ANTIMICROBIAL ACTIVITY OF SOME MARINE MICROALGAE AGAINST COMMON BACTERIAL DISEASES IN AQUACULTURE IN CHATTOGRAM, BANGLADESH

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Roll No.: 0119/07

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Master of Science in Aquaculture

Department of Aquaculture
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This is to certify that we have examined the above Master's thesis and have found that is complete and satisfactory in all respects, and that all revisions required by the thesis examination committee have been made

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List of Acronyms and Symbols Used

Abbreviation and symbols	Elaboration
mm	Millimeter
µm	Micrometer
AMR	Antimicrobial resistance
ARGs	Antimicrobial Resistance Genes
WHO	World Health Organization
EPA	Ecosapentanoic Acid
DHA	Docosahexaenoic Acid
rRNA	Ribosomal RNA
CO_2	Carbon dioxide
>	Greater than
<	Less than
CVASU University	Chattogram Veterinary and Animal Sciences
EDTA	Ethylene Diamine Tetraacetic Acid
L	Liter
^0C	Degree Celsius
mg	Milligram
gm	Gram
%	Percentage
CFU	Colony Forming Units
µl	Microliter

Abstracts

The present investigation aimed to study antimicrobial activity of indigenous marine microalgae *Tetraselmis, Chlorella* and *Nannochloropsis* against Gram-negative *Aeromonas hydrophilla* and Gram-positive *Staphylococcus* and *Lysinibacillus sphaericus* isolated from diseased fish in Chattogram, Bangladesh. The highest antimicrobial activity was found in the extract of *Tetraselmis* which exhibited an inhibitory effect against *Aeromonas hydrophilia* (1.99mm). Moreover, *Chlorella* showed the highest inhibitory activity against *Staphylococcus* (1.262mm). However, *Nannochloropsis* showed no sensitivity against any of these three bacteria. *Chlorella* showed the MIC value of 20mg/ml against *Staphylococcus* and *Tetraselmis* at 10mg/ml against *Aeromonus hydrophila*. The observed antimicrobial activities might be linked to the contents of the algal extracts containing valuable fatty acids, carotenoids, chlorophylls, hycocyanin, phycoerythrin, phycobiliprotein and phenolic compounds that need to be determined. The studied microalgae could be considered as a potential natural source of bioactive compounds with antimicrobial activities.

CHAPTER 1
INTRODUCTION

1.1 Background of the study

Microalgae are classified as unicellular which, by photosynthesis, can transform the energy of its covert sun into chemical energy like the mainland plant to produce complex organic compounds (Harun *et al.*, 2010). Microalgae can be found all over the biosphere as they have evolved to survive and withstand environmental stress such as high or low temperature, drought, salinity, photo-oxidation, anaerobicity, osmotic pressure and ultraviolet exposure (Amaro *et al.*, 2011). The two major algae species are filamentous and phytophyton algae and the four main groups are diatoms, green algae, blue-green algae and golden algae (Demirbas, 2010). Microalgae possess compounds of great importance, such as polyunsaturated fatty acids, vitamins, carotenoids and many biologically active compounds (Spolaore *et al.*, 2006). Marine microalgae constitute attractive sources of novel and active metabolites, comprising proteins, enzymes, pigments and polyunsaturated fatty acids (PUFA) that could be exploited in pharmaceutical, food, feed and cosmetic industries (Mendes *et al*., 2003). Compounds with pharmaceutical characteristics, as antioxidative, anti-inflammatory, antimicrobial or antitumoral properties, have been identified; some of them have been in the clinical trial state (Catarina *et al*., 2011). Antimicrobial activities are among the most researched features in natural extracts. They have been attributed to different compounds, including, indoles, terpene derivatives, acetogenins, phenols, fatty acids and hydrocarbons (Bhakuni *et al.*, 2006).

In 2018, global aquaculture fish production reached 82.1 million tonnes, 32.4 million tones of aquatic algae and 26000 tonnes of ornamental seashells and pearls, a total of 114.5 million tonnes have been brought in high. In 2018, aquaculture fish production was dominated by finfish (54.3 million tones- 47 million tonnes from inland aquaculture and 7.3 million tonnes from marine and coastal aquaculture). Asia dominates this production, accounting for 89 per cent by volume and 79 percent by value, with China by far the largest producer 42 percent in 2018) (Cai *et al.*, 2019).

The increased growth of aquaculture production is achieved despite facing many challenges in the aquaculture environment. Disease problems constitute the largest single cause of economic losses in aquaculture. Different diseases occur among the fishes such as bacterial, viral, infectious, and parasitic diseases. The major bacterial species in aquaculture are *Rickettsiales, Aeromonas, Enterococcus, Flavobacterium, Flexibacter, Pseudoalteromonas, Pseudomonas, Streptococcus* and *Vibrio* (Southgate, 2003). Bacterial diseases cause serious wild and cultured mortality of fish (Grisez *et al.*, 1996). The farm issues are normally resolved by avoiding disease outbreaks by using medications or chemicals to cure the real illness. Antimicrobial agent usages in aquaculture operations increased significantly. Antibiotics have been used in both human and veterinary medicine experimentally attempted prevention of bacterial fish infection. *Aeromonas hydrophila* and *Aeromonas sobria* are pathogens that most important causes of mortality of freshwater fish farmed in Bangladesh. *Streptococcus* affected the farmed fish in either salt or freshwater environments, typically in tropical regions. High stocking densities, poor water conditions and high temperatures are the most favorable conditions for *Streptococcal* outbreaks (Darwish *et al.*, 2005).

Because of the growing bacterial resistance against commercial standard and reserve antibiotics, researchers are searching for new active substances with antibacterial activity against pathogenic bacteria of increasing importance (Mundt *et al.*, 2007). Due to the increase in disease outbreak and the development of microbial resistance, there is an urgent need to prevent infectious agents and other harmful microorganisms without propagating antibiotic resistance pattern (Kemper *et al.* 2008). One of the possibilities is to use nanoparticles as antimicrobial drug in aquaculture but their potential use for disease control is not fully explored yet. Antibiotic residues remain as sediment on the base of ponds and penetrate into the soil; this will leave negative impact on the environmental toward the fishes or shrimps. Microalgae act as natural compound that might be used as an alternative way to antibiotics for the purpose of limiting the growth of bacterial pathogens (Kokou *et al.*, 2012).

1.2 Significance of the study

Farmers face severe outbreak of disease caused by bacteria pathogens which cause significant economic losses in the field of aquaculture. Basically, larvae fish or shrimp is more easily infected with various bacteria. To solve this problem, many scientists

developed various chemical that consisting of the antimicrobial agent to prevent the propagation of bacteria under aquaculture conditions. However, these chemicals which are used in the fish or shrimp farming are highly costly and the most serious problem is it may cause the chemical residues remain in the organism's body and the consequent effect to human after consumption of the contaminated fish. So, it is very important to find out the other effective or alternative ways to prevent the propagation of bacteria and it may not cause any effect to fish or shrimp. Microalgae not only are a food source for fish, but they are able to produce the bioactive compound which acts as an antimicrobial to certain bacterial species. Due to these bioactive compound present in the microalgae, it inhibits the pathogenic bacteria to infect host and consequently eliminates. Hence, microalgae have a potential to substitute the costly chemical antimicrobial agent. Microalgae can be easily cultured in huge density and commercially applied in the aquaculture or fish farming. Besides that, the microalgae have high nutrients profile like docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), arachidonic acid (ARA) and polyunsaturated fatty acids (PUFA) that needed for aquatic organisms to grow and enhance the immune system. Microalgae also use as food supplements for aquatic organisms in case of insufficiency of artificial diet.

Aims and objectives: The overall aim of this study is to demonstrate antimicrobial activities of marine micro algae on bacteria causing diseases in fish. The specific objectives are:

- To study different microalgae whether they have antimicrobial effect on bacteria causing diseases in fish.
- To analyse the degree of antimicrobial activity of microalgae as potential alternative of currently available antibiotics.

CHAPTER 2
REVIEW OF LITERATURE

2.1 Marine microalgae and their potentials in pharmaceutical field

Algae are a group of aquatic photosynthetic microorganisms which have the ability to transform carbon dioxide into biochemical that can be further processed into biofuels, food, feed and high value bioactive compounds (Walker *et al.,* 2005). Microalgae are present in both aquatic as well as terrestrial environment, signifying a large variety of species living in a wide range of habitats. It is predicted that more than 50,000 species of microalgae prevail, but only around 30,000 have been studied and analyzed (Richmond, 2004). The major requirements for growth of algae are sunlight, water, nutrients and arable land. Microalgae have the ability to fix CO_2 using solar energy with efficiency 10 times more than that of the terrestrial plants. Algae are more proficient at utilizing sunlight than terrestrial plants and they consume harmful pollutants, resource requirements are very less and do not compete with food or agriculture for precious resources (Brennan *et al.*, 2010).

Algae are heterogeneous group of plants with a long fossil history. Two major types of algae can be identified: the macroalgae (seaweeds) occupy the littoral zone, which included green algae, brown algae and red algae, and the micro algae are found in both benthic and littoral habitats and also throughout the ocean waters as phytoplankton (Garson, 1989). Phytoplankton comprises organisms such as diatoms (bacillariophyta), dinoflagellates (dinophyta), green and yellow–brown flagellates (chlorophyta; prasino-phyta; prymnesiophyta, cryptophyta, chrysophyta and rhaphidiophyta) and blue–green algae (cyano-phyta). As photosynthetic organisms, this group plays a key role in the productivity of oceans and constitutes the basis of the marine food chain (Wynno *et al.,* 1985). The extraction of metabolites with potential applications in biomedicine and pharmacology is a quite new trend in microalgae biotechnology. The natural active compounds present in the microalgal biomass is responsible for distinct biological activities, such as cytotoxic, antibiotic, antioxidant, antifungal, anti-inflammatory and antihelminthic compounds (Pulz *et al.*, 2008).

2.2 Marine microalgae available in Bangladesh

Almost all seaweed or plants that remain attached to the bottom of the sea are algae. There are microscopic algae as well, which float in water as phytoplankton. Seaweed needs a hard or rocky substratum for growth and development. Marine microalgae are abundantly found around the Cox's Bazar and St. Martin's Island of Bangladesh. 45 taxa under 17 genera of green algae have been reported from Bangladesh coasts (Islam *et al.*, 2000). They grow abundantly in winter, and spring growth is affected by rainwater runoff. The super-tidal zones or splash zone of the benthic marine environment is dominated by blue-green such as *Calothrix*. In the inter-tidal and sub-tidal zones, brown algae are dominated. Among the several green species, *Chlorella miminutissima, Tetrselmis chuii, Nannochloropsis* sp., *Arthrospira platensis, Isochrysis*sp., *Chondruscrispus, Mastocarpus stellallatus, Ascophyllum nodosum, Alaria esculentus, Sprirulina platensis, Chlorella esculentus, Nannochloropsis oculata* and *Dunaliellasalina*etc are found in St. Martin's Island of Bangladesh.

