ZAHIDUL ISLAM MASTER OF SCIENCE IN AQUACULTURE JUNE, 2020

GROWTH, PRODUCTIVITY, PIGMENTS AND PROXIMATE COMPOSITION OF INDIGENOUS MARINE MICROALGAE ISOLATED FROM COX'S BAZAR COAST

Zahidul Islam

Roll No. 0119/03

Registration No. 0693

Session: 2019-2020

A thesis submitted in the partial fulfillment of the requirements for the degree of Master of Science in Aquaculture

Department of Aquaculture

Faculty of Fisheries

Chattogram Veterinary and Animal Sciences University

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Dedicated to,

My Creator the Omnipotent "ALLAH SWT"

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Zahidul Islam
June, 2020

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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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JUNE 2020

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June, 2020

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List of Abbreviations

Words	Abbreviation
sp.	Species
CD	Cell Density
OD	Optical Density
DO	Dissolve Oxygen
ppt	Parts Per Thousand
ppm	Parts Per Million
pH	Power of Hydrogen
℃	Degree Celsius
$\mu Em^{-2} s^{-1}$	Microeinsteins per second per square meter
VP	Volumetric Productivity
LP	Lipid Productivity
AP	Areal Productivity
hr	Hour
µ	Micro
mL	Mili Liter
L	Liter
nm	Nanometer
cm	Centimeter

Abstract

Microalgal biomass has been widely reported as great source of feed, food, biodiesel, and pigments. Suitable microalgae species for particular industrial application are selected on the basis of its growth rates, productivity, and adaptability to the laboratory conditions, pigments concentrations, and proximate profiles. Understanding the growth pattern of microalgae is crucial because it is the primary steps for any kind of operational procedure mostly biomass extractions. Present study focused on four different indigenous microalgae species (*Chlorella* sp., *Chaetoceros* sp., *Nannochloropsis* sp., and *Tetraselmis* sp.) to determine their growth, productivity, pigments and proximate composition. Experimental species were collected from previously preserved sample (isolated from Cox's Bazar coast) of Department of Aquaculture, Faculty of Fisheries, Chattogram Veterinary and Animal Sciences University. Selected species were cultured in commercial Conway culture medium. Biomass was harvested at their stationary phase for each species for further analysis. Result indicated distinct growth phase for each species in terms of cell density and optical density which were significant at 1% level of significance. In addition, *Chaetoceros* sp. showed significantly ($p < 0.05$) higher volumetric productivity (0.61 ± 0.08 mg/L/Day), areal productivity (1.2 ± 0.17 mg/cm^2/Day), and lipid productivity (0.109 ± 0.003 mg/L/Day) compare to *Chlorella* sp., *Nannochloropsis* sp., and *Tetraselmis* sp. On the other hand, *Tetraselmis* sp. produced significantly highest amount of chlorophyll a and b (2.68 ± 0.04 µg/L, 1.23 ± 0.02 µg/L) where *Chaetoceros* sp. produced (0.29 ± 0.01 µg/L) highest amount in case of chlorophyll c. Moreover, in case of carotenoids *Nannochloropsis* sp. content (1.68 ± 0.05 µg/mL) significantly ($p < 0.05$) higher amount compares to *Tetraselmis* sp. (1.51 ± 0.14 µg/mL), *Chaetoceros* sp. (1.36 ± 0.2 µg/mL), and *Chlorella* sp. (0.56 ± 0.03 µg/mL). In addition, allophycocyanin reported highest amount in all the four species rather than phycocyanin and phycoerythrin. However, *Nannochloropsis* sp. content significantly ($p < 0.05$) higher amount of phycobiliproteins than other three species (*Tetraselmis* sp., *Chlorella* sp., and *Chaetoceros* sp.). Besides, the results of proximate composition in this study showed that, *Tetraselmis* sp. content significantly ($p < 0.05$) higher amount of protein (57 ± 0.66% dry weight), *Nannochloropsis* sp. content significantly ($p < 0.05$) higher amount of lipid (25 ± 1.84% dry weight), whereas *Chlorella* sp. content significantly ($p < 0.05$) higher amount of carbohydrate (23 ± 1.62% dry weight) among all other

species. For the production of valuable biomass quality microalgae are potential candidates because of their easy culture system, and capability to grow in low cost media. In that way the production cost and contamination risk also decrease. Therefore, proper selection of microalgae' species through proper characterization is necessary for various industries.

Keywords: Microalgae, growth curve, productivity, phycobiliprotein, carotenoid, chlorophyll

Chapter- 1: Introduction

Algae the green gold of Bangladesh is the base of the entire aquatic food chain. Algae are considered to be the fittest support for the production of renewable resources in near future. Therefore, it is not surprising that the microalgae which compose the phytoplankton play a vital role in the rearing of aquatic animals like mollusks, shrimp and fish. Microalgae are known as prokaryotic or eukaryotic photosynthetic microorganisms that can be found in all ecosystems both aquatic and terrestrial (Mata et al., 2010). It is narrated that 30,000 microalgae species have been marked out already among approximately 50,000 species of the nature (Richmond, 2004).

Fulfilling excess demand of transportation and food due to increasing number of world population, industrialization has been identified as the tertiary cause of recent climate change (Faried et al., 2017). Maximum yield of microalgae could reach 55tons/ha/year that can control the biodiesel production cost considerably (Wang et al., 2016; Pan et al., 2017). In today's world these photosynthetic organisms are consider as a great source of potential biodiesel stock termed as third generation feedstock (Zhou et al., 2017).

Microalgae have recently been attracted a considerable level of interest into the whole world due to their extensive application potentiality in the field of renewable energy, biopharmaceutical and nutraceutical industries (Barsanti et al., 2008). In today's world, microalgae pigments are extensively utilized in various industries, including food, nutraceutical, pharmaceutical, aquaculture, and cosmetic industry in addition with using in clinical laboratories or research laboratories, which are effective as label for antibodies and receptors (Santiago-Santos et al., 2004). Because of some other properties, these organisms have the potential to produce renewable energy and serve as alternative sources for biodiesel from nature's most plentiful resources; water and solar energy (Parmar et al., 2011).

Different microalgae can produce high amounts of protein and other valuable industrial bi-products such as carotenoids, phycobiliproteins, and asthaxanthin (Ahmed et al., 2014), poly unsaturated fatty acids as nutraceuticals (Adarme-Vega et al., 2014) or lipid for biodiesel feedstock (Sharma et al., 2014). Culture of indigenous microalgae has shown higher adaptability in local environmental condition. Advantages of using

microalgae compared to other feed source are microalgae shows higher areal productivity resulting less land use than other crops (Rodolfi et al., 2009).

Species or strain selection is the first and critical step in bioprospecting of microalgae for any commercial application. The growth phase of microalgae had significant influence on lipid content but as well as surface characteristics (Xia et al., 2017). Moreover, it was reported that biomass harvesting associated with settling, tangential flow filtration can be influenced by the growth phase of algal cells (Danquah et al., 2009), flocculation (Lee et al., 1998), and flotation (Zhang et al., 2012). However, there is currently little information available on microalgae status in our country and its potentiality.

Bangladesh is a densely populated country where most of the people are directly or indirectly depend on fisheries sector. With the development of fisheries sector a great demand of quality fish feed is creating day by day. In future, it might be possible to use microalgae as animal feed replacing the present high valued ingredients. Hence, it is important to look upon on this sector.

1.1 Objectives of the Study

Though microalgae pigments are one of the most important facts in the matter of production of various high valued products but there is no precise and detail data available in aspects of our country. Therefore, the present study is undertaken to screen out various natural pigments to identify good quality microalgae. The specific objectives of this research are as follows:

a. To determine growth curves, productivity and proximate composition of different types of microalgae.
b. To screening of different types of natural pigments from selected microalgae.

1.2 Scope of the Study

Replacing artificial colors through natural colors will add a new dimension in the food industry. It is projected that using micro-algal pigments will achieve a great revolution in the field of nutraceuticals, cosmetic and pharmaceuticals industry. It has a great feasibility in the field of ornamental fish culture for their color enhancement. Without that, result of this study will help producers to select good quality microalgae species according to their demand.

Chapter-2: Review of Literature

Before conducting any research under a definite experimental procedure, it is important to have a look on the previously conducted research activities on the related topics. Microalgae is an autotrophic microscopic organism considered as a great source of natural pigments such as chlorophyll, carotenoids and phycobiliproteins etc. A review of literature relevant to the present research work has been given below.

2.1 Microalgae

Microalgae are considered as one of the most important photosynthetic organisms that habituated in different aquatic habitats, which includes ponds, lakes, oceans, rivers, and even wastewater (Khan et al., 2018). These organisms have the ability to tolerate a wide range of salinity, temperatures and pH values with different light intensities; and conditions (Barsanti et al., 2008). Microalgae are two types which are prokaryotic and eukaryotic photosynthetic microorganism. According to the color's microalgae are classified as green, red, blue-green and brown (Graham and Wilcox, 2000). In open water, microalgae contributions are important in producing energy and essential nourishing component for proper development of aquatic organisms (Habib et al., 2003). They also become main live foods for zooplankton such as *Rotifers, Cladocerans* as well as for different fish larvae specially larvae of shrimp (Gallardo et al., 1995).

In aquaculture, microalgae play an important role in aquaculture development. Microalgae are widely used as an ineluctable food source in the field of commercial rearing of all growth stages of mollusks, larval stages of crustaceans and early growth stages of fishes (FAO, 1996). They also can be use as food additive to basic nutrients or as a food coloring. But it must be in proper size for repast, for instance for filter feeders (1-15 micrometer), for grazers (10-100 micrometer) and readily digested (Kawamura et al., 1998).

Microalgae have recently been attracted a considerable level of interest into the whole world. There extensive potential application had been reported in various filed including's biopharmaceutical, renewable energy, and nutraceutical industries (Barsanti et al., 2008). In today's world microalgal pigments application also recorded widely in industrial filed in addition with various research, clinical and pharmaceutical laboratories (Santiago-Santos et al., 2004). Because of some other properties, it creates

a vast feasibility in the field of renewable energy and substitute of biodiesel and natural energy (Parmar et al., 2011). Considering microalgae profile, it is clear that various factors are behind influenced on nutritional profile of microalgae which includes cell size (Fernandez-Reiriz et al., 1989) and ability to digest (Epifanio et al., 1981).

For this experiment, four species of tropical marine microalgae have been selected which are *Chlorella* sp., *Chaetoceros* sp., *Nannochloropsis* sp., and *Tetraselmis* sp. These marine species have been used deliberately in a huge in aquaculture industry especially for growth and larval rearing just because of its nutritional profile (Jeffrey et al., 1994).

2.1.1 *Chlorella* sp.

Figure 2.1 shows *Chlorella* sp. under 40x magnifications isolated from Cox's Bazar coast. *Chlorella* belonging to the genus Chlorophyta is a genus of single cell green algae. It is look spherical alike and ranges 2 to 10μm in diameter. It has no flagella. It is known a sack of source of Chlorophyll. Chloroplast is the source of chlorophyll a and b and in optimum condition like optimum salinity, pH, water and CO_2 condition it multiplies rapidly (Scheffler, 2007).

For photosynthetic efficiency of *Chlorella* is considered as a great source of high protein and essential stuff. It consists protein (45%), fat (20%) and carbohydrate (20%) and fiber (5%) and minerals (10%) when dried. The processed or dried *Chlorella* powder is known as superfood. Moreover, the species have the ability in control of body weight, and support one's immune system (Balasco, 1997). On the basis of growing conditions, *Chlorella* produce some oil which is a great source of poly unsaturated fatty acid, which can produce about 39.9% EPA of total lipids (Yongmanitchai, 1991).

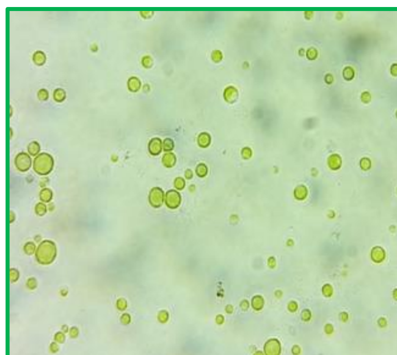

Figure 2.1: *Chlorella* sp. isolated form Cox's Bazar coast

2.1.2 *Chaetoceros* sp.

Figure 2.2 shows *Chaetoceros* sp. under 100x magnifications isolated from Cox's Bazar coast. *Chaetoceros* sp. generally is colonial bipolar centric diatoms and its size range from 2.5 to 6 µm (Khoi et al., 2006). It consists single or multiple chloroplasts in its oval or cylindrical structure. The chloroplast size also varies on basis of its cell structure. Cingulum is incomplete but mantle is complete and vertical in structure (Guiry, 2017).

Chaetoceros sp. has a great use in aquaculture sector, because of its unique compositions of nutrients which is suitable for larval rearing and suitable for different filter feeders (FAO, 1996). In shrimp hatchery the species *Chaetoceos calcitrans* is one of the most using strain. This is because of its organic nutrients and energy (Jeffrey et al., 1994). But it has to remember that, the nutritional value has influenced by its culture pattern (Whyte et al., 1989). It is also important in the concern of economical view (Coutteau and Sorgeloos, 1992). O'Connor and Heasman (1997) reported this species as the most use items in any marine hatchery system as larval feed.

Figure 2.2: *Chaetoceros* sp. isolated form Cox's Bazar coast

2.3.3 *Nannochloropsis* sp.

Figure 2.3 shows *Nannochloropsis* sp. under 100x magnifications isolated from Cox's Bazar coast. *Nannochloropsis* sp. are marine, unicellular and free-floating microalga. The cell is sub spherical, with a structure cylindrical diameter. Its chloroplast is moderate develop color tends to yellow to green (Antia and Cheng, 1982). Golgi body and Mitochondrion are common in every cell with cytoplasmic lamellate vesicles, a pyrenoid and a cell wall papilla (Hideaki, 2002). According to Ma et al. (2014), the species has plant alike plastids with very simple morphological structure of diameter 3-

8 µm. These species are mainly used for zooplankton feeding *(Artemia* or *Rotifers)* which are later use for larval feeding of fish larvae (Malcolm, 1998). *Nannochloropsis* sp. also use in nutritional supplement as it contains high of Eicosapentaenoic acid (EPA) (Wan, 2012).

Nannochloropsis sp. are commonly used in marine hatcheries regulator of water quality (Riquelme and Avendaño-Herrera, 2003). *Nannochloropsis* sp. are commercially cultured for extensive use in the aquaculture industry for growing small zooplankton such as rotifers, copepod, daphnia and *Artemia* (Banerjee et al., 2002) for feeding SPS corals and other filter-feeders. In food industry, it is well known as a source of different valuable compounds such as vitamin E (Durmaz, 2007) and pigments; chlorophyll, astaxanthin and canthaxanthin. (Lubian et al., 2000).

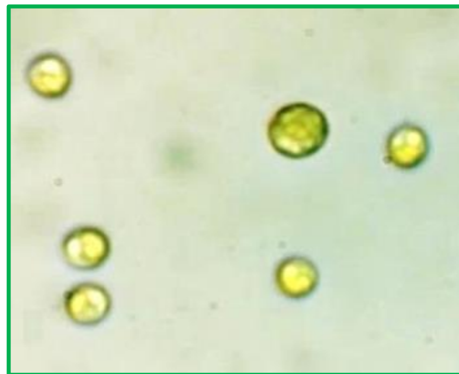

Figure 2.3: ***Nannochloropsis*** **sp. isolated form Cox's Bazar coast**

2.1.4 *Tetraselmis* sp.

