshwater species that are dormant in marine habitats) based on their germination ability, but in practical terms this is difficult to achieve. Moreover, there is an increasing tendency to identify marine fungi by molecular biological methods that do not require sexually reproducing life stages, for example sequencing of rDNA, instead of traditional approaches based on morphological characteristics (Rateb & Ebel 2011)

3.1.9.2 *Pseudohalonectria lignicola* Minoura and Muroi 1978

The marine fungal strain 165 has been taxonomically identified to be Ascomycetes belongs to the genus *Pseudohalonectria* and closely related to *P. lignicola* species. *Pseudohalonectria lignicola* was first described in 1978 by K. Minoura and T. Muroi. This was also the first description of the genus *Pseudohalonectria*. The Fungus was isolated for the first time from balsa wood, which had been dipped for three months in a freshwater lake in Japan. Based on the morphological characteristics, it was identified that the fungi belongs to Ascomycetes class. Although there was lot of confusions to place the fungi into the taxonomical position, Minoura and Muroi finally gave the introduction of the new genus *Pseudohalonectria*.

Later, Shearer et al also reported *P. lignicola* from the initial discovery of the species in the United States. In this study, branches of various hardwoods were placed in a stream

over a period of 30 months where the aim was to record all ascomycete's species had settled in the water lying wood, as well as the frequency of their occurrence. It was studied that the fungus exclusively moves towards woody tissue since it was found mostly at the bare woody ends. A. and J. Revay Gönczöl from United Kingdom gave the first report in 1990 on the occurrence *P. lignicola* from European water system.

3.1.9.3 Morphology of *P. lignicola*:

Various types of the genus *Pseudohalonectria* can differ in terms of morphological characteristic based on different disposition of their fruiting bodies. The ascomata of *P. lignicola* are 222-497 x 243-524 microns in size, lying singly or in the aggregate, from round to flattened round shape with a long, beak-shaped tapered end. First, they are pale yellow in color, but will darken with time. The paraphyses - sterile hyphae Center of the fruit body is longer than the asci and 1 to 5-fold septet

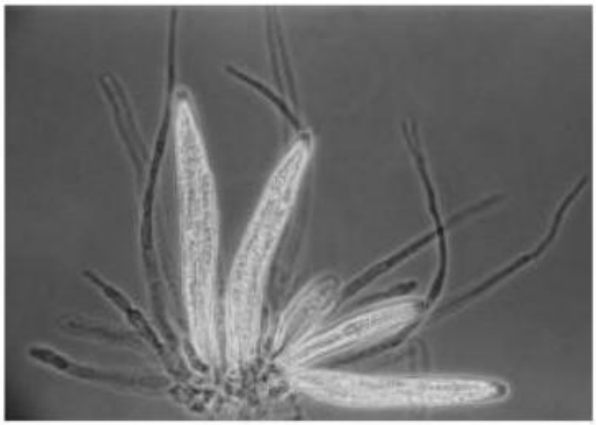


Figure 2 : Paraphyses with asci of *Pseudohalonectria lignicola*; the cylindrical, straight or sigmoid shaped unitunicaten, 90-132 X 11 to 17.6 microns large asci contain eight Ascospores. These are usually septate 5-Fold, from also Cylindrical in shape with blunt rounded ends and average 62.6 X 5.1 microns in size. Initially, they are colored yellow with time but orange to brown. Microscopical image courtesy: Cornelia Rosemann.

3.1.9.4 Taxonomy classification of *P. lignicola*:

The precise taxonomic classification of *Pseudohalonectria lignicola* is not currently possible due to the unexplained membership at the level of order. When Minoura &

Muroi introduced the genus *Pseudohalonectria*, there was no clear statement about their phylogenetic position. Instead, it was only mentioned on similarity to *Halonectria milfordensis* from the order of Hypocreales (Shearer & Bodman 1983) . Shearer has mentioned about the problem of the allocation to an order of *Pseudohalonectria* is because of morphological features represents both of Diaporthales as well as the characteristic Sordariales. Later, Shearer also proposed in 1989 based on phylogenetic results that, the genus *Pseudohalonectria* may be belongs to the family of the Lasiosphaeriaceae (Chen et al. 1995) however, in 1995, it was believed that *Pseudohalonectria* was more closely related to species of the Order of Sordariales than to those of Diaporthales (Chen et al. 1995) . In contrast, the results of phylogenetic analysis by CHEN et al. were not supporting that *Pseudohalonectria* belongs to Sordarialesea (Inderbitzin & Berbee 2001). In recent studies *P. lignicola* was found to be closely related to species of the family of the Magnaporthaceae (speciesfungorum 2015). This thesis was carried out based on the phylogenetic results obtained from the ribosomal DNA sequence blasted in NCBI database and the final results suggested that the species of strain 165 of *Pseudohalonectria* genus belongs to magnaporthaceae family. According to the present position of *Pseudohalonectria lignicola* that has been given in the phylogenic classification based on latest entry is as follows http://www.speciesfungorum.org/Names/GSDSpecies.asp?RecordID=321792 (2015)

Kingdom: Fungi
Department: Ascomycota
Class: Sordariomycetes
Subclass: Sordariomycetidae
Order: Magnaporthales
Family: Magnaporthaceae
Genus: Pseudohalonectria
Spieces: *P. lignicola*

3.1.9.5 Occurrence of genus *Pseudohalonectria* (Marine or freshwater fungi)

- *Pseudohalonectria lignicola* (Minoura & Muroi 1978)
- *Pseudohalonectria adversaria* (Shearer 1989)
- *Pseudohalonectria falcata* (Shearer 1989)
- *Pseudohalonectria lutea* (Shearer 1989)
- *Pseudohalonectria longirostrum* (Shearer 1989)
- *Pseudohalonectria phialidica* (Shearer 1989)
- *Pseudohalonectria palmicola* (Yuen et al. 1999; Hyde et al. 1999)
- *Pseudohalonectria eubenangeensis* (Yuen et al. 1999; Hyde et al. 1999)
- *Pseudohalonectria aomoriensis* (Ono & Kobayashi 2001)
- *Pseudohalonectria fuxianii (Tsui et al.* 2003)
- *Pseudohalonectria tayloriae* (HYDE & FRÖHLICH 2003)
- *Pseudohalonectria suthepensis* (Promputtha et al 2004)
- *Pseudohalonectria halophila* (Kohlmeyer et al. 2005)
- *Pseudohalonectria miscanthicola* (Shenoy et al. 2005)

Until 1999, it was assumed that Pseudohalonectria and species existence is strictly limited to freshwater habitats because all known species have been found in freshwater lakes, streams and rivers. Later in 1999, some species of Pseudohalonectria was isolated from terrestrial environment also for example two new species, *P. eubenangeensis* and *P. palmicola*, were isolated from the palm in Australia (Queensland) at the same time was the first report of species this genus of terrestrial habitats (Yuen et al. 1999; Hyde et al. 1999)

The life style of the genus Pseudohalonectria is mostly by colonization on the dead wood by which the special wood degrading activity can be performed, especially lignicola plays by the decomposition of organic material is an important role in the ecosystem. All examined Species were able to decompose wood which might have been infected by any of the other fungus also (Chen et al. 1995)

3.1.9.6 Chemical prospecting of Pseudohalonectria species studied so far:

The first studies on the biological activity of the genus Pseudohalonectria was conducted by SHEARER and employees. They noted the antagonistic activity of Pseudohalonectria genus that inhibit the growth of a plurality of filamentous Fungi (Shearer & Zare-Maivan 1988) . The growth of the yeast *Saccharomyces cerevisiae* and the Bacteria *Bacillus subtilis* and *Escherichia coli* however remained unaffected. It was one Correlation between the ability of inhibition and the degree of a yellow pigment has been observed. Unpigmented species showed considerably less inhibitory in comparison to the pigmented Similar results were obtained YUEN et al. when they had studied the *in vitro* interaction between various tropical and subtropical freshwater fungi (Yuen et al. 1999). The pigment-producing species like *P. longirostrum* was one of the most inhibiting species. Went out of their study further shown that fungi with a slow growth rate, such as *P. longirostrum* caused a stronger inhibition than fast-growing species (Yuen et al. 1999). Investigations of the identity of the pigment missing till today A growth-inhibitory effect of *P. lignicola* was compared with other fungi, also *in vivo* notice. Experiments with wood treated with *P. lignicola* was settled, showed that this fungus was able to react against the invasion by other fungus (Fryar et al. 2001; Tsui et al. 2003). Lately, in 2006 Two new Azaphilones metabolites, named pseudohalonectrin A (1) and B (2), were isolated from the culture of the aquatic fungus *Pseudohalonectria adversaria* YMF1.01019, originally separated from submerged wood in Yunnan Province, China by Dong et al. Pseudohalonectrin A and B were assessed for their nematicidal activity against the pine wood nematode *Bursaphelenchus xylophilus* and their structures were defined after spectral analysis. This is the first report of secondary metabolites from any member of the genus *Pseudohalonectria* (Dong et al. 2006). However, fungal strain 165 taken for the current study closely related to *P. lignicola* has been identified as a new producer for the well known potent cytotoxic and antimicrobial polyketide, mono-terpenoid, alpha pyrone derivatives and the major lead compound from this fungal strain is Nectriapyrone. It would be interesting to review on other

Nectriapyrone producers so far and Table-2 suggests that the same chemical potentials can be produced by diverse bio producers still to be explored:

Table 2: List of Nectriapyrone isolation from various fungal sources so far.

Organism	Compound isolated	Bioactivity	Reference	Year
Gyrostoma missouriense	Nectriapyrone	Antibiotic	MSR Nair and S T Carey	1975
Phomopsis oblonga	Nectriapyrone	Antibiotic	Claydon et al.	1985
Pestalotiopsis oenotherae	Nectriapyrone	Antibiotic	Venkatasubbaiah et al.	1991
			Xiao-Long Yang et al	2011
Gliocladium vermoesenii	Nectriapyrone	Antibiotic	Avent et al	1992
Unidentified fungi from sponge named *Stylotella sp*	Nectriapyrone	Antibiotic	Abrell et al	1994
Unidentified fungus, from a sponge of the genus *Pleiocheuta*	Nectriapyrone	Antibiotic	P.Crews et al.	1994
Scytalidum sp.	Nectriapyrone	stimulates DOPA melanin in B16-F1 melanoma cells in the absence of melanin stimulating Hormone	Eckhard Thines And Heidrun Anke	1998
Phomopsis sp	Nectriapyrone	inhibitory activity on MAO in a dose-dependent manner	Lee et al.	1999

Unidentified fungi from marine sponge called *Jaspis Johnstoni*	Nectriapyrone		Vahidulla et al	2002
Phomopsis species — endophytes of *Erythrina crista-galli L*.	Nectriapyrone	Antimicrobial activity	Weber et al.	2005
Phomopsis species — endophytes of Medicinal Plant *Eupatorium arnottianum*	Nectriapyrone		Meister et al.	2007
Glomerella cingulata isolated from *Viguiera arenaria* and *Tithonia diversifolia*	Nectriapyrone	cytotoxic activity against both human T leukemia and melanoma tumor cell lines	Guimaraes et al.	2008
Phomopsis foeniculi	Nectryapyrone	Phytotoxic activity	A . Evidente et al	2011

3.1.9.7 Unknown species of the order Pleosporales:

Taxonomy results suggests that Marine fungal strains MF010 and MF 018 are closely related to each other but complicated to identify in large heterogeneous species of the order pleosporales having close affinities to the genus Phoma.

Pleosporales having a long history since 1869 for the correct placement in the phylogenic tree and finally proposed by is the largest order in the Dothideomycetes, comprising a quarter of all dothideomycetes species (Kirk et al. 2008). The name

“Pseudosphaeriales” has been applied in different senses, thus Pleosporales (as an invalid name due to the absence of a Latin diagnosis) was proposed by Luttrell in 1955 to replace the confusing name, Pseudosphaeriales, including seven families, Müller and von Arx in 1962 however, reused Pseudosphaeriales with 12 families included but Based on morphological characters of ascomata, morphology of asci and their arrangement in locules, presence and type of hamathecium, shape of papilla or ostioles, morphology of ascospores and type of habitats characters, Luttrell (1973) included eight families, Luttrell in 1973 included eight families. Pleosporaceae and Venturiaceae plus 11 other families were accepted in Pleosporales as arranged by Barr (1979a) largely using Luttrell’s concepts, Pleosporales was formally established by Luttrell and Barr (in Barr 1987b), characterized by perithecioid ascomata, usually with a papillate apex, ostioles with or without periphyses, presence of cellular pseudoparaphyses, bitunicate asci, and ascospores of various shapes, pigmentation and septation and after many changes of family inclusion/exclusion of the order pleosporales, in the most recent issue of Myconet 2010, 28 families were included in Pleosporales as per Lumbsch and Huhndorf. Overall, the circumscription of *Pleosporales* has undergone great changes in the last half century.

In general, Species included in Pleosporales have different ecological or morphological characters like pigmentation or lifestyle for example. One important reason for the unstable circumscriptions in the traditional classification of the *Pleosporales* is that the value given to the various morpho-characters, even those used at high-level classification, has proven to be overstated. For instance, fruiting-body shapes, *i.e.* cleistothecioid, perithecioid and apothecioid, previously considered sanctum at class level classification, were found to have undergone convergent evolution (Hawksworth & Lagreca 2007) , as can be seen across *Ascomycota* (Schoch et al. 2009) **.** *A*nother important distinguishing character, ascus type, has been reported to be phylogenetically misleading in numerous natural groups (Schmitt & Lumbsch 2004a). Indeed, several DNA sequence based phylogenetic reconstructions have shown that ascospore morphology has little phylogenetic significance at familial or generic level classification (Schmitt & Lumbsch 2004b) , Consequently, an increasing number of taxa designated

only by morphological characterizations in *Pleosporales* have been reported to be polyphyletic.

Thus the overall reason not to talk much about morphological characters of the fungal strains MF 010 and MF018 and it has been suggested solely, based on the DNA sequencing results that both the unknown species are belongs to the order pleosporales having close affinities towards the genus Phoma.

3.1.9.8 Chemical prospecting of the order Pleosporales studied so far:

There are many marine derived fungal species belongs to diverse taxonomical classification but the same phylogenic order 'Pleosporales' producing verity of bioactive chemical compounds has been reviewed. The table below is the list of compounds identified so far from the order Pleosporales.

Table 3: List of compounds isolation from the order pleosporales

Biological source	Compound isolated	Novelty	Bioactivity	Reference	Year
A marine fungus *Leotosohaeria obiones* from the coastal region of Sapelo island, Georgia	Obionin A	Novel	Neurotoxin	GK Poch et al	1989
A marine fungus *Leptosphaevia ovaernavis* (LINDE) from near the mouth of Sarno river, bay of Naples	Degraded Polyketidic Lactone: Leptosphaerolide Leptosphaerodione (Precursor)	Novel	Cytotoxic Antifungal	Guerriero, A.et al	1991
A Freshwater Isolate Of The Fungus *Kirschsteiniotheli a* Sp. From chile	Naphthoquinone dimer: kirschsteinin	Novel	Cytotoxic Antimicrobial	GK Poch et al.	1992
	chlorinated diphenyl ethers	Novel	Antimicrobial		
	Napththoquinone derivatives	known	Antimicrobial		
Freshwater fungus *Anguillospora*	Anguillosporl	Novel	Antimicrobial	Harrigan et al	1995

longissimA from Illinois					
Unidentified Freshwater Fungus of the Family Tubeufiaceae from North Carolina	Dihydroaltenuene A & B	Novel	Antimicrobial	Ping Jiao et al	2006
	Dehydroaltenuene A & B	Novel	Antimicrobial		
	Isoaltenuene	Known	Antifungal and Phytotoxic		
	Altenuene	Known	Antifungal and Phytotoxic		
	5'-Epialtenuene	Known	Antifungal and Phytotoxic		
Marine derived fungus isolate CRIF2;2005;Thailand	Diketopiperazines: (*Z*)-6-enzylidene-3-hydroxymethyl-1,4-dimethyl-3-methylsulfanylpiperazine-2,5-dione	Novel	Weak Cytotoxic	Prachyawarakorn, V. et al.,	2008
	Phthalide: (3*S*,3′*R*)-3- (3′-hydroxybutyl)-7-methoxyphthalide	Novel			2008
	Phthalide: (*S*)-3-butyl-7-methoxyphthalide	Novel as natural product	Weak Cytotoxic		
	Diketopiperazines: (3*R*,6*R*)-bisdethiodi (methylthio)hyalodendrin	Known	Weak Cytotoxic		
	Diketopiperazines: bis-N-norgliovictin	Known	Weak or No Cytotoxic		
A fungal strain 222 from Greifswald Baltic coast driftwood	Naphthalene derivatives: Balticol A-F	Novel	Antiviral	Shushni et al	2009
	Altechromone A	known	Antiviral		
	Hydroxysydonic acid	Known			

The Isolate F76-1 from Florida-An fresh water fungus *Wicklowia aquatica*	1,7,11-Trihydroxy-1,3,5-bisabolatrien-15-oic acid			Hosoe, T. et al., Heterocycles	2010
	Sydonic acid (ξ)-form 3-Hydroxy-4- (1-hydroxy-1,5-dimethylhexyl)benzoic acid, 9CI	Known	Weak activity		
	Deoxodihydroepiheveadride 9-Epimer, 12-deoxo, 11ξ-alcohol	Novel	Weak activity		
	Dideoxyepiheveadride 9-Epimer, 11,12-dideoxo	Novel	Weak activity		
	Tetrahydroepiheveadride 9-Epimer, 11ξ,12ξ-diol	Novel	Weak activity		
	Folipastatin	Known	Weak activity		
	Agonodepside B	Known	Weak activity		
	Waquafranone A	Novel	Weak activity		
	Waquafranone B	Novel	Weak activity	Hosoe, T. et al., Heterocycles	2010
	Epiheveadride	Known	Antifungal		
	Deoxoepiheveadride	Known	Antifungal		
	Dihydroepiheveadride	Known	Antifungal		
Unidentified terrestrial fungus (MSX 63619)	o-pyranonaphthoquinone decaketide: Obionin B			S. Ayers et al	2011

3.2 INTRODUCTION PART-II Primary metabolites in terrestrial symbiosis

3.2.1 Earthworm symbiosis:

A wide array of interactions among plants, animals, and microorganisms occurs in nature. Some of these relationships are characterized by a close physical association among species that persists for a significant period of the life cycle. In 1879 German botanist Heinrich Anton de Bary coined the term "Symbiosis" to describe these relationships, meaning the living together of different species of organisms. Symbiotic relationships span a spectrum from beneficial to detrimental effects.

Earthworms are the most dominant soil animals and play an essential role for soil fertility: they degrade plant residues, improve soil structure, and activate soil microorganisms. From the beginning of their evolutionary success history, In particular, Lumbricid earthworms have been co-evolved since 100 years with bacterial symbionts that reside in their excretion organal part called as Nephridia (Knop, 1926; Schramm *et al*., 2003; Davidson *et al*., 2013). These bacteria belong to the genus *Verminephrobacter* (Betaproteobacteria), a monophyletic group closely related to *Acidovorax* (Schramm *et al*., 2003; Pinel *et al.,* 2008) are vertically transmitted from parent to offspring (Lund et al. 2014), and have a beneficial effect for their host by increasing earthworm reproduction. The symbionts have so far not been detected in the environment outside their host, fact that suggests a deep signal mechanism by metabolic link between the partners. Still, the partners can be separated in the lab, and stable cultures of several symbionts strains and symbionts-free earthworm species have been established, which makes this symbiosis an ideal model system to study the molecular mechanisms of symbionts transmission, host specificity, and other host-symbiont interactions. By inhabiting in the nephdrial part of earthworm, these symbionts bacteria might recycle the earthworm waste products for self survival as well as to benefit not only the host but also the embryo and growing earthworm by providing amino acids, vitamins and co-factors. However, there is no other recent update after Lund et al in 2010 said comparative fitness experiments revealed that the symbionts' presence enhances host reproduction.

Upgrading host's nutrition is a well-known function among other symbioses (Buchner, 1965; Graf *et al*., 2006; Feldhaar *et al*., 2007; Bik, 2009; Gündüz and Douglas, 2009; Hosokawa *et al*., 2010; Adams *et al*., 2011; Salem *et al*., 2012; Body *et al*., 2013; Klein *et al*., 2013; Douglas, 2014). Additionally, As these worms have reduced mouth, gut and nephridial organs, these symbionts are essential for the worms fulfilling different roles to make trash into treasure.

Symbionts might involve in the detoxification of host nitrogen waste acting on dead end nitrogen-containing compounds, like urea or ammonia as they live in nepdria, might provide the resultant newly synthesized amino acids to the host. These would not only make new nutrients available for host usage reverting possible starvation condition effects, but would also be important in preventing accumulation of excretion products that are potentially toxic for the worm (Flavia, 2015 unpublished). All in all, our results may light on the real metabolism and physiology of *V. eiseniae* revealing a symbiont well adapted to its ecological niche and to its symbiotic status and may provide new basis for further quests on the symbiotic function basis (Flavia 2015 unpublished). Although it has been analyzed via various windows like genomics and proteomics this symbiosis purpose, only metabolomics analysis were considered for the partial contribution of this current thesis.

3.2.2 *Verminephrobacter eiseniae* and *tuberculata*

Verminephrobacter is a recently established genus that is closely related to the Betaproteobacteria genus *Acidovorax* (Lund et al. 2011; N. Pinel et al. 2008). The *Verminephrobacter* species are Gram-negative, heterotrophic, flagellated and rod-shaped bacteria which occur as single cells or doublets (N. Pinel et al. 2008) are endosymbiotic bacteria located in the nephridia of Lumbricid earthworms (Lund et al. 2011) first detected by light microscopy in 1926 (Anon n.d.). *Verminephrobacter eiseniae* and *Verminephrobacter tuberculata* are the specific endosymbionts of *Eiseniae fetida* and *Aporrectodea tuberculata* respectively (Lund et al. 2011). The cells of *V. eiseniae* (1.5 x 0.6 µm) are slightly smaller than *V. tuberculata* cells (2 x 0.8 µm) despite their otherwise similar appearance (N. Pinel et al. 2008; Lund et al. 2011).

Verminephrobacter eiseniae and *Verminephrobacter tuberculata* have only been characterized in the most recent years by Pinel et al and Lund et al respectively (N. Pinel et al. 2008; Lund et al. 2011) . The fact that although they are endosymbionts they can be cultivated separately from their hosts made their explicit characterization easier. Since both species have the same natural habitat they exhibit similar preferences in their choice of nutrients and growth conditions. Both species prefer microaerophilic conditions over high oxygen levels in their growth medium which matches well with the reported prevalent conditions in the nephridia of earthworms. A variety of amino acids, glucose and pyruvate are needed for successful growth, but both species also feed on fatty acids and other sugars such as fructose, galactose, mannose and rhamnose. However, the reported optimal cultivation temperatures of the two *Verminephrobacter* symbionts are different. *V. eiseniae* shows optimal growth at significantly higher temperatures (25-28°C) than *V. tuberculata* (20°C) (Lund et al. 2011).

3.2.3 Colonization of Verminephrobacter species in Lumbricid earthworms:

Several studies were conducted to investigate how and when the earthworms are colonized by their symbionts. The findings of these studies revealed that the *Verminephrobacter* species are transmitted vertically from parent to offspring (Davidson & Stahl 2006). This vertical transmission happens as follows. Upon the mating of earthworms a mucus sheath is produced and it encases both worms. Within this sheath sperm is exchanged and subsequently a precapsule forms at the clitella of each worm. Sperm, eggs and albumin are expelled into this precapsule. After sliding off the anterior end of the worm this precapsule matures to an egg capsule with a sealed chitinous shell (Davidson & Stahl 2006). The *Verminephrobacter* symbionts are deposited within the capsule prior to its separation from the adult earthworm. Hence, the symbionts originate from the parents and not the surrounding soil although the formation of the mature capsule takes place externally making it possible for soil bacteria to enter the capsule prior to shell formation. Due to this fact a mechanism for the selective recruitment of the *Verminephrobacter* cells into the nephridia of the developing embryo becomes shown

that this recruitment of the symbionts happens during a specific time window at a certain point of the embryogenesis and is completed 1 – 2 days prior to hatching (Davidson & Stahl 2008). The juvenile earthworms are therefore fully colonized by the time they leave the egg capsule (Davidson & Stahl 2006) and additionally infection by the symbionts is no longer possible after hatching (Lund et al. 2010).

As mentioned previously the *Verminephrobacter* symbionts are located in the nephridia of the earthworms or more specifically the ampulla. The nephridia are the excretory organs of the earthworms and present in pairs in each segment of the worm. The nephridia consist of three major loops and the ampullae which are colonized by the endosymbionts are formed by a widening of the second loop. The function of the nephridia is similar to that of the human kidney. Thus, they continuously pass fluid from the coelom to the exterior to rid the worm of nitrogenous and other metabolic waste and toxic compounds and secure its osmoregulation (Davidson & Stahl 2006) .

3.2.4 Metabolomics:

Metabolomics is all about studying metabolites processed in the metabolom of a living system which is complementary information for genomics, transcriptomics and proteomics studies. Metabolomics is a relatively new member to the “Omics” family of systems biology technologies (Bino et al. 2004). The term ‘Metabolom’ was coined in 1998 and was used to describe the metabolite complement of living tissues (Oliver et al. 1998). Metabolites are considered to “act as spoken language, broadcasting signals from the genetic architecture and the environment” (Jewett et al. 2006), and therefore, metabolomics is considered to provide a direct “functional readout of the physiological state” of an organism (Gieger et al. 2008) . Thus the ideas of making batch culture of host separated symbionts and analyze the metabolites at various time points of growth stage in this current study. The rapidly emerging field of metabolomics combines strategies to identify and quantify cellular metabolites using sophisticated analytical technologies with the application of statistical and multi-variant methods for information extraction and data interpretation.

4 MATERIALS AND METHODS

4.1 PART I

All the three strains described in this study were identified by Pharmaceutical Biology department, Institute of Pharmacy, Ernst Moritz Arndt University, Greifswald, Germany. *Pseudohalonectria lignicola* (Strain 165) was isolated from drift wood collected from Bay of Baltic Sea near Greifswald, Germany in July 1999. Strain 10 which is unknown yet, was isolated from sponges collected from Red sea, Egypt. Strain 18 which are also unknown, was isolated from sponges collected from Red sea, Egypt.

4.1.1 Isolation of Fungal strains:

All the above collected fungal strains described in this study were obtained from fungal collections of Prof. Ulrike Lindequist, Pharmaceutical Biology, Institute of Pharmacy, Ernst Moritz Arndt University Greifswald which were isolated by former colleagues and maintained by Frau. Monika Matthias.

4.1.2 Isolation from Drift wood:

After sterilization of collected wood pieces with 75 % ethanol, rinsed with sterile water and placed into agar plates at room temperature to check whether the wood pieces have any residual fungal spores sticking onto the wood surface. Later, the sterile agar plate containing wood pieces were cut into small pieces and placed on agar plates containing freshly prepared Hagem agar medium (see 4.1.7). Fungal colonies growing out of the wood pieces were transferred onto a liquid Hagem medium (see 4.1.7) at pH 7.5. This process is repeated in glass tubes to make a stock culture and maintained at -80°c once the taxonomy is confirmed.

4.1.3 Isolation from Sponge material:

Fungal strains were isolated by inoculating small pieces of the inner tissue of the sponge on a solid Hagem agar medium or PDA medium at room temperature. Fungal colonies growing out of the tissue of the sponge were transferred to liquid Hagem medium (see

4.1.7) at pH 7.5 in artificial sea water. This process is repeated in glass tubes to make a stock culture and maintained at -80°C once the taxonomy is confirmed.

4.1.4 Taxonomy identification:

For identification purposes, pre cultivated fungal culture (See 3.5.3) in Petri dish containing Hagem medium (see 4.1.7) + 1.5% Agar extract and incubated at 37°c for 4 weeks were used. The selected fungal cultures were monitored for sporulation during the incubation period and it was observed that, these fungal cultures are not sporulating but growing by spreading their hyphe. So, a small hyphe section was removed in a sterile condition and taken for DNA extraction using the following protocol in order to make DNA sequencing and identify the taxonomy.

4.1.5 DNA Extraction of genomic DNA from fungal Cultures:

Cell lyses Buffer:
10 mM Tris base/Tris HCl
100 mM Nacl
1 mM Na2-EDTA
1% SDS
2% v/v Triton x-100
Make up to 1 liter with sterile Distilled H2O
No Autoclaving, storage at room temperature
Method:
Small pieces of tissue (with a bit of Agar) were removed and place it in a sterile eppendorf tube. Sterile sand (up to approx. 1ml) was added and 500µl of cell lyses buffer was also added. Vortexed for 30sec and incubate at 70°C for 15min on a heated shaker then 500µl Chloroform-isoamyl alcohol (24:1) was added and vortexed for 30 sec. The whole mixture was centrifuged at 4°C at max speed for 10min. 400µl of the upper aqueous phase was added into a new eppendorf tube and repeat once again. 300µl of the upper aqueous phase was added into a new eppendorf tube. 300µl of ice cold isopropanol was added, shaken carefully and incubated for 5min. Centrifuged at

4°C at maximum speed for 10min. The supernatant was discarded and washed with 200µl of 70% ice cold ethanol. The pellet was dried and 50µl of TE buffer (or DD H2O) was added. The extracted DNA was submitted for DNA sequencing analysis.

4.1.6 Cultivation of Fungal strains:

Equipment and chemicals:

Homogenizer HO 4 (Buehler, Tubingen, Germany)

Shaking innova 2100 (New Brunswick Scientific, Nuertingen, Germany)

Orbital Shaker Incubator OSFT-LS (TEQ, P)

Delta 340 pH meter (Mettler Toledo Ltd., Halstead, UK)

Autoclave (H + P Labortechnik GmbH, Oberschleißheim, D)

Balance BP 61 (Sartorius AG, Gottingen, Germany)

Agar-agar (Carl Roth GmbH, Karlsruhe, D)

Ammonium succinate (Darmstadt, Germany)

Iron (II) sulfate (VWR International, Darmstadt, Germany)

Iron (III) chloride (VWR International, Darmstadt, Germany)

Glucose (VWR International, Darmstadt, Germany)

Urea (VWR International, Darmstadt, Germany)

Potassium iodide (Laborchemie Apolda, D)

Magnesium sulfate (Fluka Chemie GmbH, Buchs, CH)

Malt extract R2 (Bernd Euler biotechnology Microbiology, Frankfurt, Germany)

Sea salt topSea Aqua (Aqua-Concept, Ilmenau, Germany)

Sodium EDTA (Fluka Chemie GmbH, Buchs, CH)

Zinc sulfate (VWR International, Darmstadt, Germany)

4.1.7 Media composition:

PDA medium (modified from Atlas, 2004):

Potatoes	300.0 g
Glucose	20.0 g
Agar	15.0 g
Seawater	1000 ml
(ca. 30‰ salinity)	

Hagem medium (Helmholz *et al*., 1999)**:**

Ammonium succinate	0.5g
KH_2PO_4	0.5g
$MgSO_4 \times 7 H_2O$	0.5g
$FeCl_3$ solution (1%)	0.5ml
Glucose	5.0g
Malt extract	5.0g
Water	1000ml

For the production of solid media 1.5% agar was added. For the production of sea water, 5.0 g, 20.0 g and 35.0 g of sea salt dissolved in 1000 ml Demineralised water. The medium was adjusted to pH 7.5 and then sterilized in an autoclave.

4.1.8 Preparation and maintenance of stock cultures:

The fungal strains are stored and maintained in glass tubes at -80°C maintained by Pharmaceutical Biology department staff.

4.1.9 Primary culture:

All steps were performed under aseptic conditions. The cultivation took place at room temperature (22 ± 2 ° C) with natural day-night rhythm.

For the first pre-cultivation, the fungal strains were inoculated on Petri dishes with containing Hagem medium (see 4.1.7) + 1.5% Agar extract and incubated at room temperature for four weeks.

Figure 3: Strain 165 Pseudohalonectria in Hagem agar medium

The main cultivation was made by inoculating medium sized mycelium (1-2cm pieces punched) containing well grown colonies taken from the first pre cultivation on to 50 ml liquid Hagem medium (see 4.1.7) in small Erlenmeyer flasks and incubated at room temperature for another 4 weeks.

Figure 4 : *Pseudohalonectria lignicola* main cultivation in 50ml Hagem medium

4.1.10 Large scale cultivation of the fungal strain:

All steps were performed under aseptic conditions. The cultivation took place at room temperature (22 ± 2 ° C) with natural day-night rhythm.

To obtain larger amounts of fungal mycelia, the second pre cultivation whole culture was transferred into a suitable glass container and the volume was made to be 200ml with liquid Hagem medium pH 7.5 (see 4.1.7) and homogenized for two minutes using a homogenizer. Then the homogenized and evenly mixed culture was distributed as 20ml aliquots into 10 Erlenmeyer flasks. Each 20 ml culture was diluted to 200ml with liquid Hagem medium pH 7.5 (Refer 4.1.7). Growth took place in a shaking 125 rev / min for two to four weeks, depending on the growth rate of the individual each fungal strain.

Figure 5: *Pseudohalonectria lignicola* large cultivation

The culture was finally harvested after 4 or 6 weeks depending on the growth.

4.1.11 Harvesting the fungal biomass:

After the incubation period, all the 10 Erlenmeyer flasks were removed from the shaker and filtered through muslin cloth filter in order to separate the mycelia from the medium.

Figure 6: Fungal biomass harvesting by filtration method

All the collected biomass called mycelia were pooled in a glass container and lyophilized until it was dried completely. The entire collected medium were pooled and measured to be approx. 1.5 liter. This volume was divided into a 2 x 1000ml round bottom flask to concentrate under vacuum. This whole large scale cultivation process was repeated for 10 batches each containing 10 flasks of 200ml culture. In total, around 15 liter culture medium was concentrated.

4.1.12 Extraction:

4.1.13 Equipment and chemicals:

Vacuum rotary evaporator system B 178 R 114 Rota vapor with water and B 480 (Büchi Labortechnik AG, Flawil, CH)

HS 250 basic shaker (IKA Labortechnik, Staufen, Germany)

Freeze Dryer Alpha (Christ Freeze GmbH, Osterode, Germany)

Universal Hood (Moulinex GmbH, Solingen, Germany)

Soxhlet Thimbles (Schleicher & Schuell, Dassel, Germany)

n-hexane, MeOH, ethyl acetate (all VWR International, Darmstadt, Germany)

Sodium sulfate, anhydrous (VWR International, Darmstadt, Germany)

Quartz sand (VWR International, Darmstadt, Germany)

All organic solvents were distilled before use.

4.1.14 Extraction of secondary metabolites:

Each 1.5 liter culture medium of 10 batches were concentrated to approx. 250ml and then extracted against 500ml distilled Ethyl acetate in a shaker for 24hours. This liquid-liquid extraction process was repeated for 3 times to extract completely all the secondary metabolites ranging from polar to non polar compounds secreted into the medium. All the combined organic extracts collected from 10batches were measured ranging from 1.0 to 1.3 grams in case all the three fungus.

Figure 7: Liquid-liquid extraction of fungal culture medium against ethyl acetate

The mycelium from all the 10 batches was freeze-dried and the weights noted as approx. 65.0 grams to 110.0 grams from all the three fungus. Then the mycelia was divided into two equal parts and transferred into two extraction thimble. The extraction was carried out on a Soxhlet apparatus gradually with solvents of increasing polarity in the order n-hexane, MeOH and H_2O each for 24 hrs. The extracts were concentrated in a vacuum rotary evaporator to dryness or freeze-dried.

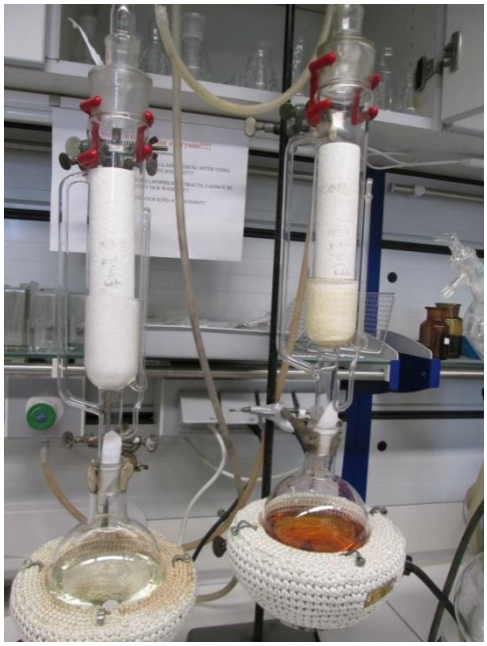


Figure 8: Soxhlet extraction against n-hexane (left) and EtOAc (right) extraction

The general flow chart of extraction is as follows:

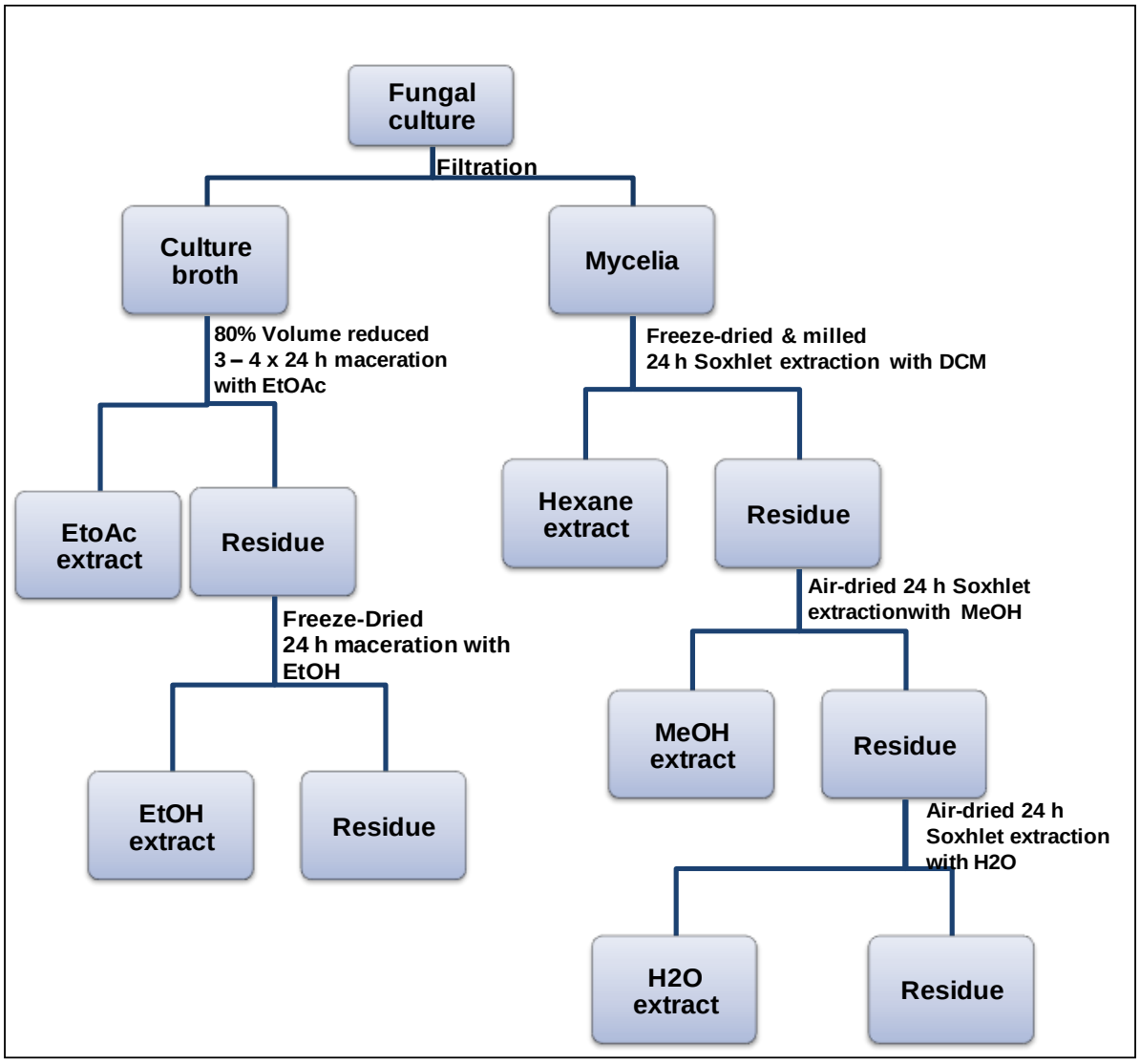


Figure 9: Work flow chart of extraction of fungal strains

4.1.15 Biological screening tests:

4.1.15.1 Antimicrobial activity screening:

The agar diffusion assay was performed according to Kirby-Bauer method as per European Pharmacopoeia. Fish pathogenic bacteria were cultivated on TSA medium

(BD-Difco™). Other bacteria and yeasts were cultivated on NA II (Nutrient Agar) medium (Merck).

For screening, aliquots of the test solution were applied into a sterile filter-paper discs (6 mm diameter) to give a final disc loading concentration of 1 mg or 2 mg for crude extracts and various concentrations (300, 100, 50 and 25 µg/disc) for pure compounds. As positive controls, Ampicillin (Merck) was used at concentration of 10 µg/disc (against *S. aureus* and *B. subtilis*), 50µg/disc (*E. coli*); Gentamicin (Biochrom) at 25 µg/disc; Oxytetracycline (Merck) at 30 µg/disc and Nystatin-dihydrat (Roth) was used at concentrations of 50 and 100 µg/disc. As a blank, solvent which was chosen to dissolve the test samples was loaded in a separate disc. The entire test including positive control and blank was prepared in duplicate to have reliable results. Agar plates were freshly prepared with suspension of loop full of test organism mixed with 20ml of hand warm liquid NA II (Nutrient Agar) medium, applied on each Petri plate. The impregnated discs were completely dried under sterile condition, placed on agar plates previously seeded with the selected test organisms.

The plates were incubated for 18-20 h at 37°C and 26°C for human and fish pathogenic bacteria, respectively. The antimicrobial activity was recorded as the clear zone of inhibition surrounding the disc at which the diameter was measured in mm. In order to improve the contrast of the inhibition zone, the plate was sprayed with INT (p-iodonitrotetrazolium chloride, SIGMA).

