ISOLATION OF MARINE FUNGI ASSOCIATED WITH *SARCOPHYTON EHRENBERGI* AND THE EFFECT OF TEMPERATURE ON THEIR PRODUCTION OF SECONDARY METABOLITES

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ISOLATION OF MARINE FUNGI ASSOCIATED WITH *SARCOPHYTON EHRENBERGI* AND THE EFFECT OF TEMPERATURE ON THEIR PRODUCTION OF SECONDARY METABOLITES

ABSTRACT

Despite limited knowledge on their ecological functions, soft coral-derived fungi produce a range of bioactive secondary metabolites, and have an impact on a diverse microbiome. Little attention has been given to soft corals and their associated fungi in Malaysia, especially the secondary metabolites they produce. Increased seawater temperature has been found to promote pathogen growth and weaken coral disease resistance, thus shifting coral-fungi association. This study isolates and identifies four fungal strains, *Aspergillus sydowii*, *Acrocalymma* sp., *Lindgomyces* sp. and *Phomatospora* sp., from a population of soft coral *Sarcophyton ehrenbergi* collected off the waters of Port Dickson in the Straits of Malacca. In addition, *Aspergillus sydowii* and *Acrocalymma* sp. were incubated at 25°C, 30°C and 35°C for two weeks to investigate the effect of increased temperatures on secondary metabolites production. The fungal biomass was extracted in ethyl acetate to obtain crude extracts, which were then analysed by TLC, HPLC and Q-TOF LCMS. Findings revealed that increased temperatures negatively affected the biomass production of both species, while the highest yields of crude extract were recorded at 35°C for *Aspergillus sydowii* and at 30°C for *Acrocalymma* sp. HPLC profiles of both species indicate an overall reduction in peak detection with an increase in temperature, despite a few unique peaks recorded at specific temperatures. On the contrary, LCMS profiles indicate that total numbers of compounds from crude extracts of both species follow a similar pattern as temperature increases, whereas the highest numbers were recorded at 25°C for *Aspergillus sydowii* and at 35°C for *Acrocalymma* sp. Detailed analysis of chemical formulas of LCMS results shows that the majority of detected chemicals were primary metabolites, which could be the high sensitivity of Q-

TOF LCMS. The investigation in secondary metabolites from soft coral-associated fungi under rising seawater temperature illustrated potential biological responses to seawater temperature rise.

Keywords: *Sarcophyton ehrenbergi*, marine fungi, climate change, secondary metabolites

PENGASINGAN KULAT MARIN YANG DIKAITKAN DENGAN SARCOPHYTON EHRENBERGI DAN KESAN SUHU KE ATAS PENGHASILAN METABOLIT SEKUNDER

ABSTRAK

Walaupun pengetahuan terhad tentang fungsi ekologi mereka, kulat yang berasal dari karang lembut menghasilkan pelbagai metabolit sekunder bioaktif, dan mempunyai kesan ke atas mikrobiom yang pelbagai. Tidak banyak perhatian telah diberikan kepada karang lembut dan kulat yang berkaitan dengannya di Malaysia, terutamanya metabolit sekunder yang dihasilkannya. Peningkatan suhu air laut didapati menggalakkan pertumbuhan patogen dan melemahkan rintangan penyakit karang, sekali gus mengubah asosiasi karang-kulat. Kajian ini mengasingkan dan mengenal pasti empat strain kulat, *Aspergillus sydowii*, *Acrocalymma* sp., *Lindgomyces* sp. dan *Phomatospora* sp., daripada populasi karang lembut *Sarcophyton ehrenbergi* perairan Port Dickson di Selat Melaka. Selain itu, *Aspergillus sydowii* dan *Acrocalymma* sp. telah diinkubasi dalam suhu 25°C, 30°C dan 35°C selama dua minggu untuk menyiasat kesan peningkatan suhu terhadap penghasilan metabolit sekunder. Biojisim kulat telah diekstrak dalam etil asetat untuk mendapatkan ekstrak mentah, yang kemudiannya dianalisis oleh TLC, HPLC dan Q-TOF LCMS. Peningkatan suhu memberi kesan negatif kepada penghasilan biojisim kedua-dua spesies, manakala hasil ekstrak mentah tertinggi dicatatkan pada 35°C untuk *Aspergillus sydowii* dan pada 30°C untuk *Acrocalymma* sp. Profil HPLC kedua-dua spesies menunjukkan pengurangan keseluruhan dalam pengesanan puncak dengan peningkatan suhu, walaupun beberapa puncak unik direkodkan pada suhu tertentu. Sebaliknya, profil LCMS menunjukkan bahawa jumlah bilangan sebatian daripada ekstrak mentah kedua-dua spesies mengikut corak yang sama apabila suhu meningkat, manakala bilangan tertinggi dicatatkan pada 25°C untuk *Aspergillus sydowii* dan pada 35°C untuk *Acrocalymma* sp. Analisis terperinci formula kimia daripada keputusan LCMS menunjukkan bahawa

majoriti bahan kimia yang dikesan adalah metabolit primer, yang mungkin disebabkan oleh sensitiviti LCMS yang tinggi. Penyiasatan dalam metabolit sekunder daripada kulat berkaitan karang lembut di bawah peningkatan suhu air laut menggambarkan potensi tindak balas biologi terhadap kenaikan suhu air laut.

Keywords: *Sarcophyton ehrenbergi*, kulat marin, perubahan iklim, metabolit sekunder

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Liu Yuanwei
Master of Philosophy
1st May, 2022

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

atm	:	Standard atmosphere
AGE	:	Agarose gel electrophoresis
AJS ESI MS Q-TOF	:	Agilent Jet Stream Electrospray Ionization Mass Spectrometry Quadrupole Time-of-Flight
ASW	:	Artificial seawater
BLAST	:	Basic Local Alignment Search Tool
BI	:	Bayesian Inference
CO_2	:	Carbon dioxide
COI	:	Cyctochrome oxidase I
DNA	:	Deoxyribonucleic acid
EF1-α	:	elongation factor 1-alpha
gDNA	:	Genomic DNA
HIV	:	Human immunodeficiency virus
HPLC	:	High performance liquid chromatography
HMGR-CoA	:	Hydroxymethylglutaryl-coenzyme A reductase
ITS	:	Internal transcribed spacer
LSU	:	Large subunit
LPS	:	Lipopolysaccharide
MCM7	:	Mini-chromosome maintenance protein 7
MEGA	:	Molecular Evolutionary Genetic Analysis
MIC	:	Minimum inhibition concentration
ML	:	Maximum Likelihood
MP	:	Maximum Parsimony
msh1	:	Mitochondrial protein-coding genes MutS homolog
NO	:	Nitric oxide
PCR	:	Polymerase chain reaction
PDA	:	Potato dextrose agar
PDB	:	Potato dextrose broth
ppt	:	Parts per thousand
PTFE	:	Polytetrafluoroethylene
Q-TOF LCMS	:	Quadrupole Time-of-Flight Liquid Chromatography Mass Spectrometry
RPB1	:	RNA polymerase II subunit 1
rRNA	:	ribosomal RNA
TAE	:	Tris-acetate-EDTA
TLC	:	Thin layer chromatography
WCPM	:	West coast of Peninsular Malaysia

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Background

Oceans, consisting of approximately 71% of the Earth surface, have been reported to host almost 178,000 species across 34 phyla, representing an extremely rich pool of biodiversity that is mostly centred in the tropical environments (Liu et al., 2021). In particular, the diversity of unknown microorganisms in each drop of seawater is 9:1 in ratio to that of humans (Zhang et al., 2018). Marine organisms are widely known to produce a wide range of natural products with unique and often highly complex structures (Zhang et al., 2019). These natural products, also known as secondary metabolites, have been reported from various living organisms such as microorganisms, plants, and animals. Many of these products have been utilized for various disease treatments and potential drug development due to their unique biological properties (Blunt et al., 2018). In fact, with diverse chemical structures, natural products show good drug-like properties and interaction with multiple cellular target proteins (Zhang et al., 2002). Raimundo et al. (2018) have also demonstrated that the discovery of chemical structures from marine organisms has been increasing throughout the last decade.

Corals (Cnidaria, Anthozoa) are the second largest source of secondary metabolites among marine animals with more than 200 novel compounds identified each year (Liu et al., 2021). Since the first study on natural products from *Pseudopterogorgia elisabethae* in the 1980s (Liu et al., 2018), the octocorals, also known as soft corals, have been regarded as promising sources of bioactive secondary metabolites, especially the production of novel terpenoids (Li et al., 2005). For example, two diterpenoids caribenols A and B, and elisapterosin B were obtained from *Antillogorgia elisabethae* (previously known as *Pseudopterogorgia elisabethae*). The compound bipinnapterolide B was isolated from *P. bipinnata* and demonstrated *in vitro* antituberculous activity to against *Mycobacterium tuberculosis* (Liu et al., 2021). Lacking effective physical protection and living in highly competitive, hostile marine conditions, the successful survival of soft corals primarily depend on their chemical defensive systems via the accumulation and release of a variety of secondary metabolites to avoid predators and to inhibit overgrowth and fouling (Changyun et al., 2008). Their effective chemical defence system relies on not only diverse terpenoids, especially di- and sesqui-terpenoids, but also polyketides, steroids, and alkaloids (Berrue et al., 2011; Rocha et al., 2011; Blunt et al., 2016). The roles of these secondary metabolites include antipredator (Lages et al., 2010), antimicrobial (Ritchie, 2006), anti-cancer (Bhanot et al., 2011), anti-fouling (Lages et al.,

2010) and allopathic (Rasher et al., 2013). Thus, octocoral-derived secondary metabolites possess enormous economic potential.

Given that traditional reports on coral-derived secondary metabolites focused on extracts from coral tissues and often ignored the production of compounds from their associated microbes, it is usually indefinable whether these natural products are produced by the corals or their associated microorganisms (Raimundo et al., 2018). However, existing findings of marine sponges and bryozoans have shown that some of their secondary metabolites have its origins in their symbiotic microbes (Davidson et al., 2001; Piel et al., 2004). In addition, current studies on novel secondary metabolites produced by marine microbes outnumber the natural products derived from marine invertebrates (Raimundo et al., 2018) Therefore, the determination of the diverse secondary metabolites from octocoral-derived microorganisms could help to trace the origin of these chemicals, whether they are synthesised by the host or the associated symbiont.

Coral-associated microbes consist of endolithic algae, endosymbiotic dinoflagellates, bacteria, fungi, alveolates, archaea, and viruses (Bourne et al., 2016; van de Water et al., 2018). The consortium of coral and its associated microbes is often termed as holobiont (Rosenberg et al., 2007). These associated microorganisms provide carbon and nitrogen sources to their host, play a part in detoxification, nutrient cycling, genetic exchange, UV protection, and chemical defence (Harvell et al., 2007; Peixoto et al., 2017; van de Water et al., 2018). While fungal diseases have impacts on gorgonian populations (Kiho et al., 2004; Smith & Weil, 2004; Kim & Rypien, 2015), relatively few investigations have been conducted on the marine fungal community derived from octocorals (van de Water et al., 2018). Furthermore, knowledge of these fungal isolates is mainly obtained from culture-based techniques, thus favouring species that are only able to be cultivated under laboratory conditions (Koh et al., 2002; Zhang et al., 2012) . Nonetheless, these cultured marine fungi have been a promising reservoir of bioactive secondary metabolites, usually with unique chemical structures, thus making octocoral-derived fungi potential sources for bioprospecting (Raimundo et al., 2018). In spite of little knowledge on the exact ecological functions of these fungal species, apart from some exhibiting potential antifungal and/or antibacterial properties, these compounds might have a role in maintaining holobiont health and regulating microbiome (Zhang et al., 2012). However, despite the long coastline and rich marine biodiversity in Malaysia, little attention has been given to the secondary metabolites produced by local soft corals and their associated fungi (Ishii, et al., 2010).

In addition to these global change in climate has made substantial shifts in the marine environment, such as increased temperatures of seawater, ocean acidification, the rising sea level, and changes in ocean salinity(Pachauri et al., 2014). The rising seawater temperatures have caused more frequent coral bleaching and even death, with some corals eventually shifting their distribution poleward (Hoegh-Guldberg & Bruno, 2010). Seawater temperature also has been found to influence pathogen growth and disease resistance of corals (Ward et al., 2007). Since marine fungi are able to adapt and maintain their important metabolic pathways under these environmental stress (Jones et al., 2013), it is important to investigate the changes in secondary metabolite production under similar conditions and evaluate its potential role as a buffer to both the host and coral. Furthermore, investigation into their production of secondary metabolites could potentially yield metabolites with potential pharmaceutical applications.

1.2 Research Questions

1) How many secondary metabolites can be determined from selected marine fungi derived from the selected octocoral species?
2) How does increase in temperature affect the production of secondary metabolites from the isolated marine fungi?

1.3 Research Objectives

1.3.1 General Objectives

This study aims to gain knowledge of fungal diversity from the soft coral, *Sarcophyton ehrenbergi*, the chemical diversity, and the impact of increased ocean temperature on secondary metabolite production.

1.3.2 Specific Objectives

1) To isolate and identify marine fungi species from *Sarcophyton ehrenbergi* through a multigene molecular phylogeny approach.
2) To determine the effects of increased temperatures (25°C, 30°C and 35°C) on biomass and secondary metabolite production of two selected *Sarcophyton ehrenbergi*-derived marine fungi
3) To analyse the secondary metabolite profiles of crude extracts obtained from the two selected marine fungi under rising temperatures (25°C, 30°C and 35°C).

4) To determine the secondary metabolites produced from the two selected marine fungi under rising temperatures (25°C, 30°C and 35°C).

1.4 Significance of Research

Despite several investigations on fungal diseases in gorgonian corals, relatively little research has been conducted on the relationship between soft corals and their associated fungal communities. In addition, marine fungi remain as 'terra incognita' compared to their terrestrial counterparts. The marine environments exert extreme physical and chemical conditions that give rise to a wide range of chemical scaffolds produced by marine adapted organisms such as corals and marine fungi. Even though corals have shown to be the second most prolific marine animals of bioactive secondary metabolite production, relatively little is known whether these bioactive compounds are produced by the corals themselves or their associated microbiomes (such as fungi). Since climate change gives rise to rising seawater temperatures, global coral communities are undergoing rapid shifts from hard coral domination to more coverage of soft corals. These changes may also influence the constitution of their microbiomes and secondary metabolite production from these microorganisms. Therefore, further investigation into their production of secondary metabolites from soft coral-derived fungi could provide insight into its adaptation under rising temperature as well as the potential environmental role of the metabolites produced. These metabolites also may exhibit potential application as pharmaceutical.

CHAPTER 2: LITERATURE REVIEW

2.1 Soft coral genus *Sarcophyton*

Octocorals (phylum, Cnidaria; class, Anthozoa; subclass, Octocorallia) are comprised of three orders; Helioporacea (often called "blue corals"), Pennatulacea (commonly known as "sea pens"), and Alcyonacea (consisting of "gorgonians" and "soft corals") (Daly et al., 2007). Soft corals have equal or more coverage than the scleractinian hard corals (Tursch & Tursch, 1982; Dinesen, 1983; Riegl et al., 1995; Fabricius, 1997), have structural complexity in coral reef ecosystems and contribute to coral reef biomass (Tursch & Tursch, 1982; McFadden et al., 2010). Therefore, they have been the target of biological studies since the nineteenth century. Soft corals include "sea fans" and "sea whips"; however, most soft corals belong to the order Alcyonacea, which comprises the families Xeniidae, Nephtheidae, and Alcyoniidae. The three most common genera within the order Alcyonacea are *Sarcophyton* Lesson 1834, *Lobophytum* Marenzeller 1886, and *Sinularia* May 1898. These genera are conspicuous in shallow offshore reef flat habitats in which they form large monospecific aggregation. Species in these genera are known to be long-lived, competitive dominants that grow slowly, resist predation, and have infrequent larval recruitment (Bastidas et al., 2004; Benayahu & Loya, 1986; Fabricius & Klumpp, 1995). All three taxa possess zooxanthellate, and as a result have been heavily impacted by recent coral bleaching events, with mortality exceeding 90% in some areas of the Indo-West Pacific (Fabricius, 1999; Bruno et al., 2001; Loya et al., 2001).

Soft corals are largely inhabited in Indo Pacific coral communities, while gorgonian octocorals are particularly populated in the north-western Atlantic Ocean and the Caribbean Sea reef environments (Li & Pattenden, 2011). In particular, soft corals of the genus *Sarcophyton* (order Alcyonacea, family Alcyoniidae) are prolific in the South China Sea and are dominant in many coral reefs (Aratake et al., 2012). Due to the lack of calcium carbonate skeletons for physical protection, soft corals depend heavily on chemical defence mechanisms in order to resist predators and prevent overgrowth and fouling, by synthesizing or accumulating a variety of secondary metabolites in their system and releasing to the environment when required (Changyun et al., 2008). They are known to produces a range of terpenoids, especially di- and sesquiterpenoids, polyketides, steroids and alkaloids (Berrue et al., 2011; Rocha et al., 2011; Blunt et al., 2017). Many of these secondary metabolites exhibit antibacterial, anticancer, anti-inflammatory, antimalaria, antiviral and neuroprotective activities (Bhanot et al., 2011; Rocha et al., 2011).

More than two decades ago, Anjaneyulu and Venkateswara Rao (1997) published the very first comprehensive review on secondary metabolites produced by soft coral species from *Sarcophyton* genus, according to the publications from 1974 to 1994. Among all the examined secondary products, terpenoids are the most frequently encountered. Liang and Guo (Liang & Guo, 2013) then reviewed on the terpenoids produced by several *Sarcophyton* species (*S. cherbonnieri*, *S. crassocaule*, *S. ehrenbergi*, *S. elegans*, *S. flexuosum*, *S. glaucum*, *S. influndibuliforme*, *S. latum*, *S. mililatensis*, *S. mole*, *S. solidum*, *S. stolidotum*, *S. tortuosum*, *S. trocheliophorum* and other unidentified species) from different geographic regions, and reported 205 terpenoids, including 7 sesquiterpenes, 165 diterpenes and 29 biscembranoids, covering the period between 1995 and July 2011. Some of these metabolites exhibit antifeedant, antifouling, antiviral and anti-inflammatory properties. However, the most recent literature review focused on new cembranoid diterpene derivatives from the genus *Sarcophyton* published from 2016 to 2018 (Rodrigues et al., 2019) because cembranoids in nature could have chemical defence properties against fish predators and/or other competitive reef organisms, bacteria and parasites (Li & Pattenden, 2011; Lai et al., 2017). Rodrigues et al. (2019) conclude that many newer cembranoid diterpenes were found in *Sarcophyton* than in other genera *Lobophytum* and *Sinularia* in the same family. Therefore, based on literary studies available to date, it is established that the genus *Sarcophyton* is a rich resource of steroids, diterpenes and tetraterpenes (Anjaneyulu & Venkateswara Rao, 1997; Zhang et al., 2006).

2.2 Marine Fungi

2.2.1 Definition of marine fungi

Studies on marine fungi have been established since Durieu de Monzonneuvo and Montagne described the very first fungal species *Sphaeria posidoniae* (now known as *Halotthia posidoniae*) from the rhizome of the sea grass *Posidonia oceanica* in Algeria in 1846 (Montagne, 1856). However, the definition of marine fungi has been varied in the literature. In the 1960s, some scientists claimed that marine fungi have the ability to grow at particular concentrations of seawater (Johnson & Sparrow, 1961; Tubaki, 1969). In contrast, other researchers defined marine fungi in terms of their physiology, whereby fungal growth requires seawater or certain concentrations of sodium chloride (Jones & Jennings, 1964; Meyers, 1968).

At present, the universally accepted definition was provided by Kohlmeyer and Kohlmeyer (1979), which states that "obligate marine fungi are those that grow and sporulate exclusively in a marine or estuarine habitat; facultative marine fungi are those from a freshwater or terrestrial mileu, able to grow and possibly also sporulate in the marine environment". Kohlmeyer and Kohlmeyer (1979) further proposed that marine fungi are able to germinate and form mycelium in natural marine environments, even though the natural conditions might vary from one species to another. If a fungus colonises an aerial part of a mangrove or other shoreline plant, it is generally considered as a representative of terrestrial fungi. By contrast, if it colonises the partly or completely submerged parts because of exposure to intertidal waters, it is commonly regarded as a marine fungus. For this reason, it is claimed that marine fungi are not classified based on taxonomy, but on physiology and ecology (Hyde & Sarma, 2000).

In the last decade, Jones et al. (2015) believed this definition was too narrow and they used the term 'marine-derived fungi' because many fungal taxa were isolated in the process of exploration for novel secondary metabolites, also known as bioprospecting (Fenical & Jensen, 1993; Fenical et al., 2000). Exhibiting asexual morphs, marine-derived fungi have been isolated from a broad variety of substrates, including off-shore habitats (such as deep sea), and have been a good reservoir of natural habitats (Jones et al., 2019). Based on many studies around the world, it is claimed marine-derived fungi are a core group of fungi as they have been consistently isolated from different substrata the marine environments (Jones et al., 2019).

Pang et al. (2016) have proposed the latest revision of the definition of 'marine fungi' and 'marine-derived fungi'. Their definition for 'marine fungi' or 'any fungi

repeatedly recovered from the marine environment' consists of three criteria: (1) their ability to grow and/or sporulate (on substrata) in marine habitats, (2) the formation of a symbiotic relationship with other marine organisms, (3) their genetic or metabolically active adaptation and evolution in the marine environment (Pang et al., 2016).

2.2.2 Number of marine fungi

The number of marine fungi has often been regarded as a recurring question (Jones, 2011). In the 1990s, at least 1.5 million of fungal species were estimated on earth (Hawksworth, 1991). However, this figure went up considerably to 5.1 million after two decades (Blackwell, 2011). The latest data on fungi diversity were provided by Hawksworth and Lucking (2017), who indicated that 1.5 million was a conservative number according to new evidence of the ratio of plant to fungi and studies on environmental sequences. They implied the number of fungi should range between 2.2 and 3.8 million (Hawksworth & Lücking, 2017). However, it is estimated that the number of fungi described is only between 120,000 to 143,273 (Hawksworth & Lücking, 2017; Wijayawardene et al., 2017), most of which belong to terrestrial fungi.

Many researchers highlighted that marine fungi are not well studied compared with the quantity of other microbes in the ocean (Jones & Richards, 2011; Raghukumar, 2017). Nevertheless, there have been approximately 1,200 species documented in a 72-year period of research into marine mycology, whereas nearly 120,000 terrestrial species were recorded over a 200-year period of research (Kirk et al., 2008). In terms of their distribution and diversity across the globe, Tisthammer et al. (2016) contended that there is very little known information about marine fungi. It is predicted that a great deal of fungal diversity can be found in anaerobic deep sediments (Drake et al., 2017). One category of these fungi is called 'the dark fungi', which have never been isolated through cultural techniques, but detected through next generation sequencing (NGS) techniques. During the exploration of marine fungal diversity, it is found that publicly available fungal DNA loci are only applicable to only 50% of the known species of marine fungi (Hassett, Vonnahme, et al., 2019). Therefore, Hasset et al. (2019) proposed a theory that this lack of availability could impede the accuracy of taxonomical classification via high-throughput sequencing with advances in this discipline. Considerable effort should be directed to sequence as many known marine fungal species as possible for the sake of enabling more scientists to identify unculturable and cryptic fungal taxa.

2.2.3 Marine fungi in coastal waters

Marine fungi can be found in a wide range of broad-scale habitats, which comprise plant-based habitats (for example mangroves and sea grasses), coastal waters, macroalgae, deep-sea subsurface sediments and polar waters (Burgaud et al., 2022). In comparison with plant-based habitats, the amount of available organic carbon is much lower in marine waters for heterotrophic organisms like fungi. Thus, it is not surprising that the open ocean has the lowest fungal diversity because of its lowest quantity of substrates. In contrast, coastal waters demonstrate a diverse range of fungal communities.

Various research has revealed that marine fungi in the subkingdom of *Dikarya*, particularly the phyla of *Ascomycota* and *Basidiomycota*, are the dominant groups in most marine environments based on culture-dependent and metabarcoding techniques (Gao et al., 2009; Li, et al., 2016; Li, et al., 2016; Duan et al., 2018; Li et al., 2018; Taylor & Cunliffe, 2016; Wang et al., 2018, 2019). A good example of this is fungal communities in coastal regions in China, where the dominant phyla are *Ascomycota* and *Basidiomycota* both in seawater and sediments with large quantities of *Agaricomycetes*, *Eurotiomycetes*, *Dothideomycetes*, *Malasseziomycetes, Tremellomycetes* and *Leotiomycetes* (Li, et al., 2016; Li, et al., 2016; Li et al., 2018; Wang et al., 2018, 2019).

Metabarcoding is the major technique used in the literature mentioned above with the focus on the internal transcribed spacer (ITS) region. When incorporating small subunit ribosomal RNA (SSU) and large subunit ribosomal RNA (LSU) genes, marine fungi in the *Chytridiomycota* phylum, an early diverging fungal phylum, were believed to represent a large proportion of the overall fungal community (Richards et al., 2015; Picard, 2017; Wang et al., 2017). In the coastal environments, the best known diatom parasites are chytrid fungi, which could serve a crucial role in transferring nutrients and organic matter in marine food webs (Gutiérrez et al., 2016; Hassett & Gradinger, 2016). However, there are fewer studies in *Chytridiomycota* which employ the ITS region. One possible explanation is that morphological characteristics are largely used in observing marine chytrids (Sparrow, 1973), which leads to the insufficient reference sequences fungi clades of *Cryptomycota* and *Chytridiomycota* in the UNITE database (http://unite.ut.ee), a databased for fungal molecular identification (Kõljalg et al., 2013).

There are three major factors which control fungal diversity in the coastal habitats, namely dissolved oxygen, salinity and the amount of nutrient. In particular, salinity is considered to be the main factor that determines the diversity of marine fungi communities (Jones, 2000; Gareth Jones, 2011; Taylor & Cunliffe, 2016; Hassett, Borrego, et al., 2019; Rojas-Jimenez et al., 2019). There were observations in transitional

changes in fungal communities with salinity shifts in Rhode Island (Burgaud et al., 2013) and the Delaware Bay (Burgaud et al., 2013). In Plymouth coastal ecosystems, the abundance and diversity of planktonic fungi showed a negative correlation with salinity, observing the highest mycoplankton α-diversity in the months of reduced salinity (Taylor & Cunliffe, 2016). In the East China Sea, water depth and dissolved oxygen have a major influence on the fungal communities because of ocean currents (Li et al., 2018). The level of nutrients, such as phosphate, ammonia and silicate, demonstrated a significant correlation with the richness of fungal operational taxonomic units or the abundance of fungal sequences in the coastal waters of Plymouth (Taylor & Cunliffe, 2016) and North Carolina (Duan et al., 2018). In addition, fungal diversity were found to be positively correlated with phytoplankton biomass in Hawaiian and North Carolina waters (Gao et al., 2009; Duan et al., 2018). There was a similar observation of the upwelling ecosystem in Chilean coastal waters (Gutiérrez et al., 2010). Priest et al. (2021) also found higher diversity and biomass of marine fungi during spring phytoplankton blooms by 18S rRNA metabarcoding technique.