Tetraselmis sp. has potential to inhibit certain bacteria because it contains antioxidative substances that is an important strain used in pharmacological studies. Based on the Kokou *et al.,* (2012) *Vibrio* bacterial strains were incubated with the*Tetraselmis* sp. and cultured for 5 days in natural photoperiod and light condition. The result of *Tetraselmis* sp. had showed the ability to inhibit the growth of six *Vibrio* bacterial strains which were *V. parahaemolyticus*, *V. anginolyticus*, *V. anguillarum*, *V. splendidus*, *V. scophthalmi* and *V. lentus*.

Nannochloropsis sp. also has the capacity to use as an alternative to replace the antibiotics. Terpenoids is an active compound contains in the *Nannochloropsis* sp. and it is most powerful antioxidants in the body and it has antibacterial activity. The functions of terpenoids are obtained through organic synthesis process and regain of body cells. Kokou *et al.,* (2012) reported that *Nannochloropsis* sp. had the ability to inhibit the growth of six *Vibrio* bacterial strains which were *V. parahaemolyticus*, *V. anginolyticus*, *V. anguillarum*, *V. splendidus*, *V. scophthalmi* and *V. lentus*.

Furthermore, *Chlorella* sp. naturally rich in proteins, vitamins, minerals and dietary fibers. Its reported benefits include boosting antibody count, promoting weight loss, and fighting cancer and other diseases. *Chlorella* contains a range of

nutrients including proteins, omega-3 fatty acids, vitamins, and minerals, including antioxidants. *Chlorella* has a tough, rigid cell wall that makes it hard to digest in its natural form but processing makes its nutrients digestible.

2.3 Characteristics of common marine microalgae species

2.3.1 *Nannochloropsis* sp.

Nannochloropsis sp. is very small unicellular organism that lives mainly in the marine environment *(*Fawley *et al.,* 2007). It acts as major food for aquaculture because the reproduction rate is very fast and retains fatty acids that do not appear in the other species of phytoplankton. It is classified as picoeukaryotic plankton because the size of *Nannochloropsis* sp. is in the range of 2 to 5 µm and it is non-motile spherical in shape (**Figure 2.1**). *Nannochloropsis* sp. acts as pigment producer that mean it is capable to produce high concentration of pigments such as astaxanthin, zeaxanthin and canthaxanthin. It is widely used in hatchery for feeding the fish fry and rotifer as it contains highly nutritional compounds such as sterols, eicosapentaenoic acid (EPA) and long chain PUFAs which promotes the good growth of fish fry.

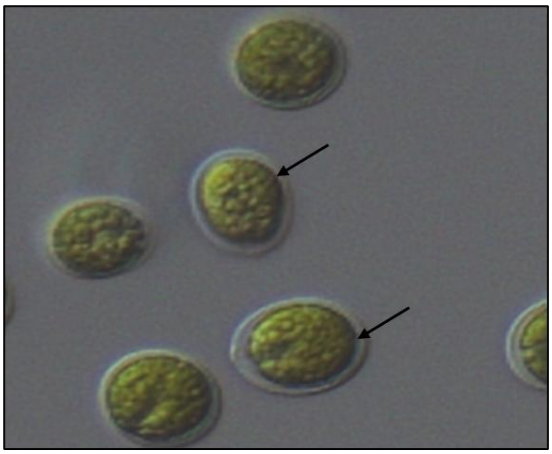

Figure 2.1 *Nannochloropsis* sp. is small, non-motile spheres.

According to the Algae Base, the taxonomy for *Nannochloropsis* sp. as below:

Domain : Eukaryota
Kingdom : Chromista
Phylum : Ochrophyta
Class : Eustigmatophyceae
Older :Eustigmatales

Family :Monodopsidaceae

Genus :*Nannochloropsis*

2.3.2 *Tetraselmis* sp.

Tetraselmis sp. is unicellular flagellate's phytoplankton that is elliptical or almost spherical in shape with green motile cells. The motile cells of *Tetraselmis* sp. are generally laterally compressed, and it forms 4 equal and homodynamic flagella in an anterior pit of the cell (**Figure 2.2**). It usually grows to 10 µm long x 14 µm wide. Thin cell wall formed by extracellular fusion of scales cover the cell which is called theca. The hairs and pentagonal scales will cover the flagella. *Tetraselmis* sp. has single chloroplasts which comprise a single eyespot and a pyrenoid that only can find in *Tetraselmis* (Arora *et al*., 2013). The taxonomic decision for *Tetraselmis* sp. is hard to decide because of the complex process of cellular characterization (Lee *et al.*, 2009). For the reproduction system, it is reproduced by asexual division when in the non-motile stage. During the non-motile stage, the new walls develop and a stalk formed by old walls accumulating as concentric rings around the cell or being polarized on one side (Guiry *et al.*, 2013). It has been used in aquaculture for decades because it acts as a source of nutrition for invertebrates (Laws *et al.*, 2011). The examples are juvenile molluscs, shrimp larvae and rotifers (Arora *et al.*, 2013).

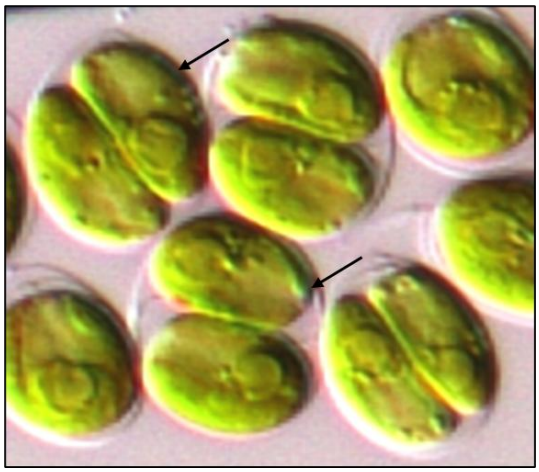

Figure 2.2 *Tetraselmis* sp. is elliptical or spherical with 4-flagella.

According to the Algae Base, the taxonomy for *Tetraselmis* sp. as below:

Domain : Eukaryota
Kingdom : Plantae
Phylum : Chlorophyta
Class :Chlorodendrophyceae
Older : Chlorodendrales
Family : Chlorodendraceae
Genus : *Tetraselmis*

2.3.3 *Chlorella* sp.

Chlorella is a genus of single-celled green algae belonging to the division Chlorophyta. It is spherical in shape, about 2 to 10 μm in diameter, and is without flagella (**Figure 2.3**). It contains the green phyotosynthetic pigments cholophyll-a and cholophyll-b in its choloplast. In ideal conditions it multiplies rapidly, requiring only CO_2, water, sunlight and a small amount of minerals to reproduce (Safi *et al.,* 2014).

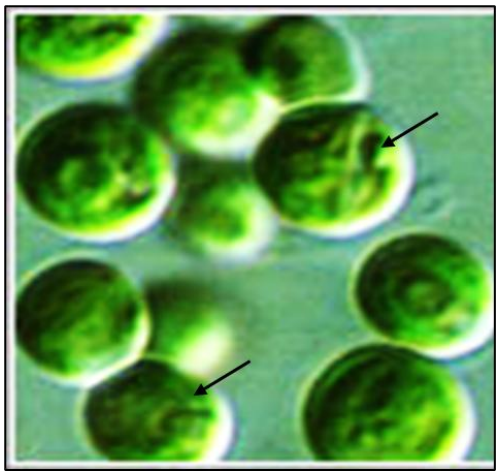

Figure 2.3 *Chlorella* sp. is spherical, single-celled green algae with no flagella.

According to the Algae Base, the taxonomy for *Chlorella* sp. as below:

Phylum : Chlorophyta
Class :Trebouxiphyceae
Older : Chlorellales
Family : Chlorollaceae

Genus : *Chlorella*

2.4 Antimicrobial activity of marine microalgae

In addition to the continuing development of antibiotic-resistance microbial pathogens, there has been a growing need for new antimicrobial compounds. One of the most interesting sources of antimicrobial compounds has been the aquatic ecosystem as various marine organisms generate bioactive metabolites in response to environmental stress and evolve chemical resistance and survival strategies (Mendiola *et al.,* 2007). From marine sources, a significant number of new active antimicrobial compounds have been isolated. However, most of these compounds have not been defined yet (Sanmukh *et al.,* 2014). Marine microalgae are valuable sources of new and active metabolites that could be used in the medicinal, fruit, feed and cosmetic industries including protein, enzymes, pigment and polyunsaturated fatty acids (PUFAs) (Mendes *et al.,* 2003). In natural extracts, antimicrobial activity is among the most researched properties. Various compounds including indoles, terpene derivatives, acetogenes, phenol, fatty acids and hydrocarbons have been traced to them (Bhakuni *et al.,* 2006).

2.5 Marine microalgae as potential alternative to antibiotics in aquaculture industry

2.5.1 Bacterial diseases of aquaculture in Bangladesh

Infectious diseases are a significant concern in the context of fish farming and can be defined as infectious bacterial, viral or fungal diseases in general. These diseases are generally associated with high rates of mortality and morbidity, leading to adverse effects on producers, consumers and the environment (Hasan *et al.,* 2005). A variety of factors are accelerating bacterial infections and diseases in fish farms, including changes in the physical and chemical parameters of pond water such as increased turbidity, temperature, salinity, pH, conductivity of water and low dissolved oxygen (Jacobs *et al.,* 2007). Over 13 bacterial genera have been reported to cause bacterial diseases in the aquaculture field worldwide (Pridgeon *et al.,* 2012). In pond-raised fish, bacterial infections are caused by motile members of the *Aeromonas* genus. *Aeromonas hydrophila, Aeromonas sorbia, Aeromonas caviae, Aeromonas schuberti* and *Aeromonas veronii* are among others (Azad *et al.,* 2001). In farmed fish, Edwardsiellosis is a serious bacterial septicemic disease and is caused by Gram-negative bacterium, *Edwardsiella* sp. (Nadirahet *et al.,* 2012). *Flavobacterium* sp. is

another bacterial pathogen in Tilapia farms and is highly contagious especially to fingerlings resulting in high mortality. The bacterial illness caused by *Streptococcus* sp. is Streptococcosis. Freshwater-cropping trout is the most susceptible fish against common pathogenic *Streptococcus* species that affects fish. *Streptococcus* sp. are gram-positive bacteria organized in strings and cocci. Another parasite affecting tilapia is *Streptococcus agalactiae* which is associated with intense breeding of brood stock (Hernandez *et al.,* 2009). Streptococcosis in tilapia farms can cause a mortality of up to 70% leading to dramatic economic loss from outbreaks (Fawzy *et al.,* 2014). A wide number of freshwater fish species including tilapia are infected by *Pseudomonas*, the etiological agent for red skin disease. It is suspected that *Pseudomonas anguilliaseptica* is one of the most effective pathogens in cultured fish (Mastan, 2013).

