# BACTERIAL DIVERSITY ASSOCIATED WITH RED SEAWEEDS, *Gracilaria manilaensis* & *Laurencia* sp., FOUND IN PENINSULAR MALAYSIA

BY

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INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA

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# ABSTRACT

Red seaweeds, Rhodophyta, are very beneficial as a good source of nutrients, collagen, and bioactive metabolites. Thus, seaweeds are consumed or harvested for various industries, including processed food and nutraceuticals. It has been debated that metabolic compounds of the seaweeds could be due to the interactions between the seaweeds and its holobiont environment which hosts microorganisms such as bacteria, protists, algal virus, fungi, and diatoms. It was the interest of this research to investigate the bacterial community profile of edible red seaweed, *Gracilaria manilaensis*, often found in coastal villages of peninsular Malaysia, and compare it against *Laurencia* sp., which is a genus prolific for the discovery and extraction of bioactive metabolic compounds. Studies of bacterial diversity associated to other Rhodophyta species have been conducted worldwide, but not extensively in Malaysia. Isolation of bacteria from marine environment is primarily done for identification of bacterial species and exploring the value of bacteria or its bioactive compounds in biotechnological application. Eight selective enrichment media were used and the bacteria isolated was also compared to the bacteria profile identified through amplicon sequencing. For the seaweed, *G. manilaensis*, there was a total colony count of 1022 on 8 media with 3 replicates, which 80 isolates were selected for *16S rRNA* identification and 43 OTUs were identified. Dominant bacteria phylum was Proteobacteria, and other isolated phyla were Firmicutes, Bacteroidetes, and Actinobacteria. The phyla profile was similar to that of the amplicon sequencing sample with 88 OTUs identified. For the red seaweed, *Laurencia* sp., 8 OTUs were isolated by bacteria culture-dependent approach and 41 OTUs were discovered by amplicon sequencing molecular approach. From the 8 culture OTUs, 3 were positive from bromoperoxidase gene. Sequences data analysis indicated that members of the Alphaproteobacteria and the Bacteroidetes which are predominant were likely to have an important role in the function of this marine bacterial community as the bacterial phyla observed are ubiquitous in seawater with some OTUs and isolates were homologous to bacteria in marine host cluster. Further investigation on these bacteria is hoped to reveal how the identified bacteria can be beneficial in a seaweed environment or for other biodiscoveries.

# خلاصة البحث

إن الطحالب الحمراء، أو رودوفيتا، مفيدة للغاية كمصدر جيد للعناصر الغذائية، والكولاجين، المستقلبات النشطة الحيوية. وبالتالي، أن الأعشاب البحرية تستهلك أو تحصد في صناعات مختلفة، بما في ذلك الأغذية المصنعة والمكملات الغذائية. وجرت مناقشة إمكانية المركبات المستقلبات للأعشاب البحرية ناتجة عن التفاعلات بين الأعشاب البحرية وبيئتها التي تضم الكائنات المجهرية مثل البكتيريا والطلائعيات والفيروس الطحلبي والفطريات والدياتومات. وأهمية هذا البحث هي دراسة خصائص المجتمع البكتيري للطحالب الحمراء الصالحة للأكل، وهي إغراسيلاريا مانيلينسيس التي وجدت كثيرا في القرى الساحلية لشبه جزيرة ماليزيا، ومقارنتها مع لورينسيا إسبي، وهي مادة غزيرة الإنتاج لاكتشاف المستقلبات النشطة الحيوية واستخلاصها. وقد أجريت دراسات على التنوع البكتيري المرتبطة بأنواع رودوفيتا الأخرى في جميع أنحاء العالم، ولكن ليست على نطاق واسع في ماليزيا. ويتم عزل البكتيريا عن البيئة البحرية أساسا لتحديد الأنواع البكتيرية واستكشاف قيمة البكتيريا أو مركباتها الحيوية في تطبيقات التكنولوجيا الحيوية. كما تم استخدام ثمانية وسائط للتخصيب الانتقائي وتمت أيضا مقارنة البكتيريا المعزولة بخصائص البكتيريا التي تم تحديدها من خلال تسلسل الأمبليكون. وبالنسبة لأعشاب البحر، إغراسيلاريا مانيلينسيس، كان هناك تعداد كلي من المستعمرات قدره ١٠٢٢ في ٨ وسائط مع ٣ حالات تكرار، أختيرت ٨٠ حالة عزلية لتحديد هوية ١٦ رمز الرَّنا الرِّيباسِيّ (rRNA) وحددت ٤٣ وحدة التصنيف التشغيلية (OTU). وشعبة البكتيريا المهيمنة كانت بكتيريا بروتينية، وغيرها من الشعب المعزولة كانت متينات الجدار، العصوانيات، والشعاويات. وكان ملف تعريف الشعب شبيها بمثال عينة تسلسل الأمبليكون مع تحديد ٨٨ OTU. أما بالنسبة للطحالب الحمراء، لورينسيا إسبي.، فقد تم اكتشاف ٨ OTU عن طريق المزرعة البكتيرية المنعزلة واكتشاف ٤١ OTU عن طريق نهج جزيئي متسلسل الأمبليكون. ومن ثمانية مستفردات OTU، كانت ثلاثة منها إيجابية من جين بروموبروكسيديز. وقد أشار تحليل بيانات التسلسل إلى أن أفراد متقلبات ألفا والعصوانيات كانت سائدة مرجحة ولها دور مهم في وظيفة هذا المجتمع البكتيري البحري حيث أنها شوهدت في كل مكان في مياه البحر مع بعض OTU والشعب كانت متماثلة مع البكتيريا في المجموعة البحرية المضيفة. ومن المأمول إجراء مزيد من الدراسة بشأن هذه البكتيريا لكشف كيفية استفادتها في بيئة الأعشاب البحرية أو في الاكتشافات البيولوجية الأخرى.

# APPROVAL PAGE

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In the name of Allah, the Most Gracious and the Most Merciful. And of His signs is that He sends the winds as bringers of good tidings and to let you taste His mercy and so the ships may sail at His command and so you may seek of His bounty, and perhaps you will be grateful (Ar-Rum: 46).

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# LIST OF SYMBOLS

| | |
|---|---|
| cm | Centimetre |
| G | Gram |
| H | Hour |
| kg | Kilogram |
| L | Litre |
| mg | Milligram |
| mg/mL | Milligram per millilitre |
| mL | Millilitre |
| nm | Nanometre |
| ng/L | Nanogram per litre |
| ng/µL | Nanogram per microliter |
| α | Alpha |
| β | Beta |
| γ | Gamma |
| δ | Delta |
| ε | Epsilon |
| µL | Microliter |
| °C | Degree Celsius |
| ± | Plus minus |
| × | Times |
| + | Plus |
| - | Minus |
| & | Ampersand (And) |
| % | Percent |

# LIST OF ABBREVIATIONS

| | |
|---|---|
| A | Absorbance |
| AIA | Actinomycetes Isolation Agar |
| BLAST | Basic Local Alignment Search Tool |
| Bp | Base pairs |
| CIPRES | Cyberinfrastructure for Phylogenetic Research |
| $C0_2$ | Carbon dioxide |
| CTAB | Cetyl Trimethylammonium Bromide |
| DNA | Deoxyribonucleic Acid |
| DMSO | Dimethyl Sulphoxide |
| FAO | Food Agricultural Organisation |
| EBI | European Bioinformatics Institute |
| EDTA | Ethylenediamine tetra-acetic acid |
| EtBr | Ethidium Bromide |
| kb | Kilobase |
| KBr | Potassium Bromide |
| KI | Potassium Iodide |
| MA | Marine Agar |
| MHC | Marine Holobiont Cluster |
| NaCl | Sodium Chloride |
| $NaNO_3$ | Sodium Nitrite |
| NCBI | National Centre for Biotechnology Information |
| $NH_4Cl$ | Ammonium Chloride |
| OTU | Operational Taxonomy Unit |
| PCR | Polymerase Chain Reaction |
| ppt | Part per thousand |
| QIIME | Quantitative Insights Into Microbial Ecology |
| RDP | Ribosomal Database Project |
| RNA | Ribonucleic Acid |
| rRNA | Ribosomal RNA |
| SDS | Sodium Dedocyl Sulphate |
| SINA | Silva Incremental Aligner |
| SWC | Seaweed Cluster |
| TAE | Tris-Acetate EDTA |
| TE | Tris-EDTA |

# CHAPTER ONE

# INTRODUCTION

## 1.1 RESEARCH BACKGROUND

In recent decades, studies on bacterial diversity are emerging as researchers have found interest in the various different bacteria that are predominant in the environment and can create conducive symbiotic ecosystems for other organisms, vegetations and living beings to live, survive, and thrive throughout our earth's biosphere (Webster, Wilson, Blackall, & Hill, 2001; Gontang, Fenical, & Jensen, 2007; Egan et al., 2013; Selvarajan et al., 2019). As red seaweeds represent one of the uniquely diverse eukaryotic algae group which are vital primary producers, "habitat engineers", and valuable economic resource in the marine ecosystem, the study of bacterial diversity associated with red seaweeds is interesting to understand the bacteria that are present in the ecology and holobiont environment of the red seaweed.

As algae, red seaweeds do not demonstrate the root characteristics of terrestrial plants and they possess photosynthetic pigments such as β-carotene, phycoerythrin, and phycocyanin that reflect red light and give the general red appearances of the red seaweed (Sampath-Wiley & Neefus, 2007). However, red seaweeds may also appear slightly greenish or brownish with reddish hues because of the presence of chlorophyll (a, b & c) pigment content. Taxonomically classified under the phylum Rhodophyta, over 7,000 pecies have been recorded in Algaebase which is the global taxonomic database for algae species with nomenclatural and distributional information. The species are divided into six classes, but the class Florideophyceae is the largest group of rhodophytes, accounting to almost 95% of the documented red seaweed species,

inclusive of the economically important harvested seaweeds for agar and carrageenan, except for the genus *Porphyra* (nori seaweed) which belongs to the class Bangiophyceae (Guiry, 2012)

Aside from being important resource species for economic values, seaweeds are first and foremost essential for the "habitat engineer" role they play in nature. Firstly, seaweeds are among the primary producers in the marine environment, oxygenic photosynthesisers that absorbs carbon dioxides in the water and produces energy-rich organic compounds with oxygen as a byproduct for other marine organisms in the food chain. This facilitates the seaweeds effects on biodiversity as a community-structuring organism by providing niches for associated and dependant species (Andreakis & Schaffelke, 2012). Secondly, seaweeds are critical for the maintenance of the marine and benthic environments and the intertidal shores by sediment stabilisation and providing protection from water loss, heat, and irradiance. Furthermore, seaweeds can regulate the movement of dissolved and particulate matters between the seawater and the shore or sediment bed through the combination of bioturbation and bioirrigation (Umanzor, Ladah, Calderon-aguilera, & Zertuche-gonzález, 2019). Thus, seaweeds are able to shape the marine landscape and modify compositions of local communities owing to its physical and biogeochemical impacts.

For the commercial values of red seaweeds, genera *Kappaphycus* and *Euchema* are harvested for phycocolloids (agar, alginates, and carrageenan), while edible genera *Porphyra*, *Gelidium*, and *Gracilaria* are farmed for food consumption. Seaweed phycocolloids are used in various industries such as in food processing, cosmetics, nutraceutical or pharmaceutical, and laboratory research for uses as thickener, hardener, or stabiliser. For consumption, red seaweeds are sources of dietary fibre as they have the ability to promote healthy circulation, lower bad cholesterol, and

regulate blood sugar levels because edible red seaweeds are high in vitamins, minerals, a rich source of calcium, magnesium, and antioxidants. Approximately 16 million tonnes of these species were produced annually with a worth of over US $3.85 billion (Food and Agriculture Organization of the United Nations [FAO], 2017).

Other red seaweeds species found distributed worldwide are investigated and explored for its bioactive secondary metabolites because marine organisms are potential sources of highly productive biochemical compounds. These isolated metabolites which demonstrated antibacterial, antifungal, anti-inflammatory, antidiabetic, antioxidant and antitumour activity often lead to the development of new pharmaceutical agents. Examples include sulphated polysaccharides from *Palmaria palmata,* halogenated monoterpenes found in genera *Plocamium, Portieria*, and *Ochtodes,* and halogenated sesquiterpenes from genus *Laurencia* (Nogueira & Teixeira, 2016).

Recently, emerging studies have postulated that the bioactive compounds and nutritional values of edible red seaweeds are due to the interaction between red seaweeds and symbiotic bacteria in the seaweed holobiont environment (Wichard, 2015; Selvarajan et al., 2019). As red seaweeds are also one the most prolific sources of bioactive compounds or secondary metabolites after sponges and cnidarians (Blunt, Copp, Munro, Northcote, & Prinsep, 2006), it has been suggested that the secondary metabolites are of bacterial origin due to the complex process of the compounds' biosynthesis. Many works of literature have documented various bacterial species associated with red seaweed worldwide (Martin, Portetelle, Michel, & Vandenbol, 2014) and the bacterial community structures included members of the dominant Proteobacteria, Cyanobacteria, and Firmicutes phyla. The bacterial communities were often investigated for its ecological and functional roles in

symbiosis with the seaweed, and these bacteria have the potential to photosynthesise, detoxify or fix nitrogen (Goecke, Labes, Wiese, & Imhoff, 2010).

However, little is known in the literature of the bacterial communities associated with edible red seaweed species *Gracilaria manilaensis* often found in coastal waters of fishing villages around Peninsular Malaysia, though it is traditionally eaten as a delicacy promoted as "kerabu sare". Previous studies on *G. manilaensis* focused on its fatty acid content (Abdullah, 2013) and its antioxidant and cytotoxic activities (Abdullah, Muhamad, Omar, & Abdullah, 2013) as edible red seaweeds are generally known to have many nutritional values by its protein, fatty acid, and dietary fibre contents and have therapeutic values with antimicrobial, antioxidant and antitumor potentials. Seaweeds are desirable culinary products because they are tasty and nutritious, hence appealing to vegetarians as a source of protein to replace meat. This creates a big prospect for Malaysian seaweeds to be exported to the global market.

Nevertheless, to commercially farm the red seaweed, *G. manilaensis*, in Malaysia, many issues need to be addressed especially the physical and chemical parameters such as the effect of light exposure, water temperature, salinity, the cost-effectiveness of the production, and the ecological aspect of the farming which aims to give favourable growth conditions for the seaweed. Studying the ecology of seaweed is challenging as it includes the interaction of the seaweeds with its environment, the nutrition required for the seaweed to grow, the predators that prey on seaweed and consequently the symbionts that live associated with the seaweeds. When investigating the health of cultivated or farmed red seaweeds, bacteria are often associated as disease-causing agents, and these studies in Malaysia were focused on *Kappaphycus* spp. farming from Sabah.

Likewise, little is known in the literature regarding the bacterial communities associated with red seaweed genus *Laurencia,* found in Malaysia or worldwide. The genus *Laurencia* is a prominently prolific genus of the Rhodophyta group as over 600 metabolites have been isolated and characterised from the red seaweed species of this genus (de Oliveira et al., 2015; Protopapa et al., 2019). The illustrious metabolite laurencin was first isolated from *Laurencia glandulifera* (Wanke et al., 2015), a C15-acetogenin, and other eminent metabolite examples include alkaloids, diterpenes, elatol, and other sesquiterpenes (Ji et al., 2007). Bioactive compounds like the acetogenins, indole alkaloids or sesquiterpenes isolated from seaweed are often halogenated which makes it even more interesting due to their significant bioactivity (Suárez-Castillo et al., 2006, Vairappan et al., 2008). As bioactive compounds have been isolated from *Gracilaria* extracts, which may be from bacteria or have bacterial origin (de Almeida et al., 2011), it was interesting to compare the bacterial communities associated with genus *Gracilaria* against the bacterial communities associated with genus *Laurencia*.

Hence, it became the interest of this study to explore the bacterial communities that are associated with *G. manilaensis* and *Laurencia* sp. through culture-dependant and molecular approaches and explore some of the roles that the microorganisms may play that could contribute to seaweed growth through PCR-based bromoperoxidase gene screening. This is because there are little studies done regarding bacterial symbionts of seaweeds in Malaysia though many studies were done on other species worldwide. Understanding the natural ecology for seaweed to grow can provide insight into cost-effective conditions for favourable farming of Malaysian seaweed that meets the global market regulations for edible seaweed and related products.

## 1.2 PROBLEM STATEMENT

Many previous research focused on bacterial community associated with red seaweeds worldwide with the interest of bioprospecting and understanding the holobiont environment. However, little is known about the bacterial communities associated with the red seaweeds species in Peninsular Malaysia, particularly *G. manilaensis* and *Laurencia* sp., as these red seaweed species in Malaysia are different from the red seaweed species available and studied in other countries. The potential to cultivate these red seaweed species for food consumption and secondary metabolite extraction is valuable; however, at present, both species are not adequately cultivated for global market demand. Hence, this study aimed to investigate the bacterial community associated with selected red seaweeds through culture-independent and culture-dependent approaches to provide insight into the potential function of the bacterial in the red seaweed holobiont environment. Knowledge gained may benefit future seaweed cultivation studies and crop management studies against microbial-induced or pathogenic diseases.

## 1.3 RESEARCH QUESTIONS

The research questions for the present study are as follows:

1. What is the identity of the red seaweed samples collected in this study from the perspectives of morphological and molecular analysis?
2. What is the bacterial diversity associated with the cultivated and wild red seaweed, *Gracilaria manilaensis*, investigated through culture-dependent and culture-independent approaches, and do they possess bromoperoxidase functional gene?

3. What is the bacterial diversity associated with the red seaweed, *Laurencia* sp., investigated through culture-dependent and culture-independent approaches, and do they possess bromoperoxidase functional gene?
4. What is the comparison between the two red seaweeds' total bacterial diversity?

## 1.4 RESEARCH OBJECTIVES

### 1.4.1 General Objectives

The main objective of this study was to identify bacterial species or symbionts associated with red seaweeds, *G. manilaensis* and *Laurencia* sp., found in Peninsular Malaysia and explore their functional profile in their host holobiont environment. This was achieved through specific objectives that were carried out in the study as listed.

### 1.4.2 Specific Objectives

The following objectives were developed for this present study:

1. To determine the identity of the red seaweed samples collected in this study from a morphological and molecular analysis.
2. To investigate the bacterial diversity associated with the cultivated and wild red seaweed, *Gracilaria* manilaensis, through culture-dependent and culture-independent approaches, to screen bromoperoxidase functional gene from the isolated bacteria and to observe the differences between the bacterial diversity.

3. To investigate the bacterial diversity associated with the red seaweed, *Laurencia* sp., through culture-dependent and culture-independent approaches, and to screen bromoperoxidase functional gene from the isolated bacteria.
4. To compare bacterial diversity between the two red seaweeds' associated bacteria through alpha and beta diversity indices.

## 1.5 SIGNIFICANCE OF RESEARCH

This present study contributes to knowledge by establishing a bacterial library for the two different red seaweeds, *Gracilaria manilaensis* and *Laurencia* sp., found in Peninsular Malaysia. Exploring the bacterial diversity associated with the selected red seaweeds hinted on how associated bacteria have functional profiles that can influence their hosts' ecology and subsequent nutrient cycling in symbiosis, which leads to the significance of these marine bacterial symbionts. The bacterial communities structure and phylogenetic analysis shed insights on bacterial evolutionary patterns and the origin of symbiosis clusters. Screening functional halogenase genes, such as bromoperoxidase, by PCR from cultivable bacteria allude to ideas on the potential role of bacteria in the halogenation process that regularly occurs in the marine environment. The knowledge and outcomes of this study are believd to be beneficial to contribute towards understanding the seaweed environment with respect to host-bacteria association and interaction for future studies in seaweed crop management and against pathogenic diseases.

## 1.6 RESEARCH FRAMEWORK

This research framework provides a better understanding of the varety of bacteria in the seaweed environment. This study was aimed to investigate the bacterial diversity associated with red seaweeds, *G. manilaensis* and *Laurencia* sp. through culture-independent and culture-dependent approaches. The research design is illustrated in Figure 1.1.

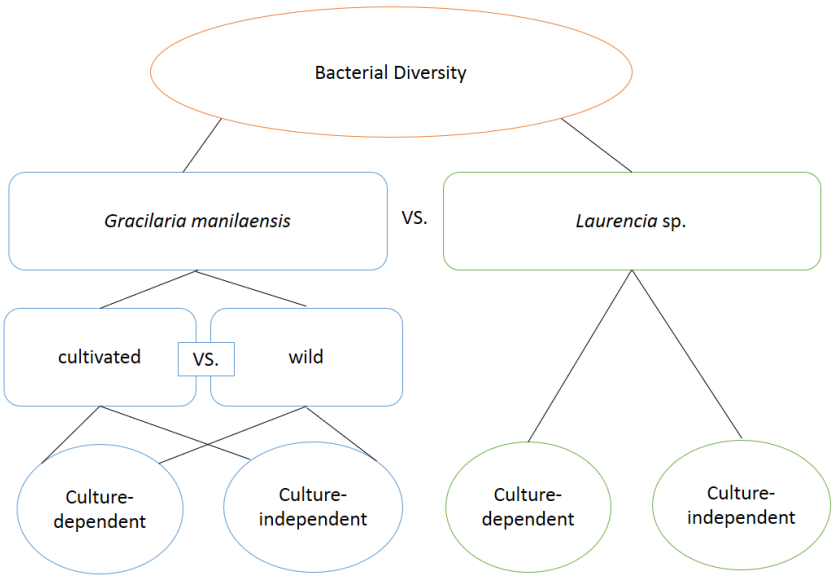



Figure 1.1 Diagram of Research Design

## 1.7 THESIS OUTLINE

This thesis has been divided into five chapters, beginning with the Introduction (Chapter 1) and ending with the Discussion (Chapter 5), to answer the four research questions. In the second chapter, which is the literature review, related publications were examined and discussed to provide the research scope. Though study on seaweed is extensive and the discovery of bacteria-algae interaction is interesting, we have

limited our research scope to focus on bacteria associated with red seaweeds, *G. manilaensis* and *Laurencia* sp., found in Peninsular Malaysia. The third chapter details the methodology employed throughout the study, while the fourth chapter presents all findings, which addressed the four research objectives; the identity of the seaweed samples collected in this study through morphological and molecular analysis, the bacterial diversity associated with red seaweeds, *G. manilaensis* and *Laurencia* sp., through culture-dependent and culture-independent approaches, and the comparison of the bacterial diversity associated with the two red seaweeds. The last chapter will discuss and conclude all the finding of this research whilst providing suggestions for future research, especially to overcome the limitations encountered within this research.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 SEAWEEDS

Seaweeds are eukaryotic, multicellular, macroscopic algae found in marine environments. They have eukaryotic cells absent of flagella and centrioles and chloroplasts lacking of external endoplasmic reticulum. As marine algae, seaweeds are photosynthetic organisms and hence, possess chlorophyll granules and photosynthetic pigments such as chlorophylls a & b for green seaweeds (Chlorophyta), β-carotene and phycoerythrin for red seaweeds (Rhodophyta), and fucoxanthin for brown seaweeds (Phaeophyta), but morphologically lack the "true" root, stem, and leaf characteristics of terrestrial plants (Sahayaraj, 2014; Baweja & Sahoo, 2015). Instead, seaweeds have holdfast and thallus components as illustrated in Figure 2.1.

The holdfast attaches the seaweed plant base to any organic substrate such as coastal rock or sediment bed, or any inorganic substrate possible in the sea, like the ropes used in seaweed farming. The thallus is the major part or body of the algae, which extends from a haptera (an extension of the holdfast) or a stipe (a stem-like structure which may or not be present in a species) to the blade of lamina which acts as a 'leaf'. Some species have a sorus which is a floatation assisting organ between the blade and the stipe (Morrison et al., 2009). However, some smaller species of seaweeds do not share these structures; they might just have a tissue consisting of filaments of cells only, and the tissue may or may not be branched. These areas, i.e. the surface of or the epi/endodermis tissues, are niches for bacteria colonisation for symbiosis and thus called the holobiont environment (Egan et al., 2013).

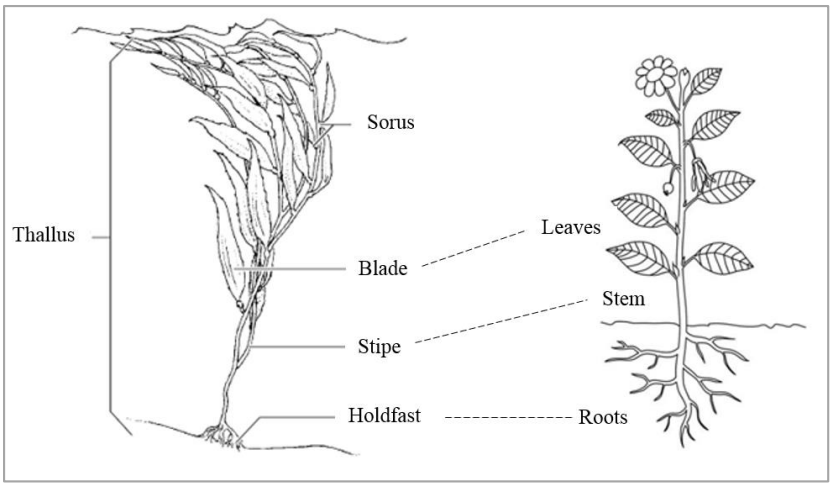



Figure 2.1 Basic Structure of Seaweed as Compared to Terrestrial Plants

The photosynthetic pigments allow seaweeds to absorb the light necessary for photosynthesis at depths with varying degrees of light intensity (Sampath-Wiley, Neefus, & Jahnke, 2008). Hence, seaweeds are found in coastal littoral zones which extend from the shoreline to the shallow seabed or submerged rock formations for the seaweeds to receive sufficient sunlight and also, fresh seawater for nutrient uptake. These pigments can be divided into three main groups; chlorophylls, phycobiliproteins, and carotenoids, and the different pigment compositions classify the seaweeds into three general classes: Chlorophyta (green seaweeds), Rhodophyta (red seaweeds), and Phaeophyta (brown seaweeds). Compared to Rhodophyta, Chlorophyta seaweeds are visibly smaller algae and greenish due to the pigments chlorophyll a, chlorophyll b, and carotenoid (Haryatfrehni, Candra, Meilianda, & Rahmawati, 2015), while Phaeophyta is the largest type of seaweed, observed brown or yellow-brown in colour because of the abundance of the fucoxanthin pigment which can conceal other pigments such as chlorophyll a and c, and β-carotene, and other xanthophylls (Dhargalkar & Kavlekar, 2004).

Red seaweeds are classified as Rhodophyta because of the presence of the pigments phycoerythrin and phycocyanin that reflect red light, thus, giving the general appearances of rhodophytes to be red (Sampath-Wiley & Neefus, 2007). However, some rhodophytes appear to be brownish, greenish or bluish because they have lesser amounts of phycoerythrin; thus, the colour appearance is influenced by the other pigments such as chlorophyll (a & b), β-carotene, and xanthophylls. Research on red seaweed has varied to include aspects of taxonomy, distribution and farming, its polysaccharide constituents, and isolated bioactive metabolites. Recent studies have shifted through molecular advancements to include expressed sequence tags and metabolic pathways of commercially important marine macroalgae, the influences of symbiotic microorganism on red seaweed health and growth, and the use of model seaweed for a multidisciplinary study approach (Chan, Ho, & Phang, 2006). These targeted studies will improve upon previous biotechnology techniques that have propagated protoplast formation, callus culture, and plant regeneration for a more robust seaweed gene line (Renn, 1997). There are 7,174 known species recorded in Algaebase under the phylum Rhodophyta, classified into six classes: Bangiophyceae, Cyanidophyceae, Pophyridiophyceae, Stylenomatophyceae, Rhodellophyceae, and Florideophyceae, with about 670 genera (Woelkerling 1990, Guiry et al., 2014). Notable examples are genera *Kappaphycus* and *Betaphycus* as important sources of carrageenan, and *Gracilaria*, *Gelidium*, and *Pterocladia* which are used in the manufacture of the all-important agar.

### 2.1.1 Ecological Importance of Seaweeds

Seaweeds are dominantly important marine or coastal vegetation involved in vital structural and functional roles in several marine habitats such as rocky shores, turfs

and kelp forests (Mineur et al., 2015). As autotrophs, seaweeds are primary producers in the food chain, providing a food source to grazing species such as sea urchins. Indirectly, seaweed provides nutrients by releasing organic matter (or dissolved organic matter) into the coastal waters for the benefit of microbial symbionts in its holobiont environment and the water nutrient cycling uptake. Symbiont bacteria such as the cyanobacteria can also play a role in the photosynthetic pigments of seaweeds or contribute to the photosynthesis in the seaweed holobiont environment (Lau, Matsui, & Abdullah, 2015). Also, as seaweeds grow in abundance, its dominant biomass provides shelter and spawning ground for juvenile fish and invertebrates (Eggertsen et al., 2017).

Interestingly, a habitat of seaweed can be suffused by several seaweed species and make up different communities, and the diversity in community species composition is beneficial for the coastal ecosystems. The species-varied seaweed environment can support high biodiversity by forming complex habitat structures for associated species which include epiphytic organisms and infaunal communities (Oskarsson, Wiklund, Thorsén, Danielsson, & Kumblad, 2014). Hence, seaweed biomass significantly determines the assemblage of associated fauna and macroinvertebrates found within the habitat by providing niches for associated and dependant species and altering competing interactions in trophic networks (Buchholz, Krause, & Buck, 2012).

The structures of seaweed habitats also influence sedimentation rates (sediment retention/stabilisation) by roots formation and as substrate filter, modify water flow and wave energy whilst protecting communities under their shelter, and changes light levels in their local environment. Through bioturbation, seaweeds can alter the biogeochemical nature and physical structure of the sediment, its stabilisation and

destabilisation, by increasing exchange rates at the sediment–water interface, while bioirrigation implicates nutrient fluxes between sediment and water column (Bouma, Olenin, Reise, & Ysebaert, 2009). Hence, as seaweeds can shape ecosystem functioning, it is dubbed as "ecosystem engineers".

## 2.2 ECONOMIC IMPORTANCE OF RED SEAWEEDS

Seaweeds are resourceful vegetation generally harvested to be consumed directly (edible seaweeds as a food source or treated for medicinal purposes) or to have its phycocolloids extracted. Seaweeds are geographically distributed towards the temperate area, although seaweeds can be found in colder waters. Hence, farming of seaweeds is more dominant in temperate Asian countries such as China which contribute towards 62.8% of the global production followed by Indonesia and the Philippines at 13.7% and 10.6%, respectively. The annual global harvest is estimated at 26 million tonnes for a revenue of about US$ 6 billion (FAO, 2017).

Seaweeds such as *Porphyra* spp. and *Gracilaria* spp. are edible and excellent sources of micronutrients including iodine, calcium, magnesium, zinc, and iron. Seaweeds also contain antioxidants and omega-3 fatty acids, DHA and EPA, aside from amino acids, proteins, carbohydrates, and vitamins $B_1$, $B_2$, $B_{12}$, and C that are necessary for the human body metabolism (FAO, 2003). The fat content of seaweeds is very low (less than 5%); hence, consuming seaweeds can help sustain a balanced nutrition intake. Previously famous in the Japanese diet, seaweeds have now become popular in European and American gastronomical ventures. *Laminaria* (kombu), *Undaria* (wakame), and *Porphyra* (Nori) are sold at US$ 2800/dry tonne, US$ 6900/dry tonne, and US$ 16,800/dry tonne, respectively (FAO, 2017).

Phycocolloids of seaweeds include agar, alginate and carrageenan which are beneficial in various industries such as food process, pharmaceutical, laboratory research, textile, paper, cosmetics, and energy resource as biofuels. The algal polysaccharides are 60% of the bioactive substance extracted from seaweeds because the phycocolloids are structural components of cell walls, which act as energy storage units, and these compounds have moisturizing and antioxidant capacity (Pereira & Costa-Lotufo, 2012; Pereira, 2018). In food, nutraceutical, cosmeceutical, and pharmaceutical ingredients, the high-value biological derivatives are useful as gelling agents, thickeners, and stabilisers in emulsions.

### 2.2.1 Red Seaweed, *Gracilaria manilaensis*, And Its Potential in Large-Scale Farming

*Gracilaria manilaensis* is a species of the genus *Gracilaria* (Gracilariales, Rhodophyta) macroalgae group. It is an important agarophyte group grown commercially worldwide to support more than 70% supply of raw agar material utilised in various industries for products such as food-grade agar and culture media agar (FAO, 2014). The seaweed group, Rhodophyta, consists of more than 300 species that are found red or greenish-brown in tropical and subtropical seas (de Almeida et al., 2011).

*G. manilaensis* is a species of the red seaweeds that externally appear with thalli characteristics which are generally cylindrical with lateral subdichotomous branches. Cross-section of the thalli would reveal the cortex and medulla, components crucial for seaweed vegetation. The seaweeds alternate between sporophytes and gametophytes in its life cycle for growth. Environmental factors such as temperature

and salinity affect seaweed *Gracilaria* growth and vegetation (Raikar, Iima, & Fujita, 2001).

Distribution of *Gracilaria* spp. became widespread because of its economic importance and ease of farming. China is the world's top commercial producer with almost 2.6 million metric tonnes per year, followed by Indonesia, Japan, and the Philippines for a collective annual estimation of 3,868,636 tonnes at an estimated value of US$ 955,724 (Redmond, Green, Yarish, Kim, & Neefus, 2014; Lim, Yang, Tan, Maggs, & Brodie, 2017). However, the presences of *G. manilaensis* have only been reported from Cebu, Philippines (Song et al., 2013), Vietnam (Lim et al., 2017), and commercial culture ponds in Kuala Muda, Kedah, Malaysia reported by Abdullah (2013) who studied on the fatty acid content of the seaweed. *G. manilaensis* is known as an edible species, but nutritional values of the seaweed are lacking in literature. Fatty acids are beneficial contents of seaweed for human diet along with protein, carbohydrate and dietary fibres. Additionally, Abdullah et al. (2013) reported the antioxidant and cytotoxicity of *G. manilaensis* extracts for its potential in healthy diet against cancer.

### 2.2.2 Red Seaweed, *Laurencia* sp., And Its Potential

The genus *Laurencia* of the Rhodophyta group is distributed worldwide and can be abundant in tropical and temperate waters attached to many substrates including rocks and other structures in the subtidal and intertidal zones. This genus includes 145 taxonomically accepted species in Algaebase (Guiry et al., 2014), and are highly prolific organisms as many important secondary metabolites were isolated from *Laurencia* spp. Secondary metabolites such as C15-acetogenins, halogenated terpenes (diterpenes and sesquiterpenes) and furanone isolated from *Laurencia* spp. have

exhibited pharmacologically relevant potential due to their strong antibiotic, anti-carcinoma, anti-inflammatory, antimalarial, and antiviral activities due to their relatively high degree of halogenation (Wanke et al., 2015; Nogueira & Teixeira, 2016; Zerrifi, Khalloufi, Oudra, & Vasconcelos, 2018). Halogenated compounds either play an active role in their ecosystem or are biologically active when extracted for potential bioactive investigations (Jesus, Correia-da-Silva, Afonso, Pinto, & Cidade, 2019).

To date, more than 1000 metabolic compounds have been discovered, isolated and characterised its biochemistry in some 600 publications for the metabolites from genus *Laurencia* since the isolation of laurencin (1) from *Laurencia nipponica* (Kladi, Xenaki, Vagias, Papazafiri, & Roussis, 2006; Lhullier et al., 2010; Kaneko, Washio, Umezawa, Matsuda, & Okino, 2014; Nogueira & Teixeira, 2016; Pereira, Da Gama, & Sudatti, 2017). Other isolated compounds include diterpenoids, triterpenoids, sesquiterpenoids, and C15-acetogenins, which have unique and diverse carbon skeleton and some compounds show antibacterial or cytotoxicity activities (Garcia-Davies et al., 2018; Kasanah, Triyanto, Seto, Amelia, & Isnansetyo, 2015; Vairappan, Kamada, Lee, & Jeon, 2013). Despite the potential use for pharmaceutical applications, studies on the biosynthesis of these halogenated compounds are limited.

However, the exploration of bioactive compounds by bioprospecting from the red seaweeds of this genus opens the possibility for sustainable biopharmaceutical exploitation of marine resources for biotechnological interest. For example, a patent was filed in Brazil to commercially use sesquiterpene (−)-elatol as an antifouling agent. However, a successful large-scale cultivation of *Laurencia* species, an optimum yield from the extraction process, and the complex total organic synthesis of (−)-elatol

in the laboratory are current obstacles to efficacious commercialisation (de Oliveira et al., 2015).