Ocean currents and riverine input have shown major influence on the coastal fungal diversity. Even though some fungi are commonly found in terrestrial habitats, for example soil and plants, these species were often isolated from coastal waters, which indicates that terrestrial fungi scatter to marine ecosystems (Richards et al., 2011; Amend et al., 2019). The occurrence of this fungal dispersal largely attributes to riverine inputs, resulting in the enrichment of planktonic fungal richness and community composition in coastal environments (Taylor & Cunliffe, 2016; Wang et al., 2019). The fungal genus *Byssochlamys*, a typical terrestrial and freshwater group, has been recorded in massive abundance in coastal environments of the East China Sea, which implies these fungi are scattered from the Yangtze River by riverine inputs (Li et al., 2018). Other scientists have also detected fungal riverine inputs to coastal waters (Wang et al., 2018, 2019). It is also suggested that ocean currents can carry planktonic fungi travelling over long distances (Burgaud et al., 2022). One example of this is that the Kuroshio current can passively disperse marine fungi, in particular *Aspergillus* species, to the shelf of the East China Sea (Li et al., 2018). This may greatly impact how marine fungi biogeographically distribute locally and even globally.

2.2.4 Adaptation of marine fungi

Previous literature has consistently emphasised that the filamentous genera *Aspergillus*, *Penicillium*, *Fusarium*, *Cladosporium* and *Trichoderma*, and the yeast genera *Cryptococcus*, *Candida*, *Hortaea* and *Rhodotorula*, are the dominant fungal groups despite a high diversity of marine fungi (Burgaud et al., 2022). It is surprising that these fungal groups are survive well in ocean environments despite being usually known as terrestrial or freshwater species. R dou et al. (2016) defined 'adaptation' as the adjustment that an organism makes to better live in a specific environment either at an expression level (through adaptive response) or at a genetic level (via adaptive evolution). The observations of microscopic features demonstrated how these activities happen in the marine ecosystem. For examples, the conidia of *Acremonium fuci* can only germinate with the existence of the algal *Fucus serratus* tissues (Zuccaro et al., 2004). Regarding ecophysiology, one example is that fungal lifestyles change from terrestrially adapted to marine-adapted on deep-subseafloor sediments (Rédou et al., 2015). The other example is that isolated deep-sea yeasts are able to grow with elevated hydrostatic pressure (Damare et al., 2006; Rédou et al., 2015). When utilising mRNA and rRNA sequencing, current high-throughput omics methods enable to discover how ubiquitous marine fungal species adapt their activity and functions in response to different marine habitats (Edgcomb et al., 2011; Orsi et al., 2013; Pachiadaki et al., 2016; Orsi, 2018; Quemener et al., 2020).

If marine habitats affect the evolution and adaptations of fungal genome, changes in up-regulation or down-regulation at a genetic or pathway level should be recorded through comparison between terrestrial and marine representatives of the same species. Insights into how fungi shift their physiological capabilities, evolve and adapt to the marine environments have been gained by comparing genomics or transcriptomics between the terrestrial and marine isolates of the same species (Burgaud et al., 2022). Comparative genomic findings on three halophilic fungal species, including *Wallemia ichthyophaga*, *Eurotium rubrum* and *Hortaea werneckii*, have shown a large quantity of (1) stress responsive genes (namely catalases and A-/B-barrel proteins), (2) protein-coding genes with a higher percentage of acidic amino acid residues, (3) genes related to the synthesis of hydrophobins and polyol, and (4) genes coding for DNA processing and damage (Lenassi et al., 2013; Zajc et al., 2013; Kis-Papo et al., 2014). The results of complementary comparative transcriptomics demonstrated that the majority of these genes were over-expressed in high salinity environments.

Another method to figure out how fungal species adapt to the marine environment is by studying their secondary metabolism. For example, compared with other terrestrial isolates, only the *Scopulariopsis brevicaulis* isolated from a sponge was able to synthesise anti-cancerous scopularides based on genomic and transcriptomic analyses, indicating these metabolites might play a role in the fungal adaptation to the marine environment (Kumar et al., 2015). According to genomic data at the Joint Genome Institute, the secondary metabolite gene clusters of *Corollospora maritima* and *Lindra thalassiae* show that three types of genes in their genome, namely polyketide synthase genes, non-ribosomal peptide synthetase genes and terpene-encoding genes, largely play an active role in producing unique secondary metabolites (Burgaud et al., 2022). This could be one possible explanation of how fungi adapt their life to the marine habitats. For this reason, metabolomics seems to be a promising method to produce solid evidence to demonstrate how marine fungi interact *in situ* through the production of a broad variety of secondary metabolites, which are different from what is produced by the same terrestrial species (Bhakuni & Rawat, 2005).

In spite of being ecologically important, marine microbiologists in large part have overlooked marine fungi since these fungi were thought to be inactive in the marine ecosystems (Burgaud et al., 2022). With previous literature on the presence, function and activity of marine fungi in various marine habitats, integrated analyses will be performed to understand how these fungi adapt to the marine environments and deal with changes in salinity, temperature and hydrostatic pressure (Rédou, et al., 2016).

Due to their adaptive capabilities, marine fungi can successfully deal with biotic and abiotic stresses in many ways, suggesting them being suitable subjects in biotechnological advances. It is possible that a large number of these capabilities can be adopted and utilised for applications in biotechnology taking the pressing socio-environmental challenges into account (Burgaud et al., 2022). To date, enzymes are recognised as the most important applications. For instance, enzymes could be applied in bioremediation by degrading complex polymers, such plastics and hydrocarbons. Besides, antimicrobial functions are another well-known characteristic of marine fungi derived secondary metabolites (Burgaud et al., 2022).

2.3 Soft corals and their holobiont

The consortium of soft corals, their associated internal and external microbiota is termed as holobiont (Rosenberg et al., 2007). The soft coral-associated microbiome consist of endolithic algae, endosymbiotic dinoflagellates, bacteria, fungi, alveolates, archaea and viruses (Bourne et al., 2016; van de Water et al., 2018). These associated microorganisms provide extra carbon and nitrogen sources to their host, as well as play a part in detoxification and nutrient cycling, genetic exchange, UV protection, and chemical defence for the coral (Rosenberg et al., 2007; Peixoto et al., 2017; van de Water et al., 2018). While fungal diseases have impact on gorgonian populations (Kiho et al., 2004; Smith & Weil, 2004; Kim & Rypien, 2015), relatively few investigations have been conducted on the marine fungal community derived from other octocorals (van de Water et al., 2018). Knowledge on the already known fungal isolates is mainly obtained from conventional culture-based techniques, thus favouring species likely to be cultivated under the laboratory conditions only (Koh et al., 2002; Zhang et al., 2012). Nonetheless, cultured marine fungi have proven to be a promising reservoir of bioactive secondary metabolites, usually with unique chemical structures, thus making octocoral-derived fungi a potential source for bioprospecting(Raimundo et al., 2018). Despite little knowledge on the exact ecological functions of these fungal species, some possess potential antifungal and/or antibacterial properties and might have a role in maintaining holobiont health and regulating microbiome (Bao et al., 2012). However, little attention has been given to the secondary metabolites produced by local soft corals and their associated fungi (Ishii et al., 2010), both globally and locally in the waters of Malaysia

2.4 *Sarcophyton* and their associated marine fungi

Marine fungi are an important constitute of coral-holobiont, the biomass of which makes of up to 0.05% of the coral weight (Raghukumar & Ravindran, 2012). According to Liu et al. (2021) and Singab et al. (2022), there have been 26 species of marine fungi across 15 genera from *Sarcophyton* soft corals to date. Table 2.1 summarises the marine fungi species isolated from the genus *Sarcophyton*. Among all the identified fungi, the most abundant genera are *Aspergillus* (with five isolated species) and *Penicillium* (with four isolated species). Similar findings were also reported for other soft corals and their marine associated fungi around the world (van de Water et al., 2018). In particular, there has been isolations of *Penicillium* spp. and *Aspergillus* spp. from the Caribbean *Gorgonia ventalina* (Toledo-Hernández et al., 2008; Zuluaga-Montero et al., 2010), the East Pacific *Leptogorgia* spp. (Soler-Hurtado et al., 2016), as well as many other soft corals in the South China Sea (Zhang et al., 2012) and Singapore (Koh et al., 2002).

Table 2.1 The marine fungi species isolated from the genus *Sarcophyton*

Soft Coral Species	Fungi	Reference
Sarcophyton ehrenbergi	*Aspergillus nomius, A. flavus*	(Singab et al., 2022)
Sarcophyton subviride	*Aspergillus terreus*	(Liu et al., 2018)
	Penicillium bialowiezense	(El-Demerdash et al., 2020)
Sarcophyton tortuosum	*Chondrostereum* sp., *Alternaria alternata, Aspergillus versicolor, Chaunopycnis* sp., *Cladosporium cladosporioides, C. dominicanum, C. sphaerospermum, Didymella* sp., *Hypocrea lixii, Microsphaeropsis* sp..	(Li et al., 2012; Huang et al., 2018)
	Paraconiothyrium cyclothyrioides, Penicillium citrinum, P. janthinellum, P. oxalicum, Phoma putaminum, Phoma sp., *Pseudocercospora* sp., *Stagonosporopsis cucurbitacearum, Talaromyces allahabadensis, Tritirachium* sp.	(Zhang et al., 2019)
Sarcophyton sp.	*Aspergillus elegans*	(Zheng et al., 2012; Agrawal et al., 2018)
	Pseudallescheria boydii	(Yuan et al., 2019)

2.5 Secondary metabolites from marine fungi

2.5.1 Definition of secondary metabolites

The growth and development of organism rely on the accessible biotic and/or abiotic recourses in the environment. Primary metabolism refers to the production of metabolites that are intrinsic to an organism's development, for example, nucleic acids for DNA, proteins for basic cellular functions, or lipids for cell membranes. Primary metabolites are vital for an organism's growth because their involvement in the structure of organisms, cell machinery and energy storage. Thus, these are common and universal metabolites produced by organisms. On the other hand, organisms respond to various stimuli by producing secondary metabolites, which are not regarded as essentials for an organism's survival. Nonetheless, secondary metabolites are generally responsive to specific needs, in order to defend against predators, adapt to environmental changes or communicate with other organisms. In fact, these metabolites can sometimes be essential for an organism's survival, such as settlement cues and mate recognition. The production of secondary metabolites is for the fulfilment of specific functions or interaction with certain biological targets (Burgaud et al., 2022).

As many secondary metabolites demonstrate valuable therapeutic properties, they attract growing interests in the pharmaceutical industry. Since 1980, over one third of the approved drugs are derived from secondary metabolites and the drug industry are still aiming for producing new drugs from natural origins (Newman & Cragg, 2020).

Fungi are of valuable importance to lead compounds and/or drug discovery. They are capable of producing a broad range of antimicrobial compounds, and since the discovery of penicillin, the screening for fungal secondary metabolites has become highly generalised (Burgaud et al., 2022). However, fungal metabolites are not limited to potential candidates for antibiotics. For example, lovastatin is used in many drugs for lowering cholesterol levels, and cyclosporine is currently used after transplantation operations to suppress the immune response (Numata et al., 1993). In ecological terms, secondary metabolites enable marine fungi to interact with other organisms in a diverse manner. For example, penicillin serves ecological functions, which defends the fungi against bacteria, whereas α-amatoxin is believed to contribute to fungal chemical defence system (Burgaud et al., 2022). In addition, gliotoxin is considered to be a virulence factor (Burgaud et al., 2022).

Secondary metabolites are synthesised from building blocks produced by primary metabolism for instance sugars, fatty acids and amino acids. Different biosynthetic

pathways have been reported to produce different categories of secondary metabolites, including peptides, alkaloids and polyketides. It is surprising that a small number of building blocks can produce a massive number of secondary metabolites. These pathways are regulated by specific chemical reactions, and many of these reactions employ specific enzymes that are encoded in the fungal genome. In fact, the majority of these fungal metabolites are encoded by biosynthetic gene clusters (Burgaud et al., 2022). An individual cluster generally comprises most genes involved in producing a specific secondary metabolite. These genes are called 'clusters' because they are typically located adjacent to each other in the genome. Biosynthetic gene clusters include (1) genes that encode proteins regulating how the backbone of the metabolite is biosynthesised, such as non-ribosomal peptide synthetases, terpene cyclases, prenyltransferases and polyketide synthases, (2) genes that encode enzymes modifying the backbone, including methyl-transferases, epimerases and hydroxylases, (3) transcription factors regulating the expression of biosynthetic gene clusters, (4) proteins participating in metabolite transport, and (5) proteins conferring resistance to secondary metabolite activities (Rédou, Vallet, et al., 2016).

According to the analyses of sequenced fungal genomes available, there is a much larger number of genes which encodes the highly conserved non-ribosomal peptide synthetases and polyketide synthases, and this finding is also applicable to those extensively studied fungal strains for natural products production (Burgaud et al., 2022). Therefore, marine fungi in general have more undiscovered secondary metabolic biosynthetic clusters than previously described in terms of their structure, identity and function.

2.5.2 Marine fungi chemodiversity

In the marine environments, fungi have developed adaptative strategies to deal with various stimuli by expressing particular biosynthetic pathways to defend or communicate. A well-known fact is that competition between marine microbes and the marine environment is extremely harsh. Taking a tropical coral reef as an example, 1,000 species can be found in one square meter, indicating the requirement to find ways to co-exist (Burgaud et al., 2022). For this reason, it is necessary to produce chemical cues and have chemical communication.

Four decades ago, there were merely 15 secondary metabolites reported from marine fungal species (Fenical & Jensen, 1993). In the last two decades, this figure has drastically increased (Bugni & Ireland, 2004; Rateb & Ebel, 2011; Blunt et al., 2016; Daletos et al., 2017). In 2020, it was believed that there were over 3,500 secondary metabolites documented on Natural Product Atlas (van Santen et al., 2019), among which nearly 22% of all fungal metabolites were isolated from marine species (Burgaud et al., 2022).

In fact, there have been many secondary metabolites produced by marine fungi with clinical importance, such as antibiotics (Blunt et al., 2018; Demain, 2014; Rateb & Ebel, 2011; Stefan Svahn et al., 2012). For example, indanonaftol A was isolated from a marine strain of *Aureobasidium* sp. (Biabani & Laatsch, 1998), and gliotoxin was isolated from a deep-sea *Aspergillus* sp. (Fan et al., 2016). Fungi synthesise secondary metabolites correspond with different stages in their life cycle (Calvo et al., 2002), and with their capabilities for inhabitation and survival in various ecosystems. Nonetheless, it can be challenging to identify fungal secondary metabolites because they need to be synthesised with similar culture conditions to the extreme habitats the fungi live in, for example in deep biosphere or the deep sea (Burgaud et al., 2022). To date, there have been only a few successfully isolated fungal secondary metabolites under elevated pressure condition (>90 atm) since constraints in technology have been posed to recover and culture piezotolerant or piezophile fungi with high temperatures (A. Kumar et al., 2015). However, bioactive compounds produced by deep-sea fungi have various biotechnological potential (Nicoletti & Trincone, 2016). The two fungal genera *Penicillium* and *Aspergillus* are the primary but not exclusive sources of bioactive molecules isolated so far (Petersen et al., 2020; Arifeen et al., 2019). These bioactive molecules have antifungal, antimicrobial, antiviral, cytotoxic and anti-inflammatory properties (Y. T. Wang et al., 2015).

Regarding secondary metabolic research into *Penicillium* and/or *Aspergillus* strains collected from deep-sea hydrothermal vents (2255 m), and from deep-sea sediments in the South China Sea (4593 m depth) and the Pacific Ocean (~5000 m depth), scientists have isolated a wide variety of (1) novel alkaloids, such as brevicompanines D - H, circumdatins F and G, meleagrins D and E, sorbicillamines and cyclopiamides, (2) steroid- and terpenoid-derivatives, including breviones and sterolic acid, and (3) polyketides, namely aspiketolactonols, aspilactonol, epiaspinonediol and aspyronol (Li et al., 2012; Chen et al., 2014; Fredimoses et al., 2014; Xu et al., 2015). Alkaloids derived from a deep-ocean *Penicillium* sp. showed weak or moderate cytotoxic activity against cancer cell lines, as well as anti-inflammatory properties (Du et al., 2009). The steroid and terpenoid metabolites exhibit cytotoxic effects against lung and breast cancer cells, and antiviral properties against HIV-1 (Li et al., 2012). In terms of *Aspergillus* sp., epiaspinonediol and spyronol, two novel C9 polyketides, together with the terpenoids wentilactones, demonstrate cytotoxicity against human leukemia cells, suggesting their potential for future antitumor agents (Xu et al., 2015). Phenolic compounds from deep-sea *Aspergillus* sp. collected from sediments in the South China Sea (from 2326 to 3002 m) exhibited antifouling properties and could regulate the activation of transcription factors involved in detoxification processes (Wu et al., 2016). Other phenolic compounds from the same fungal strain also showed potential antiviral activity against Herpes Simplex Virus 1 (Wu et al., 2016). Another fungal strain isolated from deep-sea sediments in the South China Sea (*Alternaria* sp.) could synthesise novel perylenequinones, which could inhibit cancer development-related transcriptional and epigenetic regulators (e.g., bromodomain-containing protein 4) (Ding et al., 2017).

Four other fungal strains, *Acaromyces* sp., *Cladosporium sp.*, *Engyodontium* sp. and *Simplicillium* sp., which were isolated the Indian Ocean with a depth of 3471 - 4571 m and the South China Sea with a depth of 3415 to 3739 m, were documented to produce various secondary metabolites with pronounced antitumor activities against cancer and lymphoma cells (Yao et al., 2014; Gao et al., 2016; Zhang et al., 2016). At Prydz Bay of Antarctica, a deep-sea *Penicillium* sp. was collected from deep sediments at a depth of 1393 m and from deep waters at a depth of 2284 m, and this fungal strain could produce a broad range of terpenes, including asperethers A – E, conidiogenones C and I, aspewentin A and D - H, guignarderemophilane F, spirograterpene A, and asperolides D and E (Zhou et al., 2018). These secondary metabolites demonstrated similar antiallergic properties to that of loratadine, a type of common antihistamines, and were therefore believed to participate in the inhibition of the protein tyrosine phosphatase 1B, the

production of which is implicated in type 2 diabetes (Zhou et al., 2018). In the Greenland Sea, marine fungi species belong to the Lindgomycetaceae family were collected from the deep-sea sediments, and could produce a bioactive compound named lindgomycin, which showed similar antibiotic effects to that of chloramphenicol (Wu et al., 2015). Researchers have also conducted antibacterial studies on two polypeptides, canescenins A and B, and other nitrogen-bearing hetero- cyclic compounds, such as piperazine derivatives, produced by deep-sea *Aspergillus* sp., *Penicillium* sp., and *Dichotomomyces* sp. (Fan et al., 2016; Wang et al., 2016; Dasanayaka et al., 2019). It is likely that dichotocejpins produced by *Dichotomomyces* sp. showed antifungal properties due to the inhibition of α-glucosidase (Fan et al., 2016). Polyketides and terpenes isolated from two deep-sea fungal strains *Phialocephala* sp. and *Ascotricha* sp. exhibited cytotoxicity and antifungal properties (Zhang et al., 2018; Ganesh Kumar et al., 2019). Computational studies have suggested that ascotrichin produced by the fungus *Ascotricha* sp. can bind to serotonin receptors, which indicates their potential as a drug development target in the field of central nervous system disorders (Ganesh Kumar et al., 2019).

2.6 Secondary metabolites from major fungal genera in *Sarcophyton* soft corals

Given the consistency of fungal associations in soft corals, Hou et al. (2015) also maintains that novel chemistry was found in the metabolites from the most common genera *Penicillium* and *Aspergillus*. In addition, some new or rare fungi associated with soft corals, such as the genera *Candida, Chaetomium, Hypocrea and Labyrinthula,* could produce unique and novel compounds (Hou et al., 2015).

2.6.1 Genus *Penicillium*

In 2018, the marine fungus *Penicillium bialowiezense* was isolated from *Sarcophyton subvirride* in Xisha Islands, in the South China Sea (El-Demerdash et al., 2020). The isolated *P. bialowiezense* was cultured on potato dextrose agar (PDA) and produced 11 acyclic merohemiterpenes: 8-O-methyl mycophenolic acid (**1**), 3-hydroxy mycophenolic acid (**2**), 6-(5-carboxy-3-methylpent-2-enyl)-7-hydroxy-3,5-dimethoxy-4-methylphthalan-1-one (**3**), 6-(5-methoxycarbonyl-3-methylpent-2-enyl)-3, 7-dihydroxy-5-methoxy-4-methylphthalan-1-one (**4**), 6-(3-carboxybutyl)-7-hydroxy-5-methoxy-4-methylphthalan-1-one (**5**), 6-[5-(2,3-dihydroxy-1-carboxyglyceride)-3-methylpent-2-enyl]-7-hydroxy-5-methoxy-4-methylphthalan-1-one (**6**), 6-[5-(1-carboxy-4-N-carboxylate)-3-methylpent-2-enyl]-7-hydroxy-5-methoxy-4-methylphthalan-1-one (**7**), N-mycophenoyl-l-valine (**8**), N-mycophenoyl-1-phenyloalanine (**9**), N-mycophenoyl-1-alanine (**10**) and mycophenolic acid (MPA) (**11**) (El-Demerdash et al., 2020). The structures of these compounds are shown in Figure **2.1**.

There are three compounds belonging to the mycophenolic acid family, mainly found in the genus *Penicillium*, 8-O-methyl mycophenolic acid (**1**), 3-hydroxy mycophenolic acid (**2**) and MPA (**11**). These compounds are all isolated in white crystals and commonly known for their antiviral and immunosuppressive bioactivities (Zhang et al., 2018). Compounds (**3-7**) were isolated in the form of white powders and first reported and characterised after isolation. The structures of compounds (**2-3**) are identical to each other, except the functional group at C-3, where the hydroxyl group of 3-hydroxy mycophenolic acid (**2**) was replaced by a methoxy in compound (**3**). The carboxyl group at C-6' in compound (**3**) was found to be methyl-esterified in compound (**4**) (Zhang et al., 2018). The isolated compound (**5**) bears close resemblance to euparvic acid. The skeletons of compounds (**6-7**) are also identical to each other, although the 2,3-dihydroxypropyl group in compound (**6**) was replaced by a 4-aminobutanoic acid moiety in compound (**7**) (Zhang et al., 2018). On the other hand, compounds (**8-10**) were colourless solids at the time of isolation (El-Demerdash et al., 2020).

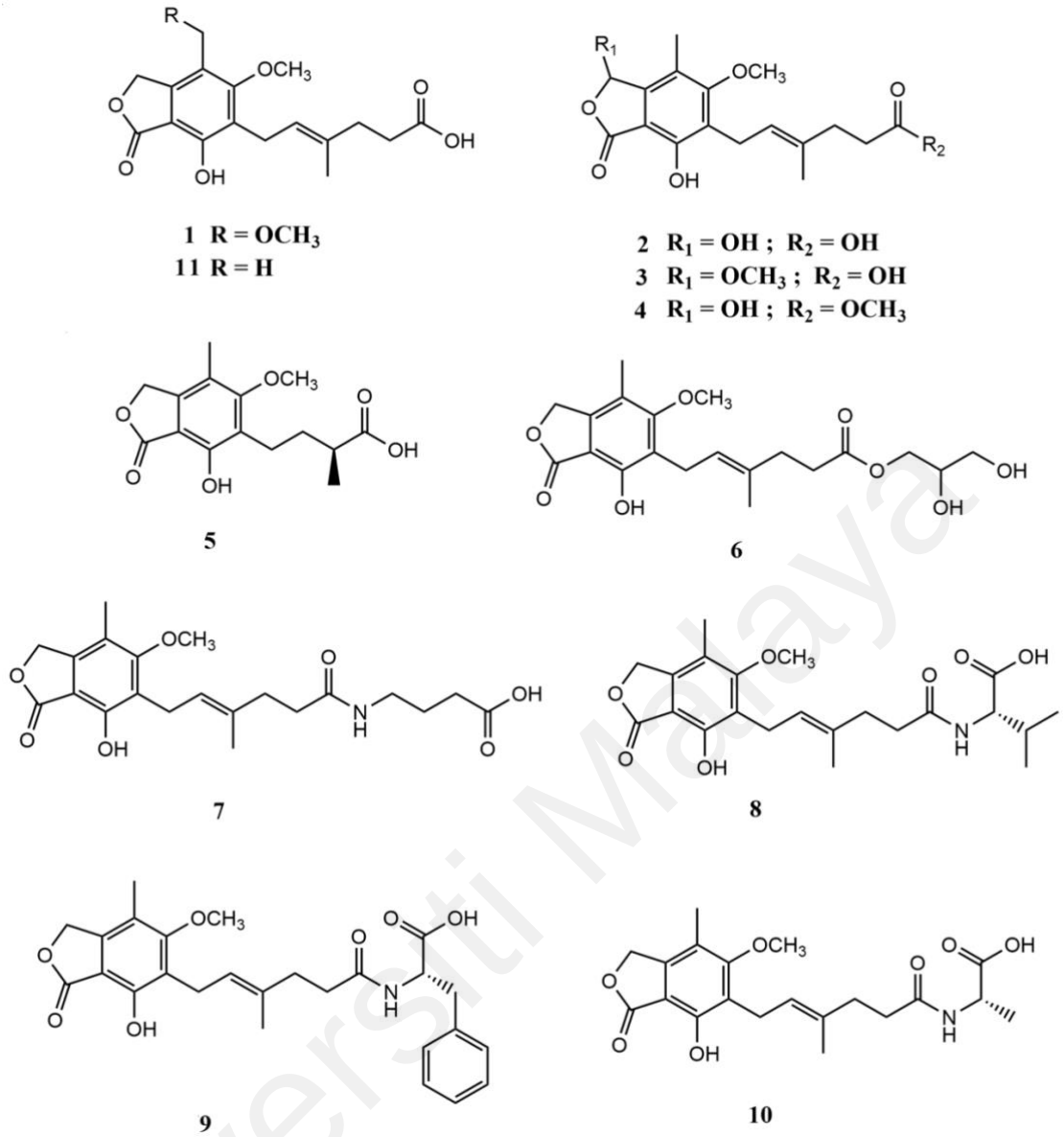


Figure 2.1 Metabolites (1-11) from *Sarcophyton*-derived *Penicillium bialowiezense*

In terms of bioactivity, compounds (**1-11**) all exhibited potent inhibition against inosine-50-monophosphate dehydrogenase (IMPDH2), which is a key enzyme in the biosynthesis of *de novo* purine nucleotide and is generally considered as an important target for developing antibacterial, antiviral and anticancer drugs (Shu & Nair, 2008). In addition, the IC_{50} values of compounds (**1-11**) ranges between 0.59 and 24.68 μM. As for immunosuppressive activity against the proliferation of T-lymphocytes *in vitro*, the IC50 values of compounds (**1-3**) ranged from 0.84 to 0.95 μM, whereas the IC_{50} values of compounds (**4-11**) varied from 3.27 to 24.68 μM.