Figure 2.4 shows *Tetraselmis* sp. under 100 x magnifications isolated from Cox's Bazar coast. *Tetraselmis* sp. are unicellular flagellate which is an ovoid body shape and curved in side views. *Tetraselmis* are measures 12-14 µm in length and 9-10 µm in width (Mehdi et al., 2015). This species is commonly spherical in shape but elliptical in sometimes with compressed and curved sight. In possesses 4 pair's flagella in both sides. Its eyespot is varying on basis of the species but present in every cases. Chloroplast is present, two in number in some cases. Two stages is observed one is motile and another is non motile (Guiry and Guiry, 2015).

Tetraselmis spp. are mainly used for zooplankton feeding *(Artemia* or *Rotifers)* which are later use for larval feeding of fish larvae (Muller-Feuga et al., 2003). *Tetraselmis* sp. becomes an important source for anti- oxidative substances in pharmacological studies (Laguna et al., 1993) and for their importance in marine eco-toxicological testing (Park et al., 2005). It has also used in plantology (Guiry and Guiry, 2015).

Furthermore, Austin et al., (1992) reported the use in antimicrobial field. In modern period is has a great potentiality as probiotics (Irianto and Austin, 2002). This species also considered as good source of vitamin, especially vitamin E and for that referred as animal diet (Carballo- Cárdenas et al., 2003).

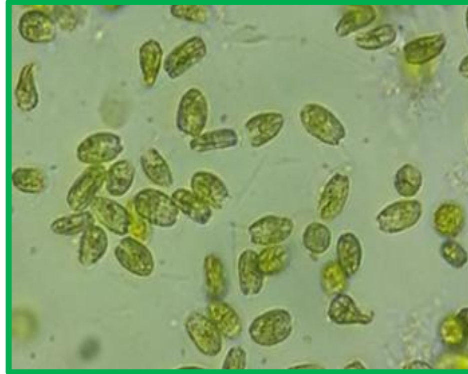

Figure 2.4: ***Tetraselmis*** **sp. isolated form Cox's Bazar coast**

2.2 Growth Factors of Microalgae

Microalgae, one of the fast-growing organisms which use sunlight for photosynthesis (about 10% solar energy) for production and multiplication with approximate yearly yield of 280 ton/ha (Formighieri et al., 2012). Kitaya et al. (2008) reported direct effect of different parameters especially environmental parameters (light, salinity, nutrient types and composition, light period, and culture pattern). According to the review article of Parmar et al. (2011) the length of lighting period and cycle (light/dark) are the most validate factors influenced most in micro-algal cultivation. Most importantly the growth of microalgae affected by some illuminating factors such as length of photoperiod, temperature, pH and light intensity (Wahidin et al., 2013). In order to optimizing microalga growth in mass culture system the above-mentioned factors must have to maintain accurately.

2.2.1 Light

Intensity of light is an important factor for microalgae cultivation. Generally, for biomass growth, microalgae depend on enough carbon source (about 40-50% carbon) and light to carry out photosynthesis process (Moheimani, 2005). Requirement varies on basis of the conditions. For an Erlenmeyer flask; 1000 Lux is suitable where 5000-10000 Lux required for larger volume (FAO, 1996). The use of fluorescence light for indoor culture can promote a better growth and cell division of microalgae (Laing,

1991). However, maximum exposure of light can become limiting factor to microalgae density. Kaewpintong (2004) reported cell growth rate increase depending on light intensity, but until a definite stage and after that the growth decrease. It is supported by Lavens and Sorgeloos (1996) that higher light intensity may result in photo-inhibition.

2.2.2 Temperature

Temperature, the 2nd most prior factor for culturing microalgae. Besides, above 27 ºC will make the algae die (Laing, 1991). Various microalgae are viable with very low temperature than its optimum (upto 15 ºC lower), but crossing limit above by 1-4 ºC can create a great damage (Teresa et al., 2010). Changing of light intensity will influence temperature which indirectly affects growth of microalgae (Huang et al., 2013). These all factors vary depending upon culture constituents, medium types, species types and strained but temperature ranged between 20-24 ºC.

2.2.3 pH

pH plays an important role for culturing microalgae. Many cellular activities disrupt in microalgae cell because of pH maintaining failure (Lavens and Sorgeloos, 1996). pH is directly related with CO_2 accessibility and for that reason it is also essential for photosynthesis. In higher concentration pH may varies and reach at limiting values pH 9 (FAO, 1996).

2.2.4 Nutrient Composition of Media

Nitrate, phosphate and silicate are some examples of macronutrients essential for microalgae growth (Lavens and Sorgeloos, 1996). Nitrogen is considered as the most important type of nutrient and most common type of nutrient in the culture medium (Thompson et al., 1989). Zinc, cobalt, boron, iron and manganese are the most commonly used trace metals (Probert and Klaas, 1999). Others are thiamin (B1), cyanocobalamin (B12) (FAO, 1996). Lourenço (2006) reports direct interaction of nutrients on growth of microalgae. Commercial media contain all types of micronutrients and macronutrients to help the growth of microalgae.

2.2.5 Salinity

Salinity has direct effect on growth of microalgae. Salinity range varies on basis of species cultured. Every microalga has a different salinity range. Change in salinity might inhibit the growth of microalgae (Takagi et al., 2006). By adding fresh water or salt we can easily control the salinity of culture medium.

2.2.6 Mixing and Aeration

For homogenizing mixing and aeration is an important factor. Kaewpintong (2004) found fine development of cell growth in an aerated culture system (bioreactor) than non-aerated system.

On the contrary, excess liquid pressure, velocity, over turbulence and excess bubbles can create stress to the cell which result damage of cell (Eriksen, 2008). It is also important to prevent sedimentation. So, to ensure better contacts with cell and nutrients it is also important to maintain homogenous conditions through balance aeration.

2.3 Growth curve

Microalgae show five phases during its growth (Lavens and Sorgeloos, 1996). These are well defined as i) Lag phase, ii) exponential phase, iii) linear growth phase iv) stationary growth phase v) decline or death phase. Under suitable condition microalgae show all that phases.

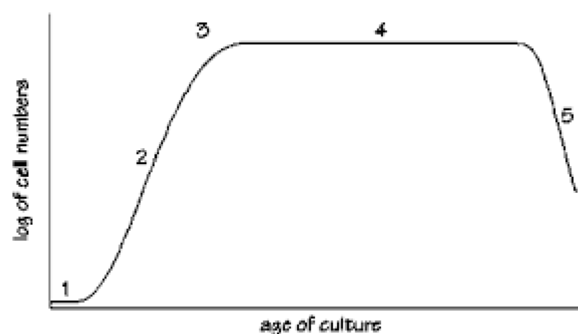

Figure 2.5: Microalgae growth curve (Teresa et al., 2010)

Here the first phase is lag phase where the cell is viable but not ready to division yet. During second phase, cell density started to increases as a function of time. Commonly microalgae double their biomass during exponential growth phase (Chisti, 2007). After they turns into stationary phase where the growth rate is balanced. In this phase microalgae have highest density than all other phase. Finally, the death phase where the

cell density started to collapse. In this phase nutrient, pH, CO_2, and other physical factors begin to limit growth (FAO, 1996).

2.4 Pigments

There are three main classes of microalgae pigments which are Chlorophylls, Carotenoids (both water insoluble) and Phycobilins (water-soluble). Chlorophylls have green pigments and Carotenoids are yellow or orange pigments (Kalidas and Loveson, 2005). Chlorophylls are surrounded by porphyrin ring. It consists free electron which can easily transfer. Because the electrons can transport freely, the porphyrin ring will likely to gain or lose electrons easily and thus energized electrons to other molecules. By this process chlorophyll absorbs the energy of sunlight. Contrariwise, carotenoid is insoluble in water but the membranes within cell. Carotenoids absorb energy through chlorophyll molecules as the can't directly participate in photosynthetic system (Kumar et al., 2009). For this reason, they are called accessory pigments.

2.4.1 Chlorophyll

The term chlorophyll refers a green photosynthetic pigment which is found in plants, algae and cyanobacteria (Humphrey, 1980). It absorbs blue and very rare of red portions of the electromagnetic spectrum, that is why its green color (Humphrey, 2004). Microalgal biomass is a good source of bioactive chlorophyll which has a great industrial value as food coloring agent (natural) and also possesses antioxidant properties (Chisti, 2007). It is found that chlorophyll are mainly two types, chlorophyll *a* and *b* and another type of chlorophyll is chlorophyll *c*. Structures of chlorophyll compounds are shown in (Figure: 2.6). Porphyrin macrocycle are the skeleton of chlorophyll molecule and it comprises of four pyrrole rings (Humphrey and Scheer, 2004). Each of the pyrrole rings contain four one nitrogen atom and carbon atoms. Mg^{2+} metal ion easily binds with all of the nitrogen atoms centrally (Scheer, 2004). In chlorophyll *b*, the methyl group in ring II of chlorophyll is replaced by a formyl group (Cubas et al., 2008). Humphrey (1980) reported the absorbance variation of chlorophyll pigments because of its structural and color different,

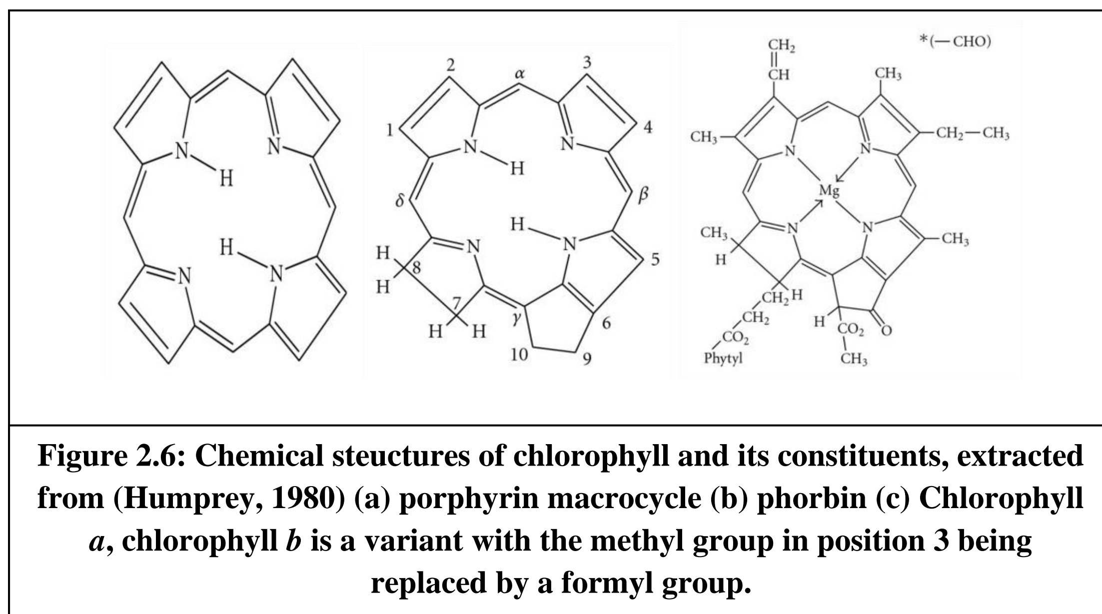

Figure 2.6: Chemical steuctures of chlorophyll and its constituents, extracted from (Humprey, 1980) (a) porphyrin macrocycle (b) phorbin (c) Chlorophyll *a*, chlorophyll *b* is a variant with the methyl group in position 3 being replaced by a formyl group.

2.4.2 Application of Chlorophyll

Chlorophyll is available, because of its complex "light harvesting'' way of photosynthesis (Humphrey, 1980). Chlorophyll is the main factor of photosynthesis produce carbohydrate through a chemical reaction which is the building block unit of all plant and produce oxygen which is necessary for the survival of animal kingdom (Humphrey, 1980). It is also important for the entire food chain.

Chlorophyll is mainly use as coloring agent which are used largely in various industry in replace of artificial colorings (Spears, 1988). Coloring is important both for consumers and manufacturers because consumers demand natural color of any foodstuff where manufacturers demand for the uniformity of any products (Timberlake et al., 1986).

It is found that chlorophyll accelerate wound healing by more than 25% and also stimulates tissue growth, inhibits bacteria growth (Carpenter, 1949; Smith et al., 1945). Chronic ulcers take significantly lengthy time for its treatment where derivatives of chlorophyll show elimination of pain after several days and also improve the appearance of the infected tissue (Cady and Morgan, 1998).

Chlrophyll *a* and its derivatives also shows various antioxidant properties but presence in very little amount in microalgae (Lanfer-Marquez et al., 2005).

2.4.1 Carotenoids

Carotenoids are found in higher plants and algae which are lipid soluble colored compounds. (Wang et al., 1994) proved that it occurs as isomers, which found as all trans, 9-cis, 13-cis, 5-cis forms. It functions as accessory pigments helps in light absorbance, and protects various photosynthetic organs from light damage (Ben-Amotz et al., 1987). This pigment are usually found in endoplasmic reticulum or in chloroplasts, or in membranes of mitochondria. More than six hundred carotenoids are known (some important are astaxanthin, β-carotene, lutein, cantaxanthin etc). Chemical structure of carotenoids is based on 40-carbon polyene which is the backbone of the molecule (Figure 2.7). (Kauar et al., 2009) mentioned that the polyene system effects on carotenoids their distinctive molecular structure and their light absorbing characteristics. The oxygenated derivatives are known as xanthophylls, whereas hydrocarbon carotenoids are carotenes. Later, (Higuera-Ciapara et al., 2006) shows that oxygen can be present as combination of both as in astaxanthin, or as OH groups (as in cantaxanthin).

At present carotenoids are produced from different types of microalgae. β–carotene is a component of photosynthetic reaction center among different carotenes. β–carotene accumulated as lipid globules in the inter thylakoid spaces of chloroplast or plastids (Vorst et al., 1994). It helps to protect organisms from damage during excessive irradiances (Telfer, 2002). In various algae (like *H. pluvialis)* carotenoid are located in cytoplasmic lipid globules (Lang, 1968). Such type of extraplastidic carotenoids are known as secondary carotenoids (Grung et al., 1992).