## 2.3 BACTERIAL DIVERSITY IN A SEAWEED HOLOBIONT ENVIRONMENT

There are many benefits that human derive from seaweed. It could be directly consumed or harvested for its polysaccharides, namely agar, alginate, and carrageenan. The seaweeds also synthesise a variety of compounds such as proteins, amino acids, lipids, saturated and polyunsaturated fatty acids, carotenoids, terpenoids, xanthophylls, and chlorophylls, besides being a resource for various bioactive metabolites such as acrylic acid with antibiotic activities, eicosanoids, and antioxidant polyphenols (de Almeida et al., 2011). This has generated many interests towards the extraction of the bioactive metabolites from the seaweed as the compounds can be applied into pharmaceutical and nutritional products. However, the production of the metabolites from seaweed *in vivo* has also gained attention as researchers believe that the bacterial diversity associated with seaweed in the seaweed's holobiont may have contributed to the production of the compounds. For example, the bacteria in seaweeds are the main producers of algal-polysaccharide-degrading enzymes that produce biologically active oligosaccharides with properties useful in maintaining human health, such as anticoagulant potentials (Pushpamali et al., 2008), anti-inflammatory (Berteau & Mulloy, 2003), antioxidant (Jiao, Yu, Zhao, Zhang, & Ewart, 2012), or immunostimulating activities (Bhattacharyya et al., 2010).

Studies on bacterial communities associated with seaweed in its holobiont environment have been documented with great interest recently. Some described the bacterial diversity associated with seaweed such as red macroalga, *Porphyra*

*umbilicalis* (Miranda, Hutchison, Grossman, & Brawley, 2013), brown alga, *Laminaria saccharina* (Staufenberger, Thiel, Wiese, & Imhoff, 2008), and green alga, *Ulva australis* (Burke, Thomas, Lewis, Steinberg, & Kjelleberg, 2010). Others reviewed over 100 studies related to 55 Rhodophyta, 46 Phaeophyta and 36 Chlorophyta distributed worldwide (Goecke et al., 2010), its chemical interactions (Egan et al., 2013; Hollants, Leliaert, De Clerck, & Willems, 2013) and the biotechnological applications of the chemical compounds produced (Bour, Ali, & Ktari, 2013; Martin et al., 2014). Seaweeds are thus known to host diverse bacterial symbionts in its holobiont environment, i.e. on the surfaces and inside the seaweeds (Selvarajan et al., 2019).

The term "holobiont" was initially introduced by Lynn Margulis in 1991 to refer to a biological unit comprised of a host and a symbiont within the host's environment, which expands on the concept "symbiosis" coined by Anton De Bary in 1879 when he first reported on the formation lichens as a result of fungi–algae association (Simon, Marchesi, Mougel, & Selosse, 2019). The holobiont concept is thus evolved as the holobiont environment is formed from the symbiotic relationship of microorganisms, mostly bacteria, within a host ecosystem and with the host, following emerging research discovering the existence of hosts and associated bacteria interactions in various biomes, especially in the marine sessile macroorganisms such as seaweeds (Egan et al., 2013; Longford et al., 2019) and sessile invertebrates such as sponges and corals (Blackall, Wilson, & van Oppen, 2015; Pita, Rix, Slaby, Franke, & Hentschel, 2018). It is suggested that a symbiotic relationship exist by which the host surfaces provide grounds and shelter to the associated bacteria while the bacterial community provides biosynthesis of metabolic compounds and other functions to the host (Penesyan et al., 2011; de Mesquita et al., 2018). Recent studies expound on the

host-associated bacterial roles in host growth, development, health, and functions due to arising realization that the ecology and phenotypic expression of the host organisms are affected by the bacterial communities in their holobiont (Wichard et al., 2015; Egan & Gardiner, 2016; Florez, Camus, Hengst, & Buschmann, 2017). Furthermore, the bacterial symbionts can either be host species-specific or ubiquitous in any host environment, giving rise to the notions of bacteria in a seaweed-specific cluster (SWC) or a marine holobiont cluster (MHC), respectively.

The bacteria associated with seaweed can be investigated through culture-dependent approach, which develops from the traditional culture method to enrichment culture strategies, and culture-independent approach, which include molecular techniques such as next-generation sequencing (Aires, Serrão, & Engelen, 2016; Serebryakova, Aires, Viard, Serrão, & Engelen, 2018). Earlier studies of bacterial species diversity were through scanning electron microscopy (Cundell, Sleeter, & Mitchell, 1977) of cultivable isolates and had progressed through time by molecular techniques including denaturing gradient gel electrophoresis (DGGE), fluorescence *in situ* hybridization (FISH), cloning, and pyrosequencing (Wahl, Goecke, Labes, Dobretsov, & Weinberger, 2012; Stratil, Neulinger, Knecht, Friedrichs, & Wahl, 2014). The culture-dependent approach is important to isolate cultivable bacteria and fungi, while the molecular approach is important to obtain data on non-cultivable microbes (Dittami, Eveillard, & Tonon, 2014). Culture-dependent approach utilises selective media and enrichment strategies such as carbon source or antibiotics, as demonstrated by Goodfellow and Fiedler (2010) and Sanchez et al. (2003) among many others. Molecular approach relies on the nucleic acid sequences comparison, either DNA, RNA, or cDNA of a microorganism against sequence data of organisms documented in databases. The molecular methods can amplify sequences

in low concentrations and thus, enabling the sensitive detection of microorganisms, viable or even non-viable, from pure cultures or complex environmental samples such as water or soil.

Hence, the strategy to utilise a culture-dependent approach or a culture-independent approach in the investigation of environmental bacteria depends largely on the objective of the research. As the culture-dependent approach relies heavily on the selective enrichment media employed, it is able to provide bacterial isolates for further study such as novel species characterisation, functional gene screening, and useful for extraction of secondary metabolite or other biochemical testing. For the culture-independent approach, it exploits molecular techniques to answer research questions of what bacteria are there, their community possible functional profile, and useful for the phylogenetic study of uncultivable bacteria. However, culture-dependent approach can be time-consuming while obtaining less bacteria for identification even though it is cheaper compared to the culture-independent approach, which is fast, expensive and can reveal bacteria in six to eleven phyla.

### 2.3.1 Bacterial Diversity Associated with Red Seaweed

Studies on red seaweeds or macroalgae and its symbiotic bacteria have led interest into the multidisciplinary research of ecology of the interactions or relationship the two organisms may have and the effect of the seaweed host-bacteria association in the environment. Published records have documented 72 red macroalgae species (Genera *Chondrus*, *Corallina*, *Gelidium*, *Gracilaria*, *Grateloupia*, *Hypnae*, *Laurencia*, *Mesophyllum*, *Polysiphonia*, *Porphyra*, and *Prionitis*) host microorganisms that contribute to the health of the algae. Bacterial species associated with seaweeds are

more functionally based than host species specific (Goecke et al., 2010; Lachnit, Meske, Wahl, Harder, & Schmitz, 2011; Hollants et al., 2013; Martin et al., 2014). Studies on *Delisea pulchra* by Penesyan et al. (2011) and Fernandes, Steinberg, Rusch, Kjelleberg, and Thomas (2012) revealed different bacterial assemblies as both studies applied different approaches, culture-dependent approach v.s. high throughput molecular approach. Penesyan et al. (2011) focused on the antimicrobial potential of the bacterial isolates which included genera *Micrcoccus*, *Phaeobacter*, *Pseudoaltromonas*, Rhodobacteraceae, *Roseobacter*, *Ruegeria*, *Schwanella*, and *Vibrio*. Fernandes et al. (2012) through pyrosequencing discovered more genera including *Rhodopirellula*, Hyphomonadaceae, Planctomycetaceae, *Haliscomenobacter*, Flavobacteriaceae, Sapospiraceae, *Marimonas*, Rhodobacteraceae, *Parvularcula*, *Aquimarina*, *Thalassomonas*, *Cellulophaga*, and Colwellliaceae which are generally associated with the degradation of complex organic materials and the hydrolysis of polysaccharides. Likewise, a study on *P. umbilicalis* (Miranda et al., 2013) revealed bacteria from 8 phyla including Bacteroidetes, Proteobacteria, Actinobacteria, Chloroflexi, Deinococcus-Thermos, and Planctomycetes that are known to digest the galactan sulfates of red algal cell walls. Consequently, Lachnit et al. (2011) discovered bacterial species associated with two different seaweed species, *Gracileria vermiculophylla* and *Delesseia sanguinea*, were similar in their functional profile though the communities were different. Bacterial phylum associated with *G. vermiculophylla* included Bacteroidetes and Alphaproteobacteria (Rhodobacterales and Rhizobiales), while bacterial phyla associated with *D. sanguinea* were Actinobacteria, Bacteroidetes (Flavobacteria), Firicutes (Bacilli), and Gammaproteobacteria. The seaweed-associated bacterial communities can be highly diverse, but a range of different bacterial groups such as

Proteobacteria and Bacteroidetes are found consistently associated with different seaweed host species.

Many studies have been conducted to understand the seaweed ecosystem including the presence of symbiotic bacteria and understanding the interaction between symbiont bacteria and seaweed host because the diversity of bacterial species associated to red seaweeds showed host species, spatial and temporal variation from brown seaweeds and green seaweeds (Staufenberger et al., 2008; Lachnit, Meske, Wahl, Harder, & Schmitz, 2010; Singh & Reddy, 2014). Research into bacteria associated with brown seaweed have revealed the phyla Proteobacteria, Bacteroidetes, and Firmicutes to be predominant from most Phaeophyta genera including *Fucus* (Lachnit et al., 2011), *Laminaria* (Bengtsson & Øvreås, 2010; Bengtsson, Sjøtun, Lanzén, & Øvreås, 2012), and *Saccharina* (Del Olmo et al., 2018). The diversity of associated bacterial species may be linked to the surface morphology of the brown seaweeds (Wahl et al., 2012; Selvarajan et al., 2019), as Phaeophytes commonly have larger fronds with metabolite exodus present on the surface area; or linked to the cultivation of macroalgae used industrially for biofuels and in aquaculture (Kilinc, Cirik, Turan, Tekogul, & Koru, 2013). For green seaweed, the genus, *Ulva* which is the most abundant representative in Chlorophyta, have profiled bacteria community including Proteobacteria (Alpha, Beta, Delta, and Gammaproteobacteria), Bacteroidetes, Planctomycetes, Pseudomonadales, Alteromonadales, and Vibrionales reported from *U. australis* (Burke et al., 2010; Tujula et al., 2010), *U. intestinalis* (Lachnit et al., 2011), *Ulva* sp. (Jung, Baek, Kim, Shin, & Lee, 2016), and *U. fasciata* (Singh, Baghel, Reddy, & Jha, 2015). Hoever, in general, the most abundant bacteria on macroalgal surfaces are the bacteria identified belonging to the Proteobacteria and Firmicutes phyla (Hollants et al., 2013). Collectively, though the bacterial

communities of seaweeds are diverse, the functional profile of the bacterial community points to the interaction of the bacteria to the host growth, health and development.

### 2.3.2 Bacterial Diversity Indices

The bacterial community of a sphere or area or sample is often analysed statistically by Species Richness (R), Evenness, and diversity indices. Species Richness (R) or Observed Species quantifies how many number of species found in a community, while Evenness is how close in numbers each species in an environment is towards each other. A low value for Evenness indicates that one or just a few species dominate the sampling area while a high value would indicate that each species in the sampled area had a relatively equal number of individuals (Morris et al., 2014).

Research on seaweed-associated bacteria such as reported by Mancuso, D'Hondt, Willems, Airoldi, and De Clerck (2016) and Amelia, Lau, Amirul, and Bhubalan (2020) used diversity indices, Shannon Index and Simpson's Diversity Index, to quantitate how many OTUs were in the samples and how evenly the OTUs were distributed. Hence, as species richness and evenness increase, the measure of diversity increases. Shannon's Index calculates richness and diversity using a natural logarithm, while Simpson's Diversity Index measures the relative abundance of the different species making up the sample richness.

In ecology, alpha diversity (α-diversity) is the mean species diversity in sites or habitats at a local scale, while the differentiation among those habitats is beta diversity (β-diversity) and the total species diversity in a landscape is gamma diversity (γ-diversity). Statistical measurements such as observed species, Chao-1, Good coverage, and PD whole tree are often employed for α-diversity description. Chao-1 is a non-

parametric estimator based on abundance which requires data of individuals belonging to a particular class in a sample and represented only singletons (Chiu & Chao, 2016), while Good's coverage of counts estimates the percentage of an entire species that are represented in a sample (Good, 1953; McCormick et al., 2016).

As β-diversity represents the explicit comparison of microbial communities based on their composition, studies often used metrics such as Unweighted Pair-group Method with Arithmetic Means (UPGMA), Unweighted Unifrac and Weighted Unifrac distance matrixes, Principal Coordinate Analysis (PCoA), Principal Component Analysis (PCA), Non-Metric Multi-Dimensional Scaling (NMDS), and Heat-map (Miranda et al., 2013; Bondoso et al., 2017). Calculation by UPGMA defined the similarity or dissimilarities between clusters as the average pair-wise distance between all their members (Quince et al., 2009). The Weighted UniFrac uses the abundance information of OTUs and phylogeny as a quantitative measure, while Unweighted Unifrac uses the presence and absence of OTUs and phylogeny as a qualitative measure (Lemos, Fulthorpe, & Roesch, 2012).

PCA is a statistical procedure that extracts principle components and structures in data by using orthogonal transformation and reducing dimensionalities of data. The more similar the composition of community among the samples are, the closer is the distance of their corresponding data points on the PCA graph. PCoA is an ordination technique, used when characters or variables are qualitative or discrete. The technique has an advantage over PCA because each ecological distance can be investigated. Beta Diversity Heat-Map uses Weighted Unifrac distance to measure the dissimilarity coefficient between pair-wise samples and is used extensively in recent microbial community sequencing projects (Amelia et al., 2020; Sachithanandam et al., 2020).

## 2.4 MOLECULAR STRATEGIES IN BACTERIAL DIVERSITY AND BIOPROSPECTING STUDIES

Identification of microorganisms associated with seaweeds by molecular techniques through DNA extraction, amplification and sequencing are rapidly emerging. Due to the non-cultivability of the major fraction of bacteria from natural environments, it is a challenge to describe the overall structure of the bacterial community (Dokić et al., 2010). Recent studies to characterise bacterial diversity have shifted to culture-independent methods which are based on genetic measures (Dittami et al., 2014). The molecular-phylogenetic perspective is a reference framework within which microbial diversity can be described and the sequences of genes can be used to identify organisms (Amann, Ludwig, & Schleifer, 1995).

A number of approaches have been developed to study molecular microbial diversity. These include DNA–DNA and mRNA-DNA hybridization, DNA cloning and sequencing, and other PCR-based methods such as Denaturing Gradient Gel Electrophoresis (DGGE) and Temperature Gradient Gel Electrophoresis (TGGE). The next-generation sequencing (NGS) technology, on the other hand, has enabled the discovery of new groups of microorganism in complex environmental systems without cultivated strains and metagenomics which help bioprospecting for secondary metabolites by functional genes (Fakruddin & Mannan, 2013). NGS is an impressive and robust platform that generates sequencing of thousands to millions of DNA molecules within the same sample simultaneously as compared to Sanger Sequencing that generates one complimentary copy to a single-stranded DNA template in each reaction, which is time-consuming, and a large amount of template DNA is needed for each read. Therefore, NGS platforms can be more cost-effective because it requires significantly less DNA sample as templates while allowing massively parallel

sequencing reactions to occur faster than modern-day Sanger Sequencing instruments that utilise capillary-based automated electrophoresis which can run 8–96 sequencing reactions analysis simultaneously (Moorthie, Mattocks, & Wright, 2011). The most used platforms for NGS are Roche 454 (Pyrosequencing) and Illumina/Solexa (e.g., Mi-Seq or Hi-Seq/amplicon sequencing, sequencing by synthesis, sequencing by ligation).

### 2.4.1 Molecular Phylogenetics

Molecular phylogenetics is the systematic analysis of genetic or hereditary molecular differences, most commonly studied by DNA sequences, to gain data on or provide evidence of an organism's evolutionary relationship to other organisms (Yang & Rannala, 2012). The molecular phylogenetic analysis has several steps or stages, and various methods or strategies can be applied to achieve each step of the analysis. The primary step is the sequence acquisition, followed by multiple sequence alignment, phylogenetic tree construction, and lastly, tree evaluation (Ajawatanawong, 2016).

ARB and MEGA are examples of analysis software that are available freely for download and use. They are capable of analysing both tree-building methodologies; examples of distance-based methods are unweighted pair group method using arithmetic mean (UPGMA) and Neighbour-joining, while examples of character-based methods are Maximum parsimony, Maximum likelihood estimation and Bayesian inference (Pavlopoulos, Soldatos, Barbosa-Silva, & Schneider, 2010).

A phylogeny tree constructed is a representation of a set of sequences that share a common ancestor; thus, it may show the trace origin of a gene, determine the relatives of the organism of interest by viewing the connection among the various organisms by the branches and evolution evidence by weight of the branches in the

tree which indicates the time between evolutions of different organisms, and also help to identify the functionality of a gene (Gaucher, Kratzer, & Randall, 2010). Hence, molecular phylogenetics analysis help to develop inferences to the evolutionary relationships of organisms that appear in a phylogenetic tree by determining the process of molecular similarities or differences which led to the species diversity.

## 2.5 SELECTIVE ENRICHMENT MEDIA IN THE CULTURE-DEPENDENT APPROACH

Selection of enrichment media for the growth of bacteria from complex sources ranged from species specific to general media. Nutrient agar and Mueller-Hinton agar are examples of general agar for broad-spectrum bacterial growth, while marine agar is generally used for samples from marine sources. Strategies such as sole-carbon-utilisation, antibiotics, and compound supplements for targeted degrading enzymes were important for encouraging the growth of rare and desired bacteria or fungi (M. Madigan, Martinko, & Parker, 2002).

Yuan, Yu, Li, Dong, & Zhang (2014) reported the use of starch casein agar, ISP2 and MR2A agar for isolation of actinobacteria with biological activities sampled from sediments in the Arctic Ocean. Others demonstrated the use of filtered natural seawater added to agar or agarose with combination of ammonium chloride or sodium nitrite to cultivate marine bacteria (Joint, Muhling, & Querellou, 2010). Isolation of the bacteria were then followed by characterisation and identification of the species either by biochemical tests or molecular techniques. Identification of cultivable species is desirable due to subsequent biotechnological applications such as fermentation of the bacteria to produce active metabolites, polysaccharides, or

biofuels (Debbab, Aly, Lin, & Proksch, 2010; Chuang & Tung, 2012; Lau et al., 2015).

## 2.6 SCREENING BROMOPEROXIDASE FUNCTIONAL GENES IN CULTIVATED BACTERIA

Many researches nowadays screened functional genes such as polyketide synthase (PKS) genes, non-ribosomal peptide synthase (NRPS) genes, and halogenase genes from isolated microorganisms to search for bioactive metabolites (Goodfellow & Fiedler, 2010; Pereira & Costa-Lotufo, 2012; Barone et al., 2014; Yuan et al., 2014). Bioprospecting minimises cost and complex processes of natural product extraction sector. Halogenase genes, for example, encode for the enzymes responsible for halogenation of organic substrates by halide ions namely Cl-, Br-, I- and F-, into halogenated compounds that are bioactive metabolites important for the growth, fitness, and chemical defences of sponges, seaweeds and other sessile marine organisms. By being able to screen and identify functional gene or gene of interest, research or experiments with the intention to extract bioactive compounds or metabolites from bacteria can be explored and designed accordingly (Wang et al., 2011; Barone et al., 2014;).

Halogenation occurs frequently in the marine environment as seawater is abundant with chlorine and bromine halide ions. The chlorination or bromination of organic substrates may occur through haloperoxidase or halogenase enzymes for which different classes require the presence of different cofactors like vanadium, flavin ($FADH_2$) or S-adenosylmethionine (SAM). While vanadium bromoperoxidases (V-BPO) are widely distributed in marine seaweeds (Carter, Beatty, Simpson, & Butler, 2002; Suthiphongchai & Boonsiri, 2008), $FADH_2$-dependent halogenases

represent the biggest class and have been found in various bacterial phyla, including the Actinobacteria, Cyanobacteria, and Proteobacteria (Hornung et al., 2007; Bayer, Scheuermayer, Fieseler, & Hentschel, 2013).

# CHAPTER THREE

# METHODOLOGY

## 3.1 INTRODUCTION

This chapter details the methodology employed throughout the study, which is guided by the four research questions posed in Chapter 1 and the literature reviewed in Chapter 2. On the basis of what was found from the literature, this chapter presents the following research methodology as illustrated in the flowchart Figure 3.1.

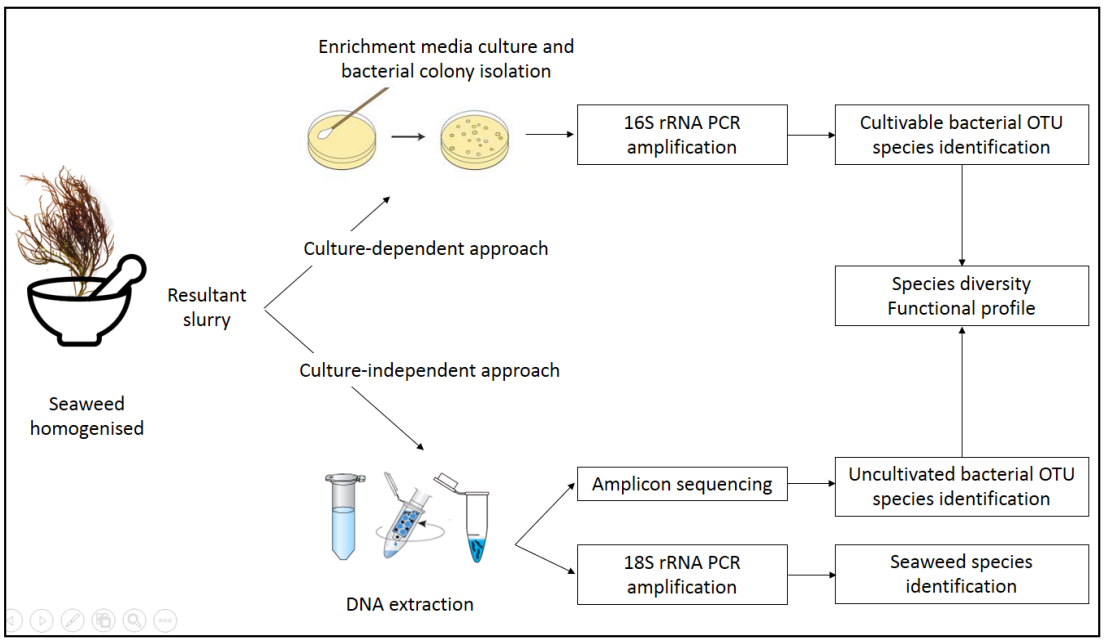



Figure 3.1 Flow Chart of Research Methodology

After seaweeds were collected, samples were processed for the respective research objectives. Seaweed samples collected and stored in ethanol were used for morphological analysis and DNA extraction for the first objective. For seaweeds samples collected and homogenised, its resultant slurry was used for the subsequent

objectives by the following methods; (1) to culture on media agar plate for bacterial isolation, and (2) to extract total genomic DNA for culture-independent approach.

### 3.2 SAMPLING LOCATIONS

There are few seaweed farms in Peninsular Malaysia; however, the farm in Kedah was selected with only two other sites were selected as the sampling locations due to financial and time constraints. Specimens of the seaweeds were collected from Pulau Bidong (Site 1, 1 specimen: SWD1), Kedah seaweed farm (Site 2, 1 specimen: SWD2) and natural coastal waters of Penang Island (Site 3, 2 specimens: SWD3 and SWD4). Location data (GPS, Figure 3.2) and physicochemical parameters (temperature, salinity, and pH) of the sampling sites were taken and recorded.

Seaweed samples collected were rinsed with sterilised seawater to remove dirt, epiphytes and other contaminants before the seaweed were stored or grinded. Seaweed samples stored were preserved with 100% ethanol in 50 mL centrifuge tubes and were kept on ice for transportation to the laboratory. The seaweed samples were transferred to -20°C freezer upon reaching the laboratory.

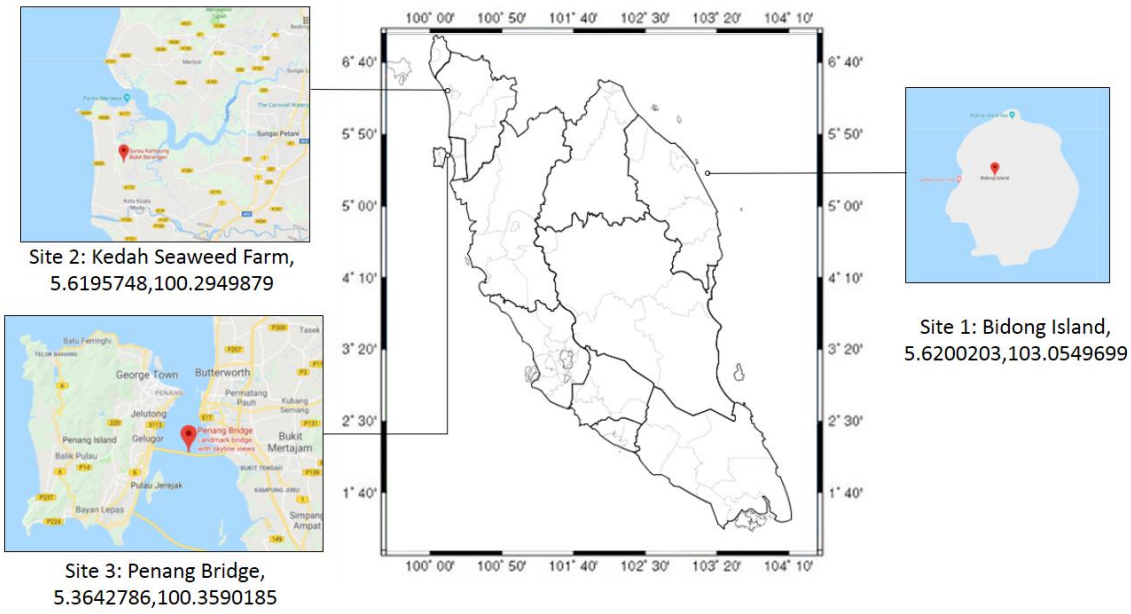



Figure 3.2 Sample Sites with GPS Coordinates

As shown in Figure 3.2, Site 3 was an island underneath the Penang Bridge for samples SWD3–SWD4 collected from the natural coastal environment to be compared with specimens of the seaweed collected from a seaweed farm located in Kedah (Site 2; SWD2) and Bidong Island (Site 1: Pantai Pasir Cina; SWD1). The island was called "Pulau Korea" (local name) and emerged as the remnant of the construction aftermath of the Penang bridge.

Figure 3.3 Seaweed Samples Collected from Pulau Pinang

(a) Pulau Korea; (b) seaweed sample.

### 3.2.1 SAMPLE COLLECTION AND PROCESSING

Seaweed samples collected were rinsed with sterilised seawater to remove dirt, epiphytes and other contaminants before the seaweed were stored or ground. Seaweed samples stored were preserved with 100% ethanol in 50-mL Falcon tubes. Seaweed samples were ground by mortar and pestle until slurry and were then transferred into 50% glycerol solution in cryogenic vials. All seaweed sample tubes were kept on ice for transportation to the laboratory. The seaweed samples in 50-mL Falcon tubes were transferred to -20°C freezer and the cryopreservation tubes were transferred to -80°C freezer upon reaching the laboratory.

## 3.3 SEAWEED INDENTIFICATION

In this section, methods of the morphological and molecular analysis of red seaweed samples are described. Some red seaweeds are ambiguous species where misidentification can occur due simple morphology and high plasticity problems. Hence, morphological and molecular analysis can be utilised concurrently to corroborate the taxonomic identities obtained from each method.

### 3.3.1 MORPHOLOGICAL ANALYSIS OF SEAWEED

For identification of the seaweed samples through morphological observation, firstly, the seaweed samples were washed with sterilised seawater and its image was captured. Cross-sectional images of the seaweed specimens were captured using Nikon Eclipse 80i microscope fixed with DS-Fi1 lense and Nikon Digital Sight Camera. Images were added scale using NIS elements 4.11 imaging software. Characteristics observation included the appearance of holdfast and thallus, the colour throughouth the anatomy of the specimen, frequency of branching, and the length of

the thallus. Taxonomic identification keys from Dhargalkar and Kavlekar (2004) were referred for anatomical characteristic of the specimen, which listed the genus *Gracilaria* as plants Rhodophyta that are branched, flattened, and structurally composed of central medulla surrounded by cortex.

### 3.3.2 MOLECULAR ANALYSIS OF SEAWEED

Seaweed DNA were extracted by InnuPrep Plant DNA Kit (AnalytikJena) according to the manufacturer's instruction. Once the seaweed was completely homogenised into a slurry, it was transferred into a 2.0-mL microcentrifuge tube. Then, 400 mL of lysis solution CBV (provided in the kit) was added into the tube with 25 mL of protein K, and the solution was mixed and incubated at 65°C overnight in a Bio TDB-100 Dry Block Thermostat (BioSan, Latvia). Precipitation was done next by adding 100 µL of Precipitation Buffer P (provided in the kit) into the tube, vortexed briefly, and incubated at room temperature for 5 min. The solution in the tube was then centrifuged at 12,000 × *g* for 1 min. The aqueous phase was transferred into a new microcentrifuge tube and 200 µL of SBS was added. The solution in the tube was centrifuged at 12,000 × *g* for 2 min, and added 600 µL MS solution for washing and centrifuged 1 min. the washing was repeated twice. Finally, to remove ethanol from the solution, the samples were centrifuged again at 12,000 × *g* for 1 min. Lastly, 100 µL elution buffer was added, incubated at RTP, and centrifuged at 8,000 × *g* for 1 min. The concentration of the extracted DNA was determined using a NanoDrop™ 1000 Spectrophotometer (ThermoScientific, USA) and the DNA was visualised through 1.5% (w/v) of gel electrophoresis.

#### *3.3.2.1 PCR Amplification, Sequencing and Species Identification*

The *18S rRNA* gene from DNA of extracted seaweed samples (SWD1–SWD4) was amplified by polymerase chain reaction (PCR) according to the following protocol volume: 25 µL PCR mixture was prepared with 10 µM universal eukaryotic primers, forward EukA (5'-CAC GTA CTA GGA CTT CGG-3') and reverse EukB (5'-GTC AGG AAT GGC AGC TTG TGA-3') (Medlin, Elwood, Stickel, & Sogin, 1988), 2X MyTaq (Applied Biosystems) PCR buffer, and 2 µL DNA template. The PCR reaction protocol using Bio-Rad T100$^{TM}$ Thermo Cycler (Bio-Rad Laboratories, USA) was as follows: initial denaturation for 5 min at 95°C; 30 cycles of denaturation for 30 s at 95°C, annealing for 45 s at 53°C, and elongation for 1.5 min at 72°C; a final extension step for 10 min at 72°C (Medlin et al., 1988). The PCR products were visualised on a 1.5% agarose gel. Amplicons with the correct expected size (~1500 bp) were sequenced with the EukA primer to Apical Scientific Sdn. Bhd. (formerly known as First BASE Laboratories Sdn. Bhd., Malaysia).

#### *3.3.2.2 Phylogenetic and Bioinformatics Analysis*

Sequence data obtained were inspected based on its sequence chromatogram for quality control, converted to fasta format, and aligned with relevant target sequences from the NCBI GenBank database, using a NCBI BLAST alignment search, obtained at 97% similarity. Phylogenetic analysis was conducted for each DNA sequence, and a multiple alignment was aligned using the FastAlign function of the alignment editor implemented in the ARB programme and refined manually. The phylogeny was inferred using MrBayes version 3.2.6 (Ronquist et al., 2012) available online at Cyberinfrastructure for Phylogenetic Research (CIPRES) server (http://www.phylo.org/). The calculation was carried out assuming the GTR (general

time reversible) phylogenetic model with 6 substitution rate parameters, gamma-distributed rates across sites and the default setting of the program. Total of $10^7$ tree generations were calculated and sampled every $1000^{th}$ generation. Points prior to convergence were determined graphically and discarded. The consensus tree was imported and edited with the ARB programme.

### 3.4 BACTERIAL DIVERSITY THROUGH CULTURE-INDEPENDENT APPROACH

To extract total genomic DNA from the seaweed samples for investigating bacteria through the culture-independent approach, three pieces of the same seaweed from different individuals previously stored in ethanol were pooled together, spun in a MiVac Pump for 5 min to remove all traces of ethanol and washed with filtered seawater before they were homogenised with the aid of liquid nitrogen using mortar and pestle. The total genomic DNA was extracted using CTAB method (Healey, Furtado, Cooper, & Henry, 2014). Starting material was taken from seaweed stored in ethanol and 5 g (wet weight) of seaweed (SWD1) was centrifuged for 5 min using miVac vacuum concentrator (SP Scientific, USA) to remove ethanol. Seaweed was homogenised using mortar and pestle in the presence of liquid nitrogen. The homogenised samples were transferred into 1.5 mL centrifuge tube and added 1 mL extraction buffer containing 100 mM Tris-HCl (pH 7.5), 25 mM EDTA, 1.5 M NaCl, 2% (w/v) CTAB, and 0.3% (v/v) β-mercaptoethanol. The mixture was incubated at 65°C for 1 h, and then cemtrifuged 5,000 × *g* for 5 min. Supernatant were decanted into a new tube and added 500 µL chloroform:isoamyl alcohol (24:1). The solution was mixed by inversion for 5 min, and then the tubes were centrifuged at 12,000 × *g*

for 10 min. Upper aqueous layer was transferred to a new centrifuge tube, added 500 µL phenol:chloroform:isoamyl alcohol (24:25:1), and centrifuged at 12,000 × *g* for 10 min. Upper aqueous layer was transferred again to a new centrifuge tube, added 500 µL chloroform:isoamyl alcohol (24:25:1), and centrifuged at 12,000 × *g* for 10 min. Lastly, the aqueous layer was transferred to a new centrifuge tube and added isopropanol (chilled to -20°C) at a ratio of 0.6 volume of transferred aqueous layer. Tubes were then incubated at -20°C overnight. After incubation, samples were centrifuged at 4°C, 9000 × *g* for 15 min. Supernatant was removed and samples were lastly washed with 300 µL 70% ethanol (chilled to -20°C) centrifuged at 9000 × *g* for 5 min. Supernatant was removed and DNA samples obtained were resuspended in 50 µL sterilised ultra-pure water. The concentration and purity of the DNA eluted were measured using NanaDrop™ 100 spectrophotometer (ThermoFisher Scientific, Germany) and visualised by 1.5% (w/v) gel electrophoresis.

DNA was then amplified by polymerase chain reaction (PCR) with *16S rRNA* universal primer, 27F (5'-AGRG TTTG ATCM TGGC TCAG-3') and 1492R (5'-TAC GGTT ACCT TGTT ACGA CTT-3') for bacteria (Weisburg, Barns, Pelletier, & Lane, 1991). PCR was conducted with MyTaq™ Red Mix (Bioline) PCR kit using 25 mL reaction volume consisting of 12.5 µL MyTaq™ Red Mix (Bioline), 10 µM of each primer, 100 ng bacterial genomic DNA, and topped off with sterile deionised water, using the following reaction protocol: initial denaturation, 95°C for 5 min, 30 cycles consisting of: primer annealing at 55°C for 40 s; primer extension 72°C for 90 s; denaturation at 95°C for 30 s, final cycle: annealing, 55°C for 40 s, extension, 72°C for 5 min (Weisburg et al., 1991; Staufenberger et al., 2008) in a Bio-Rad T100™ Thermo Cycler (Bio-Rad Laboratories, USA). Then, PCR amplicons were viewed by gel electrophoresis and sent for sequencing at First BASE Laboratories (Malaysia).

The DNA obtained was diluted into 1 ng/μL using sterilised water. The PCR reaction was set with primer set 515F/806R (Pylro et al., 2014) and High-Fidelity PCR Master Mix (New England Biolabs) along with the barcode to amplify gene in *16S rRNA* V4 region. The amplicons were detected using SYB green electrophoresed on 2% (w/v) of agarose gel. Amplicons between 400–450 bp were purified using Qiagen Gel Extraction Kit (Qiagen, Germany). Illumina HiSeq 2500 platform was used to sequence the amplicon and generate 250 bp paired end reads and the library quality was assessed by Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system.

### 3.4.1 Illumina Sequence Data Processing

Paired-end reads samples based on data generated from amplicon sequencing were divided into samples based on their unique barcode. Sequences were assembled using FLASH (V1.2.7) to merge the paired ends after the primer sequence were cut off (Magoc & Salzberg, 2011). The reads generated from the opposite ends of the same DNA fragment were overlapped and spliced. Quantitative Insights into Microbial Ecology software package (QIIME) V1.8.0 (Caporaso et al., 2010) were used to filter RAW tags into high quality tags.

Effective tags were obtained through UCHIME algorithm compared to Gold Database by removing the chimeric sequence (Edgar, Haas, Clemente, Quince, & Knight, 2011). Sequences with ≥ 97% similarity were grouped into the same OTUs by using Uparse software (Uparse v7.0.1001) (Edgar, 2013) and species annotation was screened from representative of every OTU while taxonomy information was obtained from GreenGene database according to RDP classifier (Version 2.2). Short read sequences have been deposited in GenBank short-read archive (SRA: SRX6766235).