Crude extracts, fractions and pure compounds were tested for activity against the following standard strains:

Gram-positive bacteria:
Bacillus subtilis ATCC 6051
Staphylococcus aureus ATCC 6538
Gram-negative bacteria:
Escherichia coli ATCC 11229
Pseudomonas aeruginosa ATCC 22853

Vibrio anguillarum DSMZ 11323*

Aeromonas hydrophilla DSM6173*

Pseudomonas anguilliseptica DSM12111*

*Fish pathogenic bacteria

Yeasts:

Candida maltosa SBUG 700

4.1.15.2 Bioautography Screening:

Bioautography can be considered as the most efficient assay for the detection of antimicrobial compounds in a complex mixture. The method allows the localization of antimicrobial activity on a chromatogram (Hamburger & Cordell, 1987; Rios *et al.,* 1988). In this study agar overlay or immersion bioautography was used. Amount of 500 µg of extract or 250 µg of fraction was applied on analytical TLC plate (AluO Si gel 60 F_{254}, 0.2 mm, Merck) and subsequently developed using appropriate solvent system. The TLC plates were dried properly and then put into the sterile Petri dish which was previously poured with 10 ml of agar medium as basic layer. Afterwards 20 ml of seeded agar medium (as described in 4.7 1) was applied onto the TLC plate. *B. subtilis, S. aureus* were used as test organisms.

The plates were incubated for 18-20 h at 37 ^{o}C for *B. subtilis* and *S. aureus*, while *V. anguillarum* at 26 ^{o}C. The active bands appeared as clear zones. INT was used to assist the contrast between the active bands and the background.

4.1.15.3 Cytotoxic activity screening:

Cytotoxic activity was determined by MTT Assay as per the in house protocol with the cultivated human bladder carcinoma cell line 5637 (ATCC HTB-9) and HaCaT cell lines used for the assay.

Materials used in the present study are listed as follows.

RPMI-Medium with 8 % FCS (HaCaT) or 10% FCS (5637 cell line);

1%P/S MTT Solution

Dilution of test substance (Extracts): 500 µg/ml (end concentration in the well), dilute1:2 ; possible; dilute with medium (8 dilutions)

Positive control: Etoposide

Solvent control MeOH

Seeding (96 well plate)

5637 cell line: 5000 cells per well

HaCaT cell line: 8000 cells per well

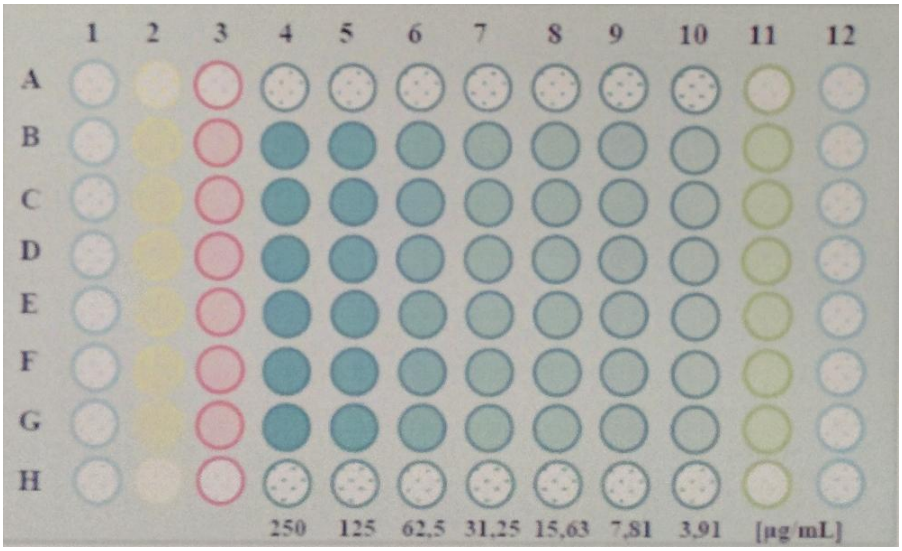


Figure 10 : Schematic representation of MTT Assay

No cells sown

Cell + Etoposid

Cell + Medium

Cell + crude extract

Cell + Methanol (blank)

Method:

- 24 h after seeding done, remove the medium, rinse with 100µl HBSS
- Add fresh medium/Extract/Solvent/Positive control
- Incubate at 37°c for 72 hours

- Wash the cells with 200µl of HBSS
- Add 100µl fresh medium with MTT in each well
- Incubate at 37°c for 3 hours
- Remove the supernatant carefully
- Add 200µl DMSO
- Vortex for 30mins at room temperature
- Measure OD at 550nm
- Calculate the cell death against positive control

4.1.15.4 Phytotoxicity test

A simple leaf puncture bioassay was performed to determine the phytotoxic activity of the fractions and of pure compounds. Since the toxicity of the culture filtrate was not host specific, three types of randomly selected leaves were used for that purpose. The test fractions and pure compounds were first dissolved in a small amount of methanol and then diluted to the desired final concentration (2 mg/ml) with sterile distilled water (3% final methanol concentration). Droplets (20 µl) of assay solutions were applied on punctured leaflets of detached leaves. One repeats consisted of 3 leaves each with one inoculation points. The inoculated leaves were incubated in a moist chamber under fluorescent light and were observed every day for symptoms. The final evaluation was recorded 3 days after application (Türkkan et al. 2011) (Evidente et al. 2011) .

4.1.16 Chromatography:

4.1.16.1 Thin layer chromatography (TLC):

TLC is to monitor the identity of each extracts and fractions, additionally to screen the qualitative purity of the isolated compound. TLC method was developed to optimize the solvent system that would be applied for column chromatography. Various semi polar solvent systems were tried.

Stationary phase:

Silica gel 60 F254DC-aluminum plates (Merck, Darmstadt, Germany)

RP-18 F254 S DC-aluminum plates (Merck, Darmstadt, Germany)

Mobile phases: Separation EtOAc crude extracts *P. lignicola*/MM010/MM018

Mobile phase 1: DCM / EtOAc / MeOH (50:50:20)

Mobile phase 2: EtOAc/Acetone/MeOH (50:50:20)

Mobile phase 3: EtOAc/MeOH (65:35)

Mobile phase 4: DCM/MeOH (65:35)

Mobile phase 5: n-hexane/EtOAc/MeOH (50:50:20)

Mobile phase 6: n-hexane/EtOAc/Acetone (50:50:10)

Mobile phase 7: n-hexane/EtOAc (50:50)

Mobile phase 8: n-hexane/EtOAc/MeOH (60:40:10)

Mobile phase 9: n-hexane/EtOAc/MeOH (40:60:10)

Mobile phase 10: n-hexane/EtOAc/MeOH (40:60:1)

Mobile Phase 11: n-hexane/EtOAc/MeOH (35:65:10)

Detection: **Detection of separated compounds by**

Daylight

UV light 254 nm and 366 nm

Vanillin sulfate reagent R, heat to 105 ° C

4.1.16.2 Glass column chromatography:

Glass column chromatography was carried out with the finalized TLC method using Silica gel 60 F254 with medium particle size 0.040 mm - 0.063 mm (Merck) was weighed around 100g and dissolved in mobile phase 11: Hexane/EtOAc/methanol (35:65:10) to become as a slurry. Appropriate sized column was chosen rinsed with mobile phase once and then filled with the slurry prepared already and allowed it to stand for 30minutes to settle. After equilibrated for two column volume with the mobile phase, the sample dissolved in 1ml of the same mobile phase was applied.

The elution from each column chromatographic separation was collected with a fraction collector (Fraction Collector 2110, Bio-Rad Laboratories, Richmond, USA). The Fraction size was 3 ml/15 min. Every fraction was assessed using TLC with mobile phase 11. Fractions having the same band distribution became larger Fractions combined.

4.1.16.3 High pressure liquid chromatography (HPLC):

Analytical HPLC was used to identify the interesting peaks from extracts and fractions as well as to evaluate the purity of isolated compounds. The gradient used started with 25:75 (MeOH : H2O) to 100% MeOH in 55 min. Peaks were detected by UV-Vis diode array detector and finally the column was by increasing the proportion of water to the starting concentration of the next run conditioned.

Analytical HPLC Instrumentation:

Make: Shimadzu

Binary pump LC20AD

Degasser DGU-20A3

Communication bus model CBM-20A

Diode Array detector SPD-m20A

Auto sampler SIL-10AF

Column oven CTO-10AS

Valve unit FCV-20AH2

Semi preparative HPLC:

Kontron HPLC UV diode array detector 432 (Kontron Instruments)

Sample injection valve (Rheodyne, Cotati, C.A., USA)

Sample loop 50µl.

LC-10AT VP Shimadzu

Shimadzu system controller SCL-10A VP

Shimadzu quaternary pump SCL-10AT

Detector diode array detector SPD-M10A

Sample loop 50µl

Semi-preparative HPLC was used for the isolation of compounds present in fractions previously separated using column chromatography. The mobile phase gradient was started with 35% methanol reached 100% in 15 mins, after 3 mins down to 35% in 1 min and 35% isocratic for 9 mins. Each injection was about 100 µl of crude extract dissolved in 1 ml of the mobile phase. The eluted peaks which were detected by the online UV diode array detector were collected separately in round-bottom flasks.

Semi preparative HPLC was performed on either a Kontron Instruments or Shimadzu Instruments and the columns were used either:

A: Analytical ACE column RP-C18 100 Å (250 × 4.6 mm)

B: Analytical Phenomenex Fusion RP- C-18 100 Å (250 ×4.6 mm)

C: Semi preparative Phenomenex Fusion RP- C-18 (250 ×4.6 mm)

4.1.17 Spectroscopy and Spectrometry:

4.1.17.1 Mass spectrometry (MS):

ESI Mass Spectra:

All the crude extracts and fractions and pure compounds were analyzed for molecular mass identification using below instrumentation.

System: Shimadzu analytical HPLC attached with Shimadzu LCMS -8030 triple quad.

Stationary phase: Phenomenex Fusion RP 4.6 x 150 mm, 5.0 micron

Mobile phase gradient: MeOH / H2O

Flow rate: 0.5 ml / min

LC detection: DAD at 216 nm, 226nm, 254nm and 280nm.

MS detection: Shimadzu8030 series; ESI Positive/negative mode, range 50-1000 amu, scan Mode.

4.1.17.2 Gas chromatography - mass spectrometry (GC-MS):

Samples were lyophilized to complete dryness at 0.5 mbar and −52°C prior to analysis. GC-MS analysis was performed by derivatization of all the samples with MeOX for 90 min at 37 C and with MSTFA for 30 min at 37°C. Chemical compounds including sugars, amino acids and fatty acids were identified in GC-MS analysis. Ribitol was used as an internal standard.

4.1.17.3 TLC-MALDI (Thin layer chromatography - Matrix assisted laser desorption ionization):

All the interesting spots from the optimized thin layer chromatography (TLC) were marked cautiously under UV long and short wavelengths, cut pieces were placed in individual eppendorf tubes containing 50µl of 60% acetonitrile: water mixture, sonicated at short intervals for 5 times, centrifuged at maximum rpm for 10 minutes. 1µl of the supernatant was pipetted, mixed with 1µl of saturated solution of DHB (Di hydroxy benzoic acid), placed carefully at the MALDI plate and left for air drying. The spots were located manually by using the MALDI software in order to analyze the molecular mass pattern of the selected spots. Measurements were carried out using AB SCIEX 4800 MALDI-TOF system.

4.1.17.4 Nuclear magnetic resonance (NMR) spectroscopy:

All the NMR spectrum of purified compounds was recorded at the University, Greifswald, Institute of Chemistry and Biochemistry.

Spectrometer: Bruker Avance spectrometer

Resonant frequencies: 1H: 600 MHz, 13C: 150 MHz

Solvent: deuterated methanol (CD3OD)

4.2 PART II

4.2.1 Mass cultivation

Cultivation condition:

Both *V. eiseniae and V. tuberculata* were cultivated in R2A broth medium under aerobic conditions at 80 rpm and 25°C. Growth was monitored by measuring the optical density (OD) at 600 nm. Different parallel cultures were established in order to obtain the samples for the different analysis. These were all highly reproducible and the determined specific values (OD and time) of one of these growth curves were considered as standard and all the other data was normalized according to the measured OD values in all experiments, four parallel cultures were grown, representing four biological replicates.

R2A Medium (per liter):

Protease peptone, 0.5g
Yeast extract, 0.5g
Casamino acids, 0.5g
Glucose, 0.5g
Soluble starch, 0.5g
Sodium pyruvate, 0.3g
K_2HPO_4, 0.3g
$MgSO_4$ $7H_2O$, 0.3g
pH adjusted to 7 with 1M HCl autoclaved.

4.2.2 Cell harvesting or sampling:

To examine growth phase-specific changes in global metabolite profiles, cells were harvested at ¼ OD_{max}, ½ OD_{max}, ¾ OD_{max}, OD_{max} as well as stationary phase as follows:

4.2.3 Intracellular metabolites

4.2.3.1 Sampling

Sampling of cells was done at temperatures below 4°C. An aliquot of 10 ml of culture broth was harvested in duplicate from all four biological replicates at each time point (t0 to t6) along with a blank medium sample as negative control and centrifuged immediately at 25.000g and 4°C for 10 min and washed three times in cold 0.9% NaCl solution. The washing solution was centrifuged at 20.000g and 4°C for 5 min to remove any medium contaminants in the pellet. The resulting pellets were frozen in liquid nitrogen and later stored at -80°C.

4.2.3.2 Extraction

The extraction of intracellular metabolites was performed adapted to a procedure described by van Gulik et al (Gulik et al. 2012). The cell pellets were thawed and 200µl of internal standard mixture (norvaline, dimethylphenylalanine, ribitol and chlorphenylalanine hydroxide) were added before extraction. 5 ml of boiling ethanol solution (75% v/v) was transferred immediately to the tubes containing cell pellets and resuspended by vortexing. To enhance better cell resuspension, ultra sound sonication was applied. Afterwards the cells in the hot ethanol solution were kept at 95°C for 3 min. This procedure effectively releases all metabolites from the cells and, at the same time, results in denaturation of the enzymes present, which prevents further conversion of metabolites in the samples. The extract was then transferred into a clean tube and store at -20°C for 2 min. The ethanol/water extract was evaporated in vacuum concentrator at 30°C.

The dried sediment was resuspended in 500µl of Milli Q water by vortexing and transferred to a tube, centrifuged at 15.000g for 5 min at 4°C. The supernatant was transferred to glass sample vials and stored at -80°C until lyophilization for further anaylsis.

4.2.3.3 Metabolite analysis

Intracellular metabolites were analyzed by GC-MS according to (Meyer et al. 2014). Samples were lyophilized to complete dryness at 0.5 mbar and −52°C prior to analysis. GC-MS analysis was performed by derivatization of all the samples with MeOX for 90 min at 37°C and with MSTFA for 30 min at 37°C. Intracellular metabolites including sugars, amino acids and fatty acids were identified in GC-MS analysis. Ribitol was used as an internal standard.

4.2.4 Extracellular metabolites

4.2.4.1 Sampling

Sampling was performed according to previously described procedures (Dörries & Lalk 2013). Shortly extracellular metabolite samples were sterile filtered (0.2 µm filter; in duplicate). A culture broth sample was taken right after inoculation (t0). More samples were taken at defined OD values starting from log phase to stationary phase. Samples were stored at -80°C.

4.2.4.2 Sample preparation for NMR analysis

Samples for the analysis of the extracellular metabolom were prepared as described previously (Dörries & Lalk 2013). To analyze the extracellular metabolites using ^{1}H-NMR spectroscopy the filtered samples were thawed. 400 µl of sample solution and 200 µl of TSP (trimethylsilylpropionic acid-d_4)-buffer consisting of 0.2 M NaH_2PO_4 / Na_2HPO_4 (pH 7) dissolved in H2O/D2O (50:50) were combined. Samples were pipetted into NMR-tubes and mixed thoroughly by turning the tube over repeatedly.

4.2.4.3 Equipment, software and chemicals

Bruker AVANCE-II 600 NMR Spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany)

Freezer (302.798-4 Privileg, Fürth, Germany)

Fridge (LABEX 282, Kirsch, Germany)

NMR-tubes (length: 178 mm, ø 5 mm) NorellTM, USA

Pipettes (Eppendorf AG, Hamburg, Germany)

Precision balance (Omnilab, Bremen, Germany)

Special accuracy balance (Sartorius, Göttingen, Germany)

Water preparation system (Merck Millipore, Darmstadt, Germany)

AMIX® Viewer Version 3.9.12

Bruker Biospin GmbH, Rheinstetten, Germany

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5 RESULTS AND DISCUSSION

5.1 PART - I

5.1.1 Taxonomy of fungal strains

Strain 165, MM010 and MM018 all the fungal strains were first taken for dissection microscopy for morphological identification. Unfortunately, there were no spores or reproductive structure was identified rather found only extensively branching mycelia. DNA sequencing was the next option. Cultivation and DNA extraction of all the three strains had been performed as per the method described in materials and methods 4.4 and 4.4.1 and submitted for DNA sequencing analysis. And the resulted DNA sequences were blasted against NCBI database. The maximum score results from the NCBI nucleotide blast suggested that, Marine derived fungal strain 165 DNA sequences matching to the genus Pseudohalonectria belongs to Magnaporthaceae family and having close affinity to the type *Pseudohalonectria lignicola.*

Sponge associated marine fungal strain isolates MM10 and MM18 positioned within the fungal order Pleosporales. More specifically, those two isolates are closely related to each other, and belong to the large and heterogenous Phoma species complex. In brief, the genus Phoma and allied genera are poorly understood, validated species concepts are widely missing, which makes species identification almost impossible. The phylogenic tree has been attached for all the three isolates in Figure 11 and 12.

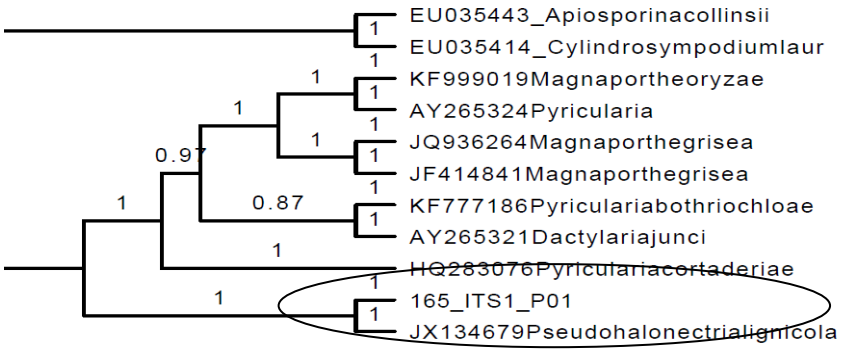


Figure 11: Position of Strain 165 in the phylogenic tree

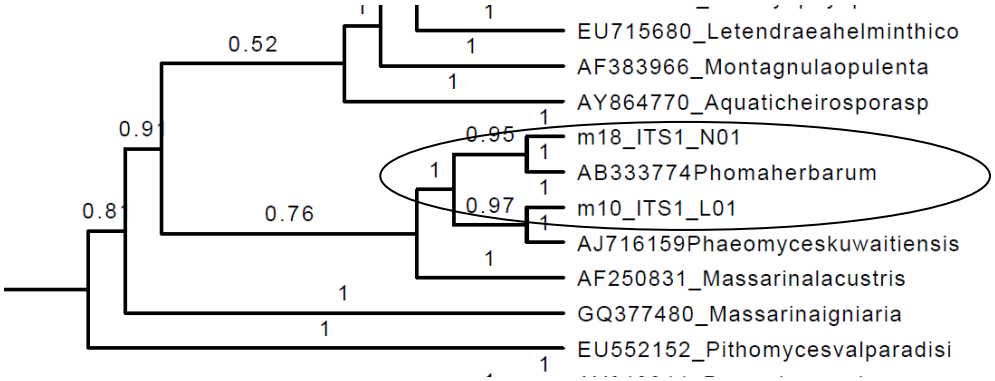


Figure 12: Position of closely similar strain MF010 and strain MF018

5.1.2 Biomass and crude extracts obtained from fungal cultivation

During continuous cultivation of all three strains, the dry weight of mycelium and the recovered amount EtOAc extract from the culture medium was determined for each batch. At irregular intervals, the antimicrobial activity of both Medium and the mycelium extract was also checked.

5.1.3 Free living fungal strain 165 : *Pseudohalonectria lignicola*

Ten batch cultures brought around 15 liters of medium, from which around 65.0g of mycelium (dried biomass) and 1.0 g of crude extract with EtOAc extraction were obtained and taken for further experiments.

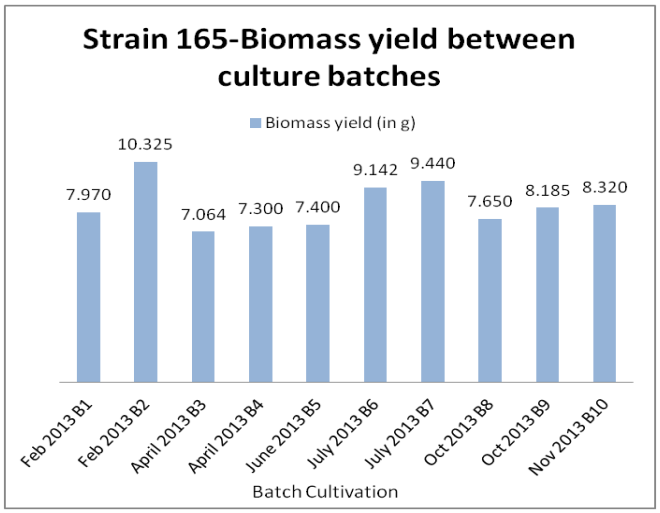


Figure 13: Strain 165 biomass yield between culture batches

The amounts of the obtained EtOAc extract had a bit of fluctuations. This ranged from 199.4 mg to 234.0 mg per every two batch cultivated (2 batches x 5 times) at once. To enhance comparability the yields based shown to one liter has been shown in Figure13 and 14.

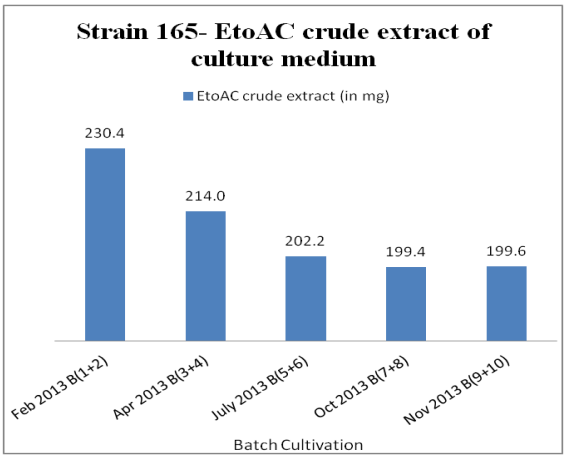


Figure 14: Strain 165 EtOAc extraction of culture medium of 10 batch cultures

As mentioned earlier the 65.0g of mycelium obtained from all the 10 batches of strain 165 cultivation was also adopted for further extraction procedures with n-hexane, methanol and water (sometimes) respectively. The water extraction was made not only for aqueous compounds but also to clear the inorganic salts present. Apparently ethyl acetate extraction of mycelium was also included after n-hexane extraction and before methanol extraction in order to check the presence of similar compounds identified from ethyl acetate crude extract of medium. The crude extracts quantities from mycelium were found to be 1.34g (n-hexane), 0.745g (ethyl acetate) and 10.2g (methanol).

5.1.4 Sponge associated strain MM010:

Total 10 individual batches made approximately in total 17 liters of culture medium to obtain around 1.0 g of ethyl acetate crude extract as well as 115.0g of mycelium (dried biomass). There was variation in crude extract quantities from batch to batch ranging from 80.0 mg to 130.0mg. To enhance comparability the yields based shown to one liter has been shown in Figure 15 and 16

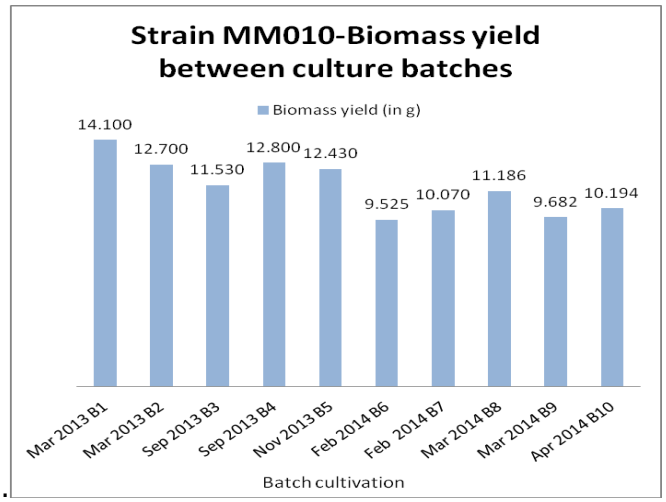


Figure 15: MM010 biomass yield between 10 batch cultures

As described in the materials and methods 4.6.2 earlier, the mycelium obtained from all the 10 batches of strain MM010 cultivation was around 115.0g also adopted for further extraction procedures with n-hexane, methanol and water (sometimes) respectively. Again, ethyl acetate extraction of mycelium was also included after n-hexane extraction and before methanol extraction in order to check the presence of similar compounds identified from ethyl acetate crude extract of medium. The crude extracts quantities from mycelium were found to be 0.876g (n-hexane), 0.5083g (ethyl acetate) and 8.281g (methanol).

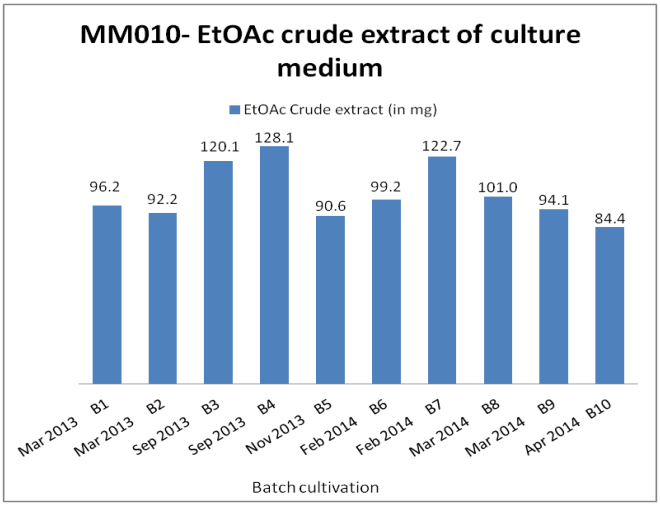


Figure 16: MM010 EtOAc extraction of culture medium 10 batch cultures

5.1.5 Sponge associated strain MM018:

Total 10 batch runs obtained roughly 18 liters of culture medium to have 0.85g of ethyl acetate crude extract as well as 114.0g of mycelium (dried biomass) for further experiments. Unlike previously described fungus, MM018 came out with a greater fluctuation in the amount of crude extract between batches ranging from 14.0mg to 150mg. To enhance comparability the yields have been shown in Figure 17 and 18.

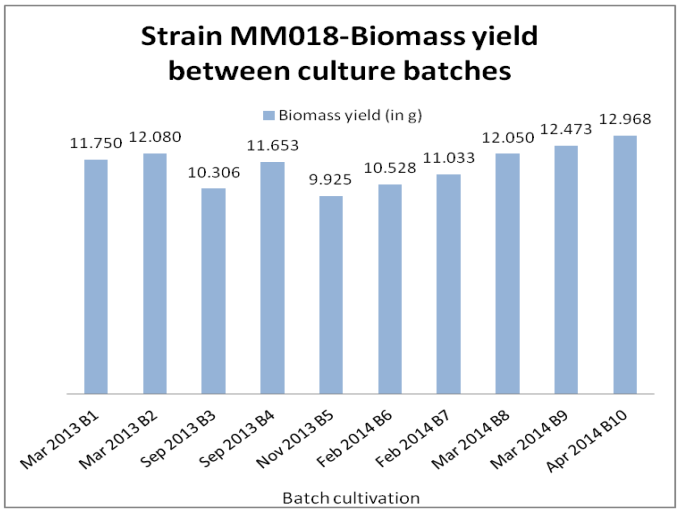


Figure 17: Strain mm018 biomass yield between 10 batches cultures

As described in the materials and methods 4.6.2 earlier, the mycelium obtained from all the 10 batches of strain MM018 cultivation was around 114.0g also adopted for further extraction procedures with n-hexane, methanol and water (sometimes) respectively. Again, ethyl acetate extraction of mycelium was also included after n-hexane extraction and before methanol extraction in order to check the presence of similar compounds identified from ethyl acetate crude extract of medium. The crude extracts quantities from mycelium were found to be 0.653g (n-hexane), 1.284g (ethyl acetate) and 14.07g (methanol) which is almost double the amount of the closely related species MM010 mycelium ethyl acetate and methanol extract.

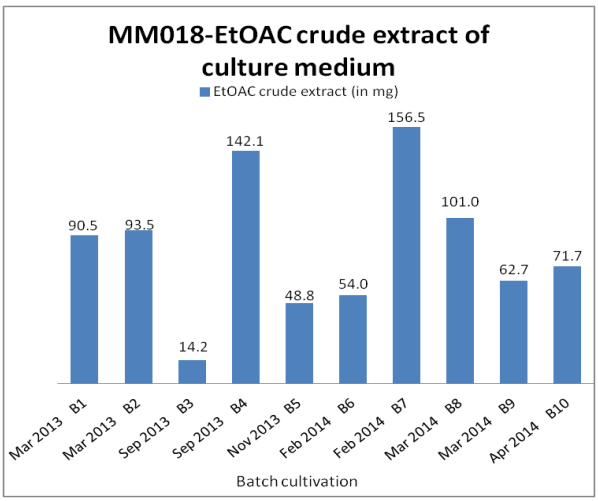


Figure 18: MM018 EtOAc extraction of culture medium

In case of overall comparison of especially ethyl acetate extracts of all the three strains both from culture medium as well as from the mycelium, almost each 15 to 18 liter culture medium approximately yields around 1g of semi polar compounds as ethyl acetate extracts.

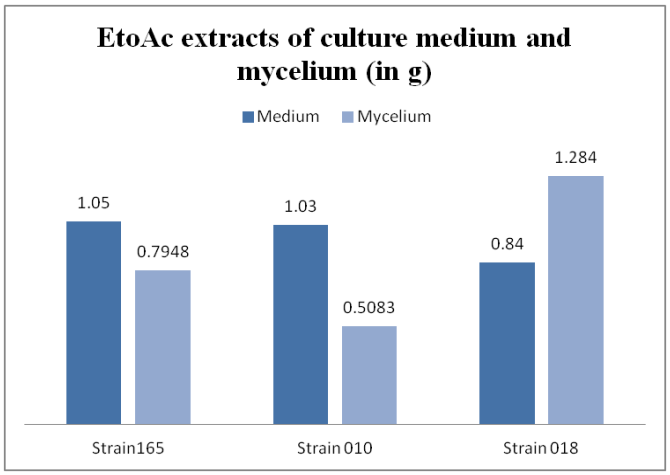


Figure 19: Compilation of EtOAc extraction of culture medium and mycelium (in g) of all three strains

Figure19 shows that the strain MM018 had yielded less quantity of ethyl acetate extract from medium compare to other two strains but the mycelium ethyl acetate extract of strain MM018 possessed the excess quantity of the same to compensate. Mycelium of

all the three strains was also extracted against n-hexane and methanol in order to obtain extremely non polar a well as extremely polar substances using hot soxhlet extraction method. Figure 20 illustrates the obtained quantities from n-hexane and methanol extraction of each strain. Comparatively, free living fungal strain 165 possess significant amount of non polar substance, in contrast sponge associated fungal strain MM018 possess comparatively high amount of polar substances and the ground reason may be or may not be correlated to the fungal habitat life style. As well as the high amount in either case cannot be considered for their bioactivity however, all the crude extract have been taken for bioactivity tests such as antimicrobial activity and cytotoxic activity. The bioactivity details have been further reported in the next sub titles.

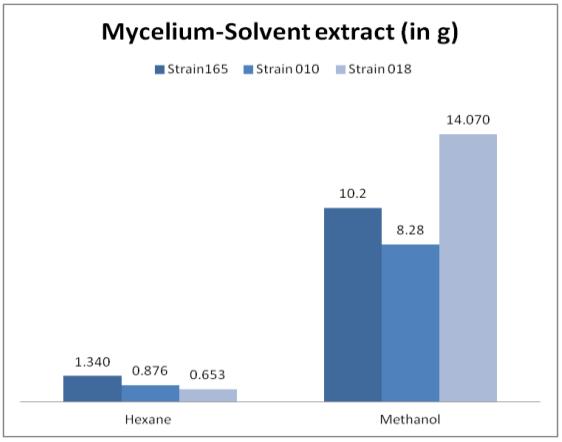


Figure 20: Compilation of n-hexane and methanol extraction of all three batches

5.1.6 Bioactivity of crude extracts

Crude extracts obtained from both culture medium and the mycelium was tested against human pathogenic bacterium as well as fish pathogenic bacterium. Antimicrobial test against human pathogenic bacterium is to find the natural product for human use. But, one may ask about the purpose of antimicrobial test against fish pathogenic bacterium.

In fact, all the three strains are marine derived candidates, it is also important as a part of the research to understand their potentials against pathogens in the marine environment. However, the crude extracts from the culture medium showed significant antimicrobial activity against test organisms compared to the crude extracts from

mycelium. Especially, ethyl acetate extract of culture medium of mostly all the three strains showed considerable activity while ethanol extract of the culture medium did not show any activity.

In case of mycelium, the extreme non polar compounds from n-hexane extraction or the extreme polar compounds extracted against methanol did not show any activity where as the ethyl acetate extract of mycelium showed again weak to moderate activity against most of the test organism (data not displayed). As the purpose of this thesis was to understand as such the bio activity of these marine habitats there was no special induction made with salinity, pH or co cultivation to alter the condition of the cultivation method in order to enhance the production of bioactive metabolites. Further subtitles describe the results of antimicrobial activity of all the three strains against each bacterium tested in this study.

5.1.7 Antimicrobial activity against Gram-positive bacteria

It has been a successful study in terms of antimicrobial test against gram-positive bacteria when compare to Gram-negative bacteria. For this study, all the crude extracts of culture medium as well as mycelium from all the three marine fungal candidates' strain 165, MF 010 and MF018 were tested against *Staphylococcus aureus* and *Bacillus subtilis* primarily. Figure 21 and 22 shows that the crude extracts of fungal culture medium of all the three strains compare to crude extracts of mycelium are significantly active against Gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis*. Noteworthy to be seen in the Figure21, ethyl acetate extract was the most active from strain 165 followed by MF010 and MF018 with IZ in the range of 18 to 21 mm in average and mycelium ethyl acetate extract of strain 165 which was moderately active.

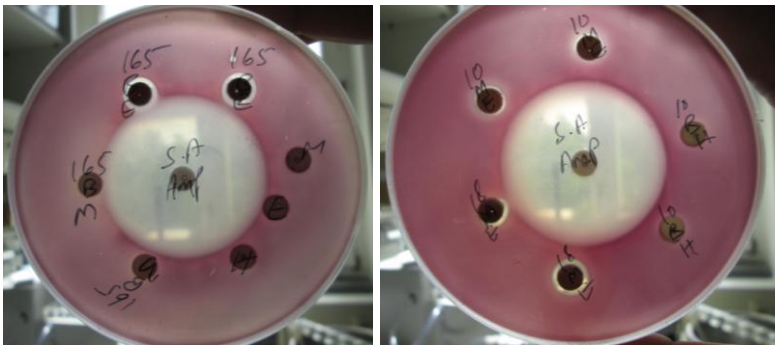


Figure 21: Inhibition zone of EtOAc extracts against Gram-positive bacteria *Staphylococcus aureus*. B-Biomass, BM-Biomass methanol extract, ME-Medium ethyl acetate extract, H-n-hexane extract, E-ethyl acetate extract

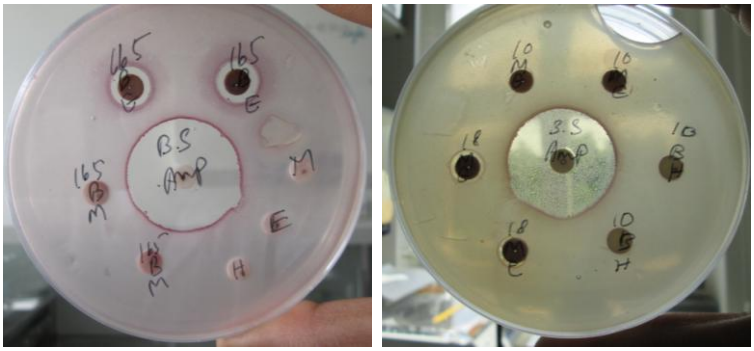


Figure 22: Inhibition zone of EtOAc extracts against *Bacillus subtilis*. B-Biomass, BM-Biomass methnol extract, ME-Medium ethyl acetate extract, H-n-hexane extract, E-ethyl acetate extract

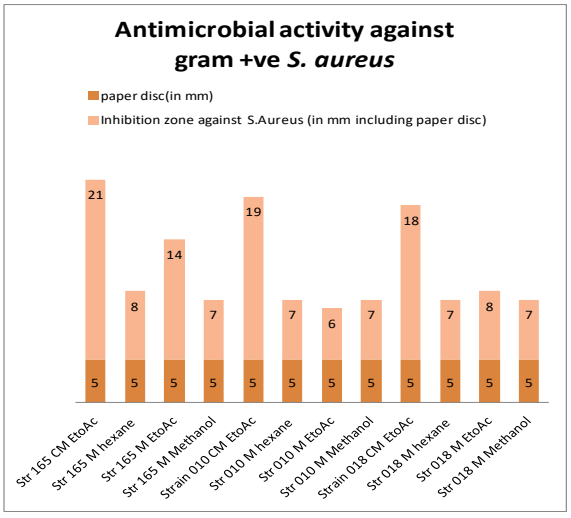


Figure 23: Antibacterial activity of EtOAc extracts against *S. aureus* (2 mg/disc)

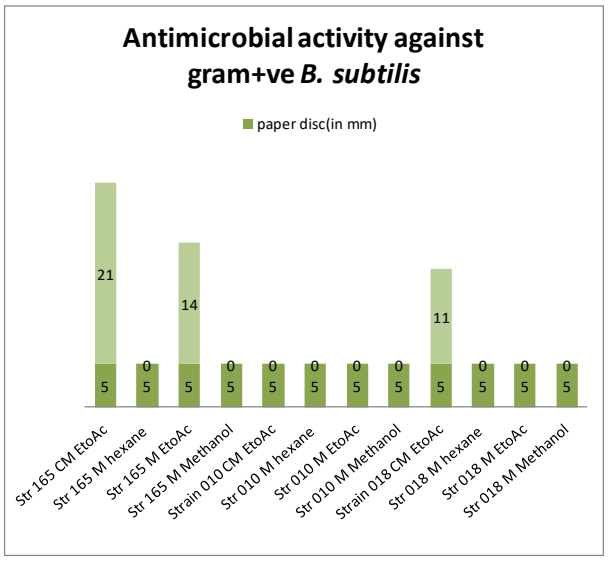


Figure 24: Antibacterial activity of EtOAc extracts against *B. subtilis* (2 mg/disc)

5.1.8 Antimicrobial activity against Gram-negative bacteria

The antimicrobial activity of ethyl acetate extracts of the culture medium of all three strains were performed against both Gram-negative human pathogenic and marine pathogenic bacteria.

Figure 25: Inhibition zone of EtOAc crude extracts against Gram-negative bacteria *Escherichia coli.* B-Biomass, BM-Biomass methnol extract, ME-Medium ethyl acetate extract, H-n-hexane extract, E-ethyl acetate extract

In case of antimicrobial activity against gram negative bacteria, Both MF strains against *Escherichia coli* totally differs from the bioactivity against *Pseudomonas aeruginosa* although both the test organisms were Gram-negative bacteria. However, strain 165 ethyl acetate extract showed no activity on both negative bacteria. Figure 25 Indicates strong bioactivity of both MF strains with IZ diameter in the range of 24 to 25 mm against *E. coli* followed by mycellium extract of ethyl acetate of strain MF018 with moderate activity with IZ diameter 13 mm. still, both MF strains shows no activity as that of strain 165 against *Pseudomonas aeruginosa* (data not shown).

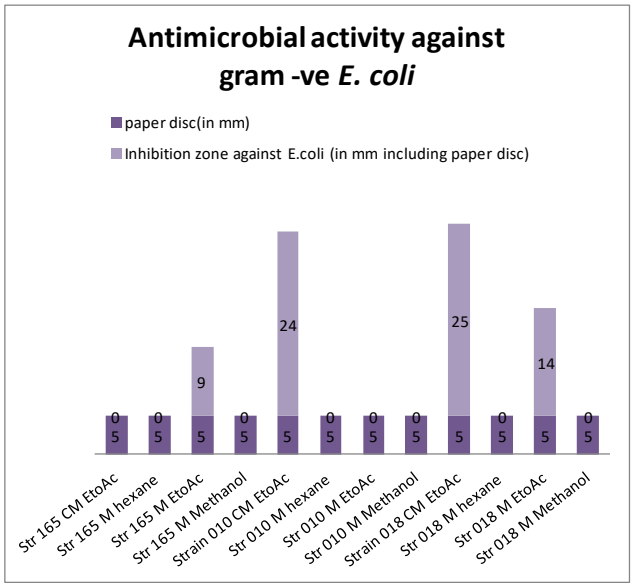


Figure 26: Antibacterial activity of EtOAc extracts against *E. coli* (2 Mg/Disc)

The ethyl acetate extracts of culture broth as well as extracts of mycelial biomass were additionally tested against Gram-negative marine pathogenic bacteria *A. hydrophilla*, *V. anguillarum* and *P. anguillaseptica* These three bacteria are well known pathogen for marine and freshwater fish. The antimicrobial activity against marine bacteria was excellently exhibited by both MF strains compare to their activity against human pathogenic bacteria.