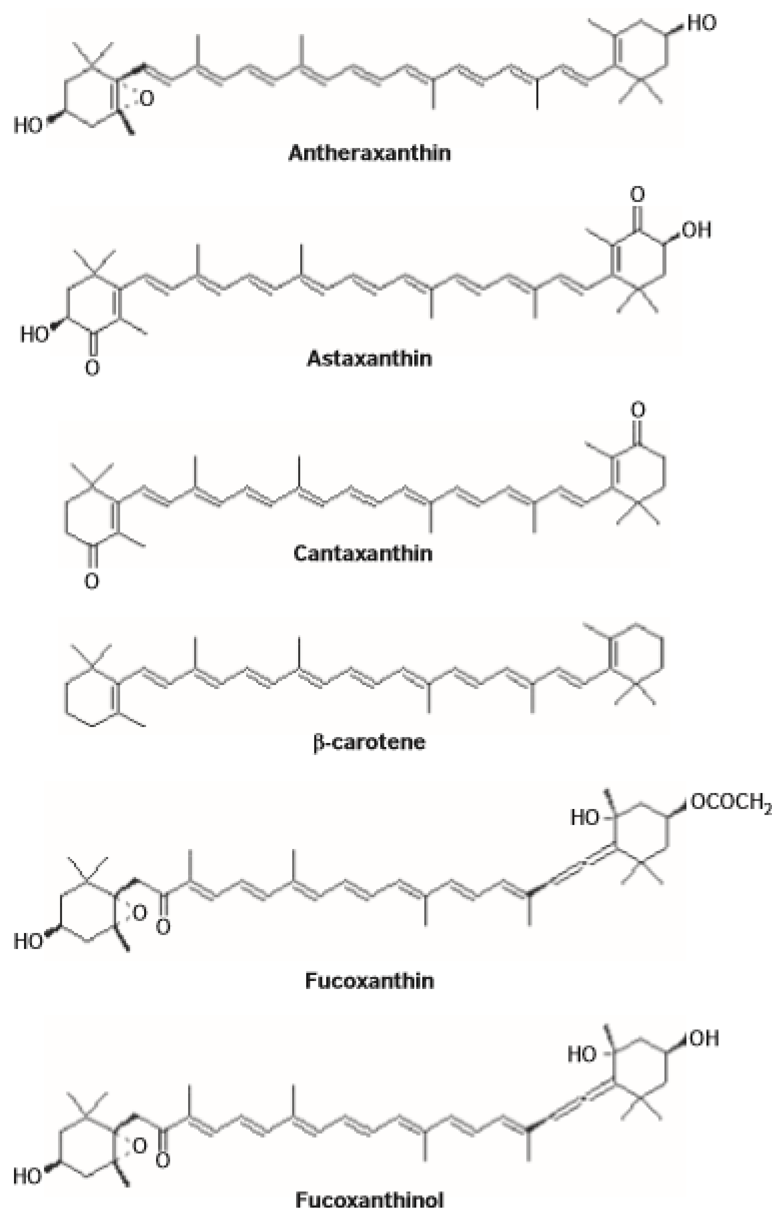

Figure 2.7: Chemical structure of Carotenoids (Antonio et al., 2019)

2.4.2.1 Applications of Carotenoids

As nutritional supplement there is an increasing demand for natural carotenoids where most carotenoids are chemically synthesized (Jin et al., 2003). This is happened because of dominating β-carotene in synthetic carotenoids and more cis forms in natural forms (Ton Laar et al., 1996). β-carotene has 10-12% less absorption rate than cis form. It is recently use in different food industry as colorant and food additives because of its provitamin activity. In addition, β-carotene has antioxidant and anticancer properties (Becker, 2004). In the field of market applications β-carotene use as provitamin A (retinol) in food and animal feed, food coloring agent, as an additive (Johnson and

Schroeder, 1995; Edge et al., 1997). In the field of nutraceuticals, cosmetics, food and feed industries xanthophyll, astaxanthin has many applications. Presently carotenoid has a major application as pigmentation source in aquaculture (Guerin et al., 2003; Cysewski and Lorenz, 2004). Recently Gurein et al. (2003) and Higuera-Ciapara et al. (2006) have been claimed carotenoid as potential element regarding health and nutritional constituents.

2.4.3 Phycobiliprotein

Phycobiliproteins are a group of photosynthetically colored proteins commonly present in cyanobacteria and red algae which possesses different functions. It consists 50% of total protein of cyanobacteria and also a great source of nitrogen reserve (Kauar et al., 2009). These have a significant anti-inflammatory, antioxidant, hepatoprotective and free radical scavenging properties which can be easily isolated and can safely be used in cosmetics and food colouring (Henrikson, 1989; Romay et al., 2000). Phycobiliproteins are present in the thylakoid membranes as phycobilisomes but not in cryptophytes (Sidler, 1994). Based on the chromophore's presence in different microalgae phycobiliproteins are classified into 3 different groups. (Gantt, 1980; Rowan, 1989; Ducret et al., 1998). These are Phycocyanin (PC), Phycoerythrin (PE), and Allophycocyanin (APC). Phycobiliproteins arise six rods of varying length which are composed of allophycocyanin and consisting phycoyanin to the proximal site and phycoerythrin to the proximal side phycobiliproteins consist allophycocyanin from which six rods arise (Figure: 2.8) (Kauar et al., 2009).

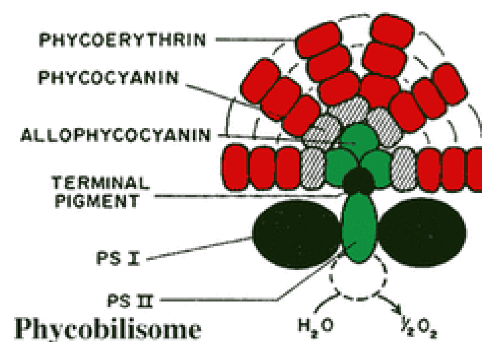

Figure 2.8: Structure of Phycobilisome (Kaur et al., 2009)

2.4.3.1 Applications of Phycobiliproteins

In today's commercial world phycobiliprotein have a great important. Primarily these are use as natural colors but so many studies show these proteins have shown a great potentiality in pharmaceutical sectors. Phycocyanin an important protein mainly uses as food items like as colorant in chewing gums, candies, soft drinks, dairy products and cosmetics like lipstick and eye liners (Santago-Santos et al., 2004). It is also use as natural colorant or dye in various industry (Batista et al., 2006). Arad and Yaron (1991) suggest that algal pigments can use in beverage and alcoholic drinks because modified pigments are stable at low pH. Pigments isolated from red algae have also use to prepare face make up, pink and purple cosmetics-eye shadow, and lipstick (Arad and Yaron, 1992). Pure phycobiliproteins also use as fluorescent labeling agents (Telfer, 2001). Both Phycocyanin and Allophycocyanin are also use as potential therapeutic agents due to their antioxidant and anti-inflammatory properties (Romay et al., 2003; Zhang et al., 2000).

2.5 Proximate Composition

Microalgae species have shown different proximate nutritional profile because of different culture pattern (Brown et al., 1997). The nutritional composition of microalgae depends on their environmental conditions, growth rates or the life cycle (Richmond, 1986). It is well known about the effect of intensity of light, fluctuation of temperature, salinity range and media types on the growth and proximate composition of microalgae (Brown et al., 1997). Overall, microalgae grown in mature harvest condition typically contain protein ranges 30-40%, lipid ranges 10-20% and carbohydrate ranges 5-20% (Renaud et al., 1999). Brown et al. (1997) give a wider range for the level of protein, lipid and carbohydrate which are 6-52%, 7- 23% and 5-23% respectively. The protein, lipid and carbohydrate value are analyzed in *Chlorella* sp., *Chaetoceros* sp., *Nannochloropsis* sp., and *Tetraselmis* sp. were determined.

2.5.1 Protein

Brown et al. (1997) states that protein composition of microalgae ranges from 6-52%. Renaud et al. (2002) found that tropical microalgae produce more 30% more of protein. There are several numbers of factors that influence the production of protein in microalgae. The optimum temperature for protein production of microalgae is within

the range of 25 ℃ and 30 ℃ (Renaud et al., 1999). Similar result also found in different studies (Oliveira et al., 1999).

2.5.2 Lipid

In an average, the percentage of lipid content ranges between 1-70% but it reached upto 90% (dry weight) in certain condition (Spolaore et al., 2006). There are several numbers of factors that influence the production of lipid in microalgae including light intensity (Yeesang and Cheirsilp, 2011), nitrogen (Illman et al., 2000) and phosphate (Reitan et al., 1994). Opute (1974) reported that, extreme high or low temperatures could reduce microalgae lipid production. Change in salinity might inhibit the growth and lipid of microalgae (Takagi et al., 2006).

2.5.3 Carbohydrate

Brown et al. (1997) stated that microalgae carbohydrate composition is range from 5-23%. According to Markou et al. (2012), several factors such as nutrient limitation and other unfavorable environmental condition might cause to the accumulation of carbohydrate in the microalgae. Carbohydrate synthesis was affected by the iron which affects the photosynthesis system (Oijen et al., 2004). Change in salinity might change the contents of carbohydrate in microalgae (Zhila et al., 2011). Previous studies have indicated that the production of carbohydrate was reduced due to the copper toxicity (Markou et al., 2012). It was also generally accepted that the increasing of the carbohydrate production was resulted from the increasing of light intensity.

Chapter-3: Materials and Methods

The present study was carried at Microalgae Laboratory, Faculty of Fisheries, Chattogram Veterinary and Animal Sciences University. During the experiment optimum condition of all parameters were maintained. The experiment was carried out from August 2019 to January 2020.

In order to achieve the objectives of the research, the following methodologies were followed,

3.1 Glassware Preparation

In dealing with life sciences, axenic condition is often maintained to prevent contamination and avoid influence from other traces of chemicals that could affect the experiment. Therefore, it is important to first clean all glassware according to the correct procedures.

Glassware made from either alumina silicate glass or borosilicate was chosen. Either of these types of glass is satisfactory for initial use of preparation and dispersing of growth media (in this experiment, growth media is referred to Conway media). Growth media are generally dispensed into flasks of a variety of sizes, depending upon the specific future uses of the media. Opening of flask should be plugged with sterile cotton, plastic closures or screw caps. Screw cap flasks are generally used to maintain sterile cultures for a long period of time for these types has been found to retard evaporations. It is important to prevent contamination of media during sterilization; therefore glassware must be washed or clean in specific ways. Glassware is often washed in a phosphate-free detergent followed by soaking in concentrated acid solution. Glassware was washed according to the procedures as follow:

Glassware was washed and cleaned using a phosphate-free detergent (Extron). They were then rinsed with tap water carefully and thoroughly. Next they were soaked in 1M HCl acid solution for at least 10 minutes, and were rinsed three times in flowing water, then another three times in double distilled. Lastly, glassware was dried in the oven overnight and after drying, opening of flasks were covered with aluminum foil and keep in dust free cabinet.

3.2. Media Preparation

3.2.1 Filtration, Sterilization and Preservation of Seawater

Seawater was collected from Saint Martin's coast of Bay of Bengal, Bangladesh. After collection, the water was preserved for the settlement of solid waste. Then water was filtered in the laboratory by using 45 mm glass microfiber filters (GF/C) mounted on filter pump in order to remove suspended solids and waste materials. Then the filtered water was autoclaved at 121 °C temperature and 15lbs pressure for 15 minutes. The filtered and autoclaved water was stored in a cold room maintained at 20-21 ºC temperature until further use (Reda et al., 2013).

3.3 Conway Medium Preparation

Now the glassware was all prepared, microalgae can be cultured however its media (Conway) has to be initially prepared. Conway medium involved the micronutrient, trace metal solution, and vitamin (Tompkin et al., 1995). Pure Conway medium was used for *Chlorella* sp., *Nannochloropsis* sp., *and Tetraselmis* sp. culture*;* however*,* Conway medium + silicate solution was used for *Chaetoceros* sp. culture. Table 3 shows the amount of different constituents. To prepare 1L Conway media, 1 mL of solution A, 0.5 mL of solution B and 0.1 mL of solution C, were added with 28-30 g/L autoclaved seawater (Table 3.1).

Table 3.1: Preparation of Conway Medium (Tompkins et al., 1995)

(A) Main Mineral Solution	
Names of Chemicals	**Quantity**
$NaNO_3/KNO_3$	100.00 g/116.00 g
Disodium EDTA ($C_{10}H_{16}N_2O_8$)	45.00 g
H_3BO_3	33.60 g
$NaH_2PO_4.4H_2O$	20.00 g
$FeCL_3.6H_2O$	1.30 g
$MnCL_2.4H_2O$	0.36 g
Trace metal solution	1.00 mL
Dissolving in deionized/distilled water and make the volume 1 L	
(B) Trace Metal Solution	
Names of Chemicals	**Quantity**
$ZnCl_2$	2.10 g
$CoCl_3.6H_2O$	2.00 g
$(NH_4)_6MO_7O_2.4H_2O$	0.90 g
$CuSO_4.5H_2O$	2.00 g
Dissolving in deionized/distilled water and make the volume 1 L	
(C) Vitamin Solution	
Names of Chemicals	**Quantity**
Thiamine, B1	0.20 g
Cyanocobalamin, B12	0.01 g
Dissolved in deionized / distilled water and make the volume 100 mL	
(D) Silicate Solution	
Names of Chemicals	**Quantity**
Sodium silicate (Na_2SiO_3)	20.00 g
Dissolving in deionized/distilled water and make the volume 1 L	

To prepare 1L Conway media 1 mL of solution A, 0.5 mL of solution B and 0.1 mL of solution C were added with 28-30 ppt autoclaved seawater.

To prepare Conway+ silica media 1 mL of solution D was added with 1 L Conway medium

3.4 Physical Parameter Analysis of Seawater and Media

Physical properties of seawater including temperature, dissolved oxygen (DO), salinity, and pH were measured using thermometer, HANNA-HI9146 DO meter, Refractrometer and pH meter (Blue lab pH pen) respectively in the laboratory.

3.5 Collection of Microalgae, Culture and Maintenance

Initially four different types of indigenous marine microalgae (*Chlorella* sp., *Chaetoceros* sp., *Nannochloropsis* sp., *and Tetraselmis* sp.) were collected from previously isolated and preserved sample at Department of Aquaculture, Faculty of Fisheries, Chattogram Veterinary and Animal Sciences University, Bangladesh. The pure sample were cultured in Conway culture medium at 25 ± 2 °C temperature with maintaining 24hours continuous light at 150 μEm^{-2} s^{-1} intensity. After that, the stock were scaled up and sub culturing was done for growth curve determination. Each species were cultured separately for maintaining pure stock and to inhibit contamination.

3.6 Determination of Growth Curve

Growth curves experiment was performed to determine the growth pattern and to fix the stationary phase of the selected microalgae species for other analysis. A total of 300 mL of culture volume was maintained in a sterile 500 mL borosilicate Erlenmeyer flask for each. Out of the 300 mL, 270 mL was culture medium and 30 mL was stock culture (Pure Conway medium for *Chlorella* sp., *Nannochloropsis* sp., *Tetraselmis* sp., and Conway medium with silicate solution for *Chaetoceros* sp.). Three replications were maintained for each species. The experiment was conducted until death phase of each species. Growth curve was determined on the basis of cell density (cells/mL), and optical density (780nm for *Chlorella* sp., 780 nm for *Nannochloropsis* sp. 480 nm for *Tetraselmis* sp. and 750 for *Chaetoceros* sp.). The culture were maintained with a constant temperature range between 25 ± 2 °C at 24 hours 150 $\mu Em^{-2}s^{-1}$ light intensity by using fluorescent light with continuous aeration by using natural sterile air pump. The experiment was continued until the death phase of each species.

3.7 Experimental Design

For growth, productivity, chlorophyll, phycobilioproteins, carotenoids and proximate composition were determined for each species, 12 autoclaved Erlenmeyer flasks (3 for each species) were filled with 1.5 L Conway culture medium (For *Chlorella* sp., *Nannochloropsis* sp., *Tetraselmis* sp.) and Conway+silica (for *Chaetoceros* sp.) medium. Then 5% of pure stock culture was inoculated in each flask. Three flasks were used for each species and cultured in separate to avoid the contamination. The culture was maintained with a constant temperature range between 25 ± 2 °C at 24 hours 150 $\mu Em^{-2}s^{-1}$ light intensity by using fluorescent light with continuous aeration by using natural sterile air pump.