Diseases caused by *Aeromonas hydrophila*

Hemorrhagic septicemia in fish caused by motile aeromonads of the *Aeromonas hydrophila* complex (Schaperclaus, 1992). In several species of freshwater fish and sometimes in marine fish and in amphibian, reptiles, livestock and humans around the world. This bacterium has been locking for culture freshwater fish (Schaperclaus, 1992) through the most important diseases exist. The bacterium is commonly spread in freshwater and organic matter containing bottom sediments as well as in the fish intestinal tract (Dumontel *et al.*, 1996). Infectious abdominal dropsy in common carp has been attributed to the *Aeromonas hydrophila was* first described in an earlier (Schaperclaus, 1992). The causative agent has since been shown to be *Rhabdovirus carpio* (spring viremia of carp) (Fijan *et al.*, 1988). During the 1960s outbreaks of red fin disease caused by *Aeromonas hydrophila* occurred frequently in cultured eels in Japan (Hayes, 2000) with concurrent infections by *Saprolegnia parasitica* (Egusa, 1978). Currently, only sporadic outbreaks of *Aeromonas hydrophila* occurs in cultured eels. *Aeromonas hydrophila* is typically recognized as an opportunistic pathogen or secondary invader (Austin *et al.*, 2012).

Diseases caused by *Lysinibacillus sphaericus*

Lysinibacillus sphaericus was thought to cause a type of human pathogenicity linked to tropical ulcer forms and dermal and/or respiratory infections in the 20th century (Wenzler *et al.,* 2015). Some researcher was of the view that *Lysinibacillus*

sphaericus infections occur only in a symbiotic relationship with several types of spirochete. Some studies have been inconclusive to confirm the existence of pathogenicity (Wenzler *et al.,* 2015). . In 2010, researchers identified the strain of *Lysinibacillussphaericus*B-1 from 16S rRNA gene (Wang *et al.,* 2010). The strain contained primarily toxin in the puffer fish (*Takifugu obscures*) (Wang *et al.,* 2010). This poison is tetradotoxin, a highly fatal neurotoxin that kills by causing paralysis in central nervous system of humans (Wang *et al.,* 2010). *Lysinibacillus sphaericus* has been shown to be susceptible to tetracycline, a common broad-spectrum antibiotic (Priest *et al.,* 1988).

Diseases caused by *Staphylococcus*

Staphylococci may be present in the fish throughout the year, but the disorder is caused by a large spike in the number of individuals. Water temperature or others variable of stress in aquatic environments are responsible. It commonly occurs in the spring and triggers summer wide problem (Vararigos, 2001). Staphylococcal infections in fish were also observed by Couch *et al.,* (1992) during specific and severe stress imposed on the fish by the environment. Exophthalmia, congestion and the usual symptoms of Staphylococcal infections in the fish and tail ulceration are observed (Kusuda *et al.,* 1998).

2.5.2 Common antimicrobials used in aquaculture industry

Aquaculture tends to be one of the last frontiers to increase contributes to food security in the developed world. In some countries it represents the fastest developing agricultural sector, with overall aquaculture production dominating freshwater aquaculture. Its global reputation is mirrored in Africa, where aquaculture provides high levels of production. Quality, low-cost food for millions of people creates money for households in agriculture and fisheries and plays a key role in many local communities (Kapetsky, 1995). Antibiotics are used as a prophylactic or treatment in aquaculture. The incidence of food-borne illness has increased. A couple of studies have demonstrated a strong connection to the use of antibiotics in foods. Animals and the rise of resistance to antibiotics in humans and pathogens of livestock (Teuber *et al.,* 1999). While in some cases, bacterial diseases are regulated by eradication, conversation of the stated health status of livestock, antimicrobial vaccination and good hygiene, nonetheless chemotherapy remains vital for treatment. Appropriate use

of antimicrobials will cure some sick animals and speed the recovery of others and improves the welfare of treated animals and reduce the spread of infection to other animals or in the case of zoonotic disease to humans. The challenges are how to use antimicrobials wisely and minimizing the risk of resistance (Kemper *et al.*, 2008). The type, frequency and quantity of antibiotics used in aquaculture are determined by a number of underlying factors, including species farmed, farming environment, production technology, farming practices, accessibility to and influence from veterinarian/fish health specialist support and implementation of food safety regulations in target markets (Henriksson *et al.,* 2017). Antibiotics are also misused due to misdiagnosis of disease. Diverse ranges of antibiotics are presently used in aquaculture, although stricter legislation has resulted in a move away from the use of medically important antibiotics. However, the same antibiotics classes are used in livestock, aquaculture and human medicine which may further increase exposure to a specific antibiotic. The WHO has different categories of antibiotic compound, varying from important to highly important to critical important (including a subgroup of highest priority antibiotic that are considered critically important for human medicine). Yet, even some of this highest priority antibiotic (e.g., quinolones) is frequently used in aquaculture. Use of 11 banned antimicrobial drugs have been identified in aquaculture in China and Vietnam, including chloramphenicol, ciprofloxacin, florfenicol, nitrofurans and enrofloxacin (Lulijwa *et al.,* 2019). The overall picture related to global use is still unclear, but results from recent reviews show that 67 different antibiotics compounds were used in aquaculture in eleven major producing countries between 2008 and 2018 (Lulijwa *et al.,* 2019). The main related species groups and belong to nine main antibiotic classes (Tetracyclines, Macrolides, Nitrofurans, Penicillins, Phenicols, Aminoglycosides, Sulphonamides, Quinonolones and Potentiated sulphonamides). Vietnam and China use the highest number of antibiotics compounds ranging from 30-40 different compounds. In other counties such as Japan and Thailand, there has been a reduction in the numbers of antibiotics used. This is encouraging, but if use is still high from a few antibiotics, the problem with resistance development may persist because of co-selection, where one antibiotic confers resistance to antibiotics from other classes.

2.5.3 Antimicrobial resistance (AMR) as a major obstacle in aquaculture sector

The aquatic environment is critical in respect of these environmental and microbial dynamics. These environments and aquaculture food production potentially important in terms of emergence, persistence and transmission of AMR (Taylor *et al.,* 2011). Inland water bodies and coastal zones have high and diverse bacterial loads and often as sinks for treated and untreated waste, agricultural runoff and pollution. Many aquatic bacteria harbor a large variety of mobile genetic elements such as plasmids, integrons and transposons that can easily move, recombine and mobilized, promoting the emergence of new mobile combinations of Antimicrobial Resistance Genes (ARGs), conferring to bacteria the capacity to rapidly adapt to new environments in which antimicrobial as present (Cabello *et al.,* 2013). Once acquired ARGs persist in the environment for long time, even after exposure has been terminated. Common fish pathogens that infect fish handlers include *Aeromonas hydrophila, Mycobacterium marinum, Streptococcus iniae, Vibrio vulificus* and *Photobacterium damselae* (Haenen *et al.,* 2013). There are some challenges facing the development of aquaculture, especially the challenges related to the use of natural resources. There are other constraints related to the limited availability of production inputs, the limited impact of research and extension, the lack and/or insufficient credits and competition with other sectors regarding the use of main resources. The challenges facing aquaculture development is currently changed from production orientation to quality orientation. Similarly, sustainable aquaculture production that considers environmental, economic and social issues is far important than quality orientated aquaculture. Despite the encouraging trends, diseases are one of constraint having negative impact on the growth of aquaculture. Although global economic losses from diseases in aquaculture have not been compiled, reports from many regions of the world have been increasing with advances in the live aquatic animal trade (Tomova *et al.,* 2015).

2.6 Spread of AMR and risks to public health

Fish and associated goods are a possible health concern from a microbiological point of view because they bear substantial human pathogenic bacteria on or within them. Via improper handling and ingestion of improperly prepared fish, bacterial infections can occur. Several bacterial genera have been isolated from fish such as *Echerichia, Listeria, Pseudomonas, Klebsilla* and *Salmonella* which may suggest multi source

contamination (Sichewo *et al.,* 2018). When treating water or other constituents in fish living conditions most individuals get contaminated by contact. The emergence of infectious diseases is often greatly influenced by the consumer's physiological state such as immune compromised or depressed persons that are very vulnerable to opportunistic infection as exemplified by people living with HIV and AIDS (Helaly *et al.,* 2007; Banquero *et al.,* 2008). Described aquatic environments can shape the evolution of potential resistance profiles as genetic reactors or hotspots for AMR genes where substantial genetic exchange and recombination can occur (Watts *et al.,* 2017). Either by mutations in their genetic material or through a mechanism called horizontal gene transfer bacteria gain tolerance. Antibiotics tolerance can be bestowed by chromosomal or mobile genetic elements and accomplished using four major techniques, including the reduction of antibiotic membrane permeability, drug inactivation and accelerated antibiotic efflux and cellular goal mutation (Baker *et al.,* 2006). In the gastrointestinal tract, much of the sharing of resistance genes takes place. Potential immune bacteria end in the soil and around food items of animal origin during excretion. These food products are an important source of human infection, leading to an increase in antibiotic use that facilitates the development of antibacterial resistance (Aminov *et al.,* 2009). In addition, the overuse of antimicrobial drugs in human and veterinary medicine to prevent or treat infections leads to the increased incidence and spread of AMR (WHO, 2007). As a consequence of metabolic deficiency fish typically succumb to opportunistic bacterial infection. In the production of fish disease, risk factors such as food shortage, poor quality water and over stocking are predisposing factors. Pathogenic bacteria widespread in fish include species of *Aeromonas* which are abundant in freshwater ecosystems (Ibrahim *et al.,* 2014). The same authors have added that in the warm tropical zones and estuarine climate, indigenous bacteria like *Aeromonas, Clostridium* and *Vibrio* are generally spread in aquatic habitats. The survival of *Salmonella, E. coli* and *Shigella* bacteria in water depends on several conditions including biological factors (interactions with other bacteria) and physical factors such as temperature. In fish bacteria *Salmonella, Shigella dysenteriae* are typically attributed to faecal infection. Owing to the potential for the transmission of resistant pathogens and commensal bacteria to the human population, the presence of antibiotic resistance in bacteria from animals has caused serious concern (Cabello *et al.,* 2006)

2.7 Alternatives to antibiotics

In land-based aquaculture crops, antibiotics are often destroyed by bypass proper hygiene and management (Singer *et al.,* 2006). Lower stocking density, vaccination better diagnostics and patient management are strategies to decrease antibiotic use. Vegetable extracts immune stimulating materials, phage therapy and quorum sensing disturbance agents may be alternatives to antibiotics (Watts *et al.,* 2016). In order to minimize the amount of antibiotics released into the atmosphere, filtering of effluent waste water is also required. The feasibility of this intervention depends on the type of device for waste disposal. As is the case for land-based animal production the need for antibiotics can be significantly minimized by disease control. A management scheme where vaccinations, irrigation and farm density reduction are safe practices for preserving safe fish and reducing the amount of antibiotics used (Guy *et al.,* 2017).

