ANTIMICROBIAL ACTIVITY OF SELECTED INDIGENOUS MICROALGAE AGAINST COMMON BACTERIA CAUSING DISEASES IN FISH AND SHELLFISH

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Roll No. 0122/01

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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 Abbreviations

Words	Abbreviation
ppt	Parts Per Thousand
ppm	Parts Per Million
rpm	Rotation Per Minute
rcf/ ×g	Relative Centrifugal Field
pH	Power of Hydrogen
ºC	Degree Celcius
µmol photons m^{-2} s^{-1}	Micromoles of photons striking a square meter per second
MT	Metric tonnes
nm	Nanometer
µg	Microgram
µl	Microliter
mm	Millimeter
cm	Centimeter
CFU	Colony Forming Unit
mL	Milli Liter
L	Liter
%	Percentage
g	Gram
lbs	Pound
v/v	Volume/Volume
Min	Minute
Sec	Seconds
Mg	Milligram
N	Normal
sp.	Species
Chl - a	Chlorophyll-a
OD	Optical Density
DO	Dissolve Oxygen

BBM	Bold’s Basal Media
AI	Activity Index
MIC	Minimum inhibitory concentration
MHA	Muller Hinton agar
ELISA	Enzyme-linked immunoassay
WHO	World Health Organization
AMR	Antimicrobial resistance
ARGs	Antibiotic resistance genes
TCBS	Thiosulfate-citrate-bile-salts-sucrose agar
EMB	Eosin methylene blue agar
TSI	Triple Sugar Iron agar
TAN	Total Ammonium Nitrogen
SRP	Soluble Reactive Phosphorus
ANOVA	Analysis of Variance
Strain 1 M1 (20)	Planktonic Marine Strain 1 (20 ppt)
Strain 2 M2 (25)	Filamentous Marine Strain 2 (25 ppt)
Strain 3 Fw1	Filamentous Freshwater Strain 1
Strain 4 Fw2	Filamentous Freshwater Strain 2
SE	Standard error
n-3	Omega-3
n-6	Omega-6
PUFA	Poly Unsaturated Fatty Acid
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
TUFA	Total unsaturated fatty acids
TFA	Total fatty acids
EAA	Essential Amino Acid
NEAA	Non-Essential Amino Acid

Abstract

Antibiotic resistance is recognized as a "One Health subject," comprising human, animal, and environmental interactions, and has become a crucial public health concern. Cyanobacteria, a form of microalgae, recently emerged as one of the most promising sources of natural and eco-friendly antibiotics due to different secondary bioactive compounds. The antibacterial activity of phenolic extracts was assessed using the disk diffusion technique on four strains of *Oscillatoria* sp. which were isolated from two marine and two freshwater stations in Bangladesh. Three Gram-positive bacteria (*Staphylococcus* sp. and *Streptococcus* sp.) and nine Gram-negative bacteria (*Escherichia coli*, *Vibrio* sp., *Salmonella* sp., *Shigella* sp., *Pseudomonas* sp., *Aeromonas* sp.) were isolated from diseased marine fish, Shrimp and Crab and evaluated. Filamentous *Oscillatoria* sp. indicated higher antibiotic activity than planktonic one. Filamentous *Oscillatoria* sp. showed the highest inhibition zone 34.06±0.08mm against *Staphylococcus* sp., while planktonic *Oscillatoria* sp. showed lower inhibition zone against *Pseudomonas* sp. about 17.11±0.18mm. Minimum inhibitory concentration (MIC) value was found to be 100µg/ml for filamentous *Oscillatoria* sp., and 150 µg/ml for planktonic *Oscillatoria* sp. These findings suggest that, Cyanobacteria contain potential antibacterial substances which could be a better substitute for existing antibiotics and efficiently serve both the aquaculture and pharmaceutical industries.

Keywords: Isolation, Microalgae, Cyanobacteria, Growth parameters, antimicrobial activity, Pathogenic bacteria

CHAPTER 1: INTRODUCTION

Global fish production surged to approximately 179 million MT in 2018, with an incredible 82 million MT originating from aquaculture production that generates a staggering value of USD 250 billion (FAO, 2020). Aquaculture has also sprouted as one of the rapidly expanding food industries globally, with an impressive annual growth rate of 5.8% (FAO, 2020). Despite its rapid growth rate, aquaculture is facing serious challenges due to the intensification of culture systems, leading to the emergence of various microbial diseases that have threatened global production and caused billions of dollars in annual economic losses worldwide (Bondad-Reantaso et al., 2012). In an attempt to control these pathogenic diseases, antibiotics have been widely used in aquaculture. In recent years, antibiotics usage in aquaculture has significantly expanded, and in some countries, the reported use rate is as high as 2,500 mg/kg of fish produced (Cabello et al., 2013). However, the prolonged use of antibiotics has driven the exposure of antibiotic-resistant bacterial strains, which has initiated an "antibiotic resistance crisis" that possess a risk to public health through zoonoses (Rossolini et al., 2014). Antibiotic Resistance is a serious global public health concern that results from the misuse and overuse of antibiotics, directing to the selection of bacteria with resistance traits that can be transferred between animals, humans, and the environment (WHO, 2015). Around 80% of antimicrobials administered in aquaculture persist in the environment, retaining their potency and promoting the emergence of antibiotic-resistant bacteria. These bacteria acquire resistance traits through genetic mutations or mobile genetic elements that carry multiple resistance determinants, which can spread to other bacteria horizontally (Cabello et al., 2016). The genomes of more than 10,000 bacteria obtained from fish and shellfish farms were scrutinized across 27 countries and the antimicrobial resistance genes were ubiquitously present in bacteria from every single country, with some nations exhibiting more pronounced levels of resistance compared to others. Furthermore, some resistance genes were universally shared among bacteria from different nations, implying that antibiotic resistance can effortlessly disseminate across borders (Zhu et al., 2021). In China, the widespread incidence of AR bacteria in fish and shrimp farms, exhibiting resistance to antibiotics including tetracyclines, sulfonamides, and quinolones. Additionally, the presence of resistant

bacteria was detected in shrimp farms in Vietnam, demonstrating resistance to antibiotics such as cefotaxime, ciprofloxacin, and gentamicin (Cabello et al., 2016). The antibiotic resistance amplitude in Bangladesh is also a growing concern. In Bangladesh, the overall frequency of antibiotic resistance was found to be 67.4%, with the highest prevalence of 89.7% attributed to amoxicillin and the lowest, 23.3% to carbapenems. The multidrug-resistant bacteria were also found in various settings, including hospitals, community clinics, and the environment (Haque et al., 2022). Therefore, there is a pressing requirement to implement efficient measures for antibiotic stewardship, infection prevention and control in order to tackle this crisis. Vaccines are widely recognized as one of the most promising alternatives to antibiotics in both human and animal health. Prebiotics, probiotics, immune modulators, phages, phytochemicals, organic acids, and antimicrobial peptides are also being investigated as potential alternatives (Cutler, 2021). However, microalgae could be a promising biological alternative to vaccines and immunostimulants in preventing and treating microbial infections. Microalgae-derived compounds, such as polyunsaturated fatty acids, carotenoids, and peptides, have been found to possess antibacterial and antifungal properties (Mehta et al., 2021). Several microalgal species, including *Nannochloropsis oculata*, *Chlorella vulgaris*, *Spirulina platensis*, *Dunaliella salina*, and *Oscillatoria terebriformis* are promising sources of antibacterial, antifungal, antiviral, and anti-irritant agents (Patai et al., 2018). Recently, Cyanobacteria have emerged as a potent natural substitute for antibiotics in aquaculture, owing to their exceptional capability to synthesize an immense array of bioactive compounds with formidable antimicrobial and immunostimulatory effects (Madhu et al., 2021). While some green and brown microalgae are known to produce antimicrobial compounds, they are generally less diverse and less potent than those produced by cyanobacteria. Furthermore, Cyanobacteria are more prevalent in aquatic environments and can be cultivated in larger quantities, which makes them a more feasible option for aquaculture purposes (Cao et al., 2019). Cyanobacterial extracts contain various bioactive compounds with antimicrobial activity including peptides, alkaloids, and polyketides such as cylindrospermopsin, microcystins, anabaenopeptins, aeruginosins, and nostocyclins (Moustafa et al., 2021; Alam et al., 2017). Cyanobacterial extracts also have the potential to control diseases in fish and shrimp without having any negative impacts of

antibiotics including the development of antibiotic resistance and the bioaccumulation of antibiotic residues in the aquatic environments (Cao et al., 2019). Cyanobacterial extracts exhibited effective antibacterial activity against various fish pathogens including *Aeromonas hydrophila* and *Yersinia ruckeri* which control bacterial infections in Rainbow trout with a significant increase in the survival rate of infected fish (Alam et al., 2017). Cylindrospermopsin, a cyanobacterial compound had potent antibacterial activity against *Streptococcus agalactiae* and *Aeromonas hydrophila* that also enhanced the immune response of Tilapia (Moustafa et al., 2021). Cyanobacterial extracts also serve as a novel source of antibiotics for pharmaceutical purposes within the fisheries industry. *Nostoc* sp. extracts exert antimicrobial activity against several pathogenic bacteria in aquaculture, including *Vibrio harveyi* and *Aeromonas hydrophila* with minimal cytotoxicity on fish cells (Velmurugan et al., 2016). *Oscillatoria* sp. extracts exhibit strong antibacterial activity against antibiotic-resistant fish pathogens such as *Edwardsiella tarda*, *Aeromonas hydrophila*, and *Pseudomonas fluorescens* (El-Sheekh et al., 2020). Additionally, a variety of multidrug-resistant bacteria were effectively combated by *Oscillatoria* species such as *Staphylococcus aureus* and *E. coli* with negligible toxicity to human cells in vitro. Thus, the cyanobacterial extracts have the potential to be a valuable resource for developing novel antibiotics to combat antibiotic-resistant infections in both fish and humans (Kim et al., 2020). However, no prior research has been found pertaining to the exploration of the antimicrobial potency and minimum inhibitory concentration of indigenous freshwater and marine Cyanobacteria isolated from Bangladesh.

The current study aims to establish the efficacy of native microalgae, especially Cyanobacteria, found in Bangladesh as a potent natural antibiotic against various pathogens. Therefore, it is imperative to harness the vast potential of microalgae, which can bring benefits not only to the fisheries sector but also to humanity in the long run. Specific objectives of the study are:

• To isolate, identify, and screen different indigenous microalgae whether they possess antimicrobial activity against bacterial diseases in aquaculture.

• To evaluate the extent of antibacterial activity of microalgae specifically Cyanobacteria as a potential substitution of the commercially available antibiotics.

CHAPTER 2: REVIEW OF LITERATURE

2.1 Microalgae

Microalgae are a type of single-celled organisms that transmute solar energy into chemical energy using photosynthesis, and possess the capability to produce complex organic compounds similar to that of terrestrial plants (Harun et al., 2010). Microalgae are also considered as a sustainable alternative to conventional crops due to their fast growth rates, high productivity, and low environmental footprint (Davies et al., 2021). Microalgae become a novel source of diversified compounds, including polyunsaturated fatty acids, carotenoids, and peptides, that indicate various therapeutic effects like antioxidant, anticancer, antimicrobial activities, and anti-inflammatory effects (Coulombier et al., 2021). Microalgae may serve as an alternative natural source of antioxidants and antibiotics in terms of phenolic compounds and pigments like carotenoids (Safafar et al., 2015). Additionally, microalgae have the capability to remove heavy metals and organic pollutants from contaminated waters, making them promising candidates for bioremediation purposes (Chen et al., 2021). Microalgae can be both unicellular and multicellular which have the potential to produce various bioproducts that are significant in the industry. This category of algae includes prokaryotic microalgae, such as cyanobacteria, as well as eukaryotic microalgae, including Chlorophyta (green algae), Bacillariophyta (diatoms), and Rhodophyta (red algae). Microalgae having simple growth requirements are ideal resources for eco-friendly alternatives like pharmaceutics and nutraceuticals (Pignolet et al., 2013).

2.2 Microalgae available in Bangladesh

Both freshwater and marine microalgae are found in Bangladesh. Marine and coastal areas of Bangladesh, such as the Sundarbans, Cox's Bazar, and St. Martin's Island, are rich in microalgae species. There have been 45 taxa belonging to 17 genera of green algae reported from the coasts of Bangladesh (Islam et al., 2000). Microalgae, such as *Nannochloropsis* sp., *Tetrselmis chuii*, *Chlorella minimutissima*, *Chlorella esculentus*, *Isochrysis* sp., *Arthrospira platensis*, *Mastocarpus stellatus*, *Chondrus crispus*, *Alaria esculentus*, *Ascophyllum nodosum*, *Spirulina platensis*, *Dunaliella salina*, and, *Nannochloropsis oculate* have been documented from the St. Martin's Island of

Bangladesh (Ahmed et al., 2016). These microalgae species have the ability to be utilized in a variety of ways such as food and feed supplements, biofuel production, and bioremediation. (Alam et al., 2021). Several species of freshwater microalgae have been recorded from Bangladesh including *Scenedesmus quadricauda*, *Desmodesmus communis*, *Nostoc* sp., and *Ulothrix* sp (Islam et al., 2017), *Nostoc* sp., *Anabaena* sp., *Oscillatoria* sp., *Scenedesmus* sp., *Chlorella* sp. (Rahman et al., 2019); *Scenedesmus quadricauda*, *Chlorella vulgaris*, *Microcystis aeruginosa*, *Desmodesmus communis*, *Nostoc* sp., and *Ulothrix* sp. (Islam et al., 2015).

2.3 Cyanobacteria

Cyanobacteria, also referred to Blue Green Algae, are a Gram-negative bacterial class with a distinctive form of oxygenic photosynthesis that is comparable to higher plants (Chakdar and Pabbi, 2012). Cyanobacteria are known to produce secondary metabolites such as cyanotoxins, which can be harmful to humans and animals if ingested in high quantities. However, many species of cyanobacteria also provide bioactive components with potential pharmaceutical as well as biotechnological applications (Carmichael, 2001.) They produce many active substances with algicidal, enzyme-inhibiting, antiviral, antibacterial, fungicidal, cytotoxic and immunosuppressive properties. These substances have been isolated from cyanobacterial biomass or laboratory cultures (Knubel et al., 1990; Mule et al., 1991). Both freshwater and marine Cyanobacteria have been isolated from Bangladesh such as *Anabaena* sp., *Nostoc* sp., *Oscillatoria* sp., and *Synechococcus* sp. (Islam et al., 2017). Islam et al. (2018) identified several marine cyanobacterial species from the Bay of Bengal, such as *Synechocystis* sp., *Synechococcus* sp., and *Microcoleus* sp.

2.4 *Oscillatoria*

2.4.1 Taxonomic Classification

Kingdom: Monera

Phylum: Cyanobacteria

Class: Cyanophyceae

Order: *Oscillatoria*les

Family: *Oscillatoria*ceae

Genus: *Oscillatoria* (Vaucher ex Gomont, 1822)

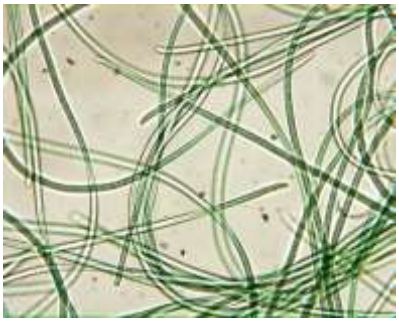


Figure 2.1: Microscopic view of *Oscillatoria* sp.

(Source: https://www.luc.edu/media/lucedu/biology111/microscopy/*Oscillatoria*.jpg)

2.4.2 Morphology

Oscillatoria is a genus of cyanobacteria that is widely distributed in aquatic environments, including freshwater, marine, and brackish water habitats (Whitton and Potts, 2000). The thallus of this organism is shiny and appears bluish-green or brownish in color. Its trichome is straight and elongated, with one end tapering briefly (Halder, 2017). The cells are shorter than broad, measuring around 15 µm in width and 2-8 µm in length (Komárek and Anagnostidis, 2005). They are confined at the cross walls and have granulated appearance. Cell contents are homogeneously light blue-green. The apical cell is hemispherical and tapered, while the cell wall is thick (Halder, 2017).

2.4.3 Bioactive Compound

Oscillatoria species are known to produce several bioactive compounds, including alkaloids, cyclic peptides, and fatty acids, with potential pharmaceutical and biotechnological applications (Mehbub et al., 2014). Some alkaloids isolated from *Oscillatoria* species have been found to have antifungal and antimicrobial properties (Hassan et al., 2012), while others have been shown to have cytotoxic and antitumor activity (Luesch et al., 2001). *Oscillatoria* sp. has been found to produce Oscillatol, which has demonstrated antimicrobial activity (Choi et al., 2007) and another phenolic compound produced by *Oscillatoria* sp. is Oscillamide Y, which has shown potential cytotoxic activity against several cancer cell lines (Kehr et al., 2005).

2.4.4 Application

Oscillatoria has potential applications in various fields such as bioremediation, biotechnology, and food industry. It is capable of removing heavy metals from contaminated water, making it a promising candidate for bioremediation purposes (Sarker et al., 2020). *Oscillatoria* has been demonstrated promising supplier of bioactive compounds that could be used in the pharmaceutical industry. In particular, Hasan et al. (2017) identified an antibiotic compound from *Oscillatoria acuariiformis* that was effective against gram-positive bacteria. Additionally, Singh et al. (2013) isolated a compound from *Oscillatoria sancta* that displayed both antioxidant and anti-inflammatory properties. Additionally, *Oscillatoria tenuis* exhibited antibacterial properties against both of the gram-positive and gram-negative bacteria (Kalla et al., 2018).

2.5 Isolation of Microalgae

Selecting the appropriate species or strain is a crucial and fundamental aspect of exploring microalgae for commercial purposes. (Barclay and Apt, 2013). The process of microalgae screening encompasses a series of critical steps, including collection of sample, isolation, identification, purification, maintenance, screening and characterization of potential products (Gong and Jiang, 2011). Determining the taxonomy of microalgae involves sequencing of the 18S ribosomal RNA genes for eukaryotic and 16S ribosomal RNA for prokaryotic microalgae (Bellinger and Sigee, 2010) based on the variety of microalgae. Despite the fact that algae isolation and molecular identification are standard practices globally, there are very few reports available on the isolation and molecular identification of algae in Bangladesh. To assess their suitability as feedstock and for biofuel production, *Chlorella vulgaris* and *Anabaena variabilis* were isolated from both natural and artificial water bodies respectively from Dhaka University and Khulna, (Tarin et al., 2016). *Spirogyra maxima* and *Pithophora polymorpha* were identified by analyzing partial 18S rDNA sequences and identified as newly isolated microalgae in Bangladesh (Alfasane et al., 2019). Recently, four type of marine microalgae were isolated from the coast of Cox's Bazar, Bangladesh named *Nannochloropsis* sp., *Chlorella sp*., *Tetraselmis* sp., and *Chaetoceros* sp. and also characterized on the basis of growth performance, proximate

composition, and pigments (Islam et al., 2021). In Bangladesh, significantly less attention has been served to indigenous freshwater and marine microalgae specifically Cyanobacteria and their potentialities.

2.6 Growth Parameters of Microalgae

The microalgal growth is affected by several factors including the length of the photoperiod, temperature, pH, and light intensity (Table 2.1). The optimum parameters for microalgae growth vary depending on the species and sometimes type of the parameter itself (Wahidin et al., 2013).

Table 2.1 Optimum growth parameters for microalgae culture

Parameters	**Optimum range**	**Reference**
Light intensity	100 to 2000 μmol photons m^{-2} s^{-1} In case of Erlenmeyer flask: 1000 lux, and for larger volume: 5000-10000 lux	Wang et al., 2021; FAO, 1996
Nutrients	nitrogen concentrations: 10-50 mg/L phosphorus concentrations of 1-5 mg/L	Kumar, 2021
Temperature	20°C to 24°C	Wang et al., 2021
pH	7.0 to 8.5	Wang et al., 2010
Carbon dioxide	2% to 10% (v/v)	Wang et al., 2021
Salinity	Some microalgae species, such as *Dunaliella salina*, require high salinity levels (up to 20%) for growth, while others prefer lower salinity levels according to their habitat.	Kumar, 2021
Aeration	Aeration and homogenized mixing are also crucial factor for promoting microalgae growth. A bioreactor with aeration produced better cell growth compared to a non-aerated system.	Kaewpintong, 2004

2.7 Growth Curve of Microalgae

During the lag phase, which lasts for 2-3 days, the algae cells adjust to the new environment and conditions, and their growth remains constant. Following this phase, the exponential growth phase occurs and lasts for approximately 4-6 days. During this phase, the cells grow rapidly due to the presence of optimal levels of light and nutrients. As nutrients become depleted and cell crowding limits light availability, cell division slows down, leading to the stationary phase. Ultimately, insufficient nutrients and light prevent further cell division, resulting in the death phase (Chan and Javaheri, 2017).

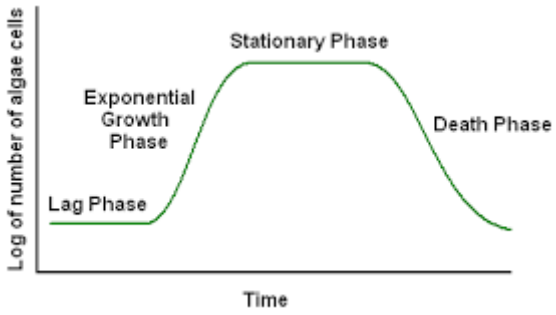


Figure 2.2: Microalgal growth curve **(**Chan and Javaheri, 2017)

2.8 Monitoring of Algae Growth

Monitoring algae growth can be done using various techniques, including optical density: This method involves measuring the absorbance of light by the algal cells, chlorophyll fluorescence, cell counting, dry weight and flow cytometry (Günerken et al., 2017). There are two main analytical methods used for analyzing microalgae growth: counting the number of cells per milliliter (Godoy-Hernández and Vázquez-Flota, 2006), and measuring the cell suspensions absorbance using spectrophotometry (Mikschofsky et al., 2009). To monitor cyanobacterial growth, various methods can be used such as cell counting using a hemocytometer or flow cytometer, optical density measurement and chlorophyll-a measurement by spectrophotometry (Sarrafzadeh et al., 2015). Fluorescence measurement also can be done as Cyanobacteria exhibit autofluorescence due to the presence of phycocyanin and phycoerythrin pigments. This fluorescence can be measured using a fluorometer and can be used as an indirect indicator of cell density (Kühl, 2005).

2.9 Chlorophyll

Cyanobacteria are photosynthetic organisms that use chlorophyll-a as their primary photosynthetic pigment (Whitton and Potts, 2000). In cyanobacteria, chlorophyll-a is found in the thylakoid membranes of the cells, where it participates in the light-dependent reactions of photosynthesis (Allen and Martin, 2017). It also involved in light acclimation and adaptation to changing light conditions according to light intensity and quality (Kehoe and Gutu, 2006). The concentration of chlorophyll-a in cyanobacteria can be used as an indicator of their biomass and growth rate (Liu et al., 2018). Few genera of cyanobacteria exhibit chlorophyll-a that involves in the activation of a regulatory protein called NtcA, which controls the expression of genes involved in nitrogen metabolism and cellular differentiation (Zinser et al., 2006). The chlorophyll content in *Oscillatoria* can vary depending on various factors such as growth conditions, light intensity, and nutrient availability. Keshavanath et al. (2014) reported that the chlorophyll a content in *Oscillatoria tenuis* ranged from 7.3 to 22.5 mg/g in dry weight, while chlorophyll B concentration varied from 1.7 to 5.8 mg/g in dry weight. The chlorophyll a content in *Oscillatoria salina* is found to be 6.18 mg/g dry weight (Abbas et al., 2021).

2.10 Phycobiliprotein

Phycobiliproteins, a group of water-soluble pigments found in cyanobacteria, absorb spectral light in the blue and green ranges, transferring energy to chlorophyll for photosynthesis (Kehoe and Grossman, 1996). The three main types of phycobiliproteins found in cyanobacteria are phycocyanin, phycoerythrin, and allophycocyanin (Singh et al., 2015). Phycocyanin retains blue light and appears blue-green, whereas phycoerythrin absorbs green-yellow light and turns red. Allophycocyanin retains orange light and appears in orange color (Grossman and Schaefer, 1993). The phycocyanin content in *Oscillatoria* sp. has been reported to range from 0.49 to 1.24 mg/g, while the allophycocyanin content ranges from 0.16 to 0.43 mg/g (Singh et al., 2014). These pigments have a crucial role in the photosynthesis of cyanobacteria and are also used in various applications such as fluorescent labeling, diagnostics, and food colorants (Grossman and Schaefer, 1993; Singh et al., 2015). Studies have shown that phycobiliproteins, such as phycocyanin and allophycocyanin, possess antimicrobial

properties against various pathogenic microorganisms such as *Staphylococcus aureus*, *Pseudomonas aeruginosa* and, *Escherichia coli* as well as fungi such as *Candida albicans* (Rajauria et al., 2016; Patel et al., 2022).

