Abstract

To utilize the enormous potentiality of indigenous microalgae, this study contains indices on isolation of some primordial freshwater microalgae and to determine their growth rate, pigments and proximate composition. For microalgae isolation, streak plating method in agar, serial dilution and capillary pipetting was performed and for the evaluation of growth performance, data were collected in terms of cell density and optical density. Selected microalgae (*Nephrocytium* sp., *Nannochloropsis* sp., *Selenastrum* sp., *Sphaerocystis* sp., *Ankistrodesmus* sp., *Monoraphidium* sp., *Pectinodesmus* sp. and *Scenedesmus* sp.) were mass cultured in commercial Bold Basal Medium (BBM), harvested at their stationary phases to determine the pigments content (chlorophyll-a, chlorophyll-b, carotenoid and phycobiliproteins) and proximate composition (protein, lipid and carbohydrate). Results showed that, onset of stationary phase (9–14 days) varied among the eight species where in the stationary phase; cell density was significantly higher ($p<0.05$) in *Nannochloropsis* sp. All microalgae showed chlorophyll-a as the main pigment component, where *Monoraphidium* sp. showed significantly highest ($p<0.05$) chlorophyll-a (11.264±0.065 µg/mL), b (2.082±0.067 µg/mL) and carotenoid content (8.05±0.07 µg/mL). Moreover, total phycobiliproteins was also significantly higher ($p<0.05$) in *Scenedesmus* sp. (6.105±0.12 mg/g) and significantly lowest ($p<0.05$) in *Pectinodesmus* sp. (1.152±0.012 mg/g) and *Sphaerocystis* sp. (1.296±0.007 mg/g). Significantly higher ($p<0.05$) amount of total protein was recorded in *Nannochloropsis* sp. (34.34±1.22 % dry weight) and significantly lower ($p<0.05$) in *Scenedesmus* sp. (21.77±2.17 % dry weight) and *Ankistrodesmus* sp. (24.26±0.78 % dry weight). Similarly, highest and lowest ($p<0.05$) amount of lipid content was recorded in *Monoraphidium* sp. (25.28±0.31 %dry weight) and *Pectinodesmus* sp. (11.79±0.27 %dry weight), respectively. In addition, carbohydrate content was highest ($p < 0.05$) in *Pectinodesmus* sp. These findings indicate that the microalgae assessed in this study have potential as multi-nutrient and pigments supplement, as well as can be manipulated for high protein, lipid and pigments production for the sustainable use of native species in aquaculture, pharmaceuticals and nutraceuticals industry.

Keywords: Isolation, Microalgae, Stationary phase, Growth, Pigments, Proximate composition

Chapter 1: Introduction

Microalgae are diverse group of unicellular and multi-cellular microscopic autotrophs/heterotrophs (Roque et al., 2018), convert solar energy into stored chemical energy (Ozkurt, 2009). Above forty thousand species of microalgae have been identified (Shakeel et al., 2018), many of which are considered as natural producers of protein, lipid, carotenoids, and fatty acids, that are important not only in human and animal nutrition but also in medicines (Khatoon et al., 2017). The increasing demand for the aquaculture species all over the world has led to the expansion of intensive aquaculture which discharge large amount of nutrient containing effluents (Gao et. al., 2016). In this case, microalgae can be considered as an alternative for the treatment of aquaculture wastewater which offers several advantages, like improving effluent quality and producing biomass with low energy costs (Guldhe et al., 2017). Furthermore, biologically active compounds derived from microalgae have paid more industrial attention (De Morais et al., 2015) and continues to expand which required new microalgal species that can produce these or many other essential compounds is a continuing area of research. Species or strain selection is the preliminary and most important step in bioprospecting of microalgae for any commercial application (Borowitzka, 2013). Monitoring of cell growth is deliberated as a primitive portion for the usage of microalgae in aquaculture (Santos-Ballardoa, 2015). Protein content of microalgae is considered as one of the crucial components influencing their nutritional value (Safi et al., 2013). Microalgae lipid content may vary from 20% to 50% of dry weight (Hu et al., 2015) and the high oil contribution of many microalgae species has been exploited in biofuel production (Hussain et al., 2017). Compared to other microalgae compounds, carbohydrates have a lesser energy value but they are the preliminary raw component for the synthesis of biofuels via biotechnological conversion (Andreeva et al., 2021). Pigments are considered as one of the most essential product from microalgae (Granado-Lorencio et al., 2009) and chlorophylls, carotenoids, and phycobilins are the significant pigment group found in microalgae (Koller et al., 2014). Chlorophyll and carotenoid play an important role in photosynthesis of algae and photosynthetic bacteria (Lamers et al., 2012) and have antioxidant property, utilized as a natural food coloring agent (Hosikian et al., 2010) and also used widely in pharmaceutical products (Bhagavathy and Sumathi, 2012). Phycobiliproteins are a group of proteins with covalently attached linear tetrapyrrole