In the same year, Zhang et al. (2019) isolated three *Penicillium* species, *P. citrinum*, *P. janthinellum* and *P. oxalicum*, from *Sarcophyton tortuosum* in the South China Sea. The crude extracts of these fungal isolates were further tested for their anti-microfouling activity by estimating the inhibitory zone (mm) to three indicator bacteria.

While *P. janthinellum* showed moderate anti-microfouling activity against three bacteria, with *Loktanella hongkongensis* (7.4 ± 0.9 mm), *Micrococus luteus* (2.3 ± 0.7 mm) and *Pseudoalteromonas piscida* (5.7 ± 0.8 mm), *P. oxalicum* only displayed a slightly higher moderate anti-microfouling activity (6.2 ± 0.4 mm) against *Micrococus luteus* (Zhang et al., 2019). Thus, Zhang et al. (2019) proposed that *P. janthinellum* and *P. oxalicum* with antifouling activity could play a protective role in their coral host against pathogens and some other marine fouling organisms. The results from this study also support the previous literature that coral-derived marine fungi are able to produce a wide range of intracellular and extracellular secondary metabolites (Blunt et al., 2018) and these metabolites could have the potential for environmentally-safe antifouling agent discovery (Shao et al., 2015).

2.6.2 Genus *Aspergillus*

At present, *Aspergillus* spp. are the most prolific producers of secondary metabolites among microbes-associated with soft corals (Raimundo et al., 2018). They can synthesise a wide variety of secondary metabolites, including alkaloids, cytochalasins, lactones and phenylalanines with antibacterial, antifouling and anti-inflammatory properties (Raimundo et al., 2018).

The most studied *Aspergillus* isolated from *Sarcophyton* spp. is *A. elegans*, which has been isolated from *Sarcophyton* sp. in the South China Sea (Zheng et al., 2013). In 2013, two novel cytochalasins, apochalasin A1 (**12**) and cytochalasin Z24 (**13**), were isolated in white powders from ethyl acetate extract of *A. elegans* cultures. These compounds belong to indole alkaloids, which are usually products of a wide range of microorganisms (Zheng et al., 2013). In fact, apochalasins are categorised as a subgroup of cytochalasins, which constitute of a macrocyclic ring, isoindolone moiety and a 2-methyl-proply group. The newly reported compounds (**12-13**) were found to have similar macrocyclic properties to other known cytochalasins. In addition, another eight known cytochalasin-derivatives (**14 -21**) were also isolated from the same study, all of which had been formerly reported from different species under the genus *Aspergillus* (Zheng et al., 2013). Although aspochalasins B (**14**) and D (**15**) were isolated from *Aspergillus niveus* derived from a marine crustacean (Gebhardt et al., 2004), aspochalasin H (16) was first reported from a strain of *Aspergillus* sp. which produced aspochalasin D (Liu et al., 2021). Aspochalasins I (17) and J (18) were initially isolated from *Aspergillus flavipes* derived from the rhizosphere of a turpentine bush *Ericameria laricifolia* (Zhou et al., 2004). Similarly, the compound aspergillin PZ (19) also originated from an *Aspergillus* species (*A. awamori*),

despite being a soil fungus (Zhang et al., 2002). Besides, zygosporin D (20) was also reported from a soil fungus *Metarrhizium anisopline* (Fujii et al., 2000), whereas rosellichalasin (21) has been reported from an *Aspergillus* strain from a marine solar saltern in China (Xiao et al., 2013). The structures of indole alkaloid compounds (12-21) are shown in Figure 2.2.

Classified as cytochalasins, the fungal alkaloid metabolites (**12-21**) could possess various bioactivities targeting cytoskeleton processes by binding to actin filaments and blocking the polymerisation and elongation of actin (Liu et al., 2021). Compound (**15**) displayed a broad variety of antibacterial abilities, especially against the following four pathogenic bacterial strains, *Staphylococcus epidermidis, S. aureus, Escherichia coli* and *Bacillus cereus* (Zheng et al., 2013). On the contrary, compound (**17**) only demonstrated moderate inhibition activity, in terms of minimum inhibition concentration (MIC), against the terristerial bacteria *S. aureus* (MIC = 10 μM) and *S. epidermidis* (MIC = 20 μM). Besides, compounds aspochalasin D and H-J (**15-18**) exhibited strong antifouling properties against the the barnacle, *Balanus amphitrite*, larval settlement (EC_{50} = 6.2, 37, 34 and 14, respectively) (Zheng et al., 2013). According to Zheng et al. (2013) the electrophilic α,ß-unsaturated carbonyl moiety in compound (**15**) takes an important part in the antifouling properties of these cytochalasins. Give that compound (**15**) had higher antifouling activity than compound (**16**), Zheng et al. (2013) deduced that the presence of a double-bond between C-19 and C-20 was the possible active site for cytochalasin antifouling activities.

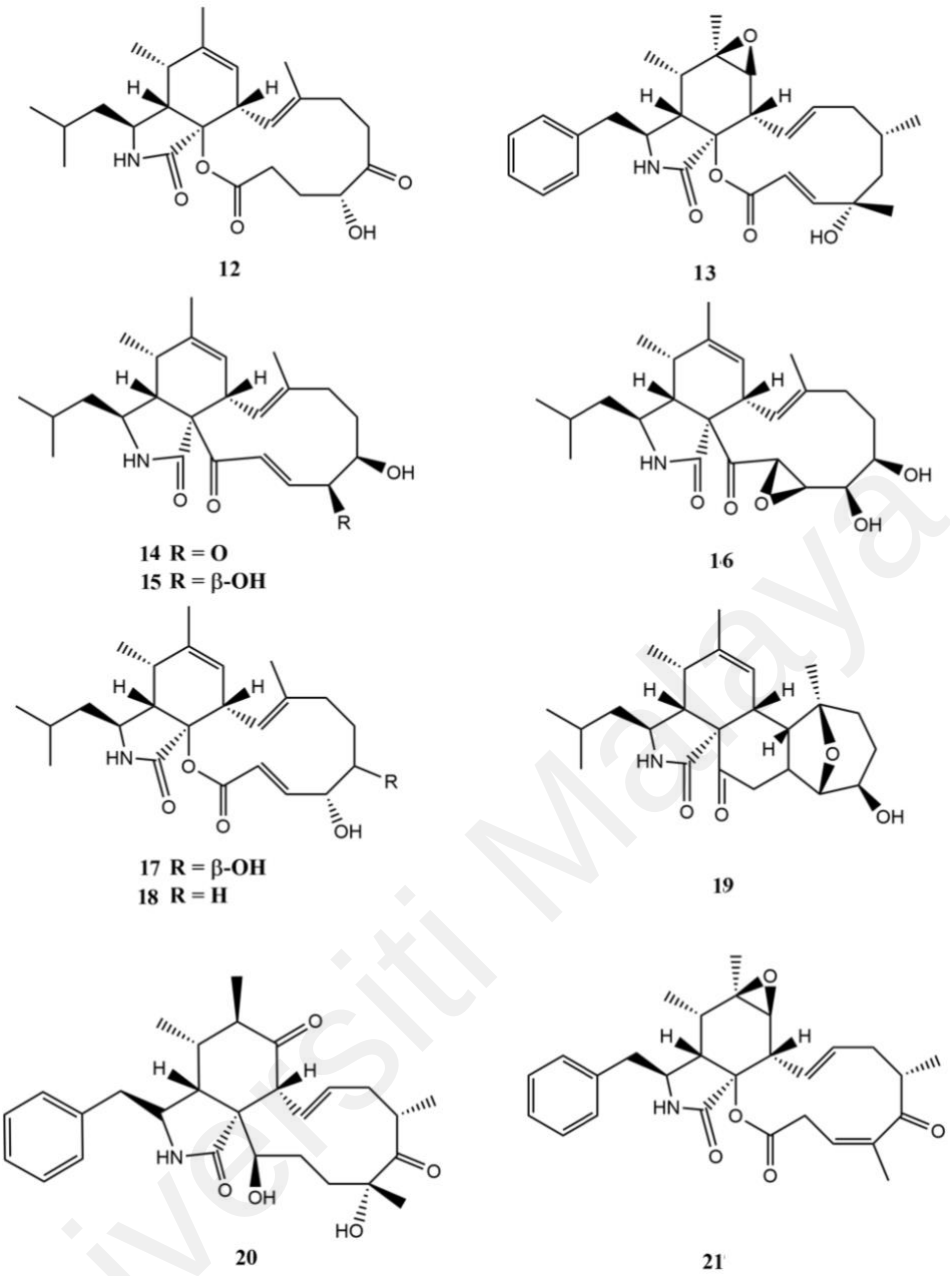
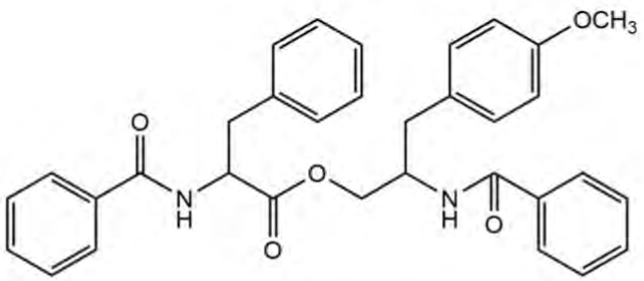
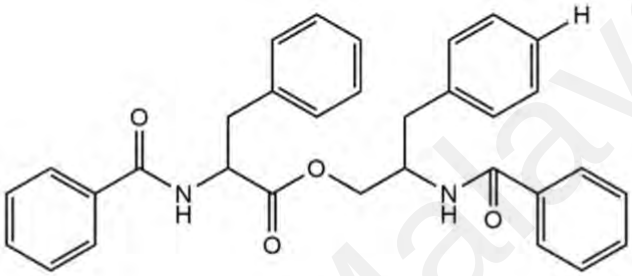


Figure 2.2 Indole alkaloid compounds (12-21) from *Sarcophyton*-derived *Aspergillus elegans*

Apart from indole alkaloids, Zheng et al. (2013) have also isolated two amino acid derivates, 4'-OMe-asperphenamate (**22**) and asperphenamate (**23**). Compounds (**22-23**) are both phenylalanine derivatives with an identical skeleton structure. Compound (**22**) was a newly identified secondary metabolite at the time of isolation, while compound (**23**) was first record of asperphenamate isolated from a marine-derived fungus (Zheng et al., 2013). The structures of compounds (**22-23**) are shown in Figure 2.3.



22 R= OCH_3



23 R = H

Figure 2.3 The chemical structures of compounds (22-23)

In 2018, *Aspergillus terreus* was isolated from the soft coral *Sarcophyton subviride* in the South China Sea, and a total of 12 secondary metabolites was isolated from the cooked rice cultures (Liu et al., 2018). In addition, these metabolites belong to a wide variety of chemical groups. This study characterised three novel compounds, luteoride E (**24**), versicolactone G (**25**) and (3E,7E)-4,8-di-methyl-undecane-3,7-diene-1,11-diol (**26**), together with nine known compounds asterrelenin(**27**), methyl 3,4,5-trimethoxy-2-(2-(nicotinamido)benzamido)benzoate (**28**), 140-hydroxyergosta-4,7,22-triene-3,6-dione (**29**), territrem A (**30**), territrem B (**31**), territrem C (**32**), lovastatin (**33**), monacolin L acid methyl ester (**34**) and monacolin L (**35**) (Liu et al., 2018). Compound (**24**), isolated as a yellow oil, was classified as a prenylated tryptophan derivative with a 3,7-disubstituted indole. Compound (**24**) displayed potent inhibition against lipopolysaccharide (LPS)-induced nitric oxide (NO) production by RAW 264.7 cells (IC_{50} = 24.64 μM). Compound (**25**) was a kind of butanolide, which was obtained as an amorphous white powder. In addition, compound (**26**), obtained as a colourless oil, was classified as a linear aliphatic alcohol. When compounds (**24-26**) were screened for α-glucosidase inhibitory activity, only compound (**25**) exhibited strong potential inhibitory ability (IC_{50} = 104.8 ± 9.5 μM) (Liu et al., 2018). Even though compounds (**24-26**) showed no antibacterial activity, they

all displayed anti-inflammatory properties against NO production with IC_{50} values ranging from15.7 to 24.6 μM. Compound (**27**), isolated as a colourless cubic crystal, is a type of alkaloids and was first isolated from *A. terreus* in 2005 (Li et al., 2005). Compound (**28**) was initially characterised from *A. terreus* cultures with 10% of salt (a high saline environment) (Wang et al., 2011). In fact, if the salt concentration is at 3% or below, the production of compound (**28**) will not be triggered. Compound (**28**) also demonstrated mild antibacterial property with an MIC = 52.4 μM against *Staphylococcus aureus* and *Enterobacter aerogene* (Wang et al., 2011). Before isolation from *A. terreus*, compound (**29**) was only obtained through synthesis, and this is the first reports on its origin in nature. In addition, compounds (**30-32**) are nitrogen lacking tremorgenic mycotoxins, which was isolated from PDA culture of *A. terreus* (Liu et al., 2018). However, this compound was previously reported from rice cultures of *A. terreus* (Li et al., 2005). Compounds (**31-32**) demonstrated strong anti-acetylcholinesterase inhibitory activity (IC_{50} = of 4.2 ± 0.6 μM and 20.1 ± 3.3 μM, respectively) (Nong et al., 2014). Compound (**33**) is a well-known fungal secondary metabolite, which was initially reported from *Aspergillus sclerotiorum*. Compound (**33**) exhibited cytotoxicity towards Vero (normal kidney) cells with IC_{50} values fall between 2.2 and 8.4 μM, and inhibition towards the hydroxymethylglutaryl-coenzyme A reductase (HMGR-CoA) activity by 42% at 200 μM (Phainuphong et al., 2016). This is because lovastatin inhibits HMGR-CoA, relates to the cause of reduced cholesterol in humans and has cytotoxicity towards MCF-7, the human cervical cancer cell line (HeLa), the human liver cancer cell line (HepG2), and the human skin melanoma cell line (B16F10) (Liu et al., 2021). Besides, the compounds territrem A (**30**) and lovastatin (**33**) displayed strong inhibitory potency against NO production with IC_{50} ranging from 5.48 to 29.34 μM (Liu et al., 2018). Lastly, compounds (**34-35**) were previously reported as a by-product from the production of a traditional Chinese food and medicine called the red yeast rice, which requires the fermentation of sterile rice using the fungal strain *Monascus purpureus* (Ma et al., 2000). The chemical structures of compounds (**24-35**) are shown in Figure 2.4.

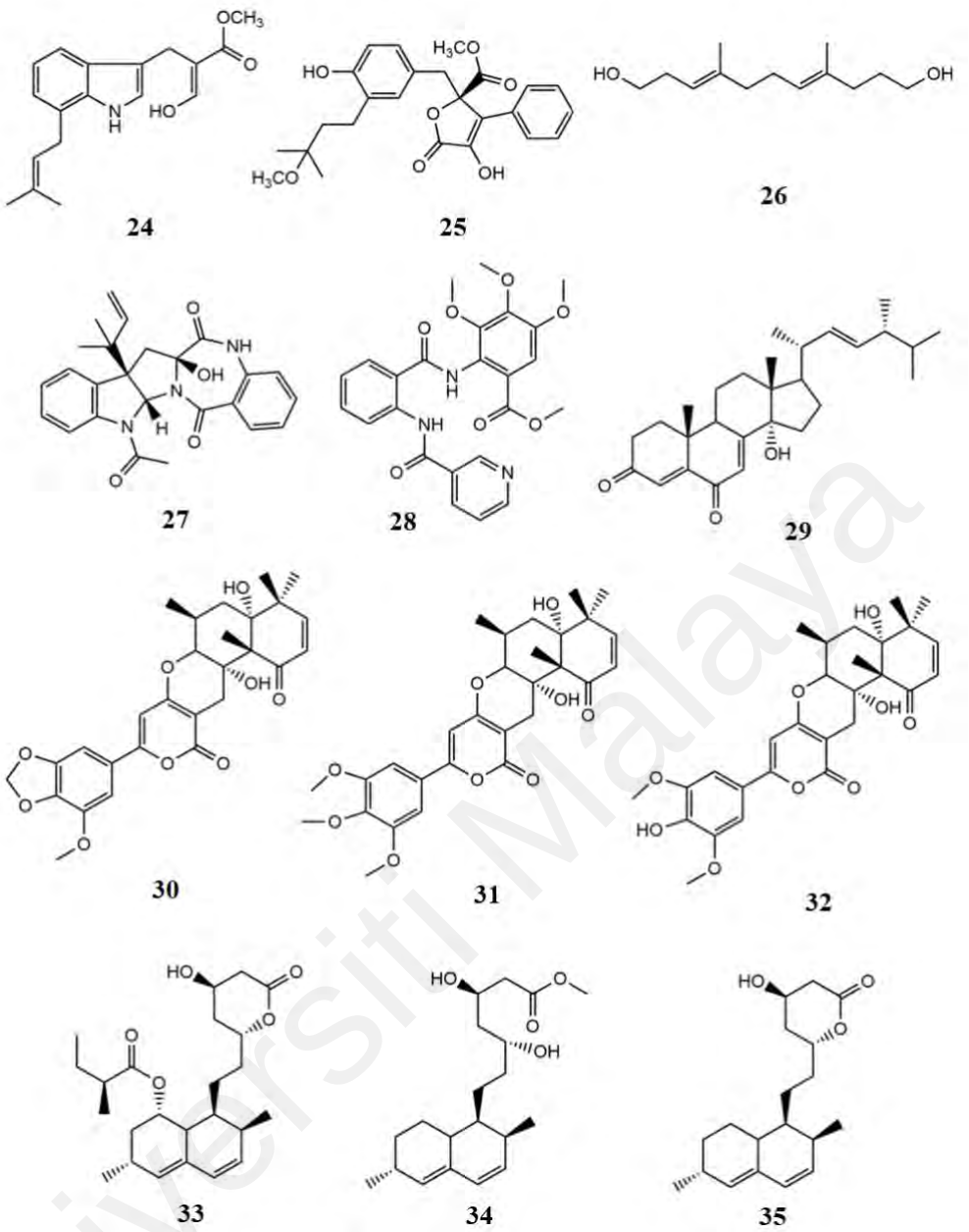


Figure 2.4 The chemical structures of compounds (24-35)

Although three species of *Aspergillus* were isolated from the soft coral *Sarcophyton* spp., the most well-known *Aspergillus* associated with soft corals, *Aspergillus sydowii*, which did not report from the genus *Sarcophyton*. The fungus *Aspergillus sydowii* (Bainier & Sartory) Thom & Church, commonly known as a food contaminant and sometimes an opportunistic pathogen in humans, is a mesophilic soil saprobe (Olutiola & Cole, 1977; Rinaldi, 1983; Ghareib & Dein, 1990). *A. sydowii* can cause a disease called aspergillosis and this disease may lead to the decline in gorgonian coral communities, the dominant reef taxa in the Caribbean oceans (Chiappone & Sullivan, 1994; Geiser et al., 1998; Kiho et al., 2004). Although aspergillosis is one of the intensively studied coral diseases, its origin, transmission mode and pathogenicity mechanisms are unknown

(Rypien et al., 2008). Similar to other opportunistic diseases, the ability of *A. sydowii* to cause diseases depends on the host's genetic composition and immune status, as well as the degree and period of exposure (Rinaldi, 1983). The health of the host is important for its survival undergoing aspergillosis. For example, compared to less frequent incidents in healthy individuals, aspergillosis induced by *A. fumigatus* is more likely to cause death in immune-comprised humans (Dixon & Walsh, 1992).

Despite successful isolation from marine environments, *Aspergillus* species (including *A. sydowii*) are not considered normal inhabitants in the ocean (Alker et al., 2001). Nevertheless, there seems to be one significant difference between terrestrial and marine strains of *A. sydowii*. Compared to those strains isolated from diseased sea fan corals, terrestrial strains were found not to be pathogenic to sea fans (Geiser et al., 1998).

There are several theories concerning the origin of this typical terrestrial fungus in marine environments. According to the Endemic Marine Hypothesis, *A. sydowii* has existed for a long period in the ocean, and only recently this species has the ability to cause diseases as a result of environmental or host immune system changes (Rypien et al., 2008). This hypothesis is supported by the presence of *A. sydowii* in water sample before the epidemic (Sparrow, 1937; Roth et al., 1964; Steele, 1967). The second theory is the African Dust Hypothesis (Rypien et al., 2008), which explains *A. sydowii* entered marine ecosystems recently through dust from the Sahara-Sahel area in Africa and then it was carried across the Atlantic Ocean by winds before deposited in the Caribbean (Shinn et al., 2000; Garrison et al., 2003). This theory is based on the discovery of viable *Aspergillus* spp. in Saharan dust (Kellogg et al., 2004), and recent increased deposition of Saharan dust in the Caribbean (Prospero & Nees, 1986; Prospero & Lamb, 2003) theory is the Terrestrial Runoff Hypothesis (Rypien et al., 2008), which proposes that *A. sydowii* entered marine environments together with local terrestrial runoff (Smith et al., 1996). This explanation is backed up by the rising amount of terrestrial sediment in the Caribbean as a result of coastal development (Burke & Maidens, 2004).

All the three theories suggest a difference in genetic diversity and relatedness patterns between environmental and infectious *A. sydowii*. In order to verify these hypotheses, a global population genetics study investigated the genetic diversity, population structure, population differentiation and morphology among these fungal isolates. The results, however, show a low level of differentiation between disease-causing and environmental *A. sydowii* isolates (Rypien et al., 2008). This study also suggests that, in view of its presence in diverse environments (including terrestrial sediments, the ocean and the atmosphere), *A. sydowii* is likely to exist, and have existed in most marine ecosystems for

many years. Therefore, it seems unlikely that specific virulence factors contribute to aspergillosis since infectious *A. sydowii* isolates draw no clear genetic distinction from environmental isolates, and any *A. sydowii* isolate is assumed to have the ability to cause aspergillosis. The variance in global aspergillosis prevalence might be on-site factors, including host density (Jolles et al., 2002), nutrient levels (Bruno et al., 2003; Baker et al., 2007), temperature (Ward et al., 2007), water movement (Kiho et al., 2004) and varied infection resistance ability of hosts (Dube et al., 2002)

2.7 Effect of temperature on fungal metabolites

Since most coral-associated microorganisms require higher temperatures than their hosts, increases in seawater temperature are forecast to alter coral-fungus interaction to favour the pathogenic ones (Alker et al., 2001). This is because the defence efficacy of gorgonian corals can be comprised under stress (Arkoosh et al., 1998; Lenihan et al., 1999; Ross et al., 1996), even though they are able to produce antifungal secondary metabolites for disease resistance (Kim et al., 2000). For corals, either higher or lower temperature changes are significant stress factors, and these changes can break down the symbiosis between the host and its zooxanthellae, such as coral bleaching (Brown, 1997).

Alker et al. (2001) have done a characterisation study on four different strains of *A. sydowii*, in which they measured the diameters of the colony under four temperatures (22°C, 25°C, 30°C and 36°C) over 10 days and tested the ability of gorgonian coral extracts to inhibit fungal growth at 25 and 30°C. They have found that temperature had a strong influence on the growth rates of *A. sydowii*, with the maximum at 30°C. The results suggest that water temperature increases in summer may lead to higher chances of aspergillosis and its pathogenicity. In addition, they found out that the efficacy of the crude extracts from *Gorgonia ventalina* was temperature-dependent, with the least active at 30°C than 25°C. Therefore, higher temperature tends to promote fungal growth and make it overcome host's defence mechanism (Alker et al., 2001).

Although there is limited research on the effects of temperature on secondary metabolism of coral-associated fungal species, similar studies have carried out in fungi from other environments. In 2013, Mathan et al. examined the impact of temperature on secondary metabolite production from an endophytic fungi *Aspergillus terreus* isolated from the seaweed *Codium decorticatum*. The results indicate that 25°C was the optimal temperature for antimicrobial metabolite production of *A. terreus*, when cultured at 15°C, 20°C, 25°C, 30°C, 35°C, 40°C and 45°C (Mathan et al., 2013). The highest growth, in terms of mycelial weight, was also observed at 25°C. However, *A. terreus* produced the

lowest biomass and bioactive secondary metabolites at 15°C and 20°C. When incubation temperature rose from 25°C to 45°C, both biomass yield and production of antimicrobial metabolites showed a gradual reduction. Thus, the production of secondary metabolites follows a linear pattern before and after the optimum growth temperature.

The findings of Mathan et al. (2013) are similar to a previous study on *A. terreus* carried out by Jain and Pundir (2011). According to Jain and Pundir, when incubated at five temperatures (20°C, 25°C, 30°C, 35°C and 45°C) the optimal biomass yield and antimicrobial metabolite production were also observed at 25°C. The difference was that *A. terreus* was isolated from various soil samples. Thus, whatever the source of *A. terreus*, this fungal species demonstrates the same optimal temperature for growth and antimicrobial production.

Regarding soil fungi, a more recent study has investigated the effect of increased temperatures (at 4°C, 10°C, 15°C and 28°C) on the secondary metabolite profiles of different polar soil fungi, in which the highest peaks of major compounds were found in *Atradidymella* sp. at 4°C, whereas higher concentrations of these compounds were recorded at 15°C for *Pseudogymnoascus* sp. and *Penicillium flavigenum* (Yogabaanu et al., 2017). In contrast, reductions in the metabolite production were observed at 10°C among all fungal isolates, suggesting that the effect of temperature on secondary metabolite production was not in a simple fashion (Yogabaanu et al., 2017).

Research in arbuscular mycorrhizal fungi (AMF) suggests that temperatures 5 - 10°C lower than the normal could significantly promote growth in fresh weight of their host (cucumber) and their host's secondary metabolite production, especially flavonoids, phenols, phenolic compounds and lignin (Chen et al., 2013). Their research also reported large increases in enzyme activities related to secondary metabolism in their hosts, thus suggesting AMF may play a role in optimization of their host's growth through regulating secondary metabolite production.

Therefore, based on those studies, temperature exerts an effect on fungal secondary metabolite production; however, this influence may not always follow a linear pattern.

CHAPTER 3: METHODOLOGY

3.1 Sampling Site

Samples were collected from Blue Lagoon in Port Dickson, Negeri Sembilan (GPS: 101°51′13.93″E, 2°25′11.95″N). The site was selected based on previous studies where healthy shallow coral reefs have been determined to be abundant (Praveena, Siraj, & Aris, 2012). Figure 3.1 shows the sampling site.