2.11 Proximate Composition

The proximate composition of dried cyanobacterial mass varies based on the species, growth conditions and the analytical method. Generally, dried cyanobacterial mass contains high amounts of protein (typically ranging from 40-60% of dry weight), carbohydrates (10-30%), and lipids (5-15%) (Safi et al., 2013; Singh et al., 2016). It also contains various minerals and vitamins such as calcium, magnesium, iron, vitamin A, and vitamin B12 (Safi et al., 2013). The approximate composition of dried *Oscillatoria* mass is: Protein: 38.7%, Carbohydrates: 19.6%, Lipids: 10.1%, Ash: 19.9% (Abdel-Raouf et al., 2012)

There is some evidence that the high protein content of cyanobacterial mass, along with its other components such as carbohydrates, lipids, and minerals, may contribute to its antimicrobial activity. Crude extract of *Phormidium tenue* exhibited potent antimicrobial properties against different Gram-positive and Gram-negative bacteria, and fungi which could be attributed to its high protein and carbohydrate content, as well as secondary metabolites including alkaloids and phenols (Kumar and Gaur, 2011). *Oscillatoria acutissima* inhibits the growth of several pathogenic bacteria, including *Escherichia coli* and *Staphylococcus aureus* which may be due to their high protein, carbohydrate content, as well as the presence of phycocyanin and other pigments (Singh et al., 2015). Crude lipid extract of *Nostoc* sp. exhibited antimicrobial activity against several pathogenic bacteria (Maghembe et al., 2020). Lipid extracts of *Anabaena* sp. and *Nostoc* sp. also showed antimicrobial activity against various bacterial strains (Singh et al., 2017).

2.12 Antimicrobial Activity of Cyanobacteria

Cyanobacteria are photosynthetic bacteria commonly found in aquatic and terrestrial environments. Some species of cyanobacteria have been found to possess antimicrobial properties, which allow them to inhibit the growth of microorganisms such as bacteria, fungi, and viruses. Several cyanobacterial compounds including cyanopeptolins,

nostocyclopeptides, and anabaenopeptins are involved in antibiotic activity (Singh et al., 2019). Antimicrobial activity of *Nostoc* sp. had antibiotic activity against pathogenic bacteria such as *E. coli*, *Klebsiella pneumoniae*, as well as fungi such as *Aspergillus fumigatus* and *Candida albicans* (Chitra and Vijayalakshmi, 2012). *Arthrospira platensis*, commonly known as *Spirulina*, is a well-known cyanobacterium with potent antimicrobial activity against an extended range of bacteria and fungi, including *E. coli*, *Staphylococcus aureus*, and *Candida albicans* (Vázquez-Sánchez et al., 2017). *Oscillatoria* and its extracts exhibit potent antimicrobial activity against a variety of pathogenic microorganisms including both Gram-negative, Gram-positive bacteria and fungi (Sharma and Bhatnagar, 2016; Karuppiah and Chelliah, 2017). The antimicrobial activity of *Oscillatoria* is attributed to the existence of various secondary metabolites, such as phenolic compounds, alkaloids, and terpenoids. For instance, some studies have identified compounds like phycocyanin, phycobiliprotein, and oscillamide as being responsible for the antimicrobial activity of *Oscillatoria* extracts (Venkateswarlu and Sateesh, 2012; Karthikeyan and Ganesan, 2016). *Oscillatoria* sp. has also been found to produce extracellular polymeric substances (EPS) that exhibit antimicrobial and antifouling properties, which may be useful in the development of new biomaterials (Wang and Liu, 2019). Fatty acids were isolated from *Oscillatoria redeki* and found to be effective against *B. subtilis*, *Micrococcus flavus*, *S. aureus*, *S. aureus* (Mundt et al., 2003). *Oscillatoria* extract also exhibited inhibitory properties against virus like Herpes Simplex Virus type 1 (HSV-1) (Cianca and Strout, 2017). So, *Oscillatoria* may have potential applications in the development of new antimicrobial agents and could be further explored for its therapeutic potential. Methanol extracts of *Oscillatoria boryana* displayed the highest zone of inhibition of 20 mm against *Vibrio cholerae*, while 18 mm and 17 mm were recorded against *Staphylococcus aureus* and *Bacillus Subtilis* respectively (Dash et al., 2022). The ethanolic extract of *Oscillatoria* sp. exhibited the highest inhibition zone against *B. subtilis*, *S. aureus*, and *E. coli* (Selim et al., 2014). The ethanolic extract of *Oscillatoria* did not exhibit any impact on both Gram-positive bacteria, such as *Bacillus subtilis* and *Staphylococcus aureus*, as well as Gram-negative bacteria, such as *Escherichia coli* and *Pseudomonas aeruginosa*. However, the bacteria were efficiently affected by the phenolic component that was isolated from the ethanolic extract. According to the results of inhibition zone

diameters, Gram-positive bacteria are more vulnerable to phenols than Gram-negative bacteria, with the maximal inhibition being 20 and 19 mm for Gram-positive bacteria and 10 and 16 mm for Gram-negative bacteria. (Al-Katib and Amin, 2020).

2.13 Minimum Inhibitory Concentration

Noscomin from *Nostoc commune* has antibacterial activity against *Escherichia coli* at MIC 128 ppm, *Bacillus cereus* at MIC 32 ppm, and *Staphylococcus epidermidis* at MIC 8 ppm equivalent to those of the conventional medicines. *Anabaena* extract was found to have antibacterial activity in opposite to *S. aureus* at MIC 32-64 g/ ml (Bhateja et al., 2006).) Minimum inhibitory concentration (MIC) value of *Oscillatoria* sp. methanolic extracts is also reported as 30 µg/ml for gram-positive bacteria and 25 µg/ml for gram-negative bacteria (Bhuyar et al., 2020).

2.14 Bacterial Diseases of Aquaculture

In recent years, microbial communities have been evaluated in farmed seawater due to the presence of different potential pathogens causing diseases (Jin et al., 2022). Marine fish diseases are associated with bacteria including *Vibrio*, *Aeromonas*, *Edwardsiella* and *Pasteurella*, streptococci, myxobacteria, mycobacteria, nocardias (Frerichs, 1989). Tilapia farming is a widespread practice in various environments, primarily in fish ponds and cages. Although tilapia is known to be a hardy fish, it is still susceptible to diseases such as Pseudomonas infection, Motile Aeromonas Septicemia, and Streptococcosis caused by bacteria like *Pseudomonas fluorescens* and *P. aeruginosa*; *Aeromonas hydrophila*; *Streptococcus agalactiae* and *S. iniae* respectively (Tahiluddin and Terzi, 2021). Southern bluefin tuna have been found to harbor *Aeromonas* and *Vibrio* spp. in their kidney and other internal organs. Additionally, disease-causing bacteria such as *Photobacterium* and *Mycobacterium marinum* have also been reported to affect Tuna fish (Munday et al., 2003). Giant yellow croaker has frequently been linked to *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, *Vibrio harveyi*, and *Pseudomonas plecoglossicida (*Shan et al., 2005; Liu et al., 2016). *Setipinna phasa* is reported to be contaminated by *Vibrio parahemolyticus* (Dutta et al., 2018).

In shellfish and finfish aquaculture, Vibriosis is one of the main disease issues. Globally, Vibriosis is the leading cause of farmed shrimp mortality (Chen et al., 2000).

Gram-negative bacteria from the Vibrionaceae family are responsible for causing vibriosis including *Vibrio splendidus*, *Vibrio harveyi*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Vibrio alginolyticus*, *Vibrio anguillarum*, *Vibrio orientalis*, and *Vibrio damsella* (Manam et al., 2019). Crabs are also susceptible by *Vibrio parahemolyticus*, *Vibrio cholerae*, and *Vibrio vulnificus* (Wang, 2011). The proliferation of these bacteria may be influenced by environmental factors that accelerate their growth. In shellfish blood, these bacteria are normally present and adapted. (Sizemore and Davis, 1985).

2.15 Antibiotics Used in Aquaculture

The majority of antibiotics are used for both therapeutic and preventative purposes. Oxytetracycline, amoxicillin, ciprofloxacin are the most frequently used antibiotics (Chowdhury et al., 2022). In aquaculture, antimicrobials are primarily used in the disease treatment. Oxytetracycline Chloramphenicol, ceftriaxone, colistin, cefalexina, cefpodoxime, moxiolin, amitriptyline, doxcycline, cefliofur, clavamox, ciprofloxacin, clavaseptin, clindamycin, clavulanic acid, enrofloxacin, dichlorophene, pirlimycin, neomycin etc are commercially available antibiotics for disease treatment. (Schwarz, 2016). Though developments have been made in diagnostic technologies to identify infectious agents for suitable treatments, small-scale farmers from low- and middle-income countries lack diagnostic capability to identify infections promptly and accurately, which may result in the overuse or abuse of antibiotics (Henriksson et al., 2018).

2.16 Antimicrobial Resistance, Spreading, and Public Health Concerns

Antimicrobial resistance (AMR) in fish pathogenic bacteria has emerged concurrently with the growth in antibiotic use in aquaculture as a part of treatment and prevention (FAO, 2005). Antibiotic resistant infections are found in several farmed fish species, including tilapia, carp, salmon, catfish, and crustaceans like shrimp. (Watts et al. 2017). The omnipresence of antibiotics in the environment and their continued usage to treat bacterial infections in aquaculture have resulted in their "pseudo-durability" and induced the emergence of selective pressure on the bacterial community (Gao et al., 2012). Under selection pressure, antibiotic-resistant bacteria can evolve into an environmental reservoir of antibiotic-resistant genes (ARGs). Since aquaculture systems have been identified as "hotspots for AMR genes," evaluating the aquaculture

resistance, or collection of AMR genes, is a crucial area of global research (Watts et al. 2017). Infections caused by resistant bacteria are more challenging to treat, less accessible, costly, and typically highly toxic medications. In rare circumstances, bacteria have developed an immunity to every type of antibiotic (ECDPC, 2015). The spread of bacteria that are resistant to antibiotics from the aquaculture system to the natural environment may lead to the development of antibiotic resistance in wild fish and the relevant food sources. Considering their direct consumption and the virtually impractical management procedures, this has been taken seriously because it affects human health (Cizek et al., 2010). The majority of antimicrobials used in animal husbandry share structural similarities with the antibiotics used in human, thus cross-resistance and co-resistance are promoted (EMA, 2014). Along with the proliferation of microorganisms that are resistant to antibiotics and genes, the presence of antibiotics in the environment is considered a type of pollution poses a persistent public health challenge worldwide (Xi et al., 2009). The types of antibiotics used in food production, together with the total amount of antibiotics utilized, also constitute a highly delicate issue. Animals often utilize some last-resort antibiotics for humans that have no substitutes, such as colistin. Numerous studies have found that a large proportion of aquatic bacteria contain plasmids, integrons, and transposons, among other mobile genetic elements that are simple to transfer, recombine, and mobilize. As a result, novel mobile combinations of antimicrobial resistance genes (ARGs) can develop, allowing bacteria to swiftly adapt to new environments containing antibiotics (Sørum, 2008; Cabello et al., 2013). Additionally, fish serve as reservoirs for zoonotic pathogens that can spread to humans through foodborne infections and direct contact with infected fish in aquaculture facilities (Gauthier, 2015). The most prevalent fish pathogens that can infect fish handlers are *Streptococcus iniae*, *Photobacterium damselae*, *Aeromonas hydrophilia*, *Vibrio vulnificus*, *Mycobacterium marinum* and (Haenen et al., 2013), while the most common foodborne pathogens are *Listeria monocytogenes*, *Clostridium* spp., and *Aeromonas* (Herrera et al., 2006; Normanno et al., 2006). The results of molecular research have demonstrated a striking connection between the ARGs found in terrestrial bacteria that cause human and animal illnesses and the AMR genes found in bacteria related to aquaculture (Smith, 2008). A wide variety of various antibiotics, including oxytetracycline, ampicillin, gentamycin, erythromycin, polymyxin B,

rifampicin, chlorotetracycline, ciprofloxacin, streptomycin, neomycin, furazolidone, and nalidixic acid were resistant to the *Vibrio harveyi* isolated from infectious shrimp farm (Tendencia and Pena 2001). Fish feeds may have a role in the entrance of resistant bacteria into the culture systems as evidenced by the recovery of oxytetracycline resistance isolates from carp feeds (Singh et al. 2009). Antibiotic resistance is recognized as a "One Health subject," including interactions between people, animals, and the environment and become a crucial public health concern (Bhushan et al., 2017). Multidrug-resistant bacteria pose a growing hazard to global health, and the efficacy of antimicrobial treatments in the future is uncertain. The majority of antimicrobial drugs that are effectively employed in clinical practice have disadvantages such as toxicity, high costs, and lack of efficacy. In addition, their regular usage might lead to the evolution of resistant bacterial strains. Therefore, there is a pressing need to create alternative, efficient, biodegradable components from diversified natural sources with minimal side effects (Jena and Subudhi, 2019).

2.17 Natural and Biological Alternatives of Antibiotics

In a wide range of ecosystems, algae produce a large number of biochemical compounds. From marine algae, more than 18,000 novel bioactive substances have so far been isolated, the majority of which are still uncharacterized. Algal extracts and extracellular components from several microalgae have been shown to have antibacterial, antiprotozoal, antifungal, antiviral, and anti-plasmodial properties. Phenols, indoles, fatty acids, acetogenins, terpenes, and some volatile halogenated hydrocarbons produced from microalgae are chemical groups that have demonstrated antibacterial action. Microalgae are intended to play a significant role as a source of raw materials in the effective manufacture of vitamins, amino acids, and other pharmaceuticals (Jena and Subudhi, 2019). In particular, polyketide synthetase or non-ribosomal peptide synthetase systems are important for the cyanobacterial secondary metabolites biosynthesis. These compounds exhibit a diverse array of biological actions, including anticancer, antiviral, antibacterial, and protease inhibitory properties. The abundance of novel entities that might result from the great level of diversified chemicals in secondary metabolites of Cyanobacteria. This may also serve as a fertile ground for the creation of novel medications (Singh et al., 2011). These microorganisms

have mostly remained an "untapped" resource due to the scant amount of study that has been done on them to date. So, it is high time to tap into the immense wealth of microalgae, which will benefit not only the fisheries sector but also humanity in the long term. Present work will provide the eligibility of indigenous microalgae commonly found in Bangladesh as a prominent natural antibiotic against various pathogens.

CHAPTER 3: MATERIALS AND METHODS

3.1 Sample Collection of Microalgae

3.1.1 Sampling Sites

Microalgae samples were procured from both freshwater and marine environments, encompassing the collection of freshwater microalgae samples from two locations in Chattogram, Bangladesh, comprising Kaptai Lake, Rangamati (22°64' N, 92°19' E), Halda River, Chattogram (22°51' N, 91°84' E). Marine microalgae samples were obtained from two stations in the Bay of Bengal including Maheshkhali Channel, Cox's Bazar (21°31' N, 91°59' E) and Naf Estuary, Teknaf, Cox's Bazar (20° 47′ N, 92° 28′ E). Samples were collected between the months of February and July.

3.1.2 Assessment of Water Quality Parameters

Surface water temperature, pH level, dissolved oxygen (DO) concentration, and salinity were determined using a glass thermometer, a handheld pH meter (pHep-HI98107, HANNA, Romania), a dissolved oxygen meter (DO-5509, Lutron), and a handheld ATC refractometer (YEGREN), respectively. All instruments were calibrated prior to use. Additionally, the levels of total ammonium nitrogen (TAN), nitrite-nitrogen (NO2-N), and soluble reactive phosphorus (SRP) were determined using analytical methods outlined by Parsons et al. (1984).

3.1.2.1 Determination of Total Ammonia Nitrogen (TAN)

Analysis of total ammonia nitrogen (TAN) was performed following the analytical method described by Parsons et al. (1984). A standard stock solution of $(NH_4)_2SO_4$ was prepared by weighing 9.343 g of anhydrous grade $(NH_4)_2SO_4$ that had been dried at 110°C for 1 hour and cooled in a desiccator before weighing. Standard solutions series (0.01, 0.03, 0.05, 0.07, 0.1, 0.3, 0.5, and 1.0 mg L^{-1}) were made from the stock solution, which contained 1000 mg L-1 of total ammonia-nitrogen. The standard solutions were made by dilution with deionized water. 10 ml water samples with standard solutions were mixed to a phenol solution (20 g of analytical grade phenol in 200 ml of 95% v/v ethyl alcohol) and sodium nitroprusside (1 g of $Na_2[Fe(CN)_5\ NO]_2H_2O$ in 200 ml of DDH_2O). After adding oxidizing solution, the mixture cooled at room temperature at 20-27°C for an hour. Test tubes were covered with parafilm to ensure stability of the

color for 24 hours after the reaction period. The absorbance was measured at 640 nm using a spectrophotometer (Nano Drop Spectrophotometer, Model-Nanoplus, Germany).

The oxidizing solution contained mixture of 100 ml alkaline reagent (5 g of sodium hydroxide and 100 g of sodium citrate and in half liter of DDH_2O) along with 25 ml of commercial hypochlorite (e.g. Clorox) that contained about 1.5 N of sodium hypochlorite.

3.1.2.2 Determination of Nitrite-Nitrogen (NO_2-N)

The determination of nitrite-nitrogen (NO_2-N) was carried out using the Parsons et al. (1984) methodology. A standard stock solution was prepared by weighing 4.93 g of anhydrous $NaNO_2$ (dried at 110°C for 1 hour and cooled in a desiccator before weighing) and dissolving it in 1000 mL of deionized water. A series of standard solutions (0.01, 0.03, 0.05, 0.07, 0.1, 0.3, 0.5, and 1.0 mg L^{-1}) were then prepared by diluting the stock solution with deionized water. Subsequently, 10 mL of samples and standard solutions were placed in test tubes, and 0.2 mL of sulfanilamide solution (prepared by dissolving 5 g of sulfanilamide in a mixture of 50 mL of concentrated hydrochloric acid and diluted to 500 mL with DDH_2O) was added. After 2-10 minutes, 1 mL of NED reagent (0.5 g of N-(1-napthyl)-ethylenediamine dihydrochloride dissolved in 500 mL of distilled water) was added and mixed immediately. The extinction was then measured at a wavelength of 543 nm using a Nano Drop Spectrophotometer (Model-Nanoplus, Germany) between 10 minutes and 2 hours after the addition of NED reagent.

3.1.2.3 Determination of Soluble Reactive Phosphorous (SRP)

Soluble reactive phosphorus (SRP) was analyzed using the method established by Parsons et al. (1984). A standard stock solution was meticulously prepared by accurately weighing 4.39 g of anhydrous potassium dihydrogen phosphate (KH_2PO_4), dried at 110°C for one hour, cooled in a desiccator before weighing, and dissolved in 1000 mL of deionized water. A series of standard solutions with concentrations ranging from 0.01 to 1.0 mg L^{-1} were then prepared by diluting the stock solution with deionized water. Subsequently, 10 mL of both the sample and standard solutions were introduced

into test tubes, followed by the addition of 1 mL of a mixed reagent. The absorbance at 885 nm was measured using a Nano Drop Spectrophotometer (Model-Nanoplus, Germany) after a reaction time of 5 minutes, ideally within the first 2-3 hours. The mixed reagent was formulated by combining 100 mL of ammonium molybdate (dissolved by adding 15 g of analytical reagent grade ammonium paramolybdate $(NH_4)6Mo_7O_{24}$ to 500 mL of distilled water), 250 mL of sulfuric acid, 100 mL of ascorbic acid (dissolved by adding 27 g of ascorbic acid to 500 mL of distilled water), and 50 mL solution of potassium antimonyl-tartrate (0.34 g of antimony potassium tartrate (tartar emetic) dissolved in 250 mL of distilled water).

3.1.3 Collection and Concentration of Sample

To collect samples, a 60 µm mesh plankton net was utilized to filter approximately 40-50L of freshwater. Filtrate was subsequently collected in a 300mL sample bottle and transported to the laboratory under refrigerated conditions. Then, the samples were concentrated by centrifugation at 3000 rpm for 5 minutes, with the supernatant being discarded and the resulting concentrate utilized for isolation.

3.2 Microalgae Isolation

3.2.1 Preparation of Agar Plate

The preparation of the agar medium involved dissolving 1.5% agar powder into 1 liter of Bold Basal Media (BBM), followed by sterilization in an autoclave at 121˚C for 15 minutes under a pressure of 150 lbs. Subsequently, the medium was cooled to nearly 50°C, and vitamin solution was added to it while gently rotating the flask to ensure nutrient homogenization and prevent bubble formation. The warm medium was then aseptically poured into sterile petri dishes, with the agar medium occupying at least ½ or ¾ of the petri dish depth. The agar medium was allowed to cool and solidify under UV light in the biosafety cabinet before being stored in plastic bags under refrigeration for subsequent use.

3.2.2 Streaking of Microalgae (Streak Plate Method)

Following the preparation of the agar plate, a concentrated microalgal sample was meticulously placed to the agar's periphery using sterile Pasteur pipette. Aseptically, the suspension was streaked in parallel fashion onto the agar using a wire nano loop.

To safeguard against unwanted environmental exposure during incubation, the plates were covered, inverted, and securely sealed with parafilm. Plates were incubated around 7-12 days under optimal light and temperature. Upon visible growth, a sterile fine needle was utilized to meticulously select the desired colony from the dish. The colony was subsequently transferred to a sterile test tube containing 3-5mL of liquid medium, and underwent microscopic examination to select colonies free of contaminants for further isolation. In instances where the colony manifested multiple microalgae, the streaking procedure was repeated to establish a pure, single colony.

3.2.3 Serial Dilution of Microalgae

To perform a serial dilution, 9 mL of the previously prepared liquid culture medium (BBM) were dispensed into test tubes labeled 10^{-1} to 10-^{10} to indicate the dilution factor. Subsequently, the test tubes were placed in a biological safety cabinet, where the cap was removed and the neck of test tube was flamed. Then, 1 mL of the enrichment sample was added to the first test tube (10^{-1}) and gently mixed. Thereafter, 1 mL of the previous dilution was transferred to the subsequent tube (10^{-2}) and similarly mixed, with the process repeated for the remaining tubes (10^{-3} to 10^{-10}). Following the completion of the dilution procedure, the test tubes were incubated under controlled temperature and light conditions. After 2-4 weeks, each dilution tube was microscopically examined by withdrawing a small, aseptic sample. A unialgal culture was considerably obtained when observed in one of the higher-dilution tubes (10^{-6} to 10^{-10}). In cases where the tubes contained two or three distinct species, the capillary pipetting method was employed to isolate a single algal species.

3.2.4 Capillary Pipetting (Picking up Method)

The capillary pickup method was employed to obtain a single algal unit directly. A capillary pipette was obtained from a Pasteur pipette by heating it in the hottest region of a flame until it reached a soft and pliable condition. The pipette was then quickly removed from the flame and a thin tube was formed by applying a gentle pull. The thin tube was subsequently bent using forceps to produce a capillary pipette. A large drop of algal suspension was placed on a glass slide, and 4-6 drops of prepared liquid media were deposited on another slide. Using an inverted microscope to observe the cells, the desired single cell from the large algal suspension drop was transferred to one of the

liquid media drops. This process was repeated by pipetting the desired algal unit from one liquid medium drop to the next until a single algal unit was found in the final drop without any contamination. The single algal unit was then transferred to an eppendorf tube containing liquid media and under desired environmental conditions, isolated microalgal units were incubated.

3.3 Morphological Identification of Microalgae

Isolated microalgae were morphologically identified using optical microscope at 40X magnification and phytoplankton identification books were used to identify microalgae on the basis of morphological characteristics (Belcher and Swale, 1976; John et al., 2002 and Bellinger and Sigee, 2010; Tomas, C. R., 2012).