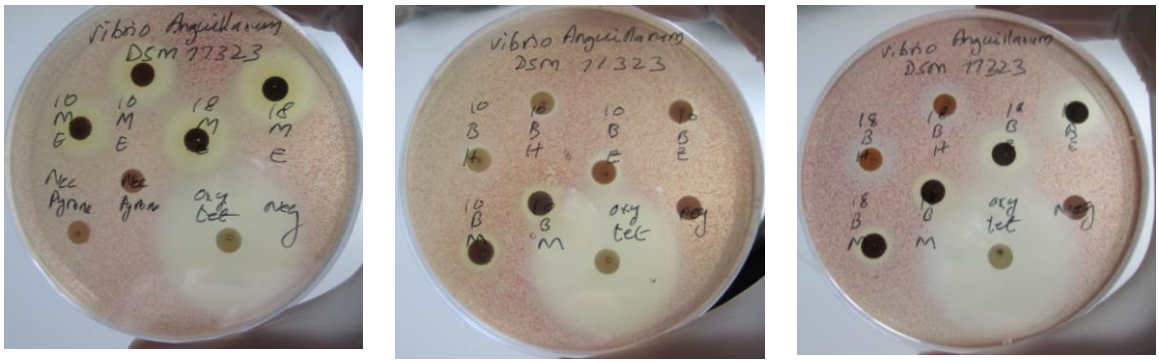


Figure 27: Inhibition zone of extracts against Gram-negative fish pathogenic bacterium *V. anguillarum*. B-Biomass, BM-Biomass methanol extract, ME-Medium ethyl acetate extract, H-n-hexane extract, E-Ethyl acetate extract.

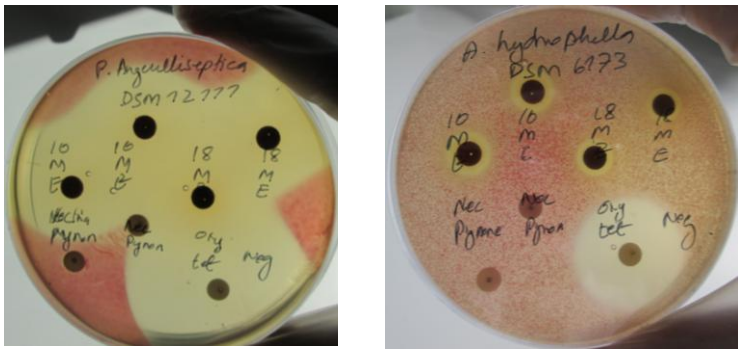


Figure 28: Inhibition zone of extracts against Gram-negative fish pathogenic bacterium *A. hydrophilla* and *P. anguillaseptica*. B-Biomass, BM-Biomass methnol extract, ME-Medium ethyl acetate extract, H-n-hexane extract, E-Ethyl acetate extract

As it is known from the Figure 27 and 28 ethyl acetate extracts of both MF strains are strongly active against *A. hydrophilla*, *V. anguillarum* and *P. anguillaseptica* where the ethyl acetate extract of the culture medium of strain 165 shows weak or no activity.

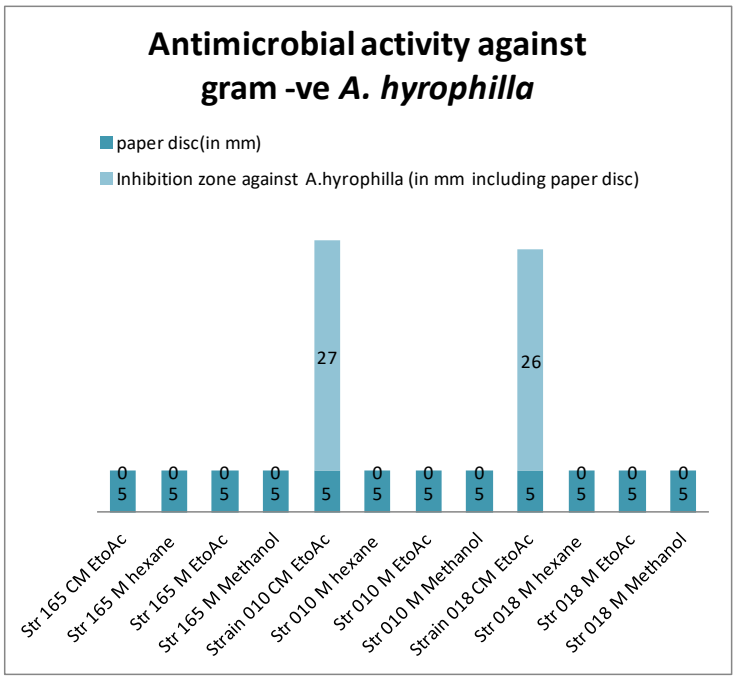


Figure 29: Antibacterial activity of EtOAc extracts against *A. hydrophill*a (2 Mg/Disc)

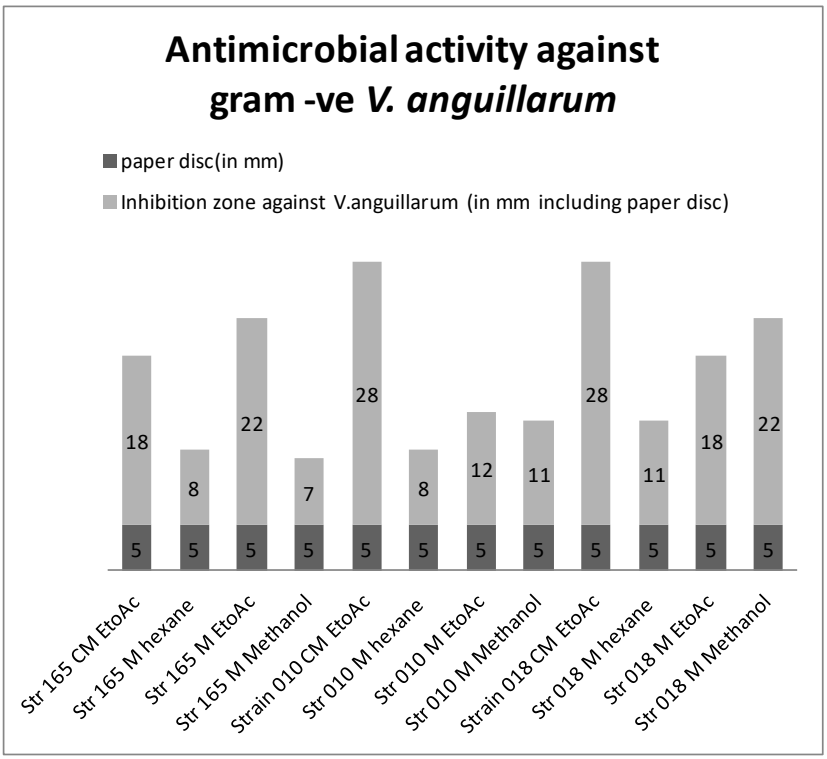


Figure 30: Antibacterial activity of EtOAc extracts against *V. anguillarum* (2 Mg/Disc)

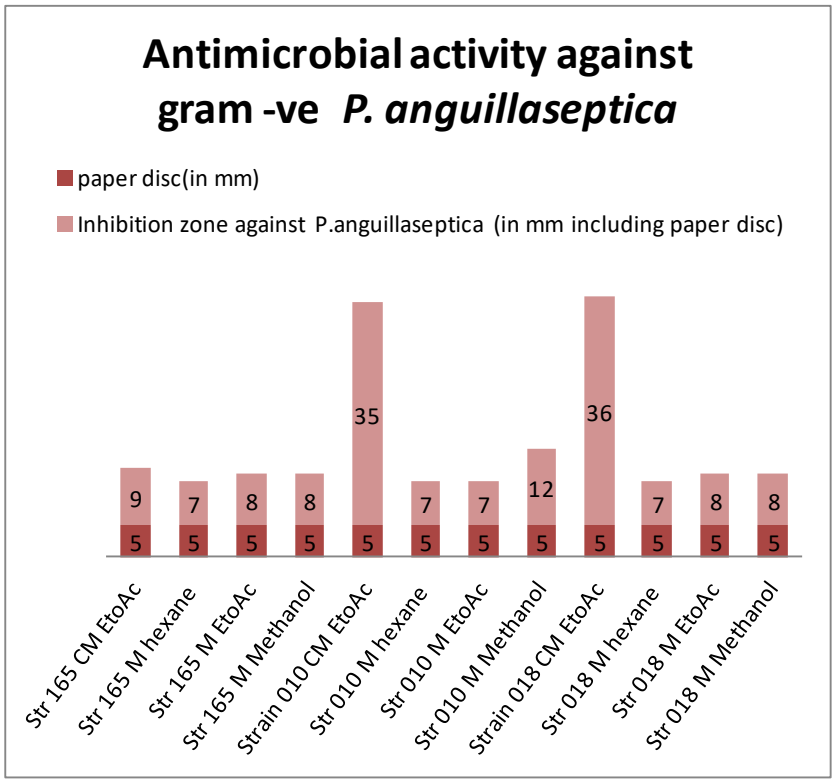


Figure 31: Antibacterial activity of EtOAc extracts against *P. anguillaseptica* (2 Mg/Disc)

5.1.9 Isolation of bioactive metabolites from free living marine fungi

Strain 165 was further investigated for active secondary metabolites as it exhibited a strong antimicrobial activity. Bioassay-guided fractionation method was followed to obtain targeted biologically active compounds. For the isolation of bioactive compounds, initially the active extract was fractioned as compound groups then the bioactive fraction was subjected to semi preparative HPLC in order to isolate the compounds which was again purified in the second attempt of semi preparative HPLC.

The flow chart of isolation process is shown in Figure 32

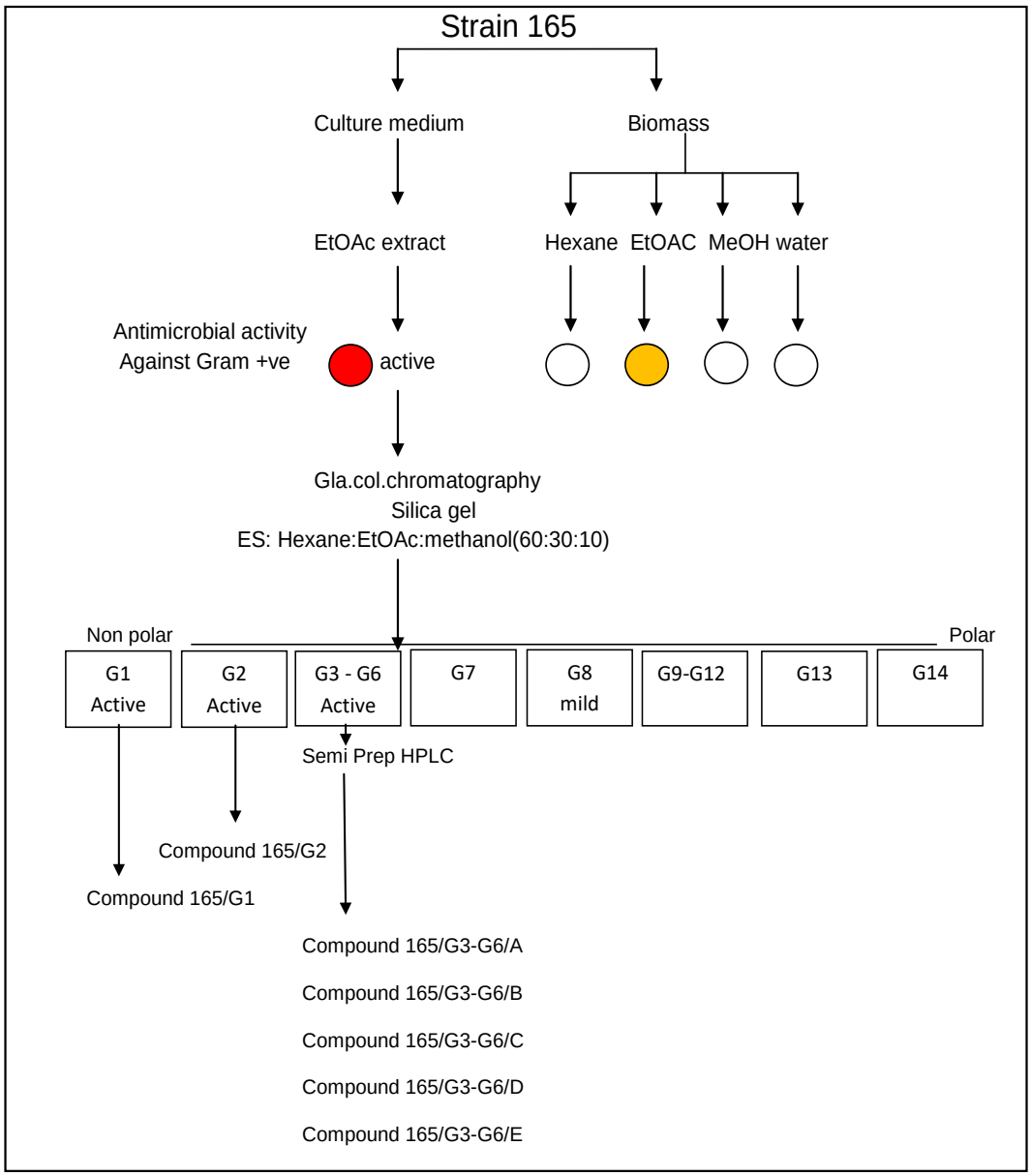


Figure 32: The flow chart of isolation process of strain 165

5.1.9.1 GC-MS Analysis of crude extracts

Crude extracts of strain 165 biomass and culture medium extracted against various solvents were dried and submitted for Gas Chromatography Mass spectroscopy

analysis (GC-MS). The results against NIST library afforded to confirm the presence below listed fatty acids, Ergosterol and its analogs considering match factor more than 700.

Table 4: List of fatty acids from strain 165 identified by GC-MS

Strain 165	NIST	Match
Biomass n-hexane extract	Palmitic acid, trimethylsilyl ester	902
	Linoleic acid trimethylsilyl esterl	800
	Oleic acid, trimethylsilyl ester	851
	Stearic acid, trimethylsilyl ester	849
	Dehydroergosterol 3,5-dinitrobenzoate	699
	22E)-3-[(Trimethylsilyl)oxy]ergosta-5,7,22-triene	839
	3-[(Trimethylsilyl)oxy]ergosta-8,24 (28)-diene	
	Dihydroisophorone	694
Biomass EtoAc extract	Glycerol, tris (trimethylsilyl) ether	888
	Butyl caprylate	600
	Palmitic acid, trimethylsilyl ester	827
	Oleic acid, trimethylsilyl ester	736
	Stearic acid, trimethylsilyl ester	798
Biomass methanol extract	α-D-Glucopyranoside, 1,3,4,6-tetrakis-O-(trimethylsilyl)-β-D-fructofuranosyl …..	746
	α-D-Glucopyranoside, 1,3,4,6-tetrakis-O-(trimethylsilyl)-β-D-fructofuranosyl 2,3,4,6-tetrakis-O-(tri….	837

5.1.9.2 TLC separation

As per the above flow chart, Thin layer chromatography was the beginning stage of Glass column chromatography where the stationary phase and the mobile phase are set for the separation of compounds. Ethyl acetate extracts of strain 165 showed a strong antimicrobial activity against Gram-positive human pathogenic bacteria. After many trial and errors, the TLC method was optimized with mobile phase11 containing ethyl acetate: n-Hexane: methanol (60:30:10) and silica gel as stationary phase. Figure 33 is the TLC outcome of this method and three major bands dominating at 366nm, 254nm

and the last one sprayed with Anisaldehyde solution and heated at 110°c. Still there are some hidden bands behind the main bands may contain biologically active compounds.

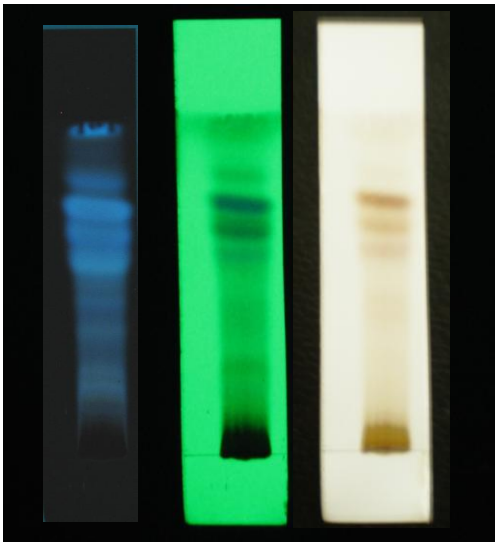


Figure 33: Thin layer chromatography result of strain 165 ethyl acetate crude extract against mobile phase11 containing ethyl acetate : n-hexane : methanol (60:30:10) and silica gel as stationary phase

Figure 34: Thin layer chromatography runs of all the five culture batches cultivated subsequently

Nevertheless, the extract used here was little bit older and the TLC was repeated with freshly cultivated batches along with old one for comparison. This time it was sprayed with vanillin sulfate reagent. Figure 34 shows many new uniform bands in all the freshly cultivated batch cultures with different intensities.

Additionally, the mobile phase components ratio was changed slightly with higher percentage of ethyl acetate in the solvent system to emphasis the non polar region. However, Figure 34 shows all the five batch cultures of strain 165 contains uniform compounds with varying in intensity. The variation may be due to the difference in climatic condition of culture time. For example Batch culture II was cultivated during winter in beginning of February, III during spring in beginning of April, IV during summer in July, and the last batch culture V during autumn in October. The following bioautography test was performed to locate the bioactive compounds in the TLC. The best TLC method was considered for further glass column chromatography.

5.1.9.3 Bioautography

In order to determine the active components of the ethyl acetate crude extracts, bioautographic assay was carried out with an old extract using above optimized TLC method. Solvent system containing ethyl acetate: n-hexane: methanol (60:30:10) was used as mobile phase to separate the crude extract of strain 165 on two TLC plates in parallel. Bands on TLC were detected under UV light at 254 nm and 366 nm and one plate was sprayed with anisaldehyde reagent. The obtained result is presented in Figure 35.

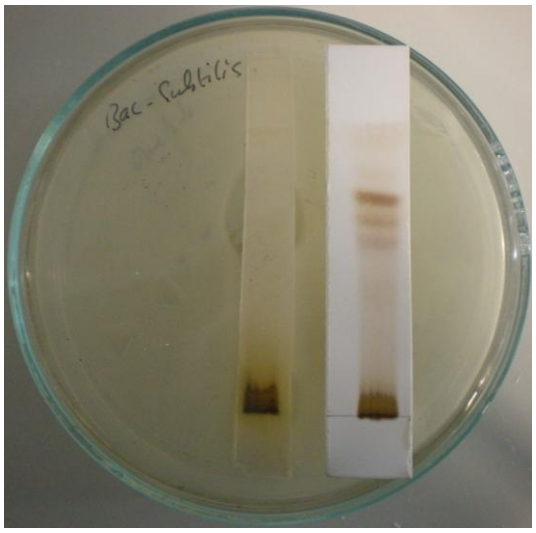


Figure 35: Bioautography test result shows three main bands responsible for strong anti microbial activity against *Bacillus subtilis*

Another TLC plate with the same solvent system was not sprayed with anisaldehyde reagent, but immersed in a petridish filled with Gram-positive test organism (*S. aureus* and *B. subtilis*) seeded agar medium for 20h incubation. Band clusters between Rf 0.75 to 0.85 exhibited antibacterial activity against the test organism. Subsequently, the same solvent system was applied on further fractionation process to isolate the active components. Bioautography test was also carried out for the additional TLC methods developed.

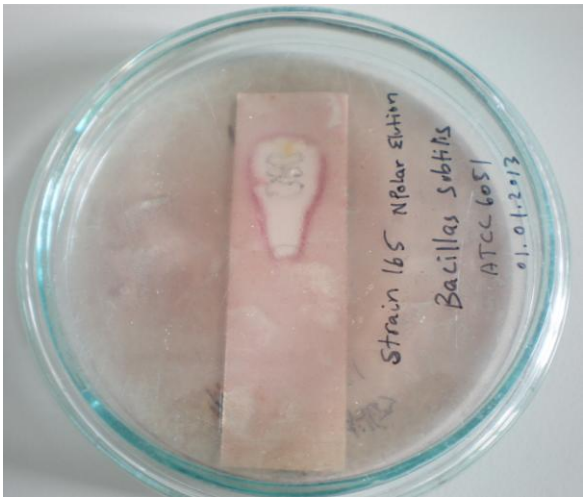


Figure 36: Bioautography test result shows three main bands responsible for strong anti microbial activity against *Bacillus subtilis*

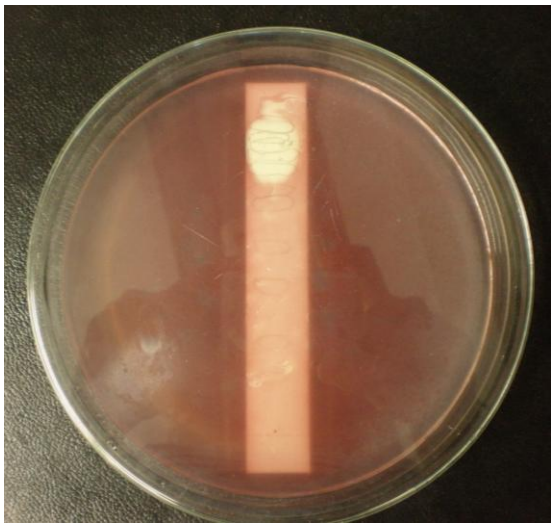


Figure 37: Bioautography test result shows three main bands responsible for strong anti microbial activity against *Bacillus subtilis*

5.1.9.4 Fractionation

Initial fractionation of compounds was performed by glass column chromatography and further purification was done in HPLC. For the glass column chromatography, mobile phase 11 (Refer 4.8.1) was chosen in relation to TLC separation and bioautography.

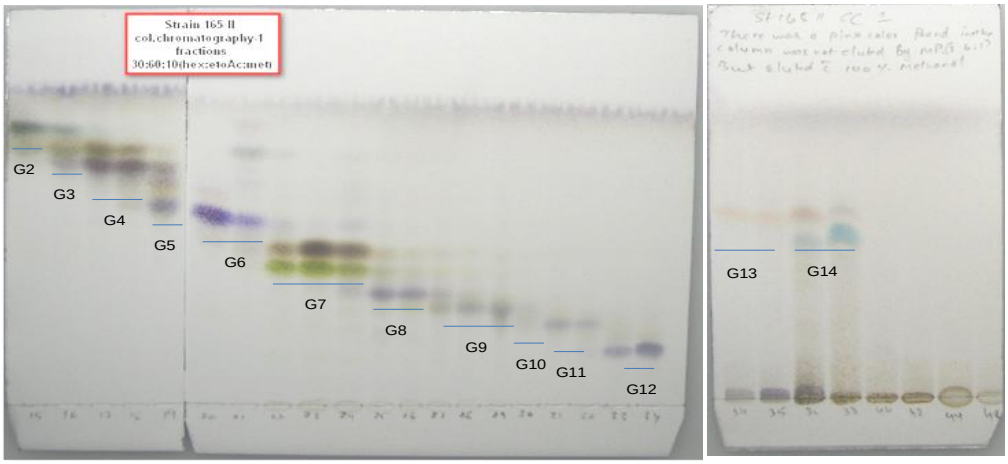


Figure 38: Thin layer chromatography traces of all the 14 groups obtained from strain 165 ethyl acetate extract fractionation

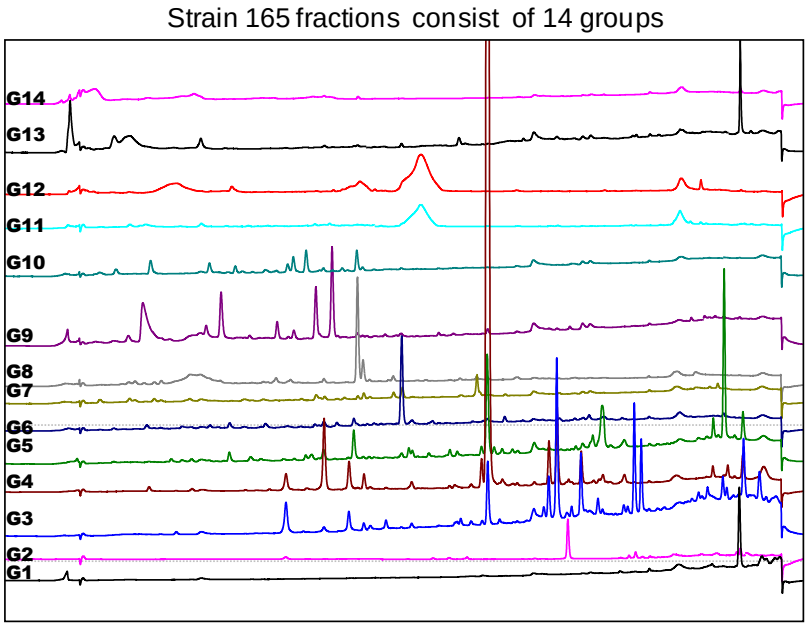


Figure 39: HPLC traces of all the 14 groups obtained from strain 165 ethyl acetate extract fractionation

Ethyl acetate crude extract of strain 165 was fractioned and the similar fractions were combined according to TLC (Figure 38) and it was 14 fractions in total. All the 14 fractions were immediately tested for antimicrobial activity to locate the fraction responsible for it. Fractions G1-G6 was strongly active against Gram-positive human pathogenic bacteria *S. aureus* and *B. subtilis*.

5.1.9.5 Cytotoxic activity

Ethyl acetate extracts of strain 165 culture medium was found to be strongly active against urinary bladder carcinoma cell lines in cytotoxic assay. Ethyl acetate extract was first fractionated by glass column chromatography and anti microbial fractions G1-G6 were tested against two types of urinary bladder carcinoma cell lines 5637 and HaCaT. As result, Fraction G3 exhibited IC50 values against HaCaT at concentration of 16.95 µg/ml and it exhibited comparatively less cytotoxic activity against 5637 carcinoma cell lines with IC50 value 22.45µl/ml. In contrast, fraction G4, G5 and G6 exhibited strong cytotoxic activity against 5637 cell lines with IC50 value 18.7µl/ml, 17.9 µl/ml and 17.3

µl/ml respectively. Same fractions exhibited less activity against HaCaT cell lines. Details are given below in Table 5.

Table 5: Cytotoxicity of strain 165 ethyl acetate extract fractions

Strain 165 EtOAc fractions	IC50 (µl/ml)	
	5637 cell lines	HaCaT cell lines
G1	Not tested	Not tested
G2	90.9	141.75
G3	22.45	16.95
G4	18.7	35.65
G5	17.9	30.2
G6	17.3	Test failed

5.1.9.6 Isolation and Purification

Based on the bioactivity exhibited from fractions G1, G2, G3, G4, G5 and G6 were taken for semi preparative chromatography to isolate the individual peaks (or compounds) by collecting them separately. Then all the individually collected compounds were purified by semi preparative chromatography in the second attempt. Fraction G1 was a yellow oily substance weighed about 2mg contained 90% single peak at R_t 27.0 min was further purified in semi preparative HPLC. Similarly, fraction G2 was also 2.0 mg of oily brownish yellow substance contained single peak at R_t 24.0 min was purified further in semi preparative HPLC. Fraction G3, G4, G5 G6 was found to be overlapping with bioactive compounds based on TLC detection. Therefore similar sub-fractions were combined and named initially as per R_t in preparative chromatography and later were labeled as G3-G6/A, B, and C...

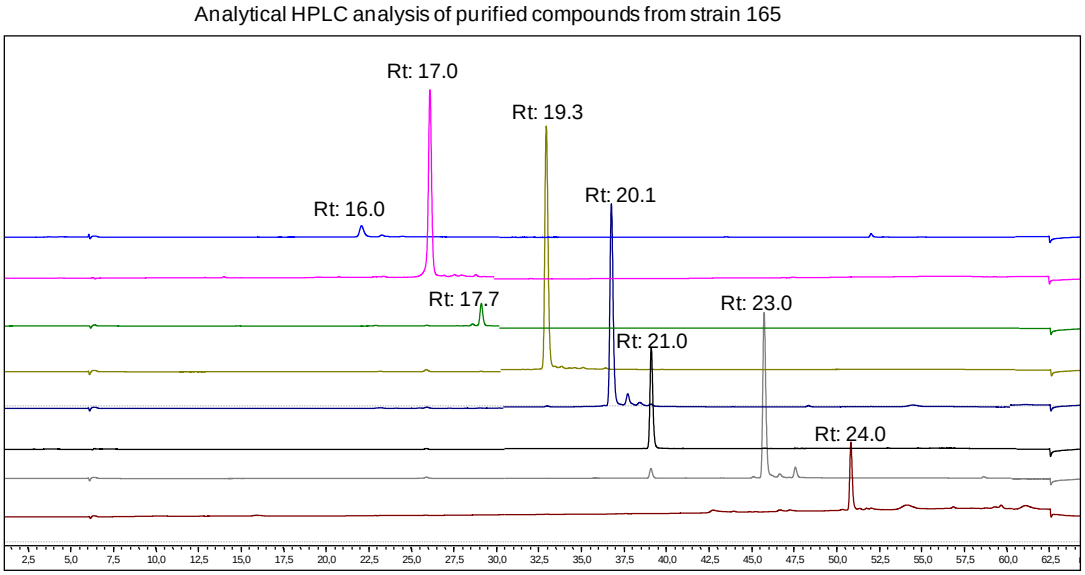


Figure 40: overlaid HPLC chromatograms of strain 165 purified compounds with retention time labeled

Total 10 compounds were Isolated besides the bioactivity and out of which low quantity sub-fractions were screened only by mass spectrometry for compound identification. Five majorly yielded compounds at R_t 9.5, 16.0, 17.0, 19.0, 21.0 were investigated further for their structure elucidation. Figure 40 is the analytical HPLC of purified compound submitted for NMR. R_t 9.5 was not included in the above figure.

5.1.9.7 Structure elucidation of compound 165/G1

The dark color band at Rf 9.5 in normal phase TLC was perfectly matching to the band obtained from pure Ergosterol (Refer Figure 41). For further confirmation, this compound was fractionated as G1 (4.0 mg; 95% white needle shape crystals) in the fractionation step, analyzed later in analytical RP HPLC, eluted at R_t 27.0 min as a single peak with λ max at 260 and 280. The molecular mass was determined as m/z 391.0 (M+H), 413.0 (M+Na), 804.0 (2M+Na) which is not matching to the molecular mass of Ergosterol reported as m/z 396.0 (M+H) in the literatures. In particular the 1H-NMR data confirmed the steroid substructure and showed all signals corresponding to Ergosterol. Due to the fact that Ergosterol is an important fungal metabolite the isolation of this compound is often described as a fungal metabolite.

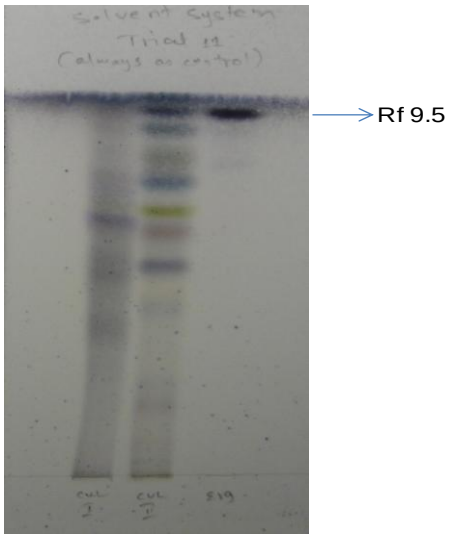


Figure 41: Thin layer chromatography comparisons of strain 165 crude extract and Ergosterol standard

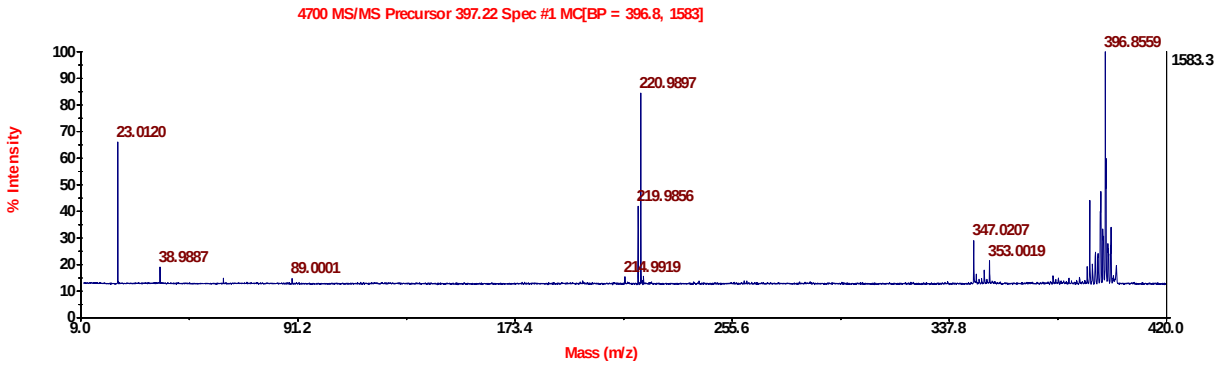


Figure 42: TLC-MALDI of MS/MS fragments obtained from strain 165 Fraction G1

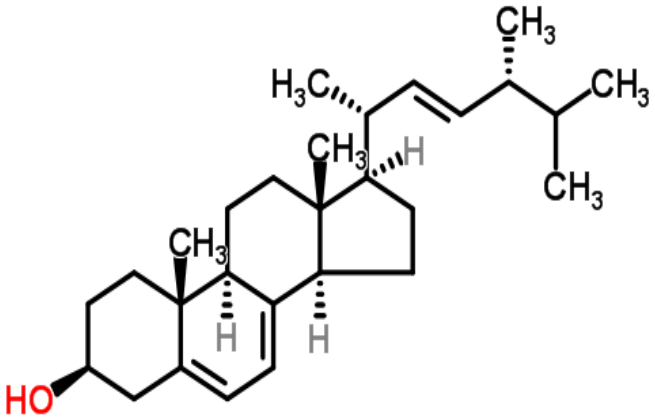


Figure 43: Chemical structure of Ergosterol obtained from literatures

5.1.9.8 Structure elucidation of compound 165/G2

The chemical compound obtained from column chromatography as fraction G2 (white powder; 2 mg; 90% purity) showed single peak in analytical HPLC. Molecular mass obtained was m/z 451.5 (M+Na), 883.0 (2M+Na). Search results from the dictionary of natural products and literatures against this molecular mass suggested this compound to be well known Ergosterol peroxide which can be expected from fungi as a secondary metabolites. All spectroscopic data of the isolated compound matched to the literature data. In particular the steroid backbone NMR signals were obvious. It has to be studied if the isolated compound is a product of fungal metabolism or secondary oxidation processes.

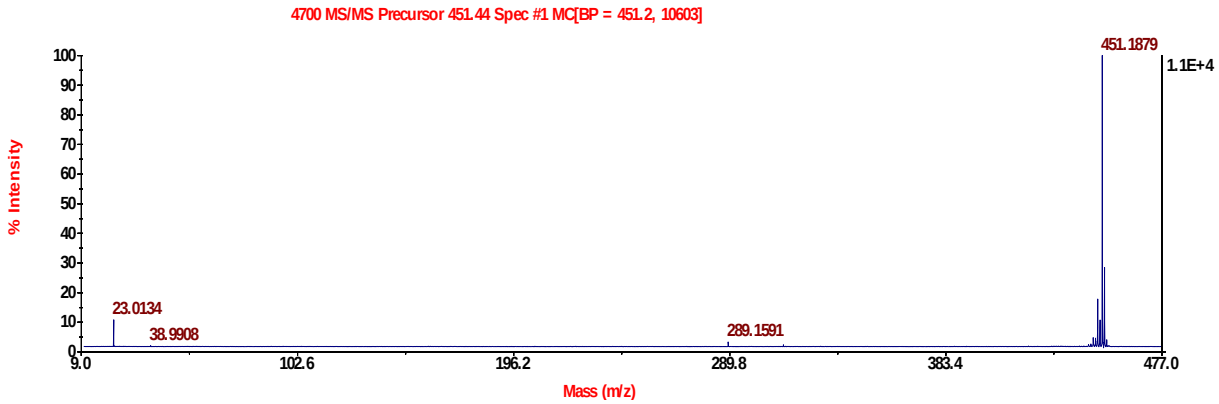


Figure 44: TLC-MALDI of MS/MS fragmentation obtained from strain 165 fraction 2

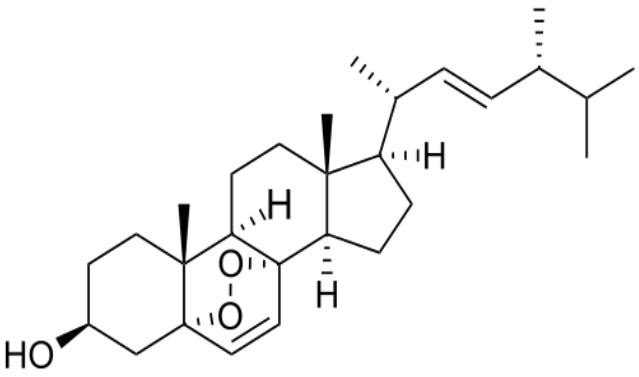


Figure 45: Chemical structure of Ergosterol peroxide obtained from literatures

5.1.9.9 Structure elucidation of compound 165/G3-G6/A

Compound 165/G3-G6/A was obtained from ethyl acetate extract fractions G3, G4, G5 when further separated in semi preparative chromatography. The substance was obtained as white solid substance; 2.0 mg. Molecular mass from ESI MS was found to be m/z 213.0 (M+H), 229.0 (M+oxygen adduct), 251.0 (M+oxygen+Na), 479.0 (2M+Oxygenated+Na) or (2M+MeOH+Na) and the UV absorbance showed λmax at 220nm and 284nm. Compound search in Dictionary of natural products against this molecular mass and UV spectra leads to suspect to be $C_{12}H_{20}O_3$, a Helicascolide A or B (Poch & Gloer 1989) which is also a polyketide α pyrone metabolite. The NMR spectroscopic data revealed a ketide substructure and matched the 1H-NMR data of Helicascolide. The conclusive 13C-NMR was not possible so far due to lack of pure compound.

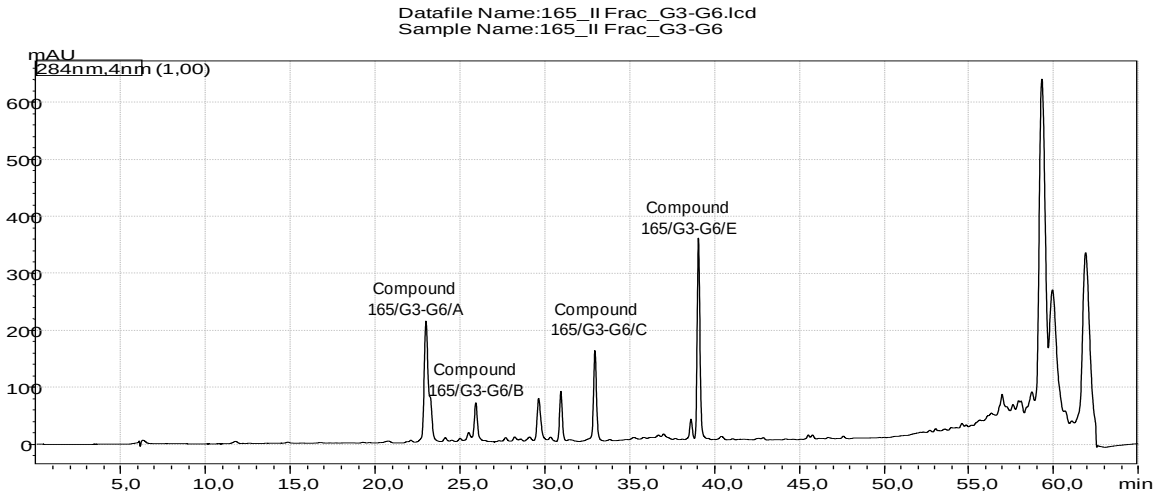


Figure 46: HPLC chromatogram obtained from strain 165 Fraction 3

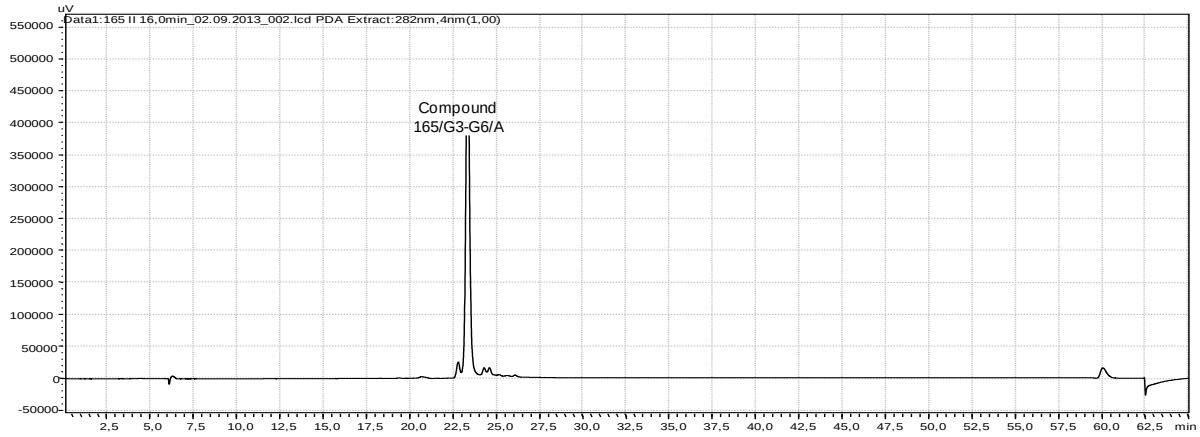


Figure 47: HPLC chromatogram obtained from isolated compound 165/G3-G6/A

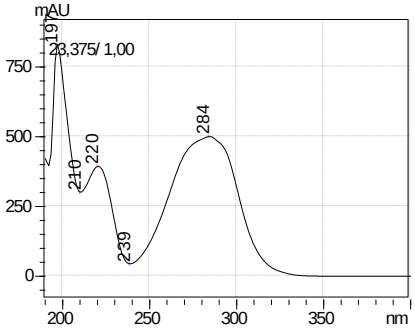
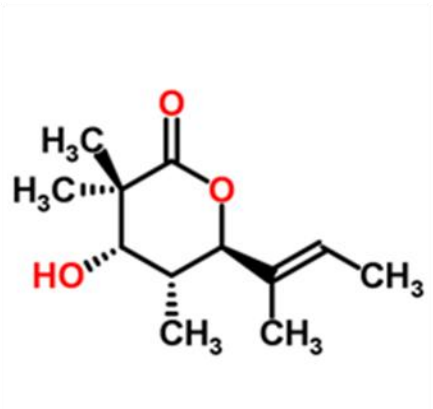
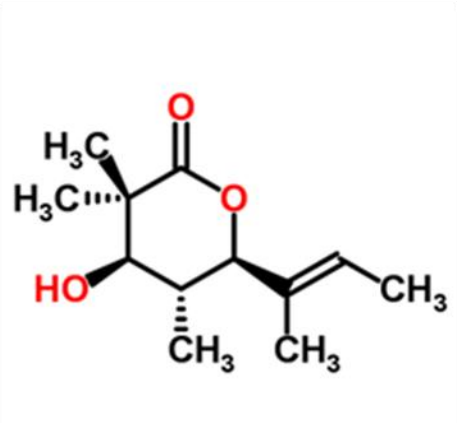


Figure 48: UV spectrum obtained from isolated compound 165/G3-G6/A



Helicascolide A



Helicascolide B

Figure 49: Chemical structure of Helicascolide A and Helicascolide B

5.1.9.10 Structure elucidation of compound 165/G3-G6/B

Compound 165/G3-G6/B was obtained from ethyl acetate extract fractions G3, G4, G5 after semi preparative chromatography. The substance was obtained as white crystals; 2.0 mg. Molecular mass from ESI MS was found to be m/z 183.0 (M+H), 205.0 (M+Na), 387.0 (2M+Na). UV absorbance showed λmax at 227nm and 334nm. Compound search in Dictionary of natural products against this molecular mass and UV spectra perfectly matching to a tetraketide compound Vermopyrone which is a co-metabolite or cleavage of Nectriapyrone previously reported by (Avent et al. 1992) for the first time. The conclusive NMR structural evidence is based on the 1H-NMR spectrum. This matches the literature data. Due to lack of more pure compound the 13C-NMR spectral confirmation is a matter of following work.