2.8 Marine microalgae as potential alternative to antibiotics

Proteins, polysaccharides, polyunsaturated fatty acids (PUFAs) particularly EPA and DHA, amino acids and antioxidents (polyphenols, flavonoides and carotenoids) are among the most essential bioactive constituents of algae with demonstrated antimicrobial capacity (Al-saif *et al.,* 2014). The quality of algae protein was considered higher than that of other plant source such as wheat, rice or beans but lower than that of sources of animal protein such as milk or meat (Mendes *et al.,* 2007). Nevertheless, interest in marine proteins can not only be specifically associated with intact protein but also the likelihood of bioactive peptides being produced. Due to their ubiquity and basic molecular structure, small peptides are commonly recognized as the most ancient antimicrobial agents. Bioactive peptides typically contain relatively different peptides. Small molecules (<10kDa or 12-50 amino acids) with no previous bioactivity released from parent protein that is intact. In particular processes such as absorption or hydrolysis as a result of the implementation of technical therapies such as elevated hydrolysis. These peptides show multiple physiological roles, including pressure processing, inhibition of vitamin, anti-hypertension or ACE, anticoagulants and antimicrobial properties (Ngo *et al.,* 2011). The antimicrobial ability against *E. coli* of many algae strains (ATCC 25322), *Staphylococcus aureus* (ATCC 29213), *Pseudomonas aerginosa* (ATCC 27853) and the higher protein content of *Enterococcus faecalis* (ATCC 2921) was directly related

to found in them (Al-saif *et al.,* 2014). Another very interesting group of algae-derived polysaccharides with a demonstrated. The antimicrobial capacity is that of Fucoidans (Phaeophyta polysaccharides) of the most important ones. The essential properties of these polysaccharides: anticoagulant, anti-thrombotic, antiviral, antitumor, anti- inflammatory, antioxidant and immune modulatory (Li *et al.*, 2013). Antioxidants are very effective devices to counteract oxidative stress and thus enhancement of the welfare status of the population (Rani *et al.*, 2013). The phenols include the largest group of known secondary metabolites in algae species. In recent year, a wide-ranging the in vitro antibacterial activity continuum was correlated not only with plant phenols but also phenols from algae especially against *Staphylococcus aureus* and *Bacillus* sp. Other antimicrobial phenolic compounds isolated from the marine environment include anthraquinones coumarins and flavonoides (Amaro *et al.*, 2011). The first antibacterial compound isolated from a microalgae *Chlorella* sp., was mixture of fatty acids. This compound was effective against both Gram-positive and Gram-negative bacteria (Vello *et al.,* 2014). The characteristics saturated and unsaturated fatty acids profile in algae with a predominance of myristic, palmitic, oleic and eicosapentaenoic acids (EPA) is a specific feature associated with the antimicrobial potential of algal species (El Shoubaky *et al.*, 2014).

CHAPTER 3
MATERIALS AND METHODS

3.1 Culture of microalgae

Three marine microalgae species were obtained from the laboratory of Fisheries Faculty, CVASU that were isolated from the coastal water of Cox's Bazar region. *Nannochloropsis* sp., *Chlorella* sp. and *Tetraselmis* sp. were cultured in an Erlenmeyer flask containing the Conway Media. Sub culturing was done every two weeks in order to maintain healthy and good stocks.

3.1.1 Conway medium preparation

Conway medium was used to culture the marine microalgae species. Preparation of Conway medium needs to prepare the stock solutions which were macronutrients, trace metal solutions and vitamins. Each of the vitamins was dissolved separately in 100 ml distilled water and stored in a refrigerator. Different reagent bottles were prepared for the solution A, B and C. For each 1ml of A, 0.5 ml of B, and 0.1ml of C was added into 1000 ml of filtered and sterilized sea water (James, 1996). The compositions of Conway medium shown in **Table 3.1**.

Table 3.1 Composition and preparation of Conway medium (James, 1996)

Constituents	Quantities
Solution-A chemicals	
Potassium nitrate	100g
Sodium orthophosphate	20g
Sodium EDTA	45g
Boric acid	33.4g
Ferric chloride	1.3g
Manganese chloride	0.36g
Distilled water	1000ml
Solution-B trace metals	
Zinc chloride	4.2g

Cobalt chloride	4.0g
Copper sulphate	4.0g
Ammonium molybdate	1.8g
Distilled water	1000ml
Acidify with HCl to obtain a clear solution	
Solution-C vitamins	
Vitamin B (Thiamin)	200mg
Vitamin B_{12} (Cyanocobalamin)	10mg

3.1.2 Mass culture of microalgae

Mass culture of selected potential isolates was done in large scale in tank using Conway medium. The culture was gradually scaled up from an initial starter culture volume of 20 ml to 20 L. Initially, 20 ml of micro algal stock cultures were mixed with 30 ml medium in each flask (total culture volume 50 ml), with batch cultures of increasing volume (250 ml, 500 ml, 1 L, 10 L) as inocula for the next step after which they were transferred to bigger container of 20 L culture medium. After that, *Nannochloropsis* sp. and *Chlorella* sp. were harvested at their stationary phase on the day12 and *Tetraselmis* sp. was harvested on the day10. The microalgae were harvested by centrifugation at 5000 rpm for 5 minutes to get rid of the water content.

3.1.3 Preparation of microalgae crude extracts

The collected algae pastes were dried at 40^0C for 24h using a hot air oven. The dried biomass was taken in clean 70 ml volume screw-crapped bottles and immersed in methanol solvents for 48h at room temperature (Senhorinho *et al.,* 2012). Via a sterile funnel and sterile Whatman filter paper No.1 the supernatants were purified. Using a 0.2µm membrane filter paper, the filter paper was sterilized (Bhakuni *et al.,* 1992). In a rotary evaporator the filtrate was then concentrated under decreased pressure. The dry extract was stored at 4^0C until use.

3.2 Isolation and identification of bacteria from fish

3.2.1 Samples collection from fish

Intestinal and gill samples were collected from Tilapia and Bata fish using sterile swabs. A total of 5 samples were collected from each fish. The samples were

inoculated into test tubes containing 10 ml of buffered peptone water (BPW) and incubated at 37^0C for 24 hours. Turbid appearance of the BPW indicated bacterial growth.

3.2.2 Preparation of bacterial culture media

Buffered peptone water (BPW)

To prepare BPW, all the solid ingredients were dissolved in 1L distilled water (**Table 3.2**). Heated the solution when necessary to dissolve the medium completely. The mixture was sterilized by autoclaving at 121^0C for 15 minutes and stored at 4°C.

Table 3.2 Ingredients and composition of buffered peptone water

Ingredients	Quantities
Protease Peptone	10.0g
Sodium Chloride	5.0g
Disodium phosphate, anhydrous	3.5g
Potassium hydrogen phosphate	1.5g
Distilled water	1L

Trypticase soy agar (TSA)

To prepare TSA, a mixture of 40g of solid ingredients including enzymatic digest of casein and soybean meal, sodium chloride and agar were dissolved into 1L distilled water (**Table 3.3**). The medium was heated with frequent agitation and boiled for 5 minutes to make sure the medium completely dissolve. After that, the medium was autoclaved at 121°C for 15 minutes. The medium was poured gently into petri dishes when ~60°C in a laminar flow cabinet. The TSA was stored at 4°C in refrigerator.

Table 3.3 Ingredients and composition of trypticase soy agar

Ingredients	Quantities
Enzymatic digest of casein	15g
Enzyme digest of soybean meal	5g
Sodium chloride	5g
Agar	15g
Distilled water	1L

Tryptic soy broth (TSB)

To prepare TSB, a total of 30g of the solid ingredients were dissolved in 1L distilled water and mixed thoroughly (**Table 3.4**). The medium was autoclaved at 121°C for 15 minutes and stored at 4°C in refrigerator.

Table 3.4 Ingredients and composition of tryptic soy broth

Ingredients	Quantities
Enzymatic digest of casein	17.0 g
Enzyme digest of soybean meal	3.0 g
Sodium chloride	5.0 g
Di-potassium phosphate	2.5 g
Dextrose	2.5 g
Distilled water	1L

Nutrient broth

Nutrient broth was prepared by mixing 13g of solid ingredients including gelatin peptone, beef extract, yeast extract, sodium chloride in 1L distilled water (**Table 3.5**). Then, the medium was autoclaved at 121°C for 15 minutes. Nutrient Broth was cooled at room temperature then stored at 4°C in refrigerator.

Table 3.5 Ingredients and composition of nutrient broth

Ingredients	Quantities
Gelatin peptone	5.0 g
Beef extract	1.0 g
Yeast extract	2.0 g
Sodium chloride	5.0 g
Distilled water	1L

Mannitol salt agar (MSA)

To prepare MSA, a total of 111.025g of the solid ingredients including proteose peptone, beef extract, sodium chloride, D-mannitol, phenol red, and agar were suspended in 1L distilled water (**Table 3.6**). The medium was heated with frequent agitation and boiling for 5 minutes to make sure the medium completely dissolved. After that, the medium was autoclaved at 121°C for 15 minutes. The medium was poured gently into petri dish when 60°C in a laminar flow. The MSA was stored at 4°C.

Table 3.6 Ingredients and composition of mannitol salt agar

Ingredients	Quantities
Proteose peptone	10.0 g
Beef extract	1.0 g
Sodium chloride	75.0 g
D-manitol	10.0 g
Phenol red	0.025 g
Agar	15.0 g
Distilled water	1L

Blood agar

Blood agar was prepared by mixing a total of 40g of the solid ingredients including agar, beef extract, peptone, and sodium chloride in 1L distilled water (**Table 3.7**). The medium was heated with frequent agitation and boil for 5 minutes to make sure the medium completely dissolve. After that, the medium was autoclaved at 121°C for 15 minutes. Then media kept into water bath at 60^0C for 15 minutes and added the blood of sheep (5%) and mixed gently. The medium was poured gently into petri dish when 60°C in a laminar flow and stored at 4°C.

Table 3.7 Ingredients and composition of blood agar

Ingredients	Quantities
Agar	15.0 g
Beef extract	10.0 g
Peptone	10.0 g

Sodium chloride	5.0 g
Sheep blood	50 ml
Distilled water	1L

3.3 Isolation and identification of bacteria

3.3.1 *Aeromonas hydrophila*

The samples were scooped from buffered peptone water and streaked onto the trypticase soy agar (TSA) plate by using inoculating loop and incubation for 24h at 37^0C. Creamy-white colonies observed at the next day confirmed *Aeromonas hydrophila*. For further confirmation, Gram staining was performed and rod-shaped Gram-negative bacilli observed under microscope. Further confirmation of the bacterial species was done by applying standard biochemical test using a VITEK 2 system.

3.3.2 *Lysinibacillus sphaericus*

By using inoculating loop samples were scooped from buffered peptone water and streaked onto the blood agar and incubation for 24h at 37^0C. The presence of *Lysinibacillus sphaericus* was demonstrated by the purple color large colonies. The single colony was stained with Gram's stain and detected the rod-shaped Gram-positive bacilli under microscope. Further confirmation of the bacterial species was done by applying standard biochemical test using a VITEK 2 system.

3.3.3 *Staphylococcus*

Using the inoculating loop samples from overnight incubated buffered peptone water was streaked onto the mannitol salt agar (MSA). On MSA, colorless or yellow, glossy, large colonies were observed. In Gram's staining, Gram-positive circular bacteria arranged in grape-liked groups were detected under light microscope. Further confirmation of the bacterial species was done by applying standard biochemical test using a VITEK 2 system.

3.4 Gram staining of bacterial samples

3.4.1 Preparation of Gram's stain

Crystal violet

Firstly, 95% ethyl alcohol was dissolved into 20ml of 2g marked crystal violet. Then 8g ammonium oxalate dissolved into 80ml of purified water**.** The two solutions were combined together and allowed to stand at room temperature (25℃) overnight then filtered in a coarse filter paper and stored at room temperature.