3.4 Sub-culture of Microalgae

3.4.1 Preparation of Liquid Media

3.4.1.1 Bold's Basal Medium (BBM) (Bischoff and Bold, 1963)

The formulation of BBM for microalgae culture involved the use of sterilized distilled water as the source of freshwater. To prepare a liter of standard BBM, 10mL of each of the six stock solutions (1-6), 1mL of stock solutions (7, 8, and 10), and 0.7mL of boric acid solution (9) were added to 940mL of autoclaved distilled water. Subsequently, 0.5mL of sterile vitamin solution (11) was added. Table 3.1 presented the composition of the various constituents of BBM (Table 3.1).

3.4.1.2 Conway Medium (Tompkins et al., 1995)

Marine microalgae culture comprises the Conway medium for growth and survival. Liquid Conway medium consists of various macronutrients, trace metals, and vitamins. To prepare the medium, solution A (1 ml), solution B (0.5 ml), and solution C (0.1 ml) are added to the 1000 ml filtered and sterilized sea water (Table 3.2).

Table 3.1 Constituents of Bold's Basal Medium (BBM)

Name of chemicals			**Quantity**
1. Potassium dihydro phosphate (KH_2PO_4)			8.75 g/500ml
2. Calcium chloride dehydrate ($CaCl_2.\ 2H_2O$)			1.25g/500ml
3. Magnesium sulfate hydrate ($MgSO_4.\ 7H_2O$)			3.75g/500ml
4. Sodium nitrate ($NaNO_3$)			12.5g/500ml
5. Dipotassium phosphate (K_2PO_4)			3.75g/500ml
6. Sodium chloride (NaCl)			1.25g/500ml
7. $Na_2EDTA.2H_2O$			10g/L
Potassium hydroxide (KOH)			6.2g/L
8. Iron (2) sulfate 7-hydrate ($FeSO_4.7H_2O$)			4.98g/L
Sulphuric acid (H_2SO_4)			1ml/L
9. Boric acid (H_3BO_3)			5.75g/500ml
10. Trace metal solution			g/L
Boric acid (H_3BO_3)			2.86g
Manganese (2) chloride tetrahydrate ($MnCl_2.\ 7H_2O$)			1.81g
Zinc sulfate hydrate ($ZnSO_4.\ 7H_2O$)			0.222g
Sodium molybdate dihydrate ($Na_2MoO_4.2H_2O$)			0.390g
Copper (2) sulfate pentahydrate ($CuSO_4.\ 5H_2O$)			0.079g
Cobalt nitrate hexahydrate ($Co(NO_3)_2.\ 6H_2O$)			0.0494g
11. Vitamins			0.5ml
Thiamine HCl	200mg	To 950 ml of dH_2O	
Biotin	1g/L	1ml from each of the two primary stocks was added then and made to 1L	
Cyanocobalamin	1g/L		

Table 3.2 Chemical constituents of Conway medium

Solution A- Macronutrients		
Chemical name	Molecular formula	Proportions
Sodium/Potassium nitrate	$NaNO_3$ /KNO_3	100.0 0 g/116.0 0 g
EDTA Disodium salt	$C_{10}H_{16}N_2O_8$	45.00 g
Boric acid	H_3BO_3	33.60 g
Sodium di-hydrogen orthophosphate	$NaH_2PO_4.4H_2O$	20.00 g
Ferric chloride hexahydrate	$FeCL_3.6H_2O$	1.30 g
Manganese (II) chloride tetrahydrate	$MnCL_2.4H_2O$	0.36 g
Deionized/distilled water	H_2O	1 L
Solution B- Trace Metal solution		
Chemical name	Molecular formula	Proportions
Zinc chloride	$ZnCl_2$	2.10 g
Cobalt (II) chloride hexahydrate	$CoCl_3.6H_2O$	2.00 g
Ammonium molybdate tetrahydrate	$(NH_4)6MO_7O_{24}.4H_2O$	0.90 g
Copper (II) sulfate pentahydrate	$CuSO_4.5H_2O$	2.00 g
Zinc chloride	$ZnCl_2$	2.10 g
Deionized/distilled water	H_2O	1 L
Solution C- Vitamin's Solution		
Chemical name	Molecular formula	Proportions
Thiamine	Vitamin B1	200 mg
Cyanocobalamin	Vitamin B12	10 mg
Deionized/distilled water	H_2O	100 mL

3.4.2 Subculture

The microalgae were initially sub-cultured in 50 ml conical flasks and then transferred to 100 ml conical flasks upon reaching the appropriate growth stage. Subsequent sub-culturing of promising microalgae strains was performed until they reached the maturation phase.

3.5 Growth Curve Determination

The isolated microalgae strains were cultivated utilizing the BBM and Conway medium respectively for freshwater and marine isolates. For each species, three replicates were prepared using sterile 500 mL borosilicate Erlenmeyer flasks, each containing a culture volume of 350 mL and inoculated with 2% pure culture stocks. The cultures were maintained under continuous light conditions of 24 hours, with an intensity of 150 μEm-2s-^{1}, gentle aeration at a rate of 4.53 ± 0.53 mg/L, and a temperature of 24 ± 1°C. The experiment was carried out until the death phase and the growth curve was completed based on chlorophyll content and optical density (absorbance).

3.5.1 Determination of Chlorophyll

3.5.1.1 Microalgae Extraction

To extract microalgae, 1ml $MgCO_3$ was filtered through a 47 mm Ø Whatman® GF/C glass microfiber filter paper using a filtering apparatus to cover most of the filter's area. Following this, 10ml of each algae sample was filtered and the edges with no residue were trimmed. The filter paper was then folded and placed in a 15ml centrifuge tube with the center facing downwards. Then, 2ml of 90% acetone was added and ground for 1 minute, followed by the addition of 8ml of 90% acetone and grinding for 30 seconds. The sample was then refrigerated in the dark for 1 hour. After 1 hour, the sample was centrifuged at 3000 rpm for 10 minutes, and the acetone extract was transferred to another centrifuge tube and centrifuged at low speed (500 rpm) for 5 minutes. Finally, the absorbance of the acetone extract was measured against 90% acetone as a blank.

3.5.1.2 Chlorophyll Quantification

Chlorophyll concentration was quantified based on Jenkins (1982) method. The clear acetone extract was carefully transferred into a 1 cm cuvette and optical density (OD)

was recorded at 750 nm, 664 nm, 647 nm, and 630 nm wavelengths. The OD values at 664 nm, 647 nm, and 630 nm were used to calculate chlorophyll concentration, while the OD value of 750 nm was used as turbidity correction factor and subtracted from each of the pigments OD values before using them in the equations. The concentrations of chlorophyll a, was calculated using the corrected OD values in the following equations, as described by Jeffrey and Humphrey (1975):

$$C_a = 11.85(OD664) - 1.54(OD647) - 0.08(OD630)$$

Where: C_a= Chlorophyll-a concentration in mg/L respectively, and OD664, OD647, and OD630 = corrected optical densities (with a 1 cm light path) at the respective wavelengths. Once the pigment concentrations in the extract were determined, the pigments' quantity per unit volume was computed using the following formula:

$$\text{Chlorophyll a } (\text{mg/m}^3) = \frac{\text{Ca (mg/L)} \times \text{extract volume (L)}}{\text{volume of sample (m}^3\text{)}}$$

3.5.2 Determination of Optical Density (OD)

To analyze the growth curve, optical density (OD) was measured daily, using respective culture media as the blank sample (BBM, Conway media). For each microalga, the maximum absorbance value was used to create the growth curve based on OD. The maximum absorbance was measured at specific wavelengths for each microalga, ranging from 443nm to 600nm, as those wavelengths showed the highest absorbance when scanned between 300 to 700 nm using a spectrophotometer (Nano Drop Spectrophotometer, Model-Nanoplus, Germany). Strain 1 M1 (20) sp. showed maximum absorbance at 600 nm, Strain 2 M2 (25) at 443 nm, Strain 3 Fw1 at 530 nm, and Strain 4 Fw2 at 475 nm.

3.6 Mass Culture of Microalgae

The large-scale or mass culture of selected microalgae isolates was conducted in tanks using both BBM and Conway medium respectively for freshwater and marine microalgae. The process involved gradually increasing the culture volume from an initial starter culture of 20 ml to 20 L. Initially, 20 ml microalgae stock were cultured in 30 ml of liquid medium in each flask, creating a total culture volume of 50 ml. The batch cultures were then incrementally scaled up to 100 ml, 250 ml, 500 ml, 1 L, and

finally 10 L-15L, serving as inoculum for the subsequent step before transferring to a 20 L culture medium. PVC pipe substrates were used for Cyanobacteria culture. After reaching their stationary phase on day 12, microalgae species were harvested by centrifugation at 5000 rpm for 5 minutes by using centrifugation machine (HERMLE Z 206A, Germany).

3.7 Preparation of Dried Biomass

The wet microalgae biomass obtained from post-centrifugation was subsequently oven-dried overnight at 40°C. A high-quality hot air oven (JSR Korea's Natural Convention Oven LNO-150) was employed for drying, and the dried biomass was crushed into tiny particles (0.4-0.5 mm diameter) using a mortar and pestle. The powdered microalgae were then stored in a standard freezer at 4°C until required for further use.

3.8 Phycobiliproteins Determination

To estimate the phycobiliproteins in the Cyanobacteria, 40 mg dried powder was mixed with 10ml phosphate buffer (pH 7.0; 0.1 M) using vortex mixture and stored for 24 hours at 4°C. Then, the samples were centrifuged at 6000 rpm for 10 min. Finally, the supernatant was collected and absorbance of the samples was measured against the phosphate buffer solution as blank at specific wavelengths (280, 562, 565, 615, 620, 650, 652, and 720 nm) using a spectrophotometer (Nano Drop Spectrophotometer, Model-Nanoplus, Germany). The absorbance at 720 nm was used to measure the cellular debris. Phycocyanin (PC), allophycocyanin (APC), and phycoerythrin (PE) were calculated from the absorbance values using established methods (Bennett and Bogorad, 1973; Siegelman and Kycia, 1978):

Phycocyanin (PC) mg/mL= $\{A_{615} - A_{720}) - 0.474 \times (A_{652} - A_{720})\} / 5.34$

Allophycocyanin (APC) mg/mL= $\{A_{652} - A_{720}) - 0.208 \times (A_{615} - A_{720})\} / 5.09$

Phycoerythrin (PE) mg/mL = $\{A_{562} - (2.41 \times PC) - (0.849 \times APC)\} / 9.62$

Total phycocyanin, phycoerythrin, and allophycocyanin (mg/g) were calculated according to Silveira et al. (2007) as follows:

$$P = (\text{Pigment concentration} \times V) / DB$$

Where, V= Solvent volume, DB= Dried biomass

Total phycobiliproteins (mg/g) were further estimated from the count of the phycocyanin, phycoerythrin, and allophycocyanin contents in dried microalgae biomass.

3.8.1 Purification Factor

The purification factor of phycocyanin, phycoerythrin, and allophycocyanin extract was determined spectrophotometrically by A 620/A 280, A 565/A 280 and A 650/A 280 ratio ((Bennett and Bogorad, 1973).

3.9 Proximate Compositions Determination

3.9.1 Protein Determination

The protein content of each sample was determined using the Lowry method (Lowry et al., 1951). Firstly, 5 mg of freeze-dried biomass was mixed with 25 mL of distilled water using a tissue homogenizer. Then, 0.5 mL of each sample was taken and mixed with 0.5 mL of 1N NaOH, followed by heating in a hot water bath at 100°C for 5 minutes. The samples were then cooled in a cold-water bath for 10 minutes. Then, 2.5 mL of a mixed reagent consisting of 50 mL of Reactive 2 (2g of Na_2CO_3 in 100 mL of 0.1 NaOH) and 1 mL of Reactive 1 (1% NP tartrate) was added to ach sample. After proper mixing using vortex mixture, 0.5 mL of Folin reagent was added to each sample and the mixture was allowed to stand in the dark for 30 minutes. The absorbance was measured at 750 nm wavelength using a spectrophotometer. To create a calibration curve, a stock solution of albumin at 2000 µg/L was prepared and diluted to various concentrations (20 µg/L, 40 µg/L, 80 µg/L, 100 µg/L and 200 µg/L). The protein content of each sample was then determined using the standard curve obtained from the absorbance readings.

3.9.2 Carbohydrate Determination

The carbohydrate content of the microalgae samples was determined using the method described by Dubois et al. (1956). Firstly, 5mg of freeze-dried biomass was taken and mixed with 25 mL distilled water using a tissue homogenizer to prepare a well-mixed microalgae solution. From this solution, 1mL was taken from each type of sample and mixed with 1 mL of 5% phenol and 5 mL of concentrated sulfuric acid (98%). The mixture was then left to react for 30 seconds and then cooled in a cold-water bath. The

solution was then analyzed using spectrophotometric measurements at a wavelength of 488 nm. To prepare the calibration graph, 1000 µg/L standard glucose stock solution and a series of standards at various dilutions (20 µg/L, 40 µg/L, 60 µg/L, 100 µg/L, and 140 µg/L) were prepared. The same carbohydrate analysis procedure was applied to the standards as described above, and a standard graph was plotted according to the standard results. Using this graph, the carbohydrate content of each type of sample was determined based on the absorbance readings obtained.

3.9.3 Lipid Determination

The lipid content of the samples was determined according to the Bligh and Dyer (1959) and Folch et al. (1957) methods. Each sample was labelled and weighed in aluminum dishes to obtain the initial weight. 50 mg sample was taken in a centrifuge tube and diluted into 5x volume using distilled water. A solution of methanol: chloroform (2:1, v/v) was added, and the mixture was homogenized using a tissue homogenizer and centrifuged at 1000 rpm for 4 minutes at 4°C. After centrifugation, the supernatants were transferred to clean tubes by Pasteur pipette and kept on ice. The remaining pellet was mixed with another solution of methanol: chloroform (2:1, v/v), centrifuged again under the same conditions, and combined with the previous supernatants. Then, 1.5 mL of 0.9% NaCl was added to the combined supernatants, and mixed thoroughly using a vortex mixer. The mixture was then refrigerated for 1 hour at 4°C, followed by centrifugation at 1000 rpm for 10 minutes at 4°C, resulting in the formation of two separate layers. The upper layer of methanol and chloroform was discarded, while the lower layer was transferred to a pre-prepared aluminum dish. The solvent was evaporated at 60°C using a hot air oven, and the final weight of the aluminum dishes was determined to obtain the lipid weight in each sample by subtracting the initial weight from the final weight.

3.10 Fatty Acid Determination

Two steps transesterification also known as 2TE method with a little modification of Griffiths et al. (2010) was used to determine the fatty acid composition. In a lipid extraction beaker, 500mg microalgae powder dissolved in 70ml diethyl ether. Digital Soxhlet Apparatus (FOOD ALYTRD40) was used for lipid extraction. Diethyl ether was removed by placing the test tubes in the Hot Air Oven at 60°c. Then, 1.5 ml of

methanolic NaOH was added into the lipid extract and mixed properly through Sonication at 80°C for 5 minutes. Upon cooling at room temperature (25°C), 2ml of BF_3 methanol was poured into the mixture and again sonicated for 30 minutes at 80°C. After cooling at 25°C, 1ml of isooctane and 5ml of saturated NaCl was poured and well mixed through shaking. Then two layers were observed. Fatty acid methyl-esters (FAMEs), an organic substance in the upper layer was transferred to a new test tube. 1ml sample from the test tube was taken into 1.5 ml Eppendorf vial for further fatty acid methyl-esters analysis through GCMS-Gas Chromatography Mass Spectrophotometry (GC-2020plus, SHIMADZU, Japan). Separation of FAMEs was done with a capillary column (30m length, 0.25mm internal diameter, 0.15 μm film thickness, and phase ratio is 250). Helium gas was used as a carrier gas with 1.42 ml/min flow rate. The column temperature program was: 180° to 280°c at 5 °c /min and then at 280°c. Detection of FAMEs were done by comparing the retention time with standard (FAME mix C8-C24; Sigma- Aldrich; Germany).

3.11 Amino Acid Determination

The Moore and Stein technique (1951) was slightly modified in order to identify amino acids. 1 g dried biomass of microalgae was first hydrolyzed for 24 hours at 110 ±2 °C in 25 mL of previously prepared acidic hydrolysis solution (6 M HCl + 0.1% phenol). The samples were stabilized using a little quantity of SDB/Na (Sample Dilution Buffer) after cooling. The samples' pH was then adjusted using a basic neutralizing agent to range between 2.1 and 2.3. The hydrolysates were then filtered and diluted with SDB/Na before being put into the injection vials. SYKAM S 433 amino acid analyzer with UV detector was used for the analysis. With a constant flow rate of 0. 5 mL/min of nitrogen gas at a temperature of 60^0C and a reproducibility of 3%, nitrogen gas was employed as the carrier gas. Sigma-Aldrich, Germany's AA-S-18 standard wease is used to measure the concentration of amino acids. The amount of amino acids was measured in mg/g, which was then converted to % of all amino acids.

3.12 Isolation, Identification and Culture of Bacteria

3.12.1 Sampling Site

Bacterial samples were collected from diseased fish namely Tuna (*Euthynnus affinis*), Rita (*Rita rita*), Gangetic Hairfin Anchovy (*Setipinna phasa*), Poa (*Otolithoides pama*), Tilapia (*Oreochromis mossambicus*) which were collected from Fishery Ghat, Chattogram. Crustaceans such as Shrimp (*Penaeus monodon*), Crab (*Scylla serrata*) were collected from the BFDC landing center and Crab farm, Cox's Bazar respectively.

3.12.2 Collection and Incubation of Sample

Sterile cotton swab sticks were used to obtain samples from the diseased fish skin, gills, intestine and other affected areas. Sterile swabs were also used to swab the abdominal flap, mouthparts, claws, and carapace region of the crab, and the joints of the appendages, uropod, endoskeleton, and cephalothorax region of the shrimp which were subsequently inoculated into nutrient broth. The samples were then incubated overnight at 37°C to allow the optimal growth and proliferation of the bacteria present in the samples.

3.12.3 Preparation of Bacterial Culture Media

3.12.3.1 Nutrient Broth

A total of 13.0gm of NB powder was dispersed in 1000 ml of distilled water and heated until the medium was completely dissolved. The resulting mixture was then sterilized by autoclaving at 121°C for 15-20 minutes.

3.12.3.2 Phosphate Buffer Saline (PBS)

9.55gm of PBS powder was suspended in 1L of distilled water then mixed and sterilized by autoclaving at 121°C for 20 minutes and subsequently stored in a refrigerator at 4°C.

3.12.3.3 Trypticase Soy Agar (TSA)

A total of 40gm of TSA agar powder was suspended in 1000 ml of distilled water and heated until the medium was completely dissolved. The resulting mixture was then sterilized by autoclaving at 121°C for 15-20 minutes, cooled down to 45-50°C, and mixed thoroughly before being poured into sterile petri plates.

3.12.3.4 Mannitol Salt Agar

111.02 g agar powder was suspended in 1L distilled water and heated until the medium was completely dissolved. Then, the mixture was sterilized by autoclaving at 121°C for 15-20 minutes, cooled down to 45-50°C, and poured into sterile petri dishes.

3.12.3.5 MacConkey Agar

49.53 gm agar powder was suspended in 1000 ml of distilled water and heated dissolving completely. Agar was sterilized by autoclaving at 121°C for 15 minutes, cooled to 45-50°C, mixed thoroughly, and then poured into sterile petri plates.

3.12.3.6 Eosin Methylene Blue (EMB) Agar

In 1000ml distilled water, 35.96 gm EMB powder was suspended and heated until the medium was completely dissolved. Agar was sterilized by autoclaving at 121°C for 15 minutes, cooled down to 45-50°C, and then shaken in order to oxidize the methylene blue and suspend the flocculent precipitate. The medium was mixed thoroughly before being poured into sterile petri plates.

3.12.3.7 Thiosulfate-Citrate-Bile-Salts-Sucrose (TCBS) Agar

In 1L distilled water, 89.08 gm TCBS agar was suspended and boiled until complete dissolving. Then it was cooled to 45-50°C, mixed and poured into sterile petri plates.

3.12.3.8 Pseudomonas Agar

46.4 gm agar powder was suspended in 1000 ml of distilled water and heated dissolving completely. Agar was sterilized by autoclaving at 121°C for 15 minutes, cooled to 45-50°C, mixed thoroughly, and then poured into sterile petri plates.

3.12.3.9 Salmonella-Shigella (SS) Agar

In 1L distilled water, 63.02 gm TCBS agar was suspended and boiled until complete dissolving. Then it was cooled to 45-50°C, mixed and poured into sterile petri plates.

3.12.3.10 Blood Agar

40 gm agar powder was suspended in 1L distilled water, and heated while stirring. Then sterilized by autoclaving at 121°C for 15 minutes, and cooled to 45-50°C in a water

bath. 5% (vol/vol) sterile defibrinated cow blood was added aseptically and mixed gently. The medium was then dispensed into sterile plates while still in a liquid state.

3.12.3.11 Muller Hinton Agar (MHA)

38 gm MHA agar powder was suspended in 1000 ml of distilled water and heated until the medium was completely dissolved by boiling. The resulting mixture was cooled down to 45-50°C, mixed well, and then poured into sterile petri plates for sensitivity testing.

3.12.4 Gram's Staining of Isolated Bacterial Samples

3.12.4.1 Preparation of the Gram's Stain Procedure

3.12.4.1.1 Crystal Violet

Solution A: 2g powder of crystal violet was mixed with 20 ml of 95% ethanol. Solution B: ammonium oxalate powder (8g) was dissolved in purified distilled water (80ml). Then solution A and B were mixed and left at for 12 hours. Afterward, the mixture was filtered using a filter paper and kept at 25°C.

3.12.4.1.2 Gram's Iodine

A mixture of 1 gm of crystalline iodine and 2 gm of potassium iodide was ground using a mortar and then mixed with 300 ml of distilled water. Gram's iodine solution was then stored in a dark bottle to shield it from light and kept at room temperature (25°C).

3.12.4.1.3 Decolorizer

500 ml of acetone, 475 ml of organic solvents (methanol or ethanol), and 25 ml of sterilized distilled water were meticulously combined and then kept at 25°C.

3.12.4.1.4 Safranin Stain

Initially, 2.5g safranin powder was added to 95% ethanol. Then, 10 ml of solution mixture was combined with 90 ml of distilled water. The resulting solution was then stored at 25°C

3.10.4.2 Gram's Staining Procedure

3.10.4.2.1 Preparation of Bacterial Slide Smear

A sterile inoculating loop was used to transfer a drop of distilled water onto a glass slide, and it was combined with a loop full of bacterial culture that had been taken from agar plates. A room-temperature air drying period was subsequently given to the resultant smear. It was momentarily placed over a flame for the fixation of bacteria.

3.10.4.2.2 Staining Protocol

The principal stain crystal violet was added to the prepared slide and left on for one minute. To eliminate of any unbound crystal violet, the slide was washed with a gentle stream of water for up to ten seconds. The mordant Gram's iodine was then used for 1 minute to attach the crystal violet to the bacterial cell wall. The slide was then rinsed with acetone for 3 seconds and washed with a gentle stream of water. Afterwards, the secondary stain safranin was applied to the slide for 1 minute and washed again with a gentle stream of water. Once the slide was air dried, Light microscopy was used to investigate it. Gram-negative bacteria displayed pink, but Gram-positive bacteria showed up as blue/purple.

3.12.5 Isolation and Identification of Bacteria by Culture and staining

Bacterial culture from nutrient broth was streaked onto MAC, MSA, TCBS, blood agar, Pseudomonas agar, SS agar and then incubated at 37°C for 24 hours. The growth of *E. coli, Vibrio* sp., *Staphylococcus saprophyticus*, and *Aeromonas hydrophila* was indicated by bright-pink large colonies in MAC agar, green and yellow color colonies in TCBS agar, medium-sized yellow colonies in MSA, and round, smooth, and glistening colonies, looking like dewdrops with or without hemolysis in Blood agar, respectively. The bacteria were identified by further confirmation through Gram's staining.

3.12.6 Biochemical tests

Bacterial samples underwent biochemical assays using the VITEK 2 system at the Marine Biotechnology Laboratory, University Malaysia Terengganu, Malaysia. *Aeromonas hydrophila*, *Staphylococcus saprophyticus*, and *E. coli* were among the bacteria that the VITEK 2 system identified. Additional biochemical tests such as

Catalase, Production of H_2S, Triple Sugar Iron, and motility tests were conducted at the Disease and Microbiology laboratory, CVASU to confirm the presence of other bacteria.