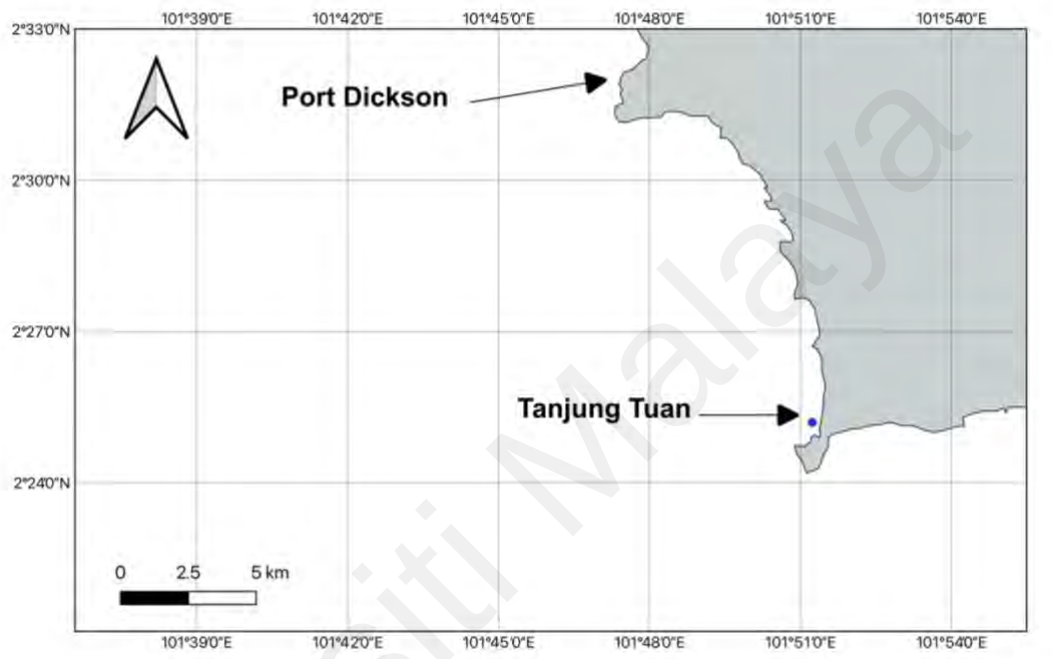


Figure 3.1 The sampling site in this study

3.2 Sample Collection

A healthy colony of *Sarcophyton* sp. was selected for this study. The colony was 10 m below the sea level and the ambient temperature at the time of collection was 29° C. Sample was documented in photographs and tagged for future identification. Soft coral sample was collected via Self-contained Underwater Breathing Apparatus (SCUBA) using a dive knife and sample collection bags. *Sarcophyton* fragments were initially cut and stored in sterile sample collection bags containing seawater. After being removed from the sea, the tissues were immediately stored separately in seawater and absolute ethanol. The former was used to isolate marine fungi, while the latter was kept for further molecular species identification. These samples were stored at under chilled condition in an ice box and then transported to the laboratory for processing.

3.3 Soft coral *Sarcophyton* sp. identification

3.3.1 Sclerite Observation

The collected soft coral samples were preliminarily identified as *Sarcophyton* sp. based on morphological characteristics as described by Verseveldt (1982) . Small tissue samples (~1 mm^3) stored in absolute ethanol were removed from the surface and interior of the polypary, and dissolved in 10% sodium hypochlorite for sclerite extraction (McFadden et al., 2006). Sclerite observation were then conducted under a compound microscope (× 4 - 40 magnification), and the determination of sclerite form was based on the definitions of Bayer et al. (1983).

3.3.2 Genomic DNA Extraction

Sarcophyton tissue was crushed using a mortar and pestle prior to DNA extraction. NucleoSpin® Tissue Kit (Machery-Nagel, Germany) was used to extract the genomic DNA of the *Sarcophyton* sample with modifications made to the manufacturer protocol. Approximately 50 mg of soft coral tissue was quantified and put into a 1.5 mL microcentrifuge tube, followed by 180 µL Buffer T1 (provided in kit) and 25 µL Proteinase K. The microcentrifuge tube was then incubated for 5 hours with brief vortexing at 30-minute intervals to obtain complete lysis. After incubation, the microcentrifuge tube was centrifuged at 11,000 × g for 5 minutes, and the clear supernatant was transferred to a new 1.5 mL microcentrifuge tube before proceeding with the rest of the kit's protocol. Extracted genomic DNA (gDNA) was stored at -20°C for further analysis. 2 µL of gDNA was used to measure the concentration and quality of DNA extracts with a NanoDrop™ spectrophotometer (Thermo Fisher Scientific Inc, USA).

3.3.3 Quality Assessment of Extracted gDNA

Agarose gel electrophoresis (AGE) was used to assess quality of extracted soft coral gDNA. A total of 0.12 g molecular grade agarose powder (NextGene, MYS) was dissolved in 15 mL of 1× Tris-acetate-EDTA (TAE) buffer (1st Base, SGP) to obtain 0.8% of agarose gel, infused with 1: 10,000 (v/v) SYBR® Safe (Thermo Fisher Scientific Inc, USA) stain. Then, 10 µL of gDNA mixed with 2 µL of 6× DNA loading dye (Thermo Fisher Scientific Inc, USA) and 5 µL of 1kb DNA ladder (Thermo Fisher Scientific Inc, USA) was individually loaded onto wells in the pre-prepared gel. AGE was carried out by an electrophoresis power supply EV243 (Consort, BEL) under 100 V for 25 minutes.

Upon completion, the gel was viewed under DLAB^{TM} GelSmart, and the fragment size and intensity of gDNA were compared with 1kb DNA ladder.

3.3.4 *Sarcophyton* DNA Marker Amplification

Soft coral molecular identification was determined based on polymerase chain reaction (PCR) amplification of the cyctochrome oxidase I (*COI*) with a short, adjacent intergenic region (*igr1*) (~ 1.1 kB), using the forward primer COII8068F (5'-CCA TAA CAG GAC TAG CAG CAT C-3') (McFadden et al., 2004) and the reverse primer COIOCTR (5'-ATC ATA GCA TAG ACC ATA CC-3') (France & Hoover, 2001). Approximately 730 bp of mitochondrial protein-coding gene MutS homolog (*msh1*) gene was amplified using the forward primers ND42599F (5'- GCC ATT ATG GTT AAC TAT TAC-3') (Sánchez et al., 2003) or ND42625F (5'- TAC GTG GYA CAA TTG CTG-3') (LePard, 2003), and the reverse primer Mut-3458R (5'-TSG AG CAA AAG CCA CTC C-3') (Sánchez et al., 2003). Table 3.1 summarise the PCR cycling parameters used in this study conducted by *Labnet MultiGene*^{TM} Gradient PCR Thermo Cycler (Labnet, USA). All PCR products were stored at -20°C for storage and then sent to Apical Scientific Sdn. Bhd. (Selangor, Malaysia) for Sanger sequencing. Obtained marker gene sequences will be verified by Basic Local Alignment Search Tool (BLAST) based on GenBank databases for species identification. The generated octocoral gene sequences will be aligned with the validated sequences on GenBank by ClustalX. Molecular Evolutionary Genetic Analysis (MEGA) version 11.0.13 will be used to analyse variation of DNA sequences, nucleotide composition and genetic distance (Kumar et al., 2016). The construction of Neighbor-Joining (NJ) phylogenetic trees will be performed by MEGA version 11.0.13 with 1000 bootstrap replicates (McFadden et al., 2001).

Table 3.1 Cycling parameters for *COI+igr1* and *msh1*

PCR Stages	***COI + igr1***		***msh1***	
	Temp	Time	Temp	Time
Initial denaturing	94°C	2 min	94°C	3 min
Denaturing	94°C	1.5 min	94°C	30 s
Annealing	58°C	1.5 min	52°C	1.5 min
Extension	72°C	1 min	72°C	1 min
Final extension	72°C	5 min	72°C	10 min
PCR cycles	35		35	

3.4 Fungal Isolation and Identification

Prior to fungal isolation, the soft coral samples were rinsed with 30 parts-per-thousand (ppt) sterile artificial seawater (ASW) three times (Li & Liu, 2006) in order to remove sediments and loosely attached microorganisms. Next, the tissues were treated with 75% ethanol for 30 seconds to sterilise the surface and washed three times with ASW. Then, the samples were cut into 1 cm^3 using sterile scalpels and were thoroughly homogenised by a blender with 10 mL ASW. After that, the homogenates were serially diluted three times (original, 1:10, 1:100) with sterile ASW and vortexed for 30 seconds.

Potato dextrose agar (PDA) was used for isolation due to its suitability for fungi (Xu et al., 2018). 200 µL of the homogenate was plated onto the agar plate (PDA) for fungal cultivation, and sterile ASW was used as negative control to test the effectiveness of surface sterilization. The isolation media were prepared with 30 ppt sterile ASW, with 1 g/L of chloramphenicol for the inhibition of bacterial growth. The agar plates were incubated at 25 °C for 1 to 2 weeks (Xu et al., 2018). Each fungal isolate was sub-cultured and purified using the single hyphal tip technique until a stable, morphologically uniform, and pure colony was obtained. The fungal isolates were kept at 4°C for long-time storage.

Fungal gDNA was extracted using 'Fungi Genomic DNA Extraction Kit (Spin-column)' (BioTeke Corporation, China) following manufacturer's instructions, in order to identify the fungal isolates. The molecular identification of fungal isolates was based on five gene markers, with final concentration of 10 µM, amplified by PCR, followed by phylogenetic analysis (Table 3.2). The internal transcribed spacer (ITS) region is a universal barcoding marker commonly used for fungal identification, due to its more defined barcoding gap and relatively high resolving power to discriminate closely related species, with high PCR and sequencing success rate across a wide variety of fungal species (Schoch et al., 2012). The 28S large subunit ribosomal RNA gene (LSU) contains D1 and D2 hypervariable domains which can facilitate species identification, when used together with ITS region (Raja, Miller, et al., 2017). In addition, elongation factor 1-alpha (EF1-α) region contains three closely spaced introns at the 5' end of the gene whose combined length was approximately 400 bp (Rehner & Buckley, 2005), while the mini-chromosome maintenance protein 7 (MCM7) is a highly conserved region to initiate eukaryotic genome replication and, when combined with LSU, this gene demonstrates enhanced phylogenetic resolution and increased nodal support for evaluation of interspecific relationships among the Ascomycota (Raja et al., 2011). Compared with LSU, most RNA polymerase II subunit 1 (RPB1) regions show more consistent phylogeny results in spite of some problematic inclusion of taxa (Matheny et al., 2002).

Table 3.2: Selected DNA markers with primer sequences for fungal identification and phylogenetic analysis

DNA Markers	Primer Names	Primer Sequence	References
ITS	f-ITS1	5' TCCGTAGGTGAACCTGCGG 3'	(Hallegraeff et al., 2014)
	f-ITS4	5'TCCTCCGCTTATTGATATGC3'	
LSU	LROR	5'ACCCGCTGAACTTAAGC3'	(Vilgalys & Hester, 1990; Rehner & Samuels, 1995)
	LR6	5'CGCCAGTTCTGCTTACC3'	
MCM7	Mcm7-709for	5'ACIMGIGTITCVGAYGTHAARCC3'	(Schmitt et al., 2009; Raja et al., 2011)
	Mcm7-1348rev	5'GAYTTDGCIACICCIGGRTCWCCCAT3'	
TEF1	EF1-983F	5'GCYCCYGGHCAYCGTGAYTTYAT3'	(Rehner & Buckley, 2005)
	EF1-2218R	5'ATGACACCRACRGCRACRGTYTG3'	
RPB1	RPB1af	5'GARTGYCCDGGDCAYTTYGG3'	(Stiller & Hall, 1997; Raja et al., 2011)
	RPB1cr	5'CCNGCDATNTCRTTRTCCATRTA3'	

Table 3.3 illustrates the modified master mix recipe used with PCR primers (Hallegraeff et al., 2014), whereas Table 3.4 and Table 3.5 show the cycling parameters of PCR reactions were conducted by *Labnet MultiGene* ™ Gradient PCR Thermo Cycler (Labnet, USA). All PCR products stored at -20°C for storage until being sent to Apical Scientific Sdn. Bhd. (Selangor, Malaysia) for DNA sequencing.

Table 3.3: Components of DNA master mix

Components of master mix	Volume
PCR water	17.8 µL
10× PCR buffer	2.5 µL
10 mM dNTPs	0.5 µL
10 µM Forward primer	1.0 µL
10 µM Reverse primer	1.0 µL
5 µg/mL *ExPrime*™ Taq DNA polymerase (GeNet Bio, Korea)	0.2 µL
Extracted DNA sample	2.0 µL

Table 3.4: Cycling parameters for ITS, LSU, MCM7 and RPB1

PCR Stages	ITS		LSU		MCM7		RPB1	
	Temp	Time	Temp	Time	Temp	Time	Temp	Time
Initial denaturing	94°C	1 min	95°C	5 min	94°C	5 min	96°C	5 min
Denaturing	94°C	1 min	94°C	30 s	94°C	45 s	94°C	30 s
Annealing	58°C	30 s	52°C	30 s	56°C	50 s	52°C	30 s
Extension	72°C	30 s	72°C	1 min	72°C	1 min	72°C	1 min
Final extension	72°C	10 min	72°C	8 min	72°C	8 min	72°C	8 min
PCR cycles	35		35		38		40	

Table 3.5: Cycling parameters for TEF1

PCR Stages		TEF1		PCR cycles
		Temp	Time	
Initial denaturing		94°C	2 min	
Phase 1 (PCR) Touchdown	Denaturing	94°C	30 s	9
	Annealing	66 – 56 °C	30 s (↓ 1°C/cycle)	
	Extension	72°C	1 min	
Phase 2 (PCR)	Denaturing	94°C	30 s	36
	Annealing	56°C	1 min	
	Extension	72°C	1 min	
Final extension		72°C	10 min	

Marker gene sequences were verified by searches against GenBank sequences via BLAST for preliminary identification of fungal isolates. Obtained fungal sequences were aligned with reference sequences based on searches from GenBank results. MEGA version 11.0.13 was used to analyse variation of DNA sequences, nucleotide composition and genetic distance (Kumar et al., 2016). The construction of NJ phylogenetic trees will be performed by MEGA version 11.0.13 (Li & Wang, 2009) with 1000 bootstrap replicates performed to support the NJ trees. While above 70% bootstrap values have a 95% possibility of real support, below 50% bootstrap values cannot be accepted as reliable values (Hillis & Bull, 1993).

3.5 Temperature Studies

Previous study on three polar soil fungi species has confirmed that culture temperatures influence their secondary metabolites production and the effects are not in a simple fashion (Yogabaanu et al., 2017). However, there has been no such research into tropical marine fungi. It is therefore important to access whether such impacts exist in tropical fungi species.

The growth rates of all four isolates were evaluated and based on the observation on growth rate, fungal isolates 1 (*Aspergillus sydowii*) and 2 (*Acrocalymma* sp.) were able to grow full plates in two weeks, whereas it took four weeks for fungal isolates 3 (*Lindgomyces* sp.) and 4 (*Phomatospora* sp.) to grow full plates. Since this study focused on the metabolite production, *Aspergillus sydowii* and 2 were eventually selected for temperature studies to maximise the production by crude extracts with higher biomass yield. A total of three 6 mm agar plugs of the fungal isolates was cultured in liquid culture medium, potato potato dextrose broth (PDB), in 12 separate 1L Erlenmeyer flasks and incubated under 3 different temperatures (25°C, 30°C and 35°C) for two weeks (14 days) on an incubator shaker to evaluate potential biochemical changes that take place as a form of adaptation. The cultures were monitored daily for changes in biomass and physical characteristics to ensure successful fermentation.

Each flask contains 250 mL culture media and was shaken at 100 rpm, and one additional negative control flask was also introduced with only PDA growth media (VanderMolen et al., 2013). Temperature preference was determined based on the current ocean surface temperature in the west coast of Peninsular Malaysia (WCPM) that ranged from 28°C to 29°C in the northeast monsoon, and reaches 30°C in the southwest monsoon (Amiruddin et al., 2011). In addition, records of surface ocean temperatures in WCPM between 1969 and 2010 show that the increases in ocean temperatures vary from 0.6°C to 1.2°C, and temperature increase is projected to be about 1.1°C to 3.6°C in 2028 to 2079 (Wong et al., 2009; Ercan et al., 2013; Sany et al., 2019). Thus, the temperatures 25°C, 30°C and 35°C were selected.

Fungal biomass was harvested from 12 Erlenmeyer flasks and filtered through Whatman® Grade 4 Qualitative Filter Paper (150 mm Circle, 25 μm). The weight of fungal biomass was measured after filtration. Fresh weight was obtained by adding up all measured biomass in each Erlenmeyer flask. In addition, dry weight was obtained by evaporating all the filtered fungal biomass in an oven.

The quantified yield of crude extracts of both fungal isolates was calculated with the formula below.

$$quantified\ yield\ of\ crude\ extract = \frac{the\ amount\ of\ crude\ extract}{the\ amount\ of\ dry\ weight} \times 100\%$$

3.6 Chemical Extraction

All the filtered fungal samples were collectively extracted in ethyl acetate for a duration of 3 to 4 days. The initial extracts were first filtered through Whatman® Grade 4 Qualitative Filter Paper (150 mm Circle, 25 µm) and final crude extracts were obtained by *in vacuo* evaporation. During concentration, the aqueous particles were removed from the extract through the addition of anhydrous sodium sulphate. Concentrated crude extract was stored in -20 ºC for further analysis (Ishii, et al., 2010) .

3.7 Chemical Profiling

Chemical profiling was conducted on the crude extract following extraction and partitioning, including thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and Quadrupole Time-of-Flight Liquid Chromatography Mass Spectrometry (Q-TOF LCMS), which were used to observe changes in metabolite production in by the cultured fungal strains in response to temperature alteration.

3.7.1 Thin layer chromatography

TLC served as a primary profiling of the crude extracts from different samples to identify the varieties of secondary metabolites contained in the samples. In order to obtain proper separation, solvents that have various polarities are used in the profiling process. Total of 10 mg crude extract was quantified and dissolved in 100 µL of 100% ethyl acetate for TLC profiling. There were three solvent systems used in TLC as the mobile phase, namely 100% chloroform, 100% toluene and hexane : ethyl acetate (3:1) .

3.7.2 High performance liquid chromatography (HPLC)

Analytical HPLC was used to analyse and generate an overall profile of crude extracts. Before HPLC analysis, the extracts were filtered through 0.22 µm polytetrafluoroethylene (PTFE) filter membrane (Labex Ltd., Hungary). 20 µm of filtered samples was profiled over a 4.6 x 100 mm 3.5-micron Agilent Zorbax Eclipse Plus C18 column conditioned at 25 °C (±1 °C). The solvents used were methanol (A) and water (B). The flow rate was maintained at 1.00 mL/min. The elution gradient was 60 % methanol (0-5 min) followed by a gradual increment to 100 % for 25 minutes.

3.7.3 Q-TOF liquid chromatography –mass spectrometry (LCMS)

The crude extracts were diluted in deionized water and filtered through a 0.45 µm nylon syringe filter. A 50 ppm of the filtrate was prepared in triplicates and used for LCMS analysis. The LCMS analysis was performed using liquid chromatography G6550A system (Agilent Technologies, Inc., USA), which consisted of a degasser, a G4220A binary pump with a pressure gradient unit, a Dual Agilent Jet Stream Electrospray Ionization Mass Spectrometry Quadrupole Time-of-Flight (AJS ESI MS Q-TOF) detector and a G4226A HiP AutoSampler. A 4.6 x 100 mm 3.5-micron G1316C Agilent Zorbax Eclipse Plus C18 column was utilized for separation (30 eV at 27°C and 20µL at 0.8 mL/min). A mobile phase of 60% of methanol and 40% of water for a separation time of 30 min was applied following the previous methods (Seto et al., 2009) with minor modifications, in which the ratio of methanol to water gradually reached 95% : 5% at 10 minute and maintained 100% of methanol and 0% of water between 20 min to 30 min. Identification of compounds by the acquisition of mass spectra was conducted in both positive and negative modes using MassHunter Qualitative Analysis software developed by Agilent Technologies.

CHAPTER 4: RESULTS

4.1 Soft coral *Sarcophyton* sp. identification

4.1.1 Sclerite Observation

The soft coral samples were observed under a compound microscope (× 4 – 40 magnification) to confirm initial identification of sample as *Sarcophyton* sp. based on the definitions of Bayer et al. (1983). Each individual soft coral has spicules shape unique to its genus.

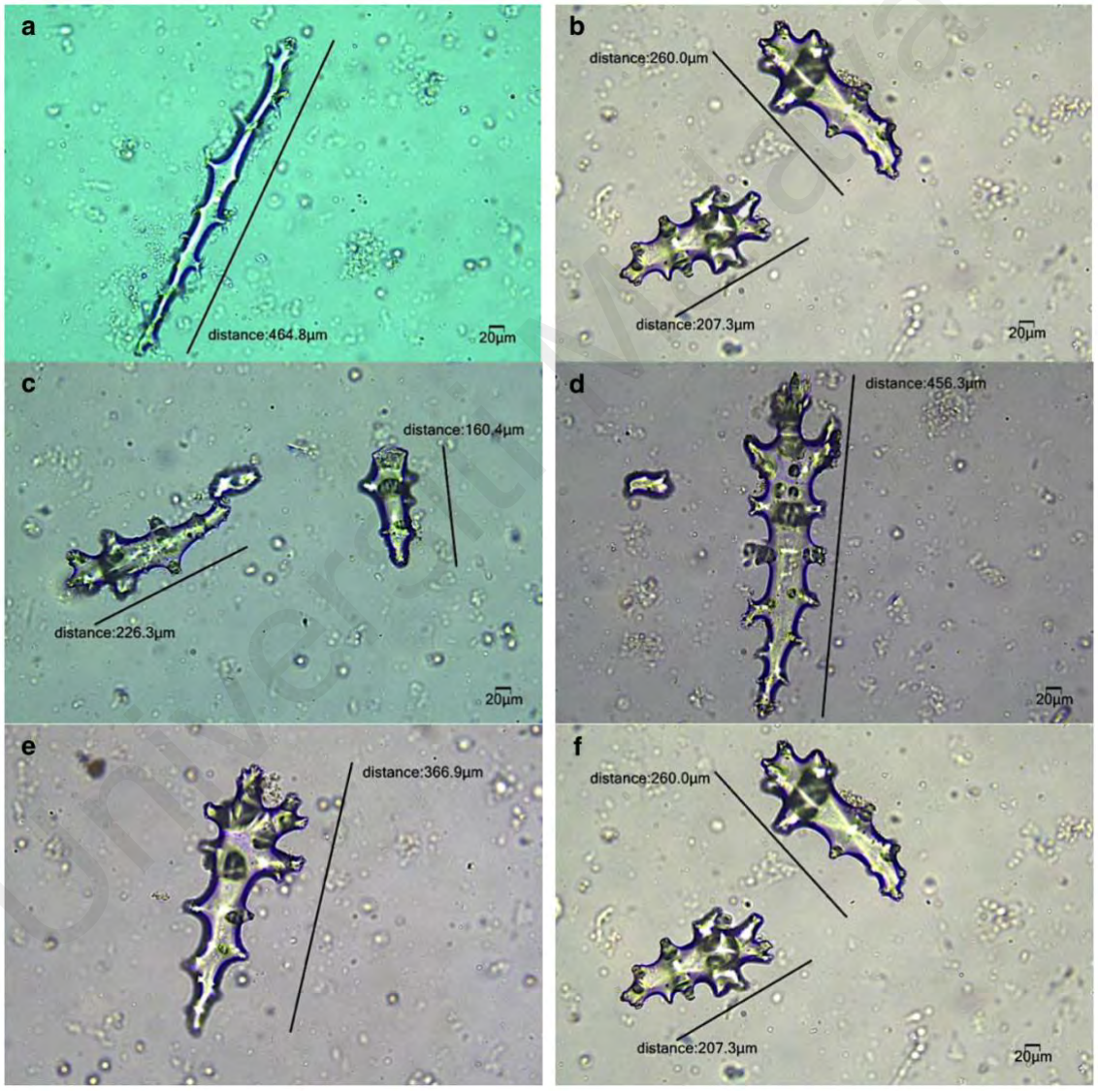


Figure 4.1 Sclerites of *Sarcophyton* sp.

The observed surface sclerites (Figure 4.1) appear to be clubs with long handles, where the heads are poorly differentiated, and simple ornamentation are sparsely distributed. The observed shape and spicules demonstrated striking resemblance to the

hand-drawn shape and spicules of the genus *Sarcophyton* (Verselveldt, 1982). On the basis of this comparison, the collected coral sample was determined to be from the genus *Sarcophyton*.

4.1.2 Genomic DNA Extraction

A quantified 50 mg of soft coral tissue material was able to yield 8.50 ng gDNA as quantified using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific Inc, USA). The quality of gDNA (2 µL) material was determined as described in Table 4.1.

Table 4.1 The concentration and purity of extracted gDNA

Sample	DNA concentration (ng/µL)	260/280	260/230
SARCO	8.50	1.41	0.45

4.1.3 Quality Assessment of Extracted gDNA

Provided that the 260/280 and 260/230 absorbance ratios indicate the purity of DNA and the presence of contaminants in the extracted DNA samples (Desjardins & Conklin, 2010; Sukumaran, 2010), Table 4.1 shows that the extracted gDNA was not 'pure' because the 260/280 and 260/230 ratios were below the generally accepted ideal 1.8 and 2.0-2.2, respectively. This means that the extracted gDNA contained contaminants.

4.1.4 Molecular analysis of *Sarcophyton ehrenbergi*

Although both *COI* and *msh1* genes were successfully sequenced for preliminary species identification, the quality of *msh1* results was poor; therefore, only the *COI+igr1* sequence was used in BLAST to confirm the soft coral species (Table 4.2), which also gives justification of the species identification of coral species in this study. According to Table 4.2, the top 10 BLAST results showed 89-98% query cover and 98.91-99.01% percent identity, and the E-values are 0.0. In addition, 7 out of the top 10 results retrieved belong to *Sarcophyton ehrenbergi* sequences. Thus, the soft coral species is preliminarily identified to be *Sarcophyton ehrenbergi*.