chromophoric groups (Ba´saca-Loya et al., 2009) and most common phycobiliproteins consist of phycoerythrin (PE), phycocyanin (PC), and allophycocyanin (APC) (Wiedenmann, 2008). Microalgae which have valuable characteristics like lipid, carbohydrate and protein, were applied in aquaculture, and have economic potential (Williams and Laurens, 2010). The high oil production of various microalgae species has been utilized in biofuel production (Hussain et al., 2017) due to their advantages over other conventional biofuel sources which is based on its non-seasonality, biodegradability, non-toxic nature and renewability (Yang et al., 2011). The biomass and product yields are well known to depend on the cultivation conditions, where theoretical biomass yield of microalgae was reported as 100–200 g dry weight $m^{-2}day^{-1}$ and the practical productivity rate was 15–30 g dry weight $m^{-2}day^{-1}$ (Subramanian et al., 2013). In addition, under stress conditions they resulted between 20% and 50% lipids in terms of the dry weight of the biomass (Wang et al., 2016) that is assumed to be between 15 and 25 t $ha^{-1}year^{-1}$, which would correspond to 4.5 and 7.5 t $ha^{-1}year^{-1}$ of lipid production (Choong et al., 2020). Moreover, several nutritional factors such as nitrogen, phosphorus, carbon and iron are recognized as one of the most important factors influencing the yield of biomass and the lipid accumulation (White et al., 2013).

A deep knowledge of the behavior of a specific microalgal strain in response to various culture conditions, like nutrient supply, is important for the optimization to mass microalgal production (Hyka et al., 2013). The availability of good quality microalgae in hatcheries is limited and different species that were used previously in commercial hatcheries have now been discarded because of their lower nutritional value (Ponis et al., 2006). Hence, developing a reliable and commercially viable process for the mass production of microalgae, selection of algal species and strain can be considered as first and most important step. Selection of the best performing tropical strains should be carried out to utilize the nutritional properties as they are well adapted to similar environment, exhibit better performance and robustness than those from a strain bank collection (Jebali et al., 2019; Abdelaziz et al., 2014). Although the measurement and monitoring of cell growth are an integral part in microalgal technology, its elaborate investigation has been highly ignored (Havlik et al., 2013). But very little research has been done on the characterization and biochemical composition of microalgae in Bangladesh.

Therefore, the aim of this study was to isolate microalgae from different south-eastern freshwater habitat of Bangladesh (Kaptai Lake, Halda River and Karnaphuli River) and to compare the growth rate, pigments content and proximate composition of isolated microalgae (*Nephrocytium* sp., *Nannochloropsis* sp., *Selenestrum* sp., *Sphaerocystis* sp., *Ankistrodesmus* sp., *Monoraphidium* sp., *Pectinodesmus* sp. and *Scenedesmus* sp.) which may contribute to select potential strains that possess fast growth, suitable pigments and high proximate profile for the sustainable use of those tropical species in aquaculture, pharmaceuticals and nutraceuticals industry.

The objectives of the research are-

1. To isolate and identify freshwater microalgae from different south-eastern freshwater sources of Bangladesh;
2. To determine the growth curve of isolated microalgae by using appropriate culture medium and;
3. To determine the pigment content and proximate composition of isolated microalgae.