### 3.4.2 Species Diversity

Alpha diversity analysed the complexity of species diversity for a sample and was applied through five indices which were Observed-species, Chao, Shannon, Simpson, and Good-coverage. All indices were calculated with QIIME (Version 1.7.0) and displayed with R software (Version 2.15.3). For beta diversity measurement, a square matrix of "distance" or dissimilarity was calculated to reflect the dissimilarity between certain samples, such as Unweighted Unifrac and Weighted Unifrac distance matices which were calculated by QIIME software (Version 1.7.0). The data in this distance matrix are visualised with Principal Coordinate Analysis (PCoA), Principal Component Analysis (PCA), and Beta Diversity Heat-Map using display by WGCNA package, stat packages, and ggplot2 package in R software (Version 2.15.3).

## 3.5 BACTERIAL INVESTIGATION THROUGH CULTURE-DEPENDENT APPROACH

Bacterial isolates were obtained from 8 selective enrichment agar plate cultures. The media included commercial type agar and custom laboratory mixture agar containing halide salts, KBr, KI, NaCl; or inorganic nitrate, $NH_4Cl$, $NaNO_3$ (Joint et al., 2010; Yuan et al., 2014). Details of the agar media composition are outlined in Table 3.1.

Table 3.1 Composition of Agar Media

| Media ID | Type | Composition (per litre) |
|---|---|---|

| | | |
|---|---|---|
| MA – Marine Agar | Commercial (Difco™) | Peptone 5.0 g,<br>Yeast Extract 1.0 g<br>Ferric Citrate 0.1 g<br>Sodium Chloride 19.45 g<br>Magnesium Chloride 8.8 g<br>Sodium Sulfate 3.24 g<br>Calcium Chloride 1.8 g<br>Potassium Chloride 0.55 g<br>Sodium Bicarbonate 0.16 g<br>Potassium Bromide 0.08 g<br>Strontium Chloride 34.0 mg<br>Boric Acid 22.0 mg<br>Sodium Silicate 4.0 mg<br>Sodium Fluoride 2.4 mg<br>Ammonium Nitrate 1.6 mg<br>Disodium Phosphate 8.0 mg<br>Agar 15.0 g |
| AIA – Actinomycetes Isolation Agar | Commercial (Difco™) | Sodium Caseinate 2.0 g<br>Asparagine 0.1 g<br>Sodium Propionate 4.0 g<br>Dipotassium Phosphate 0.5 g<br>Magnesium Sulfate 0.1 g<br>Ferrous Sulfate 1.0 mg<br>Agar 15.0 g<br>Sterile distilled water up to 1 L |
| SA – Seawater Agar | Custom laboratory mix | Agar 15.0 g<br>Sterile seawater up to 1 L |
| KBr – Potassium Bromide Halide Agar | Custom laboratory mix halide agar | Potassium Bromide (KBr) 1.20 g<br>Agar 15.0 g<br>Sterile seawater up to 1 L |
| KI – Potassium Iodide Halide Agar | Custom laboratory mix halide agar | Potassium Iodide (KI) 1.67 g<br>Agar 15.0 g<br>Sterile seawater up to 1 L |

Table 3.1 Composition of Agar Media (continued)

| Media ID | Type | Composition (per litre) |
|---|---|---|
| NaCl – Sodium Chloride Halide Agar | Custom laboratory mix halide agar | Sodium chloride (NaCl) 3.0 g<br>Agar 15.0 g<br>Sterile seawater up to 1 L |
| $NH_4Cl$ – Ammonium Chloride Agar | Custom laboratory mix inorganic nitrate agar | Ammonium Chloride ($NH_4Cl$) 5.3 g<br>Agar 15.0 g<br>Sterile seawater up to 1 L |
| $NaNO_3$ – Sodium Nitrate Agar | Custom laboratory mix inorganic nitrate agar | Sodium Nitrate ($NaNO_3$) 8.5 g<br>Agar 15.0 g<br>Sterile seawater up to 1 L |

The plates were incubated at 30°C for 2 weeks. Culture colonies were counted and sub-cultured to achieve single colony culture plates.

### 3.5.1 Colony PCR and Sequencing

Colonies from agar plates were picked using sterilised tooth picks and dissolved in 20 µL $ddH_2O$. in a 200-µL PCR tube. Tubes were then treated to heat-boiling at 98°C for 10 min. Aliquots (1.5 µL) of these samples was used to amplify the *16S rRNA* gene in a 25-µL PCR mixture with 10 µM universal bacterial primers 27F (5'-AGRG TTTG ATCM TGGC TCAG-3') and 1492R (5'-GGTT ACCT TGTT ACGA CTT-3') (Lane, 1991), and 2X MyTaq Mix (Bioline, Applied Biosystems, USA) PCR buffer containing Taq DNA polymerase, dNTPs, and magnesium chloride, $MgCl_2$. The PCR program was as follows: initial denaturation for 5 min at 95°C; 30 cycles of denaturation for 30 s at 95°C, annealing for 40 s at 55°C, and elongation for 1.5 min at 72°C; and a final extension step for 5 min at 72°C (Lane, 1991; Naim et al., 2014). The PCR products were visualised on a 1.5% agarose gel. Amplicons with the correct

expected size (~1500 bp) were sequenced with the 27F primer to Apical Scientific Sdn. Bhd.

### 3.5.2 Bacterial DNA Extraction and Sequencing

For the isolates that were not able to be identified directly from colony PCR, its DNA was extracted using the CTAB (cetyl trimethylammonium bromide) method (Joint Genome Institute). Briefly, the isolates were cultured overnight in their respective broth composition (e.g. Marine Broth for Marina Agar isolates), then centrifuged at room temperature for 5 min at the maximum speed to obtain the pellet. After centrifugation, the excess broth is decanted and the pellet was resuspended with 700 µL Tris-EDTA buffer. For lysis, 20 µL lysozyme were added into the tube and the solution was incubated for 30 min at 37°C. Later, 40 µL 10% SDS with 20 µL of protein K were added, and the solution was mixed and incubated at 56°C for 3 h in a Bio TDB-100 Dry Block Thermostat (BioSan, Latvia). Precipitation was done next by adding 100 µL 5 M NaCl into the tube, vortexed briefly, adding 100 µL CTAB/NaCl and incubated at 65°C for 10 min in the thermostat. The solution in the tube was then added 500 µL chloroform:isoamyl alcohol (24:1) and centrifuged at 16,000 × *g* for 10 min. The aqueous phase was transferred into a new microcentrifuge tube and 500 µL phenol:chloroform:isoamyl alcohol (25:24:1) was added. The solution was centrifuged again at 16,000 × *g* for 10 min. The aqueous phase was again transferred into a new microcentrifuge tube, 500 µL chloroform:isoamyl alcohol (24:1) was added, and centrifuged at 16,000 × *g* for 10 min. Then, the aqueous phase was transferred into a new microcentrifuge tube, the volume noted, and 0.6 vol (v:v, volume of aqueous phase) of chilled (-20°C) ethanol was added. The solution was incubated at -20°C for 2 h and were then centrifuged 16,000 × *g* for 15 min.

Supernatant was decanted and pellet was washed with chilled 70% ethanol and centrifuged 5 min. The washing was repeated twice. Finally, to remove ethanol from the solution, the samples were centrifuged again at 16,000 × *g* for 1 min. Pellet was suspended in 100 µL DNA-free water and stored in -20°C freezer. The concentration of the extracted DNA was determined using a NanoDrop™ 1000 spectrophotometer (ThermoScientific, USA) and DNA was visualised through 1.5% (w/v) of gel electrophoresis.

### 3.5.3 Screening Bromoperoxidase Functional Gene

For PCR-based functional halogenase gene screening, the bacteria were cultured in marine broth for 7 days before DNA extraction by CTAB method (Joint Genome Institute [JGI], 2004). After the bacterial DNA was obtained, the PCR amplification was conducted using Bio-Rad T100™ Thermo Cycler (Bio-Rad Laboratories, USA) based on the following protocols; (1) halogenase primer set, forward primer Halo-B4-FW 5′-TTCCCSCGSTACCASATCGGSGAG-3′ and reverse primer Halo-B7-RV 5′-GSGGGATSWMCCAGWACCASCC-3′ in a 25-µL PCR reaction mixture containing 12.5 µL 2× MyTaq PCR buffer (Applied Biosystems, USA), 1 µL Halo-B4-FW and Hal-B7-RV primers (30 pmol $\mu L^{-1}$) each, 2 µL genomic DNA, 8.5 µL $H_2O$, performed under standard amplification procedure with an annealing temperature of 58 °C for 1.5 min (Hornung et al., 2007); (2) sequences of two sense oligonucleotides, SZ002 (motif II) 5′-TCGGYGTSGGCGARGCGACCRTCCC-3′ and SZ003 (motif I) 5′-TSGGCGGCGGCACYGCSGGMTGGATG-3′, with antisense oligonucleotides SZ004 (motif IV) 5′-AGCATSGGRATCTTCCAGGTCCABCC-3′ and SZ005 (motif III) 5′-GCCGGAGCAGTCGAYGAASAGGTC-3′, with PCR amplification conducted by standard procedure with an annealing temperature of 63.5°C (Zehner et al., 2005);

and (3) bromoperoxidase (BPO) forward primer, fd-bpo: 5'-CCCAT GTGGA CCAC CCTT TAT-3' and reverse primer, rv-bpo: 5'-TAAG TGGT CGAT CTTG GGAAT-3' with initial denaturation at 95 °C for 2 min, 35 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s, and extension at 72 °C for 2 min; and final extension at 72 °C for 6 min (Lilles, 2011). PCR amplification was followed by gel electrophoresis (1.5%) and bands were viewed using AlphaImager.

As only the amplification of bromoperoxidase encoding genes was successfully viewed on gel, the cloning of isolates positive for BPO genes was performed using the pEASY® - T5 Zero Cloning Kit (Transgen Biotech, China) and according to the manufacturer's instruction. PCR products successfully amplified with BPO primers were purified using EasyPure® PCR Purification Kit (Transgen Biotech, China) and visualised on 1.5% agarose gel. An aliquot of the positive samples (3 µL) was ligated with 1 µL pEASY® - T5 Zero Cloning Vector (10 ng/µL) and incubated at room temperature for 15 min. The ligated product was added to 50 µL of Trans1-T1 Phage Resistant Chemically Competent Cell with gentle mixing in a 1.5-mL microcentrifuge tube. The tube was incubated in ice for 30 min and then heat-shocked at 42°C for 30 s. The tube was then transferred to ice for 2 min incubation and afterwards, 250 mL SOC media was added into the tube. Next, the tube was shaken, 200 × *g*, at 37°C for 1 h. After that, aliquots of the culture were spread onto pre-warmed 37°C LB media plates and incubated overnight.

Positive cloning was observed by the growth of white or pale blue-white colonies on the agar plates. Ten colonies were picked for analysis. The colonies were screened by colony PCR using M13 forward and reverse primers provided, temperature profile according to the manufacturer's instruction, and visualised on 1% agarose gel. Positive samples were sent for partial sequencing to First BASE

Laboratories (Serdang, Malaysia). Sequence data obtained were analysed by translation amino acid sequences by ExPASy online tools using standard genetic codes (available at https://web.expasy.org/translate/), clustered using CD-HITs Suite server (available at http://weizhongli-lab.org/cdhit_suite/cgi-bin/index.cgi), and searched in BLASTX for nearneighbour homology (ncbi website) and protein prediction (https://open.predictprotein.org/). Sequence submitted to GenBank were given the accession numbers MN551638-MN551640. The phylogenetic tree was contructed by Bayesian inference using MrBayes version 3.2.6 available online at the CIPRES server (http://www.phylo.org/), and the calculation was carried out by assuming the GTR phylogenetic model with 6 substitution rate parameters, gamma-distributed rates across sites and the default setting of the program. Total of $10^7$ tree generations were calculated and sampled every $1000^{th}$ generation. Points prior to convergence were determined graphically and discarded. The consensus tree was imported and edited with the ARB programme.

### 3.6 SEQUENCING AND PHYLOGENETIC ANALYSIS

For the *16S rRNA* sequences obtained from cultivable bacteria, the sequence chromatograms were manually inspected and curated to remove unambiguous regions using BioEdit software V7.1.9 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). All sequences were converted to FASTA format by EBI online sequence conversion tool (https://www.ebi.ac.uk/Tools/sfc/emboss_seqret/), and the compiled sequences were checked for possible chimeric origins using DECIPHER software (Wright, Yilmaz, & Noguera, 2012) available online (http://decipher.cee.wisc.edu/FindChimeras.html). Sequences with similarities of >97% were considered as 1 OTU (Operational Taxonomic Units) and were clustered by CD-HITs to obtain number of OTUs

(http://weizhongli-lab.org/cdhit_suite/cgi-bin/index.cgi). Nearest neighbours were determined by comparison to the NCBI GenBank database using BLASTn searches (7th April 2018), and sequences were deposited at the NCBI GenBank database with the accession numbers MN396133–MN396175.

From the HiSeq2500 amplicon sequencing, the paired-ends sequences were merged after the primer sequence were cut off, and reads generated from the opposite ends of the same DNA fragment were overlapped and spliced using FLASH (V1.2.7, http://ccb.jhu.edu/software/FLASH/) (Magoc & Salzberg, 2011) to obtain raw tags. Quality filtering on the raw tags were performed to obtain the high-quality clean tags according to the Quantitative Insights into Microbial Ecology software package (QIIME V1.7.0, http://qiime.org/scripts/split_libraries_fastq.html) (Caporaso et al., 2010) quality controlled process. The tags were then compared to the reference Gold database using the UCHIME algorithm (Edgar et al., 2011) (http://www.drive5.com/usearch/manual/uchime_algo.html) to detect chimera sequences which were removed to obtain the Effective Tags. Sequences with ≥ 97% similarity were grouped into the same OTU by using Uparse software (Uparse v7.0.1001) (Edgar, 2013) and species annotation was screened from representative of each OTU while taxonomy information was obtained from GreenGene database according to RDP (Ribosomal Database Project) classifier (Version 2.2). Short read sequences have been deposited in GenBank short-read archive (SRA: SRX6766236–SRX6766238). Representative sequences of OTUs obtained was run though BLASTn searches to determine nearest neighbours.

Sequences of bacterial isolates OTUs, predominant OTUs from Illumina sequencing (cutoff min 100 reads per OTU) and near neighbours the OTUs were imported into the ARB software package (Ludwig et al., 2004), aligned using the

FastAlign function of the alignment editor implemented in the ARB program and refined manually. Sequences that were not available from the SILVA database in ARB was searched from the NCBI database, and aligned together using SILVA online Silva Incremental Aligner (SINA) (Pruesse, Peplies, & Glöckner, 2012) at https://www.arb-silva.de/. Ambiguous regions of the alignment were systematically removed using the program Gblocks v.0.91b (Castresana, 2000). The default Gblocks program parameters were used, except allowing a minimum block length of 2 and gaps in 50% of the positions.

The phylogeny was inferred using MrBayes version 3.2.6 (Ronquist et al., 2012) available online at the Cyberinfrastructure for Phylogenetic Research (CIPRES) server (http://www.phylo.org/). The calculation was carried out assuming the GTR (general time reversible) phylogenetic model with 6 substitution rate parameters, gamma-distributed rates across sites and the default setting of the program. Total of $10^7$ tree generations were calculated and sampled every $1000^{th}$ generation. Points prior to convergence were determined graphically and discarded. The consensus tree was imported and edited with the ARB programme.

# CHAPTER FOUR

# RESULTS

In this chapter, the findings from this study are presented in four parts to address the research objectives. The first part describes the seaweed identification through morphological and molecular analysis. In the second part, bacterial diversity of *Gracilaria manilaensis* is explored through culture-independent and culture-dependent approaches. The third part demonstrates the bacterial diversity of *Laurencia* sp. through culture-independent and culture-dependent approaches, and lastly, the comparison of the bacterial diversity of the two seaweed genera are shown.

## 4.1 PART I: SEAWEED SAMPLES

In this section, the physico-chemical parameters of the sampling locations are presented along with the morphological and molecular analysis of the seaweed samples.

### 4.1.1 Physicochemical Parameters

Specimens of the seaweeds were collected from Pulau Bidong (Site 1, 1 specimen), Kedah seaweed farm (Site 2, 1 specimen) and natural coastal waters of Penang Island (Site 3, 2 specimens). Physicochemical parameters of the sampling sites were recorded and listed in Table 4.1.

Table 4.1 Description and Physicochemical Parameters of the Sampling Sites

| Seaweed Label | Seaweed description | Collection Area Description |
|---|---|---|
| SWD 1 | *Laurencia* sp., Red, short thallus, multibranching | Bidong Island, clear water, Salinity 30 ppt, Temp 29.0 °C, pH 7.70 |
| SWD 2 | *Gracilaria manilaensis*, polyculture | Kuala Muda Kedah polyculture with milkfish, *Chanos chanos* (Forsskål, 1775). Murky water, Salinity 16 ppt, brackish, Temp 32.6 °C, pH 7.68 |
| SWD 3 | *Gracilaria manilaensis*, wild, reddish | Pulau Korea, Pulau Pinang. Sandy shore, high biodiversity mangrove, sea grass. Salinity 30 ppt, Temp 32.0 °C, pH 7.77 |
| SWD 4 | *Gracilaria manilaensis*, wild, greenish | Pulau Korea, Pulau Pinang. Sandy shore, high biodiversity, mangroves, sea grass. Salinity 30 ppt, Temp 32.0 °C, pH 7.77 |

### 4.1.2 Morphological Characteristics of Seaweed

Seaweed specimens were found in east (Bidong Island, Terengganu, SWD1) and west of the Peninsular Malaysia (Kedah and Pulau Pinang). SWD1 formed dense patches attached to floating ropes nearshore to the island. The interchanging tides could have induced stress to the seaweed, caused by overexposure to rainfall or direct sunlight; hence, the small size of the seaweed is observed. SWD2 from a seaweed culture farm in Kedah had a thin, long hair-like, reddish-brown appearance, while SWD3 and SWD4 wild capture seaweeds had thicker thallus. Images of the respective specimens (SWD 1–4) are shown in Figures 4.1-4.4 and a summary of their observed morphological characteristics is listed in Table 4.2.

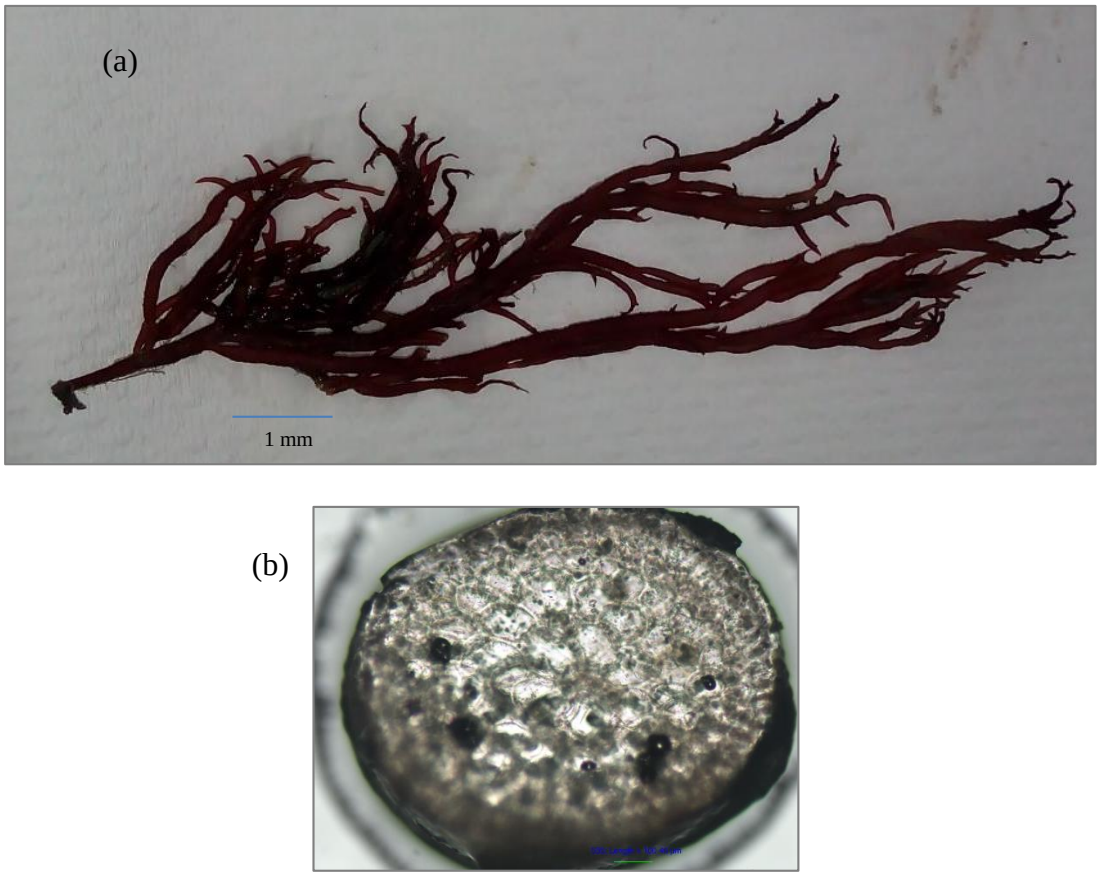



Figure 4.1 Image of Specimen SWD1 (a) Photographic image; (b) Viewed under microscope with 4X magnification.

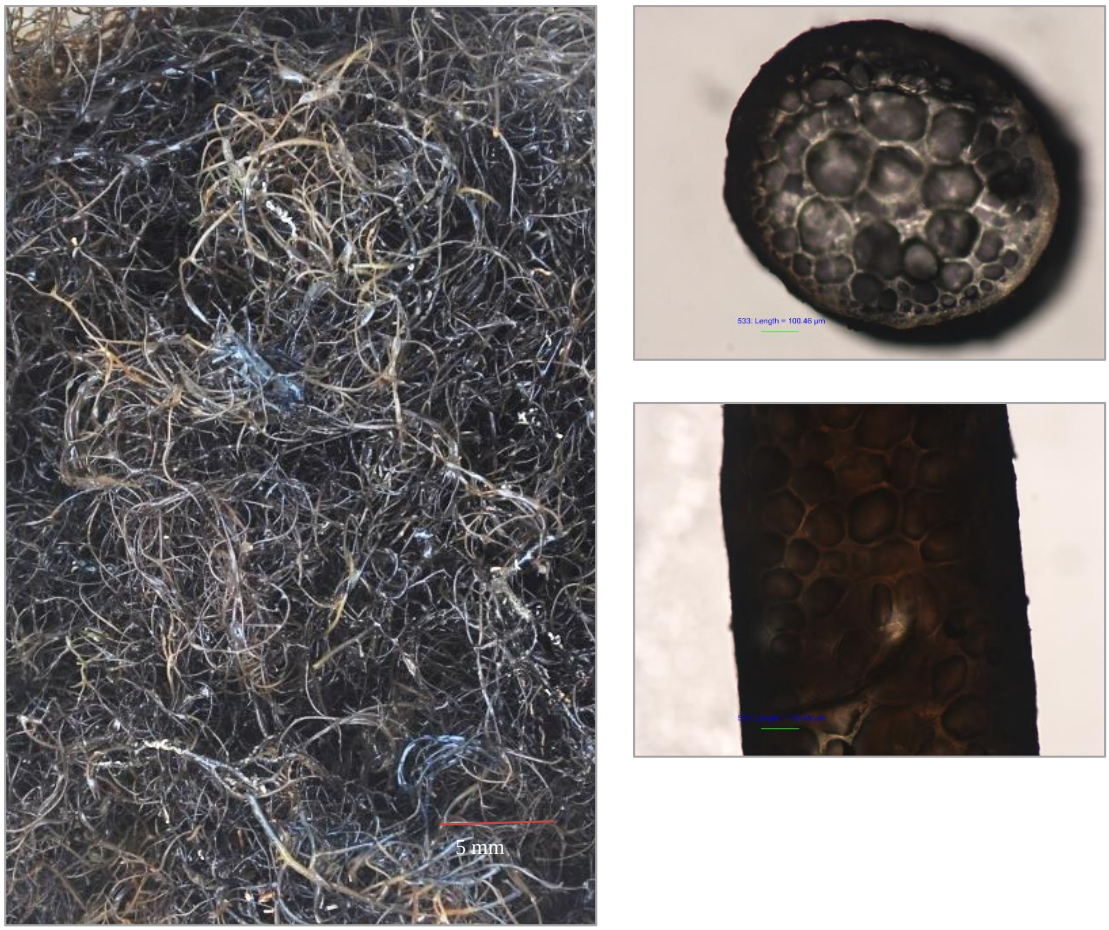



Figure 4.2 Image of Specimen SWD2 (a) Photographic image; (b) Thallus cross-section showing the medulla and cortex structure, and (c) thallus longitudinal cross-section viewed under microscope with 4X magnification.

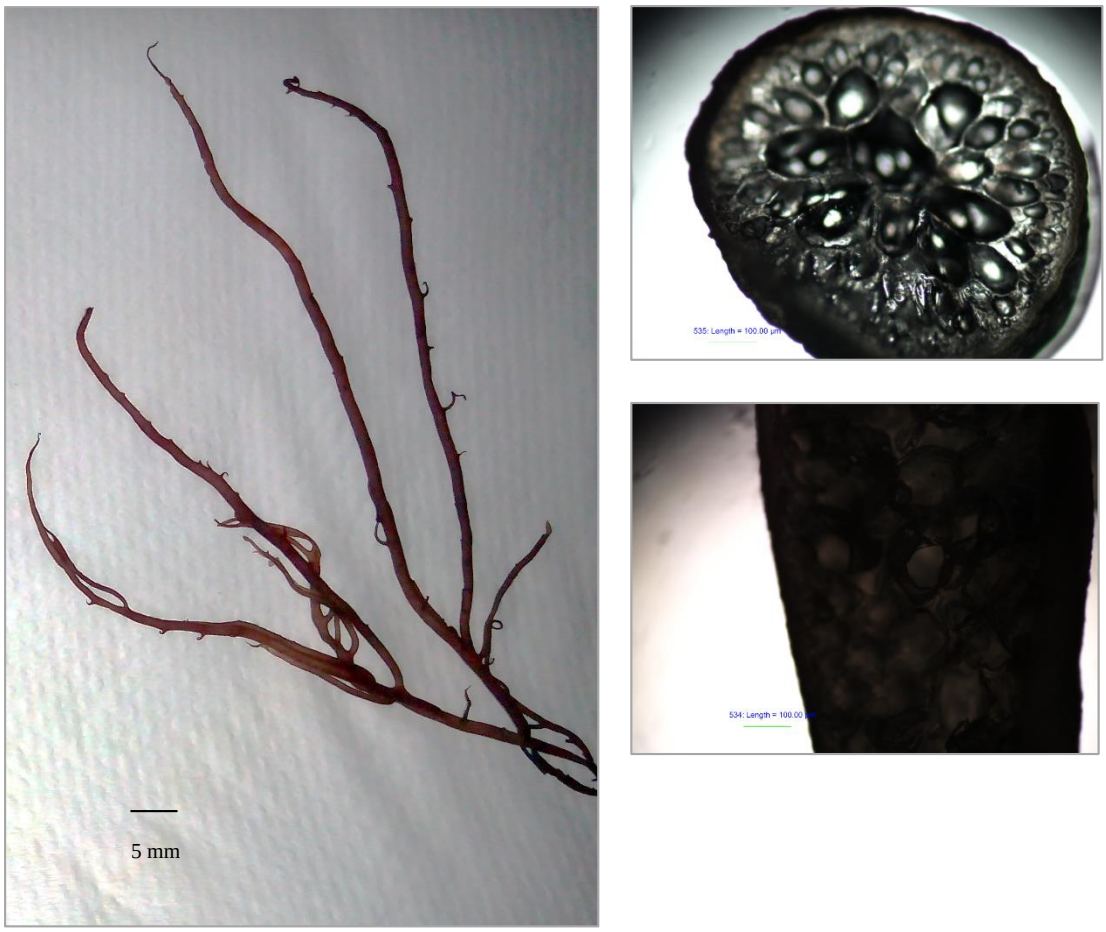



Figure 4.3 Image of Specimen SWD3 (a) Photographic image; (b) Thallus cross-section showing the medulla and cortex structure, and (c) thallus longitudinal cross-section viewed under microscope with 4X magnification.

Figure 4.4 Image of Specimen SWD4 (a) Photographic image; (b) Thallus cross-section showing the medulla and cortex structure viewed under microscope with 4X magnification, and (c) with 10X magnification.

Table 4.2 Key Morphological Features of Seaweed Specimens SWD1–SWD4

| Key Morphological Feature | SWD1 | SWD2 | SWD3 | SWD4 |
|---|---|---|---|---|
| Colour | Dark red | Dark greenish brown-red | Deep red | Greenish red |
| Main axis | | | | |
| Length | Short, measured to 100 mm | Long, measured up to 250 mm | Short, measured up to 120 mm | Long, measured up to 210 mm |
| Holdfast | Discoidal | Discoidal | Discoidal | Discoidal |
| Thallus | Segmented | Thin | Segmented | Constricted |
| Braches | Cylindrical | Cylindrical, compressed | Cylindrical, | Cylindrical, constricted at base, swelling at middle, and tapering end |
| Branching pattern | Dichotomous | Irregular | Irregular | Irregular |
| Identification | *Laurencia* sp. | *Gracilaria manilaensis* | *Gracilaria manilaensis* | *Gracilaria manilaensis* |

### 4.1.3 Molecular Approach to Seaweed Species Identification

DNA was extracted from the four seaweed samples using InnuPrep Plant DNA Kit (Analytik Jena). DNA yield and purity ($A_{260}/A_{280}$ ratio value) of seaweed samples are listed in Table 4.3.

Table 4.3 DNA Yield and Purity of Seaweed Samples

| Seaweed Sample | Yield Volume | Concentration | $A_{260}/A_{280}$ value |
|---|---|---|---|
| SWD 1 | 50 µL | 75.3 ng/µL | 2.21 |
| SWD 2 | 50 µL | 480 ng/µL | 1.85 |
| SWD 3 | 50 µL | 348.1 ng/µL | 2.05 |
| SWD 4 | 50 µL | 430 ng/µL | 2.21 |

DNA material obtained were amplified using universal eukaryotic PCR primers EukA/EukB or 18s (f/r) pair, and sent for sequencing. Sequence data obtained were curated and submitted to NCBI for BLASTn search of nearest neighbour and accession number listed in Table 4.4.

Table 4.4 Accession Number of Seaweed Samples and Nearest Neighbour by BLASTn Search

| Sample ID | NCBI Accession No. | Description | Nearest Neighbour and Accession No. | Identity (%) |
|---|---|---|---|---|
| SWD1 | MN447109 | *Laurencia sp.* | *Laurencia filiformis* (AF203894), | 97 |
| | | | *Laurencia snackeyi* (MF093932) | 97 |

Table 4.4 Accession Number of Seaweed Samples and Nearest Neighbour by BLASTn Search (continued)

| Sample ID | NCBI Accession No. | Description | Nearest Neighbour and Accession No. | Identity (%) |
|---|---|---|---|---|
| SWD2 | MN447110 | *Gracilaria manilaensis* | *Gracilaria* sp. (DQ399795) | 99 |
| | | | *Gracilaria* sp. (AF468898) | 99 |
| SWD3 | MN447111 | *Gracilaria manilaensis* | *G. vermiculophylla* (GQ292853) | 97 |
| | | | *G. gracilis* (AY617147) | 97 |
| SWD4 | MN447112 | *Gracilaria manilaensis* | *G. dura* (DQ399795) | 98 |
| | | | *G. gracilis* (AY617147) | 98 |

### 4.1.4 Phylogenetic analysis of seaweed *18S rRNA*

Sequence data of seaweeds were aligned with nearneighbour sequence data obtained from NCBI along with other seaweeds that were documented in literature, which have symbiont bacteria. The phylogenetic tree was constructed using ARB and is illustrated in Figure 4.5. The sequences of seaweed specimens SWD1-4 formed a distinct, well-supported (99% bootstrap support) clade that did not contain any other Genbank sample.

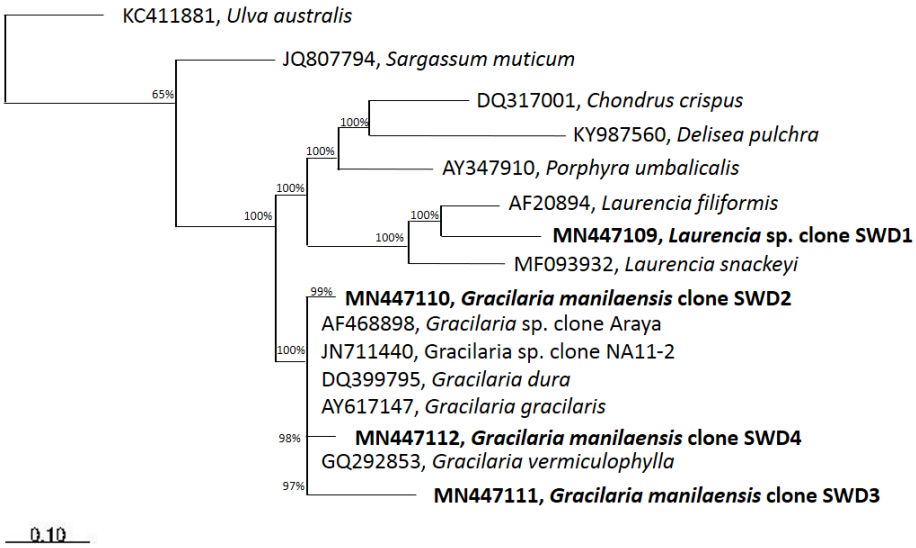



Figure 4.5 Bayesian Phylogram of Seaweed Species Based on *18S rRNA* Gene

The results demonstrated that the seaweed sample SWD1 sequence was from the genus *Laurencia*, and more specifically are not *L. snackeyi* or *L. filiformis* due to the branch length and 97% similarity to both species *18S rRNA* gene sequences. It was also indicated that the seaweed samples SWD2–SWD4 sequences were from the genus *Gracilaria*. However, there were no *G. manilaensis* sequences available in Genbank for the region of *18S rRNA* gene that was sequenced for comparison and species identity confirmation.

## 4.2 PART II: BACTERIAL DIVERSITY OF GRACILARIA MANILAENSIS

In Part II, the findings of bacterial OTUs and isolates obtained by amplicon sequencing (culture-independent approach) and through culture-dependent approach are presented.

### 4.2.1 Culture-Independent Approach Sequence Data Analysis

Total DNA was extracted from the seaweed samples (Kedah, Penang 1, and Penang 2) using the CTAB method, and the yield and purity of the DNA extracted was determined by NanoDrop™ 2000 Spectrophotometer prior to the samples sent for amplicon sequencing using the Illumina Hi-Seq 250 platform. The extracted DNA yield and purity ($A_{260}/A_{280}$ ratio) is shown in Table 4.5.

Table 4.5 Concentration of Genomic DNA Extracted

| No. | Sample Id | Volume | Concentration (Nanodrop) | $A_{260}/A_{280}$ value | Platform |
|---|---|---|---|---|---|
| 1 | SWD2 Kedah | 50 µL | 275.1 ng/L | 2.09 | Illumina HiSeq 250 |
| 2 | SWD3 Penang 1 | 50 µL | 763.0 ng/L | 1.91 | Illumina HiSeq 250 |
| 3 | SWD4 Penang 2 | 50 µL | 401.9 ng/L | 1.97 | Illumina HiSeq 250 |

Amplicon was sequenced on Illumina paired-end platform to generate 250bp paired-end raw reads (Raw PE), and then assembled and pre-treated to obtain clean tags which the chimeric sequences were detected and removed to obtain the Effective Tags (Table 4.6).

Table 4.6 Data QC statistics

| Sample | Raw PE(#) | Effective Tags(#) | Base(nt) | AvgLen (nt) | Q30 | GC% | Effective % |
|---|---|---|---|---|---|---|---|
| SWD2 | 153,660 | 148,197 | 37,444,465 | 253 | 98.59 | 45.52 | 96.44 |
| SWD3 | 145,604 | 141,028 | 35,604,108 | 252 | 98.71 | 36.92 | 96.86 |
| SWD4 | 152,644 | 148,025 | 37,364,943 | 252 | 98.70 | 39.41 | 96.97 |

*Note*: Raw PE means PE reads; Effective Tags means tags after filtering chimera; Base means base number of Effective tags; AvgLen means average length of Effective Tags; Q30 mean the percentage of base quantity that greater than 30; GC (%) means GC content in Effective Tags; Effective (%) means the percentage of Effective tags in Raw PE.