3.12.6.1 Principles of VITEK 2 Biochemical Identification Method

Based on the biochemical patterns of tested strains, the VITEK 2 compact system is a widely used integrated and automated method for bacterial identification. The ID-Gram Positive (ID-GP) cards were used to identify users in line with the manufacturer's instructions. The ID-GPC card is a plastic card containing 64 wells, comprising 18 vacant wells and 46 wells for fluorescence and inhibitory tests, which include pH change assays and derivatives to identify oxidizes and amino peptidase. The average time it takes to get results from a detection is 8 hours or less.

3.12.6.2 Preparation of Test Suspension for VITEK 2 System

Around 3 ml sterile aqueous NaCl (0.45 to 0.50%), with a pH range of 4.5 to 7, were aseptically transferred to polystyrene test tubes (12×75). Isolated sufficient colonies were then transferred from a culture with 24-hours incubation to the physiological saline (0.9%) tube using a sterile loop or swab to obtain a density equal to McFarland 0.5 to 0.63 with the VITEK 2 DENSICHEK. The culture was then tested using the VITEK 2 GP card system within 30 minutes of the suspended culture preparation. The culture tube and the VITEK 2 GP card were inserted into the VITEK 2 cassette, and the user manual provided with the instrument was consulted for guidance on the use of the instrument. The results of bacterial detection were recorded from the VITEK 2 system. A low discrimination identification, indicated by a slash line, was an appropriate outcome for the VITEK 2 GP process, as described in the VITEK 2 GP product details given to end users. Further testing may be required to improve the identification of the organism. This system shows nearly 100% probability of identifying bacterial species.

3.13 Bacterial Preservation

20% glycerol was taken in the cryovials and equal amount of purified bacterial cultures were added into the vial. Finally, the bacteria were preserved at -80°C till use. The storage period for bacterial cultures in this state is two to four years.

3.14 Preparation of Crude Cyanobacterial Extract

2g dried cyanobacterial biomass was pulverized and sieved to obtain a fine powder, which was subsequently mixed with 20 ml 95% pure ethanol (1g sample/10 ml ethanol) to yield the crude ethanolic extract (Grand et al., 1988). It was soaked in the solvent in the sterile screw-capped bottles of 100 ml volume for a day (24 h) at room temperature. (Arun et al., 2012). Then the obtained solution was fine filtered using Whatman No.1 filter paper under vacuum to remove cellular materials (Gonzalez et al., 2001).

3.15 Extraction of Cyanobacterial Phenolic Compounds

To isolate the potent extracts of phenolic compounds from *Oscillatoria* sp., filtered crude ethanolic extracts solution was concentrated under reduced pressure using a rotary evaporator at their respective boiling points. The resulting concentrated extract was then securely stored at 4 ºC until subjected to the next step of extraction. For this, an acidic degradation method was employed Harborn (1973) which involved adding 2 ml of the concentrated extract to hydrochloric acid (2M, 200 ml) and heating the mixture in a water bath (90-100ºC) with a stirrer for 30 minutes. After cooling the extract to room temperature, the solution was transferred to a separating funnel where ethyl acetate (100 ml) was added (this process was repeated twice). The two layers were then carefully separated, with the top layer comprising the ethyl portion rich in free phenolic acids. The extract was concentrated under pressure using a rotary evaporator, resulting in a precipitate which was stored at 4ºC until it was ready for use in diagnosing phenols. Meanwhile, the aqueous layer was discarded.

3.16 Antibacterial Activity of Crude and Phenolic Extracts

The disk diffusion method was used to evaluate the antibacterial activity of the ethanolic raw extract and phenolic extract of *Oscillatoria* sp. (Bauer et al., 1966), which involved Mueller-Hinton agar plates as well as the standard antibiotic disks as positive control. A turbidity of 0.5 McFarland standards was met by adjusting the bacterial suspension with 0.85% physiological saline, which corresponded to approximately 1.5×10^8 CFU/ml (Yilmaz, 2012). To assess the antimicrobial activity, six replicates of each bacterial species were inoculated onto MHA plates using sterile cotton swabs. To screen for inhibitory effects, sterile filter paper discs (6mm) were impregnated with 30

µl of the cyanobacterial extracts and then air-dried. Using sterilized forceps, the dried discs were placed on the Muller-Hinton agar plate. Colistin was used as the standard antimicrobial disk for *Aeromonas hydrophila*, *E. coli*, and *Vibrio* spp., *Salmonella*, *Shigella* sp., *Pseudomonas* spp., while Ceftriaxone was used as the positive control for *Streptococcus saprophyticus, Staphylococcus* spp. Sterile and clean Whatman filter paper was utilized as the negative control. The plates were then kept at 37°C overnight while being inverted. The extracts created the distinct, circular inhibition zones surrounding the discs. Digital slide calipers (Robotics BD shipment) were used to measure the zone of inhibition from edge to edge.

3.17 Antimicrobial Activity index (AI)

The comparison of the specific extract's zone of inhibition to that of a reference antibiotic, the activity index (AI) was determined using the following formula (Singariya et al. 2011; Dharajiya et al. 2014).

$$Activity\ index = \frac{Diamter\ of\ the\ inhibition\ zone\ of\ extract}{Diamter\ of\ the\ inhibition\ zone\ of\ the\ reference\ antimicrobial}$$

3.18 Minimal Inhibitory Concentration (MIC)

250 mg cyanobacterial power was dissolved in 1 ml 100% dimethyl sulfoxide (DMSO) and diluted with sterilised deionised water to 10% DMSO. To achieve 1% DMSO final concentrations, the stock solution was diluted with 10% DMSO. After that, the tubes were placed in a Sonicator machine for 10minutes using pulsed, high frequency sound waves to agitate, and lyse the cells. The extract concentrations were then serially diluted to 300, 250, 200, 150, and 100 µg extracts/mL of 1% DMSO. In a 96-well microtiter plate, each dilution was transferred using micropipette. Bacterial suspension was prepared and matched the standard of 0.5 McFarland. 150 µl bacterial suspensions were added in each well of a microtiter plate (96 well) from the column 1 to 12. Positive growth controls included both bacterial suspension and growth medium, whereas negative growth controls solely included growth media. Then the plates were incubated at 37°C. Using a POLARstar Omega Plate from BMG LABTECH, Offenburg, Germany, the optical density of each well was measured spectrophotometrically at 600 nm. The lowest concentration of each test solution that prevented the development of any of the microorganisms in the wells was recorded as the MIC value following an

overnight incubation. For each sample, the tests were carried out in triplicate, and the average concentration for each triplicate was determined.

3.19 Statistical Analysis

The growth curve, pigments, proximate composition, fatty acids, amino acids, antibacterial activity, and MIC content were statistically analyzed using IBM SPSS (v. 26.0). Each of the samples underwent descriptive statistics for each of the parameters, and a test for homogeneity of variance followed. One-way analysis of variance (ANOVA) was used to examine all the data that had been gathered, and Tukey's multiple comparison tests were used to determine whether there were any significant differences between the microalgal species at the 95% confidence level. Two-way analysis of variance (ANOVA) was also used for the analysis of antimicrobial activity test. Post-hoc test was utilized to discern differences between groups. The post-hoc test was used to identify group differences.

CHAPTER 4: RESULTS

4.1 Water Quality Parameters of Different Sampling Sites

The physical and chemical parameters of the water collected from various sampling sites are displayed in Table 4.1, where variances between the physical and chemical characteristics of the water were recorded.

Table 4.1: Physicochemical parameters of the sample water gathered from several freshwater and marine water sampling sites in Bangladesh:

Parameters	Halda river	Kaptai lake	Naf estuary	Sonadia island	Maheshkhali island
Temperature (˚C)	30℃	31℃	33℃	29.5℃	31℃
DO (mg/L)	7.5	7.3	7.5	7.3	7.4
pH	8.1	8.3	8.5	8.1	7.5
Salinity (ppt)	0	0	15	25	30
Total Ammonia nitrogen (TAN) (mg/L)	0.004	0.007	0.003	0.004	0.002
Soluble reactive phosphate (mg/L)	0.046	0.037	0.3365	0.005	0.022
Nitrite-Nitrogen (mg/L)	0.023	0.052	0.085	0.037	0.086

4.2 Isolated Microalgae from Different Sampling Sites:

Isolated microalgae from the freshwater and marine water sites were shown in Table 4.2. *Oscillatoria* species under this study were obtained from Naf estuary (Strain 1 M1 (20)), Maheshkhali island (Strain 2 M2 (25)), Kaptai lake (Strain 3 Fw1) and Halda river (Strain 4 Fw2).

Table 4.2: Isolated marine and freshwater microalgae from different sampling sites in Bangladesh

Halda river	Kaptai lake	Naf Estuary	Sonadia	Maheshkhali
Auxenochlorella sp.	*Coenochloris* sp.	*Gonyostomum* sp.	*Navicula* sp.	*Navicula* sp.
Chlorobotrys sp.	*Desmodesmus* sp.	*Pandorina* sp.	*Chlorella* sp.	*Oscillatoria* sp.
Tetraspora sp.	*Coelestrella* sp.	*Euglena* sp.	*Aphanocece* sp.	
Choricystis sp.	*Coccomyxa* sp.	*Chlamydomonas* sp.	*Nannochloropsis* sp.	
Scenedesmus sp.	*Choricystis* sp.	*Chlorella* sp.		
Coccomyxa sp.	*Chromochloris* sp.	*Nannochloropsis* sp.		
Carteria sp.	*Klebsormidium* sp.	*Trachelomonas* sp.		
Chlorella sp.	*Scenedesmus* sp.	*Oscillatoria* sp.		
Kirchneriella sp.	*Chlorella* sp.			
Trachelomonas sp.	*Kirchneriella* sp.			
Tetraedron sp.	*Oscillatoria* sp.			
Eudorina sp.				
Chlamydomonas sp.				
Westella sp.				
Oscillatoria sp.				

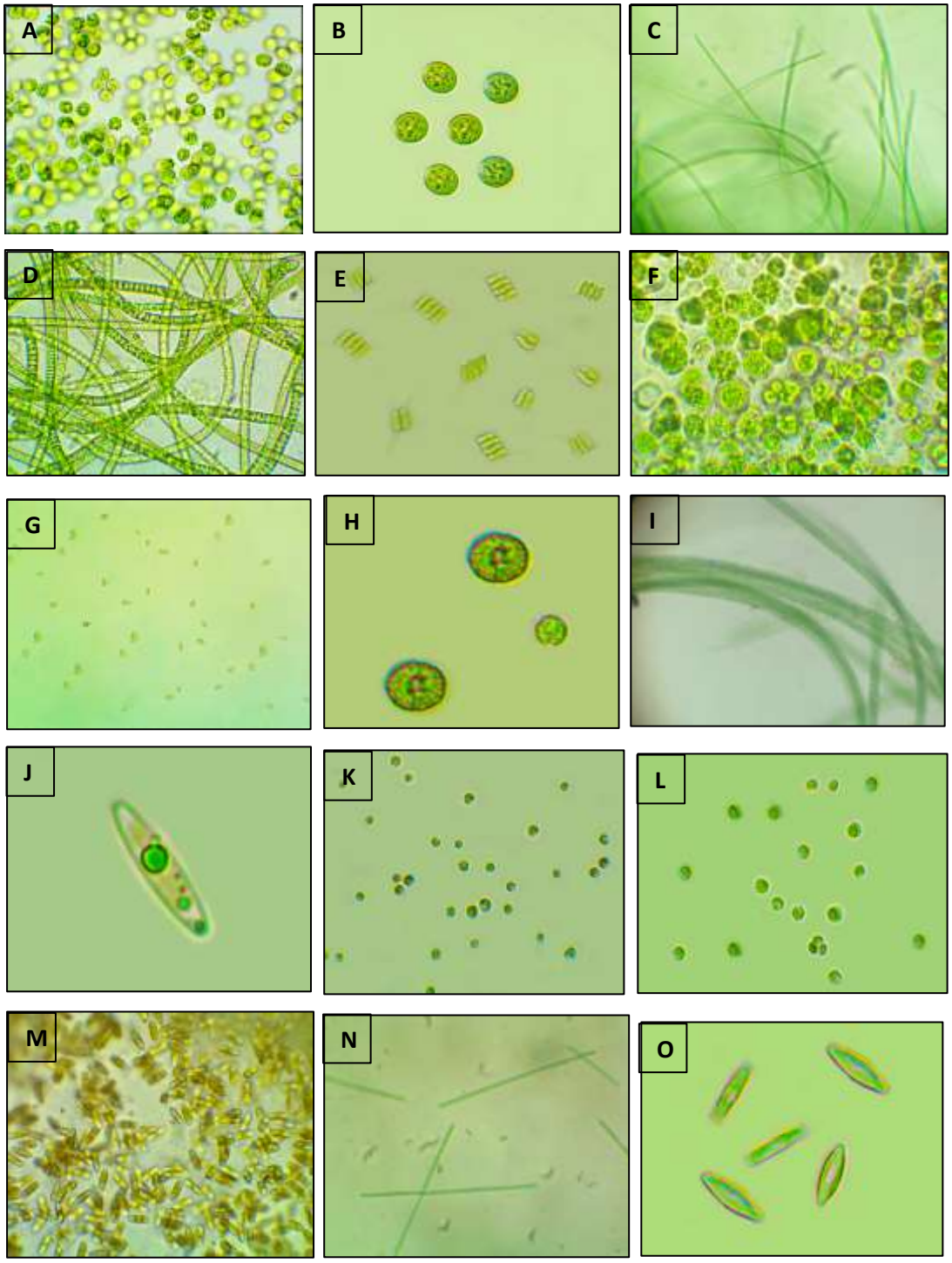


Figure 4.1: Isolated microalgae species from Halda river (A- *Chlorobotrys* sp., B- *Carteria* sp. C- *Oscillatoria* sp.), Kaptai lake (D- *Klebsormidium* sp., E- *Desmodesmus* sp., F- *Chromochloris* sp.), Naf estuary (G- *Gonyostomum* sp., H- *Chlamydomonas* sp., I- *Oscillatoria* sp.), Sonadia (J- *Navicula* sp., K- *Nannochloropsis* sp., L- *Chlorella* sp.) and Maheshkhali island (M- *Navicula* sp., N- *Oscillatoria* sp., O- *Navicula* sp.)

4.3 Characterization of *Oscillatoria* sp.

Four species of *Oscillatoria* were isolated in this study (Table 4.2). Morphological characteristics of Strain 1 M1 (20) (Figure 4.2, A -B), Strain 2 M2 (25) (Figure 4.2, C -D), Strain 3 Fw1(Figure 4.2, E -F) and Strain 4 (Figure 4.2, G -H) were depicted in Table 4.3.

Table 4.3: Morphological properties of *Oscillatoria* strains isolated from different sampling sites in Bangladesh

Microalgae species	Characteristics
Oscillatoria **Strain 1 M1 (20)**	➢ Planktonic, cylindrical, thread like structures which appeared in blue-green color. ➢ Species consists of a series of cells forming unbranched filaments or trichomes. ➢ Constricted at cross walls ➢ Cells are 5-7 μm long and 52 μm wide
Oscillatoria **Strain 2 M2 (25)**	➢ Filamentous, barrel-shaped straight structures with slight bent edges. ➢ Thallus are long and comprises dark blue-green color to blackish blue-green color. ➢ Cells are solitary or in clusters and constricted at cross walls. ➢ Cells are 6-8 μm long and 50 μm wide
Oscillatoria **Strain 3 Fw1**	➢ It contains Clusters like filaments and cells are cylindrical with tapering at the outer edges. ➢ Thallus are short and coiled and trichomes appeared as bright blue-green color. ➢ Trichomes are straight or slightly coiled, motile ➢ Cells are 3-4 μm in length and 47 μm in width
Oscillatoria **Strain 4 Fw2**	➢ Filamentous, thallus are long and visible in dark blue-green color. ➢ Trichomes are straight to slightly curved with bent outer edges ➢ Trichromes are rarely solitary and motile in nature ➢ Cells are 6-8 μm in length and 51 μm width

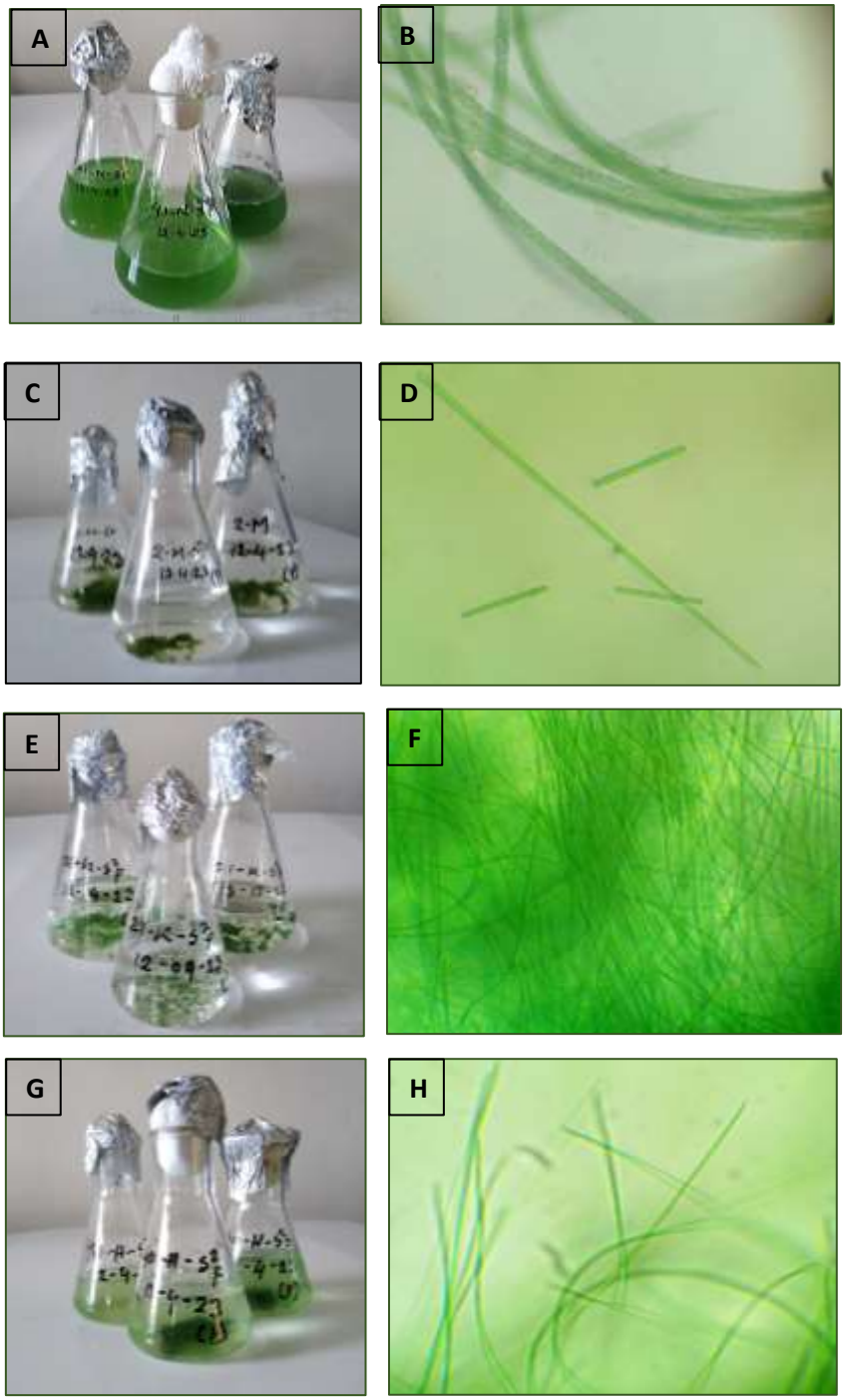
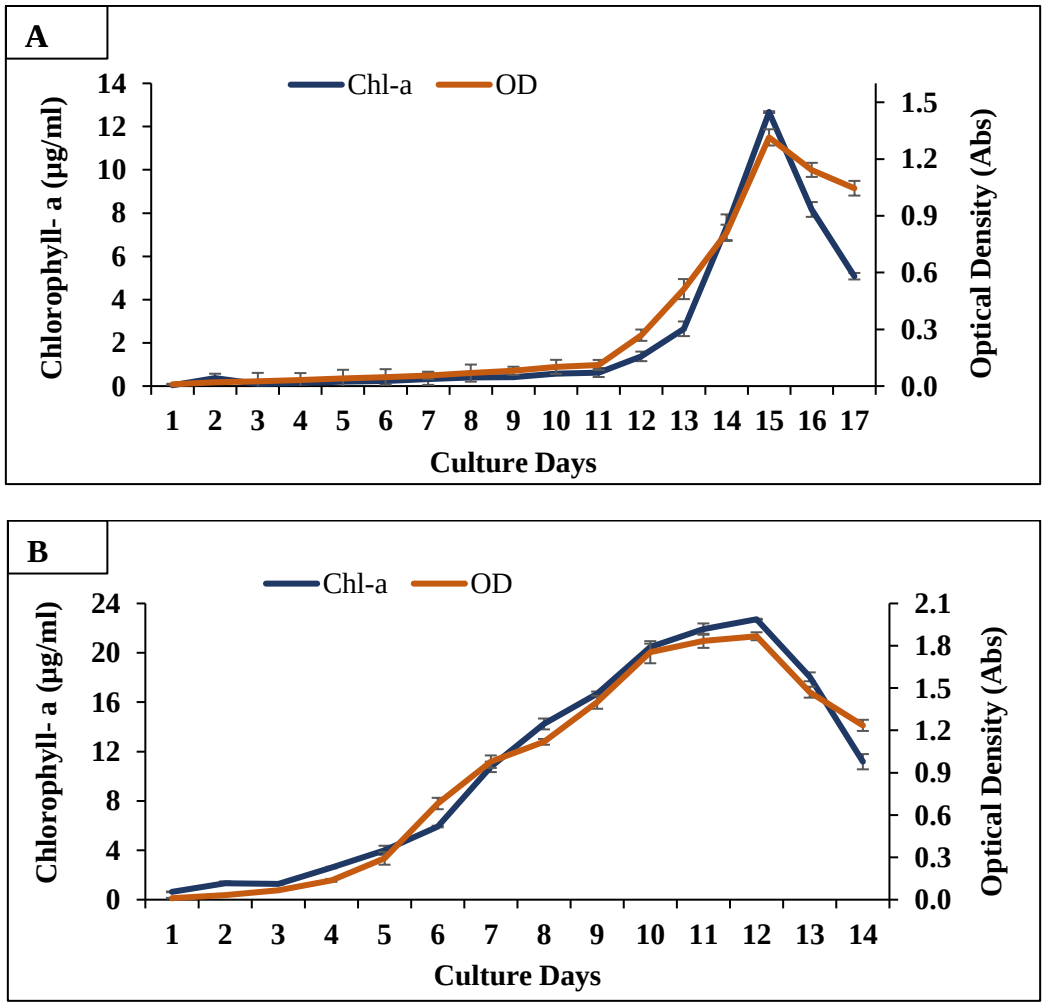


Figure 4.2 Colony structure and light microscopic pictures of isolated *Oscillatoria*, Strain 1 M1 (20) (A-Colony structure, B- microscopic view); Strain 2 M2 (25) (C-Colony structure, D- microscopic view); Strain 3 Fw1 (E-Colony structure, F-microscopic view); Strain 4 Fw2 (G-Colony structure, H- microscopic view)

4.4 Growth Curve Determination of *Oscillatoria* sp.

Differential growth phases of each *Oscillatoria* sp. were observed in this study. Growth phases were determined through chlorophyll-a content and optical density of the respective species. Figure 4.3 illustrated the chlorophyll content and optical density of each of the four species as a function of cultivation time. Strain 4 Fw2 had attained stationary phase on 9^{th} -10^{th} days followed by Strain 2 M2 (25) which remained for two days (on 10^{th} to 11^{th} day). Strain 3 Fw1 attains maturity on 10^{th} day which stayed for 11^{th} and 12^{th} day while Strain 1 M1 (20) had the stationary phase on 13^{th} and 14^{th} day. During their stationary phases, Strain 2 M2 (25) displayed significantly higher ($p < 0.05$) chlorophyll-a (22.72±0.04 µg/mL) and OD value (1.87±0.03) in the stationary phase (9^{th} to 11^{th} day) than other species. Significantly lower ($p < 0.05$) chlorophyll-a (12.67±0.04 µg/mL) and OD value (0.00±0.00) was observed in Strain 1 M1 (20) and Strain 3 Fw1 respectively.