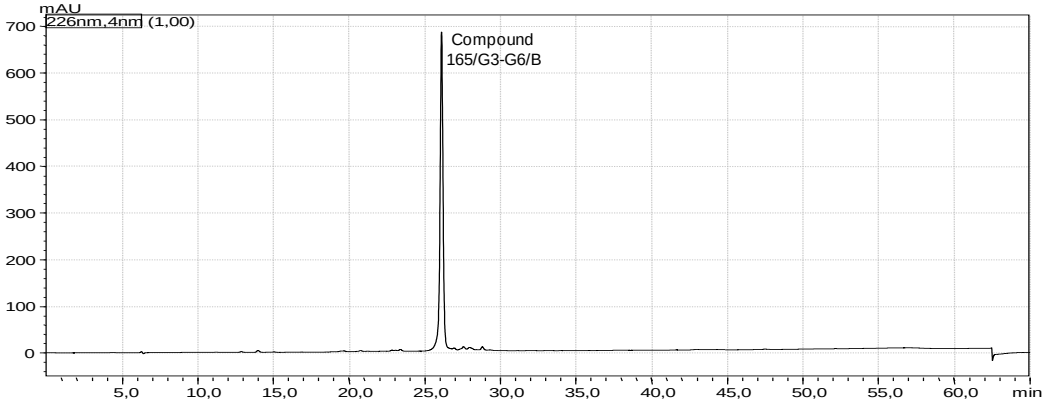


Figure 50: HPLC chromatogram obtained from isolated compound 165/G3-G6/B

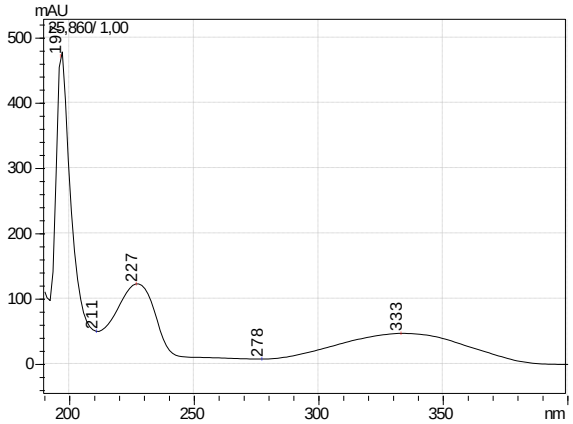


Figure 51: UV spectrum obtained from isolated compound 165/G3-G6/B

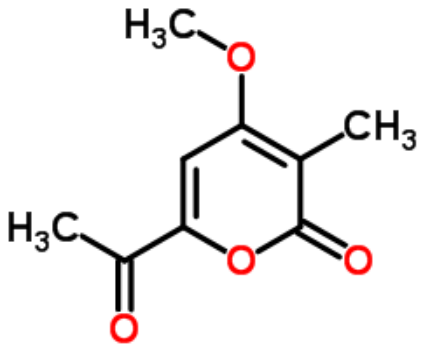


Figure 52: Chemical structure of Vermopyrone obtained from literatures

5.1.9.11 Structure elucidation of compound 165/G3-G6/C

Sub fraction at R_t 19.3 min obtained from semi preparative chromatography of ethyl acetate extract fraction G3-G6 was named as compound C. The substance was incurred as colorless crystals (2mg) which showed a prominent mass spectrum of m/z 211.0 (M+H), 233.0 (M+Na), 249.0 (M+Na+Oxygen), 443.0 (2M+Na). UV spectrum showed λ max at 216nm and 302nm. Compound based on obtained molecular mass and UV spectrum was searched in the dictionary of natural products and it was suggested to be $C_{12}H_{18}O_3$ of a known compound Helicascolide C (Tarman et al. 2012). As described for the Helicascolide A only 1H-NMR spectra were recorded. These match the literature data and could serve as another evidence for the presence of Helicascolide C in the extracts.

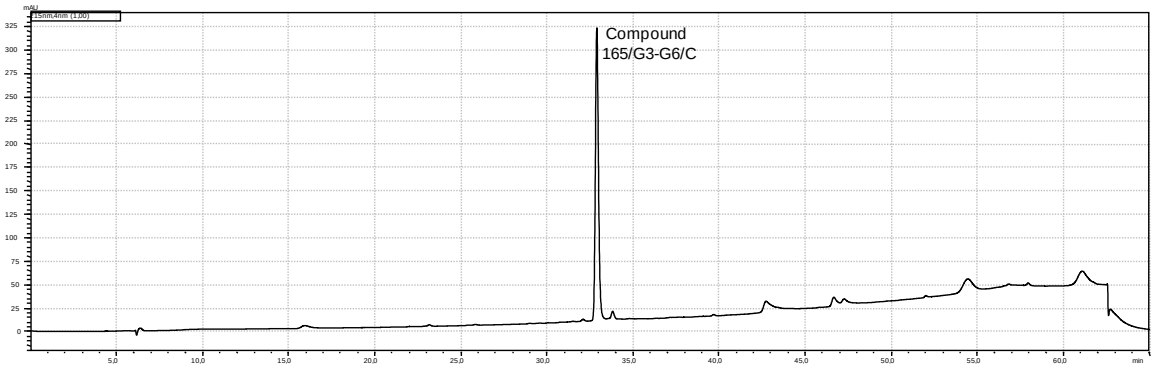


Figure 53: HPLC chromatogram obtained from isolated compound 165/G3-G6/C

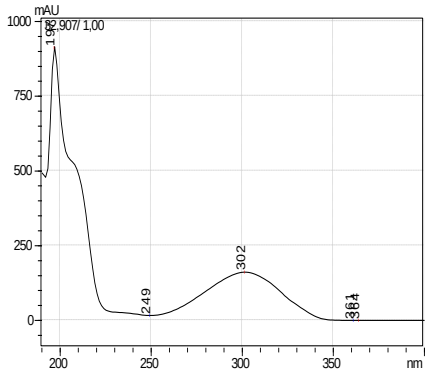


Figure 54: UV spectrum obtained from isolated compound 165/G3-G6/C

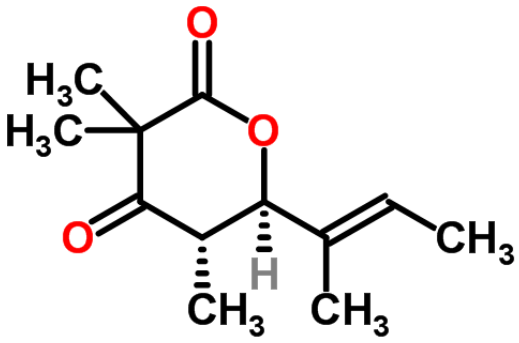


Figure 55: Chemical structure of Helicascolide C obtained from literatures

5.1.9.12 Structure elucidation of compound 165/G3-G6/E

Ethyl acetate extract fraction G3-G6 was subjected to semi preparative chromatography and compound E was isolated at R_t 21.0 (white needle crystals; 10 mg) which was found to be dominating band in TLC at R_f 0.75 corresponds to the bioactive region. The white needle shaped crystals exhibited λmax at 229nm and 330nm. Analysis using ESI MS showed the molecular peak of m/z 195.0 (M+H), 217.0 (M+Na), 389.0 (2M+H), 411.0 (2M+Na) to have the molecular formula $C_{11}H_{14}O_3$. Similar results from the previous chemical abstracts confirms the compound to be a Nectriapyrone A which is a pentaketide monoterpenoid first time isolated by (Carey et al. 1975). Nectriapyrone is a common metabolite in facultative and obligate marine fungi. The 1H-NMR data confirmed the presence as a metabolite in the present extracts.

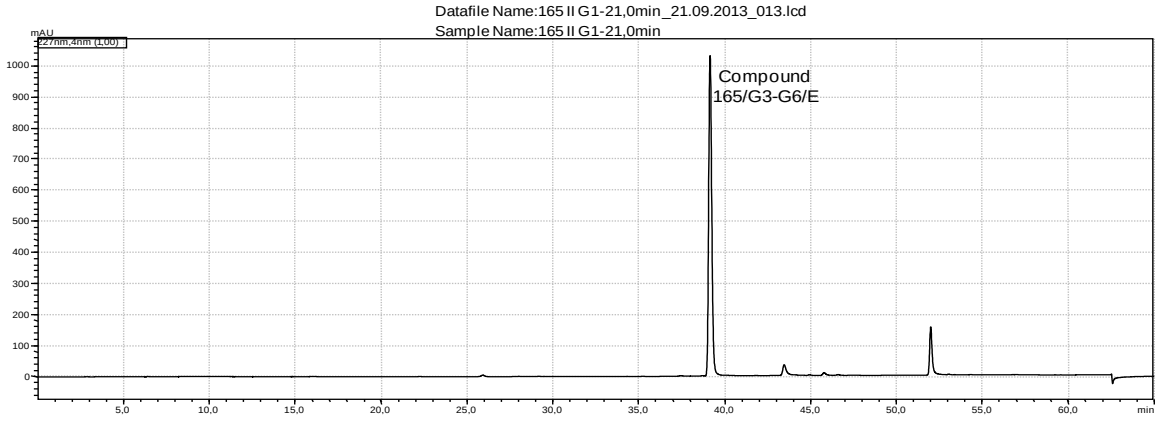


Figure 56: HPLC chromatogram obtained from isolated compound 165/G3-G6/E

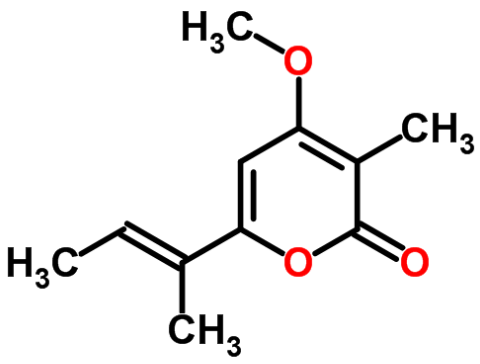


Figure 57: Chemical structure of Nectriapyrone obtained from literatures

5.1.9.13 Other metabolites isolated from strain 165

As every two batches of strain 165 were processed at a time for isolation and purification of secondary metabolites and it was observed a peak at the retention time of Nectriapyrone to be demethyl Nectriapyrone A which is an analog of Nectriapyrone

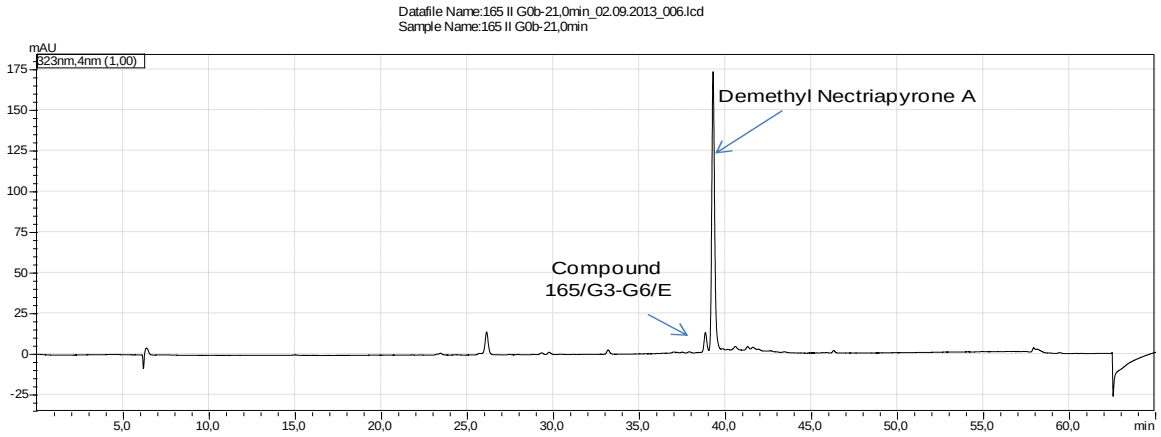


Figure 58: HPLC chromatogram obtained from the isolated compound demethyl Nectriapyrone A

formerly isolated as pestalopyrone (Venkatasubbaiah & Chilton 1992). The molecular mass spectrum was at m/z 181.0 (M+H), 199.0 (M+H2O), 221.0 (M+Na+H2O) and 361 (2M+H). Other than demethyl Nectriapyrone, there were low abundant peaks at R_t between 17.5 - 19.0 mins showed mass signal of m/z 169 and m/z 193 corresponding to

Nectriapyrone B and 5-methyl mellein which are also a pyrone analogs still, the quantity was insufficient for 1H- and 13C-NMR confirmation of structures.

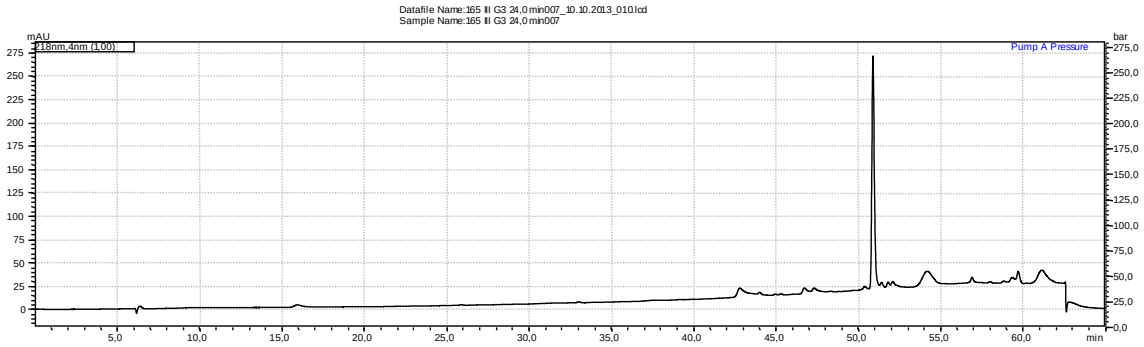


Figure 59: HPLC chromatogram obtained from the isolated compound dihydroergosterol peroxide

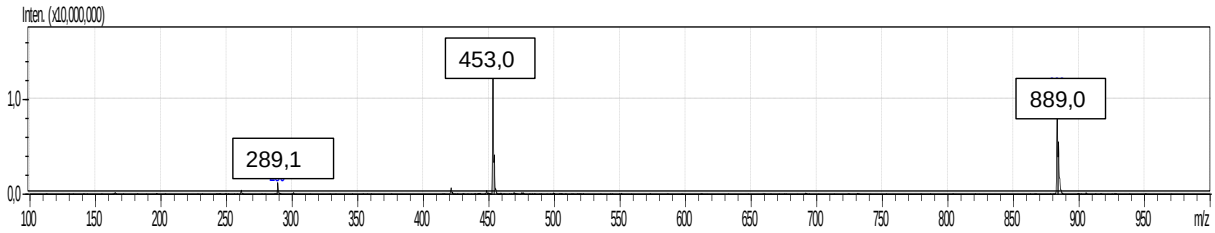


Figure 60: Mass spectrum obtained from the isolated compound dihydroergosterol peroxide

Apart from pyrone analogs, HPLC peaks in non-polar region at R_t 24 min showed a mass spectrum m/z 453 (M+22), 883 (2M+22) corresponds to Dihydroxyergosterol peroxide and another peak R_t 26.7 min showed mass signal of m/z 427 (M+H), 449 (M+Na), 409 (M-18) and 467 (M+Na+18) from ESI-MS perfectly matching to Dehydroergosterol peroxide. Nevertheless, the presence of dehydroergosterol, Dihydroxy-ergosterol along with Ergosterol has been identified by GC-MS results. Furthermore, 5-Indole carboxylic acid was also identified from ethyl acetate extract fraction 8 of strain 165 by GC-MS. But, in the course of work, none of the Indole core structures was isolated from strain 165.

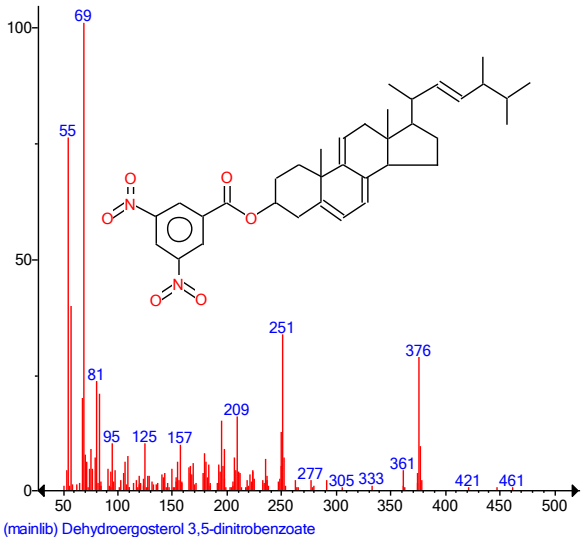


Figure 61: Mass spectrum from GC-MS analysis obtained from strain 165 biomass n-hexane extract matching to the chemical structure of Dehydro Ergosterol with 3, 5 dinitro benzoate.

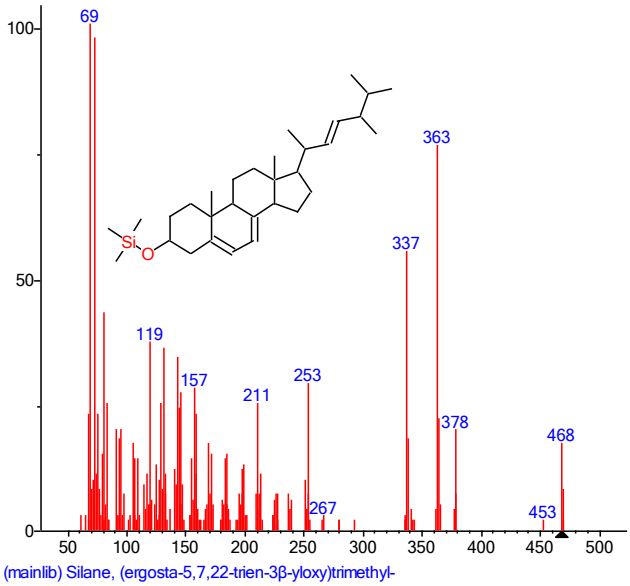


Figure 62: Mass spectrum from GC-MS analysis obtained from Strain 165 biomass n-hexane extract matching to the chemical structure of Ergosterol.

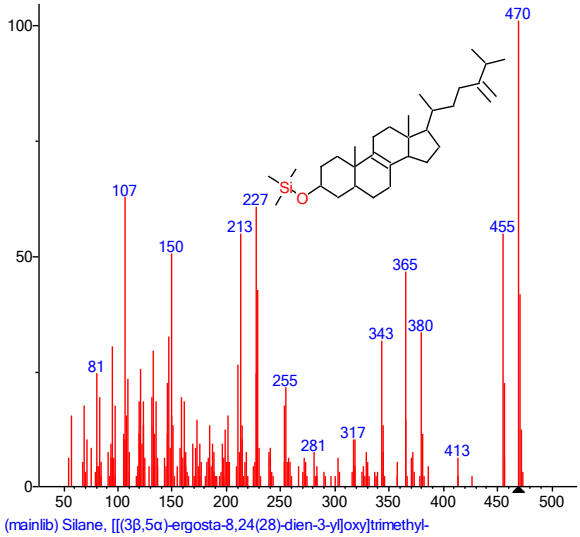


Figure 63: Mass spectrum from GC-MS analysis obtained from strain 165 biomass n-hexane extract matching to the chemical structure of dihydroxy Ergosterol.

5.1.9.14 Phytotoxicity test

As described under the section 4.7.4, a simple Phytotoxicity test was carried out on three randomly selected leaves *Moru nigra* (commonly known as mulberry), *Taraxacum officinale* (commonly known as Dandelion) and *Ribes rubru*m (commonly known as red currants) collected from the university botanical garden. This particular test was especially performed with a very small amount of purified Nectriapyrone based on earlier evidences and results obtained were in line with the previous records. Figure 64 shows the damage developed by Nectriapyrone against methanol blank. The responsible part of the Nectriapyrone for this demonstrated phytotoxic activity was found to be the methyl group at C-3 while the much lower activity of pestalopyrone showed that the methyl group at C-3 of the same ring is an important structural feature (Türkkan et al. 2011).

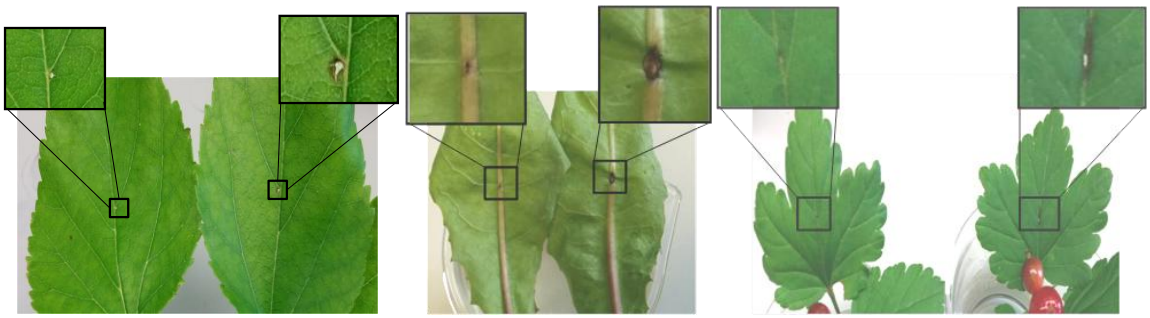


Figure 64: Phytotoxic activity tested on (from left) *Morus nigra* (commonly known as mulberry), *Taraxacum officinale* (commonly known as Dandelion) and *Ribes rubrum* (commonly known as red currants) leaves.

5.1.10 Isolation of bioactive metabolites from sponge associated fungi

Strain MM010 and MM018 were determined to be closely related sponge associated fungal strains based on taxonomy results. Nonetheless, both the strains were further investigated separately for their active secondary metabolites as they exhibited a strong antimicrobial activity against Gram-negative fish pathogenic bacteria. The HPLC profile of ethyl acetate extract showed only nine peaks so that isolation of bioactive compounds was carried out directly by semi preparative method instead of column chromatography fractionation, Initially the active extract was dissolved slightly in ethyl acetate and subjected to semi preparative HPLC in order to isolate the compounds which was again purified in the second attempt of semi preparative HPLC. The flow chart of isolation process is shown in the Figure 65.

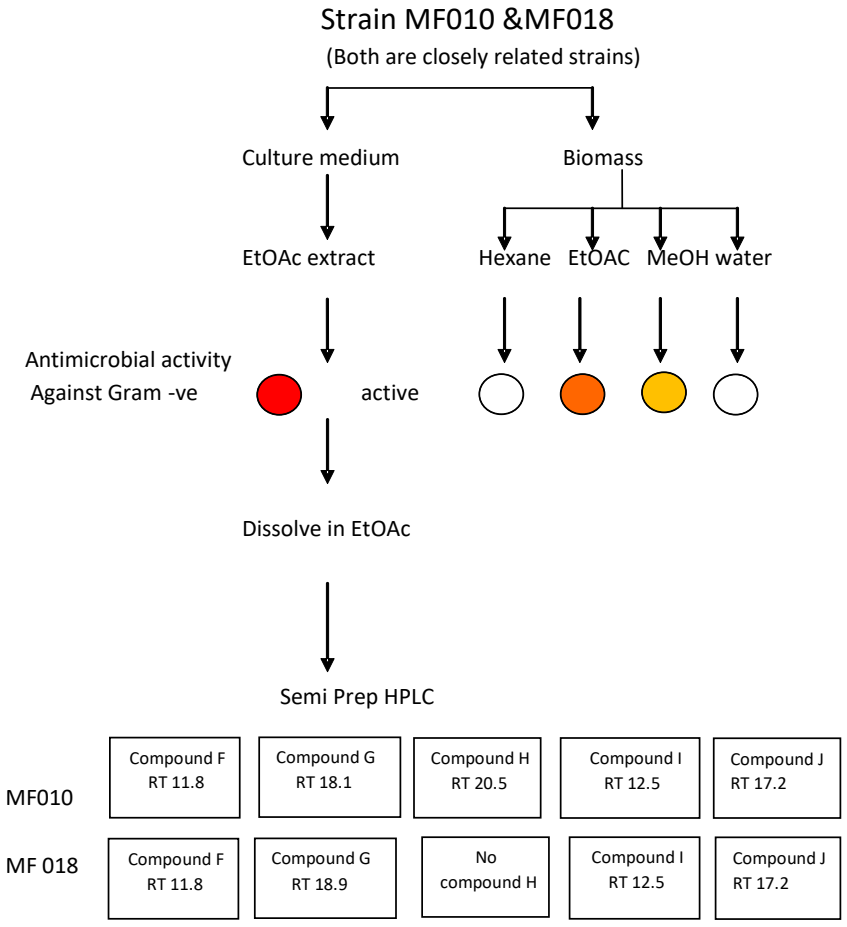


Figure 65: Work flow of Strain MF010 and MF018 compound isolation

5.1.10.1 GC-MS Analysis of crude extracts

Crude extracts of strain MF010, MF018 biomass and culture medium extracted against various solvent were dried and submitted for Gas Chromatography Mass spectroscopy analysis (GC-MS). The results against NIST library afforded to confirm the presence below listed fatty acids, Ergosterol and its analogs considering match factor more than 700.

Table 6: Fatty acids and other volatile compounds from strain MF 010 identified by GC-MS

Strain MF010	NIST	Match
Biomass n-hexane extract	Palmitic acid, trimethylsilyl ester	899
	Oleic acid, trimethylsilyl ester	864
	Stearic acid, trimethylsilyl ester	849
Biomass EtOAc extract	Dihydroisophorone	676
	Glycerol, tris (trimethylsilyl) ether	883
	Butyl caprylate	585
	Trimethylsilyl ether of glucitol	900
	Palmitic acid, trimethylsilyl ester	838
	Stearic acid, trimethylsilyl ester	572
Biomass methanol extract	Dihydroisophorone	774
	Butyl caprylate	592
	Trimethylsilyl ether of glucitol	912

	Palmitic acid, trimethylsilyl ester	792

Table 7: Fatty acids and other volatile compounds from strain MF 018 identified by GC-MS

Strain MF 018	NIST	Match
Biomass n-hexane extract	Dihydroisophorone	771
	Butyl caprylate	576
	β-Eudesmol, trimethylsilyl ether	803
	Palmitic acid, trimethylsilyl ester	841
	Oleic acid, trimethylsilyl ester	704
	Stearic acid, trimethylsilyl ester	730
	Silanol, trimethyl-, triester with boric acid (H3BO3)	919
	5-Ethyl-2-methyl-4-[(trimethylsilyl)oxy]furan-3 (2H)-one	684
	Nonanoic acid, trimethylsilyl ester	903

	Diisobutyl phthalate	925
	n-Pentadecanoic acid, trimethylsilyl ester	892
	Dehydroergosterol 3,5-dinitrobenzoate	663
	3-[(Trimethylsilyl)oxy]ergosta-8,24 (28)-diene	697
	Tetrahydrospirilloxanthin	674
	15-Hydroxy-7-oxodehydroabietic acid, trimethylsilyl ester, 15-trimethylsilyl ether	798
Biomass EtOAc Extract	Glycerol, tris (trimethylsilyl) ether	870
	Butyl caprylate	579
	Pentitol, 3-desoxy-tetrakis-O-(trimethylsilyl)-	862
	Xylitol, 1,2,3,4,5-pentakis-O-(trimethylsilyl)-	930
Biomass methanol extract	Trimethylsilyl ether of glucitol	910
	Dihydroisophorone	744
	Butyl caprylate	578
	Xylitol, 1,2,3,4,5-pentakis-O-	814

	(trimethylsilyl)-	
	Trimethylsilyl ether of glucitol	880

5.1.10.2 Thin layer chromatography

Ethyl acetate extracts of strain MF010 and MF018 showed a strong antimicrobial activity against Gram-negative fish pathogenic bacteria. After many trial and errors, the TLC method was optimized with mobile phase11 containing ethyl acetate: n-hexane: methanol (60:30:10) and silica gel as stationary phase. Figure 66 is the TLC outcome of this method showed eight distinguished bands sprayed with vanillin sulfate reagent and heated at 110°C.

Figure 66: Thin layer chromatography traces of MF010 and MF018 ethyl acetate extract

5.1.10.3 Fractionation and purification

Ethyl acetate extract of both MF010 and MF018 showed very much similar HPLC pattern except a unique compound at R_t 20.7 in MF010. However, both strains were fractionated separately; the isolated compounds were reconfirmed for the similarity and pooled when necessary. In the fractionation process, directly fractionated using semi

preparative HPLC as they showed only 8-9 clearly distinguished peaks with the crude extract HPLC profile. As a result, total six fractions were collected and tested for antimicrobial activity. F1, F3, F4 and F5, F6 were active against Gram-negative *E. coli*, *A. hydrophilla* and *P. anguilliseptica* with IZ of 12mm, 6mm, 9mm, 7mm and 7mm respectively. Accordingly, F1, F3, F4 and F5 were fractionated in the second attempt of semi preparative chromatography to isolate compound F, G, H, I and J.

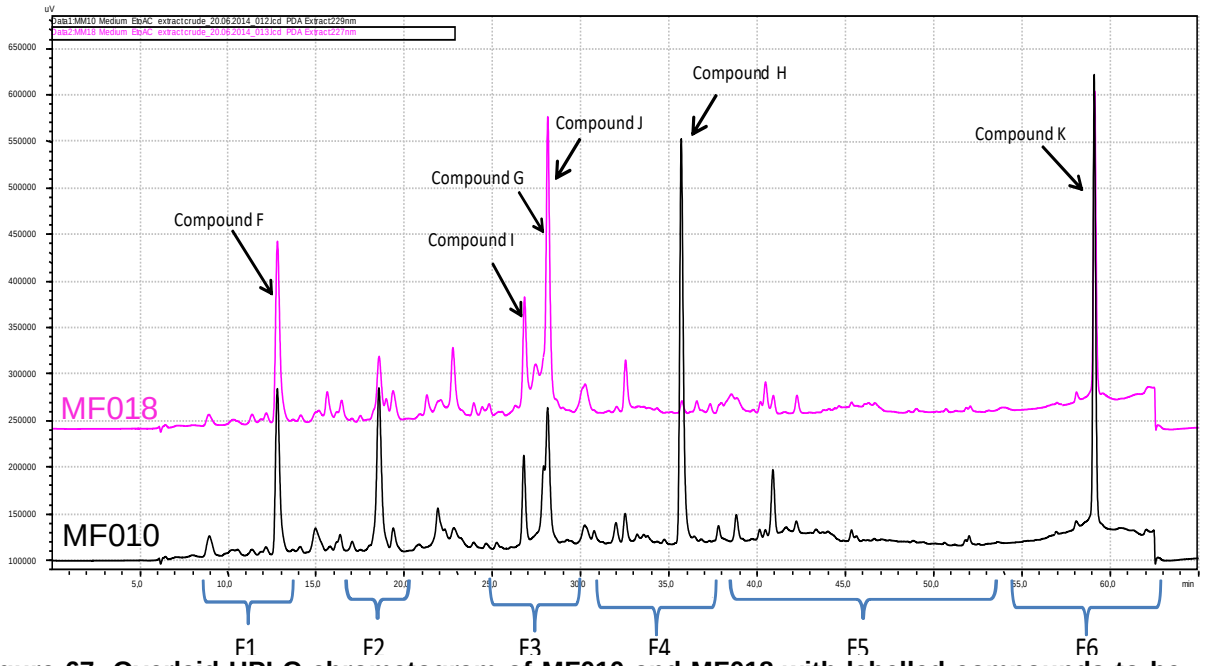


Figure 67: Overlaid HPLC chromatogram of MF010 and MF018 with labelled compounds to be isolated

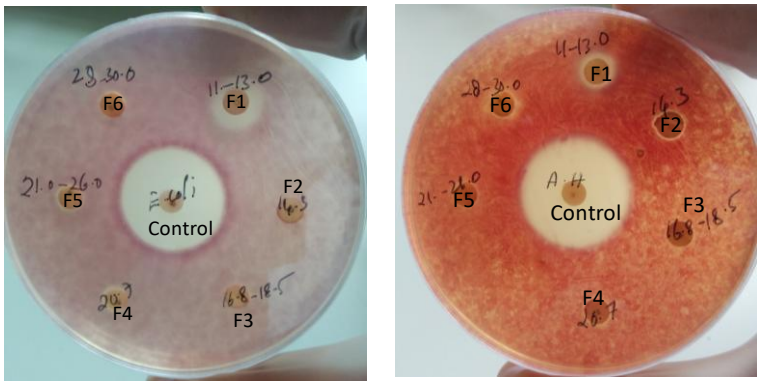


Figure 68: Inhibition Zone against *E. coli* from the ethyl acetate extract fractions obtained from MF010 and MF018

5.1.10.4 Structure elucidation of compound F

Fraction F1 of ethyl acetate extract from both the MF strains afforded to isolate compound F at R_t 11.3 min which was an oily brown substance about 2.0 mg. Molecular mass of compound F was found to be m/z 181.0 (M+H), 203.0 (M+Na), 383.0 (2M+Na) in positive mode as well as m/z 179.2 (M-H), 359.2 (2M-H). UV absorbance for this compound F was at λmax 227nm, 260nm and 315nm. NMR spectroscopic analysis is still pending due to insufficient amounts of pure compound.

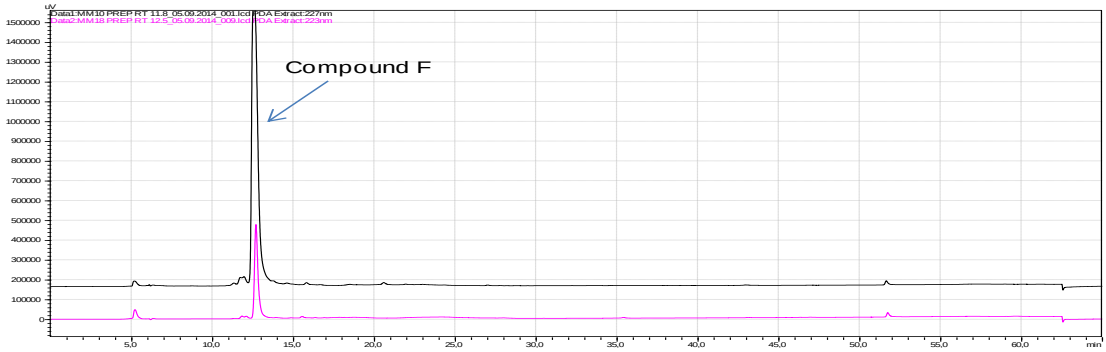


Figure 69: HPLC chromatogram obtained from the isolated compound F of strain MF010 and MF018

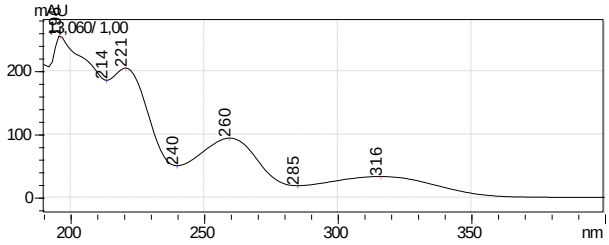


Figure 70: UV spectrum obtained from the isolated compound F of strain MF010 and MF018

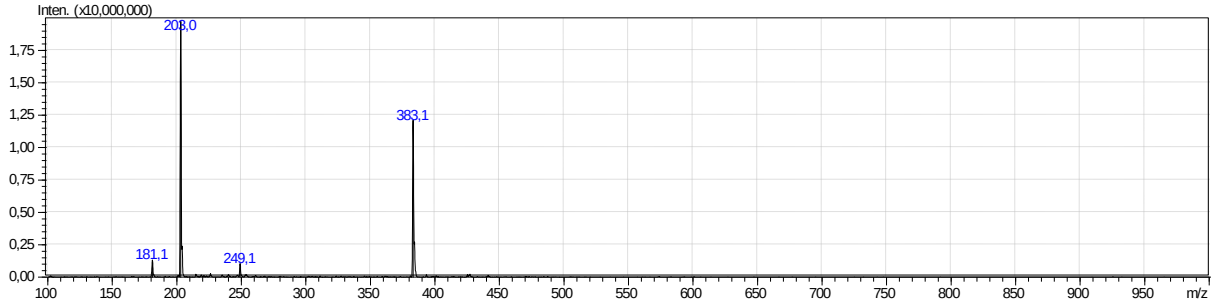


Figure 71: Mass spectrum obtained from the isolated compound F of strain MF010 and MF018

5.1.10.5 Structure elucidation of compound G

Compound G was isolated from ethyl acetate fraction F3 along with a co-eluting isomer in semi preparative chromatography at Rt 18.1 (MF010) and Rt.18.9 (MF018). Retention time of compound G was slightly varies as the MF strains were subjected into isolation process individually at different times. The obtained substance was around 2mg; oily brown color. The compound exhibited λmax at 220nm and 260nm. ESI MS showed the molecular peak at m/z 267.1 (M+H), 249.0 (M-18). Results from the database search partially matching to the previously described compound Ascosalitoxin (Evidente et al. 1993) first time isolated from *Ascochyta pisi*. The molecular formula from the literature for Ascosalitoxin found to be $C_{15}H_{20}O_4$ (264.0 Da). Nevertheless, the UV spectrum and obtained molecular mass(2 dalton in addition to Ascosalitoxin) for compound G makes hypothetical molecular formula of $C_{15}H_{22}O_4$ of Dihydro Ascosalitoxin by reduction of Aldehyde and carbonyl group in the Ascosalitoxin chemical structure. More quantity of pure substance is required for further confirmation.

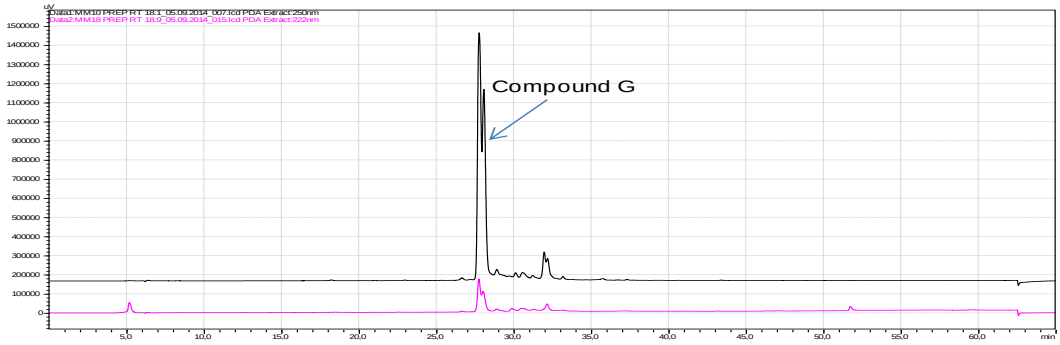


Figure 72: HPLC chromatogram obtained from the isolated compound G of MF010 and MF018

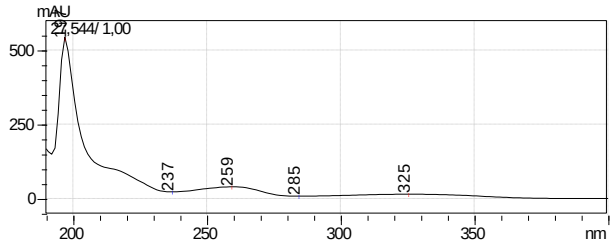


Figure 73: UV spectrum obtained from the isolated compound G of MF010 and MF018

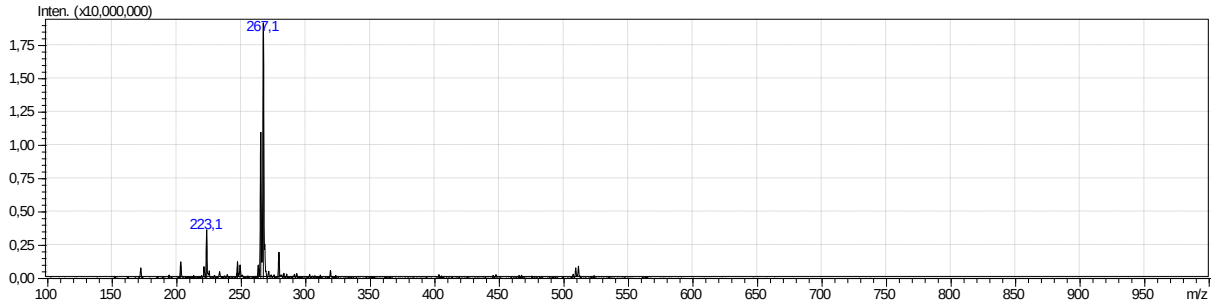


Figure 74: Mass spectrum obtained from the isolated compound G of MF010 and MF018

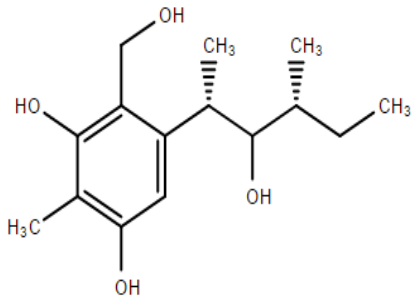


Figure 75: Hypothetical chemical structure of Ascosalitoxin dihydro derivative

5.1.10.6 Structure elucidation of compound H

Compound H was unique in strain MF 010 which was isolated as twin peak from ethyl acetate extract fraction F4(See Figure 67). UV absorbance of this compound was found to be λmax at 220nm, 238nm, 260nm, 276nm and 320nm. Molecular mass spectrum of this compound showed m/z 277.0 (100%), 409.0 (95%), 247.0 (90%), 291.0 (90%) and 295.0 (40%) in positive mode. This cluster of mass spectrum of compound H was susceptible to be Ascochitine together with Ascochital. Both Ascochitine and Ascochital have been previously isolated from *Phoma clematidina* which belongs to genus phoma (Smith & Cole 1991) as that of MF010. Ascochitin and Ascochital are chemically related compounds arising from the same core structure. They are products of the same biosynthesis pathway and differ only on the formation of a ring after reaction of the reactive aldehyde group in Ascochital. The conclusive structure elucidation is difficult

due to the chemical instability of the isolated derivatives. First 1H-NMR data suggests the presence of both compounds and is in conclusion with the literature data.