Effective Tags were clustered by 97% sequence similarity into OTUs in order to analyse the bacterial species diversity. The rarefaction curve was then produced (Figure 4.6) as a graph of the estimated species richness of each sample plotted against the size of the sample collection (sequence number). It allows for the estimation of species-abundance distribution, measuring biological diversity by counting the number of validly described taxa or OTU, and ensures that as most species taxa or OTUs have been discovered by the re-sampling of the same sample collection. Hence, the curve rapidly increases at the beginning, for most common taxa are identified, and then plateaus since lesser new taxa are plotted.

From Figure 4.6, it shows a clear saturated plateau in all three samples plotted in the graph; hence, rarefaction analysis for sequences obtained for three seaweed samples showed high sequence coverage values. The rarefaction curve for the Kedah sample had the highest gradient plotted (at 148,197 effective tags) and the rarefaction curve for the Penang 2 sample had the lowest gradient plotted (at 148,025 effective

tags), although Penang 1 site had the lowest number of effective tags (141,028) with a moderately steep gradient rarefaction curve.

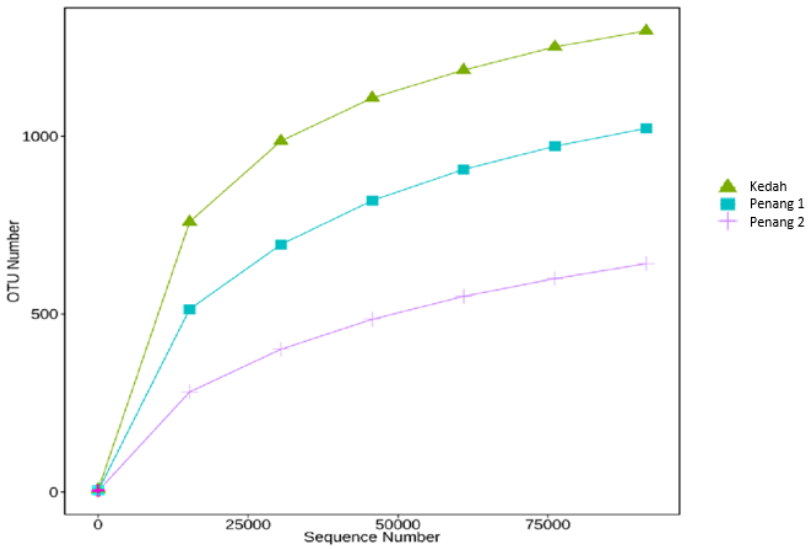



Figure 4.6 Rarefaction Curve

For Kedah and Penang 1 samples, the rarefaction curves grew rapidly at first as compared to the Penang 2 rarefaction curve. This would note that most common species are found during the initial process and then the curves converged into a plateau where samples meet saturation and only rare species could remain to be sampled. As all three rarefaction curves reached a plateau, this would suggest that the taxonomic diversity of the three sampled sites (Kedah, Penang 1, and Penang 2) were fully exploited.

#### *4.2.1.1 Alpha Diversity*

Alpha diversity is widely used to assess microbial diversity within the community. OTUs generated at 97% sequence identity were considered to be homologous in a species. Statistical indices of alpha diversity are summarised in Table 4.7, and it can

be observed, generally, that the Kedah seaweed sample had more species diversity than both Penang sites. For the Penang sites, Site 1 had more species diversity than Site 2.

Table 4.7 Alpha Diversity Indices Statistics

| Sample | Observed species | Shannon | Simpson | Chao | Goods coverage |
|---|---|---|---|---|---|
| SWD 2 (Kedah farm) | 1296 | 4.981 | 0.870 | 1489.848 | 0.997 |
| SWD3 (Penang 1) | 1022 | 3.495 | 0.756 | 1252.000 | 0.997 |
| SWD4 (Penang 2) | 642 | 2.425 | 0.662 | 890.629 | 0.997 |

In addition, a Venn diagram (Figure 4.7) was generated after reads were clustered into OTUs, the OTU table normalised, and both the common and unique information of the OTUs in the clone library for different seaweed samples were analysed. Each circle represents one seaweed sample group; blue circle for SWD2 which is the Kedah seaweed sample; green circle for SWD3 which is the Penang 1 seaweed sample; pink circle for SWD4 which is the Penang 2 seaweed sample. Values in overlapping parts represent common OTUs; total of 956 OTUs were clustered from all the samples, but only 463 OTUs were present in all 3 sample groups. SWD2 shared 320 OTUs with SWD3 and only 73 with SWD4, while SWD3 and SWD4 had 81 common OTUs. Others values are specific OTUs in each sample; SWD2 had the most specific OTUs (440), while SWD4 had the least specific OTUs (25).

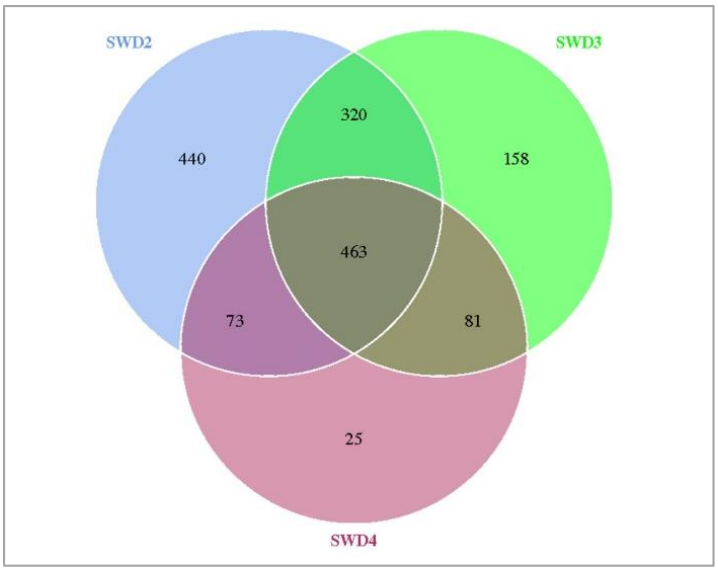



Figure 4.7 Venn Diagram of Seaweed Samples SWD2–SWD4

#### *4.2.1.2 Beta Diversity*

To study the dissimilarity among different samples, clustering analysis was applied by unweighted pair group method with arithmetic mean (UPGMA) which defines the similarity or dissimilarities between clusters as the average pair-wise distance between all their members. Weighted Unifrac distance matrix was calculated prior to UPGMA cluster analysis for quantitative measure and matrix is displayed with the integration of clustering results and the relative abundance of each sample by phylum in Figure 4.8 (a). The weighted Unifrac distance was then used to measure the dissimilarity coefficient between the sample groups in Principal component analysis (PCA) and Principal coordinates analysis (PCoA) plots, and in pair-wise samples by heat-map. The results of PCA, PCoA and heat-map analysis based on OTU abundances are shown in Figure 4.8 (b), (c), and (d), respectively.

From the weighted Unifrac distance matrix (Figure 4.8, a), the bacterial composition of SWD2 was most distantly dissimilar against SWD3 and SWD4, with the latter two groups differ distantly too. For the PCA and PCoA plots (Figure 4.8, b and c), each point represents a sample, plotted by a principal component on the X-axis and another principal component on the Y-axis, which was coloured by group. The percentage on each axis indicates the contribution value to discrepancy among samples.

The variation between the sample groups were high indicating that the bacterial compositions of each seaweed samples were significantly different irrespective the locations of sample collection being nearby to each other or further from each other and seaweed species host being the same. The heat-map representation (Figure 4.8, d) indicated that SWD2 had minor differences from SWD3 and SWD4, despite being from different locations. However, a greater difference (0.445) between SWD3 and SWD4 was observed even though the seaweed groups were sampled from close by locations.

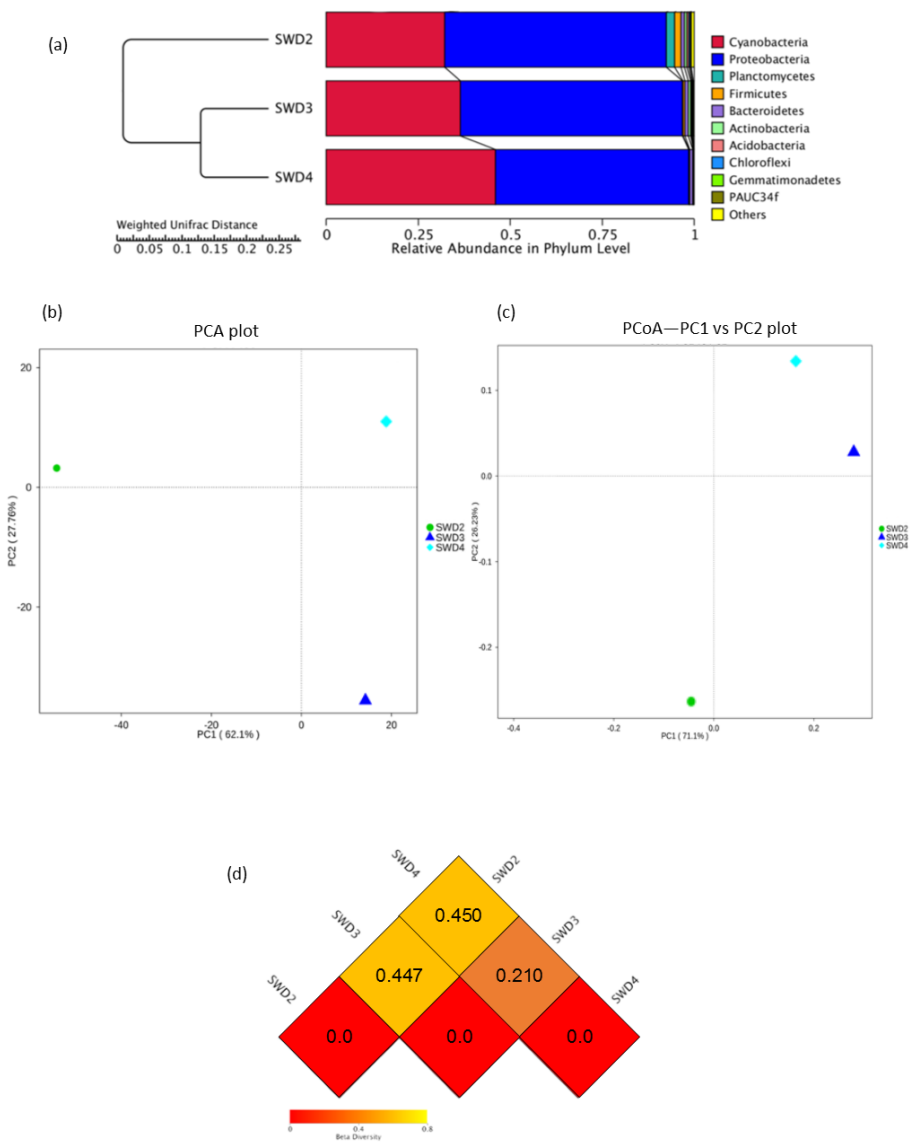



Figure 4.8 Beta Diversity Indices: (a) Weighted Unifrac Distance Matrix (SWD2: Kedah, SWD3: Penang 1, SWD4: Penang 2); (b) PCA plot; (c) PCoA plot; (d) Beta Diversity Heat-Map

#### *4.2.1.3 Predominant Bacterial OTU identification*

Bacterial species analysis was then done based on sequences normalised to 100 reads of the amplicon sequencing results output after removing mitochondrial and eukaryotic sequences (chloroplast *16S rRNA* from algal cells). A heat-map (Table 4.8) of predominant OTUs was generated to indicate species composition and abundance (% by effective tags) among the different samples. From the heat-map generated, there were 88 identified OTUs and the most abundant species overall was the Alphaproteobacteria (OTU_9, 36.11%) for *G. manilaensis* sample collected from Kedah site (SWD2), followed by Alphaproteobacteria Order Rhodobacterales (OTU_27, 10.86%) for seaweed sample collected from Penang Site 1 (SWD3), and Alphaproteobacteria Order Sphingomonadales (OTU_21, 10.74%) for *G. manilaensis* collected from Penang Site 1 (SWD4). Short read sequences of OTUs have been deposited in GenBank short-read archive (SRA: SRX6766236- SRX6766238).

Table 4.8 Heat-map for Species Composition and Abundance (In Percentage)

| #OTU ID | Consensus Lineage | SWD2 | SWD3 | SWD4 |
|---|---|---|---|---|
| OTU_9 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria | 36.11 | 0.90 | 0.91 |
| OTU_12 | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Vibrionales;f__Vibrionaceae;g__Vibrio | 7.58 | 1.60 | 1.03 |
| OTU_13 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae | 2.75 | 2.80 | 9.65 |
| OTU_14 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae | 4.67 | 0.51 | 0.30 |
| OTU_15 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae | 2.97 | 0.43 | 0.32 |
| OTU_16 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Erythrobacteraceae | 0.13 | 2.31 | 10.67 |
| OTU_17 | k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfobacterales;f__Desulfobulbaceae | 5.31 | 0.40 | 0.28 |
| OTU_18 | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__JTB255_marine_benthic_group;g__;s__ | 2.85 | 0.40 | 0.19 |
| OTU_19 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria | 2.06 | 2.02 | 5.03 |
| OTU_21 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Erythrobacteraceae;g__Erythrobacter | 0.31 | 1.89 | 10.74 |
| OTU_22 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Roseovarius | 0.08 | 0.02 | 0.26 |
| OTU_24 | k__Bacteria;p__Cyanobacteria;c__unidentified_Cyanobacteria;o__SubsectionI;f__FamilyI;g__Acaryochloris | 3.28 | 1.87 | 0.39 |
| OTU_25 | k__Bacteria;p__Planctomycetes;c__Planctomycetacia;o__Planctomycetales;f__Planctomycetaceae;g__Pir4_lineage;s__ | 2.03 | 0.12 | 0.02 |
| OTU_26 | k__Bacteria;p__Cyanobacteria;c__unidentified_Cyanobacteria;o__SubsectionIV;f__FamilyI | 0.45 | 3.13 | 0.91 |
| OTU_27 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae | 2.10 | 10.86 | 8.18 |
| OTU_28 | k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae;g__Muricauda | 1.31 | 0.66 | 1.17 |
| OTU_29 | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria | 0.76 | 0.97 | 0.65 |
| OTU_30 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae | 0.07 | 2.01 | 4.43 |
| OTU_31 | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__Alteromonadaceae;g__Alteromonas;s__ | 0.90 | 1.14 | 1.77 |
| OTU_33 | k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfuromonadales | 0.00 | 0.26 | 0.02 |
| OTU_35 | k__Bacteria;p__Cyanobacteria;c__unidentified_Cyanobacteria;o__SubsectionI;f__FamilyI;g__;s__ | 0.33 | 2.31 | 0.81 |
| OTU_36 | k__Bacteria;p__Actinobacteria;c__Acidimicrobiia;o__Acidimicrobiales | 0.02 | 3.88 | 0.05 |
| OTU_38 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae | 0.62 | 9.79 | 6.05 |

| | | | | |
|---|---|---|---|---|
| OTU_40 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Parvularculales;f__Parvularculaceae;g__;s__ | 1.59 | 0.19 | 0.14 |
| OTU_41 | k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae;g__Robiginitalea | 0.05 | 0.97 | 2.72 |
| OTU_42 | k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfobacterales;f__Desulfobulbaceae | 0.80 | 0.06 | 0.05 |
| OTU_43 | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria | 0.46 | 0.72 | 0.47 |
| OTU_44 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae | 0.64 | 0.06 | 0.05 |
| OTU_45 | k__Bacteria;p__Proteobacteria | 0.44 | 0.63 | 0.25 |
| OTU_47 | k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfobacterales;f__Desulfobacteraceae;g__Desulfatitalea;s__ | 0.65 | 0.03 | 0.04 |
| OTU_48 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae | 0.13 | 5.99 | 4.68 |
| OTU_50 | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales;f__Oceanospirillaceae;g__Reinekea | 0.06 | 0.31 | 2.63 |
| OTU_51 | k__Bacteria;p__Planctomycetes;c__Planctomycetacia;o__Planctomycetales;f__Planctomycetaceae | 0.57 | 0.00 | 0.00 |
| OTU_52 | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales;f__Alcanivoracaceae;g__Alcanivorax;s__Alcanivorax_dieselolei | 0.38 | 0.73 | 0.28 |
| OTU_55 | k__Bacteria;p__Acidobacteria;c__Subgroup_9;o__;f__;g__;s__ | 0.29 | 0.58 | 0.23 |
| OTU_56 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiaceae;g__Rhizobium | 0.36 | 0.40 | 0.32 |
| OTU_57 | k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Burkholderiaceae;g__Ralstonia;s__Ralstonia_pickettii | 0.36 | 0.27 | 0.28 |
| OTU_58 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Novosphingobium;s__Novosphingobium_aromatici | 0.34 | 0.48 | 0.35 |
| OTU_59 | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Cellvibrionales;f__Cellvibrionaceae;g__Simiduia | 0.40 | 0.36 | 0.02 |
| OTU_60 | k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfurellales;f__Desulfurellaceae;g__G55 | 0.34 | 0.25 | 0.16 |
| OTU_61 | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria | 0.02 | 0.46 | 1.59 |
| OTU_62 | k__Bacteria;p__Cyanobacteria;c__unidentified_Cyanobacteria;o__SubsectionI;f__FamilyI;g__;s__ | 0.04 | 1.72 | 0.56 |
| OTU_63 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Labrenzia | 0.14 | 0.72 | 2.45 |
| OTU_64 | k__Bacteria;p__Planctomycetes;c__Planctomycetacia;o__Planctomycetales;f__Planctomycetaceae;g__Bythopirellula;s__ | 0.49 | 0.16 | 0.00 |
| OTU_65 | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Thiotrichales;f__unidentified_Thiotrichales;g__Caedibacter;s__ | 0.05 | 2.34 | 0.09 |
| OTU_66 | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria | 0.46 | 0.48 | 0.25 |
| OTU_67 | k__Bacteria;p__Acidobacteria;c__Holophagae;o__Subgroup_10;f__TK85;g__;s__ | 0.27 | 0.34 | 0.18 |
| OTU_69 | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__Alteromonadaceae | 0.02 | 1.59 | 0.04 |
| OTU_70 | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__Pseudomonas;s__Pseudomonas_balearica | 0.35 | 0.46 | 0.28 |
| OTU_71 | k__Bacteria;p__Cyanobacteria;c__unidentified_Cyanobacteria;o__SubsectionI;f__FamilyI;g__Prochlorococcus;s__ | 0.19 | 1.32 | 0.37 |
| OTU_72 | k__Bacteria;p__Proteobacteria;c__JTB23;o__;f__;g__;s__ | 0.30 | 0.34 | 0.21 |

| | | | | |
|---|---|---|---|---|
| OTU_72 | k__Bacteria;p__Proteobacteria;c__JTB23;o__;f__;g__;s__ | 0.30 | 0.34 | 0.21 |
| OTU_73 | k__Bacteria;p__Cyanobacteria;c__unidentified_Cyanobacteria;o__SubsectionI;f__FamilyI | 0.31 | 0.25 | 0.11 |
| OTU_75 | k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae;g__Mesoflavibacter;s__Cytophaga_sp._I-545 | 0.00 | 0.10 | 2.21 |
| OTU_76 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae | 0.35 | 0.06 | 0.05 |
| OTU_77 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Rhodospirillaceae;g__;s__ | 0.25 | 0.33 | 0.21 |
| OTU_78 | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__Idiomarinaceae;g__Idiomarina;s__Idiomarina_baltica | 0.28 | 0.25 | 0.19 |
| OTU_79 | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__KI89A_clade;f__;g__;s__ | 0.00 | 0.08 | 0.19 |
| OTU_80 | k__Bacteria;p__Actinobacteria;c__Acidimicrobiia;o__Acidimicrobiales;f__unidentified_Acidimicrobiales;g__unidentified_Acidimicrobiales;s__Acidimicrobidae_ | 0.35 | 1.74 | 0.09 |
| OTU_83 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales;f__Hyphomonadaceae | 0.20 | 1.24 | 0.14 |
| OTU_89 | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria | 0.03 | 0.03 | 0.00 |
| OTU_92 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Erythrobacteraceae;g__Erythrobacter | 0.97 | 1.68 | 1.66 |
| OTU_93 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae | 0.38 | 2.23 | 0.81 |
| OTU_94 | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales;f__Oceanospirillaceae;g__Marinomonas;s__Marinomonas_communis | 0.32 | 0.19 | 0.09 |
| OTU_95 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales;f__Hyphomonadaceae;g__Hellea;s__ | 0.09 | 0.84 | 0.39 |
| OTU_98 | k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae | 0.34 | 0.31 | 0.14 |
| OTU_100 | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Moraxellaceae;g__Acinetobacter | 0.26 | 0.25 | 0.09 |
| OTU_102 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhodobiaceae;g__Anderseniella;s__ | 0.44 | 1.23 | 0.26 |
| OTU_107 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;g__Filomicrobium;s__ | 0.39 | 0.81 | 0.04 |
| OTU_108 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Erythrobacteraceae | 1.03 | 2.10 | 1.89 |
| OTU_110 | k__Bacteria;p__Gemmatimonadetes;c__BD2-11_terrestrial_group;o__;f__;g__;s__ | 0.25 | 0.21 | 0.21 |
| OTU_121 | k__Bacteria;p__PAUC34f;c__;o__;f__;g__;s__ | 0.23 | 0.45 | 0.19 |
| OTU_136 | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__CS-B046;f__;g__;s__ | 0.34 | 0.10 | 0.00 |
| OTU_138 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__AT-s3-44;g__;s__ | 0.35 | 0.37 | 0.28 |
| OTU_151 | k__Bacteria;p__Actinobacteria;c__Acidimicrobiia;o__Acidimicrobiales;f__Sva0996_marine_group;g__;s__ | 0.01 | 0.43 | 0.33 |
| OTU_188 | k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae;g__Tenacibaculum | 0.05 | 1.23 | 0.23 |
| OTU_195 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae | 0.16 | 0.02 | 0.26 |
| OTU_197 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae | 0.43 | 0.04 | 0.02 |
| OTU_199 | k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfuromonadales;f__Desulfuromonadaceae | 0.35 | 0.01 | 0.02 |

| | | | | |
|---|---|---|---|---|
| OTU_292 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Erythrobacteraceae;g__Erythrobacter | 0.12 | 0.87 | 0.28 |
| OTU_373 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae | 0.02 | 0.60 | 1.98 |
| OTU_472 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Actibacterium;s__ | 0.14 | 0.90 | 0.35 |
| OTU_473 | k__Bacteria;p__Planctomycetes;c__Planctomycetacia;o__Planctomycetales;f__Planctomycetaceae;g__Pir4_lineage | 1.43 | 0.13 | 0.19 |
| OTU_566 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae | 0.15 | 1.59 | 2.07 |
| OTU_736 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae | 0.16 | 1.50 | 1.05 |
| OTU_879 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae | 0.13 | 2.79 | 1.10 |
| OTU_941 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae | 0.16 | 0.97 | 0.28 |
| OTU_1567 | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Vibrionales;f__Vibrionaceae;g__Vibrio | 1.28 | 0.40 | 0.19 |
| OTU_1591 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae | 0.28 | 1.56 | 0.53 |

*Note*: percentage calculated by dividing PE over Effective Tags of sample based on sequences normalised to 100 reads

Proteobacteria was the dominant phylum (70%) sequenced from the seaweed sample sent for amplicon sequencing (Figure 4.9), followed by phyla Cyanobacteria (24%) and Planctomycetes (4%). Only 1 sequence (2%) was identified to belong to Bacteroidetes. Within the Phylum Proteobacteria, three groups were identified; Alphaproteobacteria was the dominant group (71.87%), followed by Gammaproteobacteria (18.75%) and Deltaproteobacteria (9.375%).

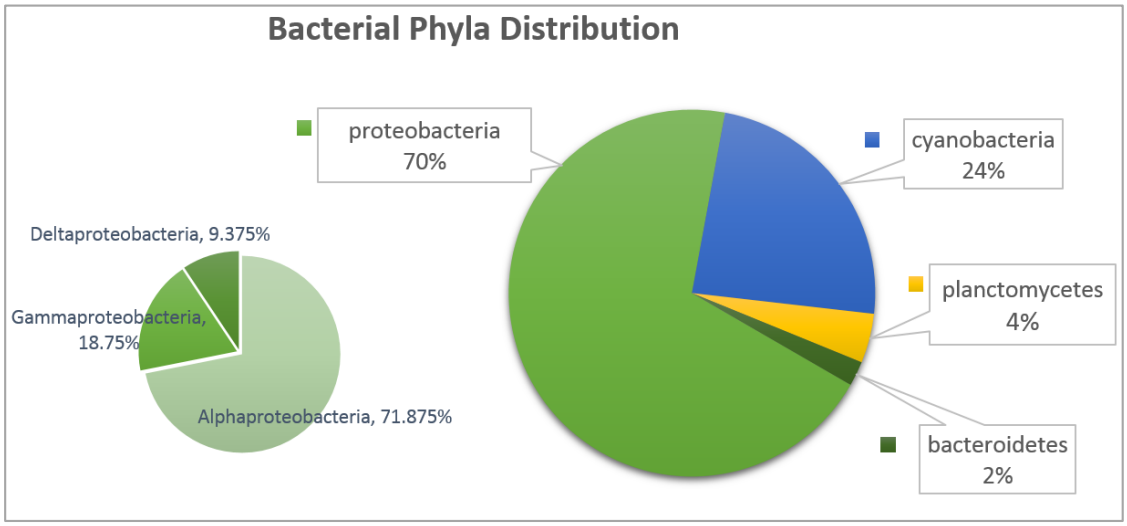



Figure 4.9 Pie-chart of Bacteria Phyla Distribution from Amplicon Sequencing

### 4.2.2 Bacterial Isolation and PCR

A total of 1022 growing colonies were observed from eight different media. Appearances of the colonies observed included circular and irregular colonies with colour pigments varying from white, yellow, orange, pink, brown, and black, as illustrated in Figure 4.10.

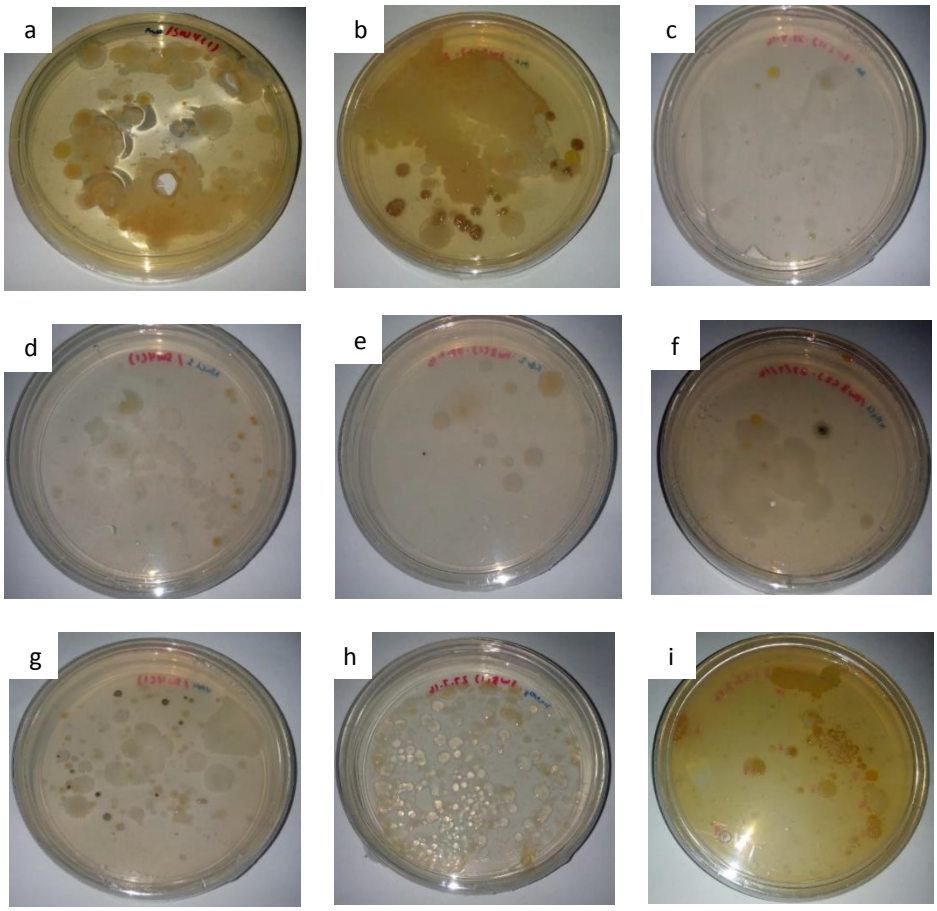



Figure 4.10 (a-i) Appearances of Colonies

From that total 1022 colony count, 83 isolates were selected based on different media source and morphologies for species identification based on *16S rRNA* gene analysis. Total number of visible colony, PCR amplicons, and OTUs based on media and seaweed source location is presented in Table 4.9.

Table 4.9 Number of Visible Colony, PCR Amplicons, and OTUs Based on Media and Seaweed Location

| No. | Media[a] | Isolation Source | Location | Visible colony (n) | No. of PCR amplicons | No. of OTU[b] |
|---|---|---|---|---|---|---|
| 1 | AIA | SWD2 | Kedah (farm) | 33 | 6 | 2 |
| | | SWD3 | Penang (1) | 39 | 6 | 3 |
| | | SWD4 | Penang (2) | 14 | 5 | 3 |
| 2 | KBr | SWD2 | Kedah (farm) | 35 | 4 | 3 |
| | | SWD3 | Penang (1) | 35 | 2 | 1 |
| | | SWD4 | Penang (2) | 23 | 1 | 0 |
| 3 | KI | SWD2 | Kedah (farm) | 16 | 3 | 3 |
| | | SWD3 | Penang (1) | 27 | 3 | 2 |
| | | SWD4 | Penang (2) | 51 | 1 | 1 |
| 4 | MA | SWD2 | Kedah (farm) | 20 | 5 | 1 |
| | | SWD3 | Penang (1) | 45 | 8 | 1 |
| | | SWD4 | Penang (2) | 3 | 4 | 3 |
| 5 | NaCl | SWD2 | Kedah (farm) | 14 | 3 | 1 |
| | | SWD3 | Penang (1) | 63 | 3 | 1 |
| | | SWD4 | Penang (2) | 94 | 1 | 1 |
| 6 | $NaNO_3$ | SWD2 | Kedah (farm) | 39 | 4 | 2 |
| | | SWD3 | Penang (1) | 33 | 3 | 2 |
| | | SWD4 | Penang (2) | 79 | 1 | 0 |
| 7 | $NH_4Cl$ | SWD2 | Kedah (farm) | 29 | 6 | 5 |
| | | SWD3 | Penang (1) | 88 | 4 | 2 |
| | | SWD4 | Penang (2) | 67 | 1 | 1 |

Table 4.9 Number of Visible Colony, PCR Amplicons, and OTUs Based on Media and Seaweed Location (continued)

| No. | Media[a] | Isolation Source | Location | Visible colony (n) | No. of PCR amplicons | No. of OTU[b] |
|---|---|---|---|---|---|---|
| 8 | SA | SWD2 | Kedah (farm) | 28 | 5 | 3 |
| | | SWD3 | Penang (1) | 42 | 3 | 2 |
| | | SWD4 | Penang (2) | 32 | 1 | 0 |

a See Section 3.5 Methodology for explanations of the different media used (names for some media have been abbreviated here; the full names (and additional components) of the media, by number, can be found in the text).

b Diversity observed at 97% sequence similarity based in different media

#### *4.2.2.1 Proteobacteria Predominates the Isolates from Red Seaweed,* **Gracilaria manilaensis**

From the total of 83 isolates selected, 53 *16S rRNA* gene amplicons were successfully amplified and sequence. A total of 43 OTUs were clustered at 97% sequence similarity (Table 4.10). Interestingly, out of 43 OTUs, there are 23 OTUs with percentage of similarity less than 97%, indicating possible novel isolates. Comparison of isolates with its near neighbours from NCBI database showed various isolation sources namely from seawater, various seaweed species, sediment, soil, seagrass and other marine invertebrates such as sponges. The sequences of isolates obtained in this study were submitted to NCBI database and assigned accession numbers MN396133–MN396175.

Dominant phylum isolated by the culture dependent approach was Proteobacteria (58.14%) as shown in Figure 4.11(a), with only Alphaproteobacteria (34.88%) and Gammaproteobacteria (23.26%) groups. Other phyla isolated were Firmicutes (27.91%), Actinobacteria (9.30%), and Bacteroidetes (4.65%). At genus level, *Bacillus* sp. predominated the isolates (17%) followed by *Thalassopir*a sp. (12%), *Labrenzia* sp. (10%) and the rest varied between 5% to 2%, shown in Figure 4.11(b).