Gram's iodine

One gram of crystalline iodine and 2g potassium iodide were grinded in a mortar and mixed in 300ml distilled water. After that, stored at room temperature (25℃) in a foil-covered bottle (to protect solution from light).

Decolorizer

To prepare decolorizer, 500ml acetone, 475ml ethanol or methanol and 25ml distilled water were mixed thoroughly and stored at room temperature.

Safranin

Firstly, 2.5g certified safranin added into 95% ethyl alcohol. Then added 10ml mixture of safranin and ethyl alcohol solution to 90ml distilled water. Then stored at room temperature.

3.4.2 Gram staining procedure

Preparation of slide smear

One loop full of bacterial broth culture was taken onto a glass slide using sterile inoculating loop. A drop of water was taken before bacterial samples taken from agar plates and mixed well. The smear was left to air dry at room temperature. The smear was fixed by holding over flame for few second.

Staining protocol

In the sample/slide, primary stain crystal violet was applied for 1 min. To washout the unbound crystal violet, rinsed the slide with a soft stream of water for up to 10 seconds. Added Gram's iodine for 1 min, this is a mordant or argent that fixes the

crystal violet to the bacterial cell wall. Rinsed the slide with acetone for 3 seconds and rinsed with a gentle stream of water. After that, secondary stain safranin was added to the slide for 1 min and washed with a gentle stream of water. After air dried, slides were examined under light microscope. Gram-positive bacteria resembled blue/purple colour while Gram-negative bacteria as pink colour.

3.5 Biochemical tests

The bacterial samples were subjected to biochemical tests, performed in the Marine Biotechnology Laboratory at the University Malaysia Terengganu, Malaysia using the VITEK 2 system. Bacterial species of *Aeromonas hydrophila*, *Lysinibacillus sphaericus* and *Staphylococcus* were confirmed by using VITEK 2 system.

3.5.1 Principles of VITEK 2 biochemical identification method

VITEK 2 compact is one of the most widely used integrated and automated systems in bacterial identification based on biochemical profiles of tested strains. The VITEK 2 was used according to the manufacturing instructions; ID-Gram positive cards (ID-GP cards) were used for identification. The ID-GPC card is a 64 wells plastic card containing 18 empty wells and 46 wells for fluorescent and inhibitory tests that include pH change tests and derivatives to detect amino peptidase and oxidizes. Results of detection are obtained in about 8h or less.

3.5.2 Materials and reagents required

- VITEK 2 system
- VITEK 2 GP test cards
- Vortex
- Incubators set to 30°C and 35–37°C
- VITEK 2 DENSICHEK kit
- DENSICHEK calibrator
- VITEK 2 cassette
- Sterile saline: aqueous 0.45 to 0.50% NaCl, pH 4.5 to 7.0
- Disposable test tubes 12 mm × 75 mm clear plastic (polystyrene)
- Sterile sticks or swabs
- Brain heart infusion (BHI) slants

- Culture media: Columbia agar with 5% sheep blood (CBA), trypticase soy agar (TSA), or trypticase soy agar with 5% sheep blood (TSAB) plates

3.5.3 Preparation of test suspension

Three millilitres of sterile aqueous 0.45 to 0.50 % NaCl, pH (4.5- 7) was aseptically moved into polystyrene test tubes (12×75). A sufficient number of colonies were moved from 24h culture on the prescribed culture medium to the saline tube using a sterile stick or swab to obtain a density equal to McFarland 0.50 to 0.63 with the VITEK 2 DENSICHEK. The culture was tested by the VITEK 2 GP card system within 30 min of the suspended culture preparation. The culture tube VITEK 2 GP card were inserted into the VITEK 2 cassette and referred for guidance on the use of the instrument to the user manual (provided with the instrument). Results of recorded detection from the VITEK 2 GP system. Slash line or low discrimination identification were appropriate outcomes for the VITEK 2 GP process, as shown in the VITEK 2 GP product details given to end users, requiring supplementary testing to better overcome the identification of the organism. Probabilities of bacteria species identification using this system is nearly 100% (**Table 3.8**).

3.5.4 Results and interpretation

The VITEK 2 interprets the performance, if a particular identifying pattern is identified; reported findings suggest a high likelihood fit for a single animal. If a potential pattern is not known the device will recommend additional tests to discriminate between 2 or 3 closely similar species or indicate the outcome as an unspecified organism (>3 species may demonstrate the pattern detected or the bio pattern is quite a typically and not shown in the database.

Table 3.8 Bacteria identification probability (%)

Bacteria	**Probability** (%)
Aeromonas hydrophilic	99
Lysinibacillus sphaericus	100
Staphylococcus	95

3.6 Antimicrobial activity test

Disk diffusion testing was done according to the 2011 guidelines of the European Communities of Antimicrobial Activity Test using standard antibiotic disks and Mueller-Hinton agar (MHA) plates. 50 mg of dried extracts were dissolved into 1ml of extraction solvents. Bacterial suspension was adjusted by adding 0.85% physiological saline to match turbidity of a 0.5 McFarland standard approximately 1.5×10^8 CFU/ml (Yilmaz, 2012). Each of the bacteria species are inoculated in three replicates into the Muller-Hinton agar plate for the use of antimicrobial activity by using cotton swabs. Sterile filter paper discs were immersed into the extraction solvents. After that, they were dried by normal evaporation in the contamination-free environment and placed on the Muller-Hinton agar plate by using the sterilized forceps. The antimicrobial agent used for the *Aeromonas hydrophila, Lysinibacillus sphaericus* and *Staohylococcus* was Ceftriaxone as standard discs positive control. A blank sterile filter paper was used as negative control. The plates were incubated in inverted position at 37°C overnight. Clear and circular zones surrounding the discs were the inhibition zones that produced by the extracts and the zone of inhibitions were measured by using of digital slide calipers (Robotics BD shipment) from one edge of the zone to the other edge. The comparison of the antimicrobial activity of the extract was a relative percentage inhibition and the following formula was used to monitor (Kumar *et al.,* 2016).

$$(X-Y) \div (Z-Y) \times 100$$

Where,

X: total area of inhibition of the microalgae extracts

Y: total area of inhibition of the blank disc

Z: total area of inhibition of the standard antimicrobial agents

For the total area of inhibition was calculated by using area = $\pi r2$; where, r = radius of inhibition zone.

In several initial experiments, it was observed that *Nannochloropsis* has no antibacterial effects on any of the three bacteria *Aeromonas hydrophila, Lysinibacillus sphaericus* and *Staphylococcus.* Therefore, during sensitivity testing and determination of minimal inhibitory concentration (MIC), only *Tetraselmis* and *Chlorella* were used.

3.7 Minimal inhibitory concentration (MIC)

The Minimal Inhibitory Concentration (MIC) of methanol extract of microalgae against bacteria was determined using a tube dilution technique (El-deen *et al.*, 2011). For preparation of *Tetraselmis* and *Chlorella* stock solutions, mixed 600 mg microalgae into 6 ml nutrient broth in an eppendorf tube. Bacterial suspension was prepared and diluted to match the 0.5 McFarland standards. 200 µl bacterial suspensions were put in each well of a 96 well microtiter plate down the column (1-7). In each of the two-replicates, microalgae stock solution was put to make the concentration as 10mg/ml, 20mg/ml, 30mg/ml, and 40mg/ml. Bacterial suspensions with the TSB act as the positive control and bacterial suspensions with antibiotic act as a negative control. Incubated the 96 well plates overnight at 37^0C temperature. The presence of bacteria in each well was determined by colour and turbidity.MIC value was determined as the minimum concentration of microalgae extract capable of inhibition of bacterial growth.

3.8 Statistical analysis

The raw data was recorded using Microsoft Excel 2007 spreadsheet. Confirmation of normal distribution of data sets was established using D'Agostino & Pearson normality test in GraphPad Prism 7 statistical software. All the data sets from different groups have passed D'Agostino & Pearson normality test and hence was compared and tested using parametric analysis of variance (ANOVA). A p value of ≤ 0.05 was considered significant.

CHAPTER 4

RESULTS

4.1 Culture of marine microalgae

In the present study, *Tetraselmis*, *Nannochloropsis* and *Chlorella* were cultured in 20 L culture jars. After harvest by centrifugation and drying, we attained about 7 grams of dry *Tetraselmis 6.7* grams of *Nannochloropsis* and 6.2 grams of *Chlorella* from each 20 L bulk culture. The dry extracts were further processed by addition of methanol, filtration etc. before stored at -20°C.

4.2 Isolation and identification of bacteria

Samples collected from Bata (*Labeo bata*) and Tilapia (*Oreochromis niloticus)* fish were placed in buffer peptone water (BPW), growth of bacteria was observed on next day when the clear BPW turned into cloudy (**Figure 4.1**) before inoculation into selective media.

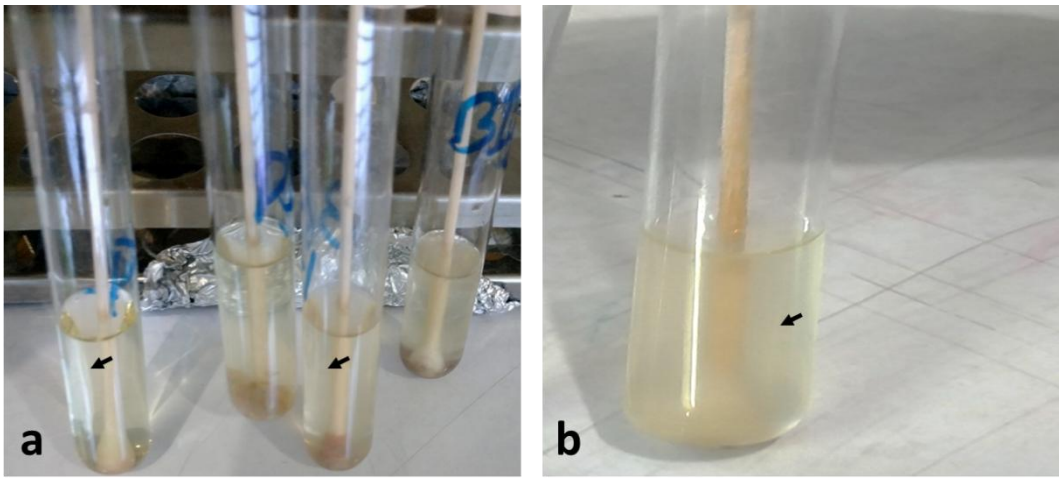

Figure 4.1 Sample collection and primary culture in buffer peptone water. a) Samples were collected using sterile cotton-swab inserted directly into the buffer peptone water (BPW, arrows indicate clear media). b) Cloudy appearance of BPW indicates growth of bacteria (arrow indicates cloudy medium).

4.2.1 *Aeromonas hydrophila*

All the samples collected from Bata and Tilapia fish were positive for *Aeromonas hydrophila*, and on trypticase soy agar (TSA) plate observed characteristics circular, creamy-white or colourless colonies (**Figure 4.2**). Gram negative rod-shaped,

pleomorphic bacilli with a monotrichous flagellum were observed in Gram staining. *Aeromonas hydrophila* species was confirmed by series of biochemical tests using a VITEK 2 system.