Chapter 2: Review of Literature

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). Although, there are over thousands or even millions of microalgae species existing in nature (Hannon et al., 2010), 30,000 species have been studied but so far not fully exploited (Mata et al., 2010) and only a few of them have been successfully produced commercially for the production of high value products (Saha and Murray 2018; Khan et al., 2018). In 1910, the first reports of the cultivation of microalgae as feed in aquaculture were published (Allen and Nelson, 1910) and since then today, the application of microalgae in aquaculture has increased intensively. Aquaculture is a growing industry, and therefore, the industry of culturing microalgae is consequently increasing where the main applications of microalgae in aquaculture could relate directly or indirectly to the various aquatic species such as rotifers and fish. However, the adaptation of the microalgae in various environmental conditions is depending on species, thus finding microalgae species that can suit well to the local environment or specific culture condition is possible (Mata et al., 2010).

2.1.1 *Nephrocytium* sp.

Taxonomic classification:

Kingdom: Plantae

Phylum: Chlorophyta

Class: Trebouxiophyceae

Order: Chlorellales

Family: Oocystaceae

Genus: *Nephrocytium* (Nägeli, 1849)

Coenobia of 4, 8 or 16 irregularly or spirally arranged cells and often retained prior to release within a thickened and well-defined gelatinizing mother cell wall, usually surrounded by a mucilaginous envelop, cells ovoid, elongate ovoid, ellipsoidal and

with obtuse apices or spindle-shaped with almost acute aspices, asymmetrical or distinctly curved. Chloroplast is parietal with 1 to several pyrenoids. Asexual reproduction occurred by 4, 8 or 16 autospores, released by gelatinization of expanded mother cell wall (John et al., 2002).

2.1.2 *Nannochloropsis* sp.

Taxonomic classification:

Kingdom: Plantae

Phylum: Ochrophyta

Class: Eustigmatophyceae

Order: Eustigmatales

Family: Monodopsidaceae

Genus: *Nannochloropsis* (Hibberd, 1981)

Nannochloropsis are small, nonmotile spheres which do not express any distinct morphological features that can be distinguished by either light or electron microscopy (Andersen et al., 1998). They have a diameter of about 2 to 3 micrometers (Kandilian et al., 2013). The algae of the genus *Nannochloropsis* differ from other related microalgae in that they have chlorophyll-a and completely lack chlorophyll-b and chlorophyll-c. In addition they are able to build up a high concentration of a range of pigments such as astaxanthin, zeaxanthin and canthaxanthin (Lubian et al., 2000). *Nannochloropsis* sp. contains about 44.3%, protein, 32% lipid in 30 ppt salinity (khatoon et al., 2014).

2.1.3 *Monoraphidium* sp.

Taxonomic classification:

Kingdom: Plantae

Phylum: Chlorophyta

Class: Chlorophyceae

Order: Sphaeropleales

Family: Selenastraceae

Genus: *Monoraphidium* (Komárková-Legnerová, 1969)

Cells are free living, without a specialized organ, if attached, more or less spindle-shaped, straignt, curved or spirally twisted, gradually or abruptly narrowing to acute aspices, thin-walled and without a mucilaginous envelope. Chloroplasts are parietal, single and without a visible pyrenoid when examined with the light microscope. Asexual reproduction is happened by 2, 4, 8 or 16 autospores, produced by oblique division and serially arranged, released by longitudinal split of mother cells or its disintegration at apices (John et al., 2002). *Monoraphidium* sp. contain 14.3 ± 0.5% of lipid after the beginning of exponential phase (after 15 days), 15.0 ± 0.2% at exponential phase (25 days) and 16.2 ± 0.38% at the starting of stationary phase (Dhup and Dhawan, 2014). *Monoraphidium* sp. contains nearly 19% - 35% lipid, 28% - 45% protein and 17% - 25% carbohydrates, in relation to the dry weight of biomass (Reyes et al., 2012).

2.1.4 *Sphaerocystis* sp.