Table 4.10 BLAST Table

| OTU | Isolate | Origin | NCBI Accession number | Nearest neighbour description & Accession number | Similarity (%) | Isolation source | Phylum |
|---|---|---|---|---|---|---|---|
| 1 | SA117 | SWD2 | MN396136 | *Labrenzia alba* isolate M2B125, partial *16S rRNA* gene [LN812985] | 98 | *Cotylorhiza tuberculata* | α-Proteobacteria |
| 2 | SA119 | SWD2 | MN396137 | *Labrenzia alba* isolate CMS163, partial *16S rRNA* gene [FR750958] | 95 | Seawater | α-Proteobacteria |
| 3 | KI125 | SWD2 | MN396136 | *Labrenzia aggregata* strain 0194, 16S ribosomal RNA gene, partial sequence [KP236323] | 92 | Sediment | α-Proteobacteria |
| 4 | KI126 | SWD2 | MN396143 | *Stappia* sp. SCS5m-7 16S ribosomal RNA gene, partial sequence [JX533670] | 95 | Seawater | α-Proteobacteria |
| 5 | MA141 | SWD2 | MN396152 | *Erythrobacter* sp. 1LE25 gene for *16S rRNA*, partial sequence [AB435653] | 96 | Seawater | α-Proteobacteria |
| 6 | AI147 | SWD3 | MN396155 | *Thalassospira* sp. URN67 gene for 16S ribosomal RNA, partial sequence [AB916875] | 97 | Seawater | α-Proteobacteria |
| 7 | SA152 | SWD3 | MN396158 | Rhodobacteraceae bacterium GUDS1112, 16S ribosomal RNA gene, partial sequence [KF282366] | 96 | Sponge, *Rhabdastrella globostellata* | α-Proteobacteria |
| 8 | KI155 | SWD3 | MN396160 | *Thalassospira* sp. SCS800m-1 16S ribosomal RNA gene, partial sequence [JX533661] | 96 | Seawater | α-Proteobacteria |

Table 4.10 BLAST Table (continued)

| OTU | Isolate | Origin | NCBI Accession number | Nearest neighbour description & Accession number | Similarity (%) | Isolation source | Phylum |
|---|---|---|---|---|---|---|---|
| 9 | KI156 | SWD3 | MN396161 | *Labrenzia* sp. strain 1334-011 16S ribosomal RNA gene, partial sequence [KY770036] | 97 | Phycosphere *Synechococcus* sp. 1334 | α-Proteobacteria |
| 10 | NN165 | SWD3 | MN396165 | *Thalassospira profundimaris* strain UMTAL06 16S ribosomal RNA gene, partial sequence [KJ721943] | 98 | Dinoflagellate culture | α-Proteobacteria |
| 11 | NN167 | SWD3 | MN396166 | Rhodobacteraceae bacterium GUDS1112, 16S ribosomal RNA gene, partial sequence [KF282366] | 98 | Sponge, *Rhabdastrella globostellata* | α-Proteobacteria |
| 12 | NH180 | SWD4 | MN396169 | Stappia sp. strain 7002-100 16S ribosomal RNA gene, partial sequence [KY770528] | 99 | Phycosphere *Synechococcus* sp. 1334 | α-Proteobacteria |
| 13 | NC181 | SWD4 | MN396170 | *Martelella* sp. strain 1334-399 16S ribosomal RNA gene, partial sequence [KY770423] | 96 | Phycosphere *Synechococcus* sp. 1334 | α-Proteobacteria |
| 14 | K1186 | SWD4 | MN396171 | *Thalassospira profundimaris* strain mj01-PW1-OH20 16S ribosomal RNA gene, partial sequence [HQ425693] | 97 | Seawater | α-Proteobacteria |

Table 4.10 BLAST Table (continued)

| OTU | Isolate | Origin | NCBI Accession number | Nearest neighbour description & Accession number | Similarity (%) | Isolation source | Phylum |
|---|---|---|---|---|---|---|---|
| 15 | MA189 | SWD4 | MN396174 | *Thalassospira tepidiphila* strain 1-1B 16S ribosomal RNA gene, partial sequence. [NR_041492] | 94 | Seawater | α-Proteobacteria |
| 16 | KB120 | SWD2 | MN396138 | *Microbulbifer variabilis* strain Ni-2088, 16S ribosomal RNA gene, partial sequence [NR_041021] | 94 | Marine macro algae | γ-Proteobacteria |
| 17 | KB122 | SWD2 | MN396139 | *Microbulbifer variabilis* strain HNS025, 16S ribosomal RNA gene, partial sequence [JN128259] | 98 | Marine sponge | γ-Proteobacteria |
| 18 | KI124 | SWD2 | MN396141 | *Alcanivorax gelatiniphagus* strain MEBiC08158, 16S ribosomal RNA, partial sequence [NR_136483] | 94 | Seawater | γ-Proteobacteria |
| 19 | NH132 | SWD2 | MN396146 | *Alcanivorax* sp. CBF L53 gene for *16S rRNA*, partial sequence [AB166953] | 94 | Sediment | γ-Proteobacteria |
| 20 | NH135 | SWD2 | MN396149 | *Microbulbifer* sp. HB09007, 16S ribosomal RNA gene, partial sequence [FJ796077] | 97 | Seawater | γ-Proteobacteria |
| 21 | MA142 | SWD3 | MN396153 | *Pseudomonas pachastrellae* strain M6-2, 16S ribosomal RNA gene, partial sequence. [AY880298] | 99 | Korea | γ-Proteobacteria |

Table 4.10 BLAST Table (continued)

| OTU | Isolate | Origin | NCBI Accession number | Nearest neighbour description & Accession number | Similarity (%) | Isolation source | Phylum |
|---|---|---|---|---|---|---|---|
| 22 | MA177 | SWD4 | MN396167 | *Alteromonas* sp. LAM-1 gene for 16S ribosomal RNA, partial sequence [AB758585] | 97 | Red alga, *Porphyra yezoensis* | γ-Proteobacteria |
| 23 | AI178 | SWD4 | MN396168 | *Alcanivorax venustensis* strain 2PR54-12, 16S ribosomal RNA gene, partial sequence [EU440953] | 98 | Seawater | γ-Proteobacteria |
| 24 | AI188 | SWD4 | MN396173 | *Alteromonas alvinellae* strain HY757, 16S ribosomal RNA gene, partial sequence [AF288360] | 95 | Polychaete host: *Alvinella pompejana* | γ-Proteobacteria |
| 25 | MA190 | SWD4 | MN396175 | *Marinobacter aquaeolei* isolate OC-9, 16S ribosomal RNA gene, partial sequence [AY669169] | 96 | Sediment | γ-Proteobacteria |
| 26 | AI112 | SWD2 | MN396133 | *Bacillus* sp. strain 70049, 16S ribosomal RNA gene, partial sequence [MF045085] | 97 | Sediment | Firmicutes |
| 27 | AIA114 | SWD2 | MN396134 | *Bacillus firmus* strain BSCS11, 16S ribosomal RNA gene, partial sequence [HQ397584] | 89 | Sediment | Firmicutes |
| 28 | KB123 | SWD2 | MN396140 | *Virgibacillus salarius* gene for 16S rRNA, partial sequence, strain: BAFBB5 [LC259996] | 96 | Coral | Firmicutes |

Table 4.10 BLAST Table (continued)

| OTU | Isolate | Origin | NCBI Accession number | Nearest neighbour description & Accession number | Similarity (%) | Isolation source | Phylum |
|---|---|---|---|---|---|---|---|
| 29 | NC127 | SWD2 | MN396144 | *Halobacillus trueperi* strain CHB-27 16S ribosomal RNA gene, partial sequence. [KT023548] | 98 | Host: *Apostichopus japonicus* | Firmicutes |
| 30 | NH130 | SWD2 | MN396145 | *Bacillus aquimaris* strain NIOT-Cu-5, 16S ribosomal RNA gene, partial sequence [KJ575054] | 97 | Sediment | Firmicutes |
| 31 | NH134 | SWD2 | MN396148 | *Solibacillus silvestris* strain Am1 16S ribosomal RNA gene, partial sequence [GU226320] | 96 | Sediment | Firmicutes |
| 32 | AI144 | SWD3 | MN396154 | *Virgibacillus pantothenticus* strain CQB-32 16S ribosomal RNA gene, partial sequence. [KR347234] | 98 | Host: *Apostichopus japonicas* | Firmicutes |
| 33 | SA151 | SWD3 | MN396157 | *Halobacillus halophilus* strain CQB-39 16S ribosomal RNA gene, partial sequence [KR347238] | 97 | Host: *Apostichopus japonicas* | Firmicutes |
| 34 | KB154 | SWD3 | MN396159 | *Bacillus cereus* gene for *16S rRNA*, partial sequence, strain: DLTA4.32 [LC208134] | 99 | Seagrass | Firmicutes |

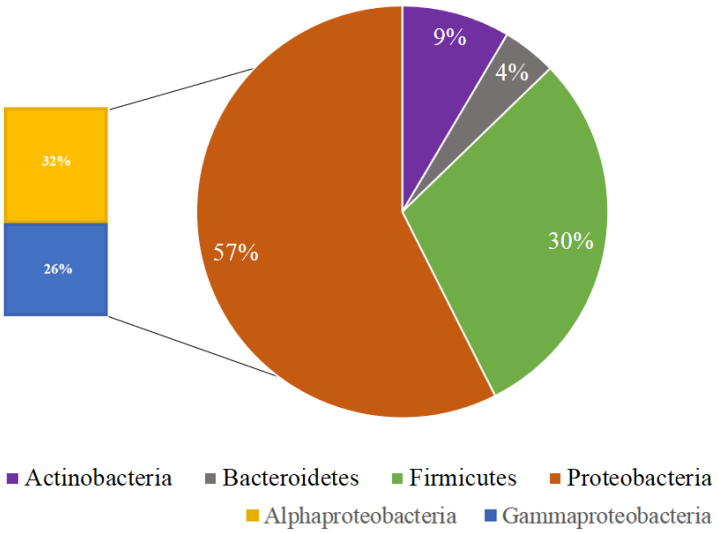
Table 4.10 BLAST Table (continued)

| OTU | Isolate | Origin | NCBI Accession number | Nearest neighbour description & Accession number | Similarity (%) | Isolation source | Phylum |
|---|---|---|---|---|---|---|---|
| 35 | NH162 | SWD3 | MN396163 | *Bacillus* sp. BAB-5784 16S ribosomal RNA gene, partial sequence [KX168057] | 92 | Soil | Firmicutes |
| 36 | NH164 | SWD3 | MN396164 | *Bacillus algicola* strain M1E7a 16S ribosomal RNA gene, partial sequence [JX501684] | 90 | Host: *Padina tetrastromatica* | Firmicutes |
| 37 | AI187 | SWD4 | MN396172 | *Bacillus cereus* strain: DLTA4.32 gene for *16S rRNA*, partial sequence [LC208134] | 94 | Seagrass | Firmicutes |
| 38 | SA116 | SWD2 | MN396135 | *Mycobacterium* sp. PRE12d 16S ribosomal RNA gene, partial sequence. KM187576 | 92 | Host: *Notophthalmus viridescens* | Actinobacteria |
| 39 | NN139 | SWD2 | MN396151 | *Streptomyces* sp. LCB 0297 16S ribosomal RNA gene, partial sequence [JF922881] | 99 | Host: *Podocarpus macrophyllus* | Actinobacteria |

Table 4.10 BLAST Table (continued)

| OTU | Isolate | Origin | NCBI Accession number | Nearest neighbour description & Accession number | Similarity (%) | Isolation source | Phylum |
|---|---|---|---|---|---|---|---|
| 40 | AI149 | SWD3 | MN396156 | *Mycobacterium* sp. 1110D-63, 16S ribosomal RNA gene, partial sequence [KF840235] | 99 | Sediment | Actinobacteria |
| 41 | NC159 | SWD3 | MN396162 | *Isoptericola* sp. BJGMM-B20 16S ribosomal RNA gene, partial sequence [JQ716226] | 95 | Soil | Actinobacteria |
| 42 | NH133 | SWD2 | MN396147 | *Flavobacterium* sp. strain Bacter-13 16S ribosomal RNA gene, partial sequence [MH671385] | 89 | Soil | Bacteroidetes |
| 43 | NN138 | SWD2 | MN396150 | Uncultured *Sphingobacterium* sp. clone DQ20 16S ribosomal RNA gene, partial sequence [KT427395] | 94 | Soil | Bacteroidetes |

(A) CULTIVABLE BACTERIA DISTRIBUTION BY PHYLUM




(B) CULTIVABLE BACTERIA DISTRIBUTION BY GENUS

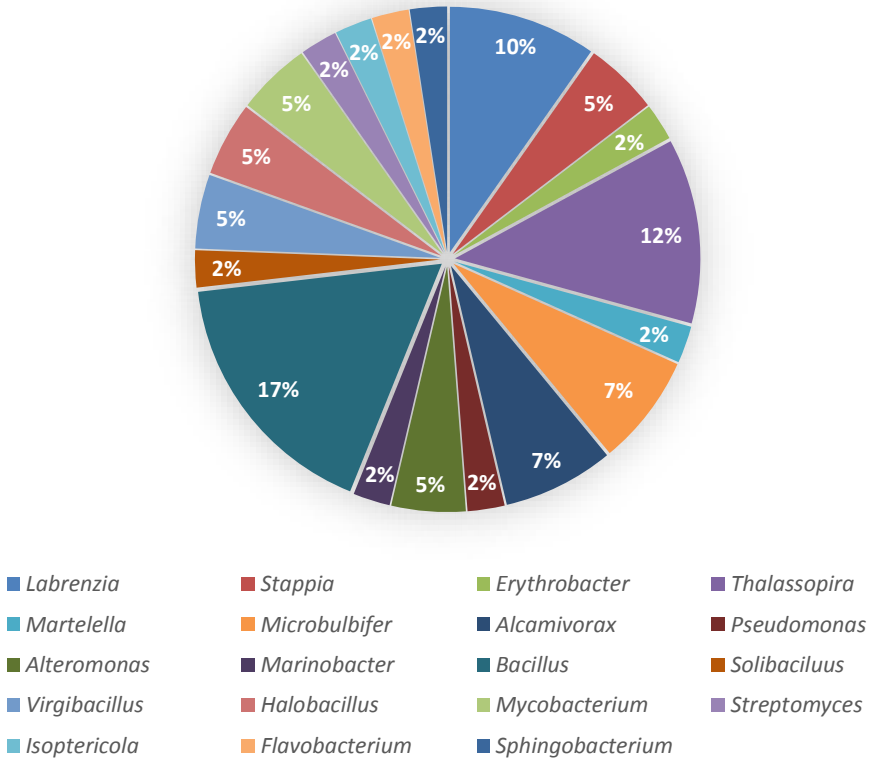



Figure 4.11 Distribution of Cultivable Bacteria Isolated from *G. manilaensis* by (A) Phylum, and (B) Genus.

The distribution of bacteria phyla (or class) is shown across the culture media types used in Figure 4.12. Alphaproteobacteria were present on all culture media types experimented except KB, and the next largest bacterial class Gammaproteobacteria grew on most culture media except NN, SA, and NC. Firmicutes did not favour the commercial marine media agar (MA), but did grew on commercial AIA, and it also favours growth on SA, KB, NC, and NH without growing on KI, and NN. Actinobacteria only grew on commercial AIA, laboratory SA, halide NC and nitrate NN, while there was no growth on halide supplemented media, KB and KI. Lastly, Bacteroidetes only grew on laboratory mixture culture media supplemented with ammonium salts, NHCl (NH) and $NaNO_3$ (NN).

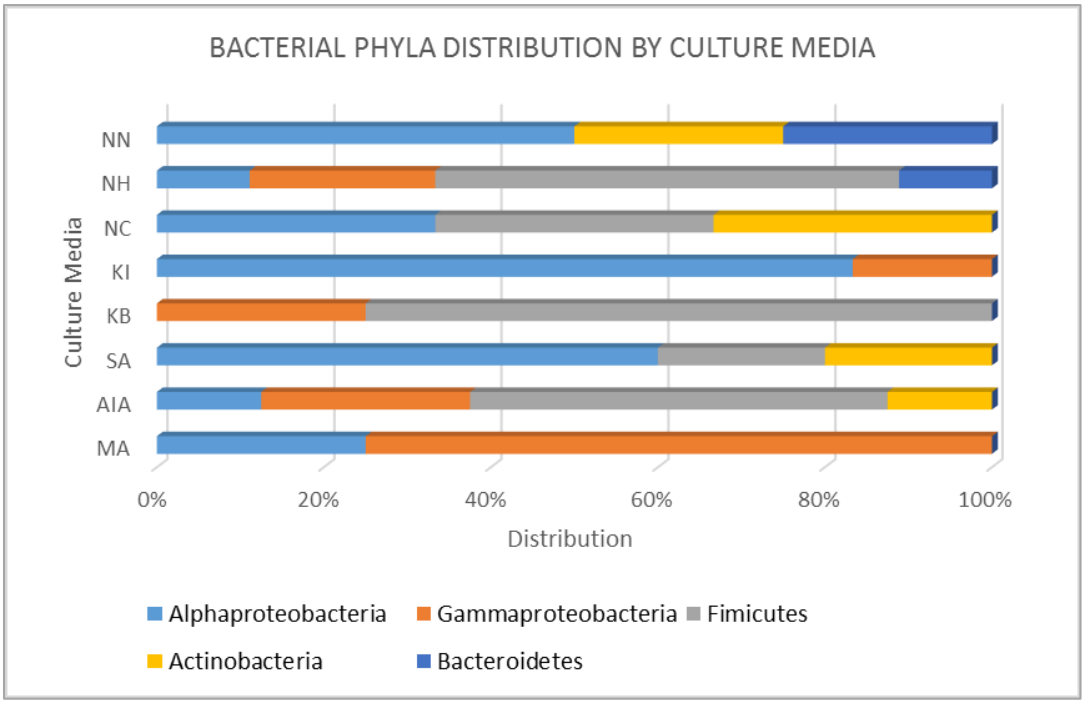



Figure 4.12 Graph of Bacteria Phyla Isolated by Media

When comparing the bacterial phyla compositions from different sites (Figure 4.13), it can be observed that all bacterial phyla were isolated more from the Penang sites than the Kedah site except for the phylum Bacteroidetes which had two

isolates from the Kedah site but none from the Penang sites. This could be due to the nature of bacteria that thrive in nutrient-rich or extreme environments that have higher salinity because the salinity recorded at the Penang site was 30 ppt, and it was much higher than the salinity recorded at the Kedah Farm site, which was 16 ppt. Other physico-chemical parameters recorded were similar; Kedah and Penang sites' water temperature were 32.6 °C with pH 7.68 and 32.0 °C with pH 7.77, respectively.

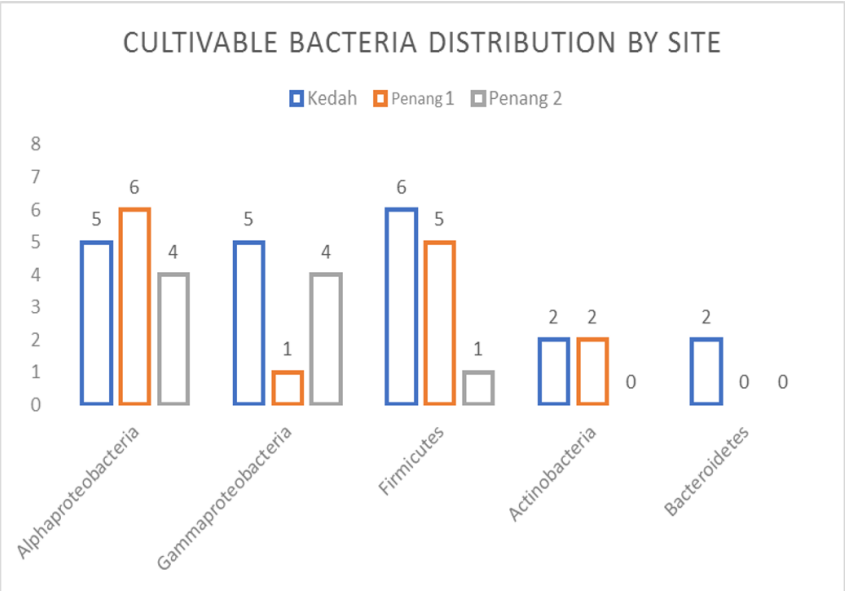



Figure 4.13 Graph of Bacteria Phyla Distribution Isolated from Sites; Kedah and Penang

### *4.2.2.2 Bromoperoxidase Gene Screening*

For the bromoperoxidase screening, only 2 isolates were able to be identified positive to have the 700-bp bromoperoxidase (BPO) gene fragment from 43 isolated OTUs, which were isolates SA116 and SA117. Sequences was BLASTx-searched for similarity of protein family from the nucleotide sequence, and the results included bromoperoxidases from *Bacillus* sp. and *Mycobacterium* sp. Sequence analysis

showed that the BPO gene screened belonged to the α/β hydrolase superfamily similar to the findings of Lilles (2011). A phylogenetic tree (Figure 4.14) was constructed using ARB software with the cloned BPO genes and bromoperoxidase genes (nearneighbours and from literature) as listed (Table 4.11) to illustrate the relationships of all the cloned BPO genes to other BPO genes with Bayesian posterior probability.

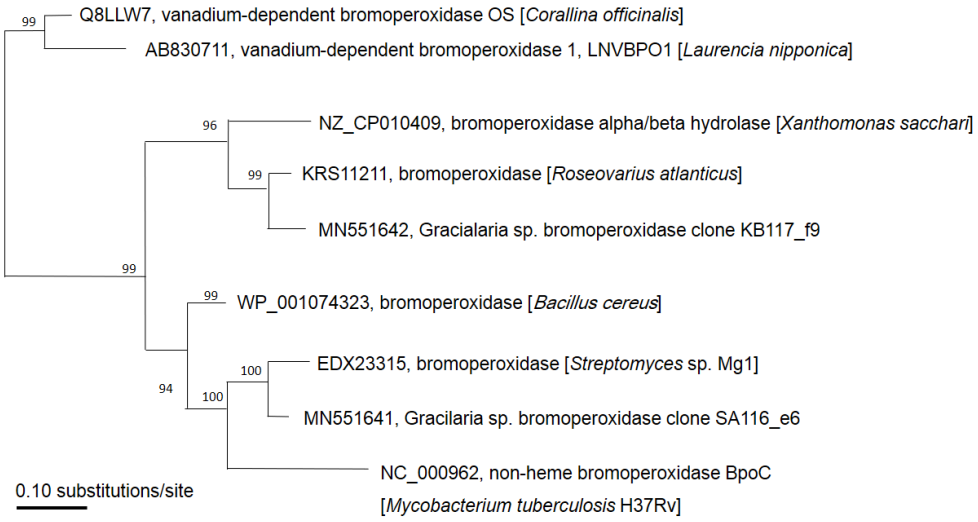



Figure 4.14 Bayesian Phylogram of Bromoperoxidase Genes Constructed Based on Protein Motif Translated Using BLASTx

From the phylogenetic tree, it indicated that the bacterial BPO gene clones were divergent from the seaweed VBPO gene sequences, and sequence data analysis indicated that we have we isolated bacteria with BPO in the alpha/beta hydrolase fold region gene family. The alpha/beta hydrolase fold includes proteases, lipases, peroxidases, esterases, epoxide hydrolases and dehalogenases, which might hint at the pathways that the bacteria may be involved in the bromination of secondary metabolites produce or regulated by seaweed-associated bacteria. With respect to that,

marine algae synthesise halogenated organics with antifouling potential, such as brominated heptanones, while most of the brominated compounds that bacteria synthesise are antibiotics (Lilles, 2011).

Table 4.11 List of GM Bromoperoxidase Genes Nearneighbours from BLASTx and Literature

| Clone | Nearneighbour description and accession number | Phylum |
|---|---|---|
| SA116_e6 | non-heme bromoperoxidase BpoC [*Mycobacterium tuberculosis* H37Rv], NC_000962 | Actinobacteria |
| | bromoperoxidase [*Streptomyces* sp. Mg1], EDX23315 | Actinobacteria |
| | bromoperoxidase [*Bacillus anthracis* str. Ames], NC_003997 | Firmicutes |
| KB117_f9 | bromoperoxidase [*Roseovarius atlanticus*], KRS11211 | Alphaproteobacteria |
| | bromoperoxidase alpha/beta hydrolase [*Xanthomonas sacchari*], NZ_CP010409 | Gammaproteobacteria |
| from literature | vanadium-dependent bromoperoxidase 1 [*Laurencia nipponica*], AB830711 | Eukarya, Rhodophyta |
| | Vanadium-dependent bromoperoxidase OS *Corallina officinalis*, Q8LLW7 | Eukarya, Rhodophyta |

### 4.2.3 Phylogenetic Analysis

Construction of the phylogenetic tree (Figure 4.15) was derived by multiple sequence alignment tool ARB with the *16S rRNA* gene sequences of the isolates, amplicon OTUs, nearest neighbours listed from the BLAST analysis of the partial *16S rRNA* gene sequences of isolated OTUs (Table 4.10, BLAST table) and SRAs (Amplicon sequencing BLAST table; Table A1, Appendix A). The tree is constructed of 9 phyla beginning with phylum Planctomycetes, which is related closest to the prokaryotic root, followed by phyla PAUC34f, Gemmatimonadetes, Acidobacteria, Firmicutes, Acidobacteria, Bacteroidetes, Cyanobacteria, and lastly, Proteobacteria (Classes α-Proteobacteria, β-Proteobacteria, δ-Proteobacteria, γ-Proteobacteria), which is illustrated as the branch most evolved from the prokaryotic root in the Bayesian phylogenetic tree.

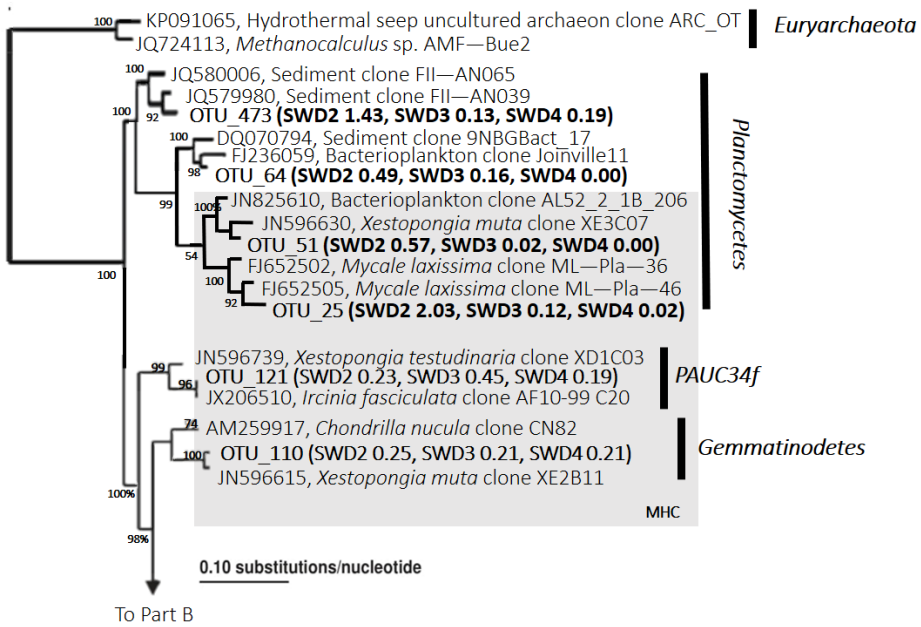



Figure 4.15 (a) Bayesian Phylogram of Bacterial Species Associated to *G. manilensis* (Part A)

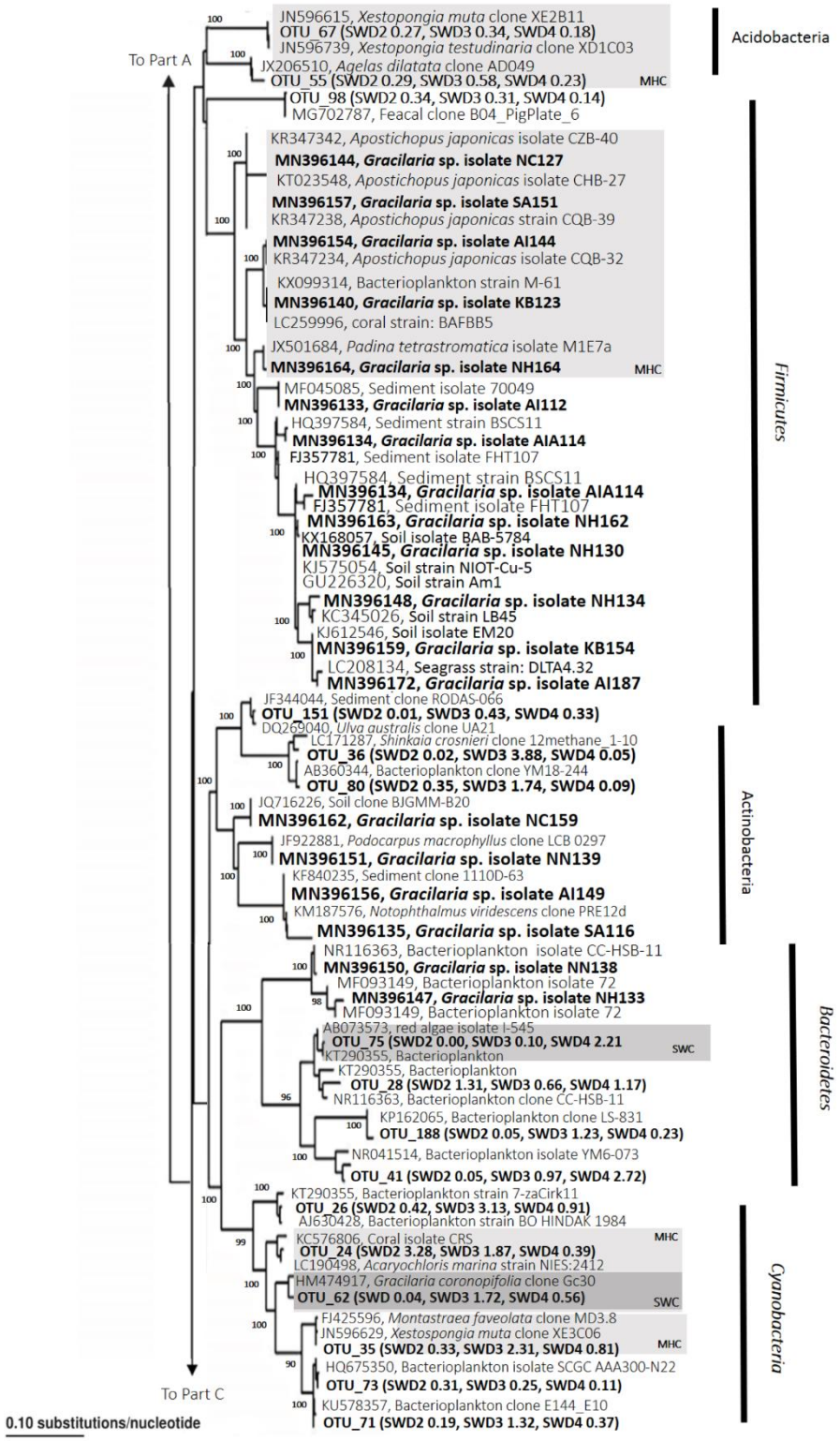



Figure 4.15 (b) Bayesian Phylogram of Bacterial Species Associated to *G. manilensis* (Part B)

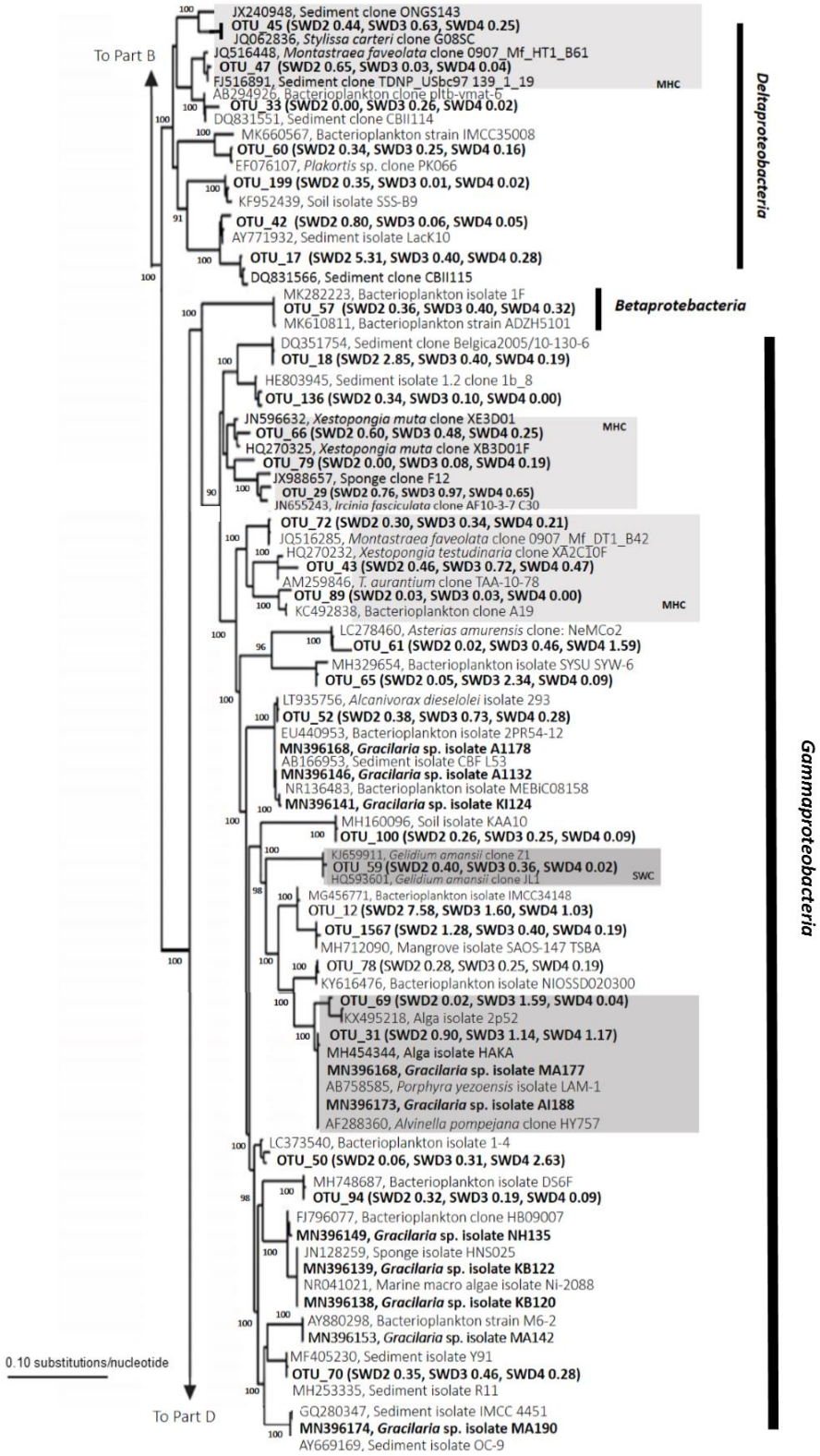



Figure 4.15 (c) Bayesian Phylogram Tree of Bacterial Species Associated to *G. manilensis* (Part C)

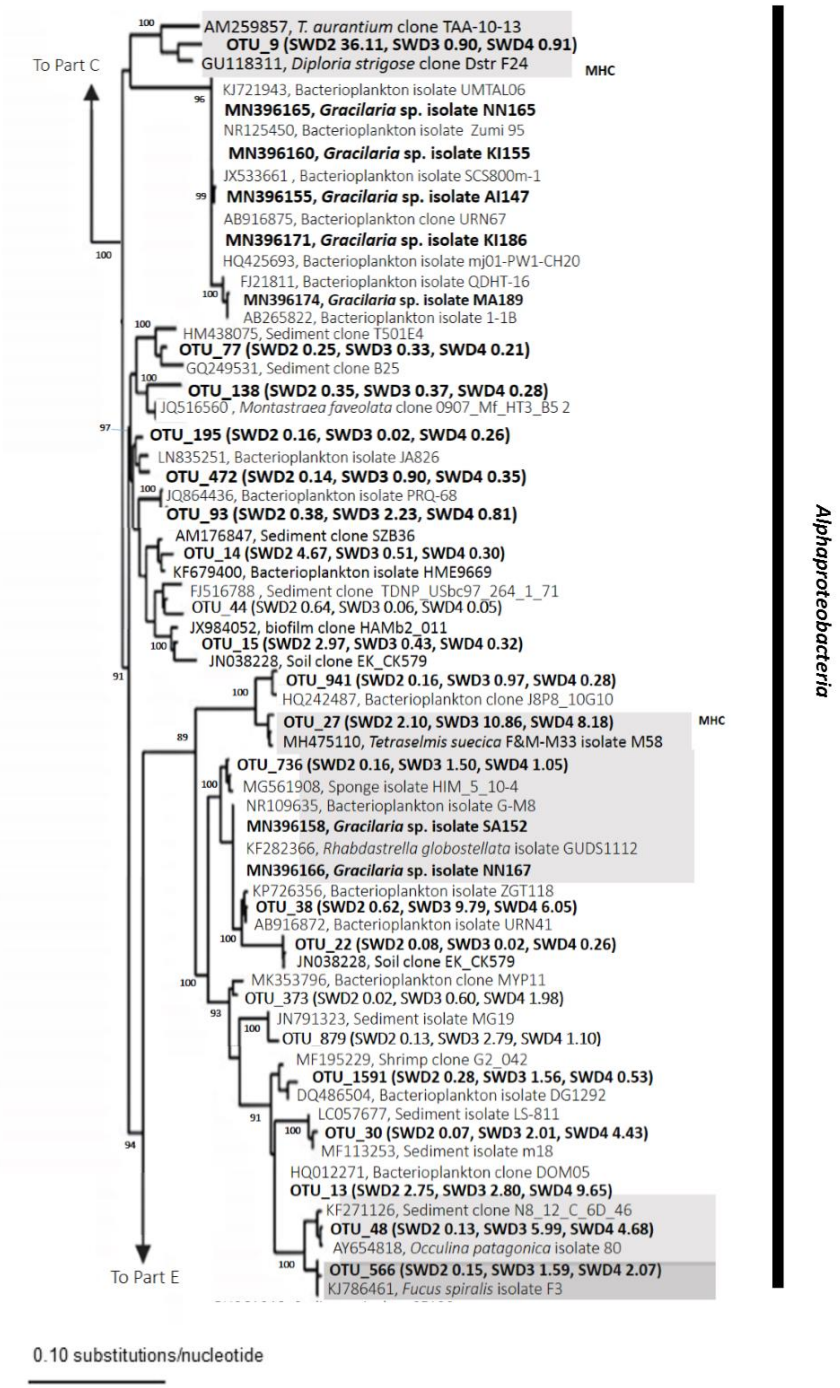



Figure 4.15 (d) Bayesian Phylogram of Bacterial Species Associated to *G. manilensis* (Part D)

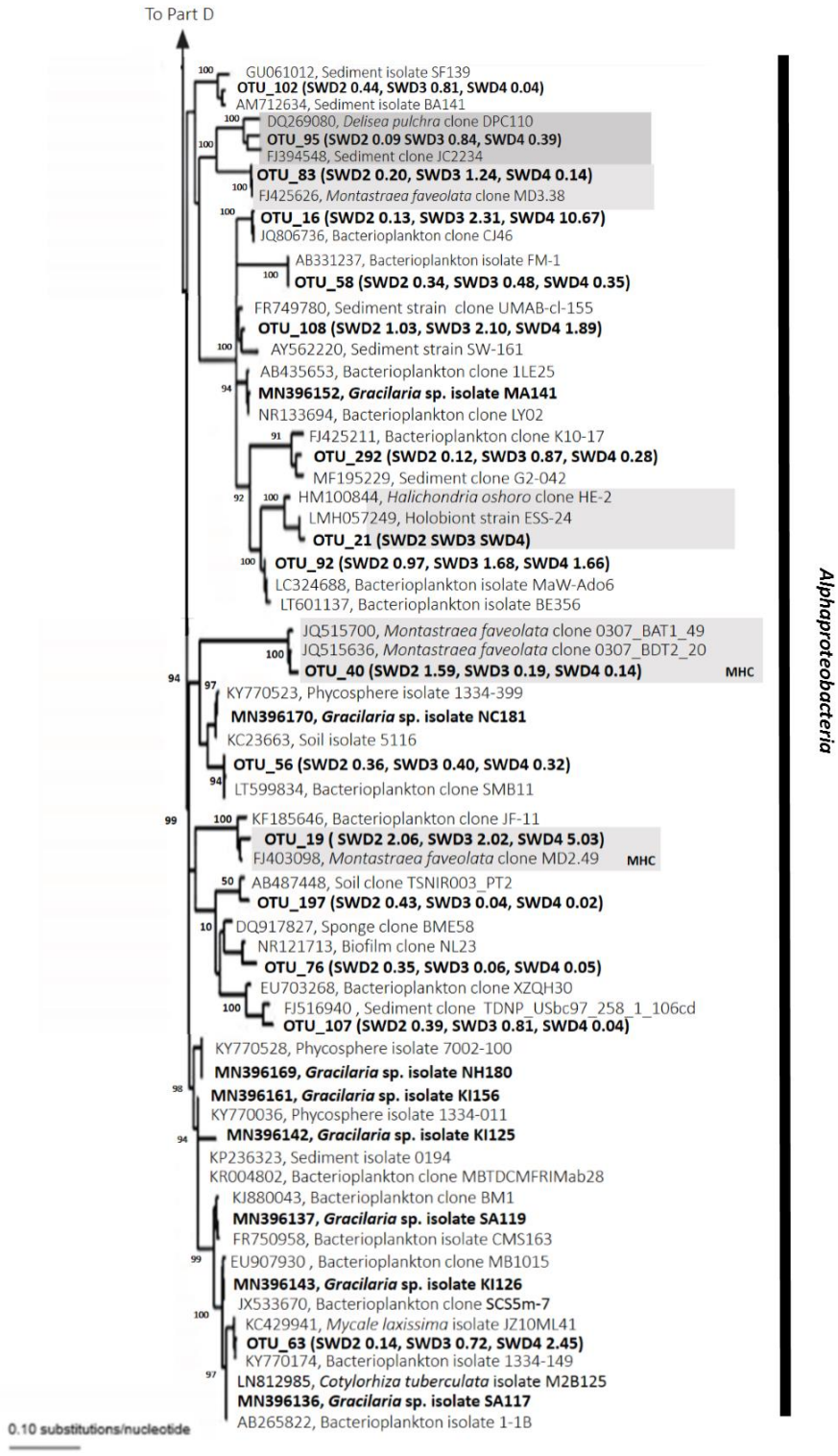



Figure 4.15 (e) Bayesian Phylogram Tree of Bacterial Species Associated to *G. manilensis* (Part E)

## 4.3 PART III: BACTERIAL DIVERSITY OF LAURENCIA SP.

The bacterial diversity of *Laurencia* sp. is presented in this section prior to the comparison of bacterial diversity of a non-edible red seaweed (*Laurencia* sp.), to that of an edible red seaweed, Gracilaria manilaensis, in the next section. Previous studies have reported that the genus *Laurencia* is prolific metabolite producer. Bacterial diversity presented could possibly give an insight to how halogenated bacterial metabolite could be produced.