Biomass was taken in every alternate days and culture volume data was recorded every day to determine productivity. For chlorophyll 10 mL of each culture were filtered by using 47 mm Ø Whatman® GF/C glass microfiber filter papers at their stationary phase. For Carotenoids 1mL aliquot solution of each culture was collected in 15mL centrifuge tube. Finally all the cultures were harvested at their stationary phase based on the growth curve experiment. For harvest, centrifuging method was used (Hitachi* High-speed Refrigerated Centrifuge, himac CR 21g-II). The biomass was dried at 60 °C temperature over night by using dry oven and preserved at normal fridge temperature for pigments and proximate analysis.

3.8 Determination of Growth curves Parameters

3.8.1 Cell Density

Microalgal cells were counted using hemacytometer every day during the data collection of growth curve. For a clear view the meter and its cover slip (Bright- line improved Neubauer hemocytometer, 0.0025 mm^2, 0.1 mm deep chambers, Assistent, Germany) were wasshed. Distill water were used for filling the hemocytometer chamber. 4x and 10x magnification (Nikon E600) were used to check the distributions of cells. For a fixation and clearance Lugol's iodine were used. In the both stages of hemocytometer's cells were counted at 40x magnification. Finally the cell density were calculated by using the following formula:

$$\text{Cell density (cell/mL)} = \frac{\text{Total number of cells counted}}{50\times4} \times 10^6$$

Here, 50 indicated the square of the the 2 chambers and 4 x 10^{-6} indicated amount(volume) of samples upon the small square areas (0.004 mm^3 (0.2 mm x 0.2 mm x 0.1 mm), expressed in cm^3 or mL).

3.8.2 Optical Density

Optical denisty of culture aliquots were measured using a spectrophotometer (UV-VIS Double beam, Model-T80, HANNA), every day during the data collection of growth curve. The culture medium for the species was used as the blanks. The absorbance were measured at the wavelength 780 nm for *Chlorella* sp., 780 nm for *Nannochloropsis* sp., 480 nm for *Tetraselmis* sp., and 750 for *Chaetoceros* sp.

3.8.3 Biomass (Dry Weight Basis)

Biomass was estimated every alternate day using 1 mL microalgae samples from each cultures, filtered through pre-weighed (Rinsed with 1 mL distilled water, and oven dried at 60 ºC for 4 hours followed by 1 hour desiccation) glass microfiber filter paper. Then the filter paper with biomass was oven dried again at 60 ºC for 4 hours followed by 1 hour desiccation. After that, the dry biomass concentration was calculated by dividing the difference between the weights of the dried filter paper (pre and post filtration) by the filtered volume (Lavens and Sorgeloos, 1996).

3.9 Productivity

Three types of productivity were calculated which are volumetric (Green et al., 1995) areal (Ugwu et al., 2008) and lipid (Benemann and Tilleta, 1987) productivity.

3.9.1 Volumetric Productivity

Productivity was calculated at the end of the stationary phase of particular microalgae. Volumetric productivity (VP) indicates the average daily productivity of a culture in terms of dry weight basis. Following equation was used to calculate the volumetric productivity:

$$VP = (X_n - X_0)/ N$$

Where, X = Final biomass, X_0 = Initial Biomass and N = Culture days

Expressed as mg/L/ day

3.9.2 Areal Productivity

Areal productivity (AP) is the productivity of an area occupied by the microalgae and it is calculated by the daily productivity of microalgae. Following equation was used to calculate Areal productivity:

$$AP = (VP \times V)/A$$

Where, VP = Volumetric Productivity

V = Total Volume of the culture

A = A = surface area occupied ground.

Expressed as $g/cm^2/day$

3.9.3 Lipid Productivity

Lipid productivity (LP) is the amount of lipids produced by microalgae in 1 day which is during early stationary phase. The lipid productivity was calculated using lipid content and volumetric productivity during early stationary phase. Following equation was used to calculate the lipid productivity:

$$LP = VP \times (\%\ lipid/100)$$

Where,

VP = volumetric productivity of the PBR and

% lipid = lipid content.

Expressed by mg/L/day

3.10 Determination of Chlorophyll a, b, and c (Trichormatic method)

3.10.1 Extraction of Microalgae for Chlorophyll Determination

For extraction 10 mL of each sample was filtered (47 mm Ø Whatman® GF/C glass microfiber filter papers.). Filtered sample placed in an airtight plastic bags and stored frozen for 3 weeks. After 3 weeks each sample filter placed in a centrifuge tube with 2-3mL 90% aqueous solution (Mixing of 90 parts of Acetone with 10 parts of $MgCO_3$ Solution) and macerated at 500rpm for 1 minute. Then the sample volume was adjusted up to 10 mL with 90% aqueous acetone solution. After this step, the samples were steeped for 2 hours at 4 °C temperature. After 2 hours the samples were clarified by centrifuging in closed tubes for 20 minutes at 500g. Then the clean extract was separated in new tubes.

3.10.2 Determination

Chlorophylls were determined According to Aminot et al. (2001). The clean extract was transferred to a 1cm cuvette and measured optical density (OD) at 750, 664, 647 and 630 nm. OD 664, 647, and 630 were used for chlorophyll determination where OD750 nm was used as turbidity correction factor. This value was subtracted from each of the pigment OD values of the other wavelengths before using them in the equations below:

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

b) $C_b = 21.03(OD647) - 5.43(OD664) - 2.66(OD630)$

c) $C_c = 24.52(OD630) - 7.60(OD647) - 1.67(OD664)$

Where, C_a, C_b, and C_c = concentrations of chlorophyll a, b, and c, respectively in µg/L, and OD664, OD647, and OD630 = turbidity corrected optical densities (with a 1-cm light path) at the respective wavelengths.

After determining the concentration of pigment in the extract, following calculation was applied to determine the amount of pigment per unit volume:

$$\text{Chlorophyll } (\mu g/L) = \frac{\text{Chlorophyll a} \times \text{Extract Volume in mL}}{\text{Volume of sample in L}}$$

3.11 Determination of Carotenoids

One mL aliquot of the algal suspension of each culture were taken at their stationary phase. Then the sample were centrifuged at 1000g for 5 minutes to obtain pellet. Afterwards, the pellet was extracted with 3 mL 2:1 of ethanol: hexane (v/v). Then the pellet with the solvent was shaken vigorously, and centrifuged again at 1000g for 5 minutes. Thus, the hexane layer was separated, and its absorbance was determined using spectrophotometer at the wavelength of 450 nm. The amount of extracted carotenoids from the samples in micrograms was determined by multiplying the absorbance (A_{450}) with 25.2 (Shaish et al., 1992).

3.12 Phycobiliproteins

3.12.1 Extraction of Phycobiliproteins

The cultures were centrifuged at 6,000 rpm at room temperature for 15 minutes to harvest the pellet. The cell pellets were rinsed 2-3 times with distilled water. These biomasses were dried in oven at 40 ºC overnight. Dried powder (40 mg) was then soaked in 10 mL phosphate buffer (pH 7.0; 0.1 M), mixed well using vortex mixture, and then stored at 4 ºC for 24 hours. Phycobiliproteins were extracted by centrifuging at 6000 rpm for 10 minutes. Finally, the supernatant was collected and absorbance was measured spectrophotometrically (UV-VIS Double beam, Model-T80, HANNA) at the wavelength 562, 615, and 652 nm; phosphate buffer was used as blank

3.12.2 Spectrophotometric Estimation of Phycobiliproteins

The concentration of phycocyanin (PC), phycoerythrin (PE) and allophycocyanin (APC) in the sample was calculated by spectrophotometer (UV-VIS Double beam, Model-T80, HANNA) and using equations and the extinction coefficients from Siegelman and Kycia (1978) as follows:

Phycocyanin (PC) mg/mL

$= \{A_{615} - (0.474 \text{ x } A_{652})\} / 5.34$

Allophycocyanin (APC) mg/mL

$= \{A_{652} - (0.208 \text{ x } A_{615})\} / 5.09$

Phycoerythrin (PE) mg/mL

$= \{A_{562} - (2.41 \text{ x PC}) - (0.849 \text{ x APC})\} / 9.62$

Total phycocyanin, phycoerythrin and allophycocyanin (mg/mL) were calculated according to Silveira et al. (2007), $\frac{\text{Pigment concentration X V}}{\text{DB}}$

Where, V= solvent volume, DB= Dried biomass

3.13 Determination of Proximate Composition

From dried preserved sample, proximate compositions were analyzed.

3.13.1 Protein Determination

Protein was determined according to Lowry et al. (1951). For each sample, 25 mL well mixed samples were prepared by using 5mg dried biomass with distilled water. 0.5 mL

from 25 mL solution was taken for each sample for protein analysis. 1 mL Reactive 1 mixed with 50 mL of Reactive 2. Then, 0.5 mL sample and 0.5 mL 0f 1N NaOH was mixed and placed in a hot water for 5 minutes bath at 100 ℃. Subsequently, the samples were cooled in a cold-water bath, and 2.5 mL of the prepared mixed reagent was added 10 minutes after cooling. After that, 0.5 mL of Falin reagent was added to the mixed reagent, and then kept in a dark place for 30 minutes. The absorbance of the mixed solution was measured using spectrophotometer at 750 nm wavelength. To develop a calibration graph, 2000 µg/L of standard (albumin) stock solution was prepared, and a series of standards were prepared (20 µg/L, 40 µg/L, 80 µg/L, 100 µg/L and 200 µg/L) from the stock solution. The same procedures as described for protein analysis were applied for the standard series; a calibration line was plotted according to the absorbance, and the protein composition for each sample was determined accordingly.

3.13.2 Lipid Determination

Lipid was determined according to Bligh and Dyer (1959), and Folch et al. (1957). For each sample, an aluminum dishes were labeled and weighted as initial weight. Then 50 mg of each sample was taken in a centrifuge tube, and diluted into 5x volume using distilled water. Then, 3 mL 2:1 of methanol:chloroform (v/v) was mixed with the sample homogenously using tissue homogenizer. After that, all the tubes were centrifuged for 4 minutes at 1000 rpm at 4 ℃; the supernatants were transferred into clean tubes by Pasteur pipette, and placed them in ice. Again, 3 mL 2:1 of methanol:chloroform (v/v) was mixed with the sample homogenously. After that, the tubes were centrifuged at same conditions again, and supernatants were transferred to the previous tubes of supernatants. In this combined supernatant, 1.5 mL of 0.9% NaCl was mixed using vortex mixture. Then the tubes were kept in the refrigerator for 1 hour at 4 ℃ temperature. After 1 hour, the tubes were centrifuged for 10minutes at 1000 rpm at 4 ℃ temperature. The upper layer of methanol and chloroform was discarded, while, the lower layer was transferred in previously made aluminum dish. The solvent was then evaporated at 60 ℃ by hot air oven. Afterwards, the aluminum dishes were weighed to get the final weight. Finally, initial weight was subtracted from the final weight to get the lipid weight in the samples.

3.13.3 Carbohydrate Determination

Carbohydrate was determined according to Dubois et al. (1956). For each sample, 5 mg freeze dried biomass was taken to prepare a 25 mL well mixed (tissue homogenizer) solution using distilled water. Afterwards, 1 mL from 25 mL solution was taken for each samples, and then 1 mL of 5% phenol solution and 5 mL of concentrated sulfuric acid were added into it. Then, the samples were placed in cold water bath. When cooled, absorbance of the solution was taken at 488 nm wavelength using spectrophotometer to estimate carbohydrate. To produce a calibration graph, 1000 µg/L of standard (glucose) stock solution was prepared, and subsequently, a series of standards at various dilution (20 µg/L, 40 µg/L, 60 µg/L, 100 µg/L, and 140 µg/L) were also prepared from the stock solution. For the standard graph the identical procedure were repeated using carbohydrate analysis and applied to standard series. A standard graph was plotted according to the standard results obtained from the absorbance, and the carbohydrate composition for every sample was determined accordingly.

3.14 Statistical Analysis

Mean and standard error of mean were calculated using MS excel. Line diagram was used to demonstrate growth curve where bar diagram was used for productivity, pigments and proximate compositions. Regression line was set to analyze the r value and level of significance of growth curves. Single factors ANOVA was set to determine the significant relationship among the species on aspects of productivity, pigments, and proximate compositions. When assumptions were met, Post Hoc significance different test was applied to find out the difference among the microalgae species. The level of significance was set 0.05. These test were performed by using IBM SPSS (v. 26.0) statistical software.

Chapter- 4: Results

4.1 Water Quality Parameters of Conway Media

The physical characteristics of media water are shown in Table 4.1. The data were recorded before and after autoclave. For physical properties, there was a slight change before and after autoclave, where pH (7.85) and dissolved oxygen (4.83 mg/L) slightly decrease to 7.72 and 4.53 mg/L respectively. On the other hand, temperature (24.6 ºC) and salinity (29.3 ppt) slightly increase to 25.2 ºC and 30.1 ppt. Overall, there is no significance difference ($p > 0.05$) for all physical properties before and after autoclave.

Table 4.1: Physical properties of media water before and after autoclave

Values are mean ± standard error (Where n=3)

Physical Properties	Before Autoclave	After Autoclave
pH	7.85 ± 0.12	7.72 ± 0.17
Temperature	24.6 ± 0.63	25.2 ± 0.70
Dissolve Oxygen	4.83 ± 0.68	4.53 ± 0.53
Salinity	29.3 ± 0.38	30.1 ± 0.11

4.2 Growth Parameter Analysis

4.2.1 Growth of *Chlorella* sp. in Conway Media

For growth curve analysis of microalgae there were two different parameters (cell density (cells/mL), and optical density) were measured to ensure the accuracy of the data. Therefore, co-relation between those parameters were analyzed for each species.

Figure 4.1 shows the growth curve of *Chlorella* sp. in terms of cell density (cells/mL) and optical density in Conway culture medium. In the figure, based on the cell density and optical density the growth was compared. *Chlorella* sp. showed its lag phase between day 1 and 2. It had shown that, between Day 3 to Day 6, the exponential phase of *Chlorella* sp. occurred. On the day 7 there was transition in the growth to the early stationary phase. On day 8, the culture was reached at their stationary phase. On Day 9 onward, *Chlorella* sp. was reached at death phase as cell density and absorbance decrease drastically. These data (cell density and optical density) are strongly positively correlated with 1% level of significance.