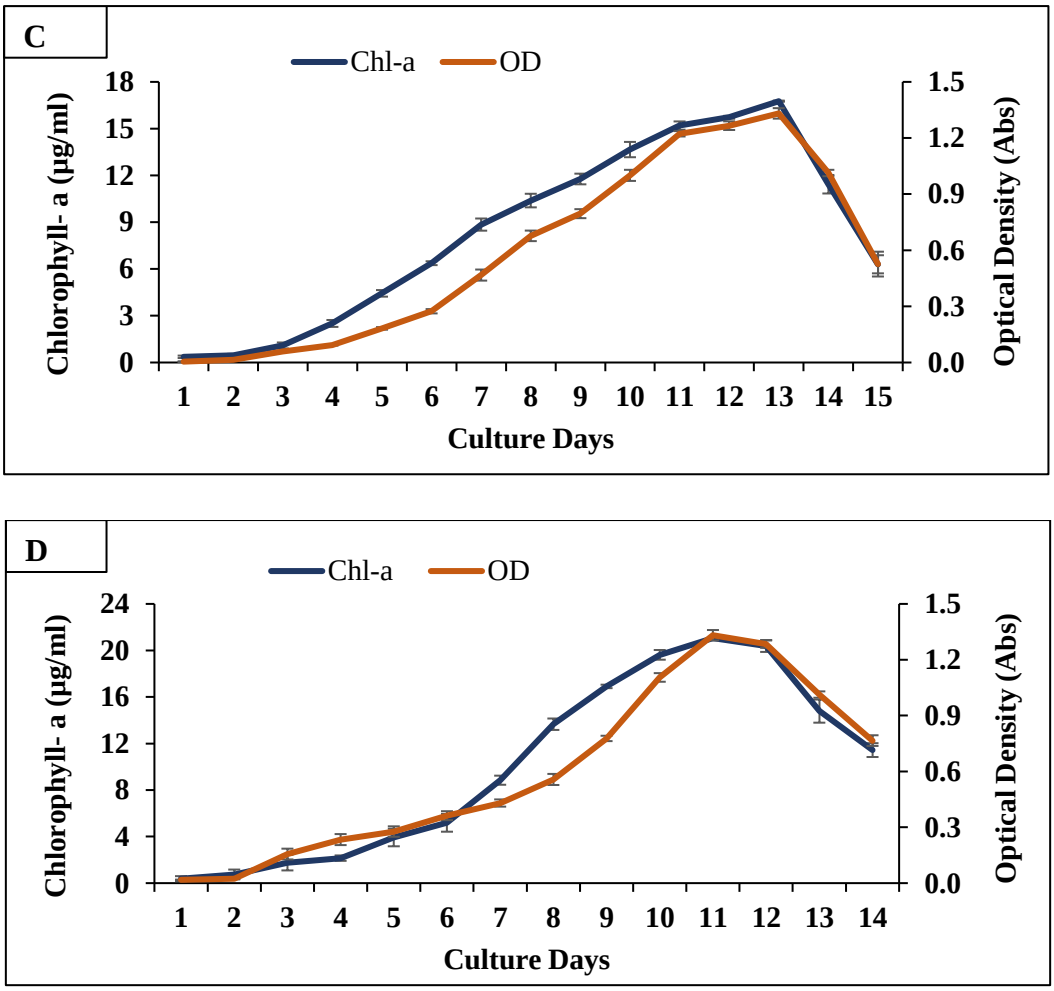


Figure 4.3: Growth curves of isolated *Oscillatoria* Strain 1 M1 (20) (A), Strain 2 M2 (25) (B), Strain 3 Fw1 (C) and Strain 4 Fw2 (D) in terms of chlorophyll-a content (µg/mL) and optical density (Absorbance) Values are means of the triplicates with standard error. Chl-a and OD represent chlorophyll-a content, and optical density respectively.

4.5 Dried Biomass of *Oscillatoria* sp.

Harvested and dried *Oscillatoria* sp. were weighed and stored. Dried powders of Strain 1 M1 (20) and Strain 2 M2 (25) were obtained from cultures of 90 L and 60 L respectively, and weighed around 9.42 g and 16.92 g each. For Strain 3 Fw1 and Strain 4 Fw2, 75 L of culture yielded 17 g and 19.75 g of dried powders respectively (Figure 4.4).

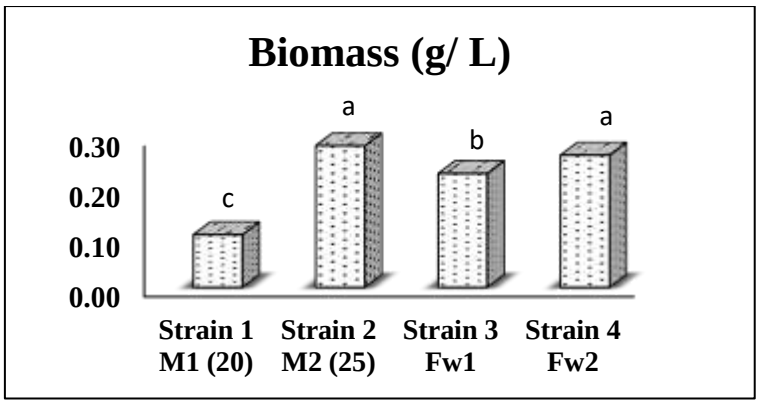


Figure 4.4: Dried biomass (g/L) of isolated marine and freshwater *Oscillatoria* strains. Values are average of the triplicates. Significant variations among the species ($p < 0.05$) are denoted by values in each series with a distinct letter.

4.6 Pigment Content of *Oscillatoria* sp.

Pigment contents such as chlorophyll, carotenoid, and phycobiliproteins were evaluated in this study. The amounts of chlorophyll-a, and carotenoid were measured in µg/mL, while the phycobiliprotein contents were measured in mg/g.

4.6.1 Chlorophyll Content

Oscillatoria exhibit significant chlorophyll content variation in Figure 4.5. Significantly highest ($p < 0.05$) chlorophyll-a content (22.72±0.04 µg/mL) was found in Strain 2 M2 (25), whereas Strain 1 M1 (20) showed significantly minimum ($p < 0.05$) amount of chlorophyll-a content (12.67±0.04 µg/mL).

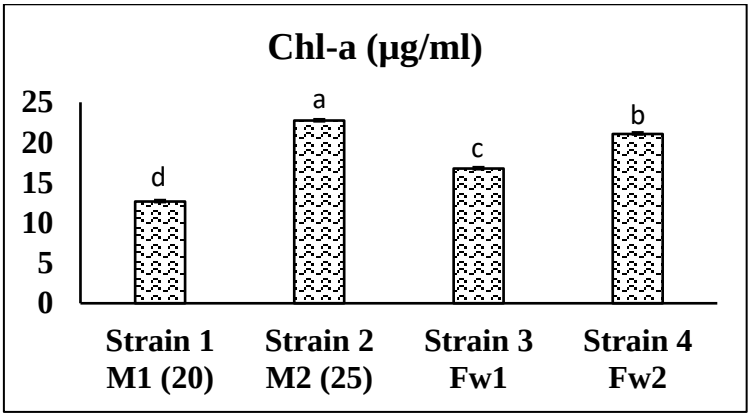


Figure 4.5: Chlorophyll-a content (means ± SE) of isolated marine (M1, M2) and freshwater (Fw1, Fw2) *Oscillatoria* strains. Significant variations among the species ($p < 0.05$) are denoted by values in each series with a distinct letter.

4.6.2 Phycobiliproteins

Phycocyanin, allophycocyanin, phycoerythrin and total phycobiliproteins of *Oscillatoria* sp. were recorded. Significantly ($p < 0.05$) highest amount of phycocyanin, phycoerythrin and total phycobiliproteins were obtained from Strain 4 Fw2 of about 93.47±0.08 mg/g, 11.82±0.03 mg/g and 121.42±0.061 mg/g respectively and allophycocyanin was obtained highest in Strain 2 M2 (25) about 18.19±0.06 mg/g (Table 4.4). Significantly ($p < 0.05$) lowest quantity of phycocyanin and total phycobiliproteins were found in Strain 3 Fw1 about 69.00±0.12 mg/g and 87.39±0.12 mg/g respectively. Strain 1 M1 (20) also exhibit significant ($p < 0.05$) lowest amount of allophycocyanin phycoerythrin of 10.78±1.00 mg/g and 0.36±0.09 mg/g consecutively (Figure 4.5).

Table 4.4: Phycocyanin (mg/g) (mean ± SE), allophycocyanin(mg/g) (mean ± SE), phycoerythrin (mg/g) (mean ± SE) content of *Oscillatoria* sp. Significant variations among the species ($p < 0.05$) are indicated by values in each series with a distinct letter.

***Oscillatoria* Species**	**Phycocyanin**	**Allophycocyanin**	**Phycoerythrin**
		(mg/g)	
Strain 1 M1 (20)	81.85 ± 0.84^{c}	10.78 ± 1.00^{b}	0.36 ± 0.14^{d}
Strain 2 M2 (25)	84.75 ± 0.12^{b}	18.19 ± 0.06^{a}	8.10 ± 0.15^{b}
Strain 3 Fw1	69.00 ± 0.12^{d}	17.00 ± 0.12^{a}	1.40 ± 0.21^{c}
Strain 4 Fw2	93.47 ± 0.08^{a}	16.13 ± 0.12^{a}	11.82 ± 0.14^{a}

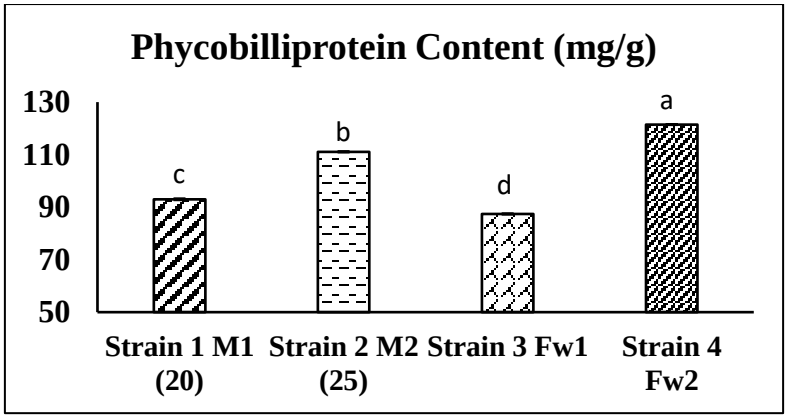


Figure 4.6: Total phycobiliprotein content (mg/g) (mean ± SE) of *Oscillatoria* sp. Values with the different letters within each series indicate significant differences ($p < 0.05$) among the species.

4.6.3 Purification Factor

The purification factor of Phycocyanin, phycoerythrin and allophycocyanin raw extract of *Oscillatoria* sp. is presented in Figure 4.7 where highest and lowest significant ($p < 0.05$) phycocyanin, phycoerythrin and allophycocyanin obtained from Strain 4 Fw2(1.00, 0.48, 0.34) and Strain 3 Fw1 (0.68, 0.28, 0.26) respectively. There is no significant ($p > 0.05$) variations in the purity of allophycocyanin raw extracts ($p = 0.188$) among the species.

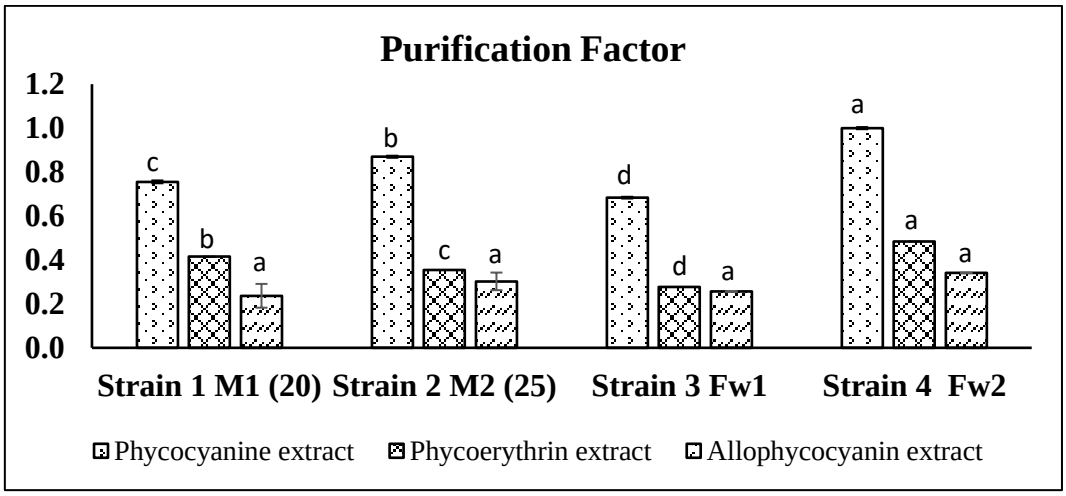


Figure 4.7: Purity of phycocyanin, phycoerythrin and allophycocyanin raw extracts of *Oscillatoria* species. Significant variations among the species ($p < 0.05$) are indicated by values in each series with a distinct letter.

4.7 Proximate Composition

The variations of the biochemical composition of *Oscillatoria* sp. is depicted in Figure 4.8 where the percentages of crude protein, lipid and carbohydrate content is measured from the dried biomass. Crude protein contents varied from 21.56±0.09 to 56.97±0.03 and found significantly ($p < 0.05$) highest in Strain 4 Fw2 and lowest in Strain 3 Fw1. Crude lipid (%) ranged from 9.07±0.07 to 17.13±0.13 where the significant ($p < 0.05$) maximum is observed in Strain 4 Fw2 and lowest in Strain 3 Fw1. Crude carbohydrate content (%) showed the range from 7.49±0.15 to 17.04±0.08 with the significant ($p < 0.05$) highest and lowest value in Strain 4 Fw2 and Strain 2 M2 (25) respectively.

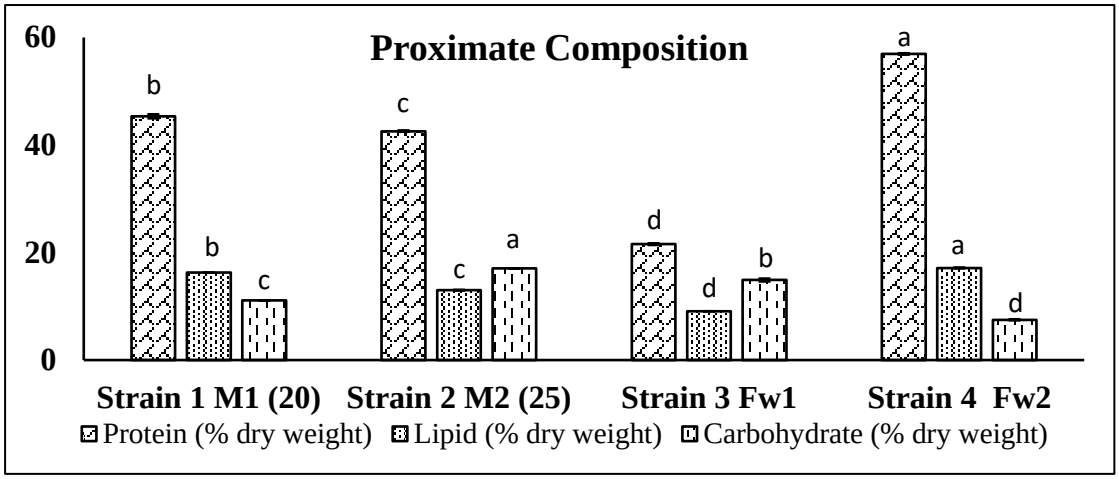


Figure 4.8: Protein content (% dry weight) (mean ± SE), lipid content (% dry weight) (mean ± SE) and carbohydrate content (% dry weight) (mean ± SE) of *Oscillatoria* sp. Significant variations among the species ($p < 0.05$) are indicated by values in each series with a distinct letter.

4.8 Fatty Acids Analysis

Variations of the fatty acids along with the total saturated and unsaturated fatty acids (mean± SE) are demonstrated in Table 4.5. Saturated fatty acids were found significantly ($p < 0.05$) highest in Strain 1 M1 (20) followed by Strain 4 Fw2, Strain 2 M2 (25), Strain 3 Fw1. Polyunsaturated fatty acids as well as n6-PUFA were found significantly ($p < 0.05$) highest in Strain 4 Fw2 followed by Strain 2 M2 (25), Strain 3 Fw1 and Strain 1 M1 (20). On the contrary, n3-PUFA had significant ($p < 0.05$) maximum in case of Strain 3 Fw1 followed by Strain 1 M1 (20), Strain 4 Fw2, Strain 2 M2 (25).

Table 4.5. Fatty acids content (% fatty acids) of different *Oscillatoria* species expressed as mean of the duplicates with standard error (mean± SE).

Carbon	Fatty Acid Methyl Esters	*Oscillatoria* Strain-1 M1 (20 ppt)	*Oscillatoria* Strain-2 M2 (25 ppt)	*Oscillatoria* Strain-3 Fw1	*Oscillatoria* Strain-4 Fw2
		Conc (ppm)			
C8:0	Methyl Octanoate	0.27±0.26	0.05±0.00	0.03±0.00	0.02±0.00
C10:0	Methyl Decanoate	0.46±0.01	4.48±0.20	1.16±1.15	3.57±0.02
C12:0	Methyl Laurate	3.30±0.06	0.04±0.00	2.02±0.00	0.23±0.00
C13:0	Methyl Tridecanoate	0.27±0.01	2.30±0.09	1.89±0.03	5.31±0.00
C14:0	Methyl Myristate	0.06±0.00	3.47±0.18	2.74±0.22	4.18±1.49
C16:0	Methyl Palmitate	14.32±0.68	9.33±0.63	12.33±0.04	9.37±0.14
C18:0	Methyl Stearate	13.85±0.70	9.14±0.03	8.53±0.10	8.59±1.04
C20:0	Methyl Arachidate	1.36±0.09	4.84±0.02	3.49±0.36	0.59±0.00
C17:0	Methyl Heptadecanoate	0.97±0.00	0.04±0.00	1.92±0.08	1.55±0.04
C21:0	Methyl Heneicosanoate	0.22±0.02	0.57±0.09	0.82±0.58	0.27±0.02
C22:0	Methyl Behenate	3.91±0.05	0.51±0.04	1.76±0.06	0.89±0.01
C23:0	Methyl Tricosanoate	ND	ND	ND	ND
C24:0	Methyl Lignocerate	ND	ND	ND	ND
	ΣSAFA	**39.00±1.18^{a}**	**35.77±1.17^{c}**	**36.42±0.58^{b}**	**34.55±0.52^{d}**
C16:1	Methyl Palmitoleate	6.43±0.03	11.17±0.09	12.24±0.51	10.97±0.05
C18:1	Methyl Oleate	48.16±0.01	42.30±0.06	46.53±0.00	46.72±0.01
C20:1	Methyl cis-11-eicosenoate	0.01±0.01	0.04±0.02	0.17±0.01	0.03±0.01
C22:1	Methyl Erucate	0.13±0.02	4.47±0.18	0.00±0.00	0.35±0.02
C24:1	Methyl Nervonate	0.00±0.00	0.21±0.11	0.09±0.04	0.05±0.01
	ΣMUFA	**54.73±0.01^{c}**	**58.19±0.29^{b}**	**59.03±0.47^{a}**	**58.12±0.08^{b}**
C18:2n-6	Methyl Linoleate	0.77±0.05	3.25±1.1	1.16±0.06	3.01±1.13
C20:3n-6	Methyl 11-14-17-Eicosatrienoate	0.44±0.03	0.15±0.06	1.22±0.04	0.22±0.09
C20:4n-6	Methyl Arachidonate	0.04±0.01	0.05±0.03	0.47±0.00	0.10±0.03
	Σn6-PUFA	**1.25±0.07^{c}**	**3.45±1.12^{a}**	**2.85±0.02^{b}**	**3.33±1.07^{a}**
C18:3n-3	Methyl Linolenate	0.89±0.03	0.25±0.00	1.22±0.41	1.23±0.00
C20:5n-3	**Methyl icosa-5,8,11, 14,17-pentaenoate**	**2.58±0.05^{a}**	**1.13±0.00^{b}**	**0.02±0.00^{b}**	**2.60±0.05^{a}**
C22:5n-3	Methyl Docosapentaenoate	1.32±1.32	0.80±0.00	0.01±0.00	0.00±0.00
C22:6n-3	**Methyl Docosahexanoate**	**0.17±0.13^{a}**	**0.14±0.00^{a}**	**0.16±0.01^{a}**	**0.15±0.03^{a}**
	Σn3-PUFA	**4.96±1.37^{a}**	**2.32±0.00^{b}**	**1.41±0.40^{c}**	**3.98±0.02^{ab}**
	ΣPUFA	**6.21±1.30^{b}**	**5.77±1.12^{c}**	**4.26±0.42^{d}**	**7.31±1.05^{a}**
	Σn3/ Σn6	**4.05±1.32^{a}**	**0.00±0.00^{b}**	**1.53±0.08^{ab}**	**0.02±0.00^{b}**
	DHA/EPA	**0.07±0.05^{c}**	**5.23±0.04^{b}**	**9.03±3.96^{a}**	**0.06±0.01^{c}**
	SAFA/TUFA	**3.05±0.40^{a}**	**0.11±0.00^{b}**	**0.62±0.04^{b}**	**0.14±0.00^{b}**
	SAFA/TFA	**0.75±0.02^{a}**	**0.10±0.00^{c}**	**0.38±0.02^{b}**	**0.12±0.00^{c}**
	TUFA/TFA	**0.25±0.02^{c}**	**0.90±0.00^{a}**	**0.62±0.02^{b}**	**0.88±0.00^{a}**

Here, Values with the different letters within each series indicate significant differences ($p < 0.05$) among the species. **SAFA** means Saturated Fatty Acids, **MUFA**= Monounsaturated fatty acids, **n6-PUFA**= ω-6 polyunsaturated fatty acids, **n3-PUFA**= ω-3 polyunsaturated fatty acids, **DHA**= Docosahexaenoic acid, **EPA=** Eicosapentaenoic acid, **TUFA**= Total unsaturated fatty acids, **TFA**= Total fatty acids.

4.9 Amino Acid Analysis:

Amino acid contents (% amino acid) are presented in the Table 4.6. While non-essential amino acids are more abundant in Strain 2 M2 (25), Strain 4 Fw2, Strain 1 M2 (20), and Strain 3 Fw1 respectively, essential amino acids are more prevalent in Strain 3 Fw1, followed by Strain 1 M2 (20), Strain 4 Fw2, and Strain 2 M2 (25).

Table 4.6 Amino acid content (% amino acids) of *Oscillatoria* sp. Here, **EAA**= Essential Amino Acid, NEAA: Non-Essential Amino Acid

Compound Name (570 nm)	**Types**	**Strain 1 M1 (20)**	**Strain 2 M2 (25)**	**Strain 3 Fw1**	**Strain 4 Fw2**
		Amount (%)			
Histidine	EAA	4.19	0	4.72	3.26
Isoleucine	EAA	2.83	3.02	2.05	2.8
Leucine	EAA	7.98	7.73	7.52	7.58
Lysine	EAA	4.54	4.23	6.05	3.64
Methionine	EAA	1.85	2.25	2.17	1.95
Phenylalanine	EAA	3.37	4.01	3.88	3.6
Threonine	EAA	5.4	5.8	5.23	5.95
Tyrosine	EAA	3.76	4.1	3.64	4.05
Valine	EAA	3.68	4.22	3.65	3.84
	ΣEAA	**37.6**	**35.36**	**38.91**	**36.67**
Alanine	NEAA	12.65	9.91	12.5	12.99
Arginine	NEAA	7.07	6.01	6.22	7.09
Aspartic acid	NEAA	12.12	13.36	11.64	13.23
Glutamic acid	NEAA	15.06	17.98	13.91	13.75
Glycine	NEAA	5.71	6.99	6.44	6.28
Cysteine	NEAA	0.07	1.72	0.21	0.12
Serine	NEAA	5.99	5.93	5.82	6.31
Proline	NEAA	3.74	2.75	4.35	3.58
	ΣNEAA	**62.41**	**64.65**	**61.09**	**63.35**
	ΣAA/ΣEAA	5.32	5.66	5.14	5.46
	ΣEAA/ΣNEAA	0.60	0.55	0.64	0.58

4.10 Isolation of Bacteria

Bacteria were isolated from the diseased marine fish and shellfish (Shrimp, Crab) (Figure 4.9). Each bacterium was cultured in selective agar and stored. Table 4.7 represents the isolated bacteria with its source, growth media and nature.