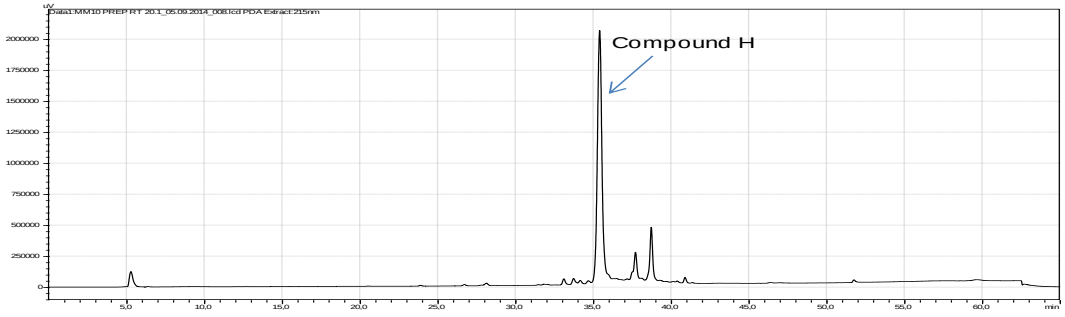


Figure 76: HPLC chromatogram obtained from the isolated compound H of MF010.

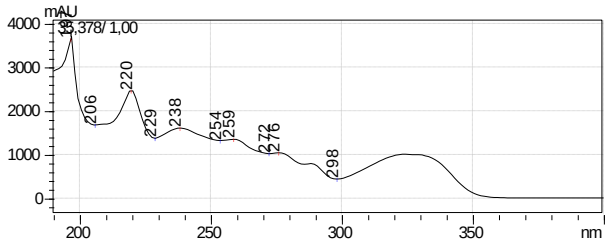


Figure 77: UV spectrum obtained from the isolated compound H of MF010.

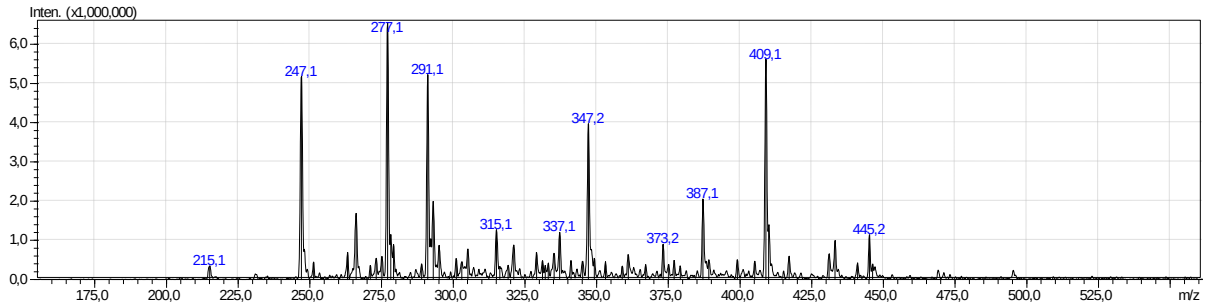


Figure 78: Mass spectrum obtained from the isolated compound H of MF010.

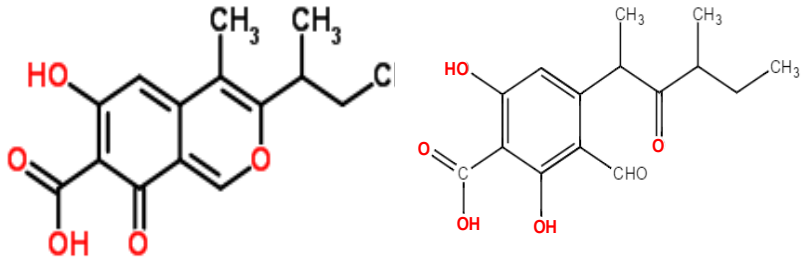


Figure 79: Chemical structure of Ascochitine (left) and Ascochital (right) obtained from the literatures

5.1.10.7 Structure elucidation of compound I

Compound I was isolated from ethyl acetate fraction F3 from MF018 as brown oily substance; 2mg at R_t 17.2 min. λmax of compound I was 215nm, 254nm, and 308nm. The molecular mass spectrum showed a prominent peak at m/z 325.0 (M+Na), 627.0 (2M+Na) in positive mode as well as m/z 301.1 (M-H), 603.2 (2M-H). This compound I was also present in MF010 but, the obtained substance not pure enough to combine with that of MF018 for structure elucidation. However, compound I isolated from MM018 submitted for NMR structure confirmation. According to the compound identified in GC-MS analysis of ethyl acetate crude extract and UV spectrum, molecular mass spectrum matching to literature suggested compound I to be 6-methoxy estrodial with molecular formula: $C_{19}H_{27}O_3$

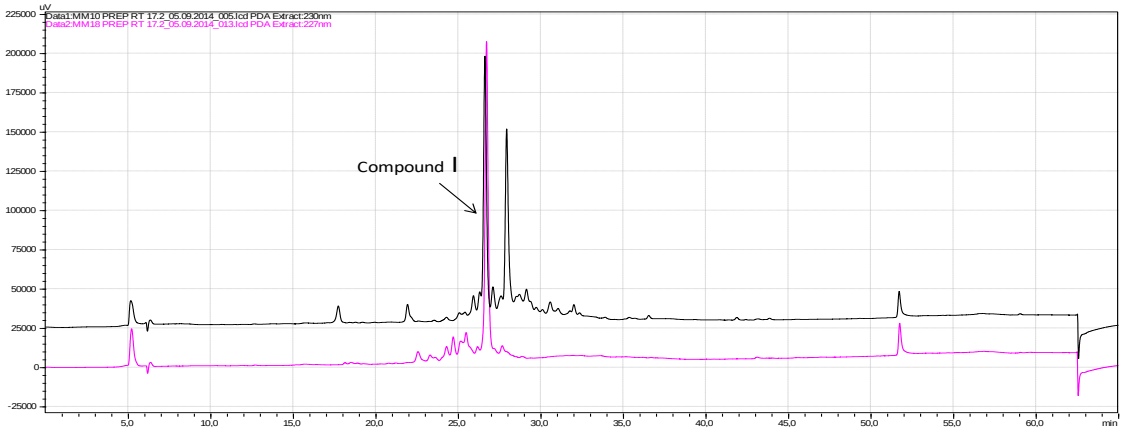


Figure 80: HPLC chromatogram of compound I from MF018 (pink) and MF010 (black)

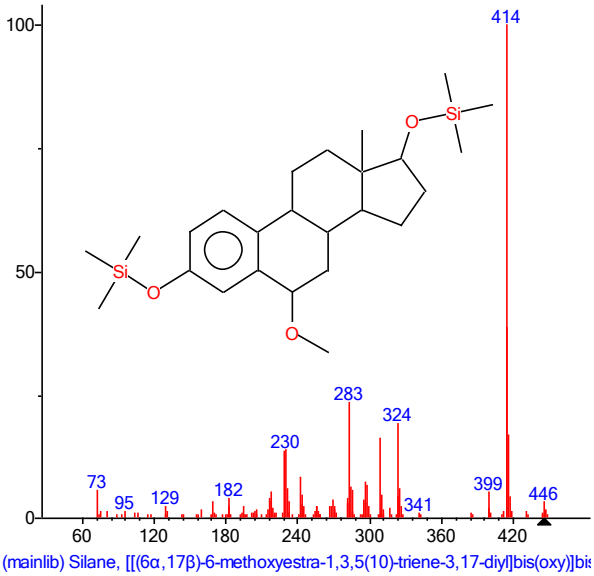


Figure 81: Gas chromatography mass spectrum and the chemical structure of 6-methoxy estrodial obtained from strain MF010 and MF018 ethyl acetate extracts

5.1.10.8 Structure elucidation of compound J

Compound J was isolated from ethyl acetate fraction F3 from MF018 as brown oily substance; 2mg at R_t 18.2 min. λmax of compound I was 210nm, 232nm, and 275nm. The molecular mass spectrum showed a prominent peak at m/z 249.0 (M-Oxygen), 265.0 (M+H) in positive mode. Compound J was present in MF010 but, the obtained substance unfortunately not pure enough to combine with the isolated compound J from MF018 for structure elucidation. However the mass spectrum and UV spectra matching to that of compound G with 2 Da less could be Ascosalitoxin as such (See section 5.1.10.5).

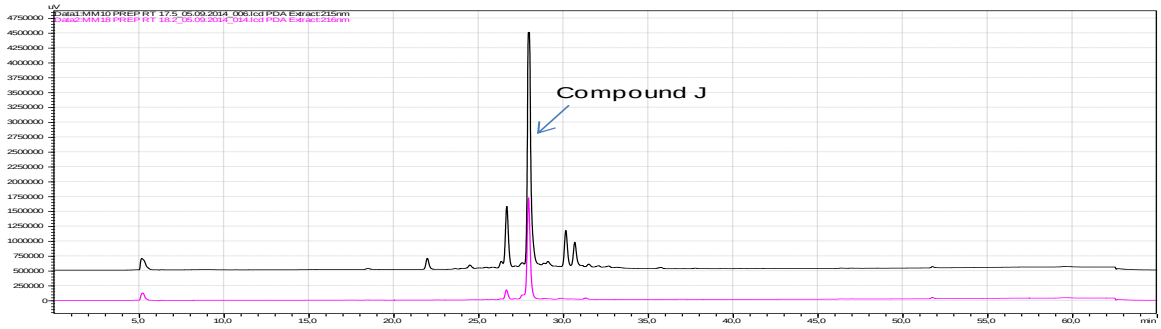


Figure 82: HPLC chromatogram obtained from the isolated compound J of MF010 and MF018

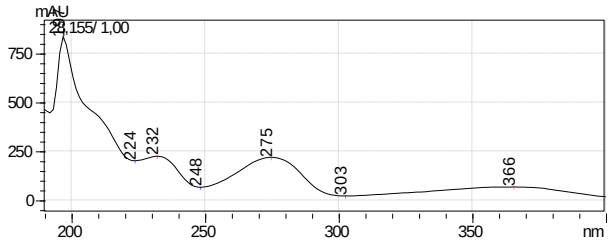


Figure 83: UV spectrum obtained from the isolated compound J of MF010 and MF018

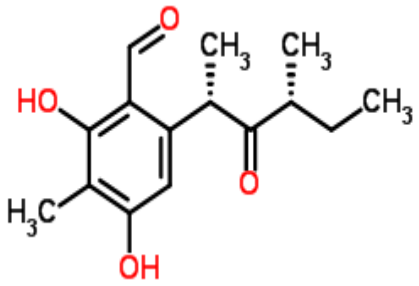


Figure 84: Chemical structure of Ascosalitoxin obtained from the literature

5.1.10.9 Structure elucidation of compound K

Fraction 6 of both MF strain ethyl acetate extract showed almost 95% of single peak at R_t 25 min to yield around 4mg of white needle crystals. Compound K was determined to be Ergosterol according to obtained molecular mass of m/z 391.0, 413.2, 783.5 and 804.1 as well as UV absorbance at λmax 262,274,283 matching to that of dictionary of natural products. NMR spectroscopic analysis confirmed the structure of Ergosterol.

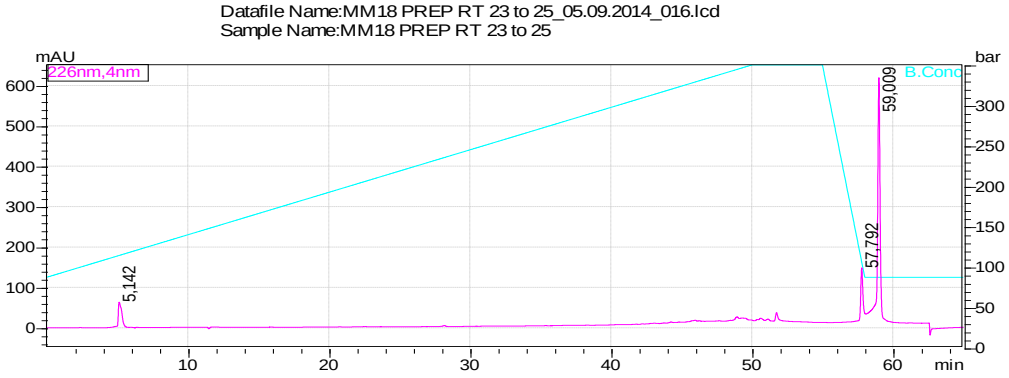


Figure 85: HPLC chromatogram obtained from the isolated compound K of MF010 and MF018

5.1.10.10 Other metabolites isolated from MF strains

GC-MS analysis of crude extracts from MF strains resulted with few sesquiterpenoid alcohols such as Beta Eudesmol and its derivatives which is unusual to isolate from marine derived fungus. Estrogen derivatives also found from GC-MS results

5.1.11 Ascomycota of fungi kingdom

Marine biodiscovery achievements mostly from Ascomycota and Actinobacteria (other than Porifera and Cyanobacteria) reported with numbers of species by World Register of Marine Species WORMS is probably yet to be fully recognized. Taxonomy analysis report of strain 165 suggested the marine derived fungus to be *Pseudohalonectria lignicola* belong to Ascomycota which was isolated in 1975 for the first time (Minoura & Muroi 1978). Other Pseudohalonectria species have been listed under section 2.8.2.3. Still, out of these 14 species of the genus Pseudohalonectria were reported already, no further chemical investigation studies have appeared in the literature to the present except two new Azaphilones metabolites, named pseudohalonectrin A (1) and B (2), were isolated from the culture of the aquatic fungus *Pseudohalonectria adversaria* YMF1.01019in 2006 (Dong et al. 2006), There are hardly any literatures available on *Pseudohalonectria lignicola* so that this 40 year old *P. lignicola* strain was focused with huge interest for the chemical investigation. Sponge associated marine fungal strains both MF010 and MF018 were also closely relative from pleosporales order belongs to Phoma genus of Ascomycota additionally investigated for chemical prospecting.

5.1.12 Workouts of candidate strains

Being a facultative fungus, all the three strains 165, MF010 and MF018 were cultivated continuously with 5 culture batches (2 x10 flasks at a time) each took 4 weeks in average as optimized at room temperature to harvest. Figure 34 shows the difference in the intensity of biosynthesized compound as the climatic condition changes with every culture batch. Every culture medium was immediately processed to isolate the interesting compounds as there could be chances of contamination while waiting for all the ten batches to pool together.

Identification of isolated fungal strain is important in the framework of Natural product discovery. Identified fungal strain could possibly be a new producer of a known bioactive compound. Fungal species is mostly determined by reproductive spore because of its stability in form and size (Kohlmeyer 1979). Even after availability of many conventional methods, molecular scientists choose to identify the fungal strain by microscopic morphological characteristics of the spores though many fungus show similar spore forms. Yet, not all the times fungal strains isolated from marine habitats easily produce spores in the artificial medium. Likewise, the study candidates strain 165, MF010 and MF018 taken for the current project was also found to be not producing spores so that, DNA extraction was submitted for the sequence analysis. Free living fungal strain 165 was identified as *Pseudonectria lignicola* species belongs to the genus Pseudohalonectria of Ascomycetes division of fungi kingdom. The other two sponge associated fungal strains are still unknown species of the complex genus Phoma belongs to pleosporales order.

In the process of fractionation and isolation, each batch culture was treated like a fresh fungal strain in case of strain 165 where it was almost equal to fractionating 5 individual fungal strains. At first strain 165 was taken for isolation of bioactive compounds. Since there was no finding of unique compound in the individual fractionating method except demethyl Nectriapyrone A found be isolated from one of the batch culture of strain 165, batch cultures were individually processed for ethyl acetate extraction, evaporated and stored while waiting for the following batch cultures. Later, the ethyl acetate extracts were pooled together by TLC profile. MF018 was also carried out similarly.

5.1.13 TLC-MALDI

Thin Layer Chromatography (TLC) combined with Matrix Assisted Lased Desorption Ionization is the soft ionization (MALDI) technique, was used in the analysis of fractions obtained from strain 165 ethyl acetate extract. As a trial all the 14 fractions were spotted using 2,5 Dihydroxy benzoic acid (DHB) and it was observed to be the mostly dominating mass signals from DHB related mass contamination listed. Apart from poor ionization, all the compounds present in the fractions may have the molecular formula

below 400.0 Da so that it is uncertain to differentiate with contamination. However, due to higher molecular mass, Ergosterol peroxide (451.0Da; M+Ma) and Ergosterol (397.0Da; M+H) presence in fraction G1 was confirmed by MS/MS in TLC-MALDI. Further there was not much method developments done in this technique with limited time and ESI MS was used for the rest of mass analysis.

5.1.14 Bioactivity of the study candidates:

While screening for bioactivities of candidate fungal isolates, total 12 extracts of 3 fungal isolates were tested. Three mycelium extracts from n-hexane, ethyl acetate and methanol and one ethyl acetate culture medium extract from each fungal isolates were screened against five human pathogenic bacterium and three fish pathogenic bacterium.

All the crude extracts showed antimicrobial activity against one bacterium at the least case. In the beginning it was believed that was may be due to the presence primary metabolite Ergosterol which is very important cell membrane component found commonly in fungal taxa. All the three ethyl acetate extracts showed strong antimicrobial activity especially, strain 165 against *Staphylococcus aureus* and MF strains against all three fish pathogenic bacterium. Most of the antibiotics are extracellular metabolites which are normally secreted in culture media (Bode et al. 2002)

Firstly, the antimicrobial activity against human pathogenic bacterium demonstrates the ability of the responded crude extract to consider for further cytotoxic activity test. Accordingly, ethyl acetate crude extract fractions of strain 165 were further tested for cytotoxic activity against Urinary bladder carcinoma 5637 and HaCaT cell lines. Total 6 fractions out of which Fraction 3 showed 50% cell death of HaCaT cell line with 16.95 µl/ml. and three fractions showed 50% cell death of 5637 cell lines with 18.7 µl/ml, 17.9 µl/ml and 17.3 µl/ml of Fraction 4, Fraction5 and Fraction6 respectively. These results suggested the presence of strong bioactive compounds to be present in strain 165 *Pseudohalonectria lignicola* and the compounds were found to be a previously reported tetraketide derived, alpha pyrone compounds majorly Nectriapyrone. Nectriapyrone has been previously reported for many other bioactivities listed under the section 2.8.2.4.

Further investigation regarding toxicity of these isolated compounds against normal cell lines was not performed due to lack of isolated substances.

Secondly, strong antimicrobial activity against fish pathogenic bacterium observed from the ethyl acetate crude extracts of sponge associated fungal strain MF010 and MF018 reveals that extra cellular metabolites support for successful life not only of the marine fungi but also benefiting the host sponges in their native environment among the predators. The compounds responsible for such bioactivity were found to be a previously reported phytotoxin Ascochitine, Ascochital, Ascosalitoxin and Hyalopyrone. Altogether, Polyketides are the large family of bioactive substances from the fungi kingdom provides natural product scientists either a new producer or new chemical structures for ecological benefits.

5.1.15 Bioactive compounds isolated

Pyrone derivatives: Nectriapyrone A was a major secondary metabolite isolated from this new producer *Pseudohalonectria lignicola*. In 1975 this compound was first isolated by Susan T Carey and MSR Nair group from Gyrostroma missouriense (Carey et al. 1975) and later isolations have been listed in Table 2under the section 3.1.9.6. isolation of the Nectriapyrone from the culture of a unidentified marine fungus of the sponge Stylotella sp. had been obtained along with analog α-pyrones Demethylnectriapyron and Nectriapyrone and B (Abrell et al. 1994). While Nectriapyrone has not been reported from any Pseudohalonectria species so far, along with Nectriapyrone other closely related pyrone analogs Vermopyrone, Nectriapyrone B, 5 methyl mellein, Helicascoide A or B and Helicascolide C have been also identified in all the culture batches of *Pseudohalonectria lignicola*. On the other hand, Demethyl Nectriapyrone was identified in only one of the culture batch. Basically, Nectriapyrone can be divided into two isoprene units and when it was first isolated by Nair and Carey 1975, it was assumed to be a monoterpenoid and it was reported to incorporate (2-‘4C) mevalonic acid. The results of feeding experiments with acetate and methionine lead to labeling patterns for Nectriapyrone which was indicative of a tetraketide biosynthesis with two C-alkylation and an O-methylation (Avent et al. 1992). However, A minor metabolite, Vermopyrone,

co-occurs with Nectriapyrone in Gliocladium vermoesenii (Avent et al. 1992). This compound possesses an acetyl side chain in place of the butenyl side-chain of Nectriapyrone. Vermopyrone has an oxygenation pattern which is not derivable by a simple polyketide biosynthesis. The metabolite may, however, be derived by cleavage of the double-bond of Nectriapyrone (Avent et al. 1992). It is interesting to note that the acetyl side chain arises from the methylation of an acetate unit and a subsequent oxidation, i.e., not all CH3CO units in polyketide are, a priori, derived biosynthetically from an acetate unit (Horst-Robert Schutte 2009).

Sterol derivatives: Ergosterol and Ergosterol peroxide were isolated from the ethyl acetate extracts of strain 165, MF 010 and MF018. GC-MS analysis of ethyl acetate crude extracts of strain 165 determined the presence of Dehydroergosterol as dehydroergosterol 3, 5 dinitrobenzoate and Dihydroxyergosterol. Compounds isolated at the nonpolar region of semi preparative chromatography with RP C18 column afforded to isolate dehydroergosterol peroxide and dihydroxyergosterol peroxide. Sterols are isoprenoid-derived molecules that have essential functions in eukaryotes in general (Benveniste 2004). It is well known that Ergosterol is one of the most important components in fungal membranes, is involved in numerous biological functions (Bard et al. 1993). Ergosterol has recently been used as a biomass indicator to compare the growth of different arbuscular mycorrhizal (AM) fungi (Hart & Reader 2002) the other study report says in contrast that Ergosterol is not a suitable biochemical marker for estimating the biomass of AM fungi and that the comparison of biomass between different fungal taxa is very difficult using any kind of currently available biochemical marker (Olsson et al. 2003). However Ergosterol has been used to indicate the fungal biomass in soil (Grant & West 1986). The Ergosterol biosynthesis pathway is a complex route in which about 20 enzymes are involved (Da Silva Ferreira et al. 2005). However, reaction sequences for Ergosterol biosynthesis downstream in the pathway from lanosterol are specific to fungal taxa (Alcazar-Fuoli et al. 2008). Ergosterol peroxide and other Ergosterol analogs are the intermediate products of Ergosterol biosynthesis.

Sesquiterpene: Beta Eudesmol was identified by GC-MS analysis of ethyl acetate extract of MF 010. Molecular mass and the UV spectrum of compound isolated from MF010 as well as MF018 by semi preparative chromatography at Rt 12.5 min (MF010) and Rt 13.1min (MF018). Due to low in quantity it was not extended for further structural elucidation. Sesquiterpenes are the C15 member of the terpenoid class of natural products, Besides its potential roles in plant defense, b-eudesmol is also known to have various beneficial effects on human health and is considered to be a lead compound for treating epileptic seizures (Chiou et al. 1997) angiogenic diseases (Tsuneki et al. 2005). Although beta eudesmol is obtained majorly from plant source, it has been previously isolated from marine derived fungus *Beauveria feline* (Vita-Marques et al. 2008) and many other marine fungus.

Azaphilones: Unidentified sponge associated fungal strains MF 010 and MF018 showed a very strong antibiotic activity against Gram-negative fish pathogenic bacteria and moderate activity against human pathogenic Gram-positive bacteria. Culture medium was extracted against ethyl acetate and the crude extract was subjected to HPLC. Remarkably, there were hardly 8-12 peaks was observed. It is also noteworthy to mention that one majorly dominating peak present in MF 010 was missing in MF018. But the antibacterial activity was equally strong by both fungal candidates with 32 mm of Inhibition zone especially against fish pathogenic bacterium. Either one of the compound or group of compounds would be responsible for such strong bioactivity. As there were only few peaks in the crude profile, it was not so necessary to involve series of separation methods like column chromatography, HPTLC followed by semi preparative chromatography. For the isolation of all eight major compounds, ethyl acetate extract was evaporated to dryness and mobile phase 11 was added gently to the top layer and tilted around to get the soluble compounds into the mobile phase. The entire compounds were isolated from this mobile phase solution by repeat injections in semi preparative chromatography. The major unique compound present in MF010 was isolated and found to be an unstable co eluting double peak in the analytical HPLC submitted for NMR. Eventually, structure elucidation of this major peak isolated from MF 010 represents the previously isolated Bicyclic Azaphilones with aliphatic side chain Ascochitine and

aromatic aldehyde Ascochital. Ascochitine is a well known phytotoxic fungal metabolite which was previously isolated from terrestrial *Ascochyta* species (Bertini 1956) (Turner & Aldridge 1983) (Beed et al. 1994) and for the first time Ascochitine was identified along with new aromatic aldehyde Ascochital from marine fungus *Kirschsteiniothelia maritime* (Kusnick et al. 2002) (Osmanova 2011). But, most recent Ascochyta species of marine fungus found to be producing Aschochita *Salicorniae Magnus*, 1902 (Seibert et al. 2006) From many studies, Ascochitine and Ascochital are known to cause necrosis in leaves, stems of the plant by a typical symptom of brown spots, damaging cell membranes (Beed et al. 1994) (Marcinkowska et al. 1991) (Kusnick et al. 2002) (Muria-Gonzalez et al. 2015) (Kim et al. 2016). Apart from Phytotoxicity, In silico experiments identified Ascochitine from the marine fungus *As. salicorniae* as a potential inhibitor of protein phosphates (Seibert et al. 2006) It was found to inhibit the enzymatic activity of mPTPB (IC50 = 11.5 μM) and PTP1B (IC50 = 38.5 μM). Protein phospha-tases have emerged as promising drug targets for a wide range of diseases. For example, protein tyrosine phosphatase 1B (PTP1B) is involved in type 2 diabetes (Johnson et al. 2002). Ascochitine producing Ascochyta species are recently being used as Bioherbicides for weeds in European corps (Cristofaro et al. 2011). Talking about the benefits of fungal sources of these Azaphilones, both terrestrial and marine fungus gets protected in their own environments by other pathogens due to the antibacterial activity of these compounds. Previous reports say that Ascochitine producing fungus has both antibacterial and antifungal activities. In contrast, MF010 showed only anti bacterial activity against Gram-negative bacteria. Also, while talking about Ascochyta genus are the major source of Ascochitine and Ascochital, in the current work, produced by strain MF010 that belongs to phoma genus of pleosporales order. Species of Ascochyta and Phoma share morphological and physiological features, and produce similar disease symptoms on plants. Due to lack of reliable morphological characters to distinguish the two genera as well as a high degree of environmental variation, systematic of these genera has never been fully resolved1. This has resulted in unclear taxonomic placement with many species having both Ascochyta and Phoma names (Kim et al. 2016).

Ascosalitoxin, a precursor of Ascochitine was also isolated from both MF010 and MF018 at Rt 18.1 and 18.9 respectively. Likewise, m/z 225 of peak eluted at Rt 14.1 matching to that of Hyalopyrone which is commonly co occurring along with Ascochitine in many species, but unfortunately due to the low quantity it was not submitted for further structure elucidation. However, it is yet unclear that in spite of the presence of Ascosalitoxin precursor and other co occurring compounds in both the MF strains, the major secondary metabolite Ascochitine and Ascochital still missing in MF018 but present in MF010.

The reason for the co-elution of Ascochitine and Ascochital in MF010 could be an unstable keto-enol tautomerism in presence of methanol. In here the mobile phase used on the top layer of ethyl acetate crude extract contained methanol moreover, the mobile phase used to semi preparative consist of methanol. Figure 86 shows the color difference in the extracts of MF strains may be due to the presence and absence of yellow pigment Ascochitine. Ethyl acetate and methanol extracts were also had the same color difference. It is interesting to note that there was a previous study has a similar statement regarding Ergosterol was produced by one isolate but was absent in another isolate of the same species, for example, in *H. appendiculata* and *Z. maritimum* (Kirk Jr et al. 1974).

Figure 86: Color difference in the n-hexane extract obtained from MF010 and MF018 mycelium indicates the presence of phytotoxic yellow pigment Ascochitine

5.1.16 Biosynthesis of polyketide isolated:

Fungal secondary metabolites are the end product of various metabolic pathways in fungal organisms. Polyketides are the large family of diverse group of secondary metabolites consisting of simple structure to maximum complex lactones with great ecological impacts. Aromatic polyketides play key role in drug discovery, pigments as well as toxins. In the current work, strain 165 found to be producing Alpha pyrone derivatives. Feeding experiments of Nectriapyrone in the fungus *Gliocladium vermoesenii* with 13 C-labeled acetate and methionine and the resulting Labeling patterns indicated the biosynthesis of a tetraketide, revealed that Nectriapyrone arising from a tetraketide core with two methylations (Carey et al. 1975) (Sympson TJ 1980) where it was believed to be a monoterpenoid by incorporating [2-‘4C]mevalonic acid. A minor metabolite, Vermopyrone co-occurs with Nectriapyrone possesses an acetyl side-chain in place of the butenyl side-chain of Nectriapyrone. Vermopyrone is not an individual secondary metabolite produced instead it could the cleavage of Nectriapyrone (Avent et al. 1992).

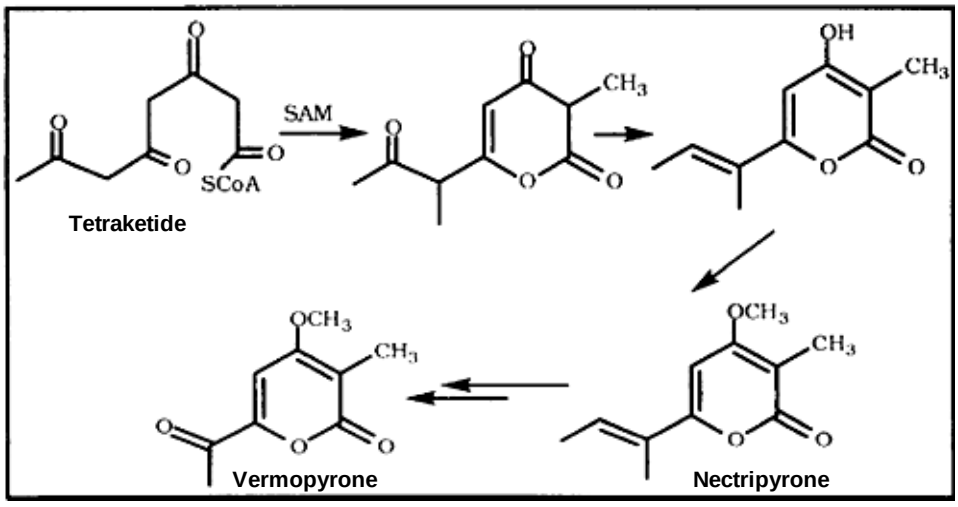


Figure 87: Schematic diagram of biosynthetic pathway of tetraketide derived Nectriapyrone and Vermopyrone. Biosynthetic pathway obtained from (Mach & Zeilinger 1998)**.**

Moreover, sponge associated fungal strain MF010 afforded to isolate a hexaketide Azaphilones Ascochitine, Ascochital, Ascosalitoxin and hyalopyrone. In terms of

Ascochital and Ascochitin, evidences presented by L.Colombo and group as represented in the Figure 88 that the first step of the biosynthesis of Ascochitine, a metabolite of *Ascochyta fabae Speg.*, involves methylation of a hexaketide, followed by cyclization, reduction to the aldehyde, dehydration, and eventually oxidation of the methyl group at C (7) to a carboxyl group (Colombo et al. 1979).

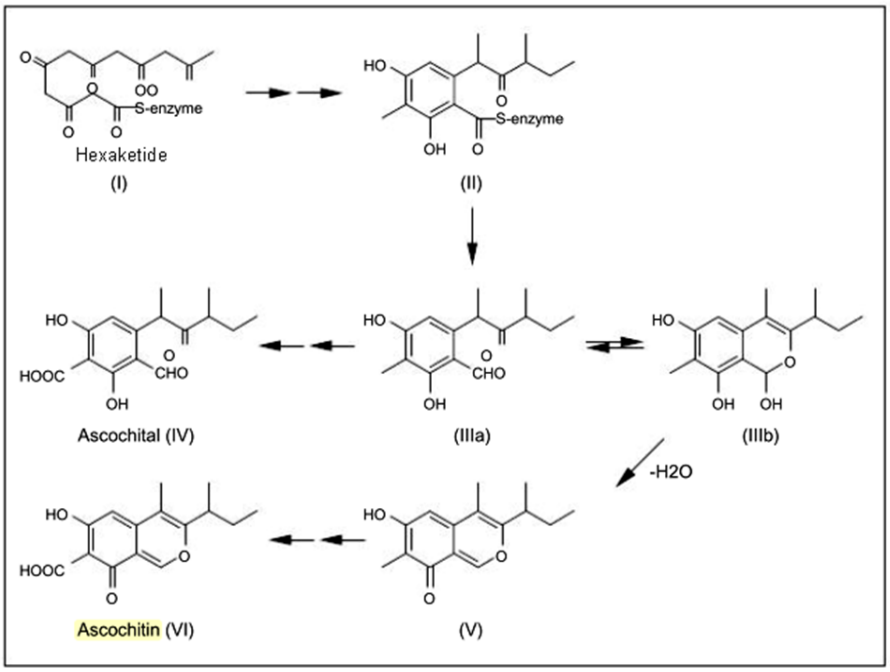


Figure 88: Schematic diagram of biosynthetic pathway of phytotoxic hexaketide derived Ascochitine and Ascochital. Pathway obtained from Colombo et al. 1980.

Due to the structural Similarity of the two compounds it can be assumed that Ascochital also follows similar biosynthetic pathways of course. The basic structure of the two substances is derived from a Hexaketide into which three-C1 Units are introduced (II). After cleavage of the enzyme results in a ketoaldehyde (IIIa) formation, from which by oxidation of the methyl group bonded cyclically to form Ascochital. Apparently, from the equilibrium reaction of the keto aldehyde with its cyclic half acetal (IIIb) is produced by dehydrating an intermediate (V). Ascochitin represents the oxidation of the methyl group. Structure confirmation by a 13C, NMR analysis of [1-13C] - and [1, 2-13C2]-acetate- and [Me-13C] methionine-derived Ascochitine confirms its origin from a single

hexaketide chain and three Cl units. An ortho-quinone-methide structure is assigned on the basis of the 13C–1H NMR coupling patterns (Colombo et al. 1980).

5.2 PART - II

5.2.1 Growth curves

The growth curves for the cultivation of *Verminephrobacter tuberculata* (*V. tuberculata*) and *Verminephrobacter eiseniae* (*V. eiseniae*) are shown in Figure 89 and Figure 90. The course of the growth of the two organisms is similar for the first 80 h of the cultivation. Past that point *V. tuberculata* shows only a slight further increase in growth up to an OD of 0.32 (medium value) while *V. eiseniae* reaches an average maximum OD of 0.74. Furthermore the course of the OD of the *V. eiseniae* samples is typical for a growth curve. There is a lag phase at the beginning of the cultivation, a log phase during which the main increase in OD occurs and a stationary phase when the growth stagnates. In comparison the graph of *V. tuberculata* shows a very short log phase and once the maximum OD is reached it decreases right away and then increases again. This course is not common for a growth curve. The anew increase of the OD might be caused by the lyses of dead cells or by restarting growth. By looking at the OD it is not possible to tell which of the two options is actually happening. In general the time required for the cultivation of both organisms is rather long for a microorganism cultivated under its intended preferred conditions. The same applies to the length of the lack phase and the maximum OD reached, even that of *V. eiseniae*, is low considering the set up was not meant to be a cultivation under stress or low-nutrient conditions.

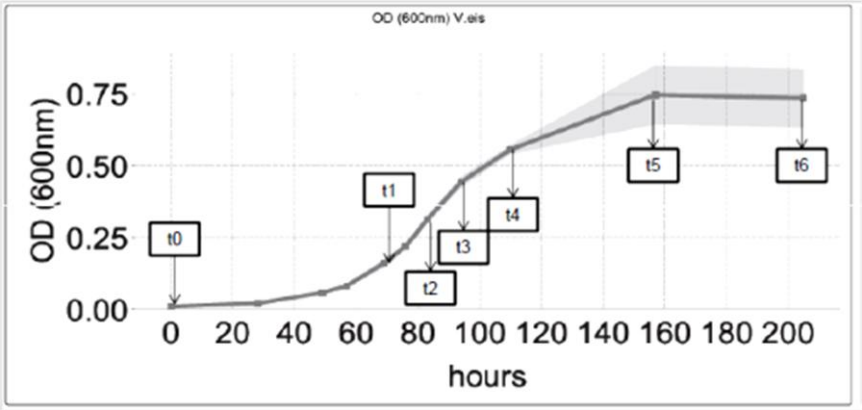


Figure 89 : Growth curves of *V. eiseniae*. The shaded area indicates the variation of the four different batches. The drawn lines represent the average values of the four batches. The arrows show the points of time at which samples were taken (t0 – t6).

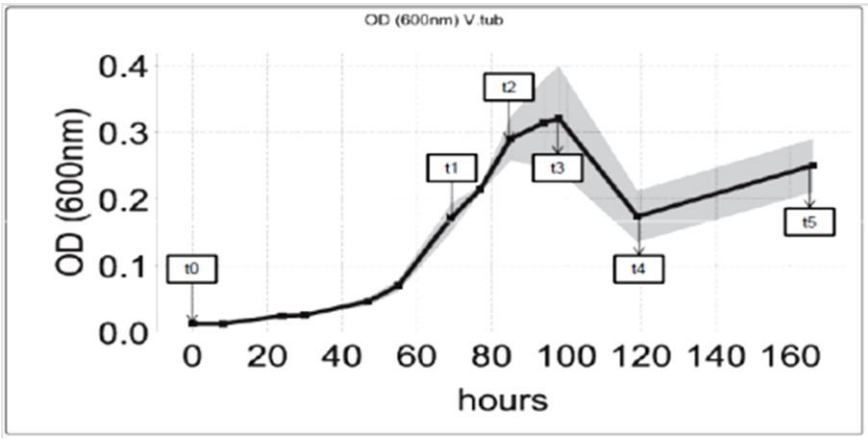


Figure 90 : Growth curves of *V. tuberculata*. The shaded area indicates the variation of the four different batches. The drawn lines represent the average values of the four batches. The arrows show the points of time at which samples were taken (t0 – t5).

5.2.2 Identified extra cellular metabolites

With the help of our in-house database 28 metabolites were identified in the extracellular samples of *V. eiseniae* and 30 in those of *V. tuberculata*. Additionally 7 and 12 unknown metabolites were found in the samples of *V. eiseniae* and *V. tuberculata* respectively.

Since the cultivation was conducted in a complex medium the evaluation of the integrals was done in a relative manner. This means no concentrations were calculated from the areas, but the areas of the integrals were plotted against the time. To improve

comparability all values were converted into a format in which they represent the area of a signal caused by one proton of the respective substance.

Table 8: List of identified and unidentified metabolites

Verminephrobacter eiseniae		***Verminephrobacter tuberculata***	
identified	**Unidentified**	**Identified**	**Unidentified**
Pyruvate	Unknown A	Pyruvate	Unknown A
Acetate	δ = 5.41 ppm	Acetate	δ = 5.41 ppm
Lactate	Unknown D	Lactate	Unknown D
Formiate	δ = 3.97 ppm	Formiate	δ = 3.97 ppm
Glucose	Unknown E	Glucose	Unknown E
Fructose	δ = 2.75 ppm	Fructose	δ = 2.75 ppm
Trehalose	Unknown G	Trehalose	Unknown G
Alanine	δ = 1.12 ppm	Alanine	δ = 1.12 ppm
Glutamic acid	Unknown K	Glutamic acid	Unknown H
Glycine	δ = 3.583 ppm	Glycine	δ = 1.10 ppm
Aspartic acid	Unknown L	Aspartic acid	Unknown K
Proline	δ = 2.93 ppm	Proline	δ = 3.583 ppm
Asparagine	Unknown Q	Asparagine	Unknown L
Leucine	δ = 1.375 ppm	Leucine	δ = 2.93 ppm
Histidine		Histidine	Unknown M
Valine		Valine	δ = 2.86 ppm
Isoleucine		Isoleucine	Unknown N
Lysine		Lysine	δ = 2.62 ppm

5-Oxoproline Methionine Threonine Tryptophan Tyrosine Phenylalanine Dihydroxyacetone Adenosine Ethanol Acetone		5-Oxoproline Methionine Threonine Tryptophan Tyrosine Phenylalanine Dihydroxyacetone Adenosine Ethanol Acetone Acetoacetate 3-hydroxybuyrate	Unknown O δ = 2.61 ppm Unknown P δ = 2.49 ppm Unknown Q δ = 1.375 ppm

5.2.3 Carbon-sources

The employed R2A medium contained multiple carbon sources which were taken up at different points of time and with differing preferences. Glucose, fructose, trehalose, pyruvate, acetate, formate and lactate are the identified carbon sources. As illustrated by the diagrams below pyruvate and acetate were taken up first by both organisms. The levels of pyruvate and acetate detected exhibit an extensive drop during the lag and starting log phase. Lactate and formate exhibit a remarkable drop as well but they are only present in very small amounts already in the initial solution (t0). When comparing the patterns of the consumption by *V. eiseniae* and *V. tuberculata* it becomes evident that they are predominantly similar for acetate, lactate and formate. However, there is a difference in the uptake of pyruvate. *V. tuberculata* consumes it faster than *V. eiseniae*

but at the end of the cultivation no pyruvate is left in the extracellular samples of both organisms.