### 4.3.1 Bacterial Taxonomic Analysis by Molecular Approach

From the Illumina reads, a total of 93,554 raw tag sequences were generated with 92,900 raw tags successfully combined. After chimera removal, a total of 91,309 were considered for analysis. After quality filtering and checking the OTUs, 75% of the tags was classified as chloroplast with a further 22% of the tags was classified as mitochondrion. As these genes are not of interest for bacterial DNA information, these tags were eliminated. Table 4.12 and Figure 4.16 highlight the statistical data generated from the amplicon sequencing of the *Laurencia* sp. sample.

Table 4.12 Statistical Data from Amplicon Sequencing *Laurencia* sp. Sample

| Parameter | Value |
|---|---|
| Raw PE | 3,554 |
| Raw Tags | 92,900 |
| Clean Tags | 91,964 |
| Effective Tags | 91,857 |
| Base (nt) | 23,510,250 |
| Avg Len (nt) | 256 |
| Q20 | 99.27 |
| Q30 | 98.57 |
| GC% | 39.70 |
| Effective (%) | 98.19 |

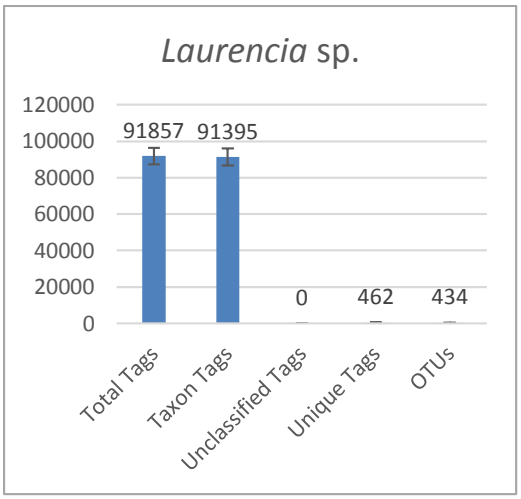



Figure 4.16 Graph of OTUs by Effective Tags

From that, 14 phyla were identified through the tag distribution and the predominant phylum was Proteobacteria (68.95%, Figure 4.17), followed by Actinobacteria (8.33%) and Cyanobacteria (7.41%). Other phyla include Chloroflexi (4.61%), Bacteroidetes, (2.52%), Planctomycetes (2.24%), Firmicutes (2.08), and Acidobacteria (1.40%), while minor bacteria distribution were identified from phyla PAUC34f (0.64%), Gemmatimonadetes (0.44%), Nitrospirae (0.20%), Poribacteria (0.12%), Tectomicrobia (0.12%), and Tenericutes (0.12%).

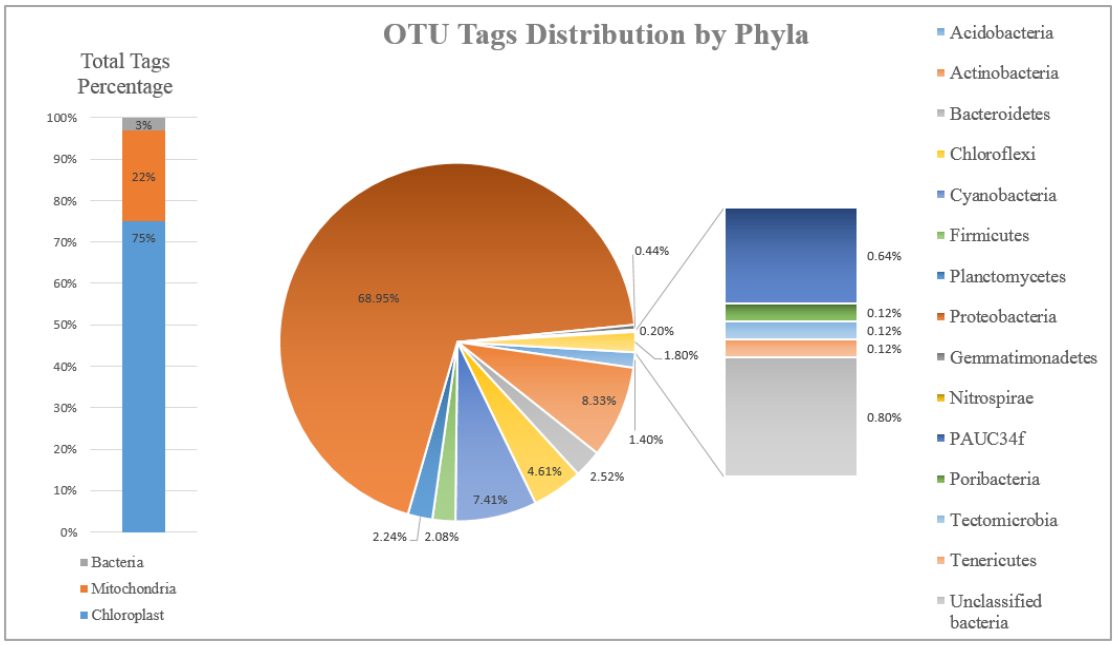



Figure 4.17 OTU Tags Distribution

However, after the sequences were normalised to 10 reads, only 1,725 sequence reads were considered as effective tags with the average length of 253 bp, and 48 OTUs were identified. A heat-map was generated to indicate species composition and abundance (percentage value (%) was generated by sequence reads divided by effective tags) in the seaweed sample (Table 4.13). The predominant bacterial species was identified under Phylum Proteobacteria, Subphyla Alphaproteobacteria (OTU_27) as the OTU sequence reads was the most abundant (8.8%).

Table 4.13 Heat-map for Species Composition and Abundance
(% of Sequence Reads Divided by Effective Tags)

| No. | #OTU_ID | Consensus Lineage | % |
|---|---|---|---|
| 1 | OTU_9 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria | 1.2 |
| 2 | OTU_12 | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Vibrionales;f__Vibrionaceae;g__Vibrio | 2.0 |
| 3 | OTU_13 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae | 3.6 |
| 4 | OTU_19 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria | 2.2 |
| 5 | OTU_21 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Erythrobacteraceae;g__Erythrobacter | 1.0 |
| 6 | OTU_24 | k__Bacteria;p__Cyanobacteria;c__unidentified_Cyanobacteria;o__SubsectionI;f__FamilyI;g__Acaryochloris | 1.4 |
| 7 | OTU_26 | k__Bacteria;p__Cyanobacteria;c__unidentified_Cyanobacteria;o__SubsectionIV;f__FamilyI | 2.7 |
| 8 | OTU_27 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae | 8.8 |
| 9 | OTU_29 | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria | 0.6 |
| 10 | OTU_30 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae | 0.9 |
| 11 | OTU_34 | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__Pseudoalteromonadaceae;g__Pseudoalteromonas | 0.9 |
| 12 | OTU_35 | k__Bacteria;p__Cyanobacteria;c__unidentified_Cyanobacteria;o__SubsectionI;f__FamilyI;g__;s__ | 2.6 |
| 13 | OTU_38 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae | 3.2 |
| 14 | OTU_43 | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria | 0.8 |
| 15 | OTU_48 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae | 2.8 |
| 16 | OTU_55 | k__Bacteria;p__Acidobacteria;c__Subgroup_9;o__;f__;g__;s__ | 0.6 |
| 17 | OTU_71 | k__Bacteria;p__Cyanobacteria;c__unidentified_Cyanobacteria;o__SubsectionI;f__FamilyI;g__Prochlorococcus;s__ | 1.4 |
| 18 | OTU_89 | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria | 6.7 |
| 19 | OTU_92 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Erythrobacteraceae;g__Erythrobacter | 0.6 |
| 20 | OTU_121 | k__Bacteria;p__PAUC34f;c__;o__;f__;g__;s__ | 0.6 |
| 21 | OTU_151 | k__Bacteria;p__Actinobacteria;c__Acidimicrobiia;o__Acidimicrobiales;f__Sva0996_marine_group;g__;s__ | 3.4 |
| 22 | OTU_152 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria | 3.8 |
| 23 | OTU_153 | k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae | 0.8 |
| 24 | OTU_155 | k__Bacteria;p__Cyanobacteria;c__unidentified_Cyanobacteria;o__SubsectionI;f__FamilyI;g__Synechococcus | 0.9 |
| 25 | OTU_175 | k__Bacteria;p__Actinobacteria;c__Acidimicrobiia;o__Acidimicrobiales | 2.5 |

| | | | |
|---|---|---|---|
| 26 | OTU_180 | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Chromatiales;f__Granulosicoccaceae;g__Granulosicoccus;s__ | 5.9 |
| 27 | OTU_187 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae | 2.8 |
| 28 | OTU_195 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae | 0.8 |
| 29 | OTU_204 | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria | 2.8 |
| 30 | OTU_207 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria | 2.7 |
| 31 | OTU_208 | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria | 2.5 |
| 32 | OTU_214 | k__Bacteria;p__Actinobacteria;c__Acidimicrobiia;o__Acidimicrobiales;f__Sva0996_marine_group | 2.6 |
| 33 | OTU_253 | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria | 3.9 |
| 34 | OTU_290 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae | 1.6 |
| 35 | OTU_295 | k__Bacteria;p__Chloroflexi;c__Ardenticatenia | 2.8 |
| 36 | OTU_303 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae | 1.4 |
| 37 | OTU_373 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae | 1.6 |
| 38 | OTU_410 | k__Bacteria;p__Chloroflexi;c__Caldilineae;o__Caldilineales;f__Caldilineaceae;g__;s__ | 1.1 |
| 39 | OTU_416 | k__Bacteria;p__Chloroflexi;c__Caldilineae;o__Caldilineales;f__Caldilineaceae | 1.1 |
| 40 | OTU_448 | k__Bacteria;p__Planctomycetes;c__Planctomycetacia;o__Planctomycetales;f__Planctomycetaceae;g__Blastopirellula;s__ | 0.6 |
| 41 | OTU_461 | k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Cryomorphaceae;g__;s__ | 0.9 |
| 42 | OTU_521 | k__Bacteria;p__Planctomycetes;c__Planctomycetacia;o__Planctomycetales;f__Planctomycetaceae;g__Planctomyces;s__ | 1.0 |
| 43 | OTU_566 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae | 1.6 |
| 44 | OTU_696 | k__Bacteria;p__Bacteroidetes;c__Sphingobacteriia;o__Sphingobacteriales;f__Saprospiraceae;g__Rubidimonas;s__ | 0.6 |
| 45 | OTU_719 | k__Bacteria;p__Actinobacteria;c__Acidimicrobiia;o__Acidimicrobiales | 1.0 |
| 46 | OTU_771 | k__Bacteria;p__Planctomycetes;c__Planctomycetacia;o__Planctomycetales;f__Planctomycetaceae;g__Blastopirellula;s__ | 0.6 |
| 47 | OTU_1574 | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria | 1.0 |
| 48 | OTU_1591 | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae | 3.0 |

After the OTUs were normalised to 10 reads, Proteobacteria still dominated the OTU distribution by 60%. Other bacterial OTUs observed were classified under phyla Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, PAUC43F and Planctomycetes as illustrated in the Phylum Distribution pie chart (Figure 4.18).

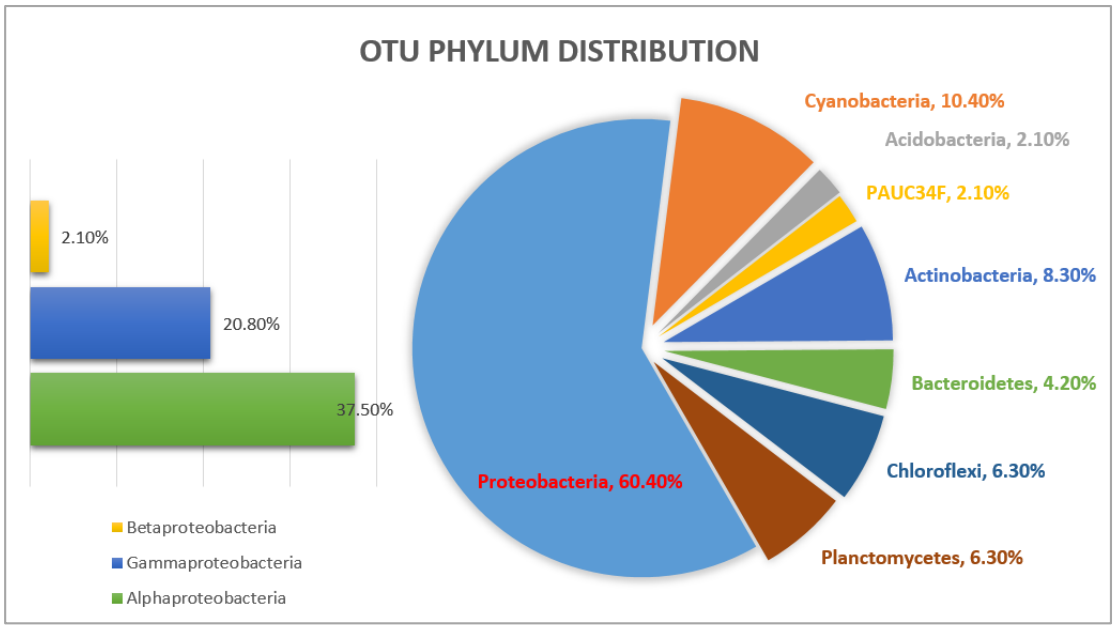



Figure 4.18 Pie-chart of OTU Phylum Distribution

The distribution of the OTUs included the classes Rhodobacterales, Sphingomonodales, Burkholderiales, Alteromonadales, Pseudoalteromonadaceae, Vibrionales, Acidimicrobiales, Chromatiales, Flavobacteriales, Caulobacterales, Acaryochloris, and Prochlorococcus, as illustrated by the clades within the phyla of the Bayesian phylogram (Figure 4.18). Archaea sequences (GCZDig39 and JQ74113) were selected as the outgroup.

### 4.3.2 Bacterial Isolation and Isolate Taxonomic Analysis

Total colony count was 36 on eight different media. From that total, 13 isolates were selected based on different media source and morphologies for species identification based on *16S rRNA* PCR protocol. PCR products were sent for partial sequencing (First BASE Laboratories Sdn. Bhd, Serdang Selangor, Malaysia) and the sequence data obtained were analysed using BioEdit software and converted to FASTA format using Seqret online tool (NCBI). FASTA format sequences were compiled and

submitted to CD-Hit Suite online tool to analyse clustering and obtain number of OTUs. Total number of visible colony, PCR products, and OTUs based on media and seaweed source location is presented in Table 4.14.

Table 4.14 Number of visible colony, PCR products, and OTUs based on media.

| No. | Media[a] | Visible colony (n) | No. of PCR products | No. of OTUs[b] |
|---|---|---|---|---|
| 1 | AIA | 16 | 3 | 3 |
| 2 | KBr | 4 | 2 | 1 |
| 3 | KI | 0 | 0 | 0 |
| 4 | MA | 4 | 2 | 1 |
| 5 | NaCl | 0 | 0 | 0 |
| 6 | $NaNO_3$ | 3 | 3 | 2 |
| 7 | NH4Cl | 7 | 2 | 1 |
| 8 | SA | 2 | 1 | 1 |

a See Section 3.5 Methodology for explanations of the different media used.

b Diversity observed at 97% sequence similarity based in different media

For the eight OTUs observed, Figure 4.19 shows a pie-chart of isolated/identified bacteria by Phyla which is predominantly Firmicutes (54%), followed by Alphaproteobacteria (31%) and Actinobacteria (15%). Only three phyla of bacteria were successfully isolated. Commercial media evidently supported the growth of *Bacillus* spp. and *Labrenzia alba*, while supplemented media supported the growth of Actinobacteria (*Micrococcus luteus*) and Alphaproteobacteria (*Labrenzia aggregata* strain 0194).

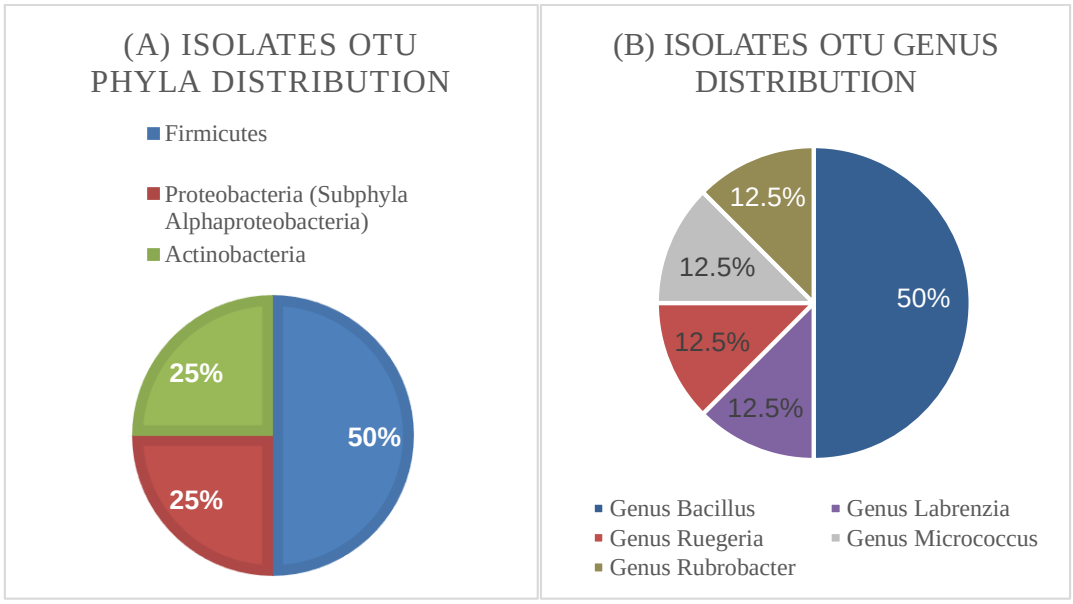



Figure 4.19 Cultivable Bacteria of *Laurencia* sp. Identified Based on *16S rRNA* Gene Similarity at (A) Phyla and (B) Genus Levels

Table 4.15 shows the similarity percentage (of identity) to the nearest neighbours BLAST-searched from NCBI (with accession number and description) for the eight OTU clustered. The OTUs showed 95-100% similarity to the nearest neighbours.

Table 4.15 Bacteria Species BLAST Table

| **Isolate ID & Accession number** | **Nuc. length (bp)** | **Nearest neighbour description & Accession number** | **Sim. (%)** | **Phylum** |
|---|---|---|---|---|
| MA98 **MN396128** | 780 | *Ruegeria mobilis* partial *16S rRNA* gene, strain SM38. **LT600615** | 100 | Proteo-bacteria |
| AIA99 **MN396129** | 1580 | *Bacillus subtilis* strain 171.1.2, 16S ribosomal RNA gene, partial sequence. **KX453939** | 95 | Firmicutes |
| AIA100 **MN396130** | 1111 | *Bacillus pumilus* strain HEP6C1, 16S ribosomal RNA gene, partial sequence. **KY608827** | 98 | Firmicutes |

Table 4.15 Bacteria Species BLAST Table (continued)

| | | | | |
|---|---|---|---|---|
| AIA101 **MN396131** | 1267 | *Bacillus altitudinis* strain SCSGAB0042, 16S ribosomal RNA gene, partial sequence. **JX315298** | 96 | Firmicutes |
| KB103 **MN396132** | 1227 | Uncultured bacterium partial *16S rRNA* gene, clone A6. **AM746684** | 96 | Actino-bacteria |
| NH106 **MN396133** | 1206 | *Micrococcus luteus* strain SSA-1, 16S ribosomal RNA gene, partial sequence. **KY486008** | 97 | Actino-bacteria |
| NN107 **MN396134** | 1175 | *Bacillus aerophilus* strain MER_159, 16S ribosomal RNA gene, partial sequence. **KT719742** | 98 | Firmicutes |
| NN109 **MN396135** | 821 | *Labrenzia aggregata* strain 0194, 16S ribosomal RNA gene, partial sequence. **KP236323** | 98 | Proteo-bacteria |

*Note*: Sim. (%) = Percentage of Similarity

#### *4.3.2.1 Bromoperoxidase Gene Screening*

From the isolated 8 OTUs, only 3 isolates were identified positive to have the 700-bp bromoperoxidase (BPO) gene fragment, which were isolates AIA99, KB103, and NN109. Sequences was BLASTx-searched for similarity of protein family from the nucleotide sequence, and the results included bromoperoxidases from *Bacillus* spp. Sequence analysis showed that the BPO gene screened belonged to the α/β hydrolase superfamily similar to the findings of Lilles (2011). Another phylogenetic tree (Figure 4.21) was constructed using ARB software with the cloned BPO genes and bromoperoxidase genes (nearneighbours and from literature) as listed (Table 4.16) to

illustrate the relationships of all the cloned BPO genes to other BPO genes with Bayesian posterior probability.

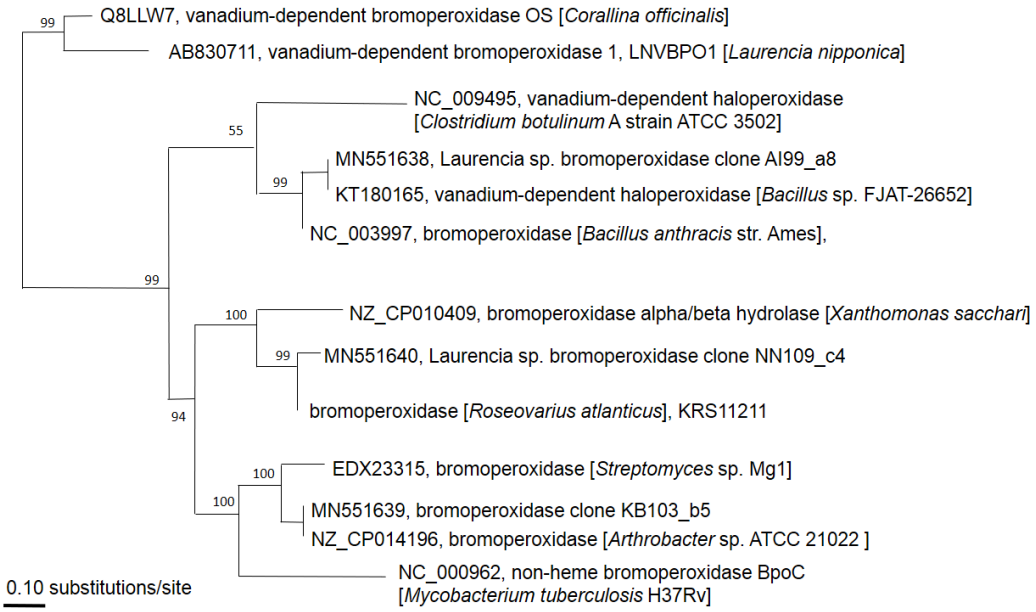



Figure 4.20 Bayesian Phylogram of Bromoperoxidase Genes Constructed Based on Protein Motif Translated Using BLASTx

From the phylogenetic tree (Figure 4.20) constructed using ARB software with the cloned BPO genes and bromoperoxidase genes (nearneighbours and from literature) as listed (Table 4.16). It was clear that vanadium-dependent bromoperoxidases (VBO) isolated from red seaweeds, *C. officialis* and *L. nipponica*, were different from the putative bromoperoxidase cloned from the bacterial isolates. As bromoperoxidases can be classified into three classes based on their cofactor requirements: vanadium-dependent, haem-containing, or those which do not have a prosthetic group (Baharum et al., 2013); it is likely that the bacterial BPO clones obtained from this study fall under a different group than the seaweed VBPO. Furthermore, we used primers from the study by Lilles (2011), who investigated the BPO primer on *Bacillus* spp. From the results obtained, it can be inferred that the BPO

is a broad range primer because isolates from Proteobacteria and Actinobacteria phyla also demonstrated positive results along with Firmicutes, and interestingly, not all Firmicute isolates showed positive bands in the BPO PCR-based screening.

Table 4.16 List of Bromoperoxidase Genes Nearneighbours from BLASTx and Literature

| Clone | Nearneighbour description and accession number | Phylum |
|---|---|---|
| AI99_a2 | vanadium-dependent haloperoxidase [*Bacillus* sp. FJAT-26652], KT180165 | Firmicutes |
| | vanadium-dependent haloperoxidase [*Clostridium botulinum* A strain ATCC 3502], NC_009495 | Firmicutes |
| | bromoperoxidase [*Bacillus anthracis* str. Ames], NC_003997 | Firmicutes |
| KB103_b3 | bromoperoxidase [*Streptomyces* sp. Mg1], EDX23315 | Actinobacteria |
| | bromoperoxidase [*Arthrobacter* sp. ATCC 21022 ], NZ_CP014196 | Actinobacteria |
| | non-heme bromoperoxidase BpoC [*Mycobacterium tuberculosis* H37Rv], NC_000962 | Actinobacteria |
| NN109_c4 | bromoperoxidase [*Roseovarius atlanticus*], KRS11211 | Alphaproteobacteria |
| | bromoperoxidase alpha/beta hydrolase [*Xanthomonas sacchari*], NZ_CP010409 | Gammaproteobacteria |
| from literature | vanadium-dependent bromoperoxidase 1 [*Laurencia nipponica*], AB830711 | Eukarya, Rhodophyta |
| | Vanadium-dependent bromoperoxidase OS *Corallina officinalis*, Q8LLW7 | Eukarya, Rhodophyta |

### 4.3.3 Phylogenetic Analysis of Bacterial OTUs

From the phylogenetic tree (Figure 4.21), it can be seen that bacteria discovered by the culture-dependent approach were more clustered into the Firmicutes phylum (4 out of 8 OTUs: AI99, AIA100, AI101, and NN107; clade cluster genus *Bacillus*), while bacteria discovered by the culture-independent approach were distributed into 8 phyla. The majority of the OTUs were clustered in Proteobacteria; 18 culture-independent OTUs with the addition of 2 culture-dependent OTUs (MA98 and NN109) in the subgroup Alphaproteobacteria (clade classes Rhodobacterales and Sphingomonodales), 10 culture-independent OTUs in the subgroup Gammaproteobacteria (clade classes Alteromonadales, Pseudoalteromonadaceae, and Vibrionales), and 1 culture-independent OTU was in the subgroup Betaproteobacteria (clade class Burkholderiales).

The phylum Actinobacteria had 2 OTUs (KB103 and NH106) identified from the culture-dependent method and 4 OTUs (OTU_151, OTU_175, OTU_214, and OTU_719) from the culture independent method. However, OTUs isolated were seen to be in different clade clusters (orders Micrococcales and Acidomicrobiales). The phyla Cyanobacteria, Chloroflexi, Bacteroidetes, Acidobacteria and PAUC34f have only OTUs identified from the molecular approach.

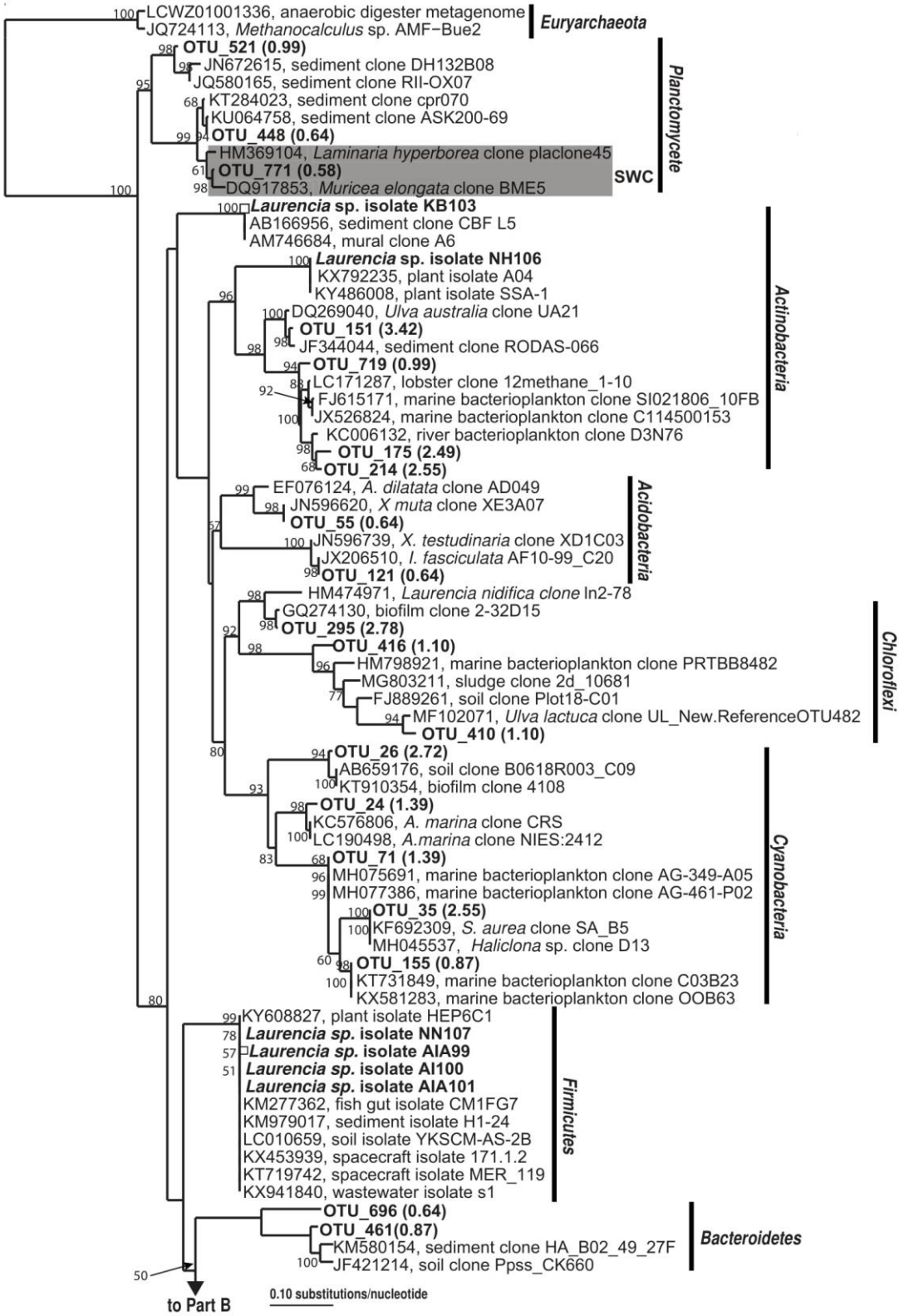



Figure 4.21 Bayesian Phylogram of Bacterial OTUs Constructed Based on *16S rRNA* Genes (1/2)

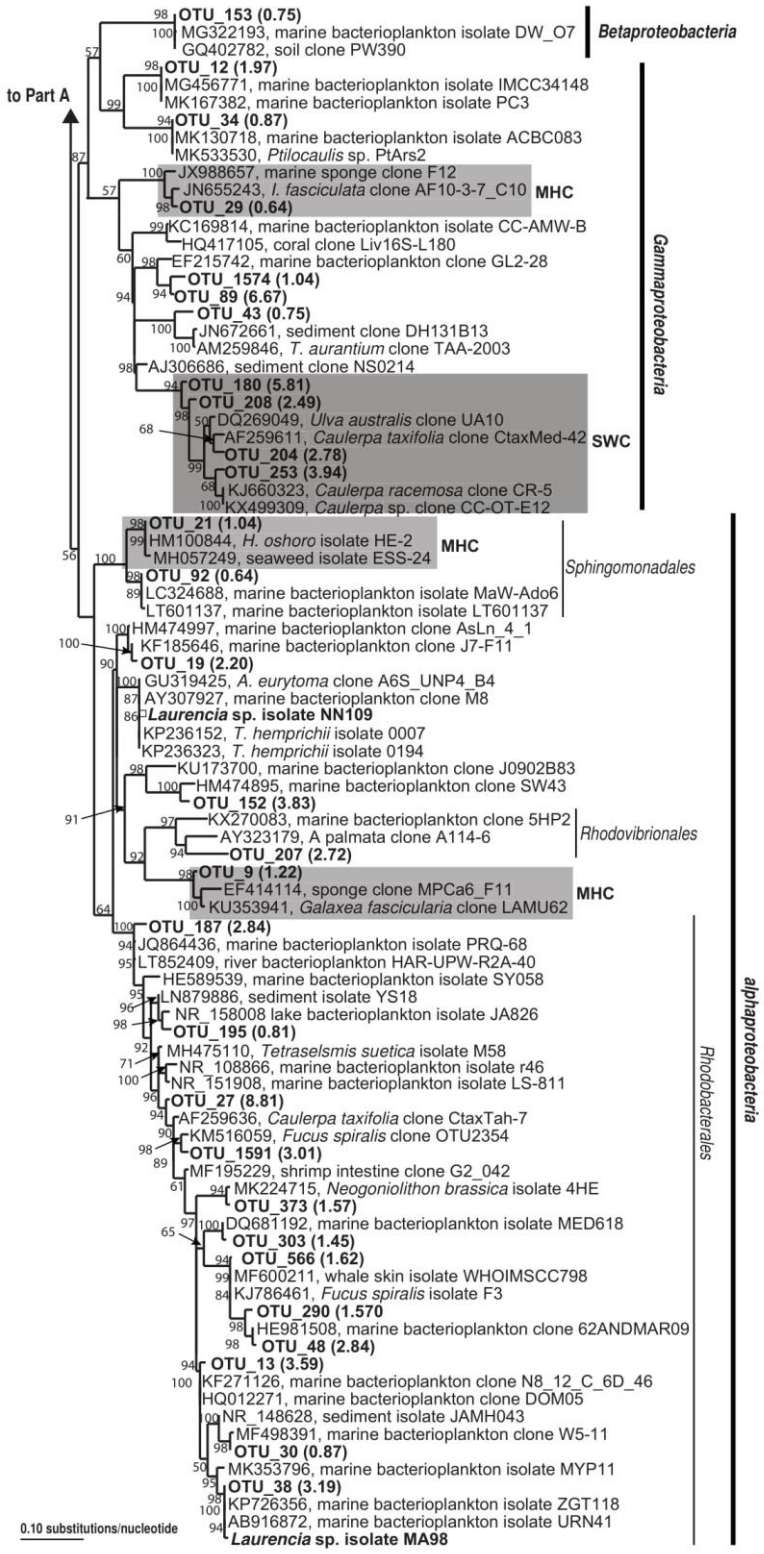



Figure 4.21 Bayesian Phylogram of Bacterial OTUs Constructed Based on *16S rRNA* Genes (2/2)

## 4.4 PART IV: COMPARISON OF BACTERIAL DIVERSITY OF THE TWO RED SEAWEEDS

Due to the holobiont environment of the seaweed, which is suitable for symbiosis, many symbionts attach to that environment, including various marine bacteria. Predominant marine bacteria groups can be divided into eleven major lineages based on their small subunit ribosomal RNA (SSU rRNA) gene, hence, the reason why most environmental or investigative bacterial studies rely on *16S rRNA* for diversity and identification. These lineages include Cyanobacteria, Proteobacteria (α, β, γ, δ, ε), Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Deferribacteres, Firmicutes, Lentisphaerae, Planctomycetes, and Verrucomicrobia (Yilmaz, Yarza, Rapp, & Glöckner, 2016). As seawater accounts for most of the marine environment, cyanobacteria in bacterioplanktons are perhaps the most apparent marine bacteria phyla, with several members having widespread distributions and are able to fix nutrients such as carbon and nitrogen in the nutrient cycle (Charpy et al., 2012). However, as symbionts for the seaweed host, Alphaproteobacteria seemed to be the most dominant group identified followed by Gammaproteobacteria, Firmicutes, Cyanobacteria, Bacteroidetes, and Actinobacteria, shown by works of Lachnit et al. (2011), Hollants et al. (2013), and Burke, Steinberg, Rusch, Kjelleberg, and Thomas (2011). Furthermore, the work in this research also showed similar trend.