Table 4.7: Isolated pathogenic bacteria from different diseased fish and Shellfish

Sl. no	**Bacteria name**	**Source**	**Growth**	**Gram Staining**
1	***Escherichia coli***	Tilapia (*Oreochromis mossambicus*)	Pink colonies on MacConkey agar	Gram-negative rod
2	***Salmonella* sp.**	Crab (*Scylla serrata*)	Opaque colonies on SS agar	Gram-negative rod
3	***Shigella* sp.**	Tuna (*Euthynnus affinis*)	Black colonies on SS agar	Gram-negative rod
4	***Pseudomonas* sp. 1**	Gangetic Hairfin Anchovy (*Setipinna phasa*)	Opaque colonies on Pseudomonas agar	Gram-negative rod
5	***Pseudomonas* sp. 2**	Rita (*Rita rita*)	Greenish-blue colonies on Pseudomonas agar	Gram-negative rod
6	***Vibrio* sp. 1**	Shrimp (*Penaeus monodon*)	Green colonies on the TCBS agar	Gram-negative curved rod
7	***Vibrio* sp. 2**	Tuna (*Euthynnus affinis*)	Greenish colonies on TCBS agar	Gram-negative curved rod
8	***Vibrio* sp. 3**	Shrimp (*Penaeus monodon*)	Yellow colonies on TCBS agar	Gram-negative curved rod
9	***Aeromonas hydrophila***	Poa (*Otolithoides pama*)	Green colonies on TCBS agar	Gram-negative rod
10	***Streptococcus* sp.**	Tilapia (*Oreochromis mossambicus*)	Opaque colonies on Nutrient agar	Gram-positive cocci
11	***Staphylococcus saprophyticus***	Poa (*Otolithoides pama*)	Yellow colonies on Mannitol Salt agar	Gram-positive clusters
12	***Staphylococcus* sp. 2**	Crab (*Scylla serrata*)	Pink colonies on Mannitol Salt agar	Gram-positive clusters

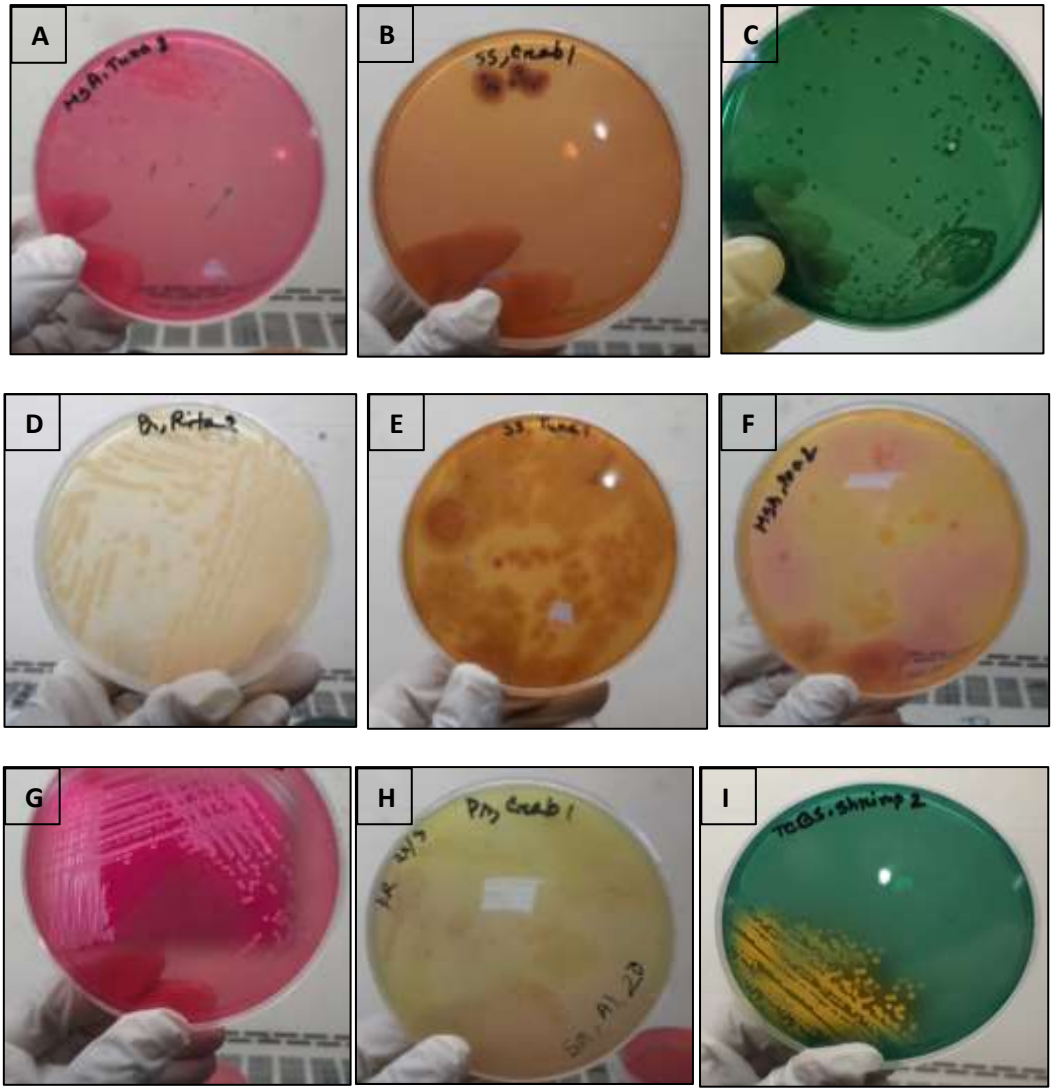


Figure 4.9: Bacterial growth on different selective culture media (A) *Staphylococcus* sp. colonies in Mannitol salt agar, (B) *Salmonella* sp. colonies in Salmonella-Shigella agar (C) *Vibrio* sp. colonies in TCBS agar (D) *Pseudomonas* sp. colonies in Pseudomonas agar, (E) *Shigella* sp. colonies in Salmonella-shigella agar (F) *Staphylococcus* sp. colonies in TCBS agar (G) *E. coli* sp. colonies in MacConkey agar, (H) *Pseudomonas* sp. colonies in Pseudomonas agar (I) *Vibrio* sp. colonies in TCBS agar

4.11 Results and Analysis of Bacteria Identified by VITEK 2

If a certain distinguishing pattern is found, the VITEK 2 analyzes the performance, and the results indicate that a single animal is likely to suit the description. The device will suggest additional tests to distinguish between two or three species that are very similar to one another if a potential pattern is unknown, or it will report the results as an unidentified organism (>3 species may demonstrate the pattern detected or the bio pattern is quite atypical and not shown in the database).

Through this mechanism, *Escherichia coli*, *Aeromonas hydrophila* and *Staphylococcus saprophyticus* were identified with 99%, 99% and 95% probability. Other bacteria are identified through morphology, gram staining and biochemical tests.

4.12 Biochemical Test of *Vibrio* sp.

Selective agar media (TCBS) was used for the growth and identification of *Vibrio* sp. Other than the selective media, biochemical tests like motility tests, TSI and catalase test, were executed to identify the genus level of the isolated bacteria (Table 4.8).

Table 4.8: Identification of *Vibrio* sp. through biochemical tests:

Biochemical test	**Properties of test**	**Outcome**	**Existence of *Vibrio* sp.**
TCBS agar	On TCBS agar, *Vibrio* appears in green, greenish or yellow colonies	Uniform green, greenish or yellow color colonies are observed in the sample	Yes
TSI test	Red slant or yellow slant in *Vibrio*	Red slant produced H_2S gas and Yellow slant may not produce H_2S gas	Yes
Catalase test	If bubbles appear the bacteria are catalase positive. If no bubbles appear, the bacteria are catalase negative.	Bubbles appeared and the isolated bacteria are catalase positive	Yes
Motility test	*Vibrio* are Motile bacteria. Positive results suggest hazy dispersed, growths that make it opaque slightly.	Slightly opaque due to hazy, diffused growth that spread across it.	Yes

4.13 Antimicrobial Activity Test

The phenolic extracts of *Oscillatoria* species were evaluated against isolated bacteria in this study. All extracts shown greater zones of inhibition. Colistin (10 µg/disc) used against gram-negative bacteria, and ceftriaxone (30 µg/disc) used as gram-positive antibiotic that exhibit clear zones when applied to bacteria. Blank disc served as the negative control and antibiotic disc served as the positive control. Both of these controls had no inhibition zone against the full test bacterial population. Comparatively, higher inhibition zone (mm) was obtained from Strain 2 M2 (25) (24.59±0.21 to 34.05±0.13) and Strain 4 Fw2 (26.80±0.21 to 33.94±0.15) whereas lower inhibition zone (mm) was recorded from Strain 3 Fw1 (21.52±0.19 to 30.20±0.18) and Strain 1 M1 (20) (17.11±0.15 to 26.27±0.15). Significant variations of *Oscillatoria* strains are demonstrated in Table 4.9 and Figure 4.10.

Table 4.9: Diameter of inhibition zone (mm) exhibited by *Oscillatoria* strains against isolated pathogenic bacteria. Values with the different letters within each series indicate significant differences ($p < 0.05$) among the species.

Bacteria	Strain 1 M1 (20)	Strain 2 M2 (25)	Strain 3 Fw1	Strain 4 Fw2	Antibiotic
E. coli	22.86 ± 0.18^{cd}	30.47 ± 0.20^{b}	26.07 ± 0.24^{d}	27.69 ± 0.22^{cde}	17.22 ± 0.16^{bc}
***Salmonella* sp.**	22.42 ± 0.19^{d}	30.13 ± 0.11^{b}	27.58 ± 0.11^{c}	29.72 ± 0.20^{b}	17.31 ± 0.11^{b}
***Shigella* sp.**	24.85 ± 0.12^{b}	34.00 ± 0.17^{a}	30.20 ± 0.18^{a}	33.94 ± 0.15^{a}	16.62 ± 0.14^{cde}
***Pseudomonas* sp. 1**	22.79 ± 0.23^{cd}	28.75 ± 0.12^{c}	27.16 ± 0.23^{c}	27.78 ± 0.18^{cd}	15.92 ± 0.19^{f}
***Pseudomonas* sp. 2**	17.11 ± 0.15^{g}	33.96 ± 0.20^{a}	26.05 ± 0.10^{d}	27.87 ± 0.19^{cd}	17.07 ± 0.13^{bcd}
***Vibrio* sp. 1**	25.10 ± 0.27^{b}	33.26 ± 0.26^{a}	25.53 ± 0.21^{de}	28.59 ± 0.14^{c}	17.49 ± 0.04^{ab}
***Vibrio* sp. 2**	20.58 ± 0.19^{e}	30.12 ± 0.19^{b}	22.57 ± 0.24^{f}	30.06 ± 0.17^{b}	16.52 ± 0.15^{def}
***Vibrio* sp. 3**	18.05 ± 0.14^{f}	33.88 ± 0.12^{a}	21.90 ± 0.21^{fg}	26.80 ± 0.21^{e}	16.38 ± 0.06^{ef}
Aeromonas hydrophila	19.84 ± 0.14^{e}	29.91 ± 0.16^{b}	21.52 ± 0.19^{g}	27.23 ± 0.17^{de}	15.97 ± 0.11^{ef}
***Streptococcus* sp.**	26.27 ± 0.15^{a}	24.59 ± 0.21^{d}	24.76 ± 0.17^{e}	27.76 ± 0.24^{cd}	14.78 ± 0.18^{g}
***Staphylococcus* sp. 1**	20.60 ± 0.21^{e}	33.57 ± 0.07^{a}	25.98 ± 0.09^{d}	29.53 ± 0.18^{b}	16.03 ± 0.1^{ef}
***Staphylococcus* sp. 2**	23.44 ± 0.14^{c}	34.05 ± 0.13^{a}	28.83 ± 0.15^{b}	33.19 ± 0.20^{a}	18.08 ± 0.10^{a}

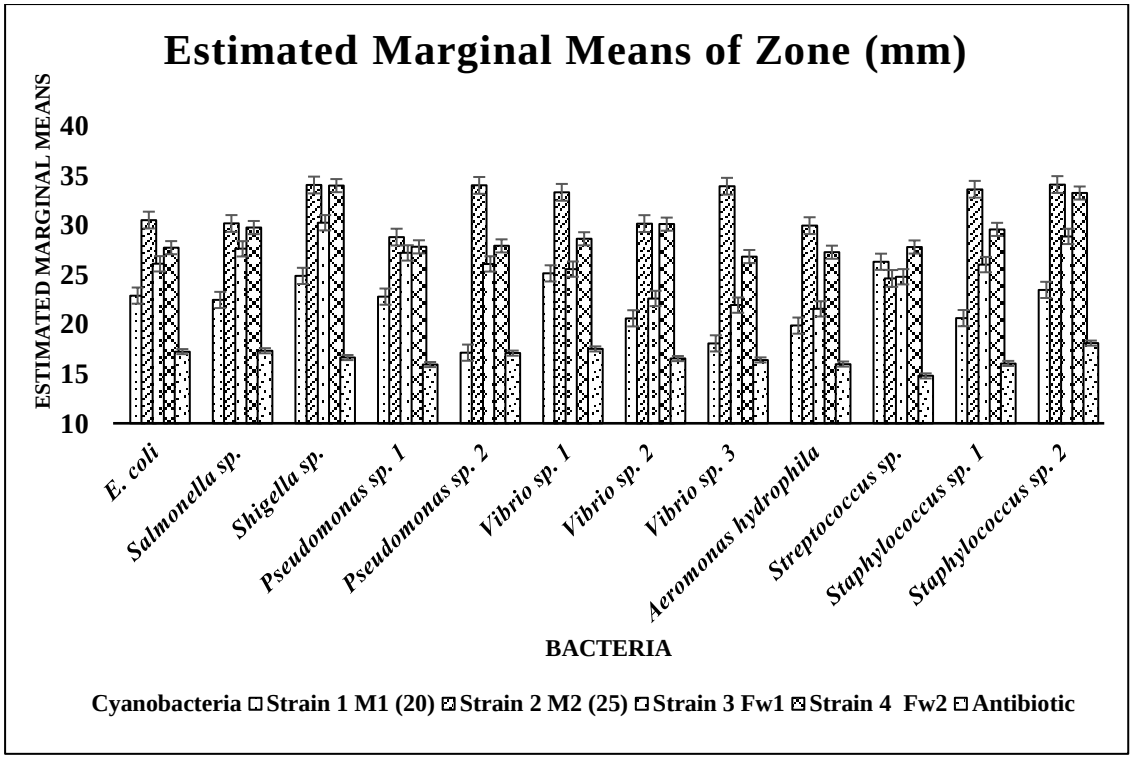


Figure 4.10: Summarized diameter of inhibition zone (mm) exhibited by *Oscillatoria* strains along with commercial antibiotic disc (Colistin and ceftriaxone) against isolated pathogenic bacteria.

4.14 Antimicrobial Activity Index

Activity index of this study shows significant ($p < 0.05$) variations among all the *Oscillatoria* strains (Table 4.10). Significantly ($p < 0.05$) higher activity index (AI) was reported from Strain 2 M2 (25) (2.10±0.03) against *Staphylococcus* sp. 1 and the lower AI value was obtained from Strain 1 M1 (20) (1.00±0.01) against *Pseudomonas* sp. 2. AI index of Strain 1 M1 (20), from 1.00±0.01 to 1.78±0.02, Strain 2 M2 (25) showed the AI of 1.67±0.03 to 2.10±0.03 range, The variations of the AI value of Strain 3 Fw1 and Strain 4 Fw2 also ranged from 1.34±0.02 to 1.82±0.02 and 1.61±0.02 to 2.04±0.02 respectively. The outcome of AI index also depicted that; gram positive bacteria showed more sensitivity against all the cyanobacterial extracts than gram negative bacteria.

Table 4.10: Antimicrobial index (AI) of *Oscillatoria* strains. against isolated pathogenic bacteria. Values with the distinct letters in each series indicate significant distinctions ($p < 0.05$) among the *Oscillatoria* species.

Bacteria	**Strain 1 M1 (20)**	**Strain 2 M2 (25)**	**Strain 3 Fw1**	**Strain 4 Fw2**
E. coli	1.33 ± 0.02^{c}	1.77 ± 0.02^{f}	1.51 ± 0.03^{de}	1.61 ± 0.02^{f}
***Salmonella* sp.**	1.30 ± 0.01^{cd}	1.74 ± 0.01^{fg}	1.59 ± 0.01^{cd}	1.72 ± 0.02^{de}
***Shigella* sp.**	1.50 ± 0.01^{b}	2.05 ± 0.02^{ab}	1.82 ± 0.02^{a}	2.04 ± 0.02^{a}
***Pseudomonas* sp. 1**	1.43 ± 0.02^{b}	1.81 ± 0.02^{ef}	1.71 ± 0.02^{b}	1.75 ± 0.02^{cd}
***Pseudomonas* sp. 2**	1.00 ± 0.01^{f}	1.99 ± 0.02^{bc}	1.53 ± 0.02^{de}	1.63 ± 0.02^{ef}
***Vibrio* sp. 1**	1.44 ± 0.02^{b}	1.90 ± 0.02^{cd}	1.46 ± 0.01^{e}	1.63 ± 0.01^{ef}
***Vibrio* sp. 2**	1.25 ± 0.02^{d}	1.82 ± 0.02^{ef}	1.37 ± 0.02^{f}	1.82 ± 0.02^{bc}
***Vibrio* sp. 3**	1.10 ± 0.01^{e}	2.07 ± 0.01^{ab}	1.34 ± 0.02^{f}	1.64 ± 0.01^{ef}
Aeromonas hydrophila	1.24 ± 0.01^{d}	1.87 ± 0.01^{de}	1.35 ± 0.01^{f}	1.71 ± 0.02^{def}
***Streptococcus* sp.**	1.78 ± 0.02^{a}	1.67 ± 0.03^{g}	1.68 ± 0.03^{bc}	1.88 ± 0.04^{b}
***Staphylococcus* sp. 1**	1.29 ± 0.02^{cd}	2.10 ± 0.03^{a}	1.62 ± 0.02^{bc}	1.84 ± 0.03^{b}
***Staphylococcus* sp. 2**	1.30 ± 0.01^{cd}	1.88 ± 0.01^{de}	1.59 ± 0.01^{cd}	1.84 ± 0.01^{bc}

4.15 Multiple Comparison

Multiple comparison was analyzed among selected *Oscillatoria* species through two-way ANOVA and it shows there are significant differences ($p < 0.05$) among the inhibition zone of *Oscillatoria* species. The comparison among the *Oscillatoria species* was significant ($p < 0.05$) in Tukey's test and presented in Table 4.11.

Table 4.11: Multiple comparison of *Oscillatoria* species in antimicrobial activity test

Dependent Variable: Inhibition Zone (mm), Tukey HSD			
(I) Cyanobacteria	**(J) Cyanobacteria**	**Mean Difference (I-J)**	**Significance**
Strain 1 M1 (20)	Strain 2 M2 (25)	-9.4008*	0.00
	Strain 3 Fw1	-3.6899*	0.00
	Strain 4 Fw2	-7.1881*	0.00
	Antibiotic	5.3747*	0.00
Strain 2 M2 (25)	Strain 1 M1 (20)	9.4008*	0.00
	Strain 3 Fw1	5.7110*	0.00
	Strain 4 Fw2	2.2128*	0.00
	Antibiotic	14.7756*	0.00
Strain 3 Fw1	Strain 1 M1 (20)	3.6899*	0.00
	Strain 2 M2 (25)	-5.7110*	0.00
	Strain 4 Fw2	-3.4982*	0.00
	Antibiotic	9.0646*	0.00
Strain 4 Fw2	Strain 1 M1 (20)	7.1881*	0.00
	Strain 2 M2 (25)	-2.2128*	0.00
	Strain 3 Fw1	3.4982*	0.00
	Antibiotic	12.5628*	0.00
Antibiotic	Strain 1 M1 (20)	-5.3747*	0.00
	Strain 2 M2 (25)	-14.7756*	0.00
	Strain 3 Fw1	-9.0646*	0.00
	Strain 4 Fw2	-12.5628*	0.00

Here, the sign (*) denotes the mean difference is significant at the 0.05 level ($p < 0.05$).

4.16 Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) value of each filamentous *Oscillatoria* Strain 2 M2 (25), Strain 3 Fw1, Strain 4 Fw2 was found to be 100 µg/ml against all the test bacteria (Table 4.9). On the contrary, planktonic Strain 1 M1 (20) requires MIC value of 100 µg/ml to be effective against all the test bacteria.

Table 4.12: Minimum Inhibitory Concentration (MIC) value of strains of *Oscillatoria*

MIC Value (µg/ml) of *Oscillatoria*				
Bacteria	**Strain 1 M1 (20)**	**Strain 2 M2 (25)**	**Strain 3 Fw1**	**Strain 4 Fw2**
E. coli	150	100	100	100
***Salmonella* sp.**	150	100	100	100
***Shigella* sp.**	150	100	100	100
***Pseudomonas* sp. 1**	150	100	100	100
***Pseudomonas* sp. 2**	150	100	100	100
***Vibrio* sp. 1**	150	100	100	100
***Vibrio* sp. 2**	150	100	100	100
***Vibrio* sp. 3**	150	100	100	100
Aeromonas hydrophila	150	100	100	100
***Streptococcus* sp.**	150	100	100	100
Staphylococcus saprophyticus	150	100	100	100
***Staphylococcus* sp. 2**	150	100	100	100

CHAPTER 6: DISCUSSION

6.1 Water Quality Parameters of the Sampling Sites

Microalgal growth is primarily driven by nutrients, CO_2 concentration, intensity and quality of light, pH, temperature salinity, and lastly aeration and mixing, which has a direct impact on all other parameters. To increase biomass productivity, it is necessary to optimize these culture conditions (Chowdury et al., 2020). To sustain effective algae and fish production, DO content higher than 5ppm (Bhatnagar and Singh, 2010) and pH values between 6.5 and 9.0 were recorded as being appropriate (Santhosh and Singh, 2007). From every sampling site, optimum pH and DO content was recorded. The temperature at the sample locations was at an appropriate level, with the majority of marine microalgae preferring a growth temperature between 20°C and 30°C (Chisti, 2008). According to certain research, most Cyanobacteria can develop to their greatest potential at a temperature of 25°C (Chang et al., 2012). The ideal range of phosphorus concentration for microalgae is 0.001 g/L to 0.179 g/L (Roopnarain et al., 2014), while the TAN concentration must be less than 0.5 mg/L and the preferred range of nitrite-nitrogen is 0-1 mg/L (Stone and Thomforde, 2004). The concentrations of nitrogen and phosphorus, which are necessary for plankton development, were optimal over every sampling location.

6.2 Isolation and Characterization of Microalgae

Numerous algae species have been found in Bangladesh's freshwater, brackish, and marine ecosystems (Ahmed et al., 2008), and these species might serve as a source of feed for the aquaculture, biofuel, pharmaceutical, and nutraceutical industries. According to Belcher and Swale (1976) as well as John et al. (2002), isolated microalgae were characterized. The four isolated strains of *Oscillatoria* species obtained from two different marine habitats and two different freshwater environments. The sample sites also yield additional green and brown algae.

6.3 Growth phases of *Oscillatoria* Species

The native microalgae and its potential have received very little attention in Bangladesh. Thus, no prior research on the expansion of *Oscillatoria* sp. has been published. In the present study, Strain 1 M1 (20) illustrated 1^{st} to 7^{th} days lag phase, 7

to 14^{th} days exponential phase, 13^{th} to 14^{th} stationary phase and the death phase on 15^{th} day. Strain 2 M2 (25) showed lag phase on days 1^{st} to 2^{nd}, the exponential phase on days 2^{nd} to 11^{th}, the stationary phase on days 9^{th} to 11^{th}, and finally the phase of death from 12^{th} day. Strain 3 Fw1almost same lag phase and exponential phase on days 1^{st} to 2^{nd} and 2^{nd} to 13^{th} day respectively. Lag phase completed in 1^{st} day, from 2^{nd} to 10^{th} day exponential phase occurred, the stationary phase on days 9^{th} to 10^{th}, and the death phase from 11^{th} day observed in Strain 4 Fw2.