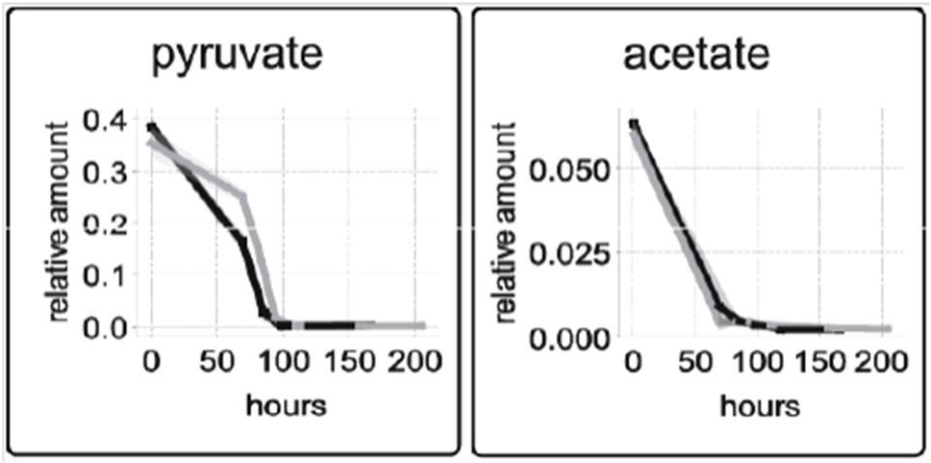


Figure 91: Relative amounts of pyruvate and acetate present in the extracellular samples of *V. eiseniae* (grey) and *V. tuberculata* (black). The relative amounts are given in unit areas. The drawn lines represent the average values of the four batches.

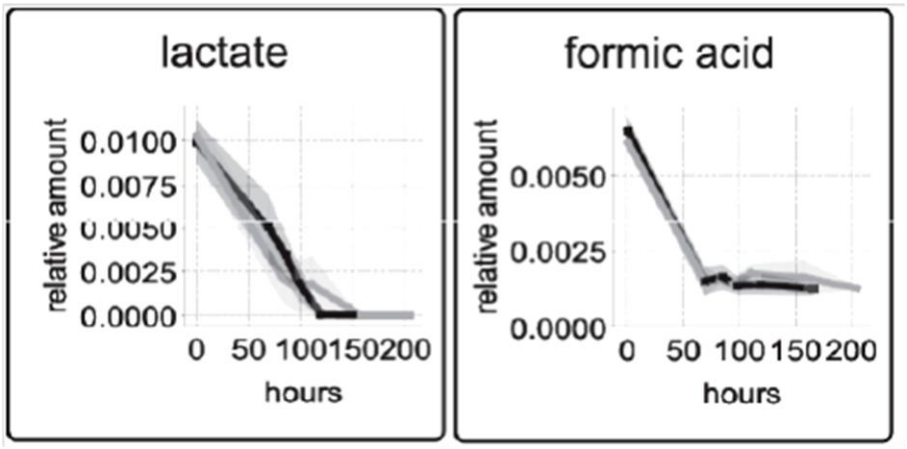


Figure 92: Relative amounts of lactate and formic acid present in the extracellular samples of *V. eiseniae* (grey) and *V. tuberculata* (black). The relative amounts are given in unit areas. The shaded area indicates the variation of the four different batches. The drawn lines represent the average values of the four batches.

The pattern of the uptake of glucose which is usually the preferred C-source does not show the remarkable drop observed for the previously discussed C-sources. But again a difference between the two organisms is exhibited upon the uptake. *V. tuberculata* starts consuming glucose right from the beginning of the cultivation. The amount of glucose absorbed during the lag and log phase is rather small, but there is a more substantial

drop during the late log phase in the amount of glucose present in the extracellular samples of *V. tuberculata* and in the samples taken at t4 and t5 basically no glucose is detected. The pattern of the glucose consumption by *V. eiseniae* is quite different. During the lag and log phase virtually no changes in the amount of glucose present in the solution are monitored. Then there is a slight drop in the last third of the log phase and a considerable drop during the late log phase, the retardation phase. But at the end of the cultivation about 25 % of the glucose observed at t0 is still present. The patterns of the uptake of fructose of both organisms are rather similar. The main phase of fructose absorption is the log phase. Up to t3 about 75 % of the fructose present in the initial solution is taken up and no more significant changes are observed from this point on until the end of the cultivation at t5 and t6 respectively.

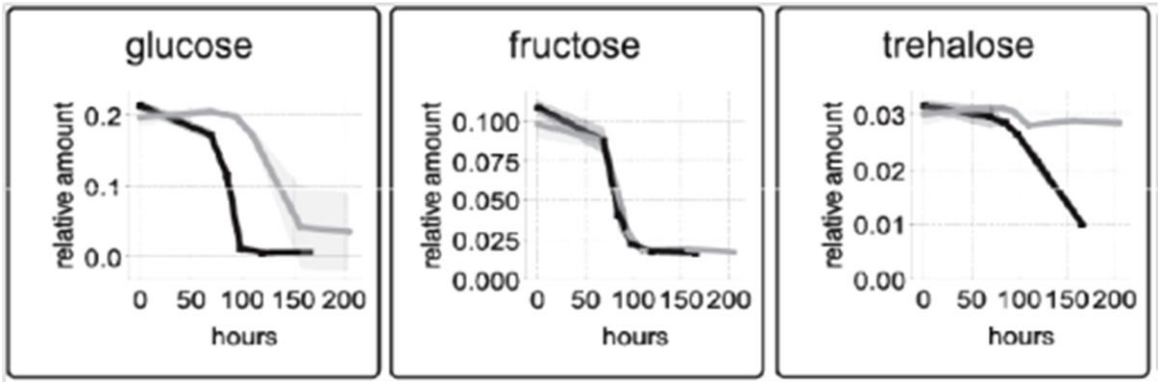


Figure 93: Extracellular samples of *V. eiseniae* (Grey) and *V. tuberculata* (Black). The relative amounts are given in unit areas. The shaded area indicates the variation of the four different batches. The drawn lines represent the average values of the four batches.

The last C-source also available to V. eis and V. tub in this medium is trehalose. The diagram shows that V. eis barely uses any of the trehalose. V. tub absorbs some of the trehalose but up to the late log phase the amount of trehalose in the solution drops not even by a third. However, between t4 and t5, the phase of the possibly restarting growth, it drops by more than a third which contradicts the assumption that the new increase in the OD between these two points of time could be caused by the lyses of dead cells.

These observations lead to the assumption that pyruvate and acetate are the preferred C-sources of both *Verminephrobacter* species. *V. tuberculata* also readily uses glucose and fructose once the amount of the preferred carbon sources drops down significantly. In contrast *V. eiseniae* favors using fructose over glucose which is surprising since glucose usually is the C-source assimilated first by microorganisms. Research in the KEGG database led to the presumption that *V. eiseniae* lacks the enzyme PTS-Glc-EIIA which is the glucose-specific IIA component of the phosphotransferase system (KEGG PATHWAY n.d.). The PTS is an active transport system for example for sugars. The lack of this enzyme might lead to difficulties in the uptake of glucose. *V. eiseniae* has the ability to generate ABC-transporters for glucose and fructose (KEGG PATHWAY n.d.). This is possibly the way how glucose is taken up by the cells at a latter point of the cultivation. The disadvantage of ABC-transporters is that they require ATP and therefore energy to carry substances across the membrane.

5.2.4 Amino Acids

Additionally to the C-sources, the medium contained several amino acids. A total of 17 amino acids were identified in the NMR spectra. The following diagrams show that all amino acids are absorbed by *V. eiseniae* and *V. tuberculata* at different levels during the cultivation.

Alanine, Glutamic acid, Glycine, aspartic acid and Proline show the most extensive drop over the course of the first sampling points. The pattern of the consumption of Ala shows that *V. tuberculata* absorbs about 80 % of the Ala present in the medium during the lag and log phase of its growth. At t3 there is almost no Ala left in the samples of *V. tuberculata*. In comparison the drop is less extensive in the samples of *V. eiseniae*. *V. eiseniae* consumes about 50 % of the initial Ala present in the solution during the lag and log phase and at t4 again almost no Ala is detected in the samples. The patterns of the uptake of Glu show a similar course. In case of *V. tuberculata* 75 % of the initial amount of Glu present is absorbed during the lag phase and the beginning log phase and nearly no Glu is detected in the samples taken at the end of the log phase. The fact that the amount of Glu present in the samples increases from t4 to t5 might be caused

by the lyses of dead cells or by the secretion of Glu by living cells. In the samples of *V. eiseniae* about 80 % of the Glu are taken up upon the lag and log phase. At the end of the log phase virtually no Glu is left in the medium. In case of Gly just under 50 % of the initial amount present in the samples was taken up during the first 70 h (lag and starting log phase) of the cultivation of *V. tuberculata*. No more Gly was detected at the end of the late log phase. In the samples of *V. eiseniae* the main drop in the amount of Gly present in the extracellular samples happened in the middle of the log phase (t2 - t3) and equaled about one third of the initial amount of Gly. From the end of the log phase onwards no more Gly was found in the *V. eiseniae* samples.

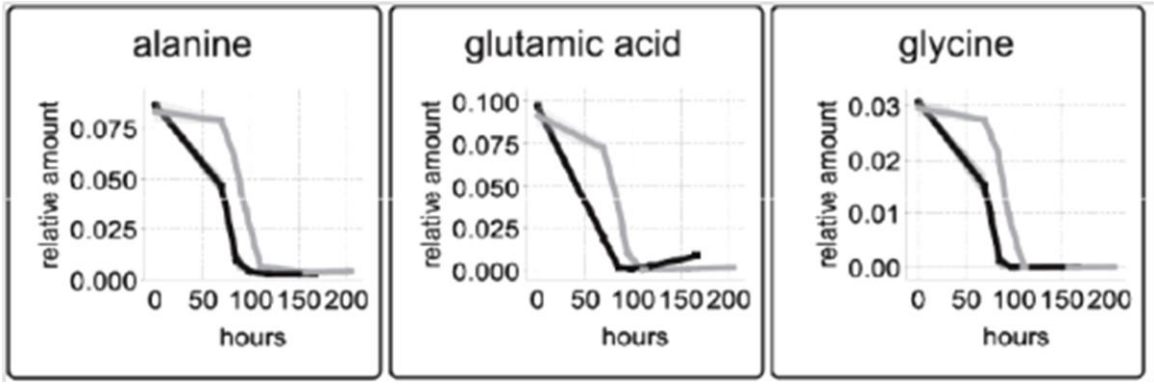


Figure 94: Relative amounts of Alanine, Glutamic acid and Glycine present in the extracellular samples of *V. eiseniae* (grey) and *V. tuberculata* (black). The relative amounts are given in unit areas. The drawn lines represent the average values of the four batches.

The absorption pattern of Asp shows that at the end of the log phase about 20 % of the primary amount of Asp is still available in the samples of *V. tuberculata*. In contrast to the previously discussed amino acids, in case of Asp small amounts of the amino acid were still found at later points of the cultivation (t3 – t5). This is true for *V. tuberculata* as well as for *V. eiseniae*. But again the pattern of the consumption differs during the first part of the cultivation. The samples of *V. eiseniae* show the most remarkable decrease in the amount of extracellular Asp during the second half of the log phase whereas the most extensive drop in the samples of *V. tuberculata* happens during its lag and log phase. The data for Proline exhibits a substantial decrease in the amount of extracellular Pro during the late log phase of *V. tuberculata*. From t3 onwards the amount stays the

same, about one fourth of the initial amount. The pattern of Pro consumption by *V. eiseniae* shows the same path.

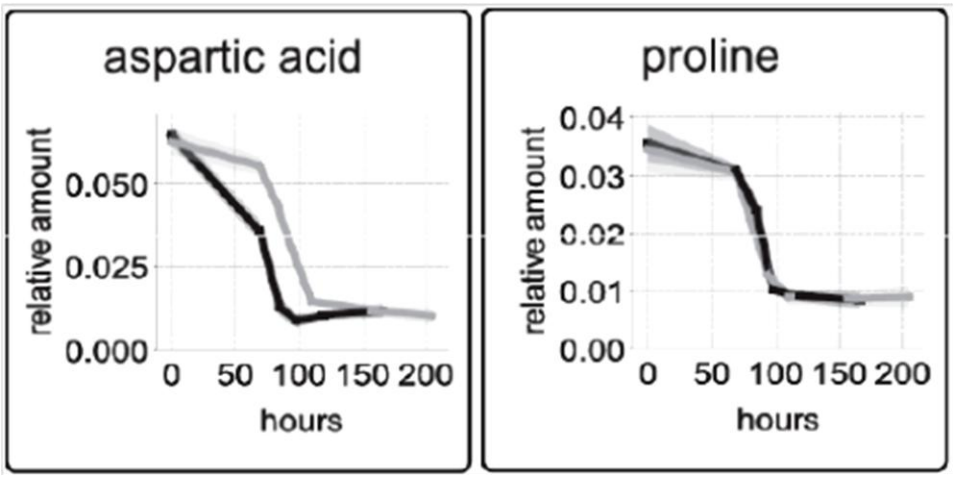


Figure 95: Relative amounts of aspartic acid and Proline present in the extracellular samples of *V. eiseniae* (grey) and *V. tuberculata* (black). The relative amounts are given in unit areas. The shaded area indicates the variation of the four different batches. The drawn lines represent the average values of the four batches.

The amount detected at t2 is slightly below that of *V. tuberculata* and the amount found at the end of the log phase is slightly above but the general pattern and the amount present at the end of the cultivation is the same.

Taken together these results show that Ala, Glu, Gly and Asp are taken up by *V. tuberculata* and *V. eiseniae* to an extensive degree during the lag phase, early log phase and mid log phase. For *V. eiseniae* the same applies to Pro, but for *V. tuberculata* Pro is different than the other amino acids discussed so far. The major uptake of Pro happens in the late log phase. Still it is obvious that these five amino acids are important for the growth of both *Verminephrobacter* species. This observation is congruent that Ala, Asp and Glu support the growth of *V. eiseniae* and *V. tuberculata* (Lund et al. 2011) . They do not comment on the effect of Gly and Pro on the growth of the two species. But at least Gly seems to be just as beneficial for the successful cultivation of *V. eiseniae* and *V. tuberculata* as the other three amino acids. A reason for the strong uptake of Ala might be the fact that it is easily converted to pyruvate (KEGG PATHWAY n.d.). Since pyruvate is apparently the preferred C-source of the two *Verminephrobacter*

species Ala might serve as an additional source of carbon. Gly can also be transformed into pyruvate via Ser (KEGG PATHWAY n.d.). Only two enzymes are involved in this conversion so Gly might too be used as a source for pyruvate or carbon respectively. It is important to notice as well that the R2A medium contains no serine. This means that Gly is not only a possible source of carbon but also the simplest way to gain access to Ser. The relevance of Glu and Asp for the growth of *V. eiseniae* and *V. tuberculata* is not surprising since they both play important roles in the conversion or generation of metabolites involved in the citrate cycle (KEGG PATHWAY n.d.), like fumarate, oxaloacetate and 2-oxoglutarate. In addition Glu has an essential function in the metabolism and especially the biosynthesis of other amino acids as it serves as the main donor and acceptor of amino groups.

The remaining 12 amino acids exhibit a different pattern of consumption. The pattern of the uptake of Asn shows just a single remarkable decrease of about 50 % in the amount present in the extracellular samples of *V. tuberculata*. This drop happens during the lag phase and the beginning of the log phase afterwards only small oscillations are observed. *V. eiseniae* absorbs Asn over a longer course of time, namely the entire lag and log phase. The amount of Asn taken up equals about 60 % of the initial amount detected. In the samples of both organisms Asn is found at a level of about 40 % of the primary amount up until the end of the cultivation. In case of Leu the extracellular amount detected drops in rather equal steps during the lag phase, log phase and starting lyses of *V. tuberculata*. In contrast *V. eiseniae* consumes Leu only slowly during the lag phase and the first two thirds of the log phase and then takes up more than 50 % of the remaining leucine upon the late log phase. At the end of the cultivations the samples of *V. eiseniae* and *V. tuberculata* both show a marginal increase in the amount of extracellular Leu and a final level of about one third of the primary amount was found. The pattern of the uptake of His shows, that in this case *V. tuberculata* absorbs little amounts of the amino acid during its lag and log phase and then more remarkable amounts during the retardation phase and the starting lyses. *V. eiseniae* also consumes only small amounts of His during the lag phase and the first half of the log phase and more extensive amounts during the second half of the log phase. The level of

extracellular His then slightly rises again in the samples of both organisms and at the end of the cultivations still about half of the initial amount is detected.

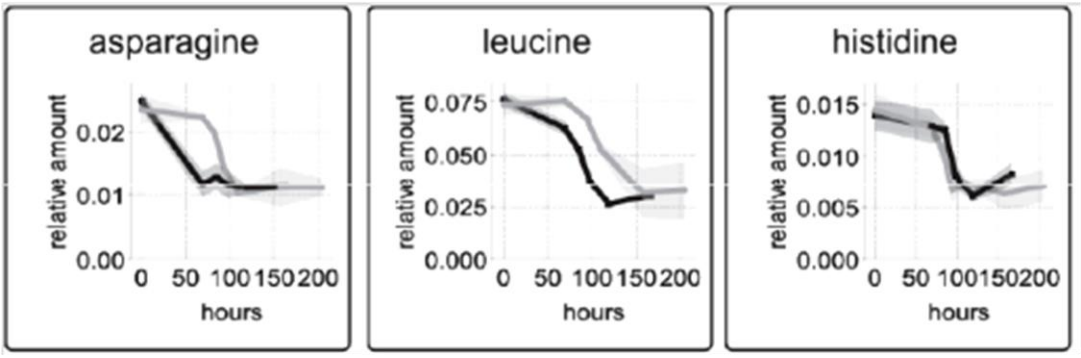


Figure 96: Relative amounts of Asparagine, leucine and Histidine present in the extracellular samples of *V. eiseniae* (grey) and *V. tuberculata* (black). The relative amounts are given in unit areas. The shaded area indicates the variation of the four different batches. The drawn lines represent the average values of the four batches.

According to the findings of Lund et al. Asn is beneficial for the growth of both *Verminephrobacter* species (Lund et al. 2011) . The fact that Asn is absorbed by *V. eiseniae* and *V. tuberculata* is congruent with their observations but a more complete uptake of the Asn available would be expected for an amino acid with a strong positive effect on the growth of a microorganism. When compared to the previously analyzed amino acids it becomes evident that leucine is not of such an importance as they are during the cultivation. Again there is the fact that it is not taken up completely or up to a very low level so apparently *V. eiseniae* and *V. tuberculata* have no need for Leu in high amounts. The fact that the uptake of Leu of *V. eiseniae* increases in the second half of the log phase might be explained by the fact, that the amounts of the preferred C- and energy sources is at a very low level at that point. Either Leu is used as an additional C-source or the biosynthesis of Leu is stopped due to low energy levels in the cells. Previous results have shown that His does not have a beneficial effect on the growth of the two *Verminephrobacter* species (Lund et al. 2011). The data presented here is in agreement with those earlier findings. The total amount of His taken in by both *V. eiseniae* and *V. tuberculata* is small despite the slightly stronger uptake during the log phase and retardation phase respectively.

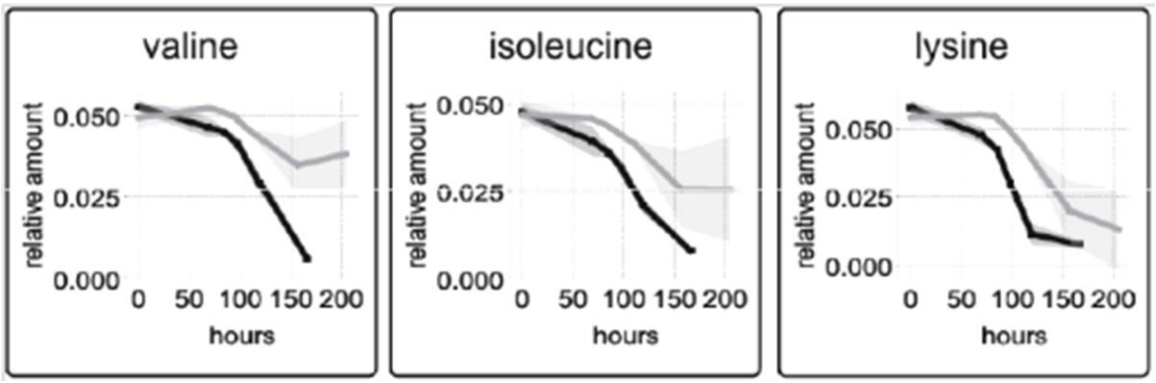


Figure 97: Relative amounts of Valine, Isoleucine and lysine present in the extracellular samples of *V. eiseniae* (grey) and *V. tuberculata* (black). The relative amounts are given in unit areas. The shaded area indicates the variation of the four different batches. The drawn lines represent the average values of the four batches.

The amino acids Ile and Val display a similar pattern in the decrease of the levels of extracellular amounts present. This is true for both species although different patterns are observed for the two different species. In case of *V. tuberculata* just a slight decrease of the levels of Val and Ile is monitored upon the lag phase, log phase and retardation phase followed by a more grave decrease by more than 50 % of the primary amounts detected during the starting lyses and possibly restarting growth. The absorption pattern of Val and Ile of *V. eiseniae* is different. *V. eiseniae* barely consumes the two amino acids upon the entire lag and log phase, but then there is a drop during the retardation phase. But even this drop equals only ~25 % of the initial amount. Val and Ile both exhibit a slight increase in their extracellular levels during the stationary phase of *V. eiseniae* growth. Also the final amounts detected in the samples are still quite high. This is not the case for the samples of *V. tuberculata*. The primary levels of Lys are comparable to those of Val and Ile but the exhibited consumption pattern of this amino acid is dissimilar for both organisms. *V. tuberculata* gradually takes up Lys upon the entire cultivation. Only the decrease in the extracellular Lys levels upon the starting lyses is somewhat larger than during the other phases of growth. In case of *V. eiseniae* basically no changes in the levels of Lys are observed upon the lag phase and the first half of the log phase. From mid-log phase onwards the levels go down gradually and as

for *V. tuberculata* there is a decrease during one phase, the retardation phase, that is slightly graver than the changes upon the other phases.

According to the previously mentioned paper by Lund et al. Isoleucine and Valine both do not have a positive effect on the growth of *Verminephrobacter eiseniae* and *Verminephrobacter tuberculata* (Lund et al. 2011). The data presented here is in agreement with that statement especially in case of *V. eiseniae*, since it takes in less than 50 % of the available Val and Ile. What is somewhat odd is the fact that *V. tuberculata* absorbs the main portion of Ile during the starting lyses and possibly restarting growth. Since lyses means that the cells are starting to die there should not be a sudden need for an amino acid. Usually the need for amino acids is the biggest during the log phase when the main part of the growth takes place. The sudden increase in the uptake of Ile only makes sense if the increase of the OD between t4 and t5 is caused by restarting growth. The fact that Val is mainly consumed upon this phase of the cultivation supports this assumption. When compared to Ile and Val the pattern of the uptake of Lys exhibits a more consistent need for Lys upon the entire cultivation. But investigations of the possible metabolic pathways of Lys did not afford any pathways of special importance or use for yielding energy, so the absorbed Lys is most likely employed in the biosynthesis of proteins. Additionally these investigations revealed that both *Verminephrobacter* species are able to synthesize Lys as long as Asp is accessible (KEGG PATHWAY n.d.). The same applies to Val, Leu and Ile, but in their cases Gly or Thr and pyruvate need to be present (KEGG PATHWAY n.d.).

The remaining six amino acids identified in the samples include the three aromatic amino acids and Thr, Met and 5-oxoproline. The absorption patterns of 5-oxoproline of *V. eiseniae* and *V. tuberculata* are very similar. The amount of 5-oxoproline decreases gradually upon all the phases of the cultivation. Only *V. tuberculata* exhibits one drop in the extracellular 5-oxoproline levels that is graver than the others and this drop happens upon the starting lyses. In total the extracellular 5-oxoproline levels decrease by more than two thirds in the samples of both species upon the entire cultivation. In case of methionine the two species exhibit different consumption patterns. *V. tuberculata* shows

a gradual decrease upon the lag phase, log phase and starting lyses and then a more extensive drop in the amount of extracellular Met between t4 and t5, the phase of the possibly restarting growth. In contrast *V. eiseniae* reveals little changes in the amount of Met present in the samples taken during the lag and log phase. Upon the retardation phase the amount of extracellular Met then decreases by about two thirds. No changes in the amount of Met present were observed during the stationary phase. In case of Thr *V. tuberculata* absorbs only little amounts during the lag and the log phase. Upon the retardation phase and the starting lyses the level of Thr in the samples then drops extensively. Just a small decrease in the Thr level is detected during the possibly restarting growth and the final amount found in the samples equals about one third of the initial amount recorded. The consumption pattern of Thr by *V. eiseniae* also shows only small changes in the amount of extracellular Thr during the lag and log phase. Then the level of Thr drops remarkably upon the retardation phase and only a bit during the stationary phase. The amount of Thr detected in the *V. eiseniae* samples at t6 (~1/2 of the initial amount) is a bit higher than that found in the samples of *V. tuberculata* at t5.

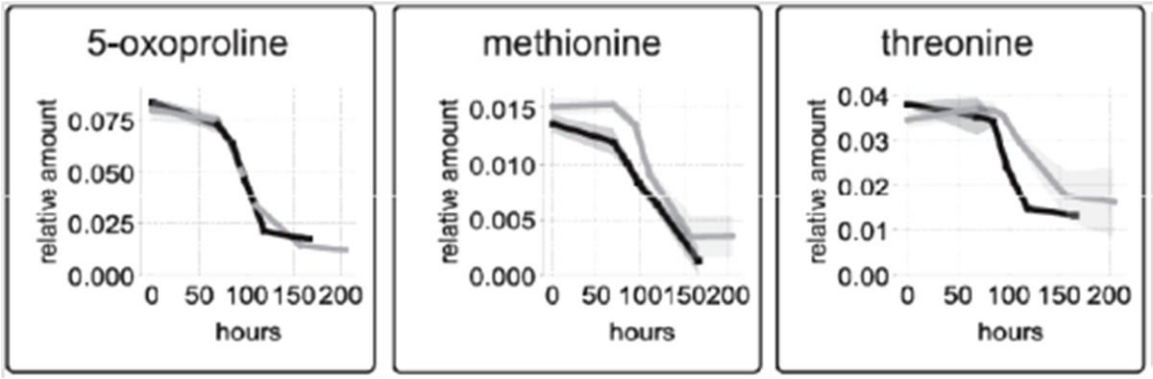


Figure 98: Relative amounts of 5-oxoproline, Methionine and Threonine present in the extracellular samples of *V. eiseniae* (grey) and *V. tuberculata* (black). The relative amounts are given in unit areas. The shaded area indicates the variation of the four different batches. The drawn lines represent the average values of the four batches.

Methionine and Threonine are two more amino acids mentioned to not be supportive for the growth of *V. eiseniae* and *V. tuberculata* (Lund et al. 2011). Since the levels of Thr and Met initially present in the medium employed here are quite low, especially those of Met, and they are still not completely absorbed upon the course of the cultivation, this

proposition is congruent with the data presented above. In comparison 5-oxoproline is taken up faster and to greater extends. Deduced from those observations it can be concluded that 5-oxoproline is either of more importance in the metabolism of the two *Verminephrobacter* species or it cannot (easily) be synthesized by them unlike Thr and Met (KEGG PATHWAY n.d.; KEGG PATHWAY n.d.). Two of the aromatic amino acids show a similar absorption pattern, but the pattern of the uptake of Trp exhibits different characteristics than the others. The initial Trp level in the samples is already quite low and it decreases by only about one third upon the entire cultivation of both *Verminephrobacter* species. The primary amount of Tyr in the samples is a bit higher than that of Trp but still low in comparison to other amino acids. In the samples of *V. tuberculata* the Tyr level drops gradually during all the phases of its growth. The final level of Tyr equals less than 25 % of the initial level. In case of *V. eiseniae* the amount of extracellular Tyr decreases just somewhat during the lag and log phase. Then a more extensive drop in the Tyr levels is registered upon the retardation phase. During the stationary phase a slight increase in the amount of Tyr present in the samples of *V. eiseniae* was recorded. At the end of the cultivation less than 30% of the primary amount of Tyr is still found in the samples. The highest primary levels of all the aromatic amino acids were recorded for Phe. In case of Phe *V. eiseniae* and *V. tuberculata* both exhibit a gradual decrease in the extracellular amounts present in the samples.

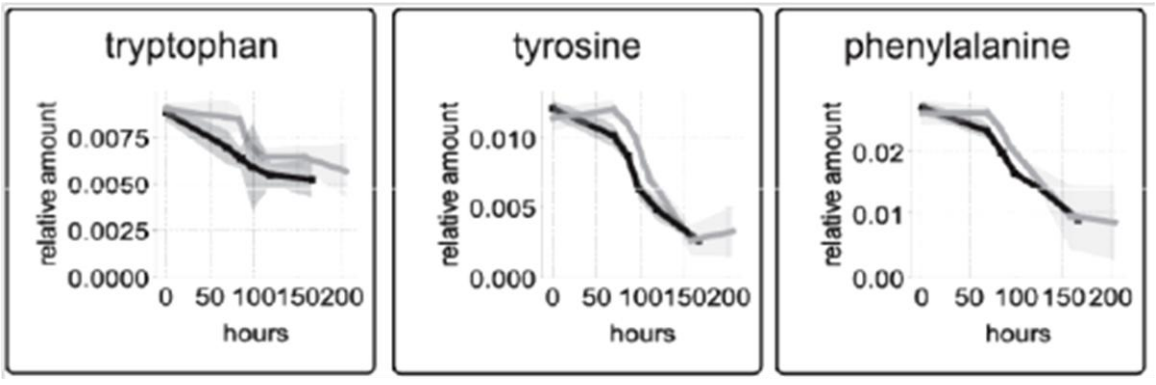


Figure 99: Relative amounts of tryptophan, tyrosine and phenylalanine present in the extracellular samples of *V. eiseniae* (grey) and *V. tuberculata* (black). The relative amounts are given in unit areas. The shaded area indicates the variation of the four different batches. The drawn lines represent the average values of the four batches.

Although the levels of Phe in the samples of *V. eiseniae* are all somewhat higher than those of *V. tuberculata* in all the samples taken during the lag and log phase, the levels found at the end of the cultivation are about the same. The extracellular samples of both *Verminephrobacter* species reveal one drop in the amount of Phe recorded that is more extensive than the others. This drop happens during the restarting growth of *V. tuberculata* and the retardation phase of *V. eiseniae* respectively. At the end of the cultivation about 30 % of the initial amount of Phe is found in the samples of *V. eiseniae* as well as *V. tuberculata*.

Since the levels of Trp do not change significantly in the samples of both species it can be concluded that Trp is not of specific relevance for the growth of *V. eiseniae* and *V. tuberculata*. Tyr and Phe seem to be more relevant since ~70 % of the available amount of both amino acids is taken up by the two organisms over the course of the cultivation. Still, in comparison to the amino acids discussed earlier (e.g. Ala, Glu, Gly, etc) which have been proven to support the growth of *V. eiseniae* and *V. tuberculata*, Phe and Tyr are taken up slower and to less extend. Those facts lead to the assumption that Phe and Tyr do not enhance growth but are consumed if available rather than wasting energy for their biosynthesis. All three aromatic amino acids can be synthesized by *V. eiseniae* (KEGG PATHWAY n.d.) and probably *V. tuberculata* as the consumption patterns of both species are quite similar.

5.2.5 Other metabolites

Besides the previously discussed substances some others have been identified in the samples of the two *Verminephrobacter* species. Dihydroxyacetone is one of those substances. The primary amount of dihydroxyacetone detected in the samples is quite low. But changes over time are still detectable. In case of *V. tuberculata* the level of dihydroxyacetone slowly decreases during the lag phase, the log phase and the retardation phase and then drops remarkably upon the starting lyses and the possibly restarting growth. The final amount of dihydroxyacetone in the samples of *V. tuberculata* equals about 25 % of the initial amount. *V. eiseniae* exhibits a different consumption pattern. A remarkable decrease is exhibited during the lag phase and the beginning of

the log phase as well as during the late log phase. Only a slight decline is registered upon the other phases of the cultivation. At the end of the cultivation about 25 % of the primary amount is still present. Another identified substance is adenosine. *V. tuberculata* gradually consumes adenosine upon the entire cultivation although the drop during the lag phase and the starting log phase is slightly bigger than the decrease upon the other phases of growth. In total only somewhat over 50 % of the adenosine available are taken up. Again a dissimilar pattern is observed for the samples of *V. eiseniae*. Only a slight decline is registered during the lag phase and the beginning log phase which is followed by a grave decrease upon the main log phase. Also after about two thirds of the log phase is completed the lowest adenosine level of ~17 % of the primary amount present in the samples is reached. Upon the late log phase and the retardation phase the level of adenosine slightly increases again up to ~29 % of the initial level. The final amount measured at t6 equals about 24 % of the primary amount. A substance that exhibits a completely different pattern of changes in the extracellular levels present in the samples than the previously discussed substances is ethanol.

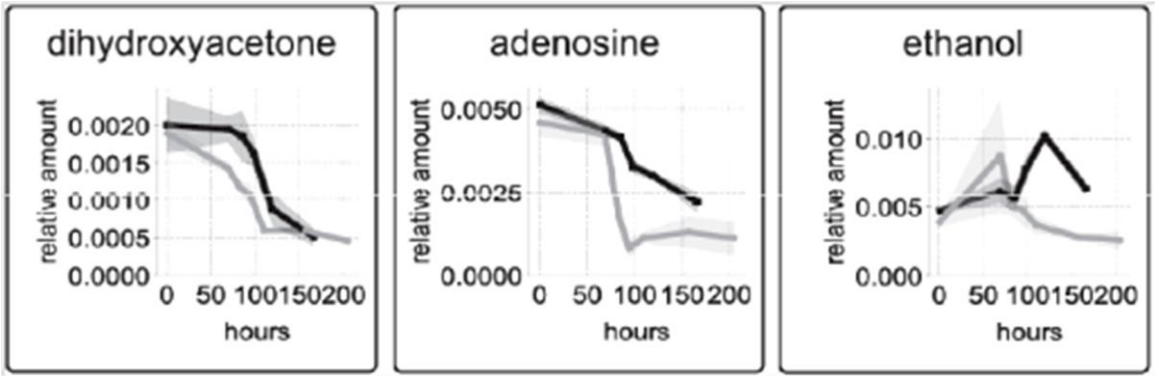


Figure 100: Relative amounts of dihydroxyacetone, adenosine and ethanol present in the extracellular samples of *V. eiseniae* (grey) and *V. tuberculata* (black). The relative amounts are given in unit areas. The shaded area indicates the variation of the four different batches. The drawn lines represent the average values of the four batches.

In case of *V. tuberculata* the amount detected in the samples increases during the lag and beginning log phase and then decreases somewhat during the main log phase followed by a strong increase upon the starting lyses and the potentially restarting growth. At t4 the level of ethanol detected is about twice as high as the initial level. Upon

the potentially restarting growth the level drops again and the final amount found in the samples is ~36 % higher than the primary amount. In the samples of *V. eiseniae* the strongest increase is exhibited during the lag and starting log phase. The amount of ethanol detected doubles upon those phases. During the main log phase the ethanol level drops again remarkably. During the second half of the log phase only a slight decrease is recorded and upon the retardation phase the amount of ethanol found in the samples of *V. eiseniae* gradually declines. Barely any changes are observed during the stationary phase and the final level of ethanol equals ~50 % of the primary level.

Although different uptake patterns are observed for dihydroxyacetone for the two *Verminephrobacter* species both exhibit a low level of dihydroxyacetone in the samples of their respective last sampling points. This indicates that dihydroxyacetone is a useful nutrient for both organisms and research in the KEGG database showed that it can be incorporated into the glycolysis (KEGG PATHWAY n.d.). However, the low initial levels and the moderate speed of uptake when compared to other nutrients like pyruvate and Glu lead to the presumption that dihydroxyacetone is not among the substances of high importance for the growth of *V. tuberculata* and *V. eiseniae*.

Adenosine can be converted into multiple compounds relevant for the metabolism of bacteria. It can either become part of the DNA or RNA biosynthesis of the organism or it can be employed in the energy storage and transport of the cell. Since ATP is the main energy currency of living cells the need for adenosine is higher than for the other nitrogenous bases. This fact might explain why adenosine is supplemented in the first place while the other nitrogenous bases are not contained in the medium. Altogether inquiries resulted in the fact that all nitrogenous bases can by biosynthesized from various amino acids and intermediates of the pentose phosphate pathway (KEGG PATHWAY n.d.; KEGG PATHWAY n.d.). In comparison the pattern of the consumption and release of ethanol indicates that *V. eiseniae* and *V. tuberculata* are capable of the synthesis and consumption of the potential C-source which is congruent with the information found in the KEGG database (KEGG PATHWAY n.d.). The increase in the extracellular ethanol levels followed by the subsequent decrease could be explained by

an afflux in the respective pathway. However, since the ethanol found in the samples might also be an artifact of the sampling instead of being of biological origin no real assumptions can be made.

Acetone, Acetoacetate and 3-hydroxybutyrate are relevant only in the samples of *V. tuberculata*. In the samples of *V. eiseniae* only acetone is detected at all and barely any changes in its extracellular levels are observed upon the entire cultivation. Acetoacetate and 3-hydroxybutyrate are recorded merely in the samples of *V. tuberculata*. In case of the extracellular levels of acetone the samples of *V. tuberculata* show a completely different pattern than those of *V. eiseniae*. During the lag and log phase basically no changes are observed as well, but during the retardation phase the amount of extracellular acetone rises a little and then upon the starting lyses it increases extensively in the samples of *V. tuberculata*. However, the gravest increase is recorded upon the possibly restarting growth. During that phase the level rises by almost two thirds. The final amount of acetone found in the samples of *V. tuberculata* is about eight times the initial amount. The pattern of the extracellular levels of Acetoacetate is somewhat similar to that of acetone. But in this case during the lag and the log phase no Acetoacetate is present at all or the amounts present are below detection limits respectively. At the end of the retardation phase a small amount of Acetoacetate is found in the samples of *V. tuberculata*.

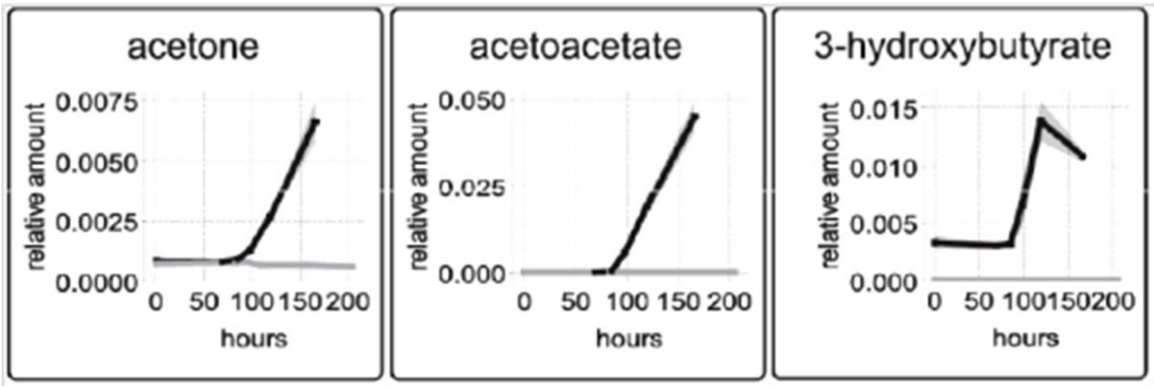


Figure 101: Relative amounts of acetone, Acetoacetate and 3-hydroxybutyrate present in the extracellular samples of *V. eiseniae* (grey) and *V. tuberculata* (black). The relative amounts are given in unit areas. The shaded area indicates the variation of the four different batches. The drawn lines represent the average values of the four batches.

The extracellular level of Acetoacetate then increases extensively by ~85 % upon the starting lyses and potentially restarting growth. In case of 3-hydroxybutyrate there is a small amount present in the samples of *V. tuberculata* right from the beginning of the cultivation. It declines somewhat during the lag and the log phase of *V. tuberculata*'s growth and then increases remarkably up to more than four times the primary amount upon the retardation phase and the starting lyses. During the potentially restarting growth the level then drops by ~16 % again.

All three compounds just discussed are part of one metabolic pathway, the degradation of ketone bodies. According to the KEGG database *V. eiseniae* is neither capable of the consumption nor of the biosynthesis of acetone which is congruent with the previously discussed observations (KEGG PATHWAY n.d.). In contrast *V. tuberculata* apparently is capable at least of the biosynthesis of acetone. Assumptions about *V. tuberculata*'s capability to degrade acetone cannot be made with the present data. Inquiries of the KEGG database also revealed the fact that *V. eiseniae* is competent of the biosynthesis of PHB (polyhydroxybutyrate) using the monomer 3-hydroxybutyrate (KEGG PATHWAY n.d.). Decrease of the 3-hydroxybutyrate in the extracellular samples of *V. eiseniae* might be the confirmation of PHB formation. The data presented here only allows for the presumption that *V. tuberculata* can synthesize Acetoacetate as well as PHB from 3-hydroxybutyrate and also degrade PHB to form back 3-hydroxybutyrate. No statement about the ability of *V. tuberculata* to degrade Acetoacetate can be made at this point.