In this study, bacteria associated with two different seaweed genera was investigated. The structure and composition of the seaweed-associated bacteria are known to differ with the geographic locations, host conditions, as well as time-factor or seasonal variation. Equally important to the physicochemical parameters of the seaweed environment that is natural or in cultivation, the host traits may also be critical in determining the community structure of associated bacteria or vice versa.

Seaweeds live in association with abundant and diverse bacteria as seaweeds rely on associated bacterial communities for diverse functions including morphological development, consumption of organic matter and nitrogen source, defense, or provision of trace nutrients and vitamins. Illustrated below is a flower venn diagram (Figure 4.22) of seaweed-associated bacteria based on the seaweed samples SWD1–SWD4.

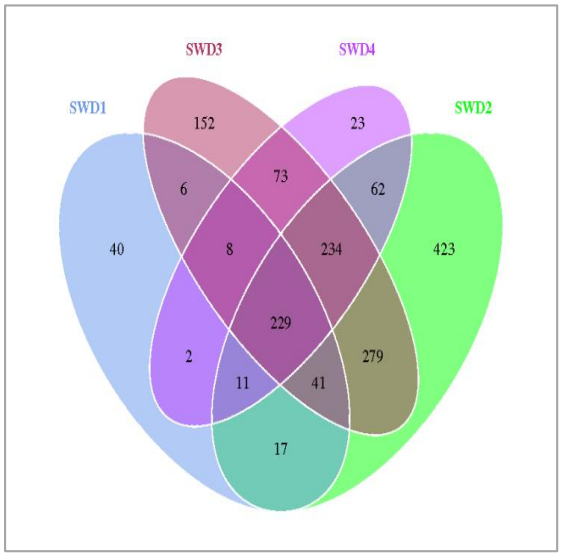



Figure 4.22 Flower Venn Diagram of Seaweed Samples SWD1–SWD4

Each colour group represents one seaweed sample group; the blue group for SWD1 which is the genus *Laurencia* seaweed sample collected from Bidong Island, Terengganu; green group for SWD2 which is *G. manilaensis* collected from a seaweed farm in Kedah; pink group for SWD3 which is *G. manilaensis* Penang 1 wild seaweed sample; and purple group for SWD4 which is *G. manilaensis* Penang 2 wild seaweed sample. Values in overlapping parts represent common OTUs; a total of 1371 OTUs were clustered from all the samples with only 229 OTUs present in all 4 sample groups. Values that represented specific OTUs in each sample were in the non-

overlapping area; SWD2 had the most specific OTUs (423), while SWD4 had the least specific OTUs (23). Overall, it can be seen that SWD2 had the most OTUs inclusive of the values that the green colour group intersects with other colour groups and SWD1 had the least OTUs. The bacterial OTU richness and diversity is supported by the alpha diversity index values presented in Table 4.17.

Table 4.17 Alpha Diversity Index of Seaweed Samples SWD1–SWD4

| Alpha diversity index | Observed species | Simpson | Shannon | Chao[1] |
|---|---|---|---|---|
| SWD1 (*Laurencia* sp.) | 354 | 1.243 | 0.400 | 396.792 |
| SWD2 (Farmed *G. manilaensis*) | 1296 | 4.981 | 0.870 | 1489.848 |
| SWD3 (Wild *G. manilaensis*) | 1022 | 3.495 | 0.756 | 1252.000 |
| SWD4 (Wild *G. manilaensis*) | 642 | 2.425 | 0.662 | 890.629 |

From Table 4.17, it can be inferred that the bacterial composition in each seaweed sample was diverse as the values for each index were high. Additionally, seaweed samples for the genus *Gracilaria* showed higher diversity that the sample for the seaweed genus *Laurencia*. Aside from the genus difference, the difference in bacterial diversity could also be due to the location of the sampling sites and the parameters of seaweed environment. Seaweed sample SWD1 genus *Laurencia* was collected from the pristine marine waters of Bidong Island, which is about 20 nautical

miles away from the mainland and boat traffic. Due to the preserved nature of the island waters, less suspension was observed, and that leads to less bacterial abundance and diversity. Conversely, the SWD2–SWD4 samples were collected at a seaweed farm or coastal area near to development, and hence, the bacterial abundance at anthropogenically-influenced area was relatively more diverse (Fernandes, Kirchman, Michotey, Bonin, & LokaBharathi, 2014).

When comparing each bacterial diversity of the seaweed samples (SWD1–SWD4), there were only subtle differences between the bacterial community composition. Generally, all *G. manilaensis* seaweed samples (SWD2–SWD4) had the same predominant OTUs identified through amplicon sequencing, which was 88 OTUs, while the *Laurencia* sp. seaweed samples (SWD1) had less, which was only 43 OTUs. However, the abundance of each of the OTU present in the seaweed varied between the samples. This can be seen from the heat-map with the percentages of the bacterial community compositions illustrated in Figure 4.22.

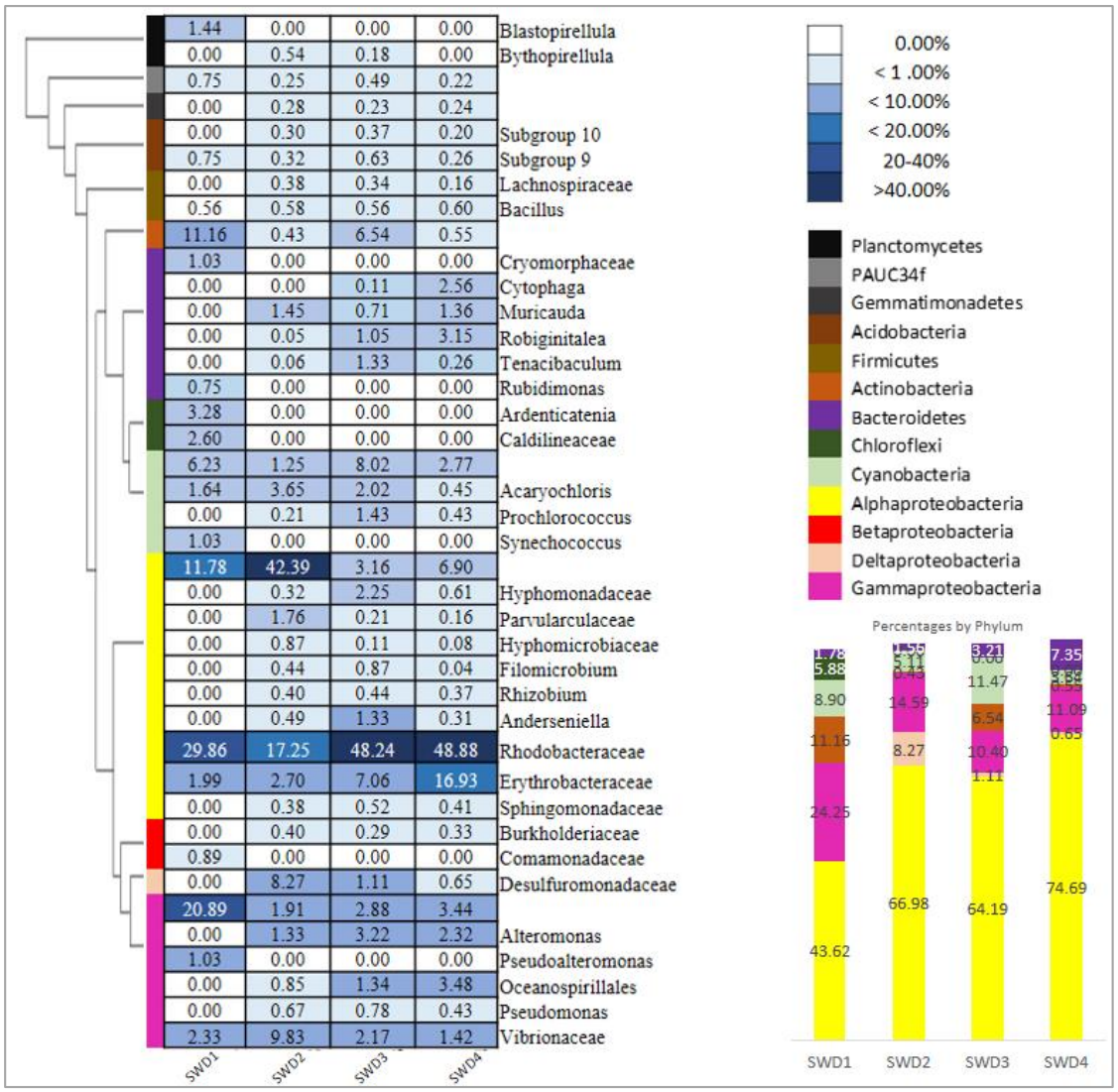



Figure 4.23 Heat-Map Based on Percentage Abundance of Bacterial OTU in Phylum (divided by subgroup, class or genus)

From the figure, it can be seen that Proteobacteria class Alphaproteobacteria was the most dominant bacterial phyla for both seaweed genera (SWD1: 43.62%, SWD: 66.98%, SWD3: 64.19%, SWD4: 74.69%), followed by the class Gammproteobacteria, (SWD1: 24.25%, SWD2: 14.59%, SWD3: 10.40%, SWD4: 11.09%), and phylum Cyanobacteria (SWD1: 8.90%, SWD2: 5.11%, SWD3: 11.47%, SWD4: 3.64%). The dominance of the bacterial groups Alphaproteobacteria, Gammaproteobacteria, and Cyanobacteria in association to sessile marine hosts is a

common observation in many research (Brodie et al., 2016; Goecke & Imhoff, 2016; Coelho et al., 2018) as the phyla are large groups consisting of many identified species and members of these phyla are ubiquitous in nature. The phylum Chloroflexi was only seen from seaweed genus *Laurencia* (SWD1: 5.88%), while the phylum Gemmatimonadetes was only observed from seaweed genus *Gracilaria* though in minor percentages (SWD2: 0.28%, SWD3: 0.23%, SWD4: 0.24%).

In the phylum Planctomycetes, the genus *Blastopirellula* was identified from seaweed genus *Laurencia*, while genus *Bythopirellula* was present in seaweed genus *Gracilaria*. Even though Planctomycetes are often identified in marine environments in association with hosts such as seaweeds (Bengtsson & Øvreås, 2010; Lage & Bondoso, 2014; Bondoso et al., 2017), the contrast in bacterial genus for seaweed hosts was interesting to note as both genera are classified under the same family Planctomycetaceae (order Planctomycetales, class Planctomycetia) but the genus *Bythopirellula* is not under the chemoorganoheterotrophic *Blastopirellula–Rhodopirellula–Pirellula* clade which is strictly aerobic, and thus indicates different functional profile. Furthermore, the only species that has been identified under this genus is an iron-oxidising bacterium, *Bythopirellula goksoyri* sp. nov, isolated from deep sea iron hydroxide deposits in a hydro-thermal vent, Arctic Mid Ocean Ridge (Storesund & Øvreås, 2013). As the seaweed *G. manilaensis*. samples were collected from areas near anthropologic development, it could be that the marine or brackish water environment had high iron to encourage the presence of bacteria genus *Bythopirellula*.

The distribution of bacteria by phyla can be seen comparable to the distribution of bacteria associated to red seaweeds in the literature (Figure 6.3). As bacteria can play a role in the degradation of seaweed polysaccharides such as cellulose, laminarin,

and pectin, this indicate that the bacterial community composition can be influenced by the seaweeds' tissue components or metabolites since certain bacterial group will dwell in niches where the seaweed polysaccharides or metabolic compounds are predominant. Bacterial members belonging to the phyla Actinobacteria, Alphaproteobacteria, and Bacteroidetes have exhibited phycocolloid-degrading properties such as agarolytic, alginolytic, carrageenanolytic, and fucoidanolytic activities (Michelou, Caporaso, Knight, & Palumbi, 2013). It is possible that the relative abundance of this bacterial group in seaweed samples is attracted to cell-wall components of the seaweeds and benefit from exudates from old and damaged seaweed tissue since these bacteria are able to utilise agar and other polysaccharides from seaweeds.

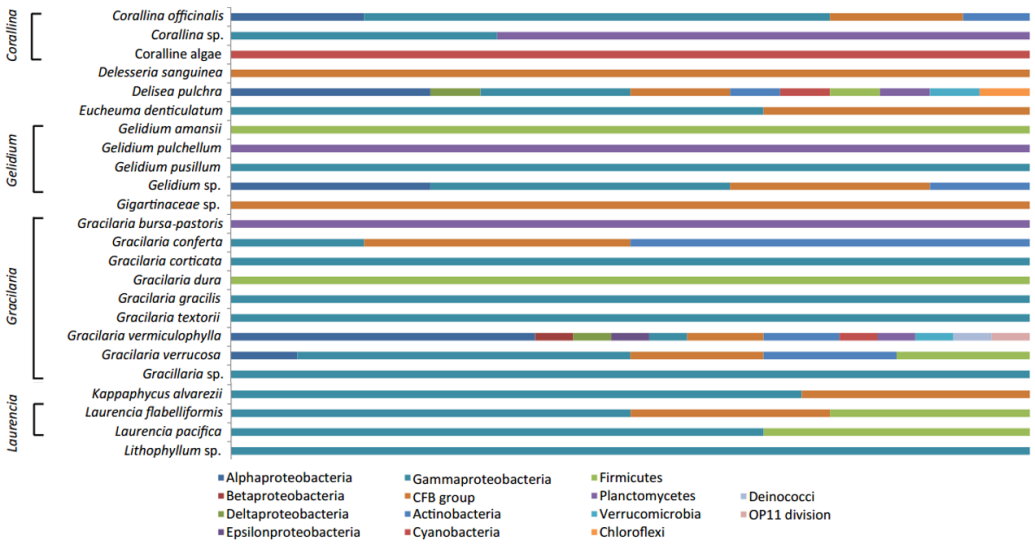



Figure 4.24 Distribution of Bacterial Phyla in Seaweed-Bacteria Research (adapted from Hollants et al., 2013)

There is no doubt that the development of molecular technics and NGS technologies has played an immense role in the recognition of microbes as key

inhabitants of macroorganisms, and as players in biological and evolutionary processes, even if less studies address functional issues. Because many host-associated microbes are uncultivable outside their hosts, environmental genomics approaches have been successfully applied to unravel the diversity and roles of microbes in both model and non-model organisms and in all ecosystems whether terrestrial, marine, or aquatic, and spanning different types of host-microbiota associations from loose to tight.

# CHAPTER FIVE

# DISCUSSIONS

## 5.1 GENERAL DISCUSSIONS

The existence of seaweed-bacterial associations is no longer doubted as various studies revealed that bacterial communities living on seaweed differ from those occurring in the surrounding seawater. These studies, however, focused on the distinctiveness of the epiphytic bacterial communities from the free-living environmental communities and never studied the specificity of the endophytic bacteria associated with seaweeds. In this study, bacteria identified from samples of seaweed Rhodophyta genera *Gracilaria* and *Laurencia* were studied with respect to their functional profile through culture-dependent and culture-independent approaches, and also potential bromoperoxidase functional gene screening of cultivable bacterial isolates. This section discusses the bacterial diversity observed from the seaweed samples through the two adopted approaches with comparison of the approaches employed, the effect of selected enrichment media utilised, and the effect of environmental physico-chemical parameter of two different sites on the bacterial diversity of seaweed of the same genus. Knowledge on bacterial diversity is hoped to shed insights on the functional profile of the symbiont bacteria in the environment.

### 5.1.1 Amplicon Sequencing Reveal More Bacterial Phyla Than Cultivable Isolates

At a glance, the bacterial community assemblage identified through this study did not reveal much difference between the two culture-dependent and culture-independent approaches for the different seaweed samples, though prior to OTUs normalisation, the initial effective tags of the amplicon sequencing (culture-independent approach) revealed 956 OTUs which is twenty-folds than the bacterial isolates OTUs. The dominant phylum identified from both approaches for both seaweed genera studied was Proteobacteria; 70% for culture-independent approach (after normalisation) and 58% for culture-dependent approach. Through the culture-independent approach nine bacterial phyla were identified collectively from both seaweed genera samples, including Firmicutes, Actinobacteria, Bacteroidetes, Cyanobacteria, Acidobacteria, Planctomycetes, Gemmatinodetes, and PAUC34f, while only four bacterial phyla were identified through the culture-dependent approach; limited to Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes. For the seaweed samples, *G. manilaensis*, the culture-independent approach through amplicon sequencing had identified 88 predominant OTUs from the nine bacterial phyla, while through the culture-dependent approach, only 43 OTUs have been identified. From the seaweed sample of *Laurencia* sp., 48 OTUs were identified from the culture-independent approach, while 8 OTUs were identified from the culture-independent approach.

The predominance of phylum Proteobacteria observed in this study was similar to previous studies because the phylum Proteobacteria or subphyla Alphaproteobacteria is commonly dominant in aquatic or marine symbiotic environment (Ortega-Retuerta, Joux, Jeffrey, & Ghiglione, 2013; Sun, Zhang, He, & Li, 2014; Dogs et al., 2017). Proteobacteria dominated the OTU distribution by 60%,

which is a common observation from seaweeds (Goecke et al., 2013; Hollants et al., 2013; Morrissey, Çavaş, Willems, & De Clerck, 2019) and marine samples, including in seagrasses (Jiang, Ingle, & Golberg, 2016), marine sponges (Alex & Antunes, 2015) and coastal waters (Xie et al., 2017), where the subphyla *Alphaproteobacteria* and *Gammaproteobacteria* were found to be the dominant groups. The large assemblage from the *Proteobacteria* phylum could be due to its members that are metabolically diverse, having many ecological roles in the seaweed environment.

Likewise, from the *G. manilaensis* samples through the culture-dependent approach, two classes were isolated, which were Alphaproteobacteria and Gammaproteobacteria, while through the culture-independent approach, two additional classes were identified, which were Betaproteobacteria and Deltaproteobacteria. As the class Alphaproteobacteria was the most abundant OTUs in the Proteobacteria phylum from the *G. manilaensis* samples, the findings were similar to other investigative bacterial studies that reveal a large number of Proteobacteria isolates, or specifically class Alphaproteobacteria, due to the members of the phylum being oligotrophs that can exist in various habitat around the world and in low-nutrient environments including from subarctic tundra soil (Kim, Park, Bhattacharya, & Yoon, 2014) and in association with hosts such as seaweeds (Hollants et al., 2011) and corals (Carlos, Torres, & Ottoboni, 2013).

In addition, orders identified under the Alphaproteobacteria group included Rhodobacterales (genus *Roseovarius*), Shingomonadales (genus *Erythrobacter*), and Parvularculales (Family Parvularculaceae). Interestingly, *Roseovarius* and *Erythrobacter* are photosynthetic bacteria symbiont to organisms in the marine environment, and Rhodobacterales have been identified in a coral holobiont (Stat et al., 2012). Family Parvularculaceae covers the genus Parvularcula which only

comprises of three bacteria that are Gram-negative, strictly aerobic, chemoheterotrophic marine bacteria extracted from seawater, coastal hot spring, and soft coral (Cavalcanti & Thompson, 2014). Thus, it is not surprising to find the OTUs among the amplicon dataset. Comparable to the cultivable bacteria isolated, the OTUs of Alphaproteobateria (family Rhodobacteraceae) was also dominant within the amplicon sequencing data.

For OTUs identified under the Gammaproteobacteria subphylum, members identified include *Vibrio*, *Alteromonas*, *Pseudoalteromonas*, and Xanthomonadales. All are Gram-negative marine bacteria except Xanthomonadales which is one of the largest groups of bacterial phytopathogens. *Stenotrophomonas* is the only genus capable of nitrate reduction within the Xanthomonadales. Alphaproteobacteria and Gammaproteobacteria are predominant in seawater, on marine substrate/organism surfaces, and in the most organic-rich coastal surface sediments due to their function/involvement in nutrient cycling. As the seaweeds samples were collected from sites near anthropogenic development, it could possibly receive a considerable supply of inorganic nitrogenous nutrients from various sources such as nearby factories, land runoff, and sewage effluents, hence influences the presence and abundance of nutrient-cycling Proteobacteria.

The deltaproteobacteria strain identified belonged to the Order Desulfuromonadales (Genus *Desulfuromonas*) and the Family Desulfobacteraceae. Members of the Order Desulfuromonadales include bacteria that are moderately thermophilic, metal reducers (iron and/or sulphur) and most were isolated from freshwater, seawater, and marine sediments (Slobodkina et al., 2012), although recently, more have been isolated from extreme environments such as the Antarctic seawater (Dickinson et al., 2016) and hot springs (Kambura et al., 2016). Sulphate-

reducing bacteria, such as member in the genus *Desulfuromonas*, are abundant in a reduced organic-rich environment or substrate surfaces. This could be due to the presence of organic nitrogen and phosphorus, as well as manganese oxides in the coastal seaweed environment. This suggests that the bacterial groups are functionally involved in the aerobic and anaerobic degradation of organic matter (Sinkko et al., 2013) and hence, the bacteria contributed to nutrient cycling for the seaweed.

Other phyla identified through amplicon sequencing were Planctomycetes, Cyanobacteria, Bacteroidetes, Firmicutes, Acidobacteria, Actinobacteria, and PAUC34f, while other phyla isolated through the culture-dependent approach were Firmicutes, Actinobacteria, and Bacteroidetes (4%). These are common phyla isolated or identified from seaweeds, such as rhodophyte *Corallina officinalis* (Brodie et al., 2016), chlorophyte *Cladophora* sp. (Zulkifly et al., 2012), and phaeophyte *Dictyota bartayresiana* (Barott et al., 2011), or marine environments as the bacterial community is associated with symbiotic or phototrophic support functional profile (Joint et al., 2010; Hollants et al., 2011; Singh & Reddy, 2016). The culture dependant and independent approaches can be said to be complementary to each other. Through amplicon sequencing, bacterial OTUs typical for marine environment and seaweed/sponge host association were detected, but a large number were not represented within the cultivable isolates. Vice versa, cultivable bacteria isolates had identified taxons that were not in the predominant amplicon OTUs. This could be due to the selectivity of the bacteria, leading to difficulties or shortcomings of strategies to culture and isolate the phyla in the laboratory. Nevertheless, current molecular techniques ease genomic detection of bacterial OTUs, especially due to widespread distribution of bacteria in various natural environments, such as seaweed, sediment,

soil, and water. Therefore, the combination of the two approaches would give a better insight of the bacterial community associated to seaweeds.

Furthermore, Cyanobacteria which is represented by OTU_24, OTU_26, OTU_35, OTU_71 and OTU_155 are found in many natural environment and also various cropping systems as they play important ecological roles in low-nutrient soils to promote plant growth by fixing nitrogen, increasing the availability of phosphorous and releasing auxins (Sharma, 2014). Chloroflexi represented by OTU_410 and OTU_416 can be found widely in aquatic and terrestrial plant ecosystems (Fierer et al., 2012) and its member *Anaerolineales* (OTUs clade cluster and its nearneighbours) bacteria are distributed in other nutrient rich environments (Yoon et al., 2010) due to the ecophysiological role of nitrogen fixation (Madigan, 1995) and nitrite oxidation (Sorokin et al., 2012). The distribution of Bacteroidetes could to a large extent be explained by host phylogenetic factors which is in contrast with the situation for Flavobacteriaceae symbionts, whose presence could be entirely explained by environmental factors.

Additionally, OTUs from the culture-dependent approach did not show any close similarities to the OTUs identified from the culture-independent approach and the bacterial composition of the culture-independent approach seemed to be more complex. Only three of cultivable isolates were in the same clades to two of the amplicon OTUs for SWD1 (Bayesian phylogram, Figure 4.21), Only three of cultivable isolates were in the same clades to two of the amplicon OTUs for SWD2–4 (Bayesian phylogram, Figure 4.15). Thus, there was only 4% similarity between the two approaches' datasets of SWD1 and 12% similarity between the two approaches' datasets of SWD2–4. The same observation in discrepancy between the culture-dependent and culture-independent approaches has been previously documented in

coralline algae bacterial symbionts (Quéré, Intertaglia, Payri, & Galand, 2019), in a Chinese medicinal plant, *Dendrobium officinale* (Pei et al., 2017), and from hydrocarbon-contaminated soils (Stefani et al., 2015) where phyla identified through culture-independent approach was more than the culture-dependent approach and isolated strains were not found in the amplicon sequences. Novel strategies could be employed for more inclusive/comprehensive isolates from the culture-dependent approach, such as the use of relatively low concentration of nutrients or non-traditional sources of carbon or complex polysaccharides, different periods or atmospheric conditions of incubation, and amendment of environmental extracts, catalase, or cations in the growth media (Kielak, Barreto, Kowalchuk, van Veen, & Kuramae, 2016). The use of a solid media in the culture-dependent approach may have enhanced the growth of certain bacteria, especially when the media have been supplemented with nutrients which favours the bacterial growth, such as those with fast growing ability even if they are not abundant in the tissues (Quere et al., 2019). This then results in a high number of cultivable isolates and isolates OTUs identified with no or little detection in the amplicon or next-generation sequencing. However, the application of amplicon sequencing which is a sequence-based approach to the investigation of the complex uncultured bacterial communities present in the Malaysian seaweed coastal environment had provided the ability to identify a greater diversity of community members than the cultivation techniques presented in this study. Hence, the two approaches could give similar results at a broad phylum observation i.e. both approaches showed Alphaproteobacteria was the dominant bacteria isolated or identified. However, at a finer taxonomic resolution, clear differences can be observed.

### 5.1.2 Selective Enrichment Media Promotes Growth of Potentially Novel Bacterial Isolates

In comparison, the culture-dependent approach yielded targeted bacteria such as Firmicutes i.e. *Bacillus* that grows easily on commercial media and the growth of bacterial species associated with the seaweed surface and/or surroundings through cultivation experimentation. Most of these bacterial species have been previously isolated from seaweed surfaces and are reported to possess several bioactive properties; examples include cell wall degrading (e.g. *Alcanivorax*, *Bacillus*, *Halomonas*, *Marinobacter*, *Pseudoalteromonas*, and *Sphingomonas*) (Lachnit, Fischer, Künzel, Baines, & Harder, 2013); pathogenic (*Halomonas* and *Pseudoalteromonas*) (Alexander, 2014); morphogenic (*Bacillus* and *Pseudoalteromonas*) (Grueneberg, Engelen, Costa, & Wichard, 2016), growth promoting (*Bacillus* and *Pseudoalteromonas*) (Seipke, Kaltenpoth, & Hutchings, 2012); and antimicrobial properties (*Bacillus*, *Microbacterium*, *Phaeobacter*, and *Pseudoalteromonas*) (Singh, Kumari, & Reddy, 2015).

Appearances of the colonies observed included circular and irregular colonies with colour pigments varying from white, yellow, orange, pink, brown, and black, and the observation of is marine agar medium plates that supported the growth of agar-degrading bacteria which is possibly involved in macroalgal diseases or defence mechanism (Weinberger, 2007). Agar-decomposing bacteria were first isolated by Gran in 1902 (Yaphe, 1957; Schroeder, Jaffer, & Coyne, 2003) and since then, several agarolytic bacterial strains were isolated from marine and other environments; some of the bacterial isolates have been assigned to the genera *Pseudoalteromonas*, *Pseudomonas*, *Cytophaga*, and *Streptomyces*. Other bacterial cultures have yet to be

described and explored and bacteria isolated from seaweed have shown great potential for bioactive compound production which are related to seaweed spore settlement, bacterial quorum sensing, anti-fouling, and morphogenesis (Armstrong, Yan, Boyd, Wright, & Burgess, 2001; Joint, Tait, & Wheeler, 2007; Wichard, 2015).

The supplemented media allowed the most growth of seaweed-associated bacteria as cultivation techniques using media with low organic matter concentrations have been demonstrated to considerably improve the bacterial cultivability, which is better than previously reported solid media could be too nutrient-rich, resulting in an overgrowth of mainly Gram-positive *Bacillus* species. Alphaproteobacteria were present on all culture media types experimented, possibly because of the abundant bacteria present in the natural environment and the group is large enough to encompass diverse varying bacteria characteristics that at least a member of the group can at least grow on any given media that replicate essential aspects of their environment in terms of nutrients, pH, osmotic conditions, and temperature (Singh & Reddy, 2016). However, the next largest bacterial class Gammaproteobacteria grew on all culture media provided except NN, a laboratory mixture culture medium containing 15% agar and 10% ammonium salt $NaNO_3$ dissolved in sterilised natural seawater (collected from the seaweed natural environment). It is inferred that just trying to mimic the natural environment or supplying the ammonium salt is not enough for the bacteria to grow because quite possibly the bacteria needs co-culture or host-associated environment i.e. seaweed to break down the ammonium salt before it can be taken up by the bacteria to grow.

Firmicutes did not favour the commercial marine media agar (MA) but did grew on commercial AIA. This could be due to the different compositions of the two media, such as the presence of peptone in MA could be a deterrent for Firmicutes to

grow. Also, AIA composition has less trace salts than MA. It is interesting to note the differences as Firmicutes are easily grown on commercial media agar. It also favours SA, KB, and NH without growing on KI, NC, and NN.

Actinobacteria only grew on AIA, SA and NN, while there was no growth on any halide group supplemented media, KB< KI< NC. Though previously halophilic and endosymbiotic Actinobacteria such as *Micrococcus*, *Micromonospora*, and *Microbacterium* have been isolated from marine sponges, this could mean that the supplemented halide ions culture media was unsuccessful to replicate the environment to encourage the growth cultivable halophilic Actinobacteria from seaweed. Bacteroidetes only grew on laboratory mixture culture media supplemented with ammonium salts, NHCl (NH) and $NaNO_3$ (NN) which could be due to the nature of the Bacteroidetes that can metabolise complex organic compounds such as ammonia in the marine environment (McIlroy & Nielsen, 2014).

Nevertheless, cultivation attempts could be further elaborated and optimised in the future to better suit the growth of symbiotic bacteria. For example, the natural environment of the endophytic bacteria, i.e. the host internal conditions, could be mimicked even more by adding photosynthetic metabolites to the culture media. Watanabe et al. (2008) developed such an effective artificial medium supplemented with organic carbon and nitrogen which imitates the nutritional conditions surrounding algae to favour the growth of photosynthesis-dependent epiphytic bacteria. In addition, the cultivation of bacteria with an intracellular metabolic pathway poses some challenges as it is difficult to identify and replicate the environmental settings necessary for metabolic biosynthesis. As several of these isolates are related to up to now uncultivated clones and possibly represent new bacterial species, they may offer great opportunities for future biodiscovery research.

### 5.1.3 Effect of Salinity and other Abiotic Factors on Bacterial Diversity

It was observed that the Kedah seaweed sample had more species diversity than Penang and Bidong Island sites; and for the Penang sites, Site 1 had more species diversity than Site 2, when comparing the relative abundance of cultivable bacteria among the sites (Kedah, Penang 1, and Penang 2) and the alpha diversity indices of amplicon dataset (refer Table 4.17). For the comparison between Kedah and the other sites, this could be due to the ambient conditions of the natural environments. Kedah had lower salinity (16 ppt) than Penang (30 ppt) and Bidong Island (30 ppt), although the temperature and pH were relatively the same (Kedah: 32.6 °C and pH 7.68; Penang: 32.0 °C and pH 7.77; Bidong: 29.0 °C, pH 7.70). In general, most studies found a trend of decreasing bacterial species diversity calculated with increasing salinity of the environmental sample (Yang, Ma, Jiang, Wu, & Dong, 2016).

Studies also revealed that salinity influenced bacterial community composition the strongest, as compared to other environmental parameters such as pH and temperature which seemed to have minor impacts on bacterial community when analysed at a considerable spatial scale. This could be due to that only some bacteria can thrive under conditions of increased salinity (Thiem, Gołębiewski, Hulisz, Piernik, & Hrynkiewicz, 2018). Gammproteobacterium *Halomonas boliviensis*, for example, was able to tolerate a salinity range of 0–25% as it was isolated from a hypersaline lake (Quillaguaman, Hatti-Kaul, Mattiasson, Alvarez, & Delgado, 2004).

A sigh salinity water environment causes salt stress in the bacterial cells. This is due to the bacteria having to defend against the salinity gradient and the osmotic pressure caused by the loss of cellular water (plasmolysis) or the recession of its cytoplasm. Most bacteria are salt-sensitive species because they could not tolerate

high salinity. In addition, high salinity could also be interrelated to high nutrient concentrations from anthropogenic sources and members in the Betaproteobacteria group can have the ability to degrade complex organic macromolecules that are often derived from anthropogenic pollutants; hence, the distribution of Betaproteobacteria, as observed in this study (Site 3), is often positively correlated with salinity (Tang et al., 2012; Zhao et al., 2018).

However, it was also noted that the different bacterial profiles observed in different sampling sites were not largely varied. It is possible that the geographic distance of each sites in this study is not so large that it affected the bacterial community hosted by the seaweed and the bacteria roles function to the host seaweed. Furthermore, as the seaweed species was similar, it supports the work of Burke et al. (2011) that have remarked bacterial profile as functionally host-specific. Additionally, some studies have shown that bacterial diversity had increased with the growth phase or age of the seaweed (Bengtsson et al., 2012) or differ with the health status of the seaweed (Zozaya-Valdes, Egan, & Thomas, 2015), which unfortunately the parameters were not observed in this study.

### 5.1.4 Bacterial OTU Phylogenetic Analysis Postulates Marine Holobiont Cluster

From the Bayesian phylogram illustrated, the bacterial diversity could be influenced by environmental, geographic, and host phylogenetic factors as associated bacteria assemblage is not a random accumulation of environmental bacteria. Associated bacteria assembly is rather a selected community through possible horizontal gene transfer and the bacterial community members are critical to the well-being of the host because the bacterial symbionts contribute to the health and development of their hosts. Most of the near neighbours (NN) of the isolates and amplicon sequencing

OTUs were either from bacterioplanktons, sediments, or sponges as most previous studies on bacterial community from marine environment focused on either water samples, sediment samples, or symbiont hosts such as sponges. Only recently, studies on symbiotic bacterial community emerged from hosts such as seaweeds, corals and sea cucumber. While bacterioplankton and sediment clones or isolates species reflect the environment of which the seaweed sample was collected, NN clones or isolates from sponges reflect the symbiosis of the bacteria to associated host. This observation postulates that associated bacteria can be clustered by host phylogenetic factors into marine holobiont cluster (MHC) and seaweed-specific cluster (SWC). Although, previous studies on sponges have expounded the Sponge Symbiont/Specific Cluster (SSC) due to the abundant studies reporting symbiotic bacteria specific to sponge host species. Notably, most sponge symbiotic bacteria NN were isolated or cloned from *Xestospongia muta*, followed by *Xestospongia testudinaria*, *Rhabdastella globostellata*, *Agelas dilatata*, *Ircinia fasciculata*, and *Thymus aurantium*. Hence, NN clones or isolates from hosts such as corals (*Monstrea faveolata*, *Oculina patagonica*, *Diploria strigosa*, and *Galaxea fascicularia*), sea cucumber (*Apostichopus japonica* and *Holothuridea leucospilota*), seagrasss, and sea star (*Asterias amurensis*) further fosters on the theme of MHC or symbiotic cluster of the bacteria.

Importantly, few OTUs had NN cloned or isolated from seaweeds. OTU_62 cyanobacteria were closely related to bacterial clones isolated from red seaweed, *Gracilaria coronopifolia*, OTU_59 Gammaproteobacteria were closely related to bacterial clones isolated from red seaweed, *Gelidium amnsii*, and isolate MA177 were closely related to bacterial clones isolated from red seaweed, *Porphyra yeozensis*. Also, there were NNs related to brown algae, *Fucus spiralis* (OTU_151, acidobacteria), *Padina tetrastomatica* (OTU_164, Firmicutes), and *Laminaria*

*hyperborea* (OTU_771); green algae *Ulva australis* (OTU_151, acidobacteria) and *Tetraselmis sueicica* (OTU_27 alphaproteobacteria). Other OTUs related were OTU_75 Bacteroidetes to red algae and OTU_69 Gammaproteobacteria, OTU_31, and isolate KB120 to algae.