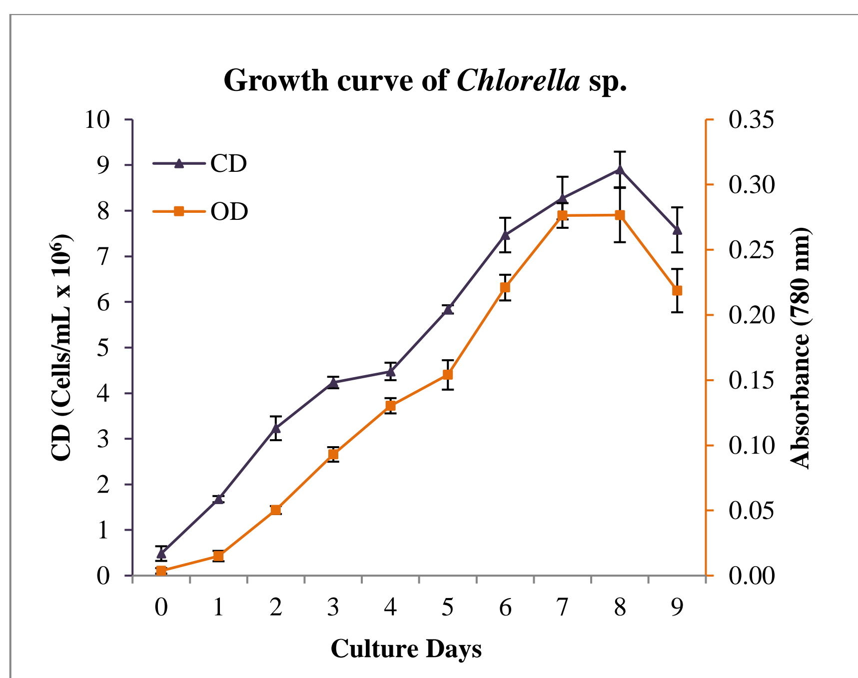

Figure 4.1: Growth curve of *Chlorella* sp. cultured in Conway media on the basis of cell density and optical density in respect of culture days.
Values are mean ± standard error (n=3)

4.2.2 Growth of *Chaetoceros* sp. in Conway Media

Figure 4.2 showed the growth curve of *Chaetoceros* sp. in respect of cell density (cells/mL) and optical density in Conway + silica culture medium. In the figure, in terms of cell density (cells/mL) and optical density the growth was compared. *Chaetoceros* sp. showed its lag phase between day 1 and 2. It had shown that, between Day 3 to Day 6, the exponential phase of *Chaetoceros* sp. occurred. At day 8, the culture was reached at their stationary phase. On Day 9 onward, *Chaetoceros* sp. was reached to death phase as cell density decrease drastically. These data (cell density and OD) are strongly positively correlated with 1% level of significance.

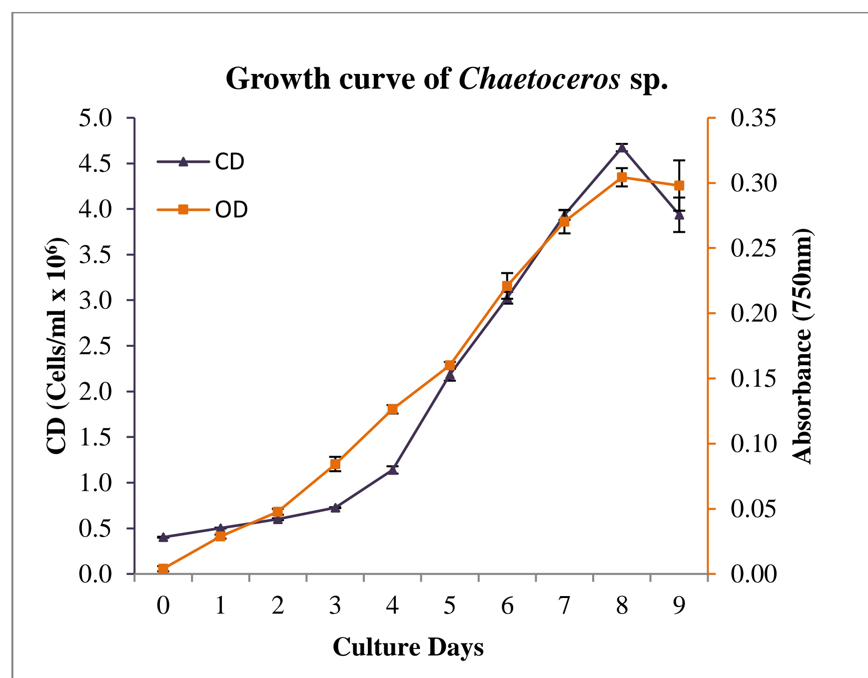

Figure 4.2: Growth curve of *Chaetoceros* sp. cultured in Conway media on the basis of cell density and optical density in respect of culture days.

Values are mean ± standard error (n=3)

4.2.3 Growth of *Nannochloropsis* sp. in Conway Media

Figure 4.3 showed the growth curve of *Nannochloropsis* sp. in respect of cell density cells/mL and optical density in Conway culture medium. In the figure, in terms of cell density (cells/mL) and optical density the growth was compared. *Nannochloropsis* sp. showed its lag phase between day 1 and 2 and exponential phase between day 3 to day 7. On the day 8 there was transition in the growth to the early stationary phase. On day 10, the culture was reached to at their stationary phase. On day 11 onward, *Nannochloropsis* sp. was reached at death phase as cell density decrease drastically. Growth parameters in terms of cell density and optical density are also strongly positively correlated with 1% level of significance.

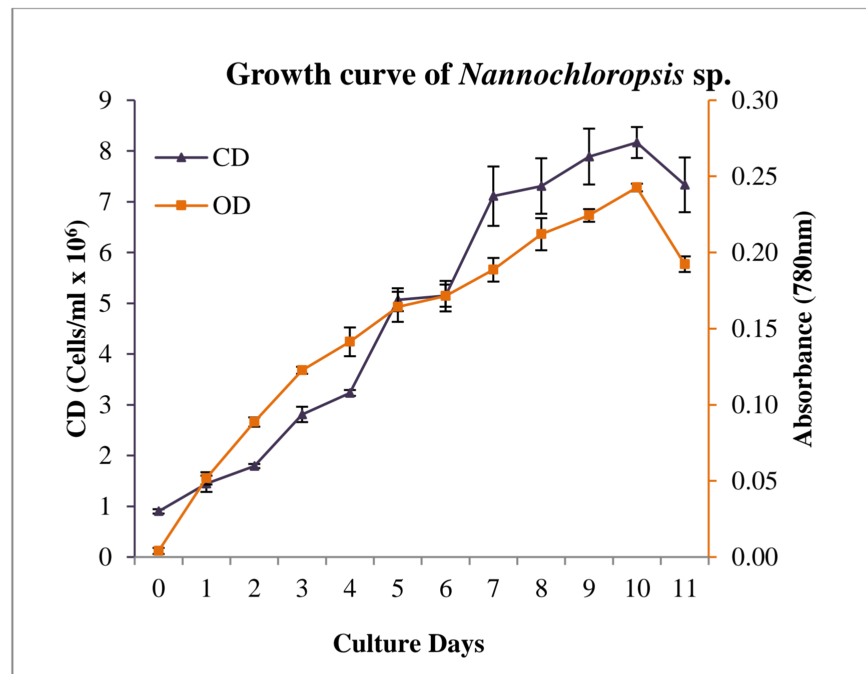

Figure 4.3: Growth curve of *Nannochloropsis* sp. cultured in Conway media on the basis of cell density and optical density in respect of culture days.

Values are mean ± standard error (n=3)

4.2.4 Growth of *Tetraselmis* sp. in Conway Media

Figure 4.4 showed the growth curve of *Tetraselmis* sp. in respect of cell density (cells/mL) and optical density in Conway culture medium. In the figure, in terms of cell density (cells/mL) and optical density the growth was compared. *Tetraselmis* sp. showed its lag phase between day 1 and 2 and exponential phase between day 3 to day 6. On day 7, the culture reached at stationary phase. On day 8 onward, *Tetraselmis* sp. reached culture was reached at their death phase as cell density decrease drastically. Cell density, biomass and optical density data are strongly positively correlated with 1% level of significance.

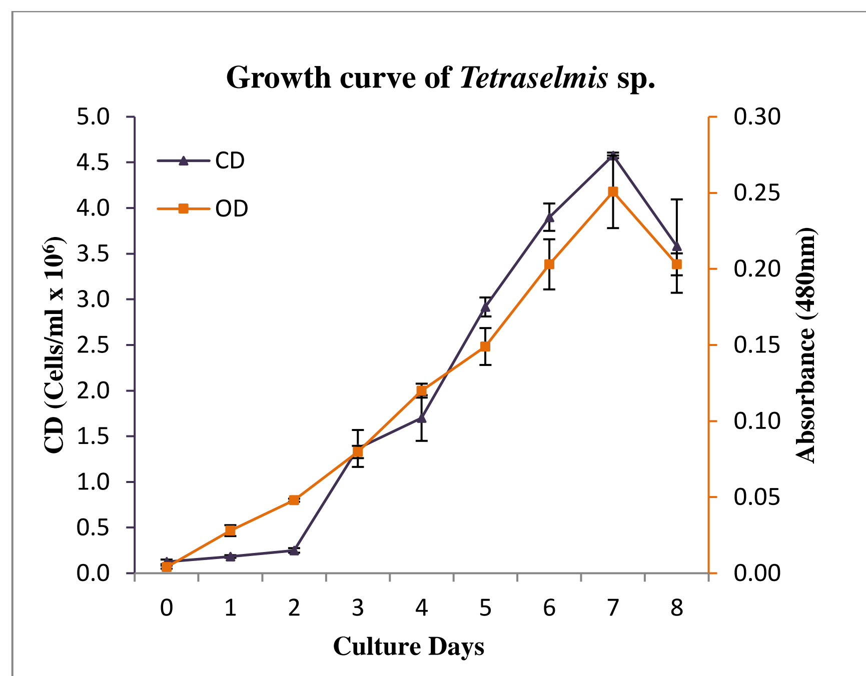

Figure 4.4: Growth curve of *Tetraselmis* sp. in cultured Conway media on the basis of cell density and optical density in respect of culture days.

Values are mean ± standard error (n=3)

4.3 Productivity

4.3.1 Volumetric Productivity

Figure 4.5 showed the volumetric productivity (mg/L/Day) of four microalgae in Conway media. Result showed that *Chaetoceros* sp. had significantly ($p < 0.05$) higher volumetric productivity (0.61 ± 0.08 mg/L/Day) compared to *Tetraselmis* sp. showed (0.57 ± 0.06 mg/L/Day), *Nannochloropsis* sp. (0.45 ± 0.04 mg/L/Day) and *Chlorella* sp. (0.39 ± 0.03 mg/L/Day).

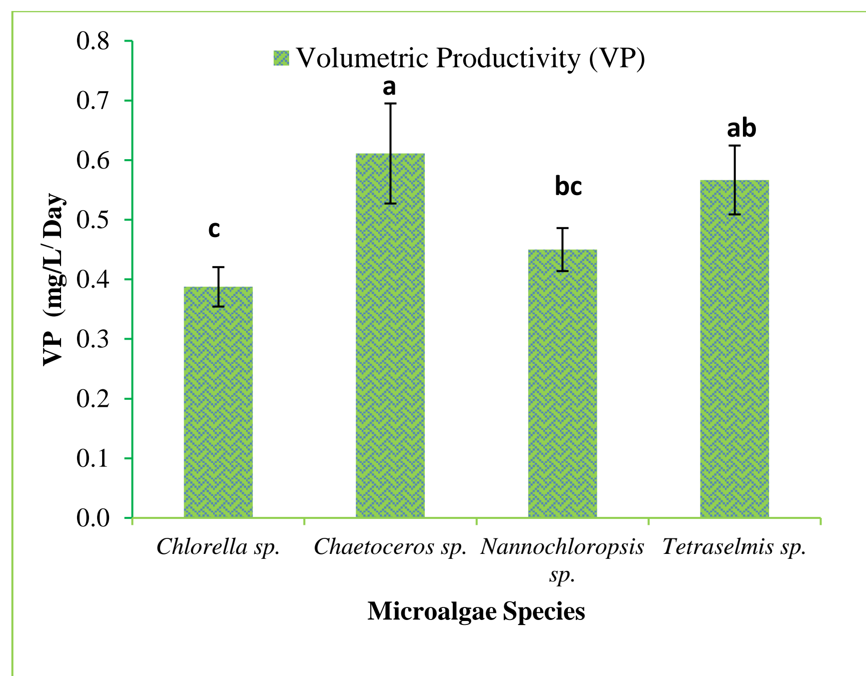

Figure 4.5: Volumetric productivity of selected microalgae cultured in Conway culture media.

Values are mean ± standard error (n=3)

4.3.2 Areal Productivity

Figure 4.6 showed the areal productivity (mg/cm^2/Day) of microalgae in Conway media. *Chaetoceros* sp. had showed significantly ($p < 0.05$) higher areal productivity (1.2 ± 0.17 mg/cm^2/Day) followed *Tetraselmis* sp. showed (1.1 ± 0.07 mg/cm^2/Day), *Nannochloropsis* sp. showed (0.89 ± 0.07 mg/cm^2/Day) and *Chlorella* sp. (0.76 ± 0.06 mg/cm^2/Day).

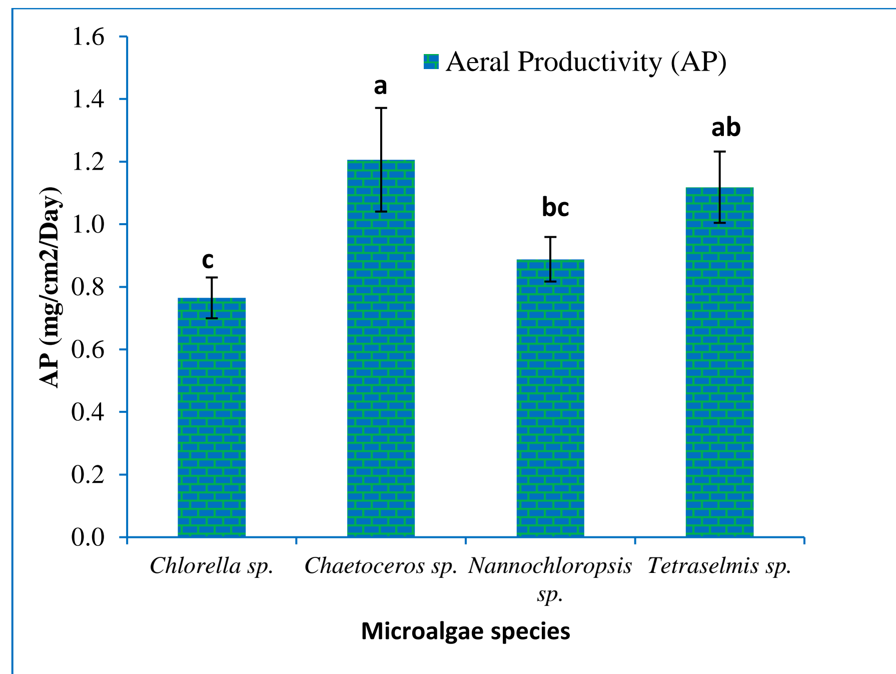

Figure 4.6: Areal productivity of selected microalgae cultured in Conway culture media.

Values are mean ± standard error (n=3)

4.3.3 Lipid Productivity

Figure 4.7 showed the lipid productivity (mg/L/Day) of microalgae in Conway media. *Chaetoceros* sp. (0.109 ± 0.003 mg/L/Day) and *Tetraselmis* sp. (0.108 ± 0.004 mg/L/Day) had significantly ($p < 0.05$) higher lipid productivity compared to *Nannochloropsis* sp. (0.051 ± 0.014 mg/L/Day) and *Chlorella* sp. (0.047 ± 0.003 mg/L/Day).