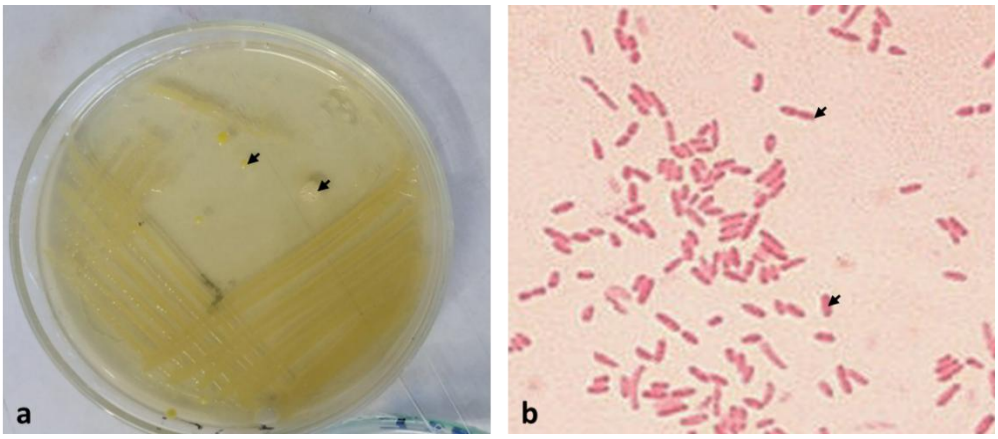

Figure 4.2 Isolation and identification of *Aeromonas hydrophila* by culture and Gram staining. a) On trypticase soy agar, *Aeromonas* was observed as circular, creamy-white colonies indicted by arrows. b) Gram-negative bacilli indicated by arrows were observed under light microscope (400×) after Gram staining.

4.2.2 *Lysinibacillus sphaericus*

To identify *Lysinibacillus*, bacterial culture from BPW was inoculated onto blood agar and characteristics purple color large colonies were observed (**Figure 4.3**). In Gram staining, Gram-positive, mesophilic, rod-shaped bacilli were observed under microscope. *Lysinibacillus sphaericus* species was confirmed by series of biochemical tests using a VITEK 2 system.

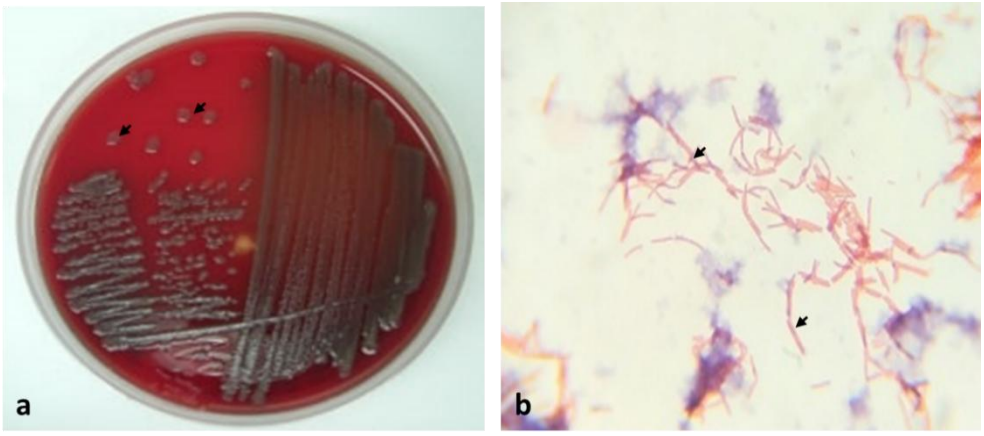

Figure 4.3 Isolation and identification of *Lysinibacillus sphaericus* by culture and Gram staining. a) On blood agar, *Lysinibacillus* was observed as purple color large colonies indicted by arrows. b) Gram-positive, rod-shaped bacilli indicated by arrows were observed under light microscope (400×) after Gram staining.

4.2.3 *Staphylococcus* sp.

Staphylococcus sp. was identified by colorless or yellow, glossy, large colonies onto mannitol salt agar (**Figure 4.4**). On Gram staining, Gram positive uniform cocci with grapes like arrangements were observed. *Staphylococcus saprophyticus* species was confirmed by several biochemical tests using a VITEK 2 system.

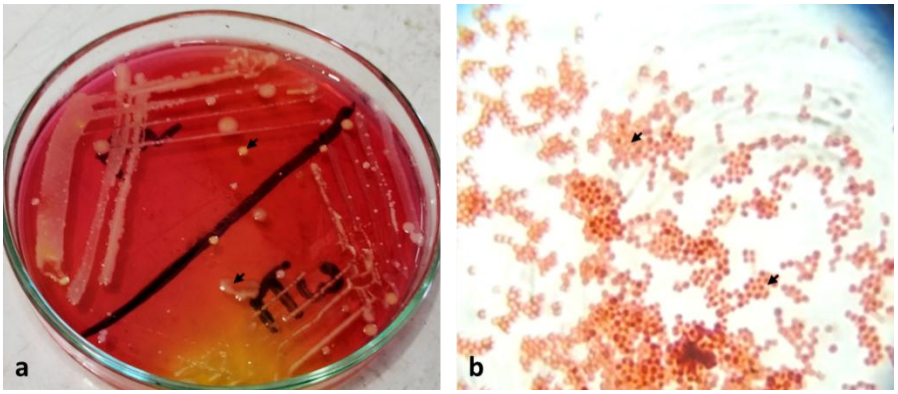

Figure 4.4 Isolation and identification of *Staphylococcus saprophyticus* by culture and Gram staining. a) On mannitol salt agar, *Staphylococcus* was observed as colorless or yellow, glossy, large colonies indicted by arrows. b) Gram-positive circular cocci with grapes-arrangement indicated by arrow were observed under light microscope (400×) after Gram staining.

4.3 Antimicrobial activity test

Initial experiments demonstrated that *Nannochloropsis* has no antibacterial effects on any of the three bacteria *Aeromonas hydrophila, Lysinibacillus sphaericus* and *Staohylococcus* (results not shown). Therefore, during antimicrobial sensitivity testing and determination of minimal inhibitory concentration (MIC), only *Tetraselmis* and *Chlorella* were tested.

4.3.1 Antimicrobial activity of microalgae against *Aeromonas hydrophila*

Antimicrobial activity of methanol extracts of *Tetraselmis* and *Chlorella* was demonstrated at variable extents against *Aeromonas hydrophila* in the current study (**Figure 4.5**).Larger inhibition zone appeared around the disc loading of extract of *Tetraselmis* with a diameter of 6.65mm ($p<0.01$).

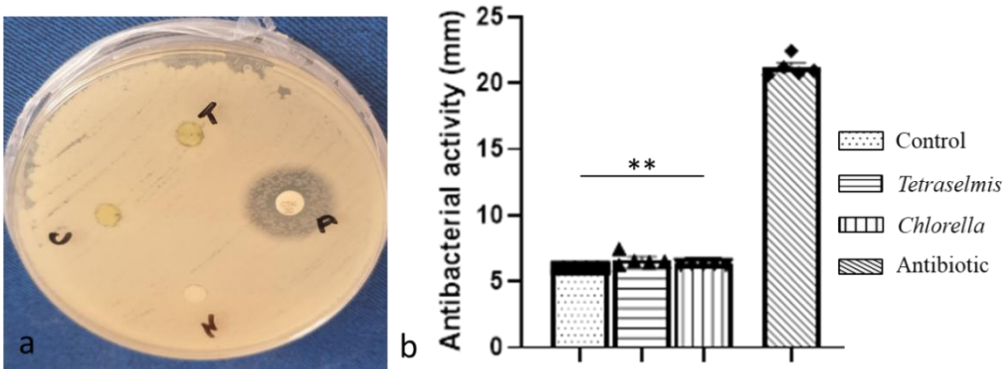

Figure 4.5 Antimicrobial activity of microalgae against *Aeromonas hydrophila*. a) Agar plate indicates clear zone of bacterial growth surrounding the antibiotic (A), *Tetraselmis* (T) and *Chlorella* (C) with no zone of inhibition around control disc (N). b) Both *Tetraselmis* and *Chlorella* showed significantly higher zone of inhibition with *Tetraselmis* the highest. Statistical tests done by ANOVA, error bars represent standard error of means, $p^{**}<0.01$.

4.3.2 Antimicrobial activity of microalgae against *Lysinibacillus sphaericus*

The antimicrobial activity of methanol extracts of *Tetraselmis* and *Chlorella* was observed against *Lysinibacillus sphaericus*(**Figure 4.6**). Both the microalgae had little effect on the bacteria though the results were not statistically significant ($p>0.05$).

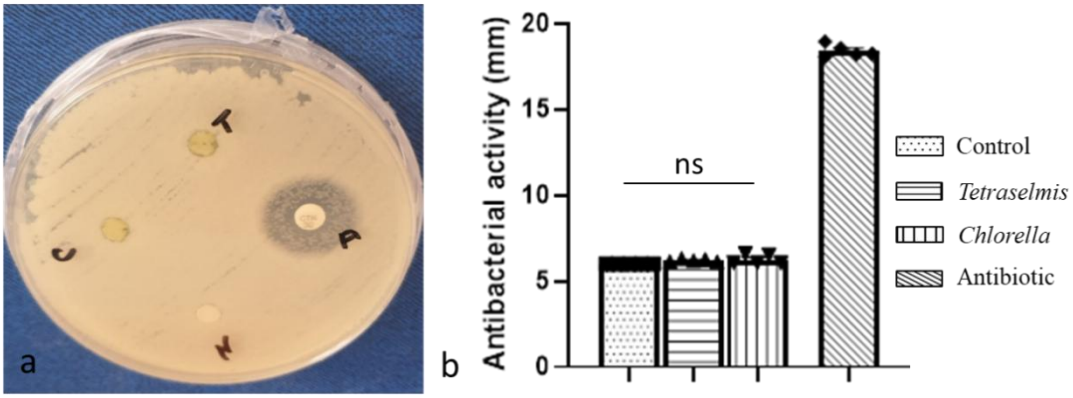

Figure 4.6 Antimicrobial activity of microalgae against *Lysinibacillus*. a) Agar plate indicates clear zone of bacterial growth surrounding the antibiotic (A), *Tetraselmis* (T), and *Chlorella* (C) with smaller inhibition zone and no zone around control disc (N). b) Statistical analysis of microalgae with control showed no significant difference in antibacterial activities. Statistical tests done by ANOVA, error bars represent standard error of means, ns: not significant.

4.3.3 Antimicrobial activity of microalgae against *Staphylococcus*

Against *Staphylococcus saprophyticus*, *Chlorella* showed greater activity compared to *Tetraselmis* with a diameter of 6.57mm ($p<0.01$) (**Figure 4.7**). Larger inhibition zone

appeared around the disc loading of extract of *Chlorella* with a diameter of 6.57mm (p<0.01).