Table 4.2 Molecular identification of the soft coral species with NCBI accession numbers

Possible species based on best matches of BLAST results					Conclusion of species identification
COI				*msh1*	
Possible species	**Query cover**	**Percent identity**	**E value**		
Sarcophyton ehrenbergi (GQ342397.1)	98%	99.01%	0.0	Not available because of poor PCR results	*Sarcophyton ehrenbergi*
Sarcophyton auritum (GU355990.1)	98%	98.58%	0.0		
Lobophytum sarcophytoides (MG701405.1)	97%	98.29%	0.0		
Sarcophyton sp. (MH152712.1)	89%	98.91%	0.0		
S. ehrenbergi (MH516375.1)	89%	98.91%	0.0		
S. ehrenbergi (MH516374.1)	89%	98.91%	0.0		
S. ehrenbergi (MH516372.1)	89%	98.91%	0.0		
S. ehrenbergi (MH516371.1)	89%	98.91%	0.0		
S. ehrenbergi (KF955155.1)	89%	98.91%	0.0		
S. ehrenbergi (JX991246.1)	89%	98.91%	0.0		

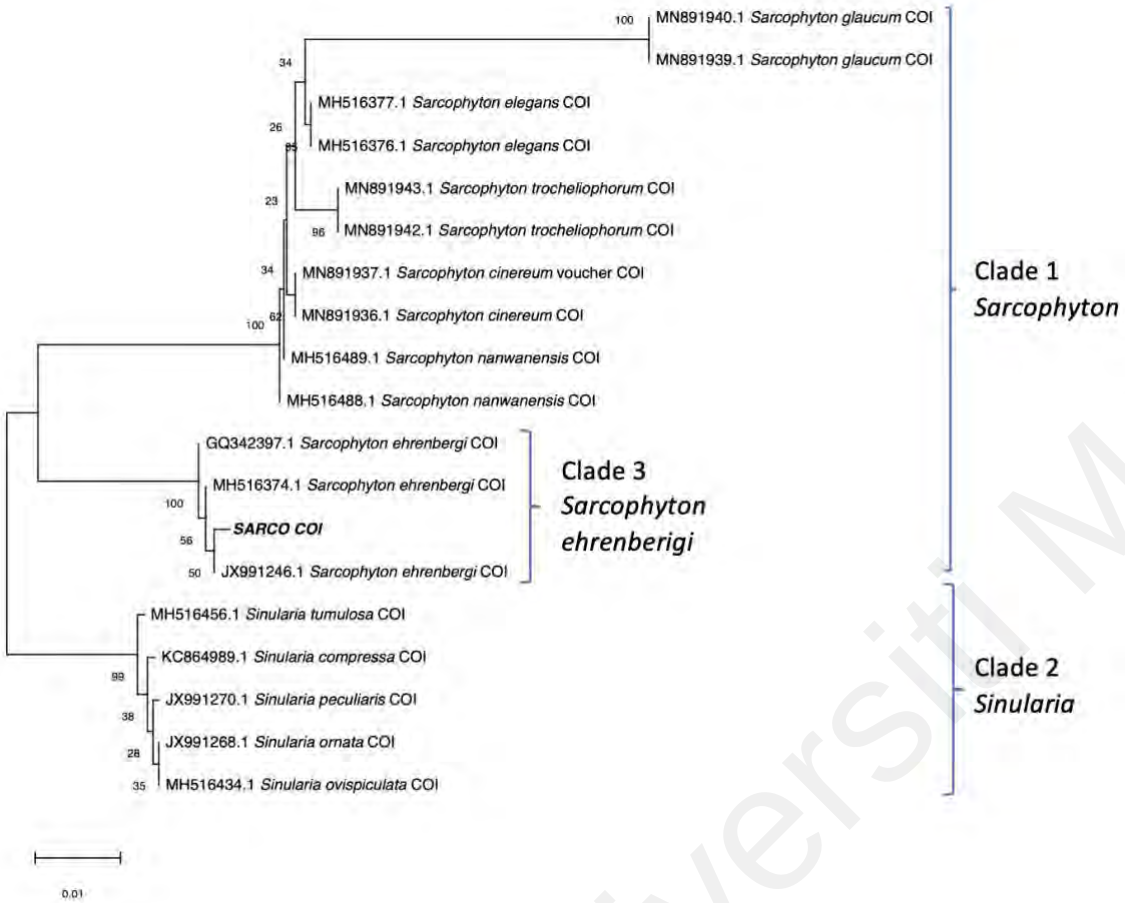


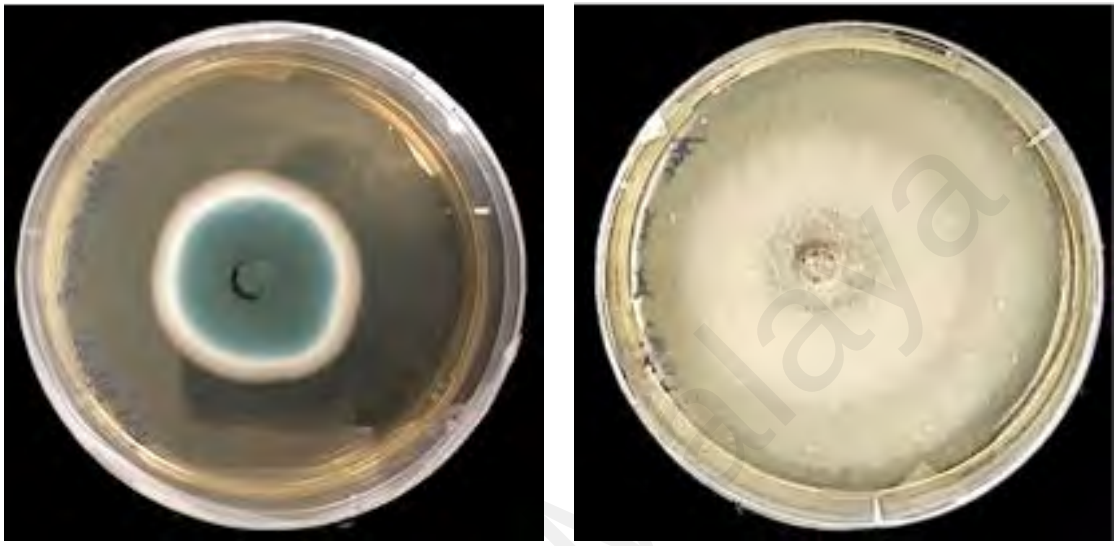
Figure 4.2 Neighbour-Joining Tree (Tamura-Nei with Gamma parameter = 0.16) showing the phylogenetic affinity of *SARCO COI* sequence with *Sarcophyton* reference *COI* sequences. *Sinularia* reference *COI* sequences were used as outgroup to root the phylogram. *SARCO COI* sequence was identified as *Sarcophyton ehrenbergi* because it strongly clusters within the *Sarcophyton ehrenbergi* clade (100% bootstrap value).

Figure 4.2 showed the phylogram using the Neighbour-Joining tree-building analysis that incorporated the Tamura-Nei substitution model with Gamma parameter = 0.16. The phylogram was rooted with *Sinularia* reference *COI* sequences, whereby the *Sinularia* outgroup comprises five *Sinularia* speices, namely *S. tumulosa*, *S. compressa*, *S. peculiaris*, *S. ornate*, *S. ovispiculata*. All *Sarcophyton* sequences clustered together (99% bootstrap value) under Clade 1 that represented the *Sarcophyton* clade. The *Sarcophyton* clade is comprised of six species, *S. glaucum*, *S. elegans*, *S. trocheliophorum*, *S. cinereum*, *S. nanwanensis* and *S. ehrenbergi*. Within the *Sarcophyton* clade, another clade (clade 3) was clearly identified which represented the *Sarcophyton ehrenbergi* clade. *SARCO COI* was found within the Sarcophyton ehrenbergi clade (100% bootstrap value). Therefore, *SARCO COI* was identified as *Sarcophyton ehrenbergi*.

4.2 Fungal Isolation and Identification

4.2.1 Fungal isolates

A total of four morphologically distinct fungal isolates, were successfully isolated in this study. Figures 4.3- 4.6 show the fungal colonies of the four isolates.



a. Fungal isolate 1 (*Aspergillus sydowii*) b. Fungal isolate 2 (*Acrocalymma* sp.)

c. Fungal isolate 3 (*Lindgomyces* sp.) d. Fungal isolate 4 (*Phomatospora* sp.)

Figure 4.3 Photos of colonies of fungal isolates 1-4

The fungal colony of isolate 1 (Figure 4.3 a) appeared as a green-blue centred colony in on PDA media, while the outer layer was white in colour. However, when the colony grows more than two weeks, the colour of the colony centre changes into a dark green or greyish-turquoise colour. The colony is in a circular form with a filiform edge. Other characteristics include a huge number of spores in the middle of the colony and white

peripheral hyphae. Based on these distinct characteristics, this isolated was suspected to be *Aspergillus sydowii*. The mean diameter of *A. sydowii* colony is 64 mm on day 14 after inoculation.

The fungal colony of isolate 2 (Figure 4.3 b) was in a white circular shape on PDA plates. As the fungal colony ages, dark black spots began to appear in the colony centre, moving to the margin. The margin of the fungal colony was filamentous, and elevation of the colony was flat. Based on the characteristics of the colony itself this isolated was close to *Acrocalymma* genus. The mean diameter of fungal isolate 2 is 55 mm day 14 day after inoculation.

The colony of fungal Isolate 3 (Figure 4.3 c) had an irregular shape on PDA plates. The colony had a dark green colour at the centre of the colony, while the margins were white and curled, with presence of droplets near the centre of the colony. The droplets disappeared slowly as the fungal colony grew. Unlike fungal isolates 1 and 2, the elevation of the isolate 3 colony was in crateriform. Based on these features, this isolate was later characterised to be potentially *Lindgomyces* sp. (Zhang et al., 2014). The mean diameter of *Lindgomyces* sp. is 43 mm on day 14 after inoculation.

In terms of form and colour, the colony isolate 4 (Figure 4.3 d) is similar to that of isolate 2. The colony appeared to have a round shape and a whitish coloured colony centre. However, it did not develop any dark spots over time. The margin of the fungal colony was entire. In addition, it had a raised elevation with the presence of many tiny spikes on the upper surface of colony, which is a distinct characteristic of *Phomatospora* genus (Senanayake et al., 2016). The mean diameter of *Phomatospora* sp. is 45 mm on day 14 after inoculation.

4.2.2 Molecular analysis of fungal isolates

All ITS, LSU, MCM7, TEF1 and RPB1 gene sequences of the four fungal isolates were successfully sequenced for preliminary species identification by BLAST, followed by methods to confirm the isolate species (Table 4.3), which also gives justification of the species identification of fungal isolates in this study. However, MCM7 and RPB1 gene sequences produced no matches after BLAST.

According to the table, the BLAST results of fungal isolate 1 showed 100% query cover and percent identity, and the E-values are 0.0. Besides, all of the top 10 results retrieved belong to *A. sydowii* sequences. Thus, this isolate was identified as *A. sydowii*.

As for fungal isolate 2, Table 4.3 illustrated that the BLAST results showed 100% query cover and E-values are 0.0, while the percent identities have a similarity from

92.53% to 93.23%. However, the sequences on GenBank and the sequence of fungal isolate 2 are not exactly the same in the ITS 1 and ITS 4 regions. Thus, this fungal isolate could only be identified as *Acrocalymma* sp.

In terms of fungal isolate 3, Table 4.3 showed that the BLAST results of LSU produce the highest confidence in the matches with 100% query cover and E-values are 0.0. Although the LSU sequences of fungal isolate 3 do not match the entire sequences of *Lindgomyces angustianscus*, *L. lemonweirenesis*,and *L. cinctosporus*, they have a high percentage of similarity, so it could be a species under the genus *Lindgomyces*. On the other hand, the BLAST results of ITS and TEF1 sequences is not reliable due to query cover less than 40%, and the maximum percent identity is only 85.64%. Thus, this isolate was only identified to genus level of *Lindgomyces* based on LSU sequence.

Regarding the BLAST result if fungal isolate 4, Table 4.3 illustrated the LSU sequence of *Lanspora coronate* have high confidence in the matches. Despite 84% query cover and 0.0 E-value, the trimmed fungal isolate sequence shows a high percentage of similarity to that of *Lanspora coronate* overall. On the contrary, the LSU BLAST results of the genus *Phomatospora* are not reliable. Although query covers ranges from 85% to 100% and E-value is 0.0, theLSU sequence of the fungal isolate constantly has some long gaps compared with the *Phomatospora* spp. sequences on GenBank, having trimmed and aligned by MEGA. However, given few *Lanspora coronata* available on GenBank, the LSU sequence of fungal isolate 4 clustered within the *Phomatospora* / *Lanspora coronata* clade in the NJ phylogenetic tree (Figure 4.10). Therefore, fungal isolate 4 could only be identified as *Phomatospora* sp.

In conclusion, the fungal isolates 1 – 4 in this study were preliminarily identified as *Aspergillus sydowii*, *Acrocalymma* sp., *Lindgomyces* sp. and *Phomatospora* sp., respectively. These species were the first reported associations with *Sarcophyton* soft corals.

Table 4.3 Molecular identification of the fungal isolates with NCBI accession numbers

Fungal Isolates	Possible species based on best matches of BLAST results												Conclusion of species identification
	ITS				LSU				TEF1				
	Possible species	Query cover	Percent identity	E value	Possible species	Query cover	Percent identity	E value	Possible species	Query cover	Percent identity	E value	
Fungal isolate 1	*Aspergillus carneus* (MT446158)	100%	100%	0.0	No match				No match				*Aspergillus sydowii*
	Aspergillu sydowii (MN413179)	100%	100%	0.0									
	A. sydowii (MN413178)	100%	100%	0.0									
	A. sydowii (MN413177)	100%	100%	0.0									
	A. sydowii (MK267403)	100%	100%	0.0									
	A. sydowii (MG309693)	100%	100%	0.0									
	A. sydowii (MG309689)	100%	100%	0.0									
	A. sydowii (MG309685)	100%	100%	0.0									
	A. sydowii (MH398045)	100%	100%	0.0									
	A. sydowii (MG976613)	100%	100%	0.0									
Fungal isolate 2	*Acrocalymma medicaginis* (KP170621)	100%	93.03%	0.0	No match				No match				*Acrocalymma* sp.
	A. medicaginis (KP170620)	100%	93.03%	0.0									
	A. medicaginis (KP170622)	100%	92.86%	0.0									
	A. medicaginis (MW081367)	100%	92.53%	0.0									
	A. vagum (KU204582)	99%	93.23%	0.0									
	A. vagum (MK729139)	100%	88.85%	0.0									
	A. sp. (MG976408)	98%	93.13%	0.0									
	A. medicaginis (MW376531)	99%	92.82%	0.0									
	A. sp. (MW060151)	99%	92.48%	0.0									
Fungal isolate 3	*Massaria campestris* (MH863729)	94%	84.99%	6e-103	*Lindgomyces angustianscus*	100%	94.38%	0.0	*Hysterobrevium mori* (GU397338)	97%	92.47%	0.0	*Lindgomyces* sp.
	M. campestris (NR_127583)	39%	84.99%	6e-103	(JX508283)				*Alternaria burnsii*	98%	92.21%	0.0	
	M. campestris (HQ599385)	39%	84.99%	6e-103	*Lindgomyces*	100%	94.83%	0.0	(XM_038928745)				
	M. vomitoria (MH863725)	37%	85.64%	2e-102	*angustianscus*				*Alternaria arborescens*	98%	92.11%	0.0	
	M. vomitoria (HQ599438)	37%	85.64%	2e-102	(NG_042721)				(XM_028650167)				
	M. vomitoria (HQ599435)	37%	85.64%	2e-102	*Lindgomyces* sp.	100%	94.83%	0.0	*Alternaria alternata*	98%	92.01%	0.0	
	M. macra (MH863723)	38%	85.41%	2e-102	(GU266247)				(MK340863)				
	M. mediterránea (NR_137764)	38%	85.41%	3e-101	*L. lemonweirenesis*	100%	94.73%	0.0	*Alternaria alternata*	98%	92.01%	0.0	
	M. platanoidea (HQ599422)	38%	85.41%	3e-101	(JF419889)				(XM_018536149)				
					L. lemonweirenesis	100%	94.73%	0.0	*Alternaria alternata*	98%	92.01%	0.0	
					(NG_042580)				(KU886536149)				
					L. cinctosporus	100%	94.64%	0.0	*Alternaria* sp.	98%	92.15%	0.0	
					(GU266245)				(DQ677911)				

Table 4.3, continued

Fungal Isolates	Possible species based on best matches of BLAST results												Conclusion of species identification
	ITS				LSU				TEF1				
	Possible species	Query cover	Percent identity	E value	Possible species	Query cover	Percent identity	E value	Possible species	Query cover	Percent identity	E value	
Fungal isolate 4	*Sordariomycetes* sp. (FJ799946)	99%	98.95%	0.0	*Phomatospora bellaminuta* (FJ176857)	100%	98.02%	0.0	*Lanspora coronata* (DQ471067)	100%	95%	0.0	*Phomatospora* sp.
	Microascales sp. (KU747567)	96%	99.82%	0.0									
	Microascales sp. (KU747863)	94%	99.82%	0.0									
	Microascales sp. (MN592960)	97%	97.90%	0.0	*Phomatospora* sp. (MT226554)	91%	99.35%	0.0					
	Microascales sp. (KU747626)	95%	99.09%	0.0									
					Phomatospora sp. (MT226548)	91%	98.37%	0.0					
					Phomatospora sp. (MT226561)	91%	97.38%	0.0					
					P. striatigera (NG_067288)	85%	98.03%	0.0					
					Lanspora coronata (U46889)	84%	98.12%	0.0					

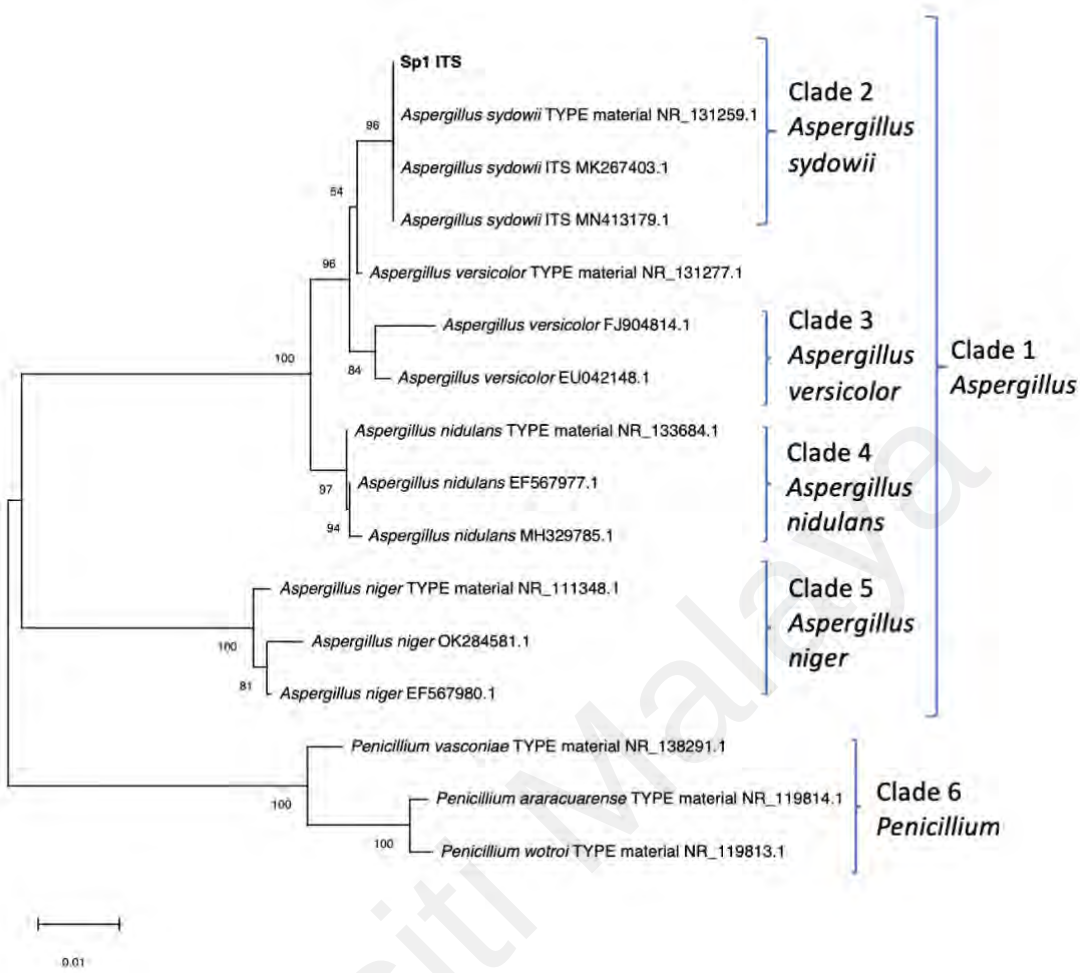


Figure 4.4 NJ Tree (Tamura-Nei with Gamma parameter = 0.05) of *Sp1 ITS* sequence and *Aspergillus* reference ITS sequences. The phylogram is rooted with *Penicillium* outgroup. *Sp1 ITS* clustered within the *Aspergillus* clade and was identified as *Aspergillus sydowii*, based on the 96% bootstrap value of the *Aspergillus sydowii* clade. The phylogenetic tree contains clades 1-6.

Figure 4.4 showed the phylogram using the Neighbour-Joining tree-building analysis that incorporated the Tamura-Nei substitution model with Gamma parameter = 0.05. The phylogram was rooted with *Penicillium* reference *ITS* sequences, whereby the *Penicillium* outgroup comprises three *Penicillium* species, including *P. vasconiae*, *P. araracuarense* and *P. wotroi*. All *Aspergillus* sequences clustered together (100% bootstrap value) under Clade 1 that represented the *Aspergillus* clade. The *Aspergillus* clade is comprised of four species, *A. sydowii*, *A. versicolor*, *A. nidulans* and *A. niger*. Within the *Aspergillus* clade, there are four clades clearly identified which represented the *A. sydowii* clade (clade 2), *A. versicolor* clade (clade 3), *A. nidulans* clade (clade 4) and *A. niger* clade (clade 5). All the *A. sydowii*, *A. versicolor* and *A. nidulans* sequences clusters in the *A. sydowii*, *A. versicolor* and *A. nidulans* clades, respectively. *Sp1 ITS* was found within the *Aspergillus*

sydowii clade (96% bootstrap value). Therefore, *Sp1 ITS* was identified as *Aspergillus sydowii*.

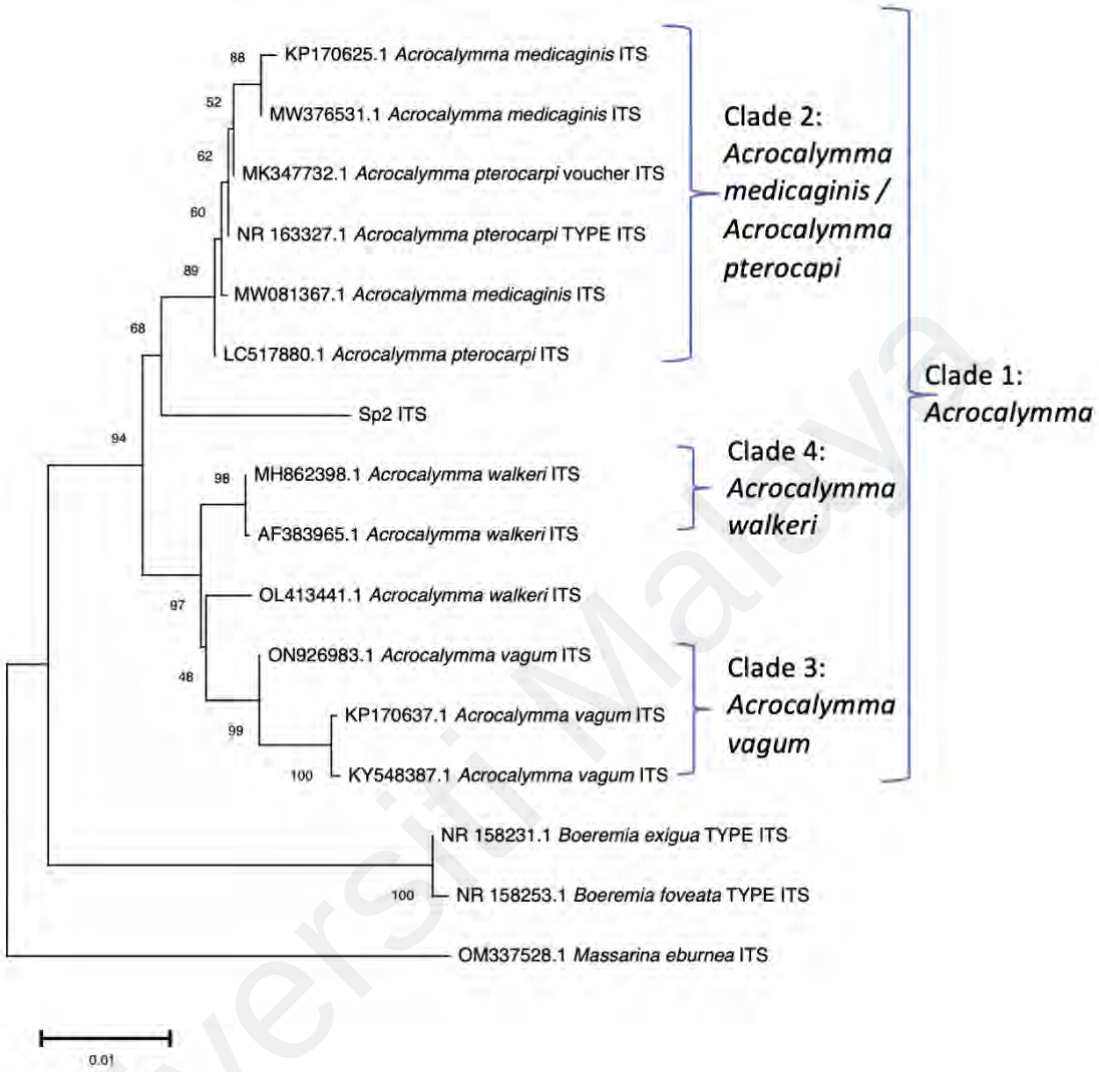


Figure 4.5 NJ Tree (Tamura-Nei with Gamma parameter = 0.05) of *Sp2 ITS* sequence and *Acrocalymma* reference *ITS* sequences. *Boeremia* and *Massarina* reference *ITS* sequences were used as outgroup. The phylogram was rooted with *Massarina51iseriata*. *Sp2 ITS* clustered within the *Acrocalymma* clade (clade 1) (94% bootstrap value) containing Clades 2–– 4. *Acrocalymma medicaginis* and *A. pterocapi* belong to Clade 2 (strong bootstrap value = 89%). Clade 3 contains only *A. vagum* (strong bootstrap value = 99%). Clade 4 contains two sequences of *A.51iserii* (strong bootstrap value = 98%). *Sp2 ITS* could only be identified as *Acrocalymma* sp. as it does not cluster within clades 2, 3 and 4.