This hinted to the symbionts in a Seaweed Cluster (SWC), although it was weak to support the argument that the bacteria were species-specific to seaweed host despite the Bacteroidetes and Gammaproteobacteria found in this study are predominant members of seaweed-associated bacteria assemblages (Burke et al., 2011; Hollants et al., 2013). Instead, the position of amplicon OTUs with respect to its nearest neighbours in the Bayesian phylogram tree indicated bacterial evolutionary patterns by which the bacteria adapt to its functionality in MHC or SWC as members of Gammaproteobacteria are important for nutrient cycling and members of Bacteroidetes can degrade polysaccharides (Hollants et al., 2013). Seaweed-bacteria associated community can respond to disturbances in the environment by being resistant to a stressor or shifting the assemblage compositional dynamics. In extreme stress conditions, the bacterial responses could lead to the host mortality (Glasl, Smith, Bourne, & Webster, 2018).

### 5.1.5 Exploration of Bromoperoxidase Functional Gene Screening

The bromoperoxidase or bromide peroxidase genes are part of the haloperoxidase functional genes possessed by seaweeds or bacteria associated with seaweeds for the ability to catalyse the conversion of metabolite produced by seaweeds to halometabolites. The systematic name of the enzyme is bromide:hydrogen-peroxide oxidoreductase, which is based on the most electronegative halide they can oxidise; chloroperoxidases (VCPO) can catalyse the oxidation of three different halides,

i.e. chloride, bromide and iodide; bromoperoxidases (VBPO) can oxidise only bromide and iodide; and iodoperoxidases (VIPO) are specific for iodide. Conversion of metabolites to halometabolites may increase the biological activity of secondary metabolites alter the compounds to become bioactive metabolites or inactivate activity of harmful metabolic by-products. Halogenated compounds have various biological functions in nature, ranging from chemical defence to signalling.

The process of halogenation may involve many different halogenating enzymes, and the halometabolites or the proteins and genes responsible for producing the halometabolites are often studied from the marine environment settings as halogen ions are readily available in the marine waters as either free ions/salts or can easily be displaced into the intended compound from its origin compound. Bromoperoxidases are often vanadium-dependent and, hence, short formed as VBPO. Other haloperoxidases include the chloroperoxidase which is also vanadium-dependent and grouped under the Vanadium haloperoxidases (VHPO) that are key enzymes that oxidise halides and are involved in the biosynthesis of organo-halogens.

Three putative VHPO genes were predicted in the genome of the Flavobacterium *Zobellia galactanivorans*, a marine bacterium associated with macroalgae. In a phylogenetic analysis, these putative bacterial VHPO were closely related to other VHPO from diverse bacterial phyla but clustered independently from eukaryotic algal VBPO and fungal VCPO. This was similar to the results obtained in our study as the VBPO gene screened (MN551638-MN551642, Figure 5.1) belonged to the α/β hydrolase superfamily similar to the algal VBPO, *Corallina officinalis*. Conservation of the tertiary structural motif and an identical arrangement of amino acid residues at the vanadium active site suggest that algal VBPO could derive from a common ancestor, sharing a common evolutionary history with bacterial VBPO in the

α/β hydrolase superfamily. A Bayesian phylogram of the bromoperoxidase (BPO) genes sequenced was constructed based on protein motif translated using BLASTx as illustrated in Figure 6.4 (a) with three BPO gene sequences cloned from bacteria isolated from *Laurencia* sp. (SWD1: MN551638-MN551640) and two BPO gene sequences cloned from bacteria isolated from *G. manilaensis* (SWD2: MN551641-MN551642).

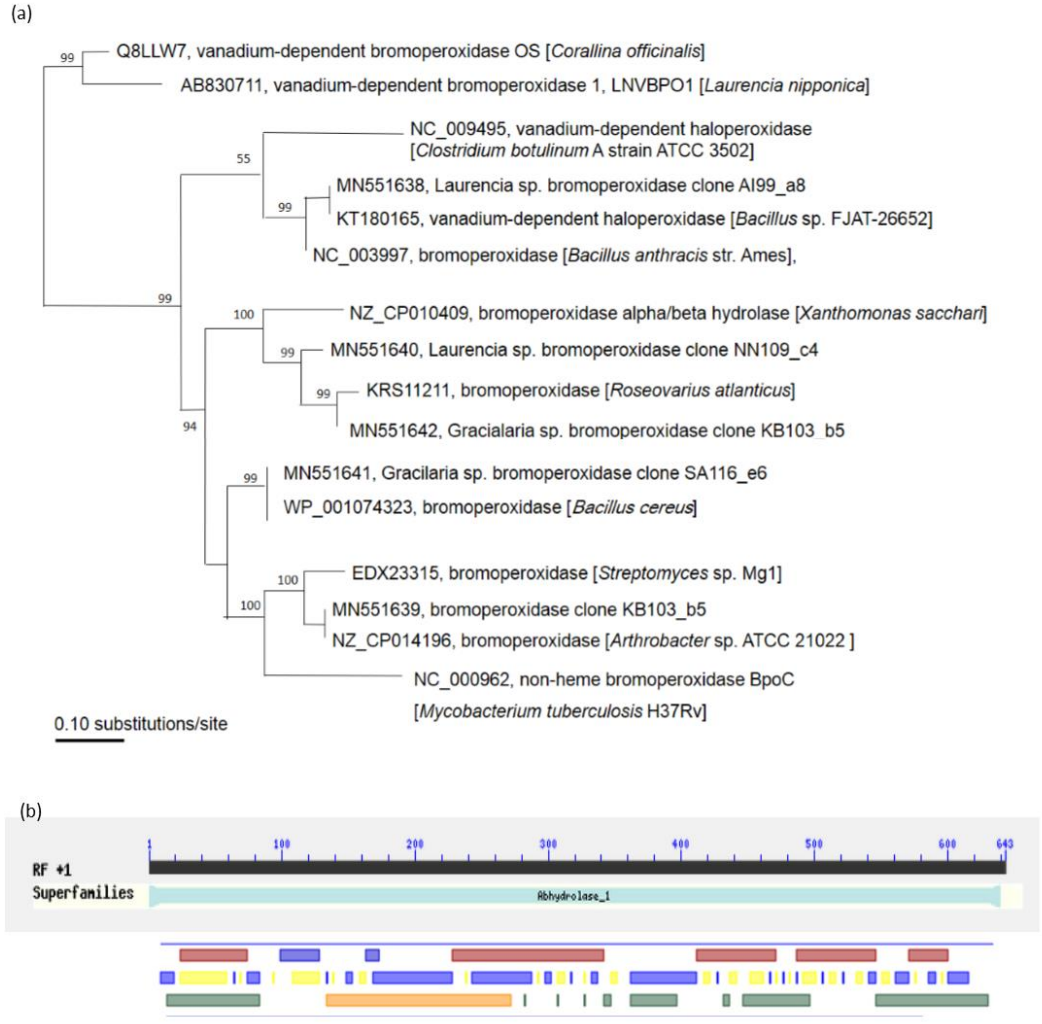



Figure 5.1 Bromoperoxidase (a) Bayesian Phylogram Constructed Based on Protein Motif Translated Using BLASTx; (b) Visual Similarity Search in BLASTp and ProteinPredict.

Figure 5.1 (b) illustrates BPO sequences MN551638-MN551642 similarity results aligned in the α/β hydrolase superfamily which consists of hydrolysing enzymes with alpha/beta hydrolase fold (containing 8 beta strands connected by 6 alpha helices). Red colour similarity indicated alignment to bromoperoxidases, blue to epoxyhydrolases, green to esterases (aryl- and Pimeloyl-ACP methyl ester carboxyl-), and yellow to chloroperoxidases, which are all members of the α/β hydrolase superfamily. Nevertheless, further study is required to establish the functions and pathway of the putative protein enzymes identified by functional genes screening.

## 5.2 STUDY LIMITATION

Much is needed to be done to identify bacterial diversity associated with the red seaweeds, *G. manilaensis* and *Laurencia* sp., including culture isolation, sanger and amplicon sequencing, sequence data analysis, taxonomic assignment and phylogenetic tree construction, and functional bromoperoxidase gene screening. Identifying the bacteria and its functional profile will help us understand the bacteria-seaweed interaction in Malaysian coastal waters. Additionally, seaweeds are potential sources of high biotechnological interest due to production of phycocolloid and a great diversity of compounds exhibiting a broad spectrum of biological activities, but there is also an urgent need for management options for a sustainable approach to the use of marine organisms as a source of food and bioactive compounds. There were several limitations encountered in this study, which included the possibility of agar media not being diverse or specific enough to grow more novel bacteria, technical difficulties where the abundant of initial culture available did not all yield DNA when extracted with different strategies (commercial kits and CTAB method), unsuccessful screening of a variant of other functional halogenase genes such as primers from the study of

Hornung et al. (2007) and Zehner et al. (2005), and limited bioinformation available in databases for the region of bromoperoxidases gene screened

## 5.3 RECOMMENDATIONS FOR FUTURE WORK

For a future outlook on marine bioprospecting where the search for seaweed molecules can be done in a sustainable manner with the conservation of biodiversity, several recommendations are proposed. These include future study to increase strategies for bacterial culture in possibly a bioassay-guided isolation approach of the seaweed-associated bacteria (e.g. antifungal, anti-tumour activity) where discovery of bioactive compounds can be achieved, to increase variety of supplemented agar media that are either nutrient-low or nutrient-rich tailored to bacteria of interest, and to optimise screening for different functional genes such as other halogenase genes, non-ribosomal protein synthase genes (NRPS), and polyketide synthase genes (PKS) with the hopes to expand knowledge on the functional profile of seaweed-associated bacteria.

Research on other sessile marine host such as sponges has shown that their bioactive compounds often possess a symbiont origin, indicating holobionts as hotspots for bacterial metabolic versatility with polyketide and terpene synthases identified in several marine bacteria symbiont to host organisms. Therefore, new technologies that focus on symbiont-driven production of seaweeds and seaweed-derived bioactive molecules are encouraged to contribute to a more sustainable blue economy while conserving the natural seaweed biodiversity.

## 5.4 CONCLUSION

This study has established a bacterial library for the two different red seaweeds, *G. manilaensis* and *Laurencia* sp., in Peninsular Malaysia. It was observed that the two red seaweeds exhibited almost similar bacteria phyla. Proteobacteria was the predominant bacterial phylum in both seaweed species samples, but the bacterial phyla diversity patterns identified in seaweed genus *Gracilaria* was broader than the phyla diversity identified in genus *Laurencia*. The difference in the symbiont patterns could be due to the seaweed hosts being classified as low-density habitat (LDH) host for genus *Laurencia* (SWD1) and high-density habitat (HDH) host for genus *Gracilaria* owing to their low and high symbiont richness, respectively.

The bacterial community structure analysis revealed that the associated bacteria have unique functional profiles that contributes to the development, health and ecology of hosts, which hinted to the postulates of Marine Holobiont Clusters (MHC) and Seaweed-specific Clusters (SWC). Nine of the OTUs screened through *16S rRNA* amplicon of HiSeq Illumina 2500 showed nearest neighbours in SWC while most others were in MHC. It is interesting to recap that the isolate MA177 were closely related to bacterial clones isolated from red seaweed, *Porphyra yeozensis*. Evidently, the phylogenetic analysis shed insights on bacterial evolutionary patterns and the origin of symbiosis clusters.

Furthermore, studies on seaweed-bacterial associations and functional roles of the bacteria in its holobiont environment could led to the discovery of beneficial bacteria in mariculture industry and the characterisation of novel bioactive compounds. The bromoperoxidase functional gene screening gave an idea of the potential of bacteria involved with seaweed hosts *Laurencia* sp. and *G. manilaensis* in producing halogenated metabolites as such compounds have been isolated from these

two genera. Bacteria from phyla Firmicutes, Actinobacteria and Alphaproteobacteria showed BPO, but they resemble minority bacteria population since they present at a very low number.

Hence, all the objectives in this study were accomplished. This study draws a wholesome picture of seaweed-associated bacteria diversity in two different red seaweeds by analysing the genomic datasets resultant from the combination of the culture-dependent and culture-independent approaches with an attempt to understand bacterial diversity and functional profile in the red seaweed environment. Limitations of this study, however, must be overcome in the future with better strategies to investigate deeper the on the cultivable bacteria and its functional potential.

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# APPENDIX A: OTU BLAST TABLE

Table A1. BLAST Table for Predominant OTUs of Amplicon Sequencing Dataset

| OTU | Len. (bp) | Nearest neighbour description | Accession number | Ident. (%) | Isolation Source |
|---|---|---|---|---|---|
| Phylum α-Proteobacteria | | | | | |
| OTU_9 | 253 | Uncultured bacterium **clone Dstr F24**, 16S ribosomal RNA gene, partial sequence. | GU118268 | 100 | *Diploria strigosa* |
| OTU_13 | 253 | Uncultured bacterium **clone N8_12_C_6D_46**, 16S ribosomal RNA gene, partial sequence. | KF271126 | 100 | Volcanic ash adding |
| OTU_14 | 321 | *Albidovulum* sp. **HME9669**, 16S ribosomal RNA gene, partial sequence | KF679400 | 99 | Seawater |
| OTU_15 | 253 | bacterium **clone HAMb2_011**, 16S ribosomal RNA gene, partial sequence. | JX984052 | 99 | biofilm |
| OTU_16 | 253 | *Altererythrobacter* sp. **CJ46** 16S ribosomal RNA gene, partial sequence | JQ806736 | 99 | Seawater |
| OTU_19 | 253 | Uncultured alpha proteobacterium clone MD2.49 16S ribosomal RNA gene, partial sequence. Taxon: 91750. | FJ403098 | 100 | *Montastrea faveolata* |
| OTU_21 | 253 | *Altererythrobacter* sp. **strain ESS-24**, 16S ribosomal RNA gene, partial sequence. Taxon: 1872480. | MH057249 | 98 | Marine holobiont |
| OUT_22 | 253 | Bacterium clone EK_CK579, 16S ribosomal RNA gene, partial sequence. | JN038228 | 98 | Soil |
| OTU_27 | 253 | *Rhodobacteraceae* bacterium **strain M58**, 16S ribosomal RNA gene, partial sequence. | MH475110 | 100 | green microalga, *Tetraselmis suecica* F&M-M33 |
| OTU_30 | 253 | *Amylibacter lutimaris* **strain m18,** 16S ribosomal RNA gene, partial sequence. Taxon: 2176002. | MF113253 | 98 | sea-tidal flat sediment |
| OTU_38 | 253 | *Ruegeria* sp. **URN41**, gene for 16S ribosomal RNA, partial sequence. | AB916872 | 100 | Seawater |
| OTU_40 | 253 | Uncultured *Parvularcula* sp. clone 0307_BHT1_49 16S ribosomal RNA gene, partial sequence. | JQ515700 | 99 | *M. faveolata* |

| OTU | Len. (bp) | Nearest neighbour description | Accession number | Ident. (%) | Isolation Source |
|---|---|---|---|---|---|
| OTU_44 | 253 | Uncultured *Rhodobacteraceae* bacterium **clone TDNP_Bbc97_264_1_71,** 16S ribosomal RNA gene, partial sequence. | FJ516788 | 98 | Biofilm |
| OTU_48 | 253 | Mucus bacterium **isolate 80**, 16S ribosomal RNA gene, partial sequence. Taxon: 284897. | AY654818 | 99 | Occulina patagonica |
| OTU_56 | 253 | *Rhizobium* sp. **strain SMB11** partial *16S rRNA* gene, **isolate 8d**. | LT599834 | 97 | Sambhar Lake |
| OTU_58 | 253 | *Novosphingobium aromaticivorans* gene for *16S rRNA*, partial sequence, **strain: FM-1**. | AB331237 | 99 | surface water of Ai River |
| OTU_63 | 253 | *Labrenzia* sp. **strain 1334-149** 16S ribosomal RNA gene, partial sequence. | KY770174 | 98 | Synechococcus sp. 1334 phycosphere |
| OTU_76 | 253 | *Hyphomicrobium nitrativorans* **NL23** 16S ribosomal RNA, complete sequence. | NR_121713 | 97 | Biofilm |
| OTU_77 | 253 | Rhodospirillaceae bacterium **clone B25** 16S ribosomal RNA gene, partial sequence. | GQ249531 | 100 | Sediment |
| OTU_83 | 253 | Uncultured *Hyphomonas* sp. **clone MD3.38** 16S ribosomal RNA gene, partial sequence. | FJ425626 | 100 | *M. faveolata* |
| OTU_92 | 253 | *Erythrobacter* sp. **MaW-Ado6** gene for 16S ribosomal RNA, partial sequence. | LC324688 | 100 | marine water |
| OTU_93 | 253 | *Oceanicella actignis* **strain PRQ-68** 16S ribosomal RNA gene, partial sequence. | JQ864436 | 100 | Seawater |
| OTU_95 | 253 | Uncultured alpha proteobacterium clone DPC110 16S ribosomal RNA gene, partial sequence. Taxon: 91750. | DQ269080 | 99 | surface of marine macro-alga, *Delisea pulchra* |
| OTU_102 | 253 | *Anderseniella baltica* partial *16S rRNA* gene, **type strain BA141T**. Taxon: 444440. | AM712634 | 99 | Baltic Sea sediment |
| OTU_107 | 253 | Uncultured Hyphomicrobiaceae bacterium **clone TDNP_USbc97_258_1_106** 16S ribosomal RNA gene, partial sequence. | FJ516940 | 99 | upper sediment |

| OTU | Len. (bp) | Nearest neighbour description | Accession number | Ident. (%) | Isolation Source |
|---|---|---|---|---|---|
| OTU_108 | 253 | *Erythrobacter gaetbuli* **strain SW-161** 16S ribosomal RNA gene, partial sequence. | AY562220 | 97 | tidal flat of the Yellow sea |
| OTU_138 | 253 | Uncultured Rhodospirillaceae bacterium **clone 0907_Mf_HT3_B5** 16S ribosomal RNA gene, partial sequence. Taxon: 352351. | JQ516560 | 99 | *Montastraea faveolata* healthy tissue |
| OTU_195 | 253 | *Rhodobacter lacus* **strain JA826**, 16S ribosomal RNA, partial sequence. | LN835251 | 97 | Lake |
| OTU_197 | 253 | Uncultured bacterium gene for 16S ribosomal RNA, partial sequence, **clone: TSNIR003_P12**. Taxon: 77133. | AB487448 | 98 | rice paddy soil |
| OTU_292 | 253 | *Erythrobacter* sp. **K10-17** 16S ribosomal RNA gene, partial sequence. Taxon: 574705. | FJ425211 | 97 | Seawater |
| OTU_373 | 253 | *Shimia* sp. **MYP11**, 16S ribosomal RNA gene, partial sequence. | MK353796 | 98 | Seawater |
| OTU_472 | 253 | *Rhodobacter lacus* **strain JA826** 16S ribosomal RNA, partial sequence. Taxon: 1641972. | NR_158008 | 99 | Lake |
| OTU_566 | 253 | *Litoreibacter* sp. **F3**, 16S ribosomal RNA gene, partial sequence. | KJ786461 | 99 | Host: *Fucus spiralis* (macroalga) |
| OTU_736 | 253 | *Ruegeria* sp. **strain HIM_5_10-4** 16S ribosomal RNA gene, partial sequence. | MG561908 | 100 | Marine sponge |
| OTU_879 | 253 | *Roseobacter* sp. **MG19** 16S ribosomal RNA gene, partial sequence. | JN791323 | 96 | Sediment |
| OTU_941 | 253 | Uncultured Rhodobacteraceae bacterium clone J8P8_10G10 16S ribosomal RNA gene, partial sequence. | HQ242487 | 89 | Seawater |
| OTU_1591 | 253 | *Amylibacter lutimaris* **strain G2_042**, 16S ribosomal RNA gene, partial sequence. | MF195229 | 98 | shrimp intestine |
| Phylum γ-Proteobacteria | | | | | |
| OTU_12 | 253 | *Vibrio tritonius* **strain IMCC34148**, 16S ribosomal RNA gene, partial sequence | MG456771 | 100 | Seawater |
| OTU_18 | 253 | Uncultured marine bacterium clone A6M_UNP3_B9 16S ribosomal RNA gene, partial sequence. | GU319341 | 99 | Seawater |

| OTU | Len. (bp) | Nearest neighbour description | Accession number | Ident. (%) | Isolation Source |
|---|---|---|---|---|---|
| OTU_29 | 253 | Uncultured bacterium **clone AF10-3-7_C30**, 16S ribosomal RNA gene, partial sequence. | JN655243 | 97 | *Ircinia fasciculata* (sponge) |
| OTU_31 | 253 | *Alteromonas macleodii* **strain HAKA** 16S ribosomal RNA gene, partial sequence. Taxon: 28108. | MH454344 | 97 | algal culture |
| OTU_43 | 253 | Uncultured gamma proteobacterium partial *16S rRNA* gene, **clone TAA-10-78**. | AM259846 | 97 | sponge, Tethya aurantium |
| OTU_50 | 253 | *Reinekea* sp. 1-4 gene for 16S ribosomal RNA, partial sequence. | LC373540 | 97 | surface seawater |
| OTU_52 | 253 | *Alcanivorax dieselolei* strain S1026 16S ribosomal RNA gene, partial sequence. | MF139137 | 100 | Oil and water mixture in Qinghai Oilfield |
| OTU_59 | 253 | *Teredinibacter* sp. JL1 16S ribosomal RNA gene, partial sequence. | HQ593601 | 100 | red algae, Gelidium amansii |
| OTU_61 | 253 | Uncultured Thiotrichales bacterium gene for 16S ribosomal RNA, partial sequence, clone: NeMCo2. | LC278460 | 100 | coelomic fluid of *Asterias amurensis* |
| OTU_65 | 253 | Fastidiosibacteraceae bacterium strain SYSU SYW-6 16S ribosomal RNA gene, partial sequence. | MH329654 | 97 | Coastal seawater |
| OTU_66 | 253 | Uncultured gamma proteobacterium clone XE3D01 16S ribosomal RNA gene, partial sequence. | JN596632 | 100 | *Xestospongia muta* (marine sponge) |
| OTU_69 | 253 | *Agaribacter* sp. 2p52 16S ribosomal RNA gene, partial sequence. | KX495218 | 97 | algae |
| OTU_70 | 253 | *Pseudomonas balearica* strain Y91 16S ribosomal RNA gene, partial sequence. | MF405230 | 100 | Sediment |
| OTU_72 | 253 | Uncultured gamma proteobacterium clone 0907_Mf_DT1_B42 16S ribosomal RNA gene, partial sequence. | JQ516285 | 100 | Montastraea faveolata |
| OTU_78 | 253 | *Idiomarina baltica* strain NIOSSD020300 16S ribosomal RNA gene, partial sequence. | KY616476 | 100 | OMZ water column |
| OTU_89 | 253 | Uncultured gamma proteobacterium **clone A19** 16S ribosomal RNA gene, partial sequence. Taxon: 86473. | KC492838 | 97 | Baltic Sea redoxcline |
| OTU_94 | 253 | *Marinomonas communis* strain DS6F 16S ribosomal RNA gene, partial sequence. | MH748687 | 100 | Camalti seawater |

| OTU | Len. (bp) | Nearest neighbour description | Accession number | Ident. (%) | Isolation Source |
|---|---|---|---|---|---|
| OTU_100 | 253 | *Acinetobacter schindleri* strain KAA10 16S ribosomal RNA gene, partial sequence. | MH160096 | 99 | decomposed soil |
| OTU_136 | 253 | Uncultured gamma proteobacterium partial *16S rRNA* gene, **isolate 1.2, clone 1b_8**. | HE803945 | 97 | Marine seabed sediment |
| OTU_1567 | 253 | *Vibrio* sp. strain SAOS-147 TSBA 16S ribosomal RNA gene, partial sequence. | MH712090 | 99 | mangrove habitat |
| Phylum β-Proteobacteria | | | | | |
| OTU_57 | 253 | *Ralstonia pickettii* strain 1F 16S ribosomal RNA gene, partial sequence. | MK282223 | 98 | River water |
| | | *Ralstonia pickettii* strain ADZH5101 16S ribosomal RNA gene, partial sequence. | MK610811 | 98 | River water |
| Phylum δ-Proteobacteria | | | | | |
| OTU_45 | 253 | Uncultured bacterium clone G08SC 16S ribosomal RNA gene, partial sequence. | JQ062836 | 99 | sponge tissue, Stylissa carteri |
| OTU_17 | 253 | Uncultured *Desulfocapsa* sp. **clone CBII115** 16S ribosomal RNA gene, partial sequence. Taxon: 218292. | DQ831556 | 99 | marine sediment |
| OTU_33 | 253 | Uncultured Desulfobacteraceae bacterium gene for *16S rRNA*, partial sequence, **clone: pItb-vmat-6**. Taxon: 218296. | AB294926 | 98 | microbial mat at a shallow submarine hot spring |
| OTU_42 | 253 | Delta proteobacterium **LacK10** 16S ribosomal RNA gene, partial sequence. | AY771932 | 98 | marine surface sediment |
| OTU_47 | 253 | Uncultured Desulfobacteraceae bacterium clone 0907_Mf_HT1_B61 16S ribosomal RNA gene, partial sequence. | JQ516448 | 99 | montastraea faveolata |
| OTU_60 | 253 | Uncultured delta proteobacterium **clone PK066** 16S ribosomal RNA gene, partial sequence. Taxon: 34034. | EF076107 | 99 | marine sponge *Plakortis* sp. |
| OTU_199 | 253 | *Pelobacter massiliensis* strain HHQ7 16S ribosomal RNA, partial sequence. | NR_104786 /FR749901 | 98 | |

| OTU | Len. (bp) | Nearest neighbour description | Accession number | Ident. (%) | Isolation Source |
|---|---|---|---|---|---|
| Phylum Firmicutes | | | | | |
| OTU_98 | 253 | Uncultured Lachnospiraceae bacterium clone B04_PigPlate_6 16S ribosomal RNA gene, partial sequence. | MG702787 | 100 | Feacal matter |
| | | Uncultured Lachnospiraceae bacterium clone G04_PigPlate_7 16S ribosomal RNA gene, partial sequence. | MG702724 | 100 | Feacal matter |
| Phylum Actinobacteria | | | | | |
| OTU_36 | 253 | Uncultured Acidimicrobiales bacterium gene for 16S ribosomal RNA partial sequence, clone: 12methane_1-10. Taxon: 310071. | LC171287 | 97 | Setae of *Shinkaia crosnieri* (crab/lobster) |
| | | Acidimicrobidae bacterium YM18-244 gene for *16S rRNA*, partial sequence. taxon:467092 | AB360344 | 97 | Marine |
| OTU_80 | 253 | Acidimicrobidae bacterium strain YM18-244 gene for *16S rRNA*, partial sequence. | AB360344 | 98 | Marine |
| OTU_151 | 253 | Uncultured actinobacterium clone Alchichica_AL52_2_1B_169 small subunit ribosomal RNA gene, partial sequence. | JN825530 | 98 | Alchichica alkaline lake |
| | | Uncultured actinobacterium **clone RODAS-066**, 16S ribosomal RNA gene, partial sequence. | JF344044 | 98 | Sediment |
| | | Uncultured actinobacterium **clone UA21**, 16S ribosomal RNA gene, partial sequence. | DQ269040 | 98 | surface of marine macro-algae Ulva australis |
| Phylum PAUC34f | | | | | |
| OTU_121 | 253 | Uncultured bacterium **clone AF10-99_C20**, 16S ribosomal RNA gene, partial sequence. | JX206510 | 100 | Ircinia fasciculata (sponge) |
| | | Uncultured *Deferribacteres* bacterium **clone XD1C03**, 16S ribosomal RNA gene, partial sequence. | JN596739 | 99 | Xestospongia testudinaria (marine sponge) |

| OTU | Len. (bp) | Nearest neighbour description | Accession number | Ident. (%) | Isolation Source |
|---|---|---|---|---|---|
| Phylum Acidobacteria | | | | | |
| OTU_55 | 253 | Uncultured *Acidobacterium* sp. **clone XE3A07**, 16S ribosomal RNA gene, partial sequence. | JN596620 | 100 | *Xestospongia muta* |
| OTU_67 | 253 | Uncultured Acidobacteria bacterium clone XD2C11 16S ribosomal RNA gene, partial sequence. | FJ269343 | 99 | *Xestospongia testudinaria* (marine sponge) |
| Phylum Bacteroidetes | | | | | |
| OTU_28 | 253 | *Muricauda lutaonensis* **strain CC-HSB-11** 16S ribosomal RNA, partial sequence. Taxon: 516051. | NR_116363 | 100 | Water |
| OTU_41 | 253 | *Robiginitalea myxolifaciens* strain YM6-073 16S ribosomal RNA, partial sequence. | NR_041514 | 99 | Seawater |
| OTU_75 | 253 | *Cytophaga* sp. I-545 gene for 16S ribosomal RNA, partial sequence. | AB073573 | 99 | red alga |
| OTU_188 | 253 | *Tenacibaculum* sp. LS-831 16S ribosomal RNA gene, partial sequence. | KP162065 | 100 | ocean water |
| Phylum Cyanobacteria | | | | | |
| OTU_24 | 253 | *Acaryochloris marina* gene for 16S ribosomal RNA, partial sequence, **culture_collection: NIES:2412**. | LC190498 | 97 | Seawater |
| OTU_26 | 253 | *Anabaena* sp. **7-zaCirk11** 16S ribosomal RNA gene and 16S-23S ribosomal RNA intergenic spacer, partial sequence. Taxon: 1840130. | KT290355 | 98 | Seawater |
| OTU_35 | 253 | Uncultured *Cyanobacterium* sp. **clone XE3C06** 16S ribosomal RNA gene, partial sequence. Taxon: 751952. | JN596629 | 100 | sponge tissue, *Xestospongia muta* |
| OTU_62 | 253 | Uncultured cyanobacterium clone Gc30 16S ribosomal RNA gene, partial sequence. | HM474917 | 100 | *Gracilaria coronopifolia* |
| OTU_71 | 253 | Uncultured bacterium **clone E144_E10** 16S ribosomal RNA gene, partial sequence. Taxon: 77133. | KU578501 | 100 | ocean water |
| OUT_73 | 253 | *Prochlorococcus* sp. isolate SCGC AAA300-N22 *16S rRNA* gene, partial sequence. Taxon: 939840. | HQ675350 | 97 | HOT Station ALOHA, subtropical ocean gyre |

| OTU | Len. (bp) | Nearest neighbour description | Accession number | Ident. (%) | Isolation Source |
|---|---|---|---|---|---|
| Phylum Gemmatimonadetes | | | | | |
| OTU_110 | 253 | Uncultured Gemmatimonadetes bacterium clone XE2B11 16S ribosomal RNA gene, partial sequence. | JN596615 | 98 | *Xestospongia muta* |
| | | Uncultured Gemmatimonadaceae bacterium partial *16S rRNA* gene, clone CN82. taxon: 246130 | AM259917 | 96 | *Chondrilla nucula* (sponge) |
| Phylum Planctomycetes | | | | | |
| OTU_25 | 253 | Uncultured Planctomycetales bacterium **clone ML-Pla-46** 16S ribosomal RNA gene, partial sequence. Taxon: 100371. | FJ652505 | 99 | marine sponge, *Mycale laxissima* |
| OTU_51 | 253 | Uncultured planctomycete clone XE3C07 16S ribosomal RNA gene, partial sequence. taxon:120965 | JN596630 | 95 | Seawater |
| OTU_64 | 253 | Uncultured *Pirellula* sp. **clone Joinville11** 16S ribosomal RNA gene, partial sequence. Taxon: 298571. | FJ236059 | 98 | drinking water |
| OTU_473 | 253 | Uncultured planctomycete **clone FII-AN065** 16S ribosomal RNA gene, partial sequence. Taxon: 120965. | JQ580006 | 99 | sediments from Figueiras Beach |

# APPENDIX B: IMAGES

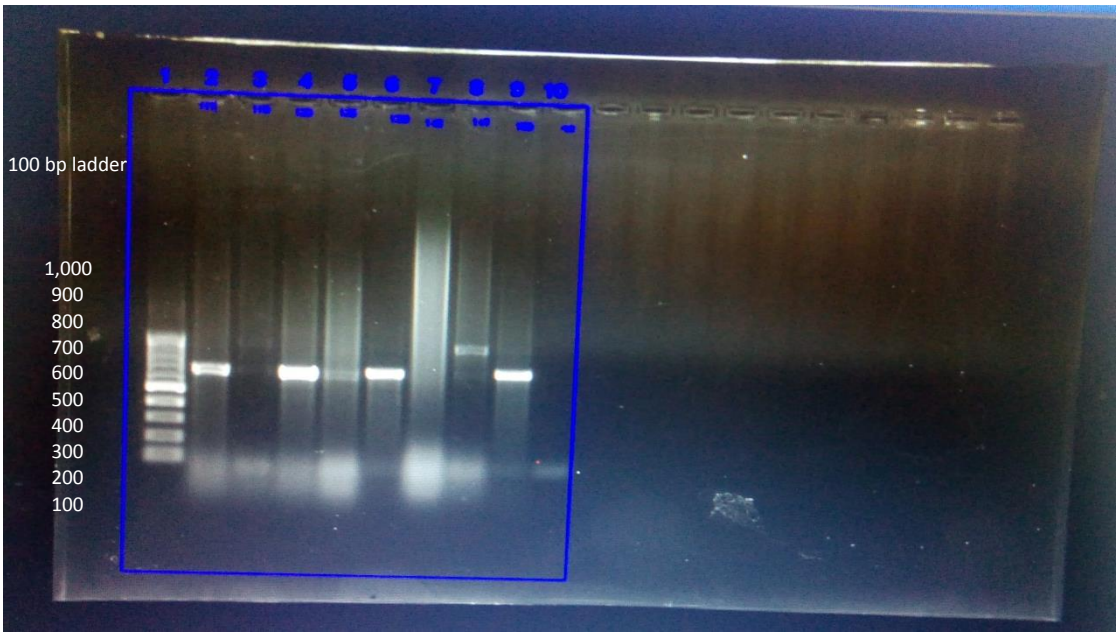


Figure B1: Gel electrophoresis visualisation of bromoperoxidase (BPO) gene primer amplification. Positive bands were estimated at 700 bp.

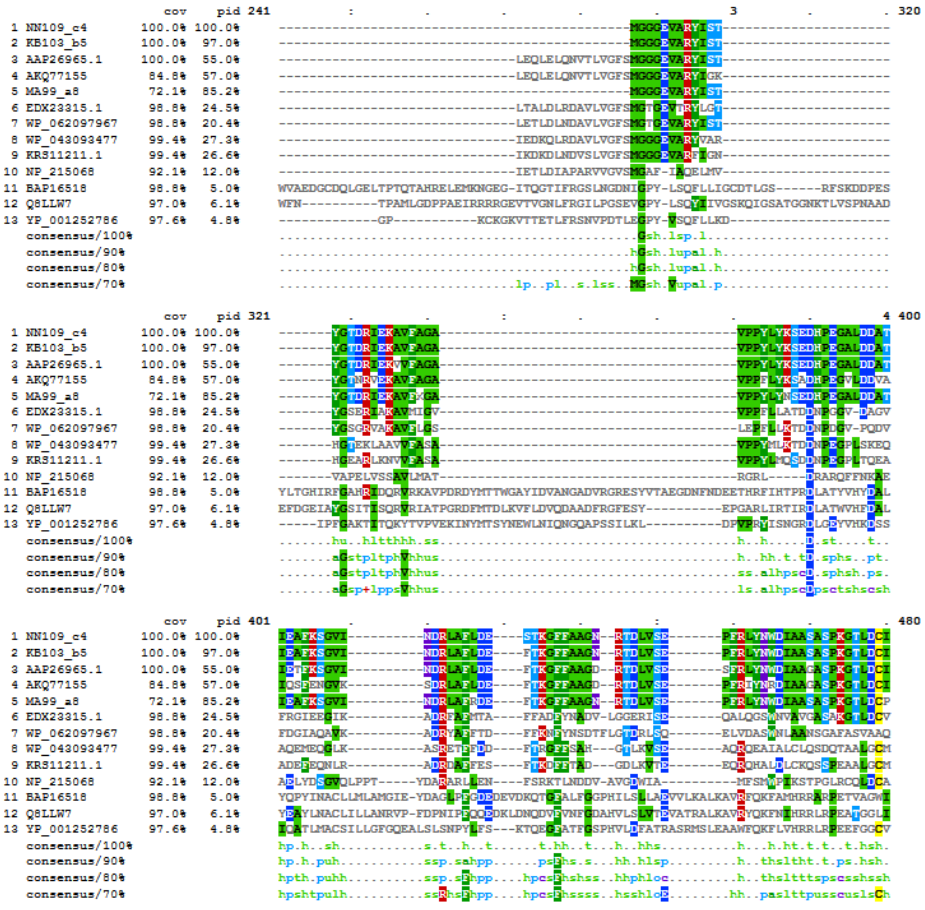

Figure B2: Bromoperoxidase sequence alignment indicating closer homology to bromoperoxidase genes screened from bacteria as compared to seaweed VBPO gene.