In India, stationary phase was obtained from the isolated *Oscillatoria subbrevis* during 18^{th} to 20^{th} day (Sarmah and Rout, 2018). According to Kasan et al. (2015), *Oscillatoria* sp. attained maturity at 8^{th} day of the 9 days culture period with highest 18 µg mL^{-1} chl-a concentration. In the present study, Strain 4 Fw2 also showed the maturity at 10^{th} day which is similar to the result obtained by Kasan et al. (2015). Strain 2 M2 (25) showed stationary phase at 9^{th} and 11^{th} days with maximum chlorophyll-a production (22.72±0.04 µg/mL). Strain 1 M1 (20) took more time to attain maturity and possess less chlorophyll-a productivity. Strain 2 M2 (25) and Strain 4 Fw2 demonstrated the maximum productivity within short duration. So, Strain 2 M2 (25) and Strain 4 Fw2 can be cultured commercially to obtain higher chlorophyll-a productivity and biomass.

6.4 Dried Biomass Content

Dried biomass of *Oscillatoria* species shows higher productivity in case of filamentous algae than planktonic one (Strain 1 M1 (20)). Marine Strain 2 M2 (25) exhibit the highest biomass level due to dense filamentous characteristics than Strain 4 Fw2 and Strain 3 Fw1. Highest dry-weight biomass 0.552 ± 0.002 g L^{-1} from *Oscillatoria sancta* was recorded by Touliabah and Refaay (2023) which is close to the Strain 2 M2 (25) biomass of 0.286± 0.004 g L^{-1} value.

6.5 Pigment Content

6.5.1 Chlorophyll

Higher amount of chlorophyll a was reported in *Oscillatoria* species. Zavřel et al., 2015 reported a cyanobacterial chlorophyll value as 22µg/ml which is similar of the chlorophyll content of Strain 2 M2 (25) (22.72±0.04 µg/ml) and Strain 4 Fw2 (21.06±0.07 µg/ml) in the present study. Strain 3 Fw1 and Strain 1 M1 (20) showed

chlorophyll-a content of 16.76±0.04 µg/ml and 12.67±0.04 µg/ml respectively which is lower than the value obtained by Zavřel et al. (2015). It also revealed that filamentous *Oscillatoria* sp. contains more chlorophyll- a content than planktonic *Oscillatoria* sp. (Strain 1 M1 (20)).

World Health Organization propose corresponding recommendation limits for cyanobacteria abundance and chlorophyll a concentration for low (<10 g/L), moderate (10 to 50 g/L), high (50 to 5000 g/L), and extremely high risk (more than 5000 g/L) situations (Chorus and Welker, 2021). In this case, all strains of *Oscillatoria* exhibited low blooming potentials ((<10 g/L). Thus, high chlorophyll-a content with low blooming potentiality in *Oscillatoria* sp. indicate the higher productivity and eco-friendly culture and biomass production prospects of the *Oscillatoria* strains.

6.5.2 Phycobiliproteins

The prevalent light-harvesting pigments also called phycobiliprotein found in Cyanophyceae and Cryptophyceae (Glazer, 1994). The phycobiliprotein content in *Oscillatoria* sp. is reported to be 182.0 ± 1.0 mg g^{-1} and 116.1 ± 1.3 mg g^{-1} in *Spirulina* sp. (Begum et al., 2020) while this study revealed the highest phycobiliprotein content of Strain 4 Fw2 about 121.42±0.06 which is close to the author's data. Phycobiliprotein content of Strain 2 M2 (25) was also found 111.04±0.06 mg g^{-1} which is nearly similar with the author's *Spirulina* data.

In commercial applications, the degree of phycocyanin purity is crucial. According to Rito-Palmares et al. (2001), purity levels between 0.7 and 4.0 are considered analytical grade, 3.9 to be reactive grade, and greater than 4.0 to be food grade. In a study, pressurized method was applied to extract phycobiliproteins from *Arthrospira platensis* in which purity index for the phycocyanin extract was 3.59 (A615/A280), and the allophycocyanin purity index was 1.72 (A652/A280). High antioxidant activity was demonstrated by the pure phycobiliprotein extract which was further demonstrated in vitro anticancer efficacy for HL60 leukemic cells. (Viana Carlos et al., 2021). The purification factor of phycocyanin, phycoerythrin and allophycocyanin raw extract of *Oscillatoria* sp. in the present study was found much lower than the results of Viana Carlos et al. (2021). Species variation and extraction procedures may be the reason behind the lower yield of phycobiliproteins in the present study. The highest value in

phycocyanin, phycoerythrin and allophycocyanin content in this study were 1.00±0.00, 0.48±0.00 and 0.34±0.00 compared to the author's results of 0.7 ± 0.2, 0.4 ± 0.2 and 0.5 ± 0.3. *Oscillatoria* sp. in the current study displayed higher purity than the purity index obtained by Begum et al. (2020) which could be due to species variation of the both studies. Thus, phycobiliprotein could be the promising natural source of pigment and may contribute in the antioxidant properties of *Oscillatoria* sp.

6.6 Proximate Composition

In earlier studies, higher quantity of protein (40–60%), carbohydrates (10-30%), and lipids (5–15%) are present in dried cyanobacterial mass (Safi et al., 2013; Singh et al., 2016). Abdel-Raouf et al. (2012) also reported the approximate composition of dried *Oscillatoria* mass to be 38.7% protein, 19.6% carbohydrates, 10.1% lipids and 19.9% ash. Protein values of *Oscillatoria* strains except Strain 3 Fw1 (21.56±0.09) found in this study supported both of the author's results. Strain 4 Fw2 showed highest crude protein (%), 56.97±0.03 followed by Strain 1 M1 (20) (45.34±0.31) and Strain 2 M2 (25) (42.57±0.03). Lipid (%) are nearly in the range except Strain 4 Fw2 (17.13±0.13). In the current study, carbohydrate (%) range (7.49±0.15 to 17.04±0.08) was found almost similar to the results obtained by both of the authors. The author's findings and the variance among the *Oscillatoria* strains in the current study might be explained by the fact that microalgae can thrive in a range of environments with different nutrient concentrations and medium compositions, which may also exhibit a variety of biochemical compositions.

6.7 Fatty Acid Composition

High amounts of -3 PUFAs are produced by microalgae, which are also prospective sources of specific fatty acids using large-scale cultivation techniques (Irmak and Arzu, 2020). PUFAs have recently attracted a lot of interest due to their multiple uses in pharmaceuticals and nutraceuticals. According to Ahlgren et al. (1992), Cyanobacteria can produce more omega-3 fatty acids than green algae. In the present study, PUFAs were found highest in Strain 4 Fw2 (7.31±1.05) followed by Strain 1 M1 (20) (6.21±1.30), Strain 2 M2 (25) (5.77±1.12) and Strain 3 Fw1 (4.26±0.42) gradually. The data analysis showed that long-chain fatty acids (LCFAs; longer than 12 carbons) and medium-chain fatty acids (MCFAs; 8 to 12 carbons) accounted nearly 92% and 12% of

the total fatty acid in the *Oscillatoria* sp. Respectively and also lacked short-chain fatty acids (SCFAs; less than 6 carbons). *Oscillatoria* sp. contain 34.74% SFA, 60.63% MUFA, 4.3% PUFA (Irmak and Arzu, 2020). All of the *Oscillatoria* strains in this study displayed somewhat higher SFA, PUFA levels as well as relatively lower MUFA levels than the author's findings, which may be due to the species variations. Balanced n-3 (3.98±0.02) and n-6 (3.33±1.07) PUFAs are found in Strain 4 Fw2 which may linked to the antimicrobial activity. Mundt et al. (2003) also narrated that, fatty acids prevented the development of the Gram-positive bacteria *Bacillus subtilis*, *Micrococcus flavus* and *Staphylococcus aureus* in an agar plate diffusion test. Thus, rich fatty acid composition may also provide the antimicrobial properties in *Oscillatoria* species.

6.7 Amino Acid Composition

Present study revealed that non-essential amino acids are found higher than essential amino acids in *Oscillatoria*. Strain 3 Fw1 possess highest essential fatty acids followed by Strain 1 M1 (20), Strain 4 Fw2 and Strain 2 M2 (25). On the contrary, Strain 2 M2 (25) and Strain 3 Fw1 showed highest and lowest percentage of non-essential fatty acid respectively. Non-essential fatty acids such as alanine, aspartic acid and were found prevalent in *Oscillatoria* sp. Strain 2 M2 (25) showed highest value of both glutamic acid (17.98%) and aspartic acid (13.36%) while Strain 4 Fw2 showed highest value of alanine (12.99%). Leucine and threonine were abundant in case of essential amino acids in all the *Oscillatoria* strains. Highest leucine (7.98%) and threonine (5.95%) value were obtained from Strain 1 M1 (20) and Strain 4 Fw2 respectively. Metcalf et al. (2021) indicated the abundance of amino acids in cyanobacterial food items and found higher percentage of glutamic acid (4.39%) followed by tyrosine (3.65%), tryptophan (2.98%), aspartic acid (2.3%), alanine (2.07), arginine (1.71%), valine (1.59%), threonine (1.4%) etc. which are lower than the result obtained by this study. It can be due to the food processing loss of amino acid and also due to the species variations.

6.7 Antimicrobial Activity of *Oscillatoria* sp.

Antimicrobial activity was carried out through disk diffusion method where the filter paper discs were the negative control and antibiotic disc acted as the positive control. Significant highest zone of inhibition was obtained from filamentous Strain 2 M2 (25) about 34.05±0.13mm against *Staphylococcus* sp. 2. Planktonic Strain 1 M1 (20) showed

significant lowest zone of inhibition of 17.11±0.15mm against *Pseudomonas* sp. 2. The possible reason for the lower value may be due to the planktonic nature of the algal strain as well as the higher pathogenicity of the bacterial strain. A 20 mm zone of inhibition against *Vibrio cholerae* was found in methanolic extracts of *Oscillatoria boryana*, while zones of inhibition of 18 mm and 17 mm against *Bacillus subtilis* and *Staphylococcus aureus* were obtained respectively (Dash et al., 2022). Mansor et al. (2013) also demonstrated that the gram-positive bacteria are more vulnerable to the methanolic extract of *Oscillatoria* sp. than the gram-negative bacteria. The diameter of the zone of inhibition (22 mm) against *Pseudomonas aeruginosa* was followed by *E. coli* (20 mm), *Bacillus subtilis* (19 mm), and *Staphylococcus aureus* (15 mm). Moreover, Al-Katib and Amin (2020) measured 20 mm and 19 mm inhibition for Gram-positive bacteria and 10 mm and 16 mm for Gram-negative bacteria where he concluded that Gram-positive bacteria are more vulnerable to phenols than Gram-negative bacteria. In this study, gram-positive bacteria also show higher susceptibility compared to the gram-negative bacteria. Inhibition zone were found higher than the respected author's result. In case of *Staphylococcus sps*, inhibition zone varied from 20.60±0.21mm to 34.05±0.13mm. *E. coli* also exhibited inhibition zone from 22.86±0.18mm to 30.47±0.20mm and 17.11±0.15mm to 33.96±0.20mm was found in case of *Pseudomonas* sp. that are higher than the result obtained by Mansor et al. (2013). This can be due to *Oscillatoria* species variability and culture condition. Inhibition zones of each *Oscillatoria* strains were found higher than the positive control. Marine filamentous Strain 2 M2 (25) shows higher degree of inhibition followed by freshwater filamentous Strain 4 Fw2, clustered filamentous Strain 3 Fw1 and finally marine planktonic Strain 1 M1 (20). The higher degree of inhibition may be caused by the phenolic compounds of *Oscillatoria* species as, phenols, indoles, fatty acids, acetogenins, terpenes, and some volatile halogenated hydrocarbons produced from microalgae demonstrated antibacterial action. So, extraction of the phenolic compound from raw ethanolic extract can be the reason behind higher antimicrobial activity.

All of the cyanobacterial strains showed statistically significant variation in the measured variables, according to the findings of the multiple comparison analysis ($p < 0.05$) of Tukey test. These notable variations among the cyanobacterial strains may have substantial ramifications for our comprehension of cyanobacterial variety and

functioning. The variances shown might be caused by a number of things, including genetic variants, environmental circumstances, or other important variables. To understand the underlying processes causing these observed disparities, more research is required. The observation of significant variations among the cyanobacterial strains also expands the knowledge about their variety and might have implications for disciplines like ecology, biotechnology, or environmental management.

6.8 Antimicrobial Activity Index

The zone of inhibition of each extract was compared to that of the reference antibiotic using the activity index (AI). Activity index is the indicator of the efficacy of an antimicrobial to inhibit the growth of pathogenic microorganisms. Less than zero activity index (AI) values demonstrated the potent impact of antibiotics on the studied pathogens, whereas more than one activity index (AI) value suggested the significant function of cyanobacterial extracts. The more AI values assessed, the more meaningful the outcomes (Awan et al., 2013). The range of the active extract's activity indices of fresh water cyanobacteria like *Lyngbya officinalis*, *Gleocapsa gelatinosa*, *Chrococcus* sp., *Anabaena* sp., *Anabaena variabilis*, *Scytonema* sp., and *Westiellopsis prolifica* was 0.33 to 1.5 (Bharat et al., 2013). Cyanobacterial extracts in this study showed excellent antibacterial efficacy. All the extracts were more effective against each of the tested bacteria than commercial antibiotics (Colistin and Ceftriaxone). The AI outcomes also varied from 1.00±0.01 to 2.10±0.03 which is also higher than the findings of Bharat et al. (2013). So, the higher activity indices indicate higher inhibition and greater efficacy of the cyanobacterial extracts.

6.9 Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration (MIC) value found 100-150µg/ml from the ethanolic extracts in this study. Bhuyar et al. (2020) found the minimum inhibitory concentration (MIC) value of *Oscillatoria* sp. 30 µg/ml for gram-positive bacteria whereas it was 25 µg/ml for gram-negative bacteria from methanolic extracts. Variances in MIC value can be due to differential species or due to different extraction solvent or method. 250 µg/ml MIC value of *Staphylococcus aureus*, 500 µg/ml MIC value of both *E. coli* and *Vibrio cholerae* of methanolic extracts are reported by Dash et al. (2022). So, the MIC value varies with the species and extraction procedure.

Chapter 6: Conclusions

In light of the findings of the present investigation, it can be stated that *Oscillatoria* species exhibited significantly higher antimicrobial activity compared to the commercial antibiotics. The variability of the strains also highlights their promising involvement in the antimicrobials industry. By harnessing their unique characteristics, these strains may prevent drug-resistant infections or overcome the challenges associated with existing antimicrobial drugs. Lower MIC value also indicates the higher efficiency of cyanobacteria for disease treatment. Higher degree of lipid and especially PUFA may also contribute to the extraction of lipid for higher production of biofuel. It also paves the way for exploring more Cyanobacterial bioactive compounds and making them potentially valuable sources for the development of novel antimicrobial agents.

CHAPTER 7: RECOMMENDATIONS AND FUTURE PROSPECTS

The antimicrobial activity test was focused on a limited microorganisms and cyanobacteria strains. So, further studies are required to investigate the broader spectrum of antimicrobial activity of diversified microalgae. Extraction, identification and characterization of more bioactive compounds should be done to observe the underlying mechanisms of the antimicrobial activity of these microalgae. Different array of microorganisms and microalgae species should be isolated and tested for the effectiveness of the process. Future attempts that can be made in this field are:

- Isolation, identification and characterization of more diversified indigenous microalgae and identify their potency as antimicrobials.
- Testing effectiveness of the microalgae against different fungal, viral and cancerous disease.
- Bacterial challenge test and utilizing microalgae as commercial products for the disease treatment.
- Extracting and identifying the phenolic compounds with their mode of action to inhibit the growth of microorganisms.
- Analyzing and characterizing the cyanotoxins as well as the bioactive compounds they possess.
- Utilizing the microalgal lipid as the basis for biofuel industry.

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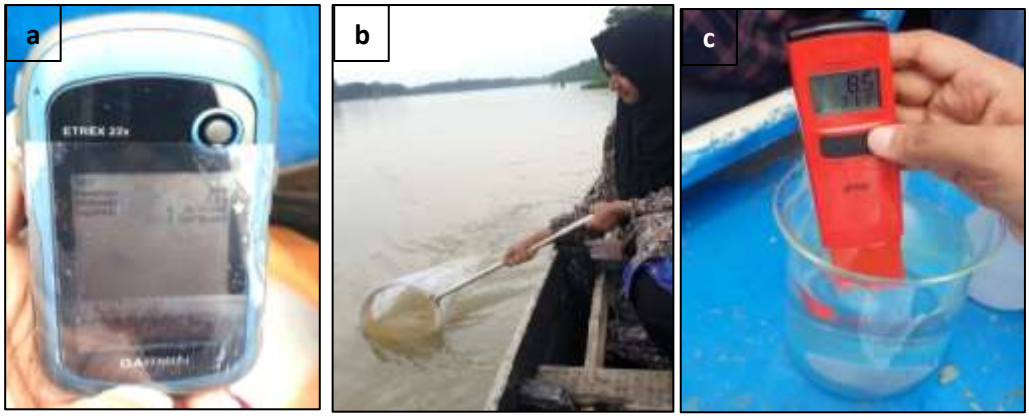
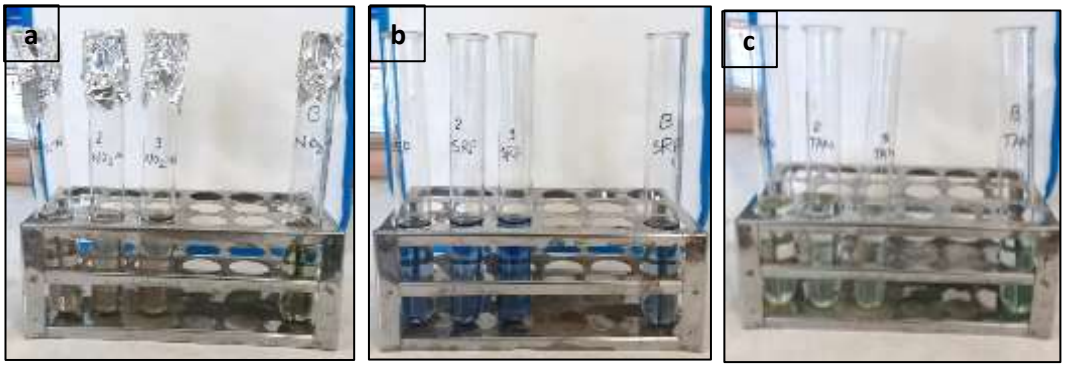
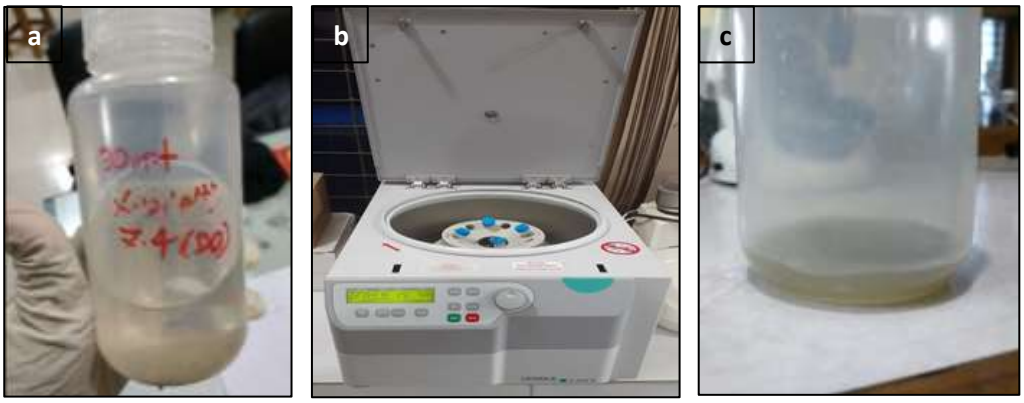
Appendices



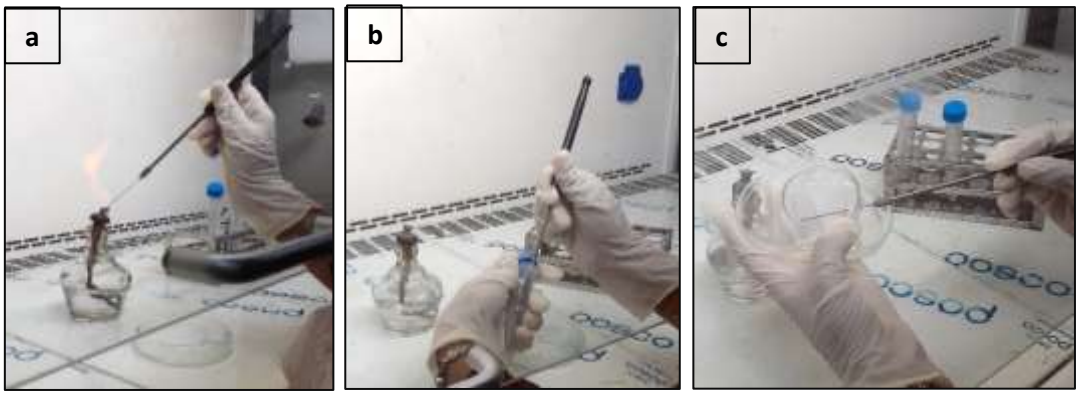
Appendix B: (a) Location tracking of the sampling site through GPS, (b) Determination of physical parameters



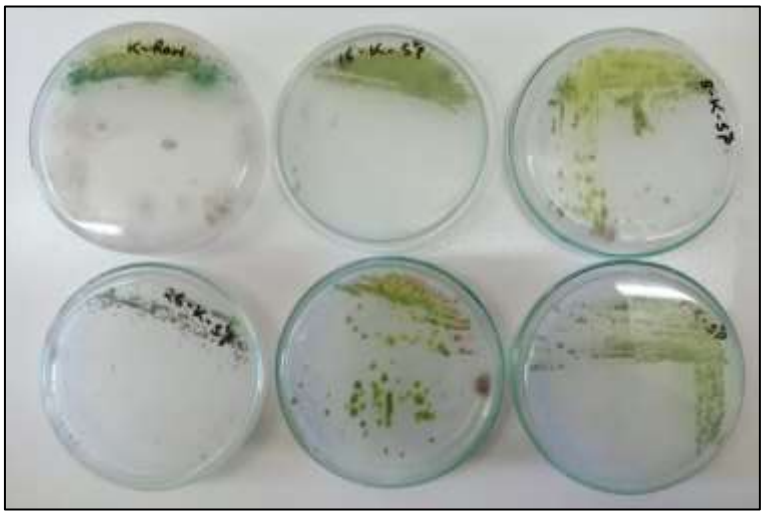
Appendix B: Water quality determination of the sampling sites. Measuring (a) nitrite-nitrogen, (b) soluble reactive phosphorus, (c) total ammonia nitrogen



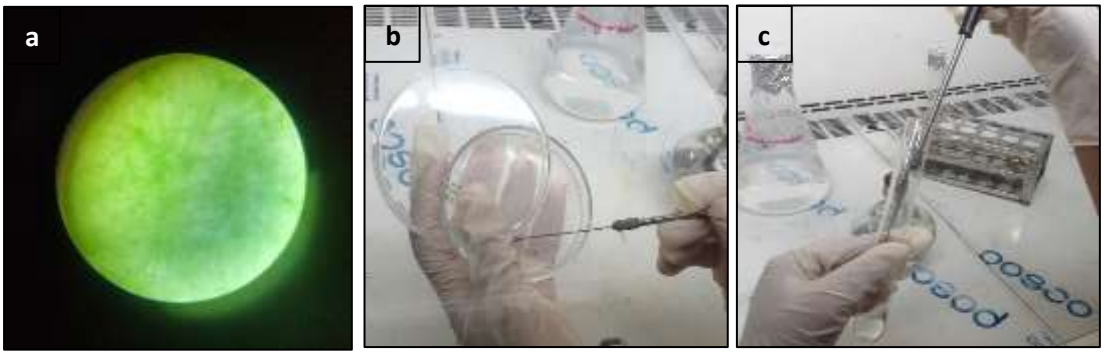
Appendix C: (a) Water sample from the sampling sites, (b) Concentration of sample, (c) Concentrated sample