Figure 4.5 showed the phylogram using the Neighbour-Joining tree-building analysis that incorporated the Tamura-Nei substitution model with Gamma parameter = 0.05. The phylogram was rooted with *Boeremia* reference *ITS* sequences, whereby the *Boeremia* outgroup comprises *B*. *exigua* and *B*. *foveate*. All *Acrocalymma* sequences clustered together (94% bootstrap value) under Clade 1 that represented the *Acrocalymma* clade. The *Acrocalymma* clade is comprised of four species, *A*. *medicaginis*, *A*. *pterocarpi*, *A*.52iseri*i* and *A*. *vagum*. Within the *Acrocalymma* clade, there are three clades clearly identified which represented the *A*. *medicaginis* / *A*. *pterocarpi* clade (clade 2), *A*.52iseri*i* clade (clade 3) and *A*. *vagum* clade (clade 4). All the *A*. *medicaginis* and *A*. and *A*. *nidulans* sequences clusters in the *A*. *medicaginis* / *A*. *pterocarpi* clade with strong bootstrap value of 80%. All the *A*. *vagum* sequences clustered under the *A*.52iseri*i* clade with very strong bootstrap value of 99%. Two of the *A*.52iseri*i* sequences (MH862398.1 and AF383965.1) cluster under the *A*. *vagum* clade with very strong bootstrap value of 98%. *Sp2 ITS* was found within the *Acrocalymma* clade (94% bootstrap value). However, this sequence does not cluster with clades 2 – 4, indicating that it does not belong to *A*. *medicaginis*, *A*. *pterocarpi*, *A*.52iseri*i* or *A*. *vagum*. Therefore, *Sp2 ITS* could be identified as *Acrocalymma* sp.

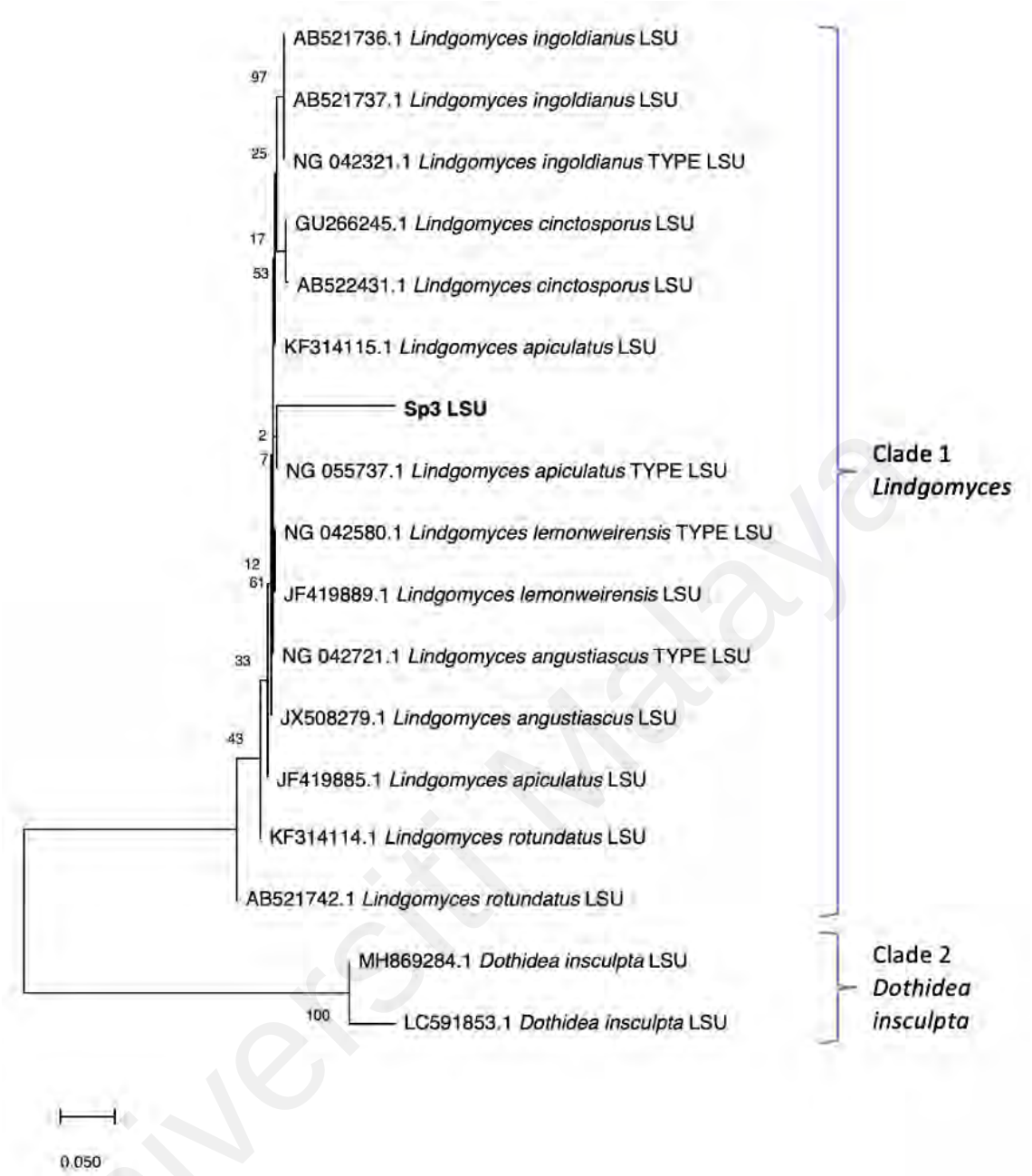


Figure 4.6 NJ Tree (Tamura-Nei with Gamma parameter = 0.53) of Sp3 LSU sequence and *Lindgomyces* reference LSU sequences. The phylogram is rooted with *Dothidea insculpta* outgroup. Sp3 LSU clustered within the *Lindgomyces* clade and could only be identified as *Lindgomyces* sp. because it does not cluster with any other *Lindgomyces* species.

Figure 4.6 showed the phylogram using the Neighbour-Joining tree-building analysis that incorporated the Tamura-Nei substitution model with Gamma parameter = 0.53. The phylogram was rooted with *Dothidea* reference *LSU* sequences, whereby the *Dothidea* outgroup comprises two *D. insculpta* sequences. All *Lindgomyces* sequences clustered together under Clade 1 that represented the *Lindgomyces* clade. The *Lindgomyces* clade consists of six species, namely *L. lemonweirensis*, *L. apiculatus*, *L. angustiascus*, *L. cinctosporus*, *L. ingoldianus* and *L. rotundatus*. Even though Sp3 LSU was found within the *Lindgomyces* clade (61% bootstrap value), this sequence does not cluster with any other *Lindgomyces* species. Therefore, Sp3 LSU could be identified as *Lindgomyces* sp.

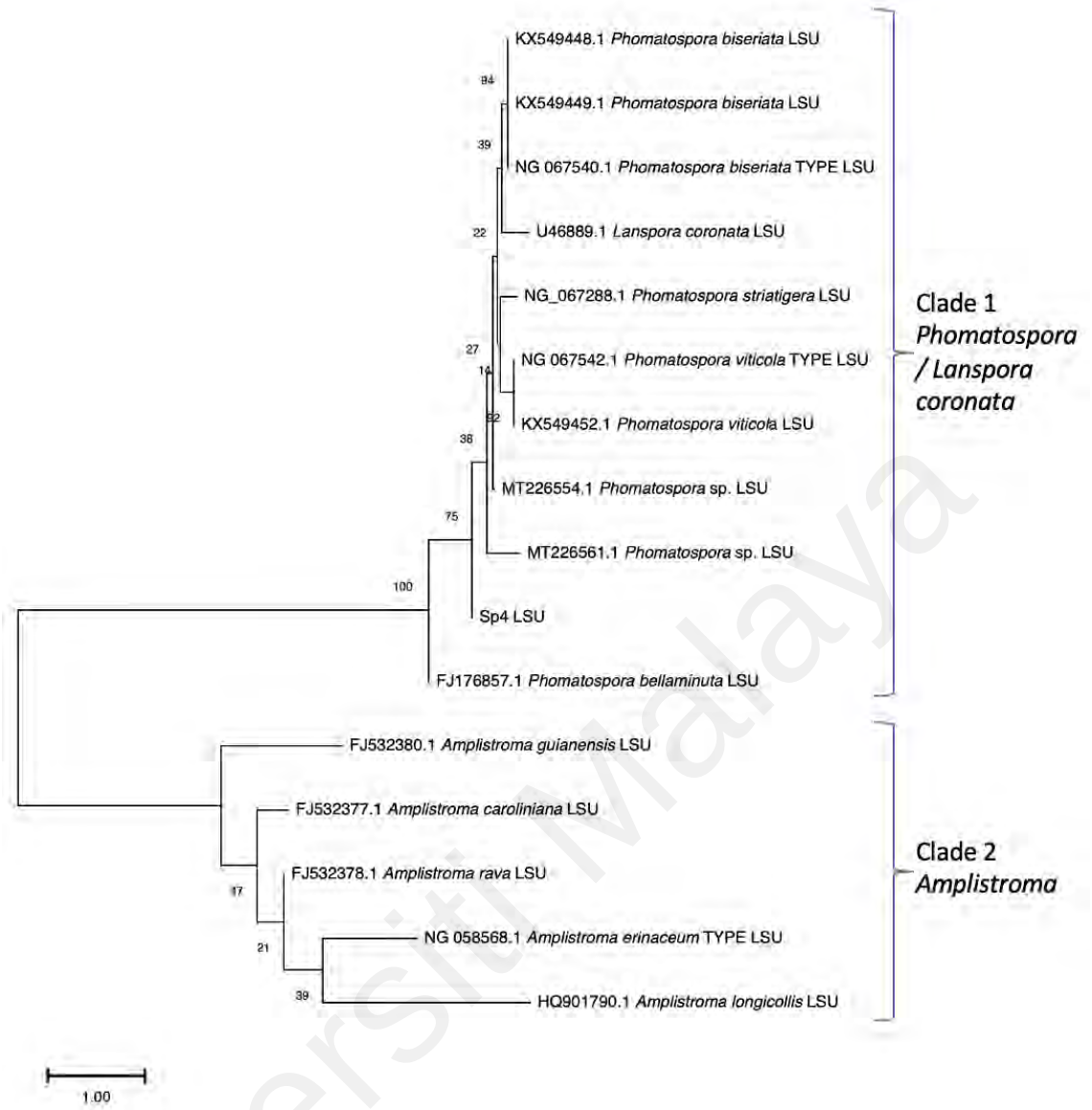


Figure 4.7 NJ (Tamura-Nei with Gamma parameter = 0.22) of Sp4 LSU sequence and *Phomatospora / Lanspora coronata* reference LSU sequences. The phylogram is rooted with *Amplistroma* outgroup. Sp4 LSU clustered within the *Phomatospora* clade and could only be identified as *Phomatospora* sp. because it is more closely related to *Phomatospora bellaminuta* (100% Bootstrap value) than *Lanspora coronata*.

Figure 4.7 showed the phylogram using the Neighbour-Joining tree-building analysis that incorporated the Tamura-Nei substitution model with Gamma parameter = 0.22. The phylogram was rooted with *Amplistroma* reference *LSU* sequences, whereby the *Amplistroma* outgroup comprises five *Amplistroma* species, *A. guianensis*, *A. rava*, *A. caroliniana*, *A. erinaceum* and *A. longicollis* sequences. All *Phomatospora* and *Lanspora coronata* sequences clustered (100% bootstrap value) together under Clade 1 which represented the *Phomatospora / Lanspora coronata* clade. The *Phomatospora / Lanspora coronata* clade is comprised of six species, *P.56iseriatea*, *P. striatigera*, *P. viticola*, *P. bellaminuta*, *Phomatospora* sp. and *Lanspora coronata*. Although Sp4 LSU was found within the *Phomatospora / Lanspora coronata* clade (75% bootstrap value), it does not cluster with any other *Phomatospora* sequences. In addition, Sp4 LSU is more closely related to *Phomatospora bellaminuta* (100% Bootstrap value) than *Lanspora coronata*. Therefore, Sp4 LSU could be identified up to the genus level as *Phomatospora* sp.

4.3 Temperature studies

4.3.1 Fresh weight

After a two-week fermentation, the respective fungal cultures were filtered, and its fresh weight was quantified to assess the effect of temperature on fungal biomass. Figure 4.8 shows the total biomass (fresh weight) of the two fungal strains cultured at three separate temperatures. Based on the data obtained, both *Aspergillus sydowii* and *Acrocalymma* sp. demonstrated a consistent reduction in a negatively proportionate trend in biomass as the temperature increased. *Aspergillus sydowii* recorded a 16.18 % reduction when there was an increase of 5°C from 25°C and further reduced 48.27% when cultured at 35°C. Similarly, *Acrocalymma* sp. responded in a similar pattern with 45.02% reduction at 30°C, followed by a 13.58% reduction at 35°C. It is clear that temperature has an effect on the fungal growth: both species yielded the highest biomass 25°C, whereas the lowest yields were at 35°C.

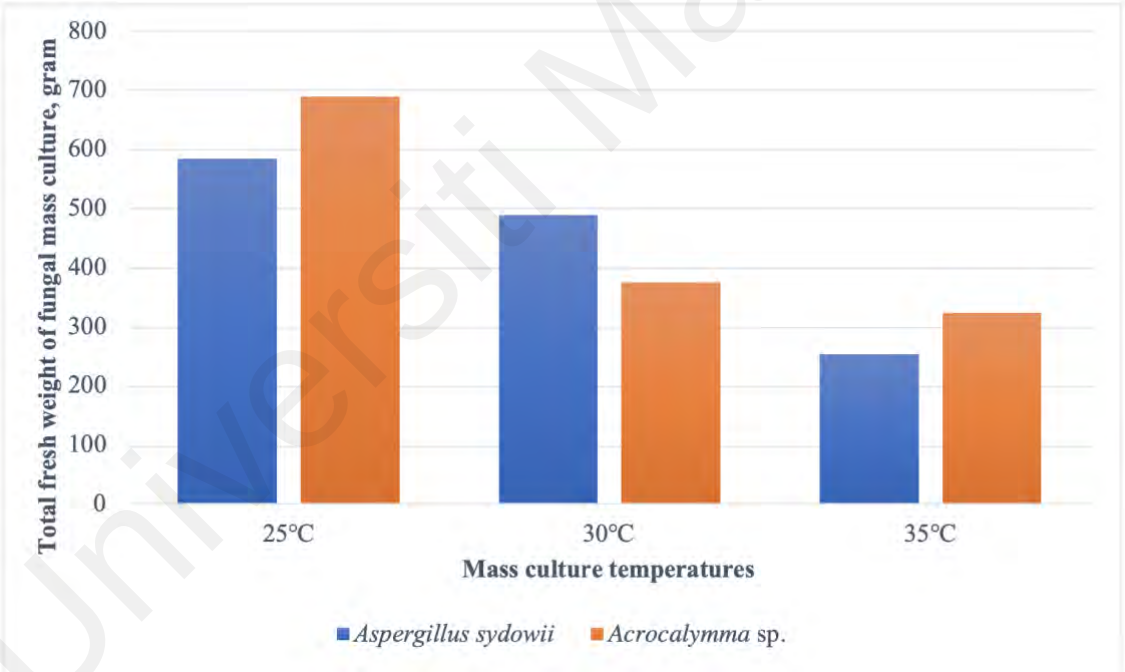


Figure 4.8 Total fresh weight (gram) of fungal mass culture at different temperatures

4.3.2 Dry weight

Figure 4.9 shows the amount of two fungal biomass (dry weight) at three temperatures. Based on the data obtained, *Aspergillus sydowii* showed a consistent reduction in a negatively proportionate trend in biomass as the temperature increased, while *Acrocalymma* sp. showed a fluctuation in biomass as the temperature rose. *Aspergillus sydowii* recorded a 29.62% reduction when there was an increase of 5°C from 25°C and further reduced 3.15% when cultured at 35°C. However, *Acrocalymma* sp. responded in a similar pattern with 61.85% reduction at 30°C, followed by a 25.53% rise at 35°C. Temperature clearly influences the fungal growth, where both species yielded the highest biomass 25°C. Compared with fresh weight, the dry weight of fungal biomass provides a more accurate measurement of fungal growth at these three temperatures.

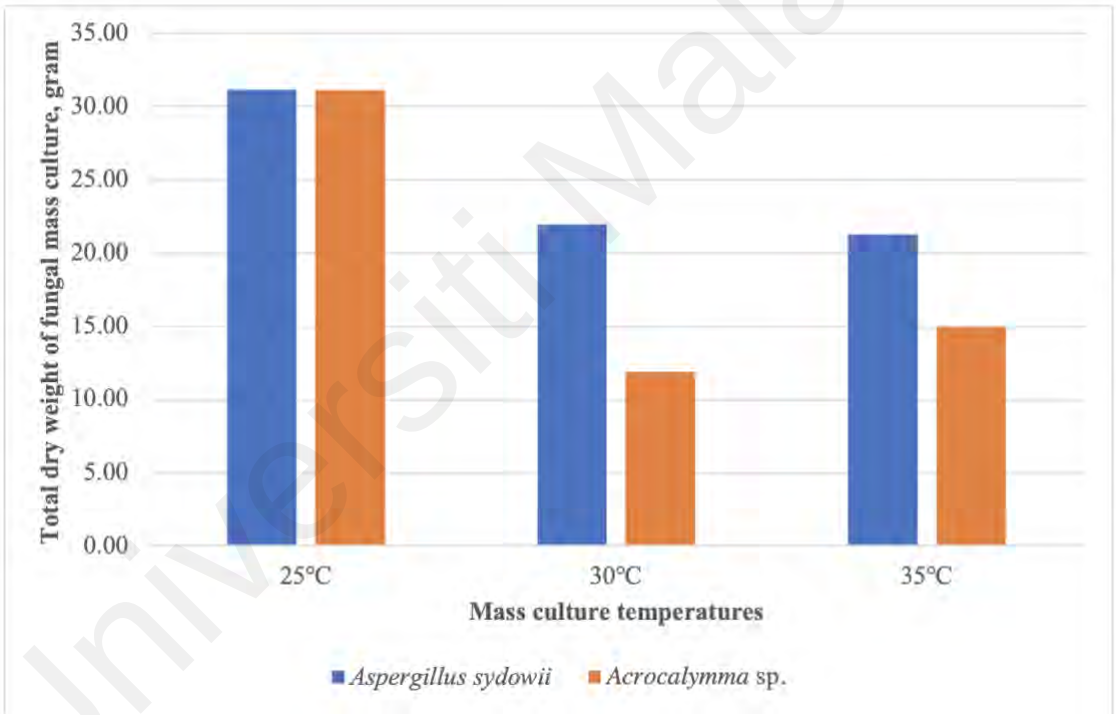


Figure 4.9 Total dry weight (gram) of fungal mass culture at different temperatures

4.4 Chemical Extraction

Figure 4.10 illustrates the quantified yield of crude extracts from fungal mass culture of the two selected fungal isolates at different temperatures.

The crude extract yield for *Aspergillus sydowii* was quantified between 9.12% and 13.03% for 25°C and 35°C respectively. The yield was low at 30°C with 9.21%. In a contrasting pattern, *Acrocalymma* sp. yielded the highest with 41.40 % at 30°C. The yield quantified at 25°C and 35°C was lowest at 3.06% and 10.39%, respectively.

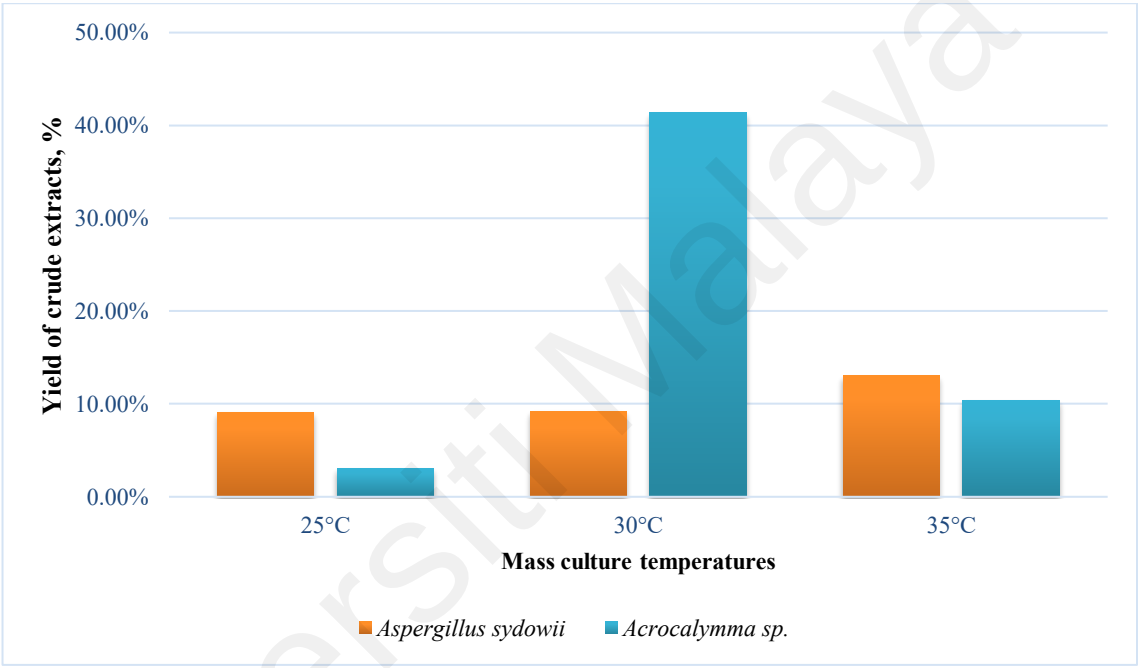


Figure 4.10 Total yield of crude extracts from fungal mass culture, %

4.5 Chemical profiling

4.5.1 Thin layer chromatography

Thin layer chromatography is the most basic form of chemical profiling to indicate presence or absence of chemical constituents in an extract. In this investigation, the crude extracts were subjected to TLC developed over three separate solvent systems of varying polarity. Mobile phase which provides the best separation will serve as a benchmark for further analysis. Figure 4.11 shows the TLC output developed by three solvent systems: hexane : ethyl acetate (H:E) = 3 : 1, 100% chloroform (Chl) and 100% toluene (Tol). Sp1 25, Sp1 30 and Sp1 35 represent the crude extracts of *Aspergillus sydowii* grown at 25°C, 30°C and 35°C, respectively; while Sp2 25, Sp 2 30 and Sp2 35 represent the crude extracts of *Acrocalymma* sp. grown at 25°C, 30°C and 35°C, respectively. Overall, the solvent system H : E = 3 : 1 seems to produce the best separation of all crude extracts. However, there is not an obvious difference in R_f values in response to elevated temperatures.

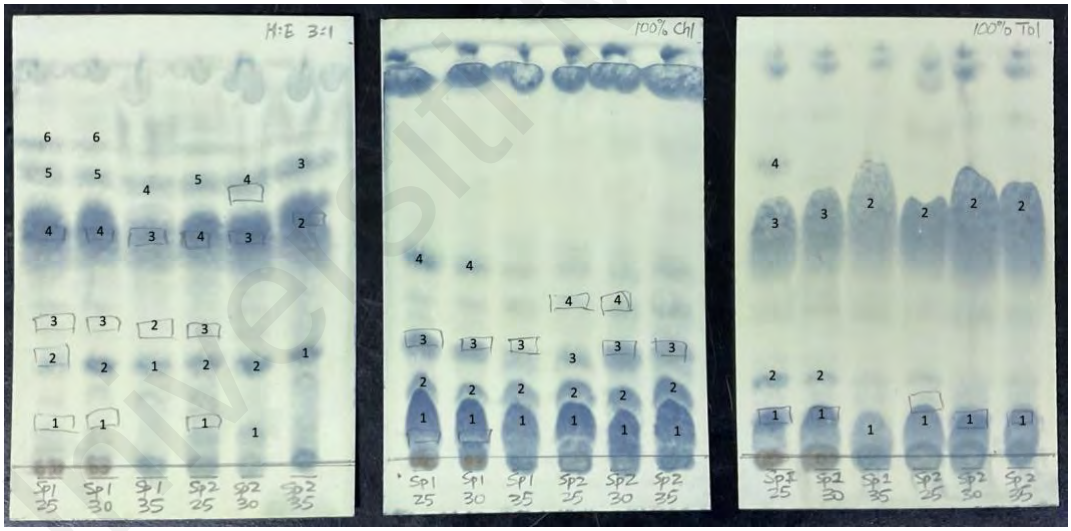


Figure 4.11 TLC results from quantified *Aspergillus sydowii* and *Acrocalymma* sp. crude extracts

4.5.2 HPLC

Based on the TLC output, there seemed to be indications of minor differences in the crude extracts across the temperature gradient for both strains cultured. To confirm, the crude extracts were subjected to HPLC analysis screened using a photo diode array detector. Figure 4.12 represents a stacked HPLC profile of *Aspergillus sydowii* at the wavelength of 215 nm. The selected wavelength revealed the most obvious differences in profile of secondary metabolites, potentially terpenoids. The HPLC profiling of extracts across temperature gradient provided indication of reduction in number as well as concentration in detected peaks suggesting that rise in temperature have an impact on the secondary metabolite production of *A. sydowii*.

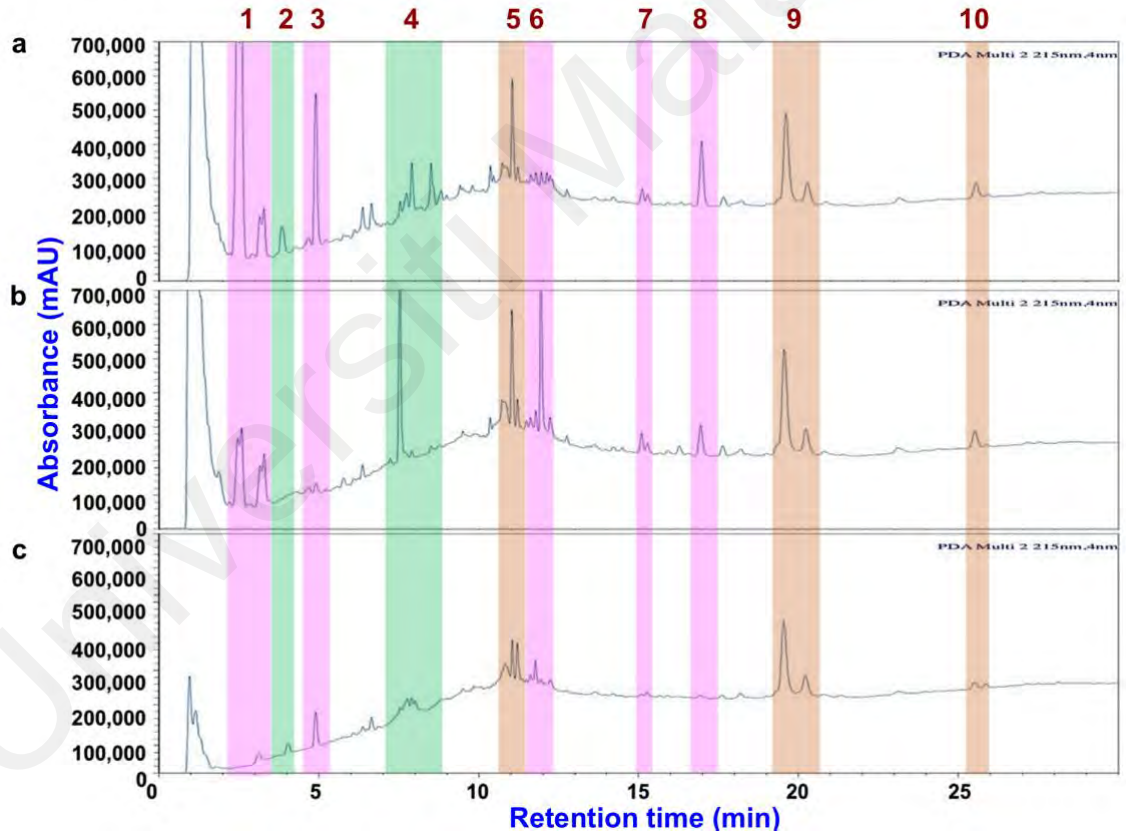


Figure 4.12 HPLC chromatograms of crude extracts of *Aspergillus sydowii* at 25℃ (a), 30℃ (b) and 35℃ (c).