5.2.6 Unidentified metabolites

Next some compounds that gave a signal in the NMR spectrum but could not be identified will be discussed. To differentiate between the different substances the unknowns were designated with different letters. Unknowns with the same chemical shift got the same letter in the sets of the two species. In the samples of *V. tuberculata* some unknowns were detected that were not present in the samples of *V. eiseniae*. This was not the case the other way around. A list of all the unidentified metabolites with their respective chemical shifts is given in Table 4.

The signal declared unknown A with a chemical shift of 5.41 ppm was observed in the spectra of both *Verminephrobacter* species. As illustrated in the diagram below barely any changes in the levels of unknown A in the samples of *V. tuberculata* and *V. eiseniae* are detected. However, the amount of unknown an available in the medium is as high as the initial amount of pyruvate and even higher than the primary amount of glucose found in the samples. This fact and the chemical shift of the signal indicate that unknown A is some sort of C-source or more precisely some kind of sugar. Nevertheless neither *V. eiseniae* nor *V. tuberculata* seems to be able to utilize this compound. The same, the chemical shift of the signal, the amount of substance detected in the samples, the lack of changes in the levels measured and the resulting interpretation applies to unknown D.

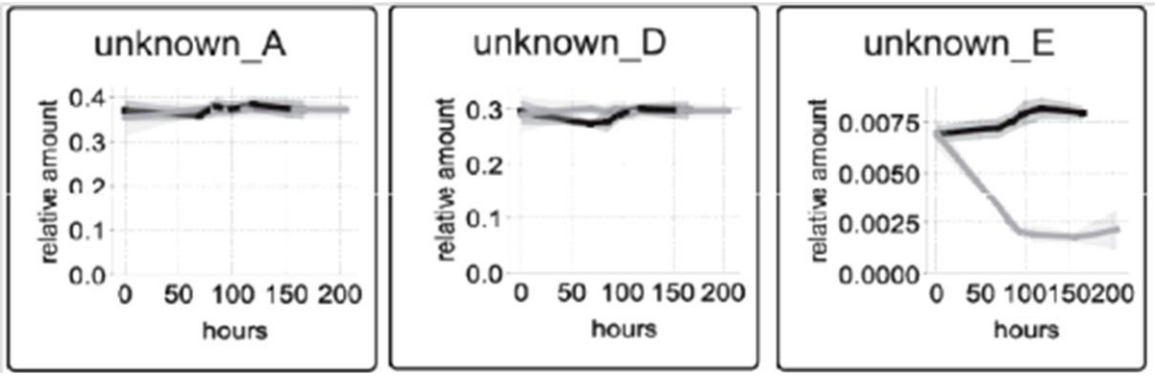


Figure 102: Relative amounts of unknown A, D and E present in the extracellular samples of *V. eiseniae* (grey) and *V. tuberculata* (black). The relative amounts are given in unit areas. The shaded area indicates the variation of the four different batches. The drawn lines represent the average values of the four batches.

The signal of unknown D is observed at δ = 3.97 ppm and it is also found in the spectra of the samples of both organisms. The third unknown detected in the samples of *V. eiseniae* and *V. tuberculata* is different. Unknown E has a chemical shift of δ = 2.75 ppm and in this case the pattern of the detected levels of the compound in the samples of *V. tuberculata* show a slight increase upon the lag, log and retardation phase and the starting lyses. Then a mere drop of the unknown E level is exhibited upon the potentially restarting growth, but the final level is still higher than the initial level. *V. eiseniae* exhibits a completely different pattern. During the lag phase and the beginning of the log

phase the level of unknown E in the samples of *V. eiseniae* drops by more than 50 %. Afterwards it declines more slowly up until the late log phase. During the retardation and stationary phase the level of unknown E only oscillates slightly. In total about 75 % of the available unknown E are taken up by *V. eiseniae*.

Derived from the two different patterns of the samples of the two *Verminephrobacter* species one can assume that unknown E is a substance that can be employed as a source of energy as well as it can be secreted into the medium in case the particular pathway it is part of is very active, causing an afflux. Thus, it presumably is a rather small, possibly an intermediate compound.

Another unidentified substance detected in the sample of *V. tuberculata* and *V. eiseniae* is unknown G with a chemical shift of δ = 1.12 ppm. In case of *V. tuberculata* the amount of unknown G registered in the samples increases remarkably during the lag and log phase and drops again extensively upon the retardation phase. The amount of unknown G detected at the end of the retardation phase is approximately the same as recorded at t0 and the level found at the end of the log phase is about four times the primary level of unknown G.

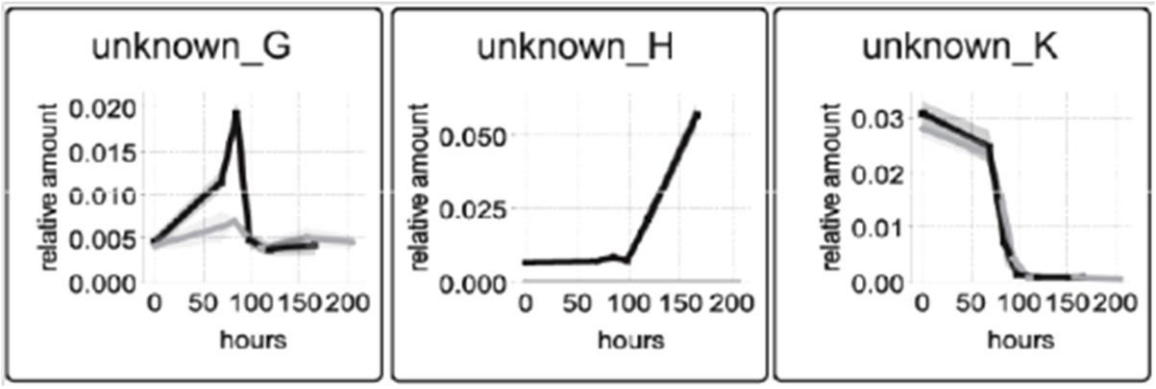


Figure 103: Relative amounts of unknown G, H and K present in the extracellular samples of *V. eiseniae* (grey) and *V. tuberculata* (black). The relative amounts are given in unit areas. The shaded area indicates the variation of the four different batches. The drawn lines represent the average values of the four batches.

Only small changes are observed upon the starting lyses and possibly restarting growth. In case of *V. eiseniae* less extensive shifts are exhibited. During the lag phase and the first half of the log phase a moderate increase in the amount of unknown G present is

observed. This increase is followed by a subsequent moderate decline in the level of unknown G in the samples of *V. eiseniae* during the second half of the log phase. Upon the retardation and stationary phase the level of unknown G merely oscillates. Unknown H is one of the unidentified compounds that were only detected in the samples of *V. tuberculata*. The level of unknown H just fluctuates somewhat during the lag, log and retardation phase. Upon the starting lyses and the possibly restarting growth the amount of unknown H found in the samples then increases extensively. The final amount is more than eight times as high as the initial amount observed. In contrast to the currently discussed unidentified substances unknown K exhibits a similar pattern for both *Verminephrobacter* species. In both cases a fast uptake of unknown K is observed during the lag and the log phase. At the end of the retardation phase of *V. tuberculata* and at the end of the log phase of *V. eiseniae* no more unknown K was detectable in the samples. Ergo it was consumed completely by both species.

Deduced especially from the pattern observed in the samples of *V. tuberculata* it can be presumed that unknown G is an intermediate or final compound of a metabolic pathway which is secreted into the medium when the metabolic rate of the respective pathway is high, resulting in an accumulation of unknown G. The reuptake of unknown G indicates that it is of use for the organism and not just a byproduct secreted into the medium as an overflow metabolite. In contrast unknown H apparently is a product of a metabolic pathway that is not valuable anymore for *V. tuberculata* or it is available in the cells to such extend that part of it is secreted. Unknown K exhibits completely different characteristics. Clearly unknown K is a nutrient of at least modest relevance for both *Verminephrobacter* species which can be concluded from the fast and complete uptake upon the lag and log phase of their growth.

The pattern of the extracellular levels of the unidentified substance unknown L is similar for *V. tuberculata* and *V. eiseniae* upon the first half of the cultivation. The chemical shift of unknown L is $\delta = 2.93$ ppm. During the lag, log and retardation phase of *V.* tub's growth the level of unknown L detected in the samples constantly rises up until a level about six times as high as the initial level is reached. In the samples of *V. eiseniae* the

level of unknown L constantly increases up until a level three times the initial level is reached. This increase happens upon the lag and log phase of *V. eiseniae*' growth. In case of *V. tuberculata* the amount of unknown L recorded in the samples then drops by ~63% upon the onset of lyses and by another ~15 % during the possibly restarting growth. In case of *V. eiseniae* the level drops by ~68 % during the late log phase and then very slightly rises again upon the retardation and stationary phase. The final levels detected in the last samples of the cultivation of both species are merely higher than the primary levels. The unidentified substance with the chemical shift δ = 2.86 ppm, unknown M, was solely found in the samples of *V. tuberculata*. During the lag and the log phase the level of unknown M detected in the samples only oscillates slightly. During the retardation phase and the starting lyses the level then increases exponentially. A ten-fold increase is observed between the amount detected at t0 and the amount present after the onset of lyses. But subsequently to the rise the level drops again extensively. The level of unknown M recorded at t5 is not even double the primary level. The metabolite unknown N has a chemical shift of 2.62 ppm and just like unknown M it was only detected in the samples of *V. tuberculata*. The level of unknown N registered in the samples slowly rises during the lag and log phase of *V. tuberculata*'s growth. Then the level drops back down upon the retardation phase to the level present at the end of the lag phase followed by a rise back up to the level observed at the end of the log phase.

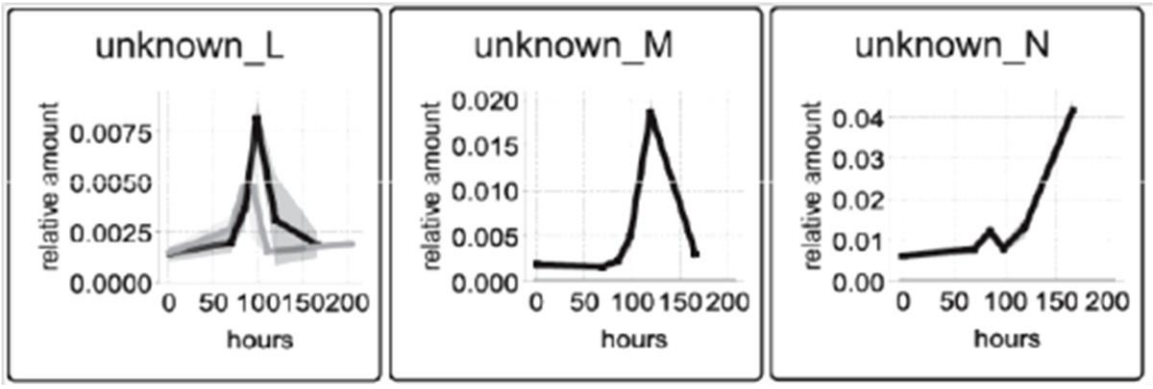


Figure 104: Relative amounts of unknown L, M and N present in the extracellular samples of *V. eiseniae* (grey) and *V. tuberculata* (black). The relative amounts are given in unit areas. The shaded

area indicates the variation of the four different batches. The drawn lines represent the average values of the four batches.

The most significant increase is detected during the phase of the potentially restarting growth. The amount found in the final samples is ~85% higher than the initial amount present. As mentioned before when unknown G was discussed the patterns of the secretion and consumption of unknown L and M suggest that the two substances are intermediate or final compounds which are secreted into the medium when the metabolic rate of their respective pathways is high, resulting in an accumulation of unknown L and M respectively. The reuptake of both substances indicates that they are not just a byproduct secreted into the medium as an overflow metabolite but are still utilized by *V. eiseniae* and *V. tuberculata*. The oscillations in the levels of unknown N suggest that the same may apply to this compound, but the extensive release of unknown N at the end of the cultivation might also be a hint that this substance is not valuable for *V. tuberculata* anymore. Especially, since it is secreted at a point when the levels of C- and energy-sources are down.

Just like unknown M and N the two unidentified compounds unknown O and P are solely detected in the samples of *V. tuberculata*. Unknown O has a chemical shift of δ = 2.61 ppm and the signal of unknown P is at δ = 2.49 ppm. Unknown O is detected at very low levels during the lag, log and retardation phase. Then the levels rise extensively upon the onset of lyses and the possibly restarting growth. The final amount of unknown O found in the samples is ~15 times as high as the initial amount detected. In case of unknown P the levels are below the detection limits during the lag, log and retardation phase of *V. tuberculata*'s growth. After the onset of lyses a considerable amount of unknown P was found in the samples and virtually no changes are observed upon the potentially restarting growth. The last unidentified substance that was observed in the samples of both *Verminephrobacter* species is unknown Q with a signal at δ = 1.375 ppm. The amount detected in the samples of *V. tuberculata* slightly drops during the lag phase and the beginning of the log phase and then rises again somewhat upon the main log phase. During the retardation phase, the starting lyses and possibly restarting growth the level of unknown Q in the samples of *V. tuberculata* gradually declines with a

slow rate. The analysis of the samples of *V. eiseniae* exhibits a very different pattern. The levels of unknown Q drop extensively by ~80 % during the lag and the log phase. Afterwards, during the retardation and stationary phase, the levels still decline, but at a much slower rate.

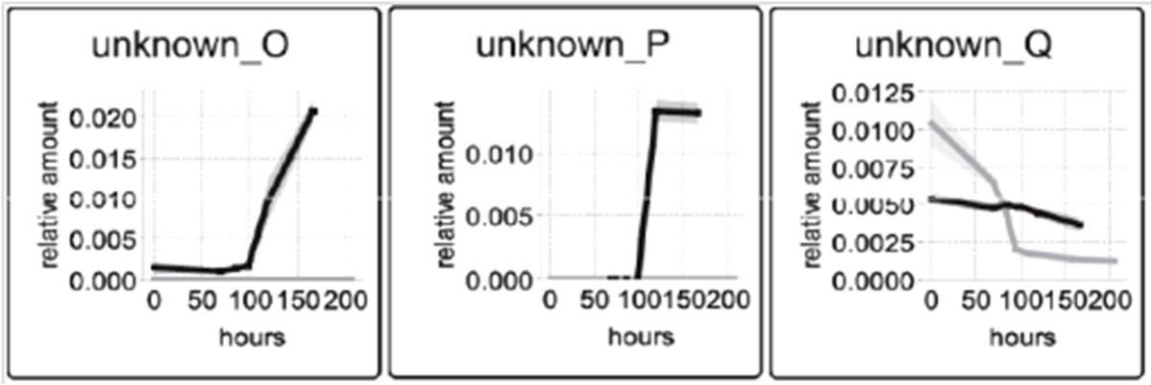


Figure 105: Relative amounts of unknown O, P and Q present in the extracellular samples of *V. eiseniae* (grey) and *V. tuberculata* (black). The relative amounts are given in unit areas. The shaded area indicates the variation of the four different batches. The drawn lines represent the average values of the four batches.

The patterns of the extracellular levels of unknown O and P indicate that the two compounds are byproducts of metabolic pathways of *V. tuberculata* and are secreted into the medium as overflow metabolites. But the sudden emergence of unknown P at t4 might also be a sign for an interrelation with the onset of cell lyses. This means that unknown P might be a product of the degradation of dead cells rather than the product of a metabolic pathway of healthy living cells. In contrast unknown Q evidently is a nutrient at least for *V. eiseniae*. The grave uptake upon the lag and the log phase is a strong indicator for that. *V. tuberculata* either does not need this compound as a nutrient or it cannot take up and/or use unknown Q as well as *V. eiseniae*.

5.2.7 Identified intra cellular metabolites:

All the intra cell samples were analyzed with three biological replicates by Gas chromatography mass spectrometer (GC-MS) and around 45 metabolites were identified in *V. tuberculata* and 40 metabolites in *V. eiseniae.* As the samples were aliquated directly from the complex media of batch cultures at time points t0 to t5 metabolites were

quantified as a relative abundance (t0-t5) against internal standard compound Ribitol dividing the average area by OD (t0-t5) value.

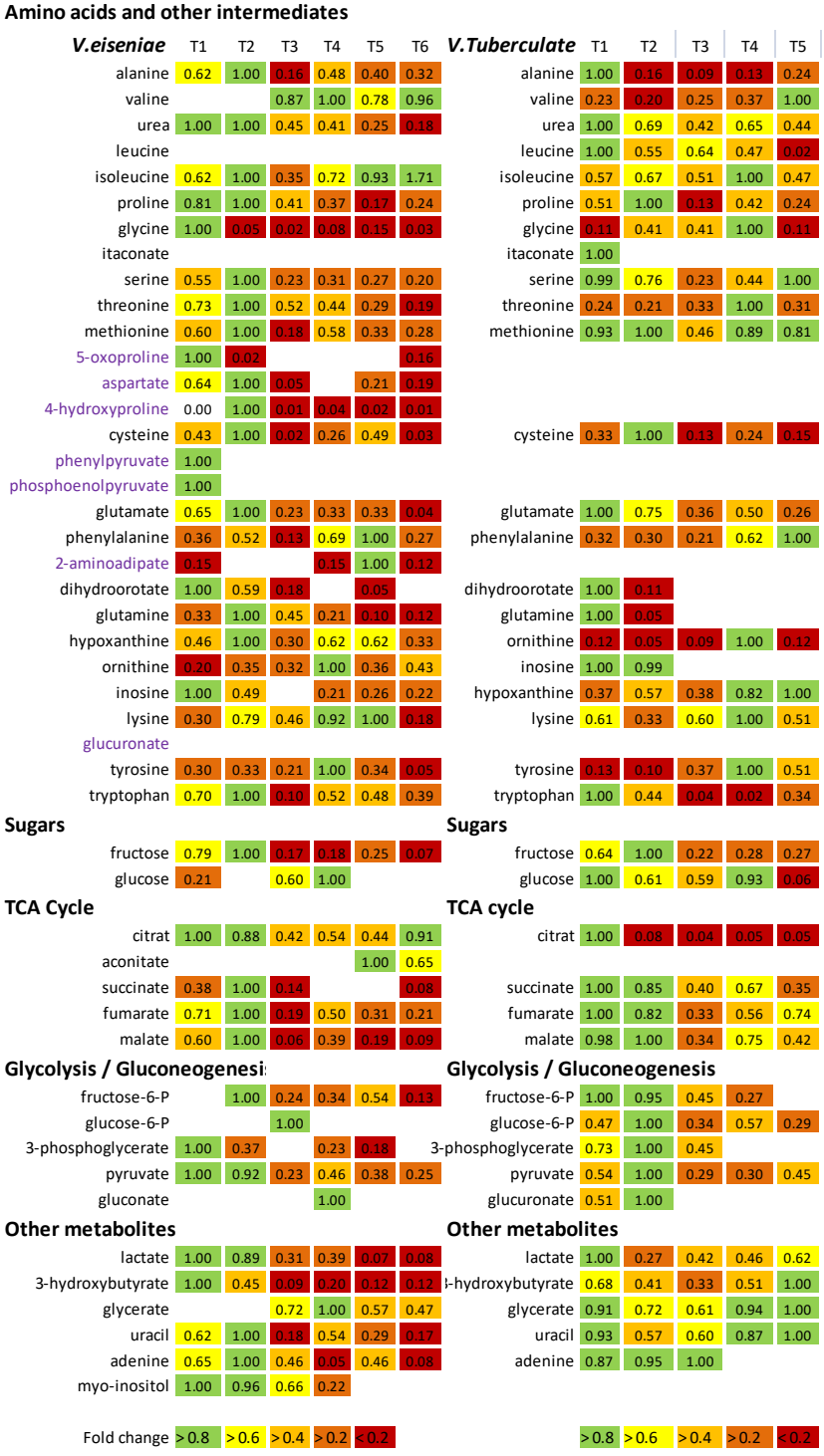


Figure 106: Heat map - metabolism pattern of *V. eiseniae* and *V. tuberculata*

The metabolism pattern of both the organisms throughout the time point are compared and presented as heat map in the Figure 106. The R2A medium contains high amounts of peptides/amino acids and carbohydrates, as well as several carboxylic acids, (e.g. pyruvate). The high concentrations of N-containing compounds partly mimics the nutritional conditions experienced by the symbionts in the nephridia. The metabolom of both *V. eiseniae* and *V. tuberculata* are compared here mainly based on Carbon metabolism and Nitrogen metabolism.

5.2.8 Carbon metabolism

Pyruvate and acetate have been detected in great amounts in the intracellular samples of both V. *eiseniae* and *V. tuberculata* bacteria and seem to be preferentially consumed by the symbionts, being depleted at t3 and t1, respectively (Figure 91). However, it seems to be slow uptake by *V. tuberculata* compare to *V. eiseniae* (Figure 91). In addition, low amount of lactate detected in the EC medium immediately utilized by the bacteria which has been noted in the IC samples at t1 further got depleted until t5. In contrast *V. tuberculata* has shown a little difference in the depletion pattern at later time points. Lactate is converted in one step to pyruvate by one of the identified lactate dehydrogenases (Veis_0025, Veis_3784; Veis_1530 and Veis_3178)* (Flavia Vianna et al. unpublished).

*Note: Proteomics data not shown here as the metabolomics data is the only relevant part to the current thesis.

Glucose and fructose were the most abundant carbohydrates in the medium, but were initially taken up by *V. eiseniae* very slowly compare to *V. tuberculata.* Beginning at t3, glucose and fructose consumption accelerated, but only about 0.5mM was consumed until late stationary phase. However, at t4 and displayed increased abundances (up to 3.15-fold).

Transaldolase B (V. eis_1779, identified and stably abundant throughout growth)* converts fructose-6-phosphate and erythrose-4-phosphate into sedoheptulose-6-phosphate and glyceraldehyde-3-phosphate. The latter is an intermediate of glycolysis,

which may proceed to final acetyl-CoA formation since all respective proteins were detected (Flavia et al. Unpublished).

Curiously, gluconeogenesis has also been implied as crucial for fitness of UPEC during infection (Alteri et al. 2009) Additionally, the same study has also proved that *in vivo* amino acids and peptides are the main carbon source for this uropathogen, suggesting a similar nutritional pattern to what was observed for *V. eiseniae* in the uptake of the different dietary sources. Additionally, the previously mentioned AUM lacks glucose or other sugars on its composition, fact that seemed to not affect its consumption by different uro-pathogen strains, suggesting that also for these bacteria sugars are not key nutrients (Brooks & Keevil 1997) (Flavia et al. Unpublished).

Talking about amino acids and peptides metabolism, it can be categorized as (i) immediately utilized (glutamate, Glycine, aspartame, Asparagine, Histidine, Proline and 5-oxoproline), (ii) moderately utilized (Methionine, Threonine, tyrosine and leucine) (iii) slowly utilized (phenylalanine, Isoleucine and lysine) and (iv) not utilized (Valine) (Fluvial et al. unpublished). But, these categories may not match in case of *V. tuberculata* as it has slow growth pattern compare to *V. eiseniae.*

Immediately utilized amino acids were taken up at a high rate and in large amounts, the majority of which being depleted when entering stationary phase. Except for Glycine and Histidine, all of these amino acids are converted via single or only two step reactions to TCA cycle intermediates (corresponding proteins identified, not shown), allowing for fast turnover in central metabolism. Glutamate was the most abundant amino acid in the culture medium. It is extensively and rapidly used by *V. tuberculata at t1* and *V. eiseniae* at t2 and half of its initial amount was already depleted at t3 in *V. eiseniae* where as in *V. tuberculata* glutamate has been depleted steadily. Glutamate is directly fed into the TCA cycle by the detected glutamate dehydrogenase (GdhA)* mediating the one-step conversion to the trycarboxylic acid (TCA) cycle intermediate 2-oxoglutarate. In addition, glutamine (not detected extracellularly) was detected intracellularly most likely produced by the detected glutamine synthetase (GlnA)* from glutamate and ammonia. Glutamine may serve as an ammonium storage or sink also for ammonia derived from

transamination reactions involving 2-oxoglutarate, which explains the presence of GlnA* throughout the whole growth. Proline and 5-oxoproline were also highly abundant amino acids in the culture medium however, Proline was not completely utilized (~40% remaining by t4) by *V. eiseniae.* In contrast, Proline has been taken up by *V. tuberculata* (~ 13% only remaining at t3). Proline is converted via L-1-pyrroline-5-carboxylate* to glutamate. 5-oxoproline may also be converted to glutamate by the detected 5-oxoprolinase (HyuAB)*, but may alternatively be channeled to glutathione biosynthesis involving the same enzyme (Van der Werf *et al*., 1971; Seddon and Meister, 1986) (Flavia et al. Unpublished). Anyway 5- oxoproline was not detected in the IC samples of *V. tuberculata.* Aspartate has been detected as maximum amount at t2 in *V. eiseniae* and immediately dropped at t3. Aspartate is converted into oxaloacetate involving the detected aspartate aminotransferase* (V. eis_2819). However, aspartate may also enter the urea cycle or may be used for cyanophycin biosynthesis (Refer 5.15 Nitrogen metabolism). IC samples from *V. tuberculata* totally did not show any aspartate. Asparagine is deaminated to aspartate by asparaginase (*V. eiseniae*_4896, identified) (Willis and Woolfolk, 1974). Glycine has been utilized by *V. eiseniae* from the culture medium immediately at t1 but in case of *V. tuberculata* Glycine can be categorized as slowly utilized amino acids as the *V. tuberculata* IC samples show maximum Glycine only at t4. Glycine is converted via serine to pyruvate involving Glycine hydroxymethyltransferase (GlyA)* and bi-functional Threonine dehydratases (IlvA)*. In addition, Glycine may also be converted to 5, 10-methylenetetrahydrofolate by the glycine cleavage complex (identified in the proteomics data not shown here) and enter the C1 metabolism. Histidine follows a series of reactions that convert it to glutamate. However, Histidine was not found in the IC samples of both organisms.

The concentration of moderately utilized amino acids remained rather constant until t3 followed by a slow decrease until depletion. For example, Threonine was consumed maximum by *V. eiseniae* at t2 itself and 40% still remaining at t4 in which already OD reached 75 %. Threonine degradation proceeds via Glycine, involving Threonine aldolase (ItaE)*, to pyruvate. Methionine is probably converted to adenosine and L-homocysteine in a series of reactions (see supplementary data for details). Even though

tyrosine seems to be utilized by *V. eiseniae* and *V. tuberculata*, the pathway for degradation of this metabolite remains to be further clarified. Tyrosine can be degraded in a series of steps into fumarate (see supplementary data for details) but none of the enzymes has been identified by proteomics methods suggesting either a different route or anabolic purposes for this compound. The branched chain amino acids leucine and Isoleucine are initially converted to the respective 2-oxo acid by the branched chain amino acid aminotransferase (IlvE)* followed by decarboxylation yielding the corresponding acyl-CoA. Nevertheless, leucine is the only amino acid was not found in *V. eiseniae* but maximum taken up by *V. tuberculata* at t1 further depleted in t2, t3 steadily.

Uptake of slowly utilized amino acids occurs only in small amounts until late stationary phase and no depletion is observed. Lysine was detected intracellularly but it remains unclear if lysine is exclusively taken up for anabolic purposes, or if it is also used for catabolism by an unknown route. The probably combined pathway for phenylalanine and tyrosine remains elusive (for details see supplementary material). However, due to the very low amount taken up, these amino acids may only serve anabolic purposes, which may also apply to the non-utilized Valine.

Besides single amino acids, the medium also contained diverse peptides, which have to be cleaved into amino acids prior to import and subsequent degradation. While small peptides may still enter the periplasmic space, cleavage of larger peptides has to take place in the extracellular space.

Amino acid metabolism is without doubt a key feature of both *V. eiseniae* and *V. tuberculata* as it seems to be for other host-associated bacteria for innumerable reasons. Serine was not part of the the culture medium but can be synthesized by the symbiont through the degradation of Threonine or Glycine, two amino acids that were actively consumed by the bacteria. That’s how Glycine has been consumed maximum by *V. eiseniae* at t1 and by *V. tuberculata* at t4 so that serine found to be maximum at t2 and t5 respectively. Additionally, amino acids seem to be the preferred carbon source for UPEC strains during host colonization indicating that these compounds can be

important for the basic metabolism of bacteria living on a urine based diet (Alteri et al. 2009) (Flavia Vianna et al Unpublished).

Amino acid prototroph has been implicated as playing an essential role in plant colonization by *Pseudomonas fluorescens* WCS365, despite the abundance of amino acids in the surrounding environment (Simons et al., 1997) and one may hypothesize a similar role for the nephridial symbionts (Flavia Vianna et al. unpublished).

5.2.9 Nitrogen metabolism

The nutritional focus on amino acid degradation results in a steadily increasing amount of intracellular nitrogen. Formation of urea is a common route of detoxification and all enzymes of the prokaryotic urea cycle were detected as well as urea within the cells. Urea can easily diffuse through the cell membranes and while some organisms excrete urea under such growth conditions (Therkildsen et al. 1997; Zech et al. 2013), Figure 103 shows in *V. eiseniae* and *V. tuberculata,* urea was only detected intracellularly displaying a decreasing internal concentration (relative abundance of 0,405 at t1 and 0,072 by t5 in *V. eiseniae* and 0,674 at t1 and 0,300 by t5 in *V. tuberculata*) suggesting that it is further metabolized. According to proteomics data, urease* was identified mediating urea cleavage to ammonia and carbon dioxide. This particular role might also be important for the symbiont establishment in the nephridia. Urea may has also been implied as a signal compound for UPEC strains to initiate colonization of the urinary tract (Withman et al. 2013) (Flavia et al. Unpublished).

In addition, urea carboxylase and amidolyase (*V. eiseniae*_3790-3792 and *V. eiseniae*_0372)* have been identified in proteomics data that convert urea to allophanate and finally ammonia and CO_2. Earthworms seem to excrete mainly ammonia and so symbionts might also play a part here in further degradation of urea to ammonia that is later excreted (Laverack, 1963) (Flavia et al. Unpublished).

Overall, in the symbiont the excess of nitrogen derived from amino acid catabolism seems to be channeled to the urea cycle that also feeds cyanophycin production. Which is a non-ribosomally syntheszied copolymer of arginine and aspartate described for

diverse Cyanobacteria and some heterotrophic bacteria (Obst and Steinbüchel, 2006). Since the amount of cyanophycin could not be determined in the metabolomics data, the balance of urea versus cyanophycin formation remains unclear at present (Flavia et al Unpublished).

5.2.10 Polyhydroxybutyrate (PHB)

PHB is a widespread energy storage compound among bacteria, normally synthesized in the limitation of inorganic nutrient conditions (Ratledge and Kristiansen 2001). The symbionts are known to be able to produce the storage compound PHB (Nicolás Pinel et al. 2008). 3-hydroxybutyrate, the monomer utilized in PHB biosynthesis was detected both extra and intracellularly. Extracellularly, the concentration of this metabolite decreases within logarithmic growth of *V. eiseniae* (t2 to t4). Still, 3-hydroxybutyrate is not completely consumed from the culture medium. Intracellularly, the concentration of this monomer decreases after t2, where only half of the initial concentration is detected confirms the formation of PHB. Conversely, *V. tuberculata* shows significant amount of 3-hydroxybutyrate at t1 and further decrease until t4 but sudden increase at t5. This may be due to the degradation of PHB by the depolymerise enzyme. At the same time, proteomics data (not shown) of *V. eiseniae* also suggests that Polyhydroxyalkanoate depolymerase (V. eis_0272), the enzyme mediating the degradation of PHB granules was also detected, suggesting the utilization and degradation of this compound when alternative energy sources are no longer available.

5.2.11 Metabolic pathways and conversions

The following Figure 104 shows the possible conversions and relations of the absorbed nutrients and secreted substances. Glucose and fructose are disintegrated to glycerinaldehyde-3-phosphate and then pyruvate upon glycolysis. The pyruvate is further converted to acetyl-CoA which usually subsequently flows into the citrate cycle where most of the energy is retrieved. Dihydroxyacetone can also become part of this pathway since it can be converted to glycerinealdehyde-3P and then pyruvate as well. Lactate can be turned into pyruvate, too. In this case the back reaction is also possible.

The same applies to Alanine. Gly can be metabolized to Ser which in turn can be transformed to pyruvate. Acetate can directly be transmuted to acetyl-CoA and then become part of the citrate cycle. Acetate and ethanol can be converted into each other via the conversion to acetaldehyde. The fairly strong rise in the levels of ethanol detected in the samples of *V. eiseniae* between T0 and T1 might be caused by this connection. The strong uptake of acetate and other nutrients that are converted to acetyl-CoA might cause an afflux in the metabolic pathways leading up to that conversion. Hence, some of the absorbed acetate possibly cannot directly be metabolized to acetyl-CoA but is transformed to ethanol and partially secreted. The acetyl-CoA that is produced from all the assimilated nutrients can either flow into the citrate cycle as mentioned before or it can be converted to Acetoacetate and then acetone or 3-hyroxybutyrate. This pathway is definitely active in the cells of *V. tuberculata*, but with the present data it is impossible to tell if *V. eiseniae* does not perform this conversions or simply just does not secret the products into the medium. The only presumption that can be made is that *V. eiseniae* cannot generate acetone since the levels of acetone do not change over the course of the cultivation and according to the KEGG database *V. eiseniae* lacks the necessary enzyme (KEGG PATHWAY n.d.).

Figure 104 shows the relations between most of the amino acids present in the medium and their possible function for *V. eiseniae* and *V. tuberculata* other than serving as building blocks in the biosynthesis of proteins. As mentioned before Glu and Asp can directly be converted to intermediates of the citrate cycle and thus can directly flow into the energy production via the just mentioned cycle. Pro can be also be integrated into this cycle since it can be transmuted into Glu. The same applies to Histidine.

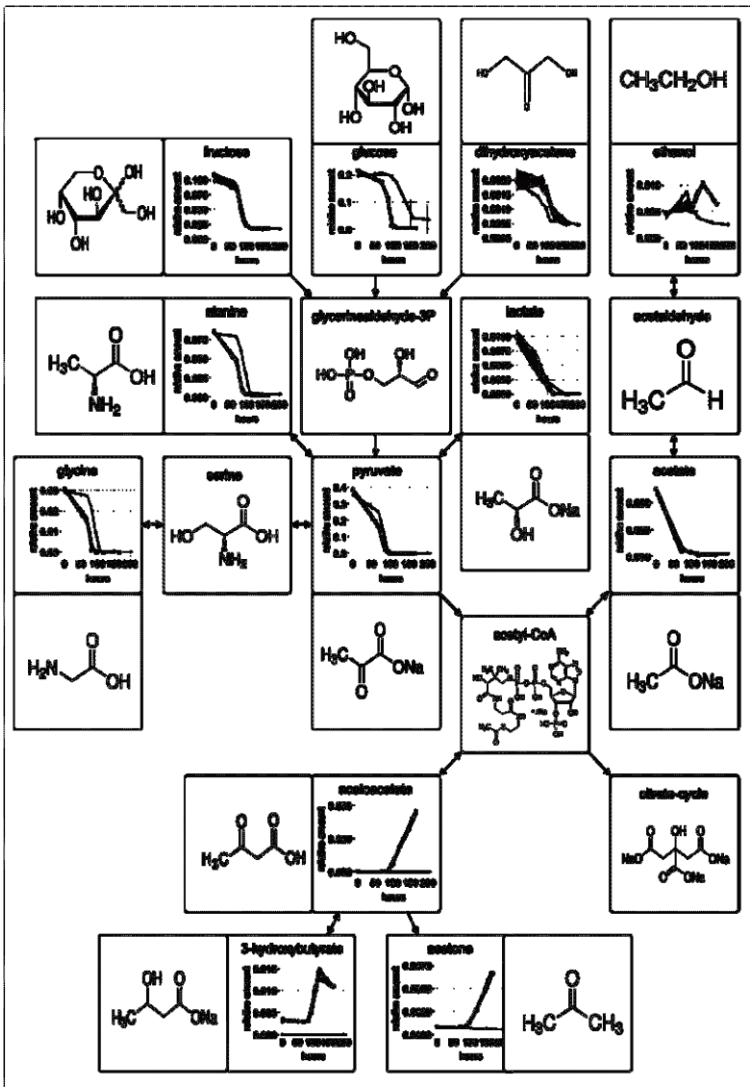


Figure 107: Possible conversions and relations of the absorbed nutrients and secreted substances *V. eiseniae* (grey) and *V. tuberculata* (black). The shown chemical structures are sourced from the websites of the KEGG database, Merck Millipore and SIGMA-ALDRICH.

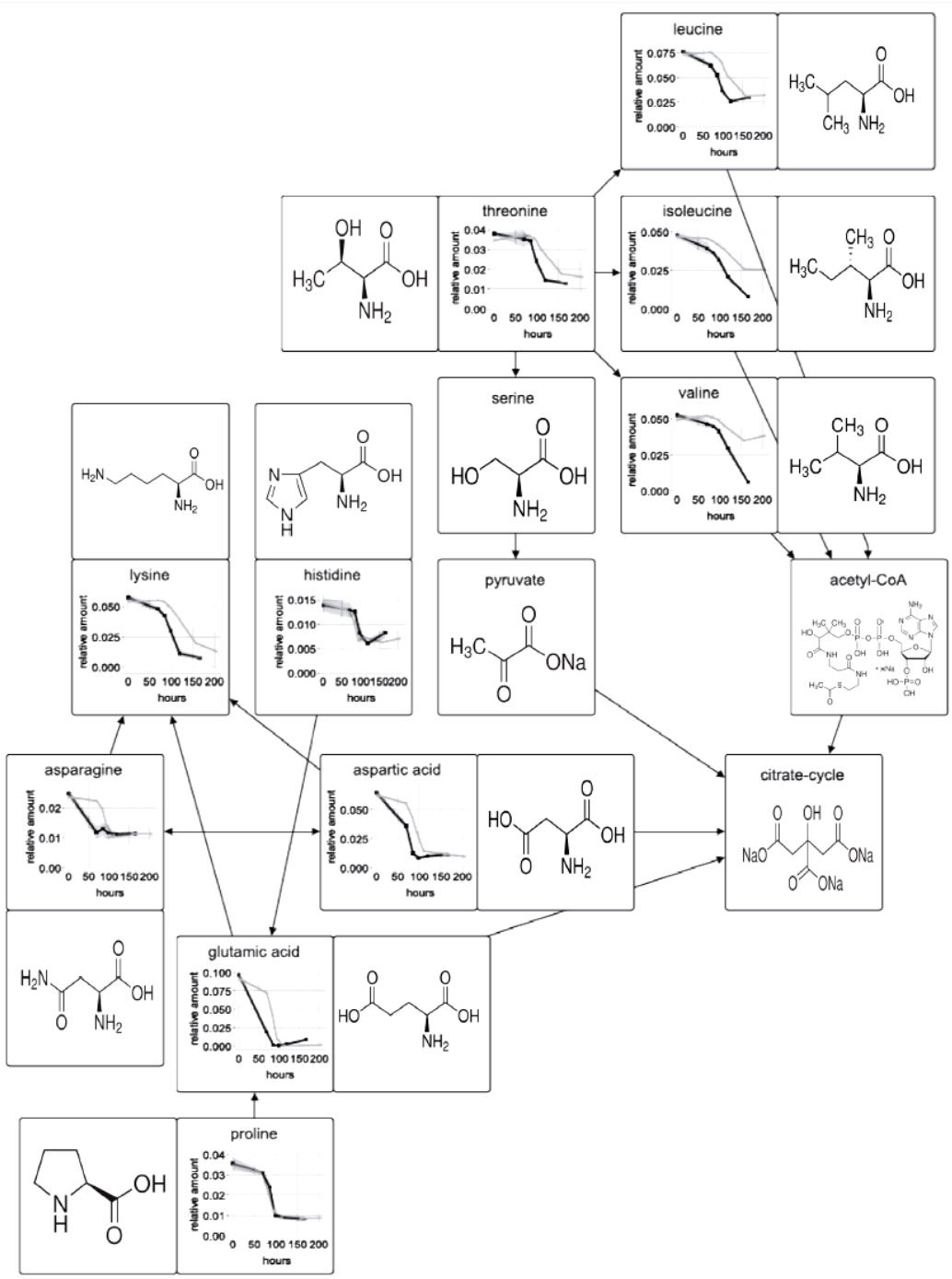


Figure 108: Possible conversions, relations and functions of some amino acids *V. eiseniae* (grey) and *V. tuberculata* (black). The shown chemical structures are sourced from the websites of SIGMA-ALDRICH.