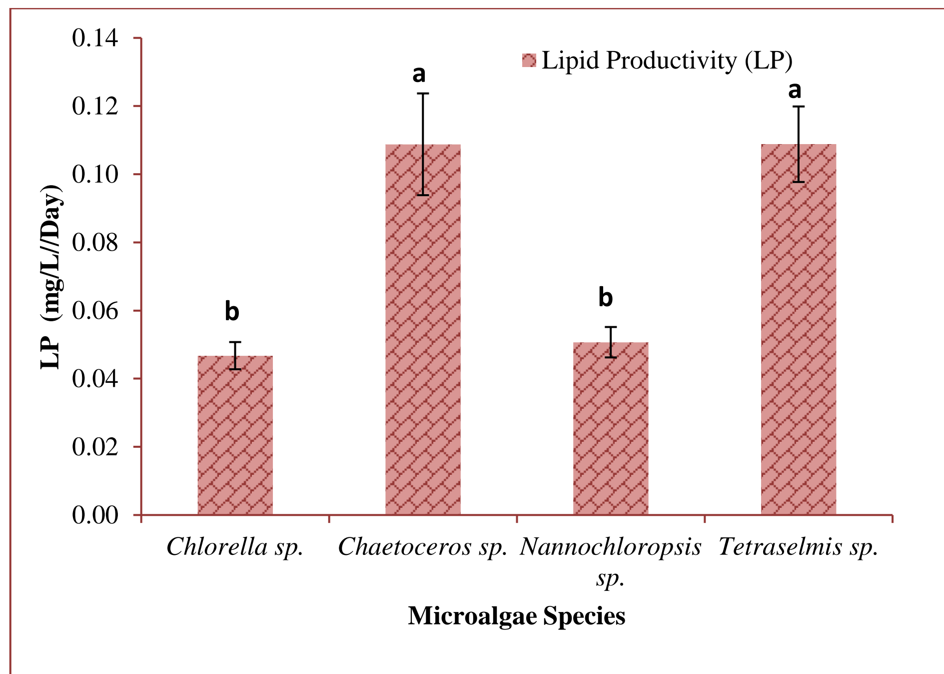

Figure 4.7: Lipid productivity of selected microalgae cultured in Conway culture media.

Values are mean ± standard error (n=3)

4.4 Chlorophyll Content of Microalgae in Conway Media

Figure 4.8 showed Chlorophyll a, b and c production (µg/L) of four species of marine microalgae cultured in Conway culture media.

Based on Figure 4.8, concentration of chlorophyll a was reported significantly ($p < 0.05$) higher (2.68 ± 0.04 µg/L) in *Tetraselmis* sp. followed by *Chaetoceros* sp. (1.3 ± 0.09 µg/L), *Chlorella* sp. (0.48 ± 0.05 µg/L) and *Nannochloropsis* sp. (0.48 ± 0.04 µg/L).

On the other hand, in case of chlorophyll b Figure 4.8 showed the same trend like chlorophyll a and it was found that *Tetraselmis* produced significantly ($p < 0.05$) higher concentration (1.23 ± 0.02 µg/L) compared to the *Chlorella* sp. (0.19 ± 0.05 µg/L) *Nannochloropsis* sp. (0.046 ± 0.003 µg/L) and *Chaetoceros* sp. (0.039 ± 0.02 µg/L).

However, in case of chlorophyll c Figure 4.8 showed that *Chaetoceros* sp. produced significantly ($p < 0.05$) higher concentration (0.29 ± 0.01 µg/L) compared to the other three species such as *Tetraselmis* sp. (0.10 ± 0.01 µg/L), *Chlorella* sp. (0.06 ± 0.01 µg/L) and *Nannochloropsis* sp. (0.01 ± 0.0 µg/L).

All the four microalgae species were produced higher chlorophyll a compare to the chlorophyll b and c.

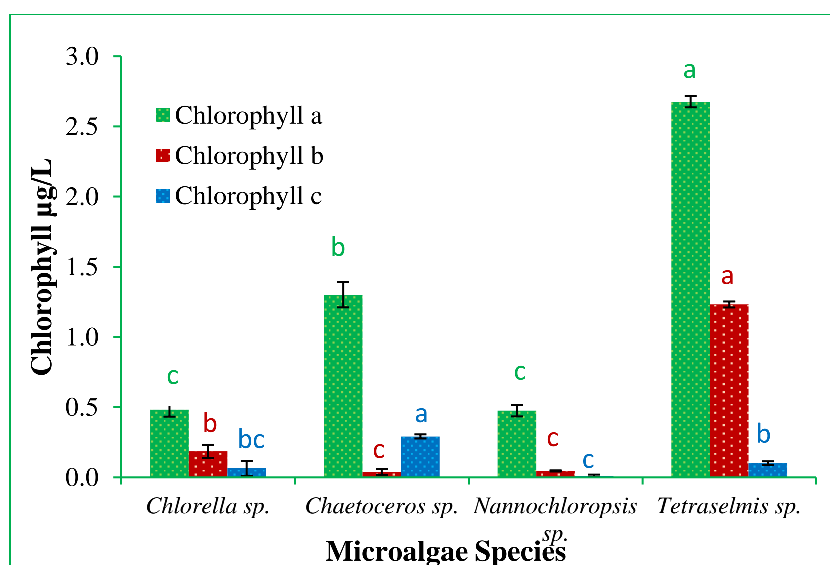

Figure 4.8: Chlorophyll a, b and c production of selected microalgae cultured in Conway culture media. Values are mean ± standard error (n=3)

4.5 Carotenoid Contents of Microalgae in Conway Media

Carotenoid content (µg/mL) in four species of microalgae cultured using Conway media are showed in Figure 4.9. Results showed that carotenoid production varies largely on the basis of the species. However, among these four species *Nannochloropsis* sp. produced significantly ($p < 0.05$) higher concentration (1.68 ± 0.3 µg/mL) of carotenoids, where *Tetraselmis* sp. produced (1.51 ± 0.05 µg/mL), *Chaetoceros* sp. (1.36 ± 0.2 µg/mL) and *Chlorella* sp. produced the lowest concentration (0.56 ± 0.02 µg/mL) of carotenoids.

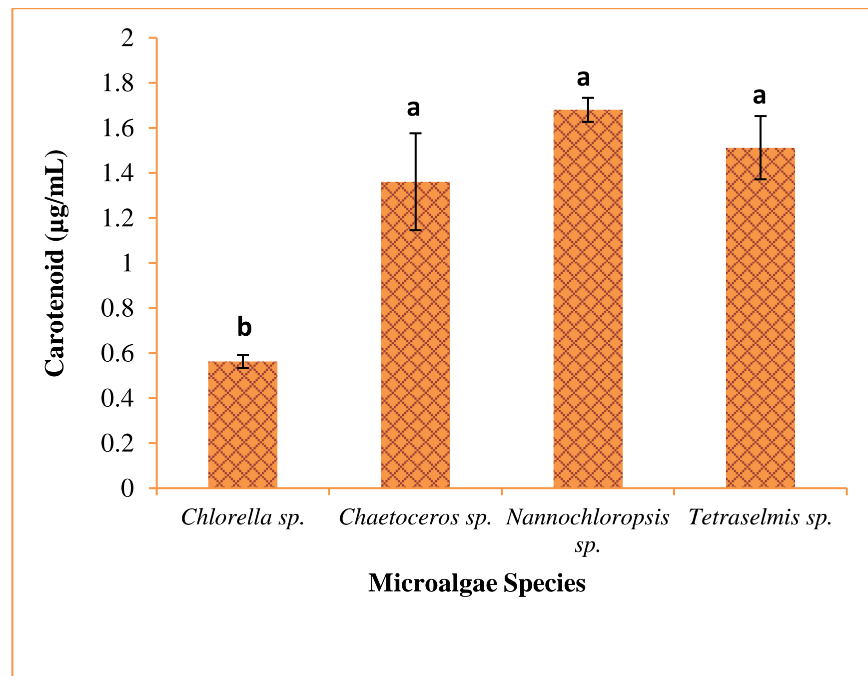

Figure 4.9: Carotenoid production of selected microalgae cultured in Conway culture media.

Values are mean ± standard error (n=3)

4.6 Phycobiliprotein Production in Microalgae

Figure 4.10 showed the phycobiliproteins (Phycocyanin, Allophycocyanin and Phycoerythrin) production (mg/mL) of four species of marine microalgae cultured in Conway media. Based on Figure 4.10, concentration of Allophycocyanin was reported significantly ($p < 0.05$) higher (0.0197 mg/mL ± 0.0006) in *Nannochloropsis* sp. rather than other species (*Tetraselmis* sp. (0.0113 ± 0.0004 mg/mL), *Chlorella* sp. (0.0103 ± 0.0005 mg/mL) and *Chaetoceros* sp. (0.0100 ± 0.0006 mg/mL). In case of phycoerythrin content there was significant differences ($p < 0.05$) among the all four species of microalgae. In this case, *Nannochloropsis* produced the highest concentration (0.0029 ± 0.0002 mg/mL) followed by *Chlorella* sp. (0.0023 ± 0.05 mg/mL) *Chaetoceros* sp. (0.0019 ± 0.0002 mg/mL) and *Tetraselmis* sp. (0.0018 ± 0.0001 mg/mL) (Figure 4.10). Finally, in case of phycocyanin there was no significant ($p > 0.05$) differences among the all four species of microalgae. However, Figure 4.10 showed that *Nannochloropsis* sp. produced higher concentrations (0.0027 ± 0.0006 mg/mL) compared to *Chlorella* sp. (0.0025 ± 0.0005 mg/mL), *Tetraselmis* sp. (0.0018 ± 0.0004 mg/mL) and *Chaetoceros* sp. (0.0017 ± 0.0005 mg/L).

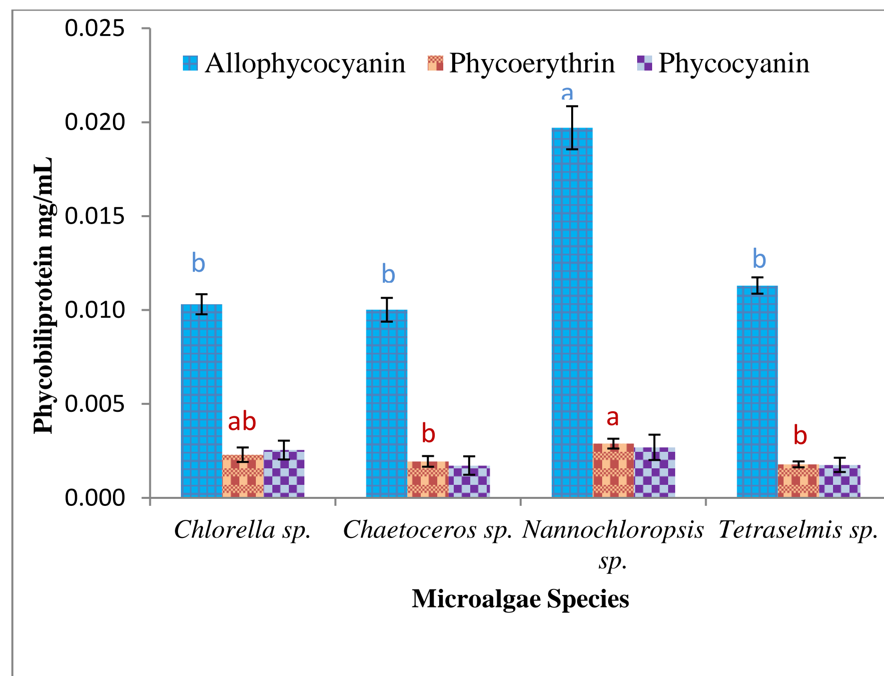

Figure 4.10: Phycobiliprotein production of selected microalgae cultured in Conway culture media. Values are mean ± standard error (n=3)

4.7 Proximate Composition of Microalgae

The proximate content (% dry weight) in four species of microalgae culture in Conway media are displayed in Figure 4.11. The protein content (% dry weight) of *Tetraselmis* sp. cultured in Conway media was significantly ($p < 0.05$) higher (57 ± 0.66% dry weight) than all other. On the other hand, protein content of *Chaetoceros* sp. was (51 ± 3.33% dry weight), *Nannochloropsis* sp. was (49 ± 2.28% dry weight) and *Chlorella* sp. was (43 ± 2.85% dry weight).

In case of lipid, *Nannochloropsis* sp. content was significantly ($p < 0.05$) higher (25 ± 1.84% dry weight) where *Chaetoceros* sp. content was (20 ± 0.14% dry weight), *Tetraselmis* sp. content was (19 ± 1.29% dry weight) and *Chlorella* sp. content was (12 ± 0.29% dry weight).

Finally, in case of carbohydrate, *Chlorella* sp. content was significantly ($p < 0.05$) higher (23 ± 1.62% dry weight) where *Nannochloropsis* sp. content was (22 ± 1.34% dry weight), *Chaetoceros* sp. content was (18 ± 1.32% dry weight) and *Tetraselmis* sp. content was (17.08 ± 0.99% dry weight).

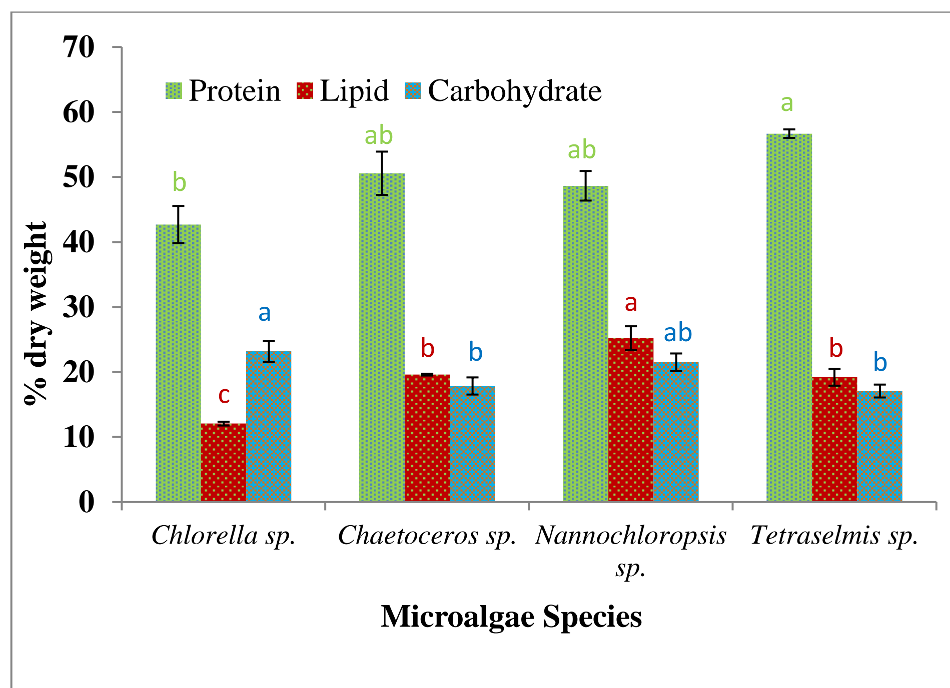

Figure 4.11: Proximate composition of selected microalgae cultured in Conway culture media.