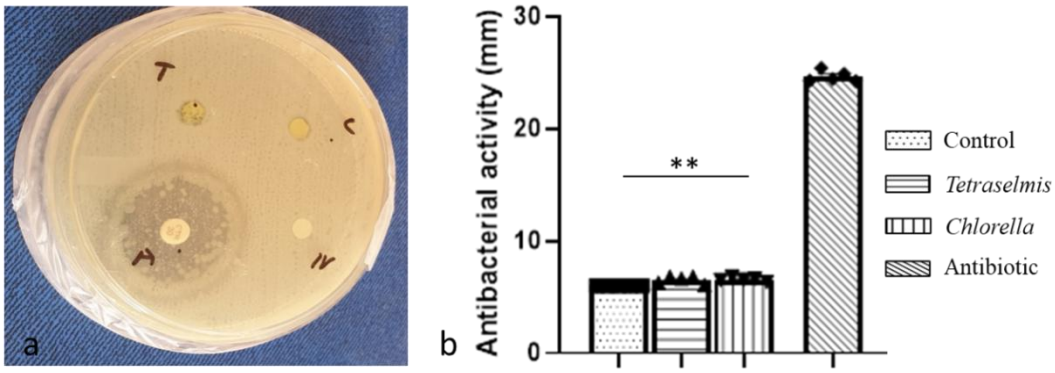

Figure 4.7 Antimicrobial activity of microalgae against *Staphylococcus*. a) Agar plate indicates clear zone of bacterial growth surrounding the antibiotic (A)*Tetraselmis* (T) and *Chlorella* (C) with no zone around the control disc (N). b) Both the microalgae showed significantly higher antibacterial activity. Statistical tests done by ANOVA, error bars represent standard error of means, p^{**}<0.01.

4.4 Antimicrobial index of microalgae extracts

Antimicrobial activity of microalgae extracts was calculated using the formula which was mentioned earlier in the Materials and Methods chapter. The relative percentage (%) inhibition zones of *Tetraselmis* extract have presented the highest against *Aeromonas hydrophila,* followed by *Staphylococcus* and the lowest against *Lysinibacillus sphaericus.* While *Chlorella* exposed relative percentage the highest against *Staphylococcus* followed by *Aeromonas hydrophilic* and *Lysinibacillus sphaericus*.

Table 4.1 Relative percentage inhibition zones of *Tetraselmis* and *Chlorella* against *Aeromonas hydrophila*, *Lysinibacillus sphaericus* and *Staphylococcus*

Selected bacteria	**Relative percentage (%) inhibition zones**	
	Tetraselmis sp.	*Chlorella* sp.
Aeromonas hydrophila	1.99	1.168
Lysinibacillus sphaericus	0.879	1.077
Staphylococcus	1.156	1.262

4.5 Minimal inhibitory concentration (MIC)

The MIC of the extracts from *Chlorella* and *Tetraselmis* grown under different conditions were determined against *Aeromonas hydrophila* and *Staphylococcus.* Due to insufficient amount, it was not possible to determine MIC value of these microalgae against *Lysinibacillus sphaericus*. The MIC value was determined as the minimum concentration of microalgae capable to prevent bacterial growth. It was observed that *Tetraselmis* has strong antimicrobial activity against *Aeromonas hydrophila*, however, *Chlorella* has against *Staphylococcus* (**Table 4.2**).

Table 4.2 MIC value of microalgae against *Aeromonas* and *Staphylococcus*

Microalgae	**MIC value (mg/ml)**	
	Aeromonas hydrophila	*Staphylococcus*
Chlorella	40	20
Tetraselmis	10	20

CHAPTER 5
DISCUSSION

Natural pigments such as carotenoids, chlorophylls, phenolics, hycocyanin, phycoerythrin and phycobiliproteins are produced by microalgae are well known. They are used in nutraceutical, pharmaceutical and in food applications (Guerin *et al.,* 2003).The antibiotic content of algal species may vary depending on many ecological and biogeographically factors. Various strains of microalgae are known to produce intracellular and extracellular metabolites with diverse biological activities such as anti algal, antibacterial, antifungal, and antiviral activity.

The present study was aimed at the screening of antimicrobial activities of three microalgae belonging to the genus *Tetraselmis*, *Chlorella* and *Nanochloropsis* against one Gram-negative *Aeromonas hydrophila* and two Gram-positive bacteria *Lysinibacillus sphaericus* and *Staphylococcus.* All the samples were collected from Bata (*Labeo bata*) and Tilapia (*Oreochromis niloticus)* fish were positive for *Aeromonas hydrophila, Lysinibacillus sphaericus* and *Staphylococcus.* Furthermore, *Tetraselmis* and *Chlorella* had variable degrees of suppressive activities against all three bacteria. Marine microalgae present many bioactive compounds such as fatty acid, acrylic acid, halogenated aliphatic compounds, terpenes, sulphur-containing hetero cyclic compounds, carbohydrates and phenols that have antimicrobial effects recognized for long time (Kannan *et al.,*2010).In this study, methanolic extracts of the marine microalgae were used to determine the antimicrobial activities. Previous study reported that, extraction of algal samples of *Chlorella* and *Nannochloropsis* was performed using methanol (Qusem *et al.,* 2016).

In the current study, bacterial samples were collected from gill and intestine of Bata (*Labeo bata*) and Tilapia (*Oreochromis niloticus*) fish and were positive for *Aeromonas hydrophila, Lysinibacillus sphaericus* and *Staphylococcus*. Previous study reported that, bacterial samples collected from eye, stomach, kidneys, gills and intestine of Tilapia (*Oreochromis niloticus*) were positive for *Lactobacillus* sp., *Bacillus firmus, Aeromonas hydrophila, Pseudomonas anguilliseptica,* and *Pseudomonas fluorescenses* (Austin *et al.,* 2012; Cruickshank *et al.,* 1975). Due to

limitations of budget, we aimed only three bacterial species and could possibly get more if explored extensively.

Present study demonstrated that *Chlorella* is effective to prevent growth of Gram-positive bacteria *Staphylococcus* than Gram-negative bacteria *Aeromonas.* The findings are in agreement with a previous study where antibacterial activity of *Chlorella vulgaris* extract was reported against *Bacillus subtilis, Staphylococcus aureus* and *Klebsiella pneumonia* (El-deen *et al.,* 2014). Mokbel and his co-workers (2005) tested the effect of butanedioic acid against some Gram-negative and positive bacteria and found that butanedioic acid has antibacterial activity against *S. aureus, B. cereus, S. enteritidis* and *E. coli. Chlorella* contains several compounds that might have inhibitory effects on the growth of pathogenic bacteria. Myristic acid, palmitic acid and butanedioic acid have good antimicrobial activity that is contained by *Chlorella*. Myristic acid, palmitic acid and fatty acids the bactericidal properties of fatty acids are well known (Kabara *et al.,* 1972; Mokbel *et al.,* 2005). On the contrary, *Tetraselmis* showed better antibacterial activities to Gram-negative bacteria *Aeromonas* followed by Gram-positive bacteria *Staphylococcus* in this study. *Tetraselmis* contains high amounts of oleic acid, fatty acids, linoleic acid and palmaitic acid which might have antimicrobial activity against the bacterial species (Maadane *et al.,* 2017). So, it can be considered that *Tetraselmis* sp. and *Chlorella* sp. have present antibacterial activity against Gram-positive and Gram-negative bacteria. An earlier study demonstrated that *Nannochloropsis oculata* had moderate antibacterial activity against *Bacillus subtilis* (Qusem *et al.,* 2016). However, the current study opposes this statement as no effect of *Nannochloropsis* was observed against any of the three-bacterial species studied.

Besides, MIC was carried to study the antimicrobial activity of *Tetraselmis* sp. against *Aeromonas hydrophila* due to the largest inhibition zone of 1.99mm compared to *Chlorella* sp. and other test bacteria. Here *Chlorella* sp. antibacterial activity against *Staphylococcus* due to the largest inhibition zone of 1.262mm compared to *Tetraselmis* sp. and other test bacteria. MIC value of *Tetraselmis* sp. was resulted at the concentration of 10mg per ml to inhibit *Aeromonas hydrophilic* and *Chlorella* sp. showed the 20mg per ml to inhibit *Staphylococcus* (1.5×10^8CFU/ml or 0.5 McFarland standards).So these extracts can control well the growth of the fish

pathogenic bacteria. Microalgal cells present different types of bioactive compounds such as bactericidal or bacteriostatic. These bioactive compounds action modes have been exposed for growth inhibition or killing of bacteria by some of the functional molecules. The effective of microalgae extracts used were sensitive to Gram-positive and Gram-negative bacteria. This is because of the difference cell wall structures of the Gram-positive and Gram-negative bacteria. The penetration, binding and antimicrobial activity of the compound was determined by the difference cell wall structures of bacteria. The cell wall structures of Gram-positive have thick peptidoglycan layer, lipid and polysaccharides that can dissolve in water that assist as a positive ion transport and carboxyl, amino acid and hydroxyl consist at a hydrophilic side of the bacteria which increase the effectiveness of antibacterial compounds to the bacteria. While for the Gram-negative bacteria, it has complex cell wall structures which are lipoprotein as outer layer, lipopolysaccharide as middle layer and the third layer of peptidoglycan. Phytochemical comprising microalgal substance may act by inducing cellular membrane perturbation, interference with certain microbial metabolic processes and modulation of signal transduction or gene expression. Free fatty acids could initiate peroxidative processes and preclude the synthesis of bacterial fatty acids. Besides free fatty acids might interact with cellular membranes of microbial cells causing leakage of molecules from these cells reduction of their nutrient uptake or inhibition of their respiration (Suresh *et al.,* 2014)

In the present study, the antimicrobial activities of *Tetraselmis* and *Chlorella* extracts were attributed to their contents of fatty acids, carotenoids and polyphenols as discussed above. These substances probably act together, either in an independent or synergistic manner. Resistance to antibiotics by bacteria is a global threat. The resistance of microbes is due to indiscriminate utilization of commercial antimicrobials. Many researchers investigated antimicrobial resistance and concluded to explore newer antimicrobial substances from several medicinal plants and algae (Algesboopathi *et al.,* 2012). In this regard, microalgae can be used as antimicrobial drug because there is no side effect and low cost.

CHAPTER 6
CONCLUSION

The current study demonstrated antibacterial activity of three-species of marine microalgae against three-species of bacteria isolated from gill and intestinal samples of Bata (*Labeo bata*) and Tilapia (*Oreochromis niloticus*) fish. It was observed that *Chlorella* has the greatest propensity to suppress *Staphylococcus*; however, *Tetraselmis* was very suppressive against *Aeromonas. Nannochloropsis* showed no sensitivity against *Aeromonas, Staphylococcus* and *Lysinibacillus*.

CHAPTER 7

RECOMMENDATIONS AND FUTURE PERSPECTIVES

According to this research work, the following recommendations may be done:

Antibiotics have high market price with side effects. Marine microalgae contain various bioactive compounds which inhibit the bacterial pathogens. The bioactive compounds may be suitably used for the other bacterial pathogens. Farmers may get good result when using of marine microalgae particularly, *Tetraselmis* and *Chlorella* sp. When fish are attacked by bacterial diseases then they can use marine microalgae because it is low cost, no side effect and high antimicrobial properties. Further studies are directed to identify the specific components of microalgae that are responsible for the sensitivities against bacterial infections.