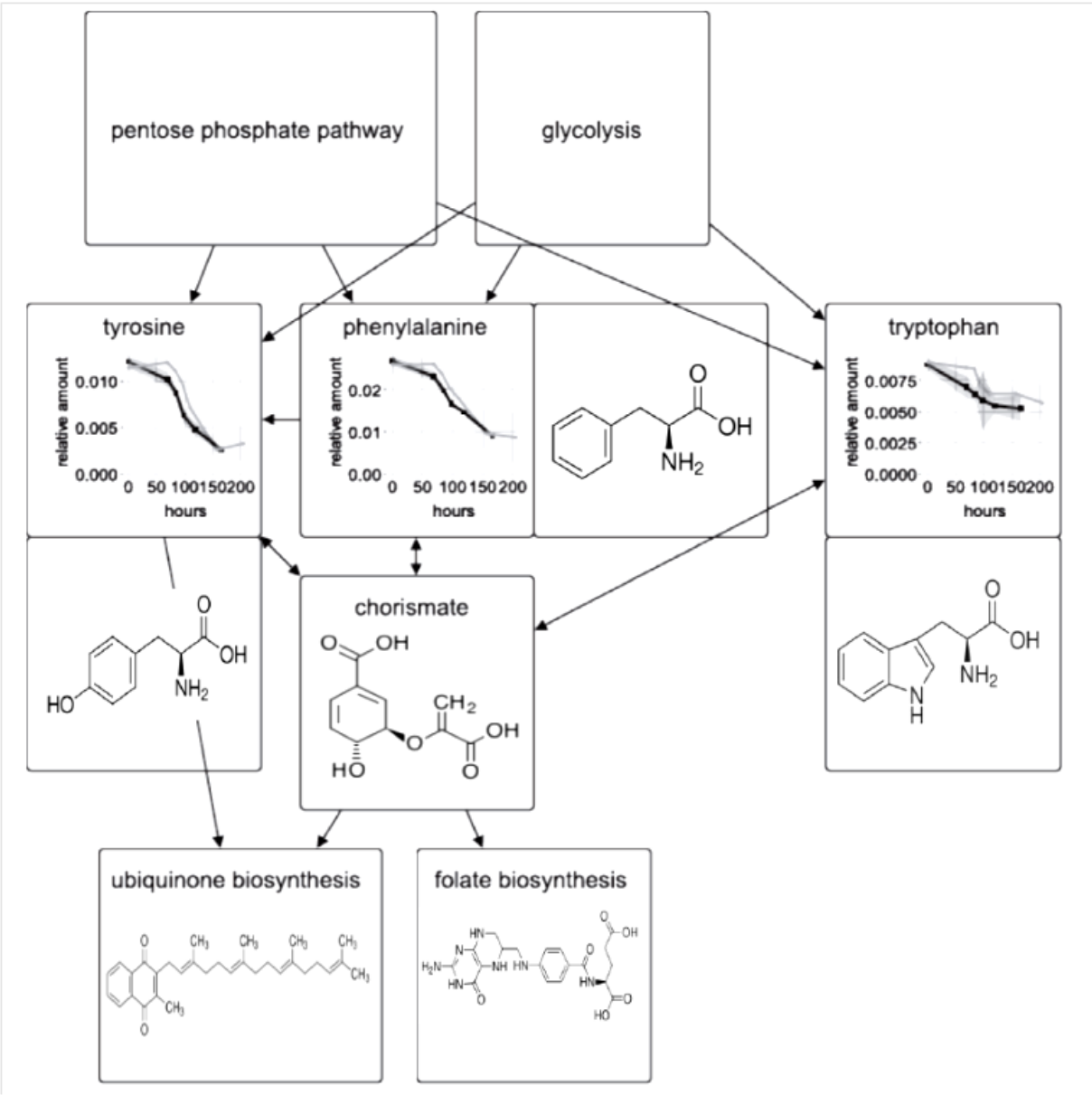


Figure 109: Possible conversions and functions of the aromatic amino acids *V. eiseniae* (grey) and *V. tuberculata* (black). The shown chemical structures are sourced from the websites of the KEGG database and SIGMA-ALDRICH. The structure shown under the title "ubiquinone biosynthesis" (menaquinone) is one of several possibly synthesized compounds. The same applies to the structure under the title "folate biosynthesis" (tetrahydrofolate).

Because of the possible interconversion of Asn and Asp, Asn can also flow into the citrate cycle and therefore be used for the retrieval of energy. According to the KEGG database *V. eiseniae* is only capable of converting Lys into cadaverin. That is the reason why Figure 105 only shows that Lys can be synthesized from Asn, Asp and Glu and does not show any metabolites that can be derived from Lys. It is not possible to tell what Lys is employed for inside the cells besides the biosynthesis of proteins. Leu, Ile and Val can be converted to acetyl-CoA and that way flow into the citrate cycle. Thr can either be incorporated into the biosynthesis of Ile, Leu and Val or become part of the citrate cycle via the conversion to serine and the subsequent transformation to pyruvate.

Since the aromatic amino acids are part of different pathways asparate Figure 106 indicates their possible roles in the metabolism of *V. eiseniae* and *V. tuberculata*. First of all three aromatic amino acids can be synthesized using intermediates of the glycolysis and the pentose phosphate pathway. Additionally all three can be converted into one another via the transformation to chorismate. Phe is directly convertible to Tyr and Tyr can directly flow into the biosynthesis of ubiquinone. Chorismate can also be integrated into the synthesis of ubiquinone and since Phe, Tyr and Trp can all be turned into chorismate, they can all flow into the biosynthesis of ubiquinone. Chorismate can also be used for the biosynthesis of folate.

5.2.12 Metabalome vs. Symbiosis mechanism

The results of the extracellular and intra cellular samples of the cultivation of *V. eiseniae* and *V. tuberculata* have been discussed with regard to the metabolism of the bacteria. Previous studies have shown that the nephridial symbionts are beneficial for the fitness of the earthworms and lead to a higher hatching success (Lund et al. 2010). However, so far the reasons for the increased fitness and higher hatching success have not been identified. In fact *V. eiseniae* might be secreting numerous substances of possible relevance for its host but it is not possible to detect them if they are secreted in low concentrations. In case of *V. tuberculata* some more metabolites were secreted in concentrations high enough for the detection via NMR spectroscopy. However, another issue is the lack of identification of some of the metabolites and the assumption of their

role. In case of *V. tuberculata* those are acetoacetate, acetone and 3-hydroxybutyrate. All three metabolites are ketone bodies and acetoacetate and 3-hydroxybutyrate can possibly be employed by *Aporrectodea tuberculata* to produce energy by reconverting them to acetyl-CoA which subsequently can flow into the citrate cycle. Acetoacetate and 3-hydroxybutyrate might also become part of the fatty acid metabolism of the earthworm and that way are possibly used for storing energy. However, when talking about supplying the symbiotic partner with nutrients another assumption suggests for itself. *V. eiseniae* and *V. tuberculata* both prefer pyruvate, acetate and lactate as a C-source over glucose and fructose. A reason for that possibly is the fact that inside the nephridia of the earthworms the concentration of these products of the glycolysis might be higher than that of intact sugars. Consequently the two bacteria potentially evolved over the time to prefer for these substances. It might be one of the reasons for *Verminephrobacter* species colonize in their hosts while they are actually able to survive and grow outside the earthworms, as was proven with the in vitro cultivation conducted for this thesis.

A potential reason for the increased fitness and hatching success of colonized earthworms that has been mentioned previously in the literature is the possibility that the *Verminephrobacter* symbionts produce essential vitamins for their hosts (Lund et al. 2010). Riboflavin is one of those vitamins the symbionts are thought to provide for the earthworm hosts. It is not possible to gain any new insights on whether this assumption is true or not with the data collected during the work for this thesis. One reason for that is the fact that even if riboflavin or other vitamins are secreted by the symbionts their concentrations would most likely be below the detection limits of NMR spectroscopy. However, inquiries of the KEGG database revealed that at least *V. eiseniae* is definitely capable of the synthesis of riboflavin out of products of the pentose phosphate pathway (KEGG PATHWAY n.d.).

Recent studies about the species *Variovorax paradoxus* a member of the same family of soil bacteria as *Verminephrobacter eiseniae* and *Verminephrobacter tuberculata* revealed that this soil bacteria species is capable of the degradation of several toxic

compounds as well as turning sulfur-containing organic substances into easier accessible and digestible sulfate (Satola et al. 2012). Making sulfur easily accessible for their hosts could be a possible reason for the symbiosis of *V. eiseniae* and *V. tuberculata* and their respective earthworm species. The entry about the sulfur metabolism of *V. eiseniae* in the KEGG database shows that this scenario is unlikely, though. *V. eiseniae* does not seem to be capable of converting a lot of different sulfur-containing organic substances into sulfate or other easier accessible forms of sulfur (KEGG PATHWAY n.d.). Some of the previously mentioned toxic compounds were halogenated hydrocarbons which are present in the soil due to their application as herbicides or industrial solvents. Since earthworms constantly ingest soil they are likely exposed to toxic compounds. Hence, a potential benefit of the symbiosis for the earthworm hosts could be the ability of the *Verminephrobacter* species to degrade and detoxify those substances. Since no compounds like that were present in the medium used for the cultivation, the collected data does not contain any information about the capability of *V. eiseniae* and *V. tuberculata* to degrade them. However, according to the KEGG database *V. eiseniae* is capable of degrading certain chloro- and fluoro-hydrocarbons. Thus, it is possible that the competence to degrade certain possibly toxic compounds is one of the reasons for and benefits of the symbiosis.

Lately conducted investigations about the purifying selection and molecular adaption in the genome of *Verminephrobacter* has shown that genes involved in membrane transport exhibit a low degree of relaxation in the purifying selection. This indicates a high significance of metabolite uptake and secretion for the symbiotic relation of the bacteria and their hosts (Kjeldsen et al. 2012).

6 Summary

Chemical signals are found to be the oldest communication method in the microbial world (Schmidt et al. 2017). Especially, potential secondary metabolites support the microbes to survive in the confrontational environment as well as the primary metabolites play key role in the communication mechanism with other life forms. With this motivation, the current research thesis focused on primary and secondary metabolites individually as two parts with specific research aims. Part-I deals with isolation of potential secondary metabolite from marine fungus for human use and part-II deals with investigation of primary metabolites and their role in the invertebrate symbiosis by comparative metabolomics approach.

The reason for choosing marine fungi for the isolation of bioactive compounds is that the unique chemistry of the ocean compare to land so that it is anticipated for distinctive bioactivity of secondary metabolites. The fungi kingdom of diverse phylogeny surviving in highly challenging environment compare to terrestrial lifestyle. Especially freshwater, estuaries are much more exigent for the existence of marine fungi with change in pH, force of water flow, unstable salt concentration and predators. Assorted class of secondary metabolites from marine fungi than terrestrial fungi has been studied for various clinical uses. Likewise, in the present study, one free living marine fungus (strain 165) and two sponge associated marine fungus (MF010& MF018) were taken for the study for their bioactive secondary metabolites as Part I.

As that of the selected study candidate were facultative fungal strains, they were cultivated as culture batches under optimized conditions. The extra cellular secondary metabolites secreted in the culture medium was extracted against ethyl acetate solvent, tested for bioactivity and the compound responsible for such bioactivity was further focused to isolate and the chemical structure was elucidated based on NMR and MS results matching to the chemical library. Bottom of the line, strain 165, MF010 and MF018 have been identified as a new producer of previously reported Alpha pyrones,

Azaphilones with potential pharmacological activity and other well known ergo steroidal derivatives also have been isolated.

Strain 165 was found to be taxonomically close to the species *Pseudohalonectria lignicola*, showed antimicrobial activity against human pathogenic Gram-positive bacterium *Staphylococcus aureus* and *Bacillus subtilis*. Strain 165 also possesses cytotoxic activity against Urinary bladder carcinoma cell lines. Strain MF010 & MF018 were found to be closely relative species belong to phoma genus showed antimicrobial activity against fish pathogenic Gram-negative bacterium *Vibrio anguillarum*, *Aeroginosa Hydrophilla* and *Pseudomonas Anguilliseptica*.

Bioassay guided fractionation method afforded to isolate bioactive compounds of strain 165, found to be a tetraketide derived alpha pyrones Nectriapyrone, Vermopyrone, demethyl Nectriapyrone, Helicascolide A, B and C along with Ergosterol and other Ergosterol derivatives.

Direct fractionation method afforded to isolate bioactive compounds of strain MF010, found to be a potentially bioactive (also known for phytotoxic) Azaphilones Ascochitine, Ascochital which is missing in the closely relative MF018 strain. Nevertheless, Ascosalitoxin and its dihydro derivative found to be present in both strains and the hypothetical chemical structure of Dihydro Ascosalitoxin which could be a new compound in the chemical library. However, further NMR structure elucidation is needed to confirm the structure elucidation. Other bioactive metabolites beta eudesmol, 6 methoxy estrodial along with Ergosterol and Ergosterol peroxide have been isolated from both the MF strains.

Terrestrial environment unsurprisingly corroborates the symbiosis of living beings stay together for mutual benefits as well as ecological benefits. Earthworm symbiosis was taken as a model to study the symbiosis mechanism by metabolomic approach. Lumbricid earthworm let a selective endosymbionts bacterium species called as *Verminephrobacter* for living in their Nephridia. The symbionts are vertically transmitted via the cocoon during early embryonic development, and have co-speciated with their

host for about 100 million years (Viana et al. 2016). In the present study, metabolism pattern of endosymbionts isolated from two contrasting ecological types of earthworm hosts. *Verminephrobacter Eiseniae* (of the fast reproducing *earthworm Eiseniae fetida* with high population density*)* and *Verminephrobacter tuberculata* (of the slow reproducing earthworm *Aporrectodea tuberculata* with low population density) have been studied by comparative metabolomic approach in part II.

Both the endosymbionts were isolated from the respective host earthworm and cultivated further as batch cultures with three biological replicates. Extra cellular and intra cellular samples were collected in duplicate from time point T1 to T5, former one was analyzed by NMR and the later one by GC-MS.

Results were compiled and compared fewer than three main categories, Carbon metabolism, Nitrogen metabolism and amino acid metabolism. fact that slow uptake of Glucose and fructose in both endosymbionts species suggesting that for these bacteria sugars are not key nutrients rather Amino acid metabolism is without doubt a key feature of both *V. eiseniae* and *V. tuberculata* as it seems to be for other host-associated bacteria for innumerable reasons. The other study has also proved that *in vivo* amino acids and peptides are the main carbon source for this uropathogen (Alteri et al. 2009), suggesting a similar nutritional pattern to what was observed for *V. eiseniae* in the uptake of the different dietary sources. amino acids seem to be the preferred carbon source for UPEC strains during host colonization indicating that these compounds can be important for the basic metabolism of bacteria living on a urine based diet(Alteri et al. 2009) (Flavia et al Unpublished).

In terms of nitrogen metabolism, earthworms seem to excrete mainly ammonia and so symbionts might also play a part here in further degradation of urea to ammonia that is later excreted (Laverack, 1963) (Flavia et al. Unpublished). 106 shows in *V. eiseniae* and *V. tuberculata,* urea was only detected intracellularly displaying a decreasing internal concentration (relative abundance of 0.405 at t1 and 0.072 by t5 in *V. eiseniae* and 0.674 at t1 and 0.300 by t5 in *V. tuberculata*) suggesting that it is further metabolized. According to proteomics data, urease* was identified mediating urea

cleavage to ammonia and carbon dioxide. This particular role might also be important for the symbiont establishment in the nephridia. Urea may has also been implied as a signal compound for UPEC strains to initiate colonization of the urinary tract(Withman et al. 2013) (Flavia et al. Unpublished). Also, Glutamine may serve as an ammonium storage or sink for ammonia derived from transamination reactions involving 2-oxoglutarate, which explains the presence of GlnA* throughout the whole growth.

Furthermore, strong rise in the levels of ethanol detected in the samples of *V. eiseniae* between T0 and T1 might be caused by the following connection. Acetate can directly be transmuted to acetyl-CoA and then become part of the citrate cycle. Acetate and ethanol can be converted into each other via the conversion to acetaldehyde. The acetyl-CoA that is produced from all the assimilated nutrients can either flow into the citrate cycle as mentioned before or it can be converted to Acetoacetate and then acetone or 3-hyroxybutyrate.

7 Future Direction

Investigation on secondary metabolites with all the three fungal candidates strain 165, MF010and MF018 found to be certainly motivating, need to be reanalyzed for their taxonomical identification as it is yet to confirm. In the bioactive region, along with mentioned alpha pyrones, other pyrone analogs like Nectriapyrone B methyl mellein also likely to be present in strain 165 which need to be focused further. Apart from Cytotoxicity against Urinary bladder carcinoma cell lines, it must be further screened for genotoxicity in order to limit the drug dosage in the pharmacological studies. It is also very exciting to focus on closely related strains MF010 & MF018 on why it is missing Ascochitine and Ascochital in MF018 although both showed similar bioactivity against fish pathogenic bacterium. Antimicrobial compound F isolated from both MF strains need to be further studied for the chemical structure which may be new in the chemical library.

Investigation on primary metabolites of Lumbricid earthworm endosymbionts suggesting that besides genetic and proteomics level, metabolites also play a vital role for the host

fitness, at times with antimicrobial property as hypothesized. Especially in terms of nitrogen metabolism, although excess of nitrogen derived from amino acid catabolism seems to be channeled to the urea cycle in the *Verminephrobacter* endosymbionts species that also feeds cyanophycin production which is a non-ribosomally synthesized copolymer of arginine and aspartate described for diverse Cyanobacteria and some heterotrophic bacteria (Obst and Steinbüchel, 2006). Since the amount of cyanophycin could not be determined in the metabolomics data, the balance of urea versus cyanophycin formation remains unclear at present (Flavia et al unpublished)

In terms of amino acids, even though tyrosine seems to be utilized by *V. eiseniae* and *V. tuberculata*, the pathway for degradation of this metabolite remains to be further clarified. Lysine was detected intracellularly but it remains unclear if lysine is exclusively taken up for anabolic purposes, or if it is also used for catabolism by an unknown route.

Citrate cycle pathway is definitely active in the cells of *V. tuberculata*, but with the present data it is impossible to tell if *V. eiseniae* does not perform this conversions or simply just does not secret the products into the medium. The only presumption that can be made is that *V. eiseniae* cannot generate acetone since the levels of acetone do not change over the course of the cultivation and according to the KEGG database *V. eiseniae* lacks the necessary enzyme(KEGG PATHWAY n.d.).

A potential reason for the increased fitness and hatching success of colonized earthworms that has been mentioned previously in the literature is the possibility that the *Verminephrobacter* symbionts produce essential vitamins for their hosts (Lund et al. 2010). the symbionts may provide vitamins or other essential co-factors [e.g., for pyrroloquinoline quinone (PQQ) and riboflavin] which have been identified in the genome of *V. eiseniae* (N. Pinel et al. 2008) and *V. aporrectodeae* ssp. tuberculata (AFAL00000000). Alternatively, the symbionts could protect the developing embryos from pathogens; potential antimicrobial properties of Verminephrobacter sp. are yet to be investigated (Lund et al. 2014). It is further need to gain any new insights as the fact that even if riboflavin or other vitamins are secreted by the symbionts their concentrations would most likely be below the detection limits of NMR spectroscopy.

8 Bibliography

Abrell, L.M., Cheng, X.-C. & Crews, P., 1994. New nectriapyrones by salt water culture of a fungus separated from an Indo-Pacific sponge. *Tetrahedron Letters*, 35(49), pp.9159–9160.

Alcazar-Fuoli, L. et al., 2008. Ergosterol biosynthesis pathway in Aspergillus fumigatus. *Steroids*, 73(3), pp.339–347.

Alteri, C.J., Smith, S.N. & Mobley, H.L.T., 2009. Fitness of Escherichia coli during urinary tract infection requires gluconeogenesis and the TCA cycle. *PLoS pathogens*, 5(5), p.e1000448.

Anon, Bakterien und bakteroiden bei oligochäten - Springer.

Anon, Global Markets for Marine-Derived Pharmaceuticals - PHM101A.

Anon, Species Fungorum - Search Page. Available at: http://www.speciesfungorum.org/names/names.asp?strGenus=Pseudohalonectria&GSD=Yes [Accessed June 23, 2015c].

Avent, A.G., Hanson, J.R. & Truneh, A., 1992. The biosynthesis of nectriapyrone and vermopyrone. *Phytochemistry*, 31(10), pp.3447–3449.

Bard, M. et al., 1993. Sterol synthesis and viability oferg11 (cytochrome P450 lanosterol demethylase) mutations inSaccharomyces cerevisiae andCandida albicans. *Lipids*, 28(11), pp.963–967.

Beed, F.D., Sue, R.E. & Strange, R.N., 1994. Variation in the production of ascochitine by Ascochyta fabae. *Mycological Research*, 98(9), pp.1069–1076.

Beedessee, G., Ramanjooloo, A. & Marie, D.E.P., 2015. Marine natural products research in Mauritius: Progress and challenges. *Marine Chemistry*, 170, pp.23–28.

Benveniste, P., 2004. Biosynthesis and accumulation of sterols. *Annu. Rev. Plant Biol.*, 55, pp.429–457.

Bertini, S., 1956. Su di un composto ad azione antibiotica prodotto da

Ascochyta pisi Lib. *Ann. Sperim. Agrar.(NS)*, pp.545–556.

Bhatnagar, I. & Kim, S.K., 2010. Immense essence of excellence: Marine microbial bioactive compounds. *Marine Drugs*, 8(10), pp.2673–2701.

Bino, R.J. et al., 2004. Potential of metabolomics as a functional genomics tool. *Trends in Plant Science*, 9(9), pp.418–425.

Blunt, J.W. et al., 2015. Marine natural products. *Natural product reports*, 22(1), pp.15–61.

Blunt, J.W. et al., 2016. Marine natural products. *Natural product reports*, 28(2), pp.196–268.

Bode, H.B. et al., 2002. Big effects from small changes: possible ways to explore nature's chemical diversity. *ChemBioChem*, 3(7), pp.619–627.

Brooks, T. & Keevil, C.W., 1997. A simple artificial urine for the growth of urinary pathogens. *Letters in applied microbiology*, 24(3), pp.203–206.

Carey, S.T., New, T. & Mywk, I., 1975. Tetrahedron Lattera No. 19, pp 1655 - 1658, 1975. Fwgawn PreLlr. Printed in Great Britain,. *Tetrahedron Letters*, 166(19), pp.1655–1658.

Chen, W., Shearer, C. & Klopp, J., 1995. Phylogenetic ordinal placement based on rDNA sequences of the freshwater genera Ophioceras and Pseudohalonectria. *Experimental mycology*.

Chiou, L.-C., Ling, J.-Y. & Chang, C.-C., 1997. Chinese herb constituent β-eudesmol alleviated the electroshock seizures in mice and electrographic seizures in rat hippocampal slices. *Neuroscience letters*, 231(3), pp.171–174.

Colombo, L. et al., 1979. Biosynthesis of ascochitine: incorporation studies with advanced precursors. *Journal of the Chemical Society, Chemical Communications*, (11), pp.492–493.

Colombo, L. et al., 1980. Biosynthetic origin and revised structure of ascochitine, a phytotoxic fungal metabolite. Incorporation of [1-13C]- and [1,2-13C2]-acetates and [Me-13C]methionine. *Journal of the Chemical Society, Perkin Transactions 1*, (0), pp.675–676.

Cristofaro, M. et al., 2011. The use of Ascochyta caulina phytotoxins for the

control of common ragweed. In *Proc. XIII Symp*.

D.S.Bhakuni & D.S. Rawat, *Bioactive Marine Natural Products*,

Davidson, S.K. & Stahl, D.A., 2008. Selective recruitment of bacteria during embryogenesis of an earthworm. *The ISME journal*, 2(5), pp.510–518.

Davidson, S.K. & Stahl, D.A., 2006. Transmission of Nephridial Bacteria of the Earthworm Eisenia fetida. *Applied and Environmental Microbiology*, 72(1), pp.769–775.

Dong, J. et al., 2006. New nematicidal azaphilones from the aquatic fungus Pseudohalonectria adversaria YMF1.01019. *FEMS Microbiology Letters*, 264(1), pp.65–69.

Dörries, K. & Lalk, M., 2013. Metabolic footprint analysis uncovers strain specific overflow metabolism and D-isoleucine production of Staphylococcus aureus COL and HG001. *PloS one*, 8(12), p.e81500.

Elhady, S. & El-Halawany, A., 2016. A New Bioactive Metabolite Isolated from the Red Sea Marine Sponge Hyrtios erectus. *Molecules*.

Elsebai, M.F., Ghabbour, H.A. & Mehiri, M., 2016. Unusual Nitrogenous Phenalenone Derivatives from the Marine-Derived Fungus Coniothyrium cereale. *Molecules (Basel, Switzerland)*, 21(2), p.178.

Evidente, A. et al., 1993. Ascosalitoxin, a phytotoxic trisubstituted salicylic aldehyde from Ascochyta pisi. *Phytochemistry*, 34(4), pp.995–998.

Evidente, A. et al., 2011. Phytotoxic polyketides produced by Phomopsis foeniculi, a strain isolated from diseased Bulgarian fennel. *European Journal of Plant Pathology*, 130(2), pp.173–182.

Fryar, S. et al., 2001. The influence of competition between tropical fungi on wood colonization in streams. *Microbial ecology*.

Fu, P. & MacMillan, J.B., 2015. Thiasporines A–C, Thiazine and Thiazole Derivatives from a Marine-Derived Actinomycetospora chlora. *Journal of Natural Products*, 78(3), pp.548–551.

Gieger, C. et al., 2008. Genetics meets metabolomics: a genome-wide association study of metabolite profiles in human serum. *PLoS genetics*, 4(11), p.e1000282.

Grant, W.D. & West, A.W., 1986. Measurement of ergosterol, diaminopimelic acid and glucosamine in soil: evaluation as indicators of microbial biomass. *Journal of Microbiological Methods*, 6(1), pp.47–53.

Gulik, W.M. Van et al., 2012. Microbial Systems Biology. , 881.

Hart, M.M. & Reader, R.J., 2002. Taxonomic basis for variation in the colonization strategy of arbuscular mycorrhizal fungi. *New Phytologist*, 153(2), pp.335–344.

Hawksworth, D.L. & Lagreca, S., 2007. New bottles for old wine: fruit body types, phylogeny, and classification. *Mycological research*, 111(Pt 9), pp.999–1000.

Horst-Robert Schutte, 2009. *Progress in Botany*.

Hu, Y. et al., 2015. Statistical Research on the Bioactivity of New Marine Natural Products Discovered during the 28 Years from 1985 to 2012. *Marine Drugs*, 13(1), pp.202–221.

HYDE, K. & FRÖHLICH, J., 2003. Nigramammilla calami gen. et sp. nov. and Arecomyces calami, A. licualae and Pseudohalonectria palmae spp. nov. from palms. *Cryptogamie. Mycologie*.

Hyde, K., Taylor, J. & Fröhlich, J., 1999. Two new species of Pseudohalonectria from palms. *Mycologia*.

Ibañez, E. et al., 2012. *Marine Bioactive Compounds*.

Inderbitzin, P. & Berbee, M., 2001. Lollipopaia minuta from Thailand, a new genus and species of the Diaporthales (Ascomycetes, Fungi) based on morphological and molecular data. *Canadian Journal of Botany*.

Jewett, M.C., Hofmann, G. & Nielsen, J., 2006. Fungal metabolite analysis in genomics and phenomics. *Current opinion in biotechnology*, 17(2), pp.191–7.

Johnson, T.O., Ermolieff, J. & Jirousek, M.R., 2002. Protein tyrosine phosphatase 1B inhibitors for diabetes. *Nature Reviews Drug Discovery*, 1(9), pp.696–709.

journal of Oncology, 2016. Investigational Sarcoma Drug GPX-150 Gets Orphan Drug Designation. , pp.2–3.

KEGG PATHWAY, KEGG PATHWAY: ABC transporters - Verminephrobacter eiseniae. [Accessed January 4, 2016a].

KEGG PATHWAY, KEGG PATHWAY: Alanine, aspartate and glutamate metabolism - Verminephrobacter eiseniae. [Accessed January 4, 2016b].

KEGG PATHWAY, KEGG PATHWAY: Cysteine and methionine metabolism - Verminephrobacter eiseniae. [Accessed January 4, 2016c].

KEGG PATHWAY, KEGG PATHWAY: Glycine, serine and threonine metabolism - Verminephrobacter eiseniae. [Accessed January 4, 2016d].

KEGG PATHWAY, KEGG PATHWAY: Glycolysis / Gluconeogenesis - Verminephrobacter eiseniae. [Accessed January 4, 2016e].

KEGG PATHWAY, KEGG PATHWAY: Inositol phosphate metabolism - Verminephrobacter eiseniae. [Accessed January 4, 2016f].

KEGG PATHWAY, KEGG PATHWAY: Lysine biosynthesis - Verminephrobacter eiseniae. [Accessed January 4, 2016g].

KEGG PATHWAY, KEGG PATHWAY: Phenylalanine, tyrosine and tryptophan biosynthesis - Verminephrobacter eiseniae. [Accessed January 4, 2016h].

KEGG PATHWAY, KEGG PATHWAY: Purine metabolism - Verminephrobacter eiseniae. [Accessed January 4, 2016i].

KEGG PATHWAY, KEGG PATHWAY: Pyrimidine metabolism - Verminephrobacter eiseniae. [Accessed January 4, 2016j].

KEGG PATHWAY, KEGG PATHWAY: Riboflavin metabolism - Verminephrobacter eiseniae. [Accessed January 4, 2016k].

KEGG PATHWAY, KEGG PATHWAY: Sulfur metabolism - Verminephrobacter eiseniae.

KEGG PATHWAY, KEGG PATHWAY: Synthesis and degradation of ketone bodies - Verminephrobacter eiseniae. [Accessed January 4, 2016m].

KEGG PATHWAY, KEGG PATHWAY: Valine, leucine and isoleucine biosynthesis - Verminephrobacter eiseniae. [Accessed January 4, 2016n].

Khalil, Z.G. et al., 2015. Aranciamycins I and J, Antimycobacterial Anthracyclines from an Australian Marine-Derived Streptomyces sp. *Journal of Natural Products*, 1(Table 1), p.150319084316006.

Kim, W. et al., 2016. Use of metabolomics for the chemotaxonomy of legume- associated Ascochyta and allied genera. *Nature Publishing Group*.

Kirk Jr, P.W. et al., 1974. Metabolites of higher marine fungi and their possible ecological significance. *Beroff. Inst. Meeresforsch. Bremerhaven Suppl*, 5, pp.509–518.

Kjeldsen, K.U. et al., 2012. Purifying Selection and Molecular Adaptation in the Genome of Verminephrobacter, the Heritable Symbiotic Bacteria of Earthworms. *Genome Biology and Evolution*, 4(3), pp.307–315.

Kohlmeyer, J., 1979. Marine fungal pathogens among Ascomycetes and Deuteromycetes. *Cellular and Molecular Life Sciences*, 35(4), pp.437–439.

Kohlmeyer, J., Volkmann-Kohlmeyer, B. & Tsui, C.K., 2005. Fungi on Juncus roemerianus. 17. New ascomycetes and the hyphomycete genus Kolletes gen. nov. *Botanica Marina*.

Kornprobst, J. & Barre, S. La, 2014. New Trends in Marine Natural Products. *Oceanography: Open Access*, 2(3), pp.2–3.

Kusnick, C. et al., 2002. Ascochital, a new metabolite from the marine ascomycete Kirschsteiniothelia maritima. *Pharmazie*, 57(7), pp.510–512.

Lund, M.B. et al., 2010. Beneficial Effect of Verminephrobacter Nephridial Symbionts on the Fitness of the Earthworm Aporrectodea tuberculata. *Applied and Environmental Microbiology*, 76(14), pp.4738–4743.

Lund, M.B. et al., 2011. Verminephrobacter aporrectodeae sp. nov. subsp. tuberculatae and subsp. caliginosae, the specific nephridial symbionts of the earthworms Aporrectodea tuberculata and A. caliginosa. *Antonie*

van Leeuwenhoek, 101(3), pp.507–514.

Lund, M.B., Kjeldsen, K.U. & Schramm, A., 2014. The earthworm-Verminephrobacter symbiosis: An emerging experimental system to study extracellular symbiosis. *Frontiers in Microbiology*, 5(MAR), pp.1–6.

Mach, R.L. & Zeilinger, S., 1998. *Genetic transformation of Trichoderma and Gliocladium*,

Marcinkowska, J., Klos, B. & Shcherbakova, A., 1991. Ascochitine production by fungi responsible for Ascochyta diseases of pea. *Journal of Phytopathology*, 131(3), pp.253–258.

Martins, A. et al., 2014. Marketed marine natural products in the pharmaceutical and cosmeceutical industries: Tips for success. *Marine Drugs*, 12(2), pp.1066–1101.

Mayer, A.M. et al., 2016. The marine pharmacology and pharmaceuticals pipeline in 2015. *FASEB Journal*, 30(1).

Mayer, A.M.S. et al., 2013. Marine pharmacology in 2009-2011: Marine compounds with antibacterial, antidiabetic, antifungal, anti-inflammatory, antiprotozoal, antituberculosis, and antiviral activities; affecting the immune and nervous systems, and other miscellaneous mechanisms of . *Marine Drugs*, 11(7), pp.2510–2573.

Mayer, A.M.S. et al., 2010. The odyssey of marine pharmaceuticals: a current pipeline perspective. *Trends in pharmacological sciences*, 31(6), pp.255–65.

Meyer, H. et al., 2014. A time resolved metabolomics study: the influence of different carbon sources during growth and starvation of Bacillus subtilis. *Molecular bioSystems*, 10(7), pp.1812–23.

Micco, S. Di, Chini, M.G. & Riccio, R., 2012. *Handbook of Marine Natural Products*, Available at: http://link.springer.com/10.1007/978-90-481-3834-0.

Minoura, K. & Muroi, T., 1978. Some freshwater ascomycetes from Japan. *Trans. Mycol. Soc. Japan*.

Muria- Gonzalez, M.J. et al., 2015. The past, present and future of secondary metabolite research in the Dothideomycetes. *Molecular plant pathology*, 16(1), pp.92–107.

Newman, D.J. & Giddings, L.A., 2014. Natural products as leads to antitumor drugs. *Phytochemistry Reviews*, 13(1), pp.123–137.

Oliver, S.G. et al., 1998. Systematic functional analysis of the yeast genome. *Trends in biotechnology*, 16(9), pp.373–378.

Olsson, P.A. et al., 2003. Ergosterol and fatty acids for biomass estimation of mycorrhizal fungi. *New Phytologist*, 159(1), pp.7–10.

Ono, Y. & Kobayashi, T., 2001. Notes on new and noteworthy plant-inhabiting fungi from Japan (1). *Mycoscience*.

Osmanova, N.P. thesis, 2011. Screening of antimicrobial effects of selected fungi and studies on antibiotic constituents of Bulgaria inquinans (Pers .) Fr . (Bulgariaceae) and Meripilus giganteus (Pers .: Fr .) P . Karst . (Meripilaceae) DISSERTATION In fulfilment of the requir.

Pinel, N., Davidson, S.K. & Stahl, D.A., 2008. Verminephrobacter eiseniae gen. nov., sp. nov., a nephridial symbiont of the earthworm Eisenia foetida (Savigny). *INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY*, 58(9), pp.2147–2157.

Pinel, N., Davidson, S.K. & Stahl, D. a, 2008. Verminephrobacter eiseniae gen. nov., sp. nov., a nephridial symbiont of the earthworm Eisenia foetida (Savigny). *International journal of systematic and evolutionary microbiology*, 58(Pt 9), pp.2147–57.

Poch, G.K. & Gloer, J.B., 1989. No Title. , (Table I), pp.3483–3486.

R.Long, 2009. Regulating Marine Biodiscovery in Sea Areas Under Coastal State Jurisdiction.

Rateb, M.E. & Ebel, R., 2011. Secondary metabolites of fungi from marine habitats. *Natural product reports*, 28(2), pp.290–344.

Ruiz, B. et al., 2010. Production of microbial secondary metabolites: regulation by the carbon source. *Critical reviews in microbiology*, 36(2), pp.146–167.

Satola, B., Wübbeler, J.H. & Steinbüchel, A., 2012. Metabolic characteristics of the species Variovorax paradoxus. *Applied Microbiology and Biotechnology*, 97(2), pp.541–560.

Schmidt, R. et al., 2017. Fungal volatile compounds induce production of the secondary metabolite Sodorifen in Serratia plymuthica PRI-2C.

Schmitt, I. & Lumbsch, H.T., 2004a. Molecular phylogeny of the Pertusariaceae supports secondary chemistry as an important systematic character set in lichen-forming ascomycetes. *Molecular phylogenetics and evolution*, 33(1), pp.43–55.

Schmitt, I. & Lumbsch, H.T., 2004b. Molecular phylogeny of the Pertusariaceae supports secondary chemistry as an important systematic character set in lichen-forming ascomycetes. *Molecular phylogenetics and evolution*, 33(1), pp.43–55.

Schoch, C.L. et al., 2009. A class-wide phylogenetic assessment of Dothideomycetes. *Studies in mycology*, 64, p.1–15S10.

Seibert, S.F. et al., 2006. Polyketides from the marine-derived fungus Ascochyta salicorniae and their potential to inhibit protein phosphatases. *Organic and Biomolecular Chemistry*, 4(11), pp.2233–2240.

Shearer, C. a, 1989. in Freshwater.

Shearer, C. & Bodman, S. Von, 1983. Patterns of occurrence of ascomycetes associated with decomposing twigs in a midwestern stream. *Mycologia*.

Shearer, C. & Zare-Maivan, H., 1988. In vitro hyphal interactions among wood-and leaf-inhabiting ascomycetes and fungi imperfecti from freshwater habitats. *Mycologia*.

Shenoy, B., Vijaykrishna, D. & Cai, L., 2005. Pseudohalonectria miscanthicola sp. nov. and three interesting fungi from tropics. *Cryptogamie.*

Da Silva Ferreira, M.E. et al., 2005. The ergosterol biosynthesis pathway, transporter genes, and azole resistance in Aspergillus fumigatus. *Medical Mycology*, 43(sup1), pp.313–319.

Smith, G.R. & Cole, A.L.J., 1991. Phoma clematidina, causal agent of leafspot and wilt of Clematis in New Zealand. *Australasian Plant Pathology*, 20(2), pp.67–72.

Sympson TJ, 1980. *Biosynthesis of polyketides.*,

Tarman, K. et al., 2012. Helicascolide C, a new lactone from an Indonesian marine algicolous strain of Daldinia eschscholzii (Xylariaceae, Ascomycota). *Phytochemistry Letters*, 5(1), pp.83–86.

Therkildsen, M., Isaksen, M. & Lomstein, B., 1997. Urea production by the marine bacteria Delaya venusta and Pseudomonas stutzeri grown in a minimal medium. *Aquatic Microbial Ecology*, 13, pp.213–217.

Tsui, C., Hyde, K. & Fukushima, K., 2003. Fungi on submerged wood in the Koito River, Japan. *Mycoscience*.

Tsuneki, H. et al., 2005. Antiangiogenic activity of β-eudesmol in vitro and in vivo. *European journal of pharmacology*, 512(2), pp.105–115.

Türkkan, M. et al., 2011. Phytotoxins produced by Pestalotiopsis guepinii, the causal agent of hazelnut twig blight. *Phytopathologia Mediterranea*, 50(1), pp.154–158.

Turner, W.B. & Aldridge, D.C., 1983. Fungal metabolites, vol II. *Paris: Academic*, pp.257–259.

Twelves, C. et al., 2010. A phase III study (EMBRACE) of eribulin mesylate versus treatment of physician's choice in patients with locally recurrent or metastatic breast cancer previously treated with an anthracycline and a taxane. *ASCO Meeting Abstracts*, 28(18_suppl), p.CRA1004.

Venkatasubbaiah, P. & Chilton, W.S., 1992. Phytotoxins Produced By Tubakia-Dryina. *Mycopathologia*, 120(1), pp.33–37.

Viana, F. et al., 2016. *Earthworm ecology affects the population structure of their Verminephrobacter symbionts*,

Vijaykrishna, D., Jeewon, R. & Hyde, K., 2006. Molecular taxonomy, origins and evolution of freshwater ascomycetes. *Fungal Diversity*, 23, pp.351–390.

Vita-Marques, A.M. de et al., 2008. A multi-screening approach for marine-

derived fungal metabolites and the isolation of cyclodepsipeptides from Beauveria felina . *Química Nova* , 31, pp.1099–1103.

Withman, B. et al., 2013. Transcriptional responses of uropathogenic Escherichia coli to increased environmental osmolality caused by salt or urea. *Infection and immunity*, 81(1), pp.80–9.

Yuen, T., Hyde, K. & Hodgkiss, I., 1999. Interspecific interactions among tropical and subtropical freshwater fungi. *Microbial ecology*.

Zech, H. et al., 2013. Adaptation of Phaeobacter inhibens DSM 17395 to growth with complex nutrients. *Proteomics*, 13(18–19), pp.2851–68.

9 Appendix

The appendix of this thesis is saved on the enclosed CD. It contains the raw data collected by ^{1}H-NMR spectroscopy and the patterns written for the quantification of the metabolites employing the integration tool of the AMIX® Viewer. Furthermore it contains the excel-tables used for the calculations to convert the values into a format in which they represent the area of a signal caused by one proton of the respective substance and also the excel-tables used as input-files for VANTED to generate the diagrams displayed in this thesis. Furthermore the data includes all HPLC-MS, GC-MS, MALDI-TOF and NMR raw data for the fractionation of marine fungi.

List of publication and other scientific achievement

Investigation of bioactive metabolites present in marine fungi

Nithyakalyani Srirangan, Stefanie Schneider, Martina Wurster, Ulrike Lindequist, Michael Lalk. 2014 UF Metabolomics workshop and conference by Southeast Center of Integrated Metabolomics (Poster presentation)

Cytotoxic activity of aquatic fungus Pseudohalonectria lignicola on two different human cell lines

S Schneider 1, N Srirangan 1, M Wurster 2, U Lindequist 2, M Lalk 1

62nd International Congress and Annual Meeting of the Society of Medicinal Plant and Natural Product Research (Poster presentation)

Cytotoxic activity of aquatic fungus Pseudohalonectria lignicola on two different human cell lines

S Schneider 1, N Srirangan 1, M Wurster 2, U Lindequist 2, M Lalk 1 Planta Med 16 · Volume 80 · Oktober 2014 (Book of selected Abstracts)

Marine derived fungi Pseudohalonectria lignicola- The new producer of the Nectriapyrone analogs

N Srirangan, M Wurster , U Lindequist , M Lalk (Manuscript under preparation)

Unknown sponge associated marine fungus produce previously reported Azaphilones

N Srirangan, M Wurster , U Lindequist , M Lalk (Manuscript under preparation)

Erklärung

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der MathematischNaturwissenschaftlichen Fakultät der Ernst-Moritz-Arndt-Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.

Ferner erkläre ich, dass ich diese Arbeit selbstständig verfasst und keine anderen als die darin angegebenen Hilfsmittel und Hilfen benutzt und keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen habe.

Unterschrift des Promovenden

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Curriculum vitae

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Education and Professional Experience

Dec 2010 – Dec 2011	Group Leader - Vimta labs, Proteomics dept, Advance molecular biology division, Hyderabad, India.
Dec 2009 – Dec 2010	Assistant Manager in Dr Reddys Laboratories Ltd, Biologics division, Hyderabad, India.
Jun 2006 – Nov 2009	Scientist in Biocon Limited, Research and Development, Bangalore, India.
Oct 2002 – May 2006	Sr. Research associate in Micro labs Ltd, Bangalore, India.
2007 – 2010	MS Pharmaceutical technology, Vinayaka mission deemed university, Salem, India
1998 - 2002	Bachelor of Pharmacy, Tamilnadu Dr. MGR University, Chennai, India.

வெள்ளத் தனைய மலர்நீட்டம் மாந்தர்தம்
உள்ளத் தனையது உயர்வு.

-**Thirukkural**
(World ethics and moral written by a Tamil poet between 3rd and 1st centuries BCE)

Explanation: The stalks of water-flowers are proportionate to the depth of water; so is men's greatness proportionate to their minds.