Values are mean ± standard error (n=3)

Chapter -5: Discussion

In the present experiment, four different indigenous microalgae species (*Chlorella* sp., *Chaetoceros* sp., *Nannochloropsis* sp., and *Tetraselmis* sp.) were cultured with maintaining optimum culture condition. In this experiment, the growth, productivity, pigments (chlorophyll, carotenoids and phycobiliprotein) and proximate composition were determined.

5.1 Physical Parameters of Conway Medium

According to Food and Agriculture Organization (FAO), the ranges of physical parameters for culturing microalgae are as follows; temperature (16-27 °C), salinity (12-40 ppt) and pH (7-9). Considering Table 4.1, the physical parameters of prepared culture medium after autoclaved were salinity (30.1ppt), and pH (7.72), and culture condition was maintained at optimum temperature (24 ℃) which were within the recommended range of FAO. In addition, FAO (1996), reported that the optimal temperature range for phytoplankton culture ranges between 20-27 ℃, but this may vary on the basis of culture condition, species or strain cultured. Chisti (2008) also mentioned the optimal temperature range for microalgae between 20 to 30 ℃. Different types of microalgae can tolerate temperature fluctuation up to 15 ℃ lesser than their optimum where growth may be reduced but a temperature of only a few degrees higher than optimum can cause cell death (Mata et al., 2010). The culture was maintained with continuous artificial light at 150 $\mu Em^{-2}s^{-1}$ light intensity by using fluorescent light. For optimum growth phototrophs activities, it is must of uptaking sufficient light (Radmer et al., 1987). In the present study, all the physical parameters such as temperature, salinity and light intensity were in recommended range for culturing microalgae also in accordance with (Laven and Sorgeloss, 1996).

5.2 Growth Parameters of Microalgae in Conway Medium

Growth curve indicates the growth pattern of microalgae including various growth phases such as lag phase, exponential phase, declining growth phase, stationary phase and death phase, which is essential before doing any kind of microalgae work. In this experiment growth of *Chlorella* sp., *Chaetoceros* sp., *Nannochloropsis* sp., and *Tetraselmis* sp. was determined in terms of cell density and optical density. These two parameters (cell density and optical density) were considered to ensure and verify the consistency of the result because each technique has some of its limitations. For

example, because of similar structure and color, rupture cell or death cell can cause incorrect count during cell counting. To minimize this problem, an additional method optical density was used which is practical and easy to determine (Sanjoy et al., 2011). This two-growth analysis provides full information about the growth of the species cultured.

In this experiment the microalgae showed similar growth trends particularly. The Figure 4.1, 4.2, 4.3 and 4.4 showed the growth pattern of *Chlorella* sp., *Chaetoceros* sp., *Nannochloropsis* sp., and *Tetraselmis* sp. respectively where considering each figure there were no significant differences in terms of cell density and optical density. If the relation between cell number and optical density showed similar trends or direct relation that indicates an appropriate culture condition (Nur et al., 2008). All the microalgae species showed distinct growth phases (log phase, lag phase, growth phase, stationary phase and death phase). Viable microalgae also need a definite time period to physiologically adjust and adapt with the new environment (Barsanti and Gualtieri, 2006). These growth parameters depend mainly on photoperiod and genus or species, pH, temperature, nutrient composition of the medium (Borowitzka et al., 1979; Payer et al., 1975; Oh-Hama and Miyachi, 1988; Richmond, 1988), and turbulence (Richmond, 2004).

In the following experiment every species took more or less 2 days to undergo lag phase. Chopin et al. (2012) reported a same type of microalgae growth pattern in the commercial medium.

5.3 Productivity of Microalgae in Conway Medium

In the present study, volumetric, areal and lipid productivity of *Chlorella* sp., *Chaetoceros* sp., *Nannochloropsis* sp., and *Tetraselmis* sp. were determined. Several factors might cause in productivity such as salinity, media types, temperature, pH, dissolve oxygen, light intensity, nutrients etc. High lipid production is associated with high growth of microalgae (Woertz et al., 2009). In this experiment, *Chaetoceros* sp. and *Teraselmis* sp. showed higher growth trends in terms of cell multiplication rates and biomass production. Besides these two species also showed higher productivity. Woertz et al. (2009) found the same. Christi (2007) found that in different adverse condition various external stress factors can increase lipid productivity. Without that, nutrient concentration also effects on productivity.

Various studies found that, growth and productivity are correlated with each other. It is common that species with high growth rates results high productivity of microalgae (Sacristan de Alva et al., 2013). Species growth and productivity depend on nutrient concentration in the culture environment. Arrendondo-Figueroa et al. (1998) reported that culture using treated wastewater shows a different growth and productivity compare to commercial medium.

5.4 Chlorophyll Production in Microalgae in Conway Culture Medium

The chlorophyll content of the cultures was evaluated in same growth conditions. Chlorophyll was quantified by using biomass from the nearly same growth phase. Though, it was reported in earlier studies that chlorophyll a concentration is same in all algae groups (Donkin, 1976; Martin et al., 1991; Grung et al., 1992). But in the present study different result is observed. According to figure 4.8 *Tetraselmis* sp. showed higher amount of Chlorophyll a and b than the other three species where *Chaetoceros* sp. produced highest amount of chlorophyll c than the other three. According to Danesi et al. (2011), higher chlorophyll concentration resulted where the cell concentration is high, but, considering chlorophyll c the opposite pattern had observed.

Chlorophyll a is higher considering each species chlorophyll concentrations since chlorophyll a is the main pigment where chlorophyll b and care accessories pigments may or may not be related with chlorophyll a (Lavin, 2000). In the present study, it was also found that all the four species content higher amount of chlorophyll a.

The photosynthetic rates of living organisms were significantly affected by the alteration of light intensity and light regime which consecutively influence its growth (Pandey et al., 2010). Increased availability of light also may cause a decrease in the content of chlorophyll a and carotenoid (Alves de Oliveira et al., 2014). Control condition was maintained in the present study.

The variations in the amounts of culture nutrients also effect on chlorophyll concentrations. In addition, there are other factors that can influence the chlorophyll contents of microalgae such as, light, temperature, water quality, and cell extraction method. Furthermore, solvent for extraction has direct effect on chlorophyll concentrations and chlorophyll concentration varies on basis of solvent (Wellburn, 1994).

5.5 Carotenoids of Microalgae in Conway Culture Medium

The biomass content has direct effect on carotenoids biosynthesis (Velichkova, 2014). In this study, *Nannochloropsis* sp. produced highest amount of carotenoids (1.68 ± 0.3 µg/mL) among four species where *Tetraselmis* sp. produced (1.51 ± 0.05 µg/mL), *Chaetoceros* sp. (1.36 ± 0.2 µg/mL) and *Chlorella* sp. produced the lowest concentration (0.56 ± 0.02 µg/mL) of carotenoids. Melina et al., (2016) found that *Tetraselmis* sp. produced 2.6µg/mL of carotenoids which is almost double than this findings. Using different culture medium Sirakov and Vekichkova (2014) found that *Nannochloropsis maculate* produced 0.836 µg/mL carotenoids. That indicates carotenoids production varies on the basis of the culture medium. In addition, carotenoids concentration also varies of various environmental parameters, chlorophyll, solvent used for extraction and species (Techetel and Ruppel, 1992; Rise et al., 1994; Sartory and Grobbbelaar, 1984).

However, the selection of method and solvent used for extraction according to the species would give a useful result.

5.6 Phycobiliprotein in Microalgae in Conway Culture Medium

Many studies had been done on microalgae considering its multidisciplinary functions especially for their organic biomass. Unique light harvesting feature of microalgae known as phycobiliproteins which have a great demand in today’s world. Parmar et al. (2011) reported different dried biomasses of algae are grinded and milled to produce commercial pigments and neutrceuticals. Jensen et al. (2001) and Soni et al. (2009) also found diversified therapeutic effect and antidiabetogenic effect of microalgae. Worldwide eleven major companies are involved in production and sale of these proteins (Sekar and Chandramoha, 2008).

In the present experiment, four different microalgae (*Chlorella* sp., *Chaetoceros* sp., *Nannochloropsis* sp., and *Tetraselmis* sp.) were cultures in Conway culture medium to measure their phycobiliproteins concentrations. In this experiment, phycobiliprotein content differ according to the species and it was found that *Nannochloropsis* sp. showed maximum production compared to other species Result showed that phycobiliproteins production varies among species. Most abundant pigment found is allophycocyanin compared to the phycocyanin and phycoerythrin. It can be, due to the levels of pigments tend to be reduced in high light exposure to prevent from photo-

oxidation damage caused by the production of free radicals. Lee (2008) stated that, phycobiliprotein production varies in species in light regime due to chromatic adaptation. Chen et al. (2013) narrated that, microalgae' cell growth and pigment production affected due to environmental change. It is reported that phycobiliproteins production was higher when the light availability was low (Alves de Oliveira, 2014). Meanwhile the phycoerythrin has been found to occur in small concentration due to its highly dependence on pH, regardless the contribution of light (Cuellar-Bermudez et al., 2014). Phycocyanin production also tends to lower probably due to artificial lighting (Reichert et al., 2006).

5.7 Proximate Composition of Microalgae in Conway Media

The main components of algae cells are proteins, carbohydrates and lipids (Becker, 1994). Environmental parameters are the most important considerations, which have direct effect on the quantity and quality of proximate compositions of microalgae (Renaud et al., 1994). Considering environmental factors, selection of microalgae those have a rich nutritional profile is very important for a microalgae-based industry (Lv et al., 2010).

Present study showed that *Tetraselmis* sp. produced highest protein (57% dry weight) and *Chlorella* sp. produced least protein (43% dry weight). Proximate content of the present study is similar with the range of protein content in marine microalgae which contain 30-50% protein, 10–20% lipid, and 5–15% carbohydrate (Brown et al., 1998; Renaud et al., 1999). However, *Tetraselmis* sp. produced slightly higher amount beyond the range. Another study showed that protein content can be as little as 15% to as much as 60 ± % dry weight (Oh-Hama and miyachi, 1988). *Chaetoceros* sp. produced high amount protein which also showed dissimilarities than present study (Khatoon et al., 2016). Nutritional value or protein also varies on the basis of the method of processing, and as well as microalgae strains (Becker, 1988; and 1986). These differences can be due to variations in some factors such as light intensity (Discontinuous or continuous), nutrients availability, harvesting methods and growth phase etc. (Gonzalez Lopez et al., 2010; Hempel et al., 2012). On the other hand, compared to the present study higher protein content were found in *Tetraselmis* sp.; *Chlorella* sp. (Brown, 1991; Becker, 2007; Christaki, 2011). Some difference can also be in the measurement of proteins because of the method of protein determination (Gonzalez Lopez et al., 2010).

Lipid in microalgae became important studies now a day as promising alternative source for the production of biodiesel. Microalgae naturally produce lipids as part of the structure of the cell (e.g. in cell membranes and as signaling molecules), and as a storage compound, similar to fat stores in animals and plants (Tsukahara and Sawayama, 2005). In the present study in case of lipid, *Nannochloropsis* sp. produced the highest amount of lipid 25% followed by *Tetraselmis* sp. 19% and *Chaetoceros* sp. 20%. The lowest lipid producer among these species was *Chlorella* sp. 12%. The lipid contents measured in the following study were within the range (10-20% dry weight) for marine microalgae reported by Renaud et al., (1999). For *Nannochloropsis* sp. higher lipid content (25% dry weight) was measured which is dissimilar than previous studies. Lipid content can be changed because of growth conditions, generally it has seen that lipid contents changes because of nutrient fluctuations, UV radiation, and temperature fluctuations (Boyd, 1973).

In the present study, *Chlorella* sp. produced highest amount of carbohydrate 23% where *Nannochloropsis* sp. produced lowest amount 22% which is similar with the study by Brown et al., (1998), Renaud et al., (1999) except *Nannochloropsis* sp. These differences can be due to the variations in some factors such as light intensity (Discontinuous or continuous), nutrients availability, harvesting methods and growth phase etc. (Gonzalez Lopez et al., 2010; Hempel et al., 2012). The accumulation of carbohydrate was mainly caused by the turning of protein metabolic pathway to the carbohydrate pathway (Markou et al., 2012). Carbohydrate synthesis was activated by 3-phosphoglycerate and inhibition of inorganic phosphorus. Phosphorus starvation might have big impact on carbohydrate production by the microalgae.

Moreover, these all are our indigenous species; which could be reason for such dissimilarities with the previous data.

Chapter- 6: Conclusion

Chlorella sp.; *Chaetoceros* sp.; *Nannochloropsis* sp.; and *Tetraselmis* sp. these all species were considered as potential in the aspect of growth, productivity biomass and pigment productions. Literature review as mentioned in Chapter 2 also has elaborated the positive characteristics of selected species. Based on the preliminary growth curve experiment, *Nannochloropsis* sp. tends to have the longest culture period (days) followed by *Chlorella* sp,; *Chaetoceros* sp.; and *Tetraselmis* sp. They were subjected to commercial Conway culture medium with maintaing constant controlled light temperature, pH and salinity. Based on productivity, *Chaetoceros* sp. showed the highest productivity followed by *Tetraselmis* sp.; *Nannochloropsis* sp.; and *Chlorella* sp. Productivity is important factor to get higher amount of biomass. In aspects of chlorophyll concentrations, *Tetraselmis* sp. produced the highest amount of chlorophyll a and b than other species. Chlorophyll has a higher demand in the sector of food producing industry as coloring agent in replacement of artificial colors. In aspects of carotenoids, *Nannochloropsis* sp. produced maximum amount of carotenoids among all species. Because of provitamin activity of carotenoid it has a major application in aquacluture industry. Without that carotnoids are used widely in cosmetics and food industries in today's world. Considering the proxiamte profile, *Chaetoceros* sp. produced higher amount protein where *Nannochloropsis* sp. produced the highest amount of lipid and carobohydrate. Selection of potential algal strains in terms of productivity and nutrient composition is the prime considerations for any microalgae-based feed industry. In today's world, microalgae can be great source to fulfill the rising protein demand. Based on characterization, allophycocyanin was found to be present as the major crude extract of overall species among all phycobiliproteins. Phycibiliproteins have many nutrceuticals importance and through further exploration it can be utilized more. Considering each factor (productivity, pigments and nutritional value) all species are imporatant depending on its use. However application of thsese species in various sectors requires more research and careful optimization. It should be not that this study was designed to chaeacterize and directly compare potential species.

Chapter-7: Recommendation and Future Perspectives

Present study showed growth, productivity, chlorophyll, carotenoids, phycobilproteins concentrations, and proximate compositions of four different indigenous microalgae *(Chlorella* sp., *Chaetoceros* sp., *Nannochloropsis* sp., and *Tetraselmis* sp.*)* isolated from Cox's Bazar coast. Result showed comparatively potential species among them in terms of growth, productivity, pigments, and proximate profile. Through analyzing these data, a commercially potential species among them could select easily on basis of their using purpose. As various food producing company trying to introduce natural pigments of microalgae' in replacement synthetic colors, on that circumstances the selected microalgae can play a dynamic role. In addition, protein enriched microalgae can be good source of feedstock for various feed producing industry. Moreover, higher lipid producing microalgae can be a good stock for biodiesel production.