LIMITATIONS

Isolation and identification of microalgae directly from the marine sources was not possible in the current study due to time limitation. We used the microalgae that we already have in our laboratory stored. A larger bacterial sample size could increase the sensitivity and specificity of the results. We also unable to grow enough microalgae for the antimicrobial sensitivity testing and determination of MIC values.

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APPENDIX

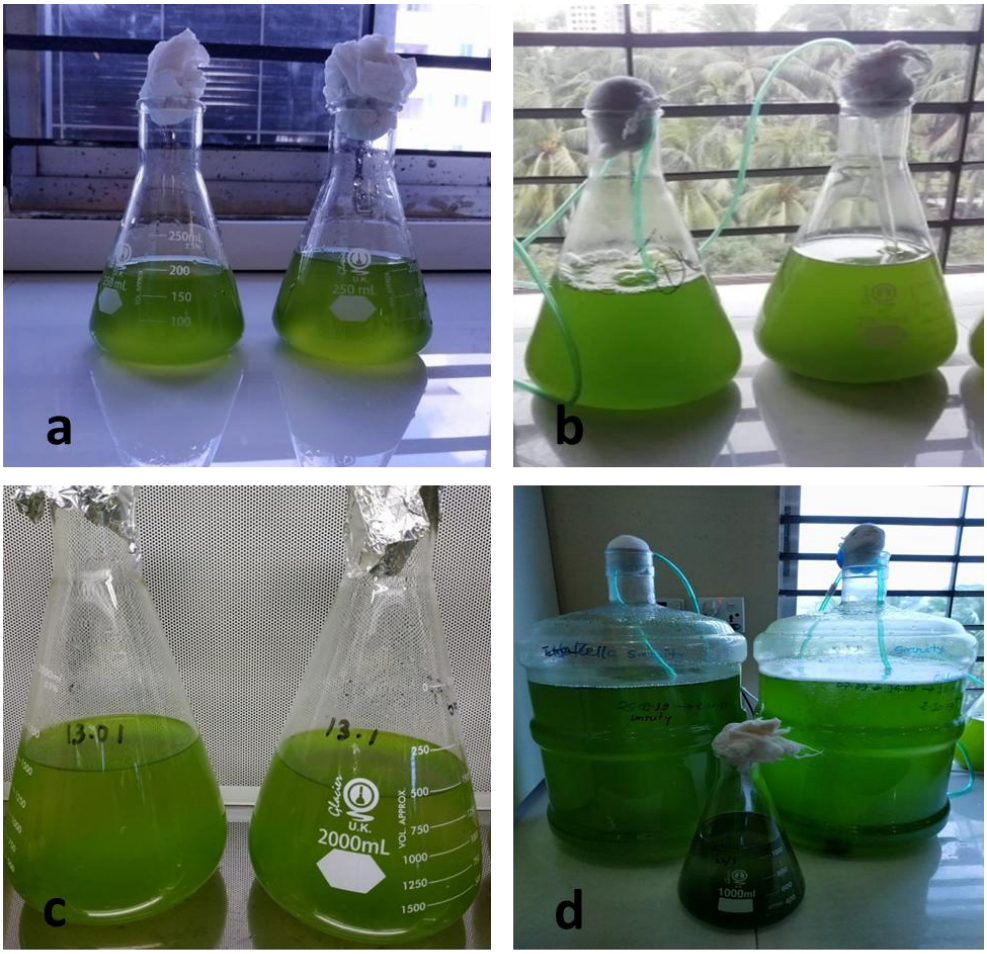

Figure 1 Culture of microalgae. Small-volume culture of a) *Chlorella*, b) *Nannochloropsis* and c) *Tetraselmis*. d) Mass-culture of microalgae in 20L jars.

Figure 2 Laboratory activities regarding culture of microalgae. a) mass-culture of microalgae and addition of culture media, b) drying microalgae extracts in a hot air Oven at 60^0C for 12h, c) harvesting microalgae by centrifugation.

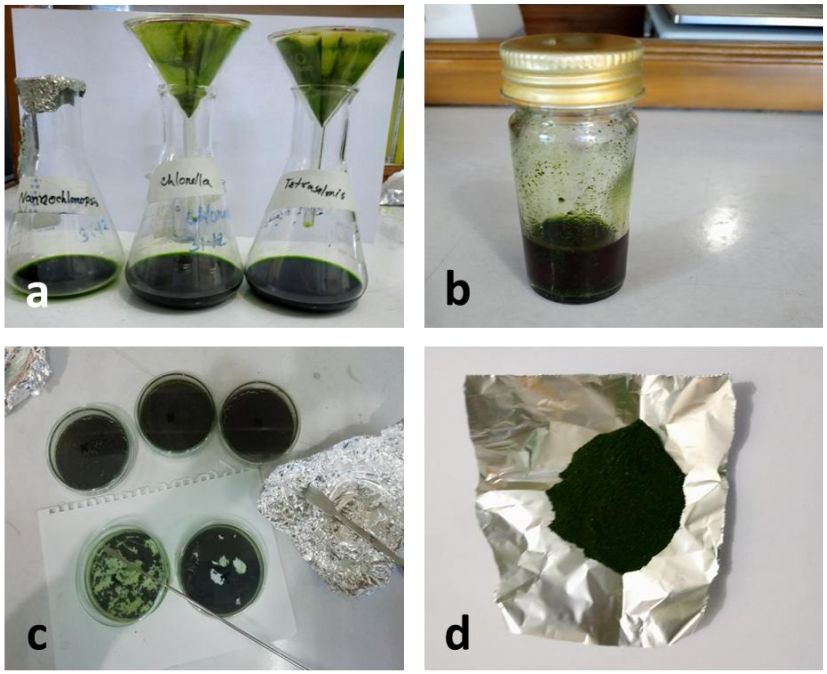

Figure 2 Harvesting cultured microalgae and preparation of dry extracts. a) filtration using Whatman filter paper 1, b) microalgae soaked in methanol, c) evaporated microalgae, d) dry extract ready to use.

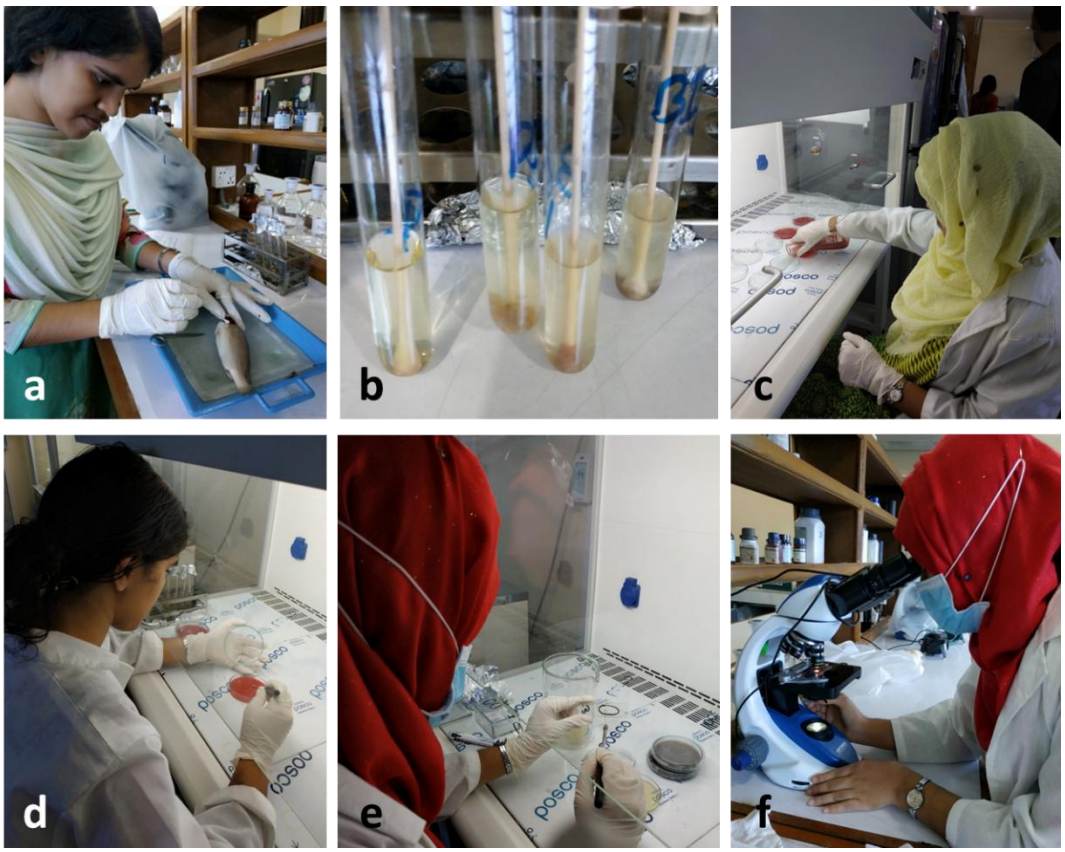

Figure 3 Sample collection and isolation of bacteria. a) sample collection from fish, b) bacterial samples in buffered peptone water, c) preparation of agar plates, d) streaking agar plates, e) slide preparation for Gram staining, f) microscopic examination of slides.

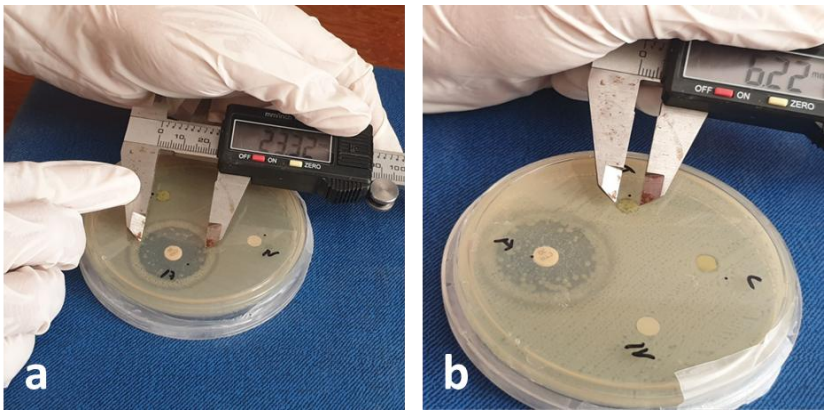

Figure 4 Measurement of zone of inhibition. a) measuring bacterial inhibition zone surrounding antibiotic, b) measurement around microalgae.

BIOGRAPHY

The author Nahida Jaman Smrity;daughter of Md. Maniruzzaman Rikabder and Rabeya Khatun from Shibpur upzilla under Narsingdi district of Bangladesh. She pursued the Secondary School Certificate Examination in 2011 and Higher Secondary Certificate Examination in 2013 from Shaheed Asad Collegiate Girl's High School and College. She obtained her B.Sc. Fisheries (Hons.) degree in 2018 from Faculty of Fisheries, Chattogram Veterinary and Animal Sciences University (CVASU), Chattogram, Bangladesh. Now, she is a candidate for the degree of M.Sc. in Aquaculture under the Department of Aquaculture, Faculty of Fisheries, Chattogram Veterinary and Animal Sciences University, Chattogram, Bangladesh.