The chromatogram had been divided into 10 regions, according to obvious changes in peak detection in response to temperature. The brown regions (5, 9 and 10) represent peaks detected in all three temperatures, while the pink regions (1, 3, 6, 7 and

8) represent peaks detected in two temperatures. On the other hand, the green regions (2 and 4) are peaks only detected in one temperature. Region 1 of Figure 4.12 observed a reduction in metabolite detection. The first detection with retention time (RT) approximately 2.5 min was highest in absorbance at 25°C but reduced greatly at 30°C to reveal a potential overlap of two metabolites. The second detection in region 1 at RT 3 min seemed to be two overlapping metabolites, was present at an identical concentration in both 25°C and 30°C spectrum. However, at 35°C all peaks were absent. Similarly in region 2, at 5 min RT a single peak was detected at 25°C but was absent in 30°C only to reappear at 35°C at a height three times (3x) lower than initially detected. In region 4, several minor peaks were only detected at 25°C only to be replaced by sharp single peak detected at 7 min RT. The spectrum of 35°C did not capture any significant detection. Similar trend was observed for region 5 where the profile at 30°C revealed two prominent peaks as compared to one in 25°C which was identical at 11 min. These detections were vastly suppressed at 35°C. In region 6, the highest peak was detected at 12 min at 30°C, while the detections at 25°C and 35°C were much smaller. Regions 7 – 10 revealed detections at 15, 17, 19, 20 and 26 mins, all of which appeared with a consistent reducing concentration across the temperature profile. In conclusion, the HPLC analysis clearly revealed that more secondary metabolites were found at 25°C and 30°C, often with slightly higher concentration compared to 35°C, where most of the detections were suppressed suggesting possible deterioration or reduction of biochemical functions to adapt to the extreme temperature.

Figure 4.13 represents a stacked HPLC profile of *Acrocalymma* sp. at the wavelength of 215 nm. The selected wavelength revealed the most obvious differences in profile of secondary metabolites, potentially terpenoids. On balance, the HPLC profiling of extracts across temperature gradient provided an indication of reduction in number as well as concentration in detected peaks suggesting that rises in temperature, similar to *A. sydowii*, also have an impact on the secondary metabolite production of *Acrocalymma* sp. Nevertheless, there were unique peaks detected at specific temperatures.

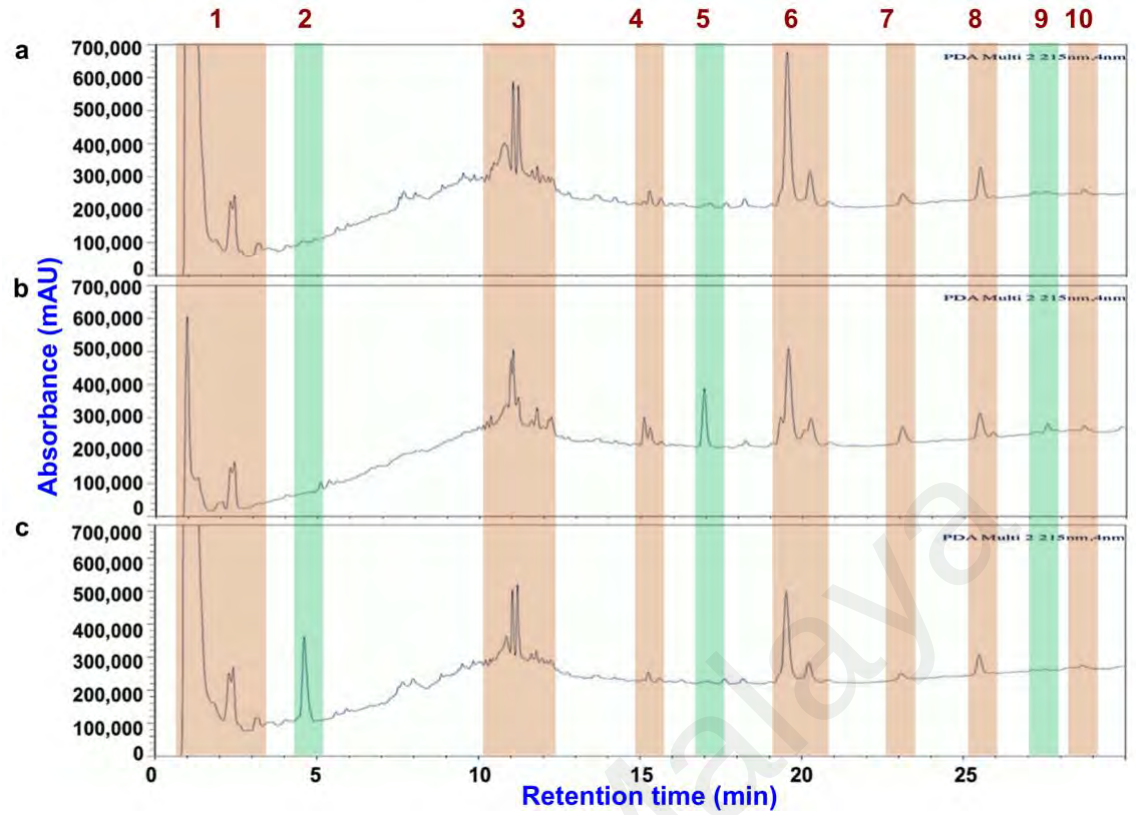


Figure 4.13 HPLC chromatograms of crude extracts of *Acrocalymma* sp. at 25°C (a), 30°C (b) and 35°C (c).

Similar to the chromatogram of *A. sydowii*, the chromatogram had been divided into 10 regions, based on obvious changes in peak detection in response to temperature. The brown regions (1, 3, 4, 6, 7, 8 and 10) represent peaks detected in all three temperatures, whereas the pink regions (1, 3, 6, 7 and 8) represent peaks detected in two temperatures. On the other hand, the green regions (2, 5 and 9) are peaks only detected in one temperature. The first detection with RT approximately 1 min was lowest at 30°C in absorbance but increased too greatly to detect at 25°C and 35°C, possibly revealing a single metabolite. The second detection in region 1 at RT 2.5 min was the highest at 35 °C in absorbance but decreased slightly at 25°C and 30°C to reveal an overlap of two metabolites. However, only one prominent peak was detected at 35°C with an RT nearly 4.5 min in region 2. Similarly, in region 5 only one single prominent peak was detected at 30°C at RT approximately 17 min. However, the first detection at RT approximately 10.7 min in region 3 was the highest at 25°C, which decreased slightly when temperature increased to 30°C and 35°C. The second and third detection in region 3 at approximately 11 min and 11.2 min respectively showed a similar pattern to that of the fist detection in this region, where the highest detection was found at 25°C and the peak dropped slightly as the temperature rose to 30°C and 35°C. On the other hand, the detection and changes

in region 4 were less prominent. The first detection in region 4 at RT around 15.1 min was only detected at 30°C, while the second detection at RT about 15.4 were detected across three temperatures with 30°C being the highest. Other prominent changes were observed in region 6. The first detection in this region at RT about 19.2 min was only detected at 30°C, although this detection might overlap with the second detection in this region at 25°C and 35°C. This detection could overlap at the other two temperatures. The third detection with a RT of nearly 20.3 min at 30°C in this region was similar to that of the first detection in terms of the only presence at 30°C. However, the second detection in region 6 was detected at RT approximately 19.5 min across three temperatures, where the height at 25°C was one-and a-half times (1.5x) higher than the later detected at 30°C and 35°C. The last detection at RT around 20.4 min was similar across three temperatures, indicating that temperatures have little impact on the production of this metabolite. Similarly, this pattern was also observed in the first detection at RT about 28.8°C in region 10, the production of which was little influenced by temperature changes. However, in region 7 with an RT of approximately 23.3 min was the highest at 30°C, which decreased slightly when the temperature rose or dropped by 5°C. In contrast, the first detection at RT around 25.5 min was the highest, and the height decreased consistently as temperature rose to 30°C and 35°C. The second small detection in region was only detection at RT 25.9 min at 30°C, absent at 25°C and 35°C, suggesting this metabolite production might be suppressed when temperatures increased or decreased from the ambient.

4.5.3 Q-TOF LCMS

4.5.3.1 Number of identified compounds

HPLC data revealed an obvious reduction in detection of metabolites as the experimental temperature was increased. Therefore, in order to attempt characterization of metabolites in the extracts of both fungal strains, Q-TOF LCMS analysis was done. Data from LCMS analysis revealed the total number of compounds and/or fragments detected by the mass spectrometer. Figure 4.14 illustrates the total number of detection from crude extracts of *A. sydowii* across the three temperatures evaluated. Based on this data, increment in temperatures have an influence on the total detections, although this impact is not in a linear pattern. Overall, more compounds were captured in positive mode than in negative mode. In line with HPLC data, higher detection were captured in the crude extract for sample cultured at 25°C. However, interestingly significant number of detections were recorded at 35°C as well, compared to the ambient temperature (30°C). The highest number of compounds was found at 35°C with 28 compounds identified, while the least number of compounds was detected at 30°C with 19 compounds identified. It is hypothesized that the high detection at 35°C could be attributed to the degradation of metabolites that were produced thus being captured as fragments by the LCMS due to its much higher sensitivity as compared to the HPLC.

Figure 4.15 shows the total number of detections from crude extracts of *Acrocalymma* sp. across the three temperatures evaluated. Based on this data, increment in temperatures have an influence on the total detections, although this impact is not in a linear pattern. Overall, significantly more compounds were captured in positive mode than in negative mode. In line with HPLC data, the highest detections were captured in the crude extract for sample cultured at 25°C. However, compared to the ambient temperature (30°C), slightly higher number of detections were recorded at 35°C as well. The highest number of compounds was found at 25°C with 56 compounds identified, while the least number of compounds was detected at 30°C with 26 compounds identified. It is hypothesized that the high detection at 25°C could be attributed that the fungal strains were less stressed than 30°C and 35°C, since this aligns with HPLC data.

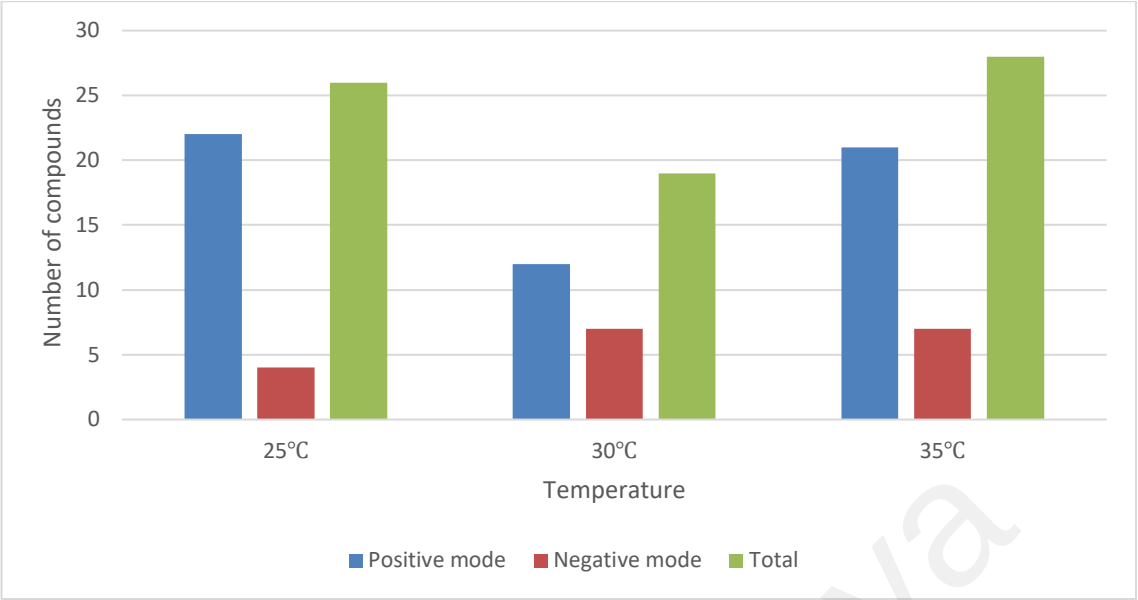


Figure 4.14 Number of compounds from *Aspergillus sydowii* at different temperatures

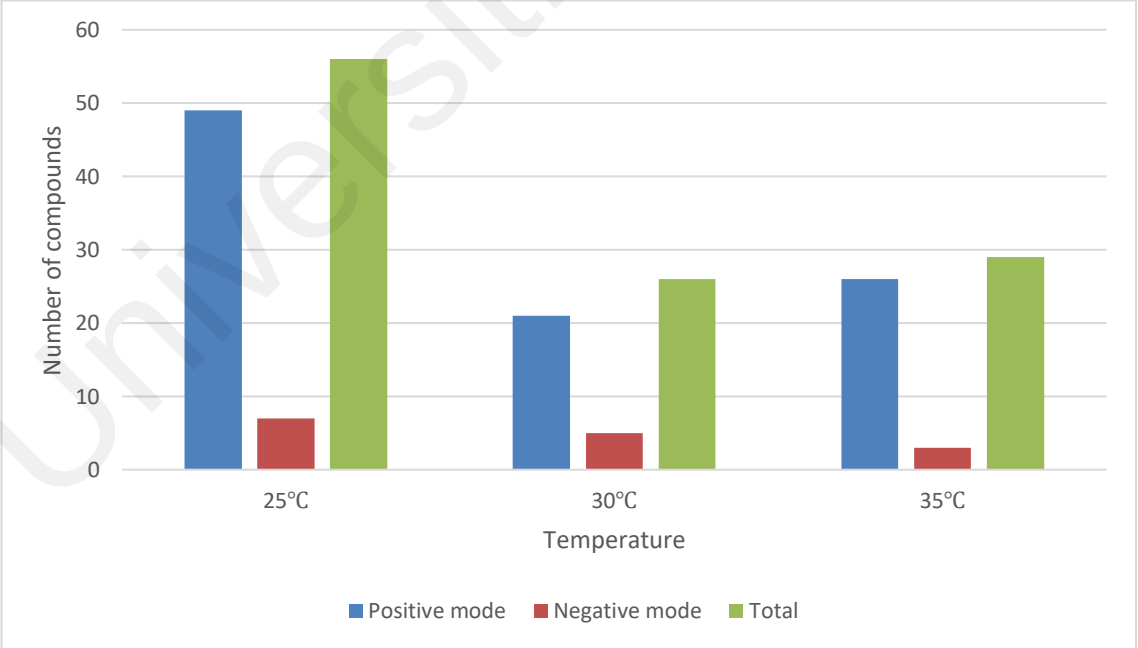


Figure 4.15 Number of compounds from *Acrocalymma* sp. at different temperatures

Tables 4.4 - 4.7 show the top five highest amounts of chemical compounds from *Aspergillus sydowii* and *Acrocalymma* sp. in Q-TOF LCMS positive and negative modes. The same colour in a table represents the same compound (chemical formula) detected at different temperatures, while the grey columns indicate that these compounds were not detected (N/A: not available). According to Table 4.4, the abundance of $C_{16}H_{44}N_9O_5S$ was higher (at 25°C) when the temperature is lower than the ambient in the positive mode of LCMS, suggesting a lower temperature leads to its production. In addition, with the increase of temperature, *A. sydowii* produced more compounds with higher exact mass on average. From Table 4.5, there were more common chemical compounds found across the three temperatures in the LCMS negative mode. The compounds $C_{18}H_{36}O_2$ and $C_{18}H_{34}O_2$ were the only compound found in all three temperatures, both of which showed a decreased abundance when temperature was higher (at 35°C) or lower (at 25°C) than the ambient (at 30°C). However, the compounds of $C_{18}H_{36}O_2$ and $C_{16}H_{32}O_2$ were only detected at the temperatures of 25°C and 30°C, both of which illustrated a higher abundance at 25°C, indicating these compounds could be induced by a lower temperature. Based on Table 4.6, the compounds of $C_{36}H_{63}N_{16}O$ and $C_{43}H_{93}N_{11}S$ were found in the crude extract from the unidentified fungal species at 25°C and 30°C. When temperatures dropped, the abundance of $C_{36}H_{63}N_{16}O$ was higher, whereas the compound $C_{43}H_{93}N_{11}S$ demonstrated a lower abundance at 25°C. However, the compound $C_{38}H_{69}N_{10}O_3$ was found in 30°C and 35°C, the abundance of which also decreased when the temperature increased. From Table 4.7, in the negative mode the compounds $C_{18}H_{32}O_2$ and $C_{16}H_{32}O_2$ were only present at 25°C and 35°C, and their abundances were higher at 35°C, suggesting they could be produced by the fungal species to cope with temperature stress. Although the compound $C_{10}H_{30}N_7O_2$ had a higher abundance when temperature was lower than the ambient, the compound $C_{18}H_{36}O_2$ showed a lower abundance when temperature was higher than the ambient.

Table 4.4 Exact mass, MS m/z, abundance, and retention time of the top five chemical compounds from *Aspergillus sydowii* crude extract in the positive mode

Temperature	Molecular formula	Determined Exact Mass	Calculated Exact Mass - Positive Mode $(M+H)^+$	Determined positive mode MS m/z $(M+H)^+$	Abundance / Height	Retention time /min
25°C	$C_{16}H_{44}N_9O_5S$	474.3185 u	475.326438 u	475.3257 - 475.3258	5060.5 ± 3973.23	1.508 - 2.341
	$C_{17}H_{44}N_{10}O_2S$	452.3368 u	453.344767 u	453.3441	5690	2.316
	$C_{28}H_{63}N_{10}O_7S$	683.4603 u	684.468017 u	701.4941	5215	2.825
	$C_{19}H_{38}N_7O_{10}S$	556.2401 u	555.232265 u	557.2472-557.2475	4556.5 ± 904.39	3.751 - 3.983
	$C_{20}H_{53}N_{24}O_2$	661.4786 u	662.486155 u	679.5122	4052	2.896
30°C	$C_{36}H_{63}N_{16}O$	735.5378 u	736.544899 u	736.5449	26048	18.154
	$C_{38}H_{69}N_{10}O_3$	713.5559 u	714.563235 u	736.5452	18576	18.154
	$C_{16}H_{44}N_9O_5S$	474.31895 u	475.326438 u	475.3258 -475.3265	4043 ± 633.92	1.168 - 1.437
	$C_{36}H_{73}N_5O_{10}$	735.5361 u	736.543570 u	736.5432 - 736.5436	1982.5 ± 1246.63	19.509 - 19.711
	$C_{15}H_{38}N_{16}S$	474.319 u	475.326431 u	475.3261	2417	1.521
35°C	$C_{43}H_{93}N_{11}S$	795.7330125 u	796.741436 u	796.7394 - 796.7406	17889.38 ± 11530.70	60.54 - 81.73
	$C_{38}H_{84}N_{17}$	778.707 u	779.717382 u	796.7406	36806	65.57
	$C_{39}H_{75}N_3O_8$	713.5555 u	714.563242 u	736.5449	35149	18.084
	$C_{41}H_{89}N_{11}S$	767.7017833 u	768.710136 u	768.7084 - 768.7095	15852.16 ± 13212.54	62.75 - 69.49
	$C_{33}H_{72}N_{12}O_9$	780.5556 u	781.562348 u	803.5449	28923	77.45

Table 4.5 Exact mass, MS m/z, abundance, and retention time of the top five chemical compounds from *Aspergillus sydowii* crude extract in the negative mode

Temperature	Molecular formula	Determined Exact Mass	Calculated Exact Mass - Negative Mode $(M-H)^-$	Determined negative mode MS m/z $(M-H)^-$	Abundance / Height	Retention time /min
25°C	$C_{16}H_{32}O_2$	256.2404	255.232405 u	255.233- 255.2333	154928.5 ± 27119.67	12.047 - 12.064
	$C_{18}H_{32}O_2$	280.2403	279.232404 u	279.2329 - 279.2334	82477.67 ± 66988.52	8.744 - 11.306
	$C_{18}H_{34}O_2$	282.2557	281.248055 u	281.2484 - 281.2486	5043.5 ± 4458.31	12.157 - 12.173
	$C_{18}H_{36}O_2$	284.2714	283.263705 u	283.2641 - 283.2643	48885.5 ± 12228	13.621 - 13.638
	N/A	N/A	N/A	N/A	N/A	N/A
30°C	$C_{18}H_{32}O_2$	280.2414333	279.232404 u	279.2339 - 279.2345	222941.67 ± 174382.94	11.298 - 11.306
	$C_{18}H_{36}O_2$	284.2721333	283.263705 u	283.2645 - 283.2655	21977.67 ± 15230.52	13.52 - 13.654
	$C_{18}H_{34}O_2$	282.2566	281.248055 u	281.2492 - 281.2495	12820 ± 14803.99	12.134 - 12.172
	$C_7H_5O_6S_3$	280.9266	279.917006 u	279.9194	228297	11.146
	$C_{16}H_{32}O_2$	256.2410333	255.232405 u	255.2337 - 255.2339	117195.33 ± 101117.88	12.041 - 12.072
35°C	$C_{13}H_{30}N_5$	256.2475	257.257945 u	255.2402	72167	11.981
	$C_{15}H_{32}N_5$	282.26335	281.257945 u	281.2557 - 281.2564	57247.5 ± 14073.55	12.07 - 12.074
	$C_{18}H_{34}O_2$	282.2559	281.248055 u	281.2486	61187	12.071
	$C_{10}H_{30}N_7O_2$	280.2474	279.238273 u	279.2402	31370	11.33
	$C_{18}H_{32}O_2$	280.2404	279.232404 u	279.2331	23661	11.33

Table 4.6 Exact mass, MS m/z, abundance, and retention time of the top five chemical compounds from *Acrocalymma* sp. crude extract in the positive mode

Temperature	Molecular formula	Determined Exact Mass	Calculated Exact Mass - Positive Mode $(M+H)^+$	Determined positive mode MS m/z $(M+H)^+$	Abundance / Height	Retention time /min
25°C	$C_{36}H_{63}N_{16}O$	735.5377	736.544899 u	736.5447 - 736.545	61686 ± 26277.50	18.113 - 18.165
	$C_{43}H_{93}N_{11}S$	795.7330333	796.741436 u	796.7399 - 796.7404	16603.5 ± 26277.50	25.831 - 27.99
	$C_{18}H_{31}N_4$	303.25495	304.262696 u	304.262 - 304.2624	18176 ± 22958.34	11.109 - 11.154
	$C_{41}H_{89}N_{11}S$	767.701625	768.710136 u	768.7083 - 768.7093	16681.5 ± 13539.61	28.068 - 28.916
	$C_{40}H_{89}N_{22}OS$	925.7308	926.738865 u	948.7193	26737	13.839
30°C	$C_{38}H_{65}N_{13}O_2$	735.5383	736.546242 u	736.5455	54290	18.054
	$C_{38}H_{69}N_{10}O_3$	713.556	714.563235 u	736.5452	54316	18.064
	$C_{36}H_{63}N_{16}O$	735.5379	736.544899 u	736.545	46167	18.077
	$C_{43}H_{93}N_{11}S$	795.7330125	796.741436 u	796.7398 - 796.7408	30301.75 ± 6824.48	25.895 - 26.879
	$C_{18}H_{31}N_4$	303.25505	304.262696 u	304.2621 - 304.2623	27634 ± 11508.87	11.025 - 11.055
35°C	$C_{38}H_{69}N_{10}O_3$	713.55565	714.563235 u	736.5447 - 736.5451	34593 ± 28620.85	18.105 - 18.164
	$C_{18}H_{43}N_5O_6S$	457.2932	458.301232 u	475.327	39274	1.435
	$C_{37}H_{69}N_9O_6$	735.5372	736.544906 u	736.5445	38910	18.111
	$C_{35}H_{76}N_{17}O_2S$	798.6085	799.616685 u	816.6422	35888	13.983
	$C_{16}H_{44}N_9O_5S$	474.3192	475.326438 u	475.3263 - 475.3266	14421.75 ± 14433.73	1.199 - 1.431

Table 4.7 Exact mass, MS m/z, abundance, and retention time of the top five chemical compounds from *Acrocalymma* sp. crude extract in the negative mode

Temperature	Molecular formula	Determined Exact Mass	Calculated Exact Mass - Negative Mode $(M-H)^-$	Determined negative mode MS m/z $(M-H)^-$	Abundance / Height	Retention time /min
25°C	$C_7H_5O_6S_3$	280.9259	279.917006 u	279.9186	110084	11.329
	$C_{18}H_{32}O_2$	280.24125	279.232404 u	279.2338 - 279.2343	64337.5 ± 54206.1	11.295 - 11.302
	$C_{10}H_{30}N_7O_2$	280.2476	279.238273 u	279.2404	60010	11.306
	$C_{16}H_{32}O_2$	256.2415	255.232405 u	255.2342	58374	12.043
	$C_{13}H_{30}N_5$	256.247	255.242295 u	255.2397	25630	12.047
30°C	$C_5H_8Cl_3N_2O_3S$	280.9323	279.924299 u	279.925	105080	11.332
	$C_{13}H_{30}N_5$	256.24725	255.242295 u	255.2395 - 255.2404	58626.5 ± 13916.57	11.979 - 11.984
	$C_{10}H_{30}N_7O_2$	280.2476667	279.238273 u	279.2402 - 279.2407	35617.33 ± 1319.28	11.331 - 11.335
	$C_{18}H_{36}O_2$	284.2711	283.263705 u	283.2638	16560	13.555
	$C_{16}H_{34}N_3O$	284.271	283.262362 u	283.2638	13.558	12400
35°C	$C_{16}H_{32}O_2$	256.2411	255.232405 u	255.2333 - 255.2341	78502.67 ± 20753.41	12.035 - 12.041
	$C_{18}H_{32}O_2$	280.2413	279.232404 u	283.2654 - 283.2655	78502.67 ± 20753.41	11.294 - 11.3
	$C_{18}H_{36}O_2$	284.2727	283.263705 u	279.2337 - 279.2343	3326 ± 1305.32	13.643 - 13.648
	N/A	N/A	N/A	N/A	N/A	N/A
	N/A	N/A	N/A	N/A	N/A	N/A

CHAPTER 5: DISCUSSION

5.1 Occurrence and distribution of *Sarcophyton ehrenbergi*

5.1.1 Identification of *Sarcophyton ehrenbergi*

The soft coral genera *Sarcophyton* and *Lobophytum* are of universal prominence and ecological importance through the Indo-Pacific shallow-water reef communities (McFadden et al., 2006). However, the only authoritative morphological guidelines for the identification of these two genera were described by Verselveldt almost four decades ago (Verselveldt, 1982, 1983). His examination and illustration were mostly based on a single specimen, while multiple specimens were included when variants were present. In addition, a small number of morphological characters were used, such as colony growth form, spacing between siphonzooids and autozooids on the surface of colony and the subjectively judged size and shape of sclerites, which were taken from the surface and interior of the polypary, as well as the surface and interior of the stalk or base. His description of each species includes a photograph of the specimen preserved and maximum six to eight hand-written illustrations of sclerites from different parts of each specimen. Identification of species from these two genera requires matching sclerite samples to the restricted illustrated specimens, indicating an exact match being almost impossible (since no two sclerites are identical) and rather subjective (since what is considered to be resembled might vary from one person to another). A more recent study pointed out some drawbacks in Verseveldt's descriptions (McFadden et al., 2006). On the one hand, smaller sclerites were lost in his method of slide preparation. On the other hand, he did not always include the entire range of sclerites on a slide, and some omitted scelerites might not assist species identification. Therefore, molecular identification must be included to confirm the soft coral *Sarcophyton* species in this study.