Hence, further studies could be done especially with various extraction and determination method. In addition, more optimization of various indigenous microalgal species is needed especially that has high market demand in different industry such as aquaculture, pharmaceuticals, nutraceuticals and biodiesel production.

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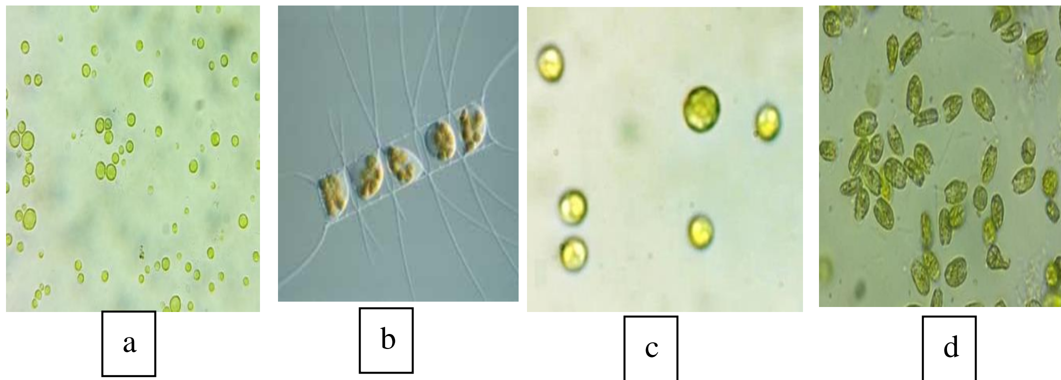
Appendices

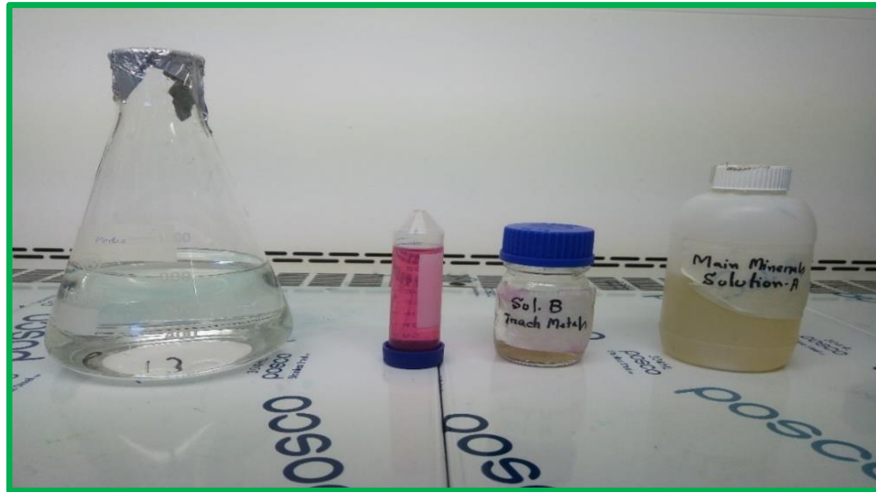


Appendix A: Experiential Species

(a) *Chlorella* sp., (b) *Chaetocesos* sp., (c) *Nannochloropsis* sp., (d) *Tetraselmis* sp.

Appendix B: Collection of Seawater

Appendix C: Filtration of Water

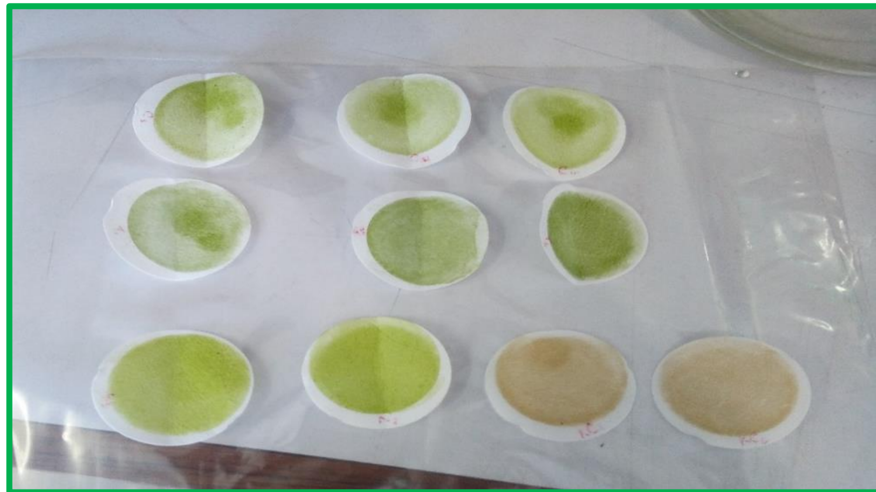


Appendix D: Media Preparation

Appendix E: Culture Unit of Microalgae

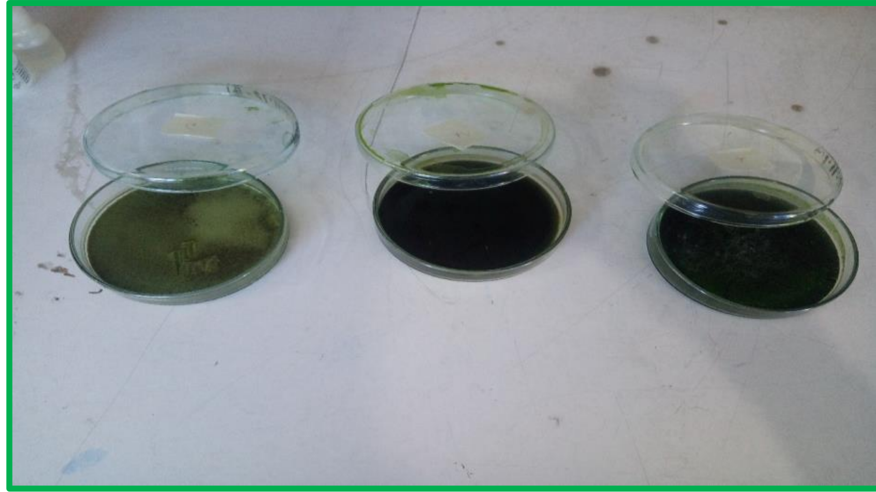
Appendix F: Cell Count of Microalgae

Appendix G: Mass Culture of Microalgae



Appendix H: Determination of Chlorophyll Content

Appendix I: Extraction of Carotenoids

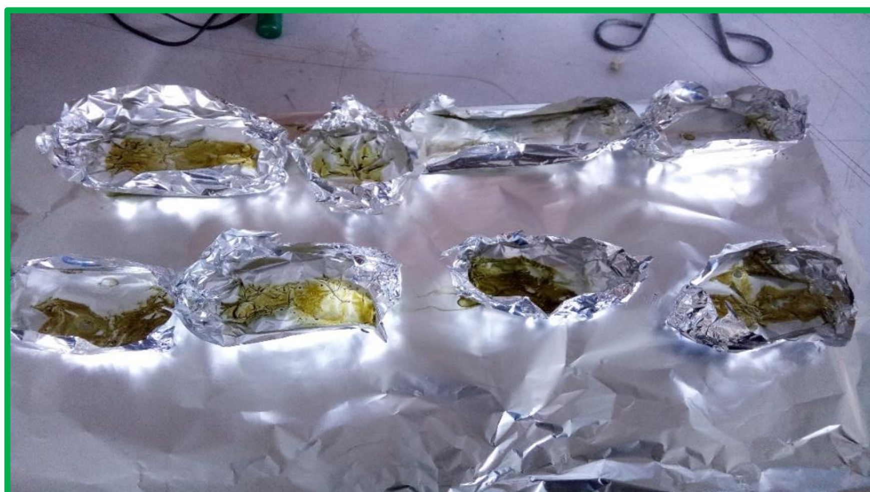


Appendix J: Biomass Extraction

Appendix K: Determination Phycobiliprotein Content

Appendix L: Determination of Carotenoids Content

Appendix M: Determination of Protein Content



Appendix N: Determination of Lipid Content

Appendix O: Determination of Carbohydrate Content

Appendix 1: Independent Sample T-Test of pH Before and After Autoclave.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	**Sig.**	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
pH before and after UV treatment	Equal variances assumed	.134	**.713**	.657	4	.547	.12000	.11779	-.41916	.67916
	Equal variances not assumed			.657	3.930	.548	.12000	.11779	-.42306	.68306

Appendix 2: Independent Sample T-Test of Temperature Before and After Autoclave.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	**Sig.**	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
Temperature before and after UV treatment	Equal variances assumed	.060	**.821**	-.603	4	.579	-.63333	1.05093	-3.55117	2.28450
	Equal variances not assumed			-.603	3.922	.580	-.63333	1.05093	-3.57414	2.30747

Appendix 3: Independent Sample T-Test of Dissolved Oxygen Before and After Autoclave.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
Salinity before and after UV treatment	Equal variances assumed	3.852	**.123**	-2.148	4	.098	.380000	.51208	-2.52175	.32175
	Equal variances not assumed			-2.148	2.232	.151	.380000	.51208	-3.09745	.89745

Appendix 4: Independent Sample T-Test of Salinity Before and After Autoclave.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
Salinity before and after UV treatment	Equal variances assumed	3.852	**.123**	-2.148	4	.098	.380000	.51208	-2.52175	.32175
	Equal variances not assumed			-2.148	2.232	.151	.380000	.51208	-3.09745	.89745

Appendix 5: Regression Analysis between CD and OD of *Chlorella* sp. Growth Parameters.

Regression Statistics	
Multiple R	0.986555
R Square	0.973292
Adjusted R Square	0.969953
Standard Error	0.498333
Observations	10

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*
Intercept	1.235713	0.281399	4.391318	0.002313	0.586806	1.884621	0.586806
OD	27.6624	1.620117	17.07432	1.41E-07	23.92641	31.3984	23.92641

Appendix 6: Regression Analysis between CD and OD of *Chaetoceros* sp. Growth Parameters.

Regression Statistics	
Multiple R	0.98043
R Square	0.961243
Adjusted R Square	0.956398
Standard Error	0.34605
Observations	10

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*

Intercept	-0.09321	0.191044	-0.48788	0.638724	-0.53375	0.347341	-0.53375
OD	14.27984	1.01377	14.08588	6.27E-07	11.94208	16.61759	11.94208

Appendix 7: Regression Analysis between CD and OD of *Nannochloropsis* sp. Growth Parameters.

Regression Statistics	
Multiple R	0.953103
R Square	0.908406
Adjusted R Square	0.899247
Standard Error	0.861516
Observations	12

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*
Intercept	-0.52933	0.594738	-0.89003	0.39436	-1.85449	0.795826	-1.85449
OD	35.78824	3.593633	9.958792	1.65E-06	27.78113	43.79536	27.78113

Appendix 8: Regression Analysis between CD and OD of *Tetraselmis* sp. Growth Parameters.

Regression Statistics	
Multiple R	0.988251
R Square	0.97664
Adjusted R Square	0.973303
Standard Error	0.282565
Observations	9

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*
Intercept	-0.30468	0.167597	-1.81792	0.111909	-0.70098	0.091626	-0.70098
OD	19.66564	1.149546	17.1073	5.72E-07	16.94739	22.38388	16.94739

Appendix 9: ANOVA Table for Volumetric Productivity.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.095664063	3	0.031888021	9.993018	0.004417	4.066181
Within Groups	0.025528241	8	0.00319103			
Total	0.121192303	11				

Appendix 9: ANOVA Table for Areal Productivity.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.372590334	3	0.124196778	9.993018	0.004417	4.066181
Within Groups	0.099426843	8	0.012428355			
Total	0.472017177	11				

Appendix 11: ANOVA Table for Lipid Productivity.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.010066117	3	0.003355372	35.16697	5.9E-05	4.066181
Within Groups	0.000763301	8	9.54126E-05			
Total	0.010829418	11				

Appendix 12: ANOVA Table for Chlorophyll a

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	9.669666634	3	3.223222211	919.8649	1.72E-10	4.066181
Within Groups	0.028032136	8	0.003504017			
Total	9.69769877	11				

Appendix 13: ANOVA Table for Chlorophyll b

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.974627998	3	0.991542666	1300.339	4.31E-11	4.066181
Within Groups	0.00610021	8	0.000762526			
Total	2.980728208	11				

Appendix 14: ANOVA Table for Chlorophyll c

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.134117023	3	0.044705674	55.00449	1.1E-05	4.066181
Within Groups	0.006502112	8	0.000812764			
Total	0.140619136	11				

Appendix 15: ANOVA Table for Carotenoids

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.20417092	3	0.73472364	42.19959	3E-05	4.066181
Within Groups	0.13928544	8	0.01741068			
Total	2.34345636	11				

Appendix 16: ANOVA Table for Allophycocyanin

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.000191559	3	6.38529E-05	116.7131	6.07E-07	4.066181
Within Groups	4.37674E-06	8	5.47092E-07			
Total	0.000195935	11				

Appendix 17: ANOVA Table for Phycoerythrin

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.1604E-06	3	7.20134E-07	8.935918	0.006202	4.066181
Within Groups	6.4471E-07	8	8.05887E-08			
Total	2.80511E-06	11				

Appendix 18: ANOVA Table for Phycocyanin

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.34802E-06	3	7.82674E-07	2.868233	0.103747	4.066181
Within Groups	2.18301E-06	8	2.72877E-07			
Total	4.53104E-06	11				

Appendix 19: ANOVA Table for Protein

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	298.5219934	3	99.50733112	8.005316	0.008579	4.066181
Within Groups	99.44125656	8	12.43015707			
Total	397.9632499	11				

Appendix 20: ANOVA Table for Carbohydrate

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	76.54565147	3	25.51521716	7.143805	0.01188	4.066181
Within Groups	28.57324959	8	3.571656199			
Total	105.1189011	11				

Appendix 21: ANOVA Table for Lipid

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	260.73	3	86.91	33.64258	6.95E-05	4.066181
Within Groups	20.66666667	8	2.583333333			
Total	281.3966667	11				

Brief Biography of the Author

Zahidul Islam, the fourth son of Md. Saleh Uddin and Jibon Nesa was born and grown up in Alexander, Lakshmipur. He had achieved his Secondary School Certificate from Alexander Pilot High School and Higher Secondary Certificate from Noakhali Govt. College. He had received his B. Sc Fisheries (Hons.) degree from Faculty of Fisheries, Chattogram Veterinary and Animal Sciences University. Now, he is a candidate of Master of Science in Aquaculture under the Department of Aquaculture, Chattogram Veterinary and Animal Sciences University. He was assigned as a research assistant from January 2019 to December 2020 under the project "Isolation, identification and screening of indigenous tropical marine microalgae for production of natural pigments" implemented by Department of Aquaculture, Faculty of Fisheries, Chattogram Veterinary and Animal Sciences University and funded by Bangladesh Fisheries Research Institute. He has research experience on "Survival of crab and prawn larvae". His research interest includes microalgae (isolation, culture, culture variation analysis, biomass production, pigment extraction, product formulation, feeding, antimicrobial sensitivity test etc.), improved aquaculture and indoor aquafarming. Presently he is working as a scientific officer at "Center for research on fish drying and improved aquaculture", Bangladesh Council of Scientific and Industrial Research. He is passionate to qualify himself as a skilled researcher to contribute his beloving country and the world as well.