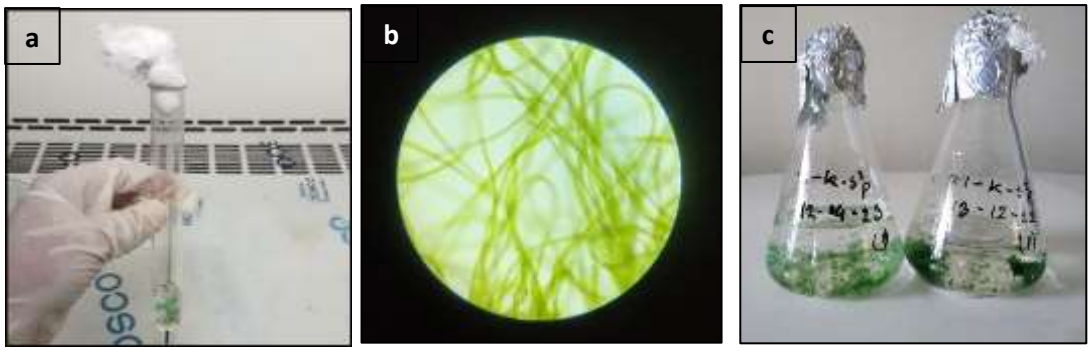
Appendix D: (a) Red heat incineration of the inoculating loop, (b) Obtaining sample, (c) Sample inoculation on the agar medium



Appendix E: Microalgal growth on agar medium after 7-12 days incubation



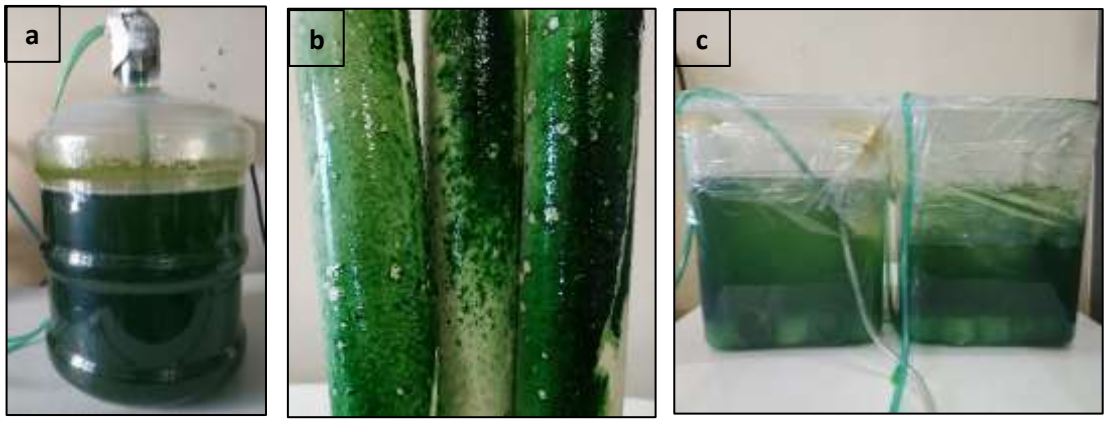
Appendix F: (a) Microscopic observation of a microalgae colony, (b) Picking a microalgal colony, (c) Colony inoculation in the liquid medium



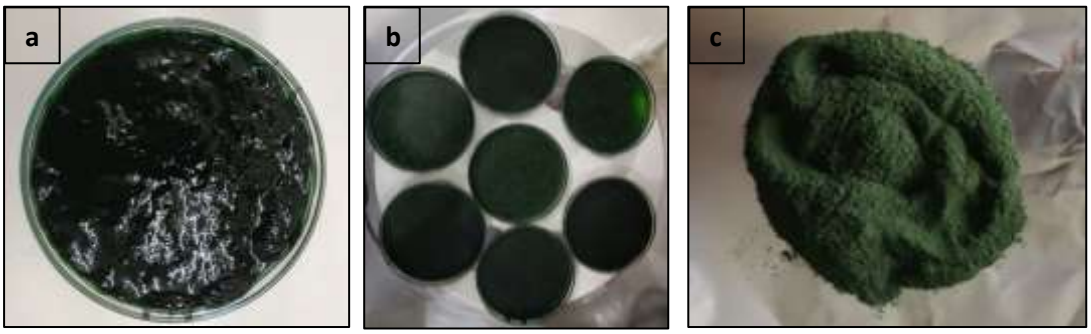
Appendix G: (a) Microalgal growth in test tube, (b) Microscopic observation of the cell, (c) *Oscillatoria* sp. Isolate in conical flask

Appendix H: Growth curve experiment (a) Setting up the experiment, (b-c) Filamentous *Oscillatoria* sp. growth phase

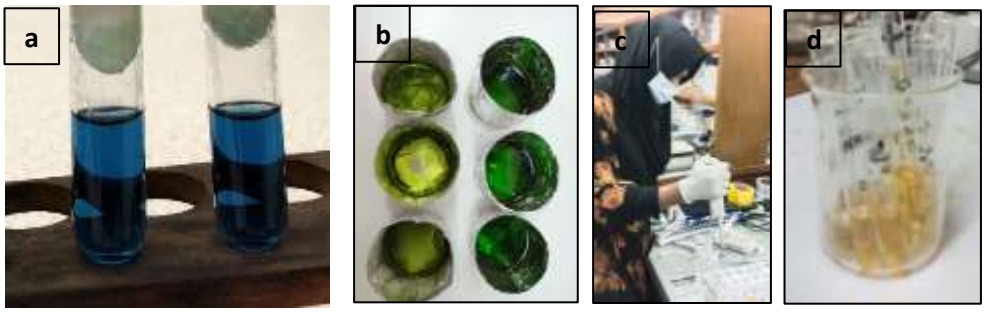
Appendix I: Chlorophyll content estimation (a) Homogenizing the sample, (b) Centrifugation of the sample (c) Spectrophotometric analysis



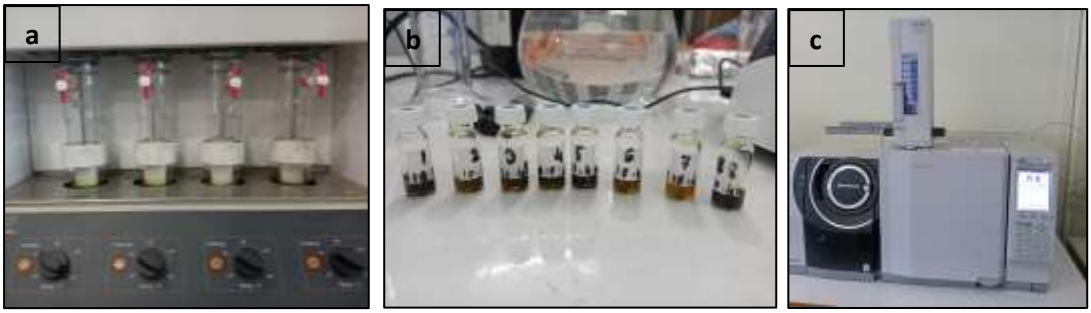
Appendix J: Mass culture of *Oscillatoria* sp. (a) Mass culture in plastic jar, (b) Concentrated *Oscillatoria* growth in PVC pipe substrate (c) Mass culture in plastic bucket



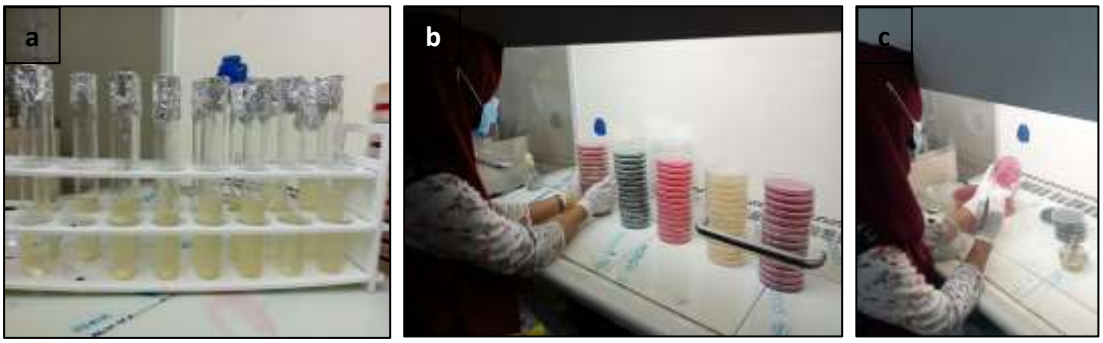
Appendix K: (a) Centrifuged wet biomass of *Oscillatoria* sp., (b) Oven dried biomass in desiccator (c) Fine *Oscillatoria* sp. powder



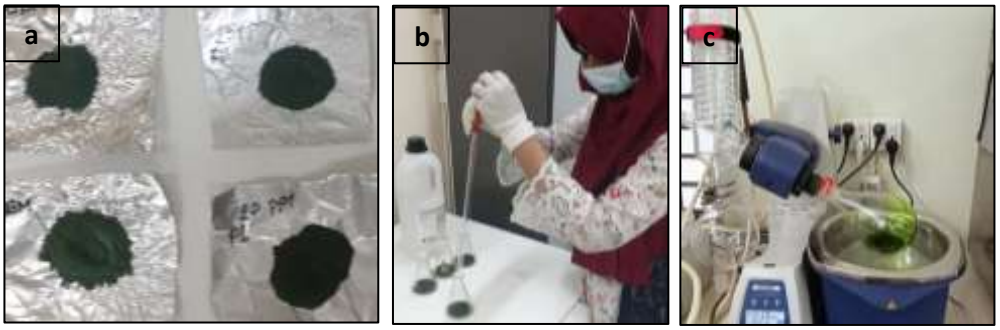
Appendix L: Proximate Composition estimation of *Oscillatoria* sp. (a) Protein content, (b) Lipid content (c) Addition of chemical in test tube (d) Carbohydrate content



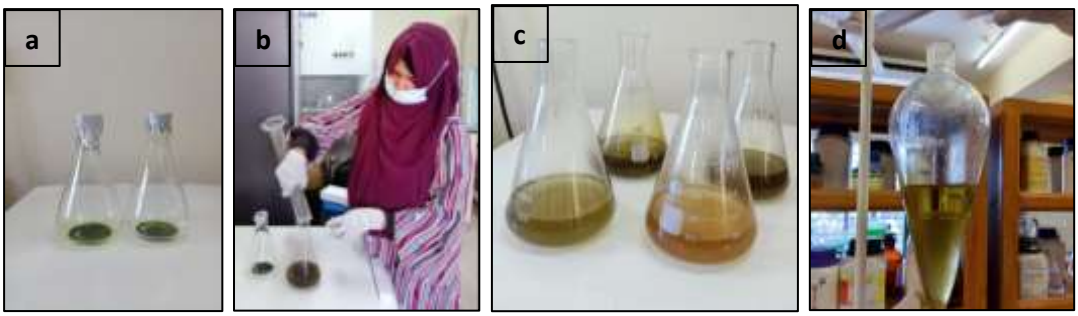
Appendix M: Fatty acid analysis of *Oscillatoria* sp.



Appendix N: Bacteria isolation (a) Bacterial growth in nutrient broth, (b) Selective agar media (c) Bacterial streaking on agar medium



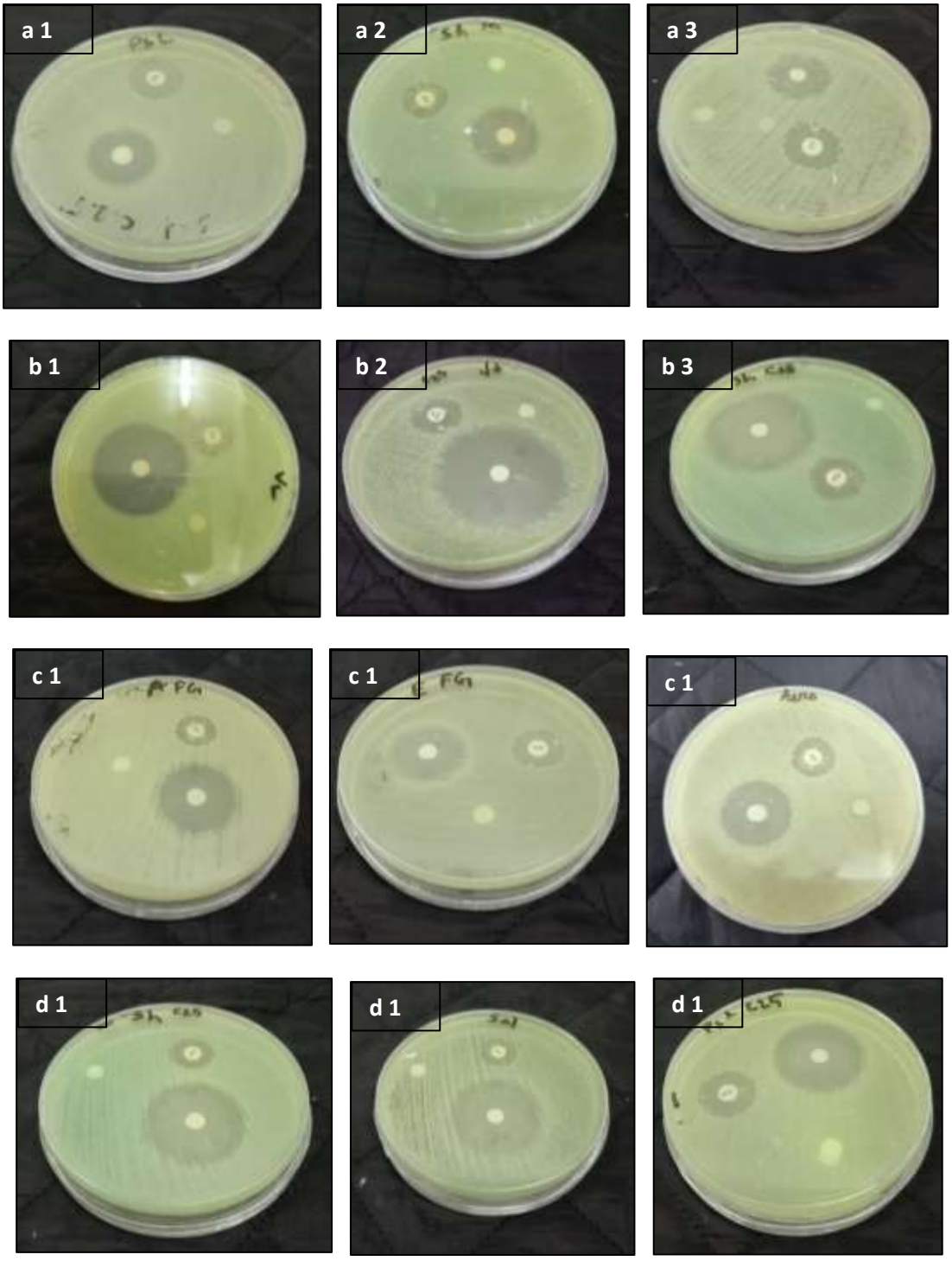
Appendix O: *Oscillatoria* sp. raw extract (a) *Oscillatoria* sp. dried biomass, (b) Addition of ethanol in the dried powder (c) Ethanolic extract Concentration in Rotary evaporator



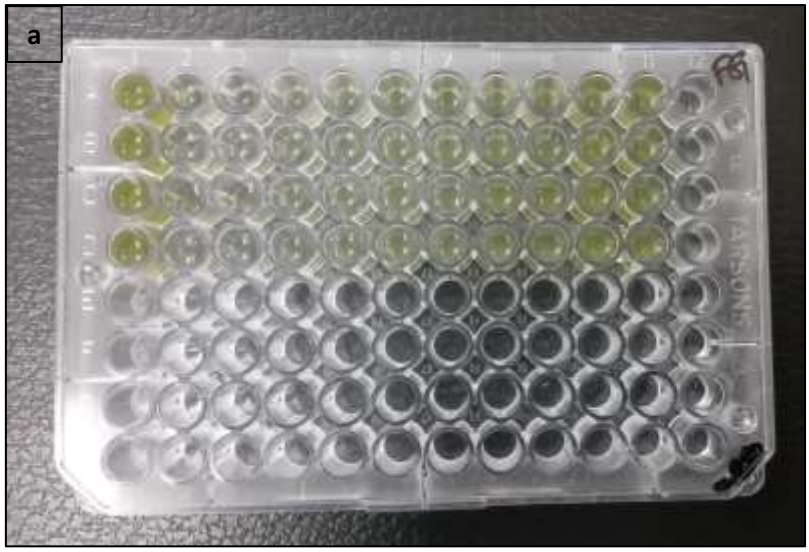
Appendix P: (a) Concentrated *Oscillatoria* sp. ethanolic extract (b) Addition of HCl in the ethanolic extract, (c) Acid decomposed extracts (d) Separate layers of upper phenolic and lower aqueous extracts

Appendix Q: (a) Concentration of *Oscillatoria* sp. phenolic extract (b) Addition of phenolic extract to blank disc, (c) *Oscillatoria* sp. phenolic extract filter paper disc

Appendix R: (a) Antimicrobial activity test preparation (b) Antimicrobial activity test, (c) Diameter of the zone of inhibition measurement of *Oscillatoria* sp.



Appendix S: Antimicrobial activity of *Oscillatoria* spp. (a) Strain 1 M1 (20) against *Pseudomonas* sp., *Shigella and Aeromonas* sp. (b) Strain 2 M2 (25) against *Pseudomonas* sp., *Vibrio* sp. *and Shigella* sp. (c) Strain 3 Fw1 against *Pseudomonas* sp., *Vibrio* sp. *and Shigella* sp. (d) Strain 4 Fw2 against *Staphylococcus* sp., *Salmonella* sp. *and Pseudomonas* sp. respectively.



Appendix T: (a) Cyanobacterial extracts in microtiter plate (96-well), Minimum inhibitory concentration determination of *Oscillatoria* Strain 2 M2 (25) (b) and Strain 4 Fw2 (c) in ELISA machine

Appendix 1: One-way analysis of variance (ANOVA) of the growth parameters of *Oscillatoria* sp.

Source of Variation		Sum of Squares	df	Mean Square	F	Sig.
Protein	Between Groups	1957.863	3	652.621	8383.583	.000
	Within Groups	.623	8	.078		
	Total	1958.486	11			
Lipid	Between Groups	120.667	3	40.222	1340.741	.000
	Within Groups	.240	8	.030		
	Total	120.907	11			
Carbohydrate	Between Groups	160.158	3	53.386	512.033	.000
	Within Groups	.834	8	.104		
	Total	160.992	11			
Chl-a	Between Groups	183.588	3	61.196	8505.753	.000
	Within Groups	.058	8	.007		
	Total	183.646	11			
Phycocyanine Content	Between Groups	923.911	3	307.970	556.257	.000
	Within Groups	4.429	8	.554		
	Total	928.340	11			
Allophycocyanin Content	Between Groups	96.408	3	32.136	41.300	.000
	Within Groups	6.225	8	.778		
	Total	102.633	11			
Phycoerythrin content	Between Groups	269.569	3	89.856	11089.31	.000
	Within Groups	.065	8	.008		
	Total	269.633	11			
Total Phycobiliprotein	Between Groups	2242.140	3	747.380	13114.18	.000
	Within Groups	.456	8	.057		
	Total	2242.596	11			

Purity of phycocyanin	Between Groups	.172	3	.057	4791.548	.000
	Within Groups	.000	8	.000		
	Total	.172	11			
Purity of phycoerythrin	Between Groups	.070	3	.023	10279.35	.000
	Within Groups	.000	8	.000		
	Total	.070	11			
Purity of allophycocyanin	Between Groups	.020	3	.007	2.030	.188
	Within Groups	.026	8	.003		
	Total	.045	11			
Biomass	Between Groups	.058	3	.019	688.788	.000
	Within Groups	.000	8	.000		
	Total	.058	11			
Carotenoid	Between Groups	.283	3	.094	446.250	.000
	Within Groups	.002	8	.000		
	Total	.285	11			
ΣSAFA	Between Groups	660.668	3	220.223	131.031	.000
	Within Groups	6.723	4	1.681		
	Total	667.391	7			
ΣMUFA	Between Groups	99.806	3	33.269	217.053	.000
	Within Groups	.613	4	.153		
	Total	100.420	7			
Σn6-PUFA	Between Groups	93652.04	3	31217.34	25882.29	.000
	Within Groups	4.825	4	1.206		
	Total	93656.86	7			
Methyl icosa-5,8,11,14,17-pentaenoate	Between Groups	13.198	3	4.399	1868.912	.000
	Within Groups	.009	4	.002		
	Total	13.207	7			
Methyl Docosahexanoate	Between Groups	.001	3	.000	.041	.988
	Within Groups	.036	4	.009		
	Total	.037	7			

Σn3-PUFA	Between Groups	50.395	3	16.798	16.527	.010
	Within Groups	4.066	4	1.016		
	Total	54.461	7			
ΣPUFA	Between Groups	90503.373	3	30167.79	14302.69	.000
	Within Groups	8.437	4	2.109		
	Total	90511.810	7			
Σn3/ Σn6	Between Groups	21.769	3	7.256	8.271	.034
	Within Groups	3.509	4	.877		
	Total	25.278	7			
DHA/EPA	Between Groups	114.348	3	38.116	71.629	.001
	Within Groups	2.129	4	.532		
	Total	116.476	7			
SAFA/TUFA	Between Groups	11.800	3	3.933	49.743	.001
	Within Groups	.316	4	.079		
	Total	12.117	7			
SAFA/TFA	Between Groups	.554	3	.185	414.245	.000
	Within Groups	.002	4	.000		
	Total	.556	7			
TUFA/TFA	Between Groups	.554	3	.185	414.245	.000
	Within Groups	.002	4	.000		
	Total	.556	7			

Appendix 2: One-way analysis of variance (ANOVA) of the growth antimicrobial activity of *Oscillatoria* sp.

ANOVA of Antimicrobial activity

		Sum of Squares	df	Mean Square	F	Sig.
Strain 1 M1 (20)	Between Groups	526.234	11	47.839	242.754	.000
	Within Groups	11.824	60	0.197		
	Total	538.058	71			
Strain 2 M2 (25)	Between Groups	566.380	11	51.489	292.312	.000
	Within Groups	10.569	60	0.176		
	Total	576.949	71			
Strain 3 Fw1	Between Groups	471.386	11	42.853	212.267	.000
	Within Groups	12.113	60	0.202		
	Total	483.499	71			
Strain 4 Fw2	Between Groups	346.054	11	31.459	144.747	.000
	Within Groups	13.040	60	0.217		
	Total	359.095	71			
Antibiotic	Between Groups	51.902	11	4.718	41.249	.000
	Within Groups	6.863	60	0.114		
	Total	58.765	71			

Appendix 3: One-way analysis of variance (ANOVA) of the growth antimicrobial activity of *Oscillatoria* sp.

ANOVA of Activity Index (AI)						
		Sum of Squares	df	Mean Square	F	Sig.
Strain 1 M1 (20)	Between Groups	2.571	11	.234	59.783	.000
	Within Groups	.088	60	.001		
	Total	2.659	71			
Strain 2 M2 (25)	Between Groups	1.243	11	.113	52.055	.000
	Within Groups	.130	60	.002		
	Total	1.373	71			
Strain 3 Fw1	Between Groups	1.507	11	.137	66.312	.000
	Within Groups	.124	60	.002		
	Total	1.631	71			
Strain 4 Fw2	Between Groups	1.116	11	.101	40.996	.000
	Within Groups	.149	60	.002		
	Total	1.265	71			

BRIEF BIOGRAPHY OF THE AUTHOR

Jannatul Nayeem is the daughter of Md. Shahinur Rahman and Rehana Akter was born in Bogura and grown up in Chattogram. She passed the Secondary School Certificate Examination in 2014 from Chittagong Government Girls' High School, Chattogram and Higher Secondary Certificate Examination in 2016 from Chittagong Government Girls' College, Chattogram. She has also achieved B.Sc. Fisheries (Hons.) degree from Faculty of Fisheries, Chattogram Veterinary and Animal Sciences University. She is now a candidate of Master of Science in Department of Aquaculture, Chattogram Veterinary and Animal Sciences University. Her research interest includes microalgae, cyanobacteria (isolation, identification, mass production, performance characterization, antimicrobial activity, pharmaceuticals and nutraceuticals industry of microalgae and cyanobacteria), zooplankton and seaweed. She is passionate to qualify herself as a competent researcher, and thus to serve the aquaculture sector of Bangladesh.