DNA barcoding has become a reliable system to aid identification of unidentified specimens by means of comparing with a reference database of molecular sequences (Hebert et al., 2003; McFadden et al., 2011). Although *COI* fragments have been proved to be an effective barcoding gene in identifying vertebrates and arthropods, their utility in species identification of invertebrates, especially the anthozoan cnidarians, can be limited due to lack of mitochondrial gene variation (Hebert et al., 2003). On the other hand, Shearer et al. (2002) suggested that the sub-class Octocorallia members of anthozoans showed slow rates of mitochondrial gene divergence similar to other anthozoans. Regarding *COI* in octocorals, low genetic divergence rates were reported at

the 3' region of the gene not the 5' 'Folmer region', thus being adopted as a universal barcode (France & Hoover, 2002).

Based on morphology and molecular methods, the soft coral in this study was identified as *Sarcophyton ehrenbergi*.

5.1.2 Occurrence of *Sarcophyton ehrenbergi*

Abou El-Ezz et al. (2013) stated that species from *Sarcophyton* genus demonstrated wide distribution in tropic and subtropic oceans. Table 5.1 also highlights that the distribution of *S. ehrenbergi* can generally be considered quite specific from the tropic to the sub-tropic regions. However, this species is the most prominent in South China Sea regions (Strychar et al., 2005; Cheng et al., 2009; Cheng et al., 2014; Yuan et al., 2019; Li et al., 2020). Other studies have shown that this species was also found in Mandapam coast, South India (Bhujanga Rao et al., 2014), Great Reef Barrier regions, Australia (Bowden et al., 1978; Fleury et al., 2000; Konig & Wright, 1998; Strychar et al., 2005), and the Red Sea (Fishelson, 1970; Hegazy et al., 2017).

Hong and Sasekumar (1981) reported the presence of *S. ehrenbergi* from Tanjung Tuan, the Straits of Malacca, being the very first record of this species in Malaysia. In the present study, this species was also recorded from the same sampling site. The presence of this species was also recorded in several earlier studies carried out in three neighbouring South-East Asian countries, namely Thailand (Chanmethakul, 2009), Indonesia (Cervino et al., 2012) and Vietnam (Ngoc et al., 2021; Ninh Thi et al., 2021).

Table 5.1 Global distribution of *Sarcophyton ehrenbergi*

Eilat, the Red Sea	(Fishelson, 1970)
Pioneer Bay, Australia	(Bowden et al., 1978)
Cape Rachado (now known as Tanjung Tuan), Malaysia	(Hong & Sasekumar, 1981)
Old Reef, Australia	(Konig & Wright, 1998)
One Three Island, Australia	(Fleury et al., 2000)
Barren Island, Australia	(Strychar et al., 2005)
Gulf of Thailand, Thailand	(Chanmethakul, 2009)
Dongsha Island, Taiwain	(Cheng et al., 2009)
Wakatobi archipelago, Indonesia	(Cervino et al., 2012)
Mandapam coast, southern India	(Bhujanga Rao et al., 2014)
Yang Meikeng, China	(Cheng et al., 2014)
San-Hsian-Tai, Taiwan	(Hou et al., 2015)
North Reef, China	(Tang et al., 2016)
Hurghada, Egyptian Red Sea Coast	(Hegazy et al., 2017)
Weizhou Island, China	(Li et al., 2020)
Van Phong Bay, Vietnam	(Ngoc et al., 2021; Ninh Thi et al., 2021)

5.2 Marine fungi associated with *Sarcophyton ehrenbergi* from Port Dickson

As foundational members, octocorals play an important role in the benthic community. Van de Water and his colleagues (2018) pointed out that they provide refuge and habitats to a rich fauna because of the structural complexity of their ecosystems by forming three-dimensional structures. Additionally, their ecological roles also include the regulation of the primary and secondary productions of the coastal food webs because they exchange energy, mass and nutrients between benthic and pelagic habitats, and transfer energy between plankton and benthos (Gili & Coma, 1998).

As all holobiont entities, octocorals also interact in a complex and intricate manner with a wide range of microorganisms, namely dinoflagellates, bacteria, fungi, viruses and archaea (Knowlton & Rohwer, 2003). These symbiotic microbes perform key roles in their host in terms of health, e.g. to supply nutrients and protect from pathogens, and adaptation to environmental changes, e.g. to degrade toxins (Bourne & Webster, 2013; McFall-Ngai et al., 2013).

According to previous studies, there have been 26 species of marine fungi isolated from the genus *Sarcophyton* worldwide (Li et al., 2012; Yuan et al., 2019; Zheng et al., 2013; Agrawal et al., 2018; Huang et al., 2018; Liu et al., 2018; El-Demerdash et al., 2020; Liu et al., 2021). Previous studies have indicated that the genus *Aspergillus* is one of the most major fungal genera associated with *Sarcophyton* spp., in particular *Aspergillus terreus* was isolated from *Sarcophyton subviride* (Liu et al., 2018), *A. versicolor* was found in *Sarcophyton tortuosum* (Zhang et al., 2019) and *A. elegans* was isolated from *Sarcophyton* sp. (Zheng et al., 2013), in which all the soft coral samples were collected from the South China Sea. *Aspergillus oryzae* and *A. flavus* are the only two *Aspergillus* species associated with *S. ehrenbergi*, which was collected from in Hurghada, the Egyptian coast of Red Sea (Singab et al., 2022). However, there has been no record on the isolation of *Aspergillus sydowii*, fungal isolate 1 in the present study, therefore making this as the first report from *Sarcophyton*. The very first record of this fungal species in Malaysia dates back to 1972, and it was isolated from soil samples in the oil palm plantation regions (Varghese, 1972). This fungal species has also been consistently found in various locations in Malaysia from Peninsular Malaysia to Sabah since 1977, and their association ranges from different soil samples to various plants, i.e. *Zea mays* (cornflour), *Triticum aestivum* (common wheat), species in the class of Magnoliopsida and other species under the genera of *Metroxylon* and *Burmannia* (Hawksworth, 2013). Therefore, the isolation of *Aspergillus sydowii* in this study is not

only the first report from the soft coral *Sarcophyton*, but also the very first isolation from aquatic environment in Malaysia, particularly marine ecosystems.

In addition, the remaining identified fungal isolates in this study*, Acrocalymma sp.*, *Lindgomyces* sp. and *Phomatospora* sp., have not been isolated from the genus *Sarcophyton* as well. However, these species could only be identified on a genus level.

Fungal isolate 2 was identified under the genus *Acrocalymma*, which was only circumscribed more than four decades ago (Alcorn & Irwin, 1987). The first identified species was *Acrocalymma mediginis*, previously known as *Stagonospora meliloti*, causing root and crown diseases of *Medicago sativa* in Australia. This species was also found in the rattan palm spines in the tropical forests of Peninsular Malaysia (Azuddin et al., 2021). *Acrocalymma aquatica* had also been isolated from northern Thai waters (H. Zhang et al., 2012). The endophytic fungus *Acrocalymma vagum* was found to be associated with leaves and roots of *Centella asiatica* plant in Bengkuku, Indonesia (Radiastuti et al., 2019). In view of their associations, the genus *Acrocalymma* could thrive in a range of environments and more species under this genus could be identified in the future.

Fungal isolate 3 was identified under the genus *Lindgomyces*, which is a newly established freshwater ascomycete genus, from the family Lindgomycetaceae, belonging to in the order Pleosporales, under the class Dothideomycetes (Hirayama et al., 2010). There has been only one reported species in Malaysia, namely *Lindgomyces ingoldianus* (previously known as *Massarina ingoldiana*), isolated from submerged wood in Sungai Lentang, Pahang (Shearer & Hyde, 1997) and from freshwater in Gombak, Selangor (Hyde, 1998). However, previous records of fungal species under this genus were mostly found in the submerged wood in temperate freshwaters, although there were some reports from the tropical and subtropical regions in the world (Raja, et al., 2017). There had been nine species under this genus until 2017, and most species were found in France, Japan and the USA, indicating this genus prefers to live in temperate regions (Raja, et al., 2017). Despite mostly identified in the freshwater environment, this study has been the first report on its isolation from a soft coral host, thus indicating that this genus could have a wider geographic distribution and survive in different environments.

Fungal isolate 4 was identified as *Phomatospora* sp. The fungal genus *Phomatospora* was first reported in the guts of Malayan grasshoppers (Monk & Samuels, 1990). The first marine origin of *Phomatospora* species was *P. nypae* from leaves of submerged *Nypa fruticans* in the intertidal zones in Kuala Selangor, Malaysia (Hyde, 1993). However, there are limited reports on *Phomatospora* in Malaysia. Our finding

suggests that this genus could have association with more than one organism in the neighbouring geographic region.

Research into fungal communities related to corals has primarily focused on culturable fungal strains, and in this study, there were four isolated fungal species associated with the soft coral *Sarcophyton ehrenbergi*. Previous isolation of fungi from soft corals was carried out in six different gorgonians from the South China Sea (Zhang et al., 2012). This study successfully isolated 41 fungal species across 20 genera, in which *Aspergillus* and *Penicillium* were the dominant genera and had the most diverse species. More fungal strains were isolated because a total of six growth media was used to isolate marine fungi, including glucose peptone agar (GPA), glucose peptone starch (GPSA), glucose yeast malt agar (GYMA), glucose yeast peptone agar (GYPA), PDA and starch yeast agar (SYA). In this study, however, PDA was the only growth media used to isolate marine fungi associated with *Sarcophyton ehrenbergi* because most fungal species has been proved to be isolated on this media (Zhang et al., 2012).

Although there were three newly identified fungal isolates from *Sarcophyton* in the present study, the species isolated are mainly culture-dependent fungal species. Studies have shown that there could be a greater fungal diversity when culture-dependent techniques coupled with culture-independent methods, such as NGS techniques (Zhang et al., 2012; van de Water et al., 2018).

Due to the limitation of morphological identification, molecular markers were used to assist identification of the isolated marine fungi. Despite being the standard barcoding gene marker, ITS shows low resolution in some fungal groups as a result of high variability of sequence length, for example, marine fungi in the genera *Cladosporium*, *Penicillium* and *Fusarium* have a shorter ITS regions (about 400 bp) (Reich & Labes, 2017).

In 2008, Peterson used DNA sequences in four loci to analyse the phylogenetic relationship among *Aspergillus* species, including beta tubulin (BT2), calmodulin (CF), ITS and lsu rDNA (ID) and RNA polymerase II (RPB2). According to his research, BT2 gene marker was not suitable to analyse groups that contain the amplified regions with different physical structures because of strong discordance (Peterson, 2008). A further study in the identification of *A. sydowii* used three DNA sequences (variable BT2, ITS and the conservative LSU genes), in which 99% - 100% matches were obtained to unambiguously identify the fungal species being *A. sydowii*. Therefore, BT2 could be used to assist fungal identification in this study, although a more common gene marker was required to identify the rest of fungal isolates.

In this study, the second fungal isolate was not able to be identified since there were not enough 100% matches of ITS and LSU on Genebank. Similar results of ITS and LSU genes were found in the BLAST results of fungal isolates 3 and 4. This suggests that more research could be done in terms of marine fungi identification.

The remaining three gene markers selected for this study were MCM7, RPB1 and TEF1, all of which generated much fewer matches, compared to ITS and LSU. This might be because these gene markers are not standard barcoding genes and are usually used to assist identification and confirmation of fungal species (Luo & Zhang, 2013).

Thus, this study has identified three new fungal associations with *S. ehrenbergi*, and thereby could expand the fungal communities related to the soft coral genus *Sarcophyton*. Meanwhile, it could possibly provide future comparison between fungal isolates from *S. ehrenbergi* in Southeast Asian regions and that of Red Sea regions.

5.3 Effect of temperature on fungal metabolites

Filamentous fungi produce a broad variety of secondary metabolites. Secondary metabolism is often associated with fungal growth and development stages, in which fungal growth slows down because of limited nutrients (Moore, 1998). It is believed that once carbohydrates are no longer required in growth as the result of slow mycelia growth, they are metabolised and fungi produce and accumulate secondary metabolites (Deduke et al., 2012). Although they may not possess specific functions as primary metabolites, secondary metabolites provide fungi with selective advantages due to their ecological functions (Deduke et al., 2012). In the natural environment, fungal secondary metabolites can function as (1) signalling molecules (Rodríguez-Urra et al., 2012; Yim et al., 2007), (2) virulent factors in facilitating pathogenic lifecycles (Coméra et al., 2007; Proctor et al., 1995; Stanzani et al., 2005), (3) microbial inhibitors to gain competitive advantages over other microbes in crowded environments (König et al., 2013; Losada et al., 2009), and (4) defensive mechanism against frugivorous predators (Rohlfs et al., 2007; Calvo & Cary, 2015).

Environmental stress, including CO_2 concentration, temperature, micronutrient, ultra-violet radiation, pH, salinity and water availability, play an important role in regulating the physiology and metabolic pathways of fungi (Kubicek & Druzhinina, 2007). The production and regulation of fungal secondary metabolites are, therefore, complex matters linked to environmental factors and stimulants in their development (Fox & Howlett, 2008), which could directly affect the transcription of polyketide synthase or indirectly initiate complex signal transduction cascades. Fox and Howlett (2008)

suggested this complicated system indicate the difficulty in separating environmental parameters from development phases and other factors. Later studies revealed that the production of secondary metabolites is closely connected with signals in the environment (Brakhage et al., 2009; Calvo & Cary, 2015). For example, *Aspergillus parasiticus* only produce aflatoxin at 28°C, not at the optimal growth temperature at 37°C (Feng & Leonard, 1998). Moreover, there can be varied effects of temperature on the secondary metabolite production: *Aspergillus nidulans* produces much higher quantity of sterigmatocystin at 37°C than 28°C, which demonstrates a reverse pattern to the closely linked chemical aflatoxin produced by *Aspergillus parasiticus* (Feng & Leonard, 1998).

In the present study, the result suggested that temperatures affect the overall biomass and in turn the production of crude extracts of the fungi. Although the highest biomass was recorded at 25°C, the highest crude extract was observed at 25°C for *Aspergilus sydowii* and 35°C for *Acrocalymma* sp. The biomass data are in complete accordance with previous studies on *Aspergillus terreus* (Jain & Pundir, 2011; Mathan et al., 2013). On the other hand, the production of crude extract for a *Acrocalymma* sp. was different from the studies on *A. terreus*, even though the highest amount of crude extract was also recorded at 25°C (Jain & Pundir, 2011; Mathan et al., 2013).

In addition, the HPLC profiles also indicate that the highest concentrations of compounds were recorded at 25°C for both fungal isolates. However, *Acrocalymma* sp. produced more types of compounds at 35°C than that of *A. sydowii*. In terms of Q-TOF LCMS profiles, the highest number of compounds was recorded at 25°C for *A. sydowii* and 35°C for *Acrocalymma* sp., thus suggesting their metabolite profile changes in response to rising temperatures. Combining the HPLC and LCMS profiles could provide a broader spectrum of compounds produced by the fungal isolates.

Brakhage (2012) stated that fungal secondary metabolites are mainly derived from polyketides or non-ribosomal peptides, whereas a few demonstrate mixed polyketide-non-ribosomal peptide origins, and the rest derive from other biosynthetic pathways. Gene expression involving in secondary metabolite synthesis and secretion is controlled by a complex network of master regulators in response to diverse environmental stimuli (Brakhage, 2012). Even though most master regulators controlling secondary metabolism are known to be activated by individual environmental stimulus, these regulators are likely to regulate the production of secondary metabolites in combination for the purpose of fine-tuning the fungal metabolic profile responding to environmental changes (Lind et al., 2016).

According to Lind et al. (2016), temperature controls the global production of secondary metabolites in *Aspergillus fumigatus* and the light-responsive master secondary metabolite regulator, a Velvet protein complex (VeA), also responds to temperature changes. The results support the hypothesis that the occurrence of regulatory secondary metabolite production is responsive to various environmental cues. In their study, *A. fumigatus* was cultured at 30°C and 37°C, and the fungal transcriptomes were examined at both temperatures. Among the 1,101 identified expressed genes, 402 were overexpressed (expressed at a higher level), while 699 were underexpressed (expressed at a lower level) at 37°C in comparison with 30°C. In particular, the underexpressed genes were enriched for cell adhesion, toxin metabolic process, oxidoreductase activity and secondary metabolic process. Thus, temperature shift from 30°C to 37°C have a marked effect on gene expression because of significant changes in the expression level of all genes (~10%) (Lind et al., 2016). In addition, there is a disproportionate effect on expression of gene clusters involved in secondary metabolism based on studies on 37 previous identified secondary metabolic gene clusters (Inglis et al., 2013; Lind et al., 2015). The results revealed that 13 out of 37 secondary metabolite gene clusters showed lower levels of expression at 37°C than 30°C, in which 10 of these clusters encode the conidial melanin pigment, endocrine, fumigaclavine, fumagillin, fumipyrrole, fumiquinazoline, fumitremorgin, gliotoxin, pseurotin, trypacidin, and the rest of three encode no known metabolites (Lind et al., 2016). In contrast, only 3 gene clusters, which encode no known products, exhibited higher levels of expression at 37°C than 30°C (Lind et al., 2016). As previously reported, endocrine is not produced when temperature was higher than 35°C (Berthier et al., 2013); therefore, this could be the result of changes in gene expression. Lind et al. (2016), also examined the expression of two backbone biosynthesis genes, namely gliP and psoA of the gliostoxin and pseurotin gene clusters, by qRT-PCR in temperature-shift experiments. The results showed increased expressions of both genes when temperature decreased from 37°C to 30°C. Therefore, the findings provide further support for the conclusion that temperature modulates secondary metabolite gene expression.

Lind et al. (2016) also deleted the two key Velvet protein complex members, *veA* and *laeA*, from *A. fumigatus* strains and cultured them at different temperatures. They found out that both veA and laeA regulate genes in many secondary metabolite gene clusters at *A. fumigatus* optimum growth temperature (37°C). Compared with a list of computationally predicted and experimentally characterised gene clusters (Inglis et al., 2013; Lind et al., 2015), the results revealed that VeA regulates many gene clusters

through transcription, which are similar to a previous study that VeA is required to synthesise fumigaclavine, fumitremorgin and fumagillin at 37°C (Dhingra et al., 2013). However, VeA and LaeA have distinct regulatory roles because six secondary metabolite gene clusters showed differential regulations by VeA at different temperature conditions (Lind et al., 2016). It has been implicated that Velvet protein complex comprised of LaeA, VeA and VelB functions a secondary metabolism regulator in many fungi (Bayram et al., 2008; Calvo, 2008; Wiemann et al., 2010; Bayram & Braus, 2012; Chettri et al., 2012). The findings by Lind et al. (2016) also support the belief that VeA and LaeA play functionally distinct regulatory roles in secondary metabolite clusters (Bayram & Braus, 2012; Lin et al., 2013). Given that the regulation of secondary metabolites by VeA is temperature-dependent, temperature is one of the environmental factors to which VeA respond (Lind et al., 2016).

Compared to marine fungi, there are more studies on the effect of temperature on secondary metabolites produced by fungi from other environments, for example, AMF (Chen et al., 2013) and soil fungi (Jain & Pundir, 2011; Mathan et al., 2013; Yogabaanu et al., 2017). Thus, it is important to study the response of marine fungi to rising seawater temperature. The metabolic profile changes showed in the present study provide evidence of how marine fungi alter their secondary metabolism under temperature-shift conditions. In comparison with their growth, the changes in fungal metabolite production and profiles do not always show a simple, linear fashion. The data also suggest that the quantity of the same metabolite changes under different temperatures, this might be attributed to overexpressed or under expressed temperature-dependent secondary metabolite gene clusters, such as VeA (Lind et al., 2016) .

5.4 Analysis of QTOF-LCMS profile

The QTOF-LCMS profiles provided a breakdown of the chemical formulas of the identified compounds from the crude extracts (see Appendixes A and B). Based on the chemical formulas, these metabolites were preliminarily categorised into primary and secondary metabolites, and secondary metabolites were further classified as terpenes and alkaloids (see Appendix C). Interestingly, the majority of compounds detected by QTOF-LCMS were classified as primary metabolites, which could be its high sensitivity.

5.5 Limitations of research methods

In the present study, fungal isolation from the soft coral *Sarcophyton ehrenbergi* was only carried through culture-dependent method using serial dilution on PDA plates. However, such method can only be used to isolate culturable fungal species from the soft coral host. Although PDA was shown to isolate the largest number of fungi from various soft coral species, a greater fungal diversity could be achieved by employing more than one growth media since certain fungal strains can only be cultured on particular growth media, such as GPA or GPSA (Zhang et al., 2012).

Regarding the identification of fungal isolates in this study, two out of four fungal strains could be identified at the species level, while the rest two could only be identified up to a genus level; even though five gene markers were used to facilitate molecular identification, only three were able to generate valid sequences after BLAST. This is because of the limited fungal DNA sequences available on the NCBI database. In fact, this limitation has been pointed out by Hassett et al. (2019), who argued that the unavailability of DNA loci of nearly half of the known marine fungi could become a hinderance to the identification of marine fungi through gene sequencing techniques. Furthermore, morphological identification could have been used to aid the identification of fungal isolates because traditionally morphological characters were used in the studies of endophytes in tropics by observing mycelia (Guo et al., 2000).

In terms of assessing the effect of temperatures on secondary metabolites from the two marine fungi species, the present study only compared the TLC, HPLC and LCMS metabolic profiles. Although it is confirmed that temperatures do alter the HPLC profiles, it is still unknown what compounds have increased or decreased production under the elevation of temperatures. Therefore, specific elutes from HPLC could be collected and isolated through high-performance thin-layer chromatography to obtain a pure compound with the aid of nuclear magnetic resonance spectroscopy.

5.6 Future Studies

The findings in this study may provide some background knowledge for further studies into the soft coral *Sarcophyton*-associated marine fungi, their metabolite profiles and their response to climate change. Immediate recommendations for improvement include (1) the use of different growth media to isolate more fungal species or using molecular methods like NGS to identify more marine fungi related to *Sarcophyton* sp., (2) the use of more barcoding gene markers to identify some of the less common fungal genera associated with *Sarcophyton* sp., and (3) more comprehensive metabolites study to identify the compounds.

CHAPTER 6: CONCLUSION

In the event of rising global seawater temperature, coral reefs around the world had been hit by mass levels of bleaching resulting in the destruction of reefs worldwide. Soft corals are not spared from the wrath of this global event though they are able to recover to a certain extent with time. The soft coral holobiont is understood to play a vital role in the survival during environmental changes due to their symbiotic relationship. Coral associated microbes, mainly fungi have been reported to produce mycosporin amino acids (MAAs) which acts as a layer of protection towards UV rays. Since not much is known on the role of these coral associated fungi, this study attempted to investigate the effects of temperature on isolated soft coral associated marine fungi. This led to the isolation of four marine fungi from the soft coral *Sarcophyton ehrenbergi*, namely *Aspergillus sydowii*, *Acrocalymma* sp., *Lindgomyces* sp. and *Phomatospora* sp. Only *Aspergillus sydowii* was identified up to the species level based on molecular identification methods by using five gene markers. However, due to the limitation of what is available on the database, not all species could be identified based on molecular method alone. Regardless, this investigation was indeed unique having isolating *Aspergillus sydowii* for the first time from *Sarcophyton ehrenbergi*.

The other three fungal isolates were only identified to a genus level. In spite of its wide distribution, *Acrocalymma* sp. was first reported in the soft coral *S. ehrenbergi*. Thus, this genus exhibit in wide geographic regions, ranging from tropical rainforest to soft coral hosts. Despite being considered as a freshwater fungi species, *Lindgomyces* was isolated and identified in this study. This may shed a light on the fungal community related to *Sarcophyton* spp. because there has been quite little research into the fungal association with this soft coral genus. Although other *Phomatospora* species were found in Malaysian grasshoppers and submerged palms, this genus was first reported in the soft coral species.

The growth evaluation of *Aspergillus sydowii* and *Acrocalymma* sp. from the *S. ehrenbergi* tissue under varying temperature conditions confirms that temperature has an effect on growth of fungi and the production of secondary metabolites as exhibited in the chemical profiles of HPLC and Q-TOF LCMS analyses. Increasing temperature was observed to be extreme for both fungal strains retarding their growth, eventually resulting in a reduction in biomass content. High temperatures also decreased metabolite production and concentration, but rather higher detection was recorded by the mass spectrometer in LCMS profiling is suspected to be remnants of degraded compound

fragments. Unfortunately, this investigation did not isolate any compound from the fungal strains. Therefore, future metabolite studies could further exploit the kinds of compounds from these fungi isolates and their bioactivity. On the whole, this experimental study could provide good baseline data to guide further in-depth investigation into fungal adaptation towards temperature stress.

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