Taxonomic classification:

Kingdom: Plantae

Phylum: Chlorophyta

Class: Chlorophyceae

Order: Chlamydomonadales

Family: Sphaerocystidaceae

Genus: *Sphaerocystis* (Chodat, 1897)

Sphaerocystis sp. are microscopic, consisting of 4, 8 or 16(-32) regularly arranged cells within a spherical to cllipsoidal and homogeneous mucilaginous envelope, cells spherical with thin and smooth walls. Chloroplasts are parietal, cup-shaped, with a single pyrenoid. Reproduce asexually by biflagellate zoospores and autospore, autospores 4 or 8(-16) per cell and zoospores 2, 4, 8 or 16 per cell, both often with an eyespot, autospores and zoospores released by gelatinization of mother cell wall which persists (John et al., 2002).

2.1.5 *Selenastrum* sp.

Taxonomic classification:

Kingdom: Plantae

Division: Chlorophyta

Class: Chlorophyceae

Order: Sphaeropleales

Family: Selenastraceae

Genus: *Selenastrum* (Reinsch, 1867)

Coenobia of 2, 4, 8 or 16 cells connected equatorially by convex sides, rarely single-celled, enclosed within a mucilaginous envelop, cells spindle, sickle or half-moon shaped or so curved as to form an almost complete circle, gradually narrowing to acute aspices. Chloroplasts are parietal, single and with a pyrenoid. Asexual reproduction occurred by 2, 4 or 8 (16) autospore, if 8 then in two tetrads one above the other, release by breakage of mother cell in middle to leave 2 horn-like wall remnants connected to daughter cells (John et al., 2002). *Selenastrum capricornutum* showed 13% and 17.47% of lipid in glass flask reactor and photobioreactor (Pugliese et al., 2020). *Selenastrum* sp. showed 19.2% crude protein when cultured in 50% BG11 medium supplemented with phosphorus (Ma et al., 2012).

2.1.6 *Pectinodesmus* sp.

Taxonomic classification:

Kingdom: Plantae

Division: Chlorophyta

Class: Chlorophyceae

Order: Sphaeropleales

Family: Scenedesmaceae

Genus: *Pectinodesmus* (Schoch et al., 2020)

Cells are 25–60 × 5–12 μm in size, straight to slightly bent, cell length to cell width 5–8:1, coenobia with 4–8 linear or slightly alternately arranged cells. Cells under the SEM with longitudinal ridges and on top of the cells many bristles were excreted (Hegewald et al., 2013). The protein, lipid and carbohydrate content in *Pectinodesmus* sp. is more than 15%, 21.27% and 20.78% respectively in total of the dry weight in BG-11 broth (Samadhiya et al., 2021).

2.1.7 *Ankistrodesmus sp.*

Taxonomic classification:

Phylum: Chlorophyta

Class: Chlorophyceae

Order: Sphaeropleales

Family: Selenastraceae

Genus: *Ankistrodesmus* (Corda, 1838)

Cells are usually very elongate, narrowly spindle-shaped to cylindrical, straight, gradually or abruptly narrowing to acute apices. Chloroplast parietal, single and covering most of cell with or without a pyrenooid (John et al., 2002). protein content of *A. gracilis* is more than 50% of dry weight in NPK (Sipaúba-Tavares and Pereira, 2008) and carbohydrate content of *A. falcatus* tends to reduce (14.5% and 13.5%, respectively) under stress conditions (i.e., salinity) in BG-11 and BBM (Sun et al., 2014). *Ankistrodesmus falcatus* contain about 46.41 ± 0.57% protein, 23.22 ± 0.56% lipid and 32.99 ± 0.26% carbohydrate content in BBM (Okomoda et al., 2021).

2.1.8 *Scenesdesmus* sp.

Taxonomic classification:

Kingdom: Plantae

Division: Chlorophyta

Class: Chlorophyceae

Order: Sphaeropleales

Family: Scenedesmaceae

Genus: *Scenedesmus* (Meyen, 1829)

Flat colonies of 2, 4 or 8 elongated cells arranged in a row, some species have spines at the corners of the colonies. Cells are 5-30 μm long (Hilary Belcher and Erica Swale, 1976). *Scenedesmus* sp. is an economically available microalgae, where *Scenedesmus obliqus* produced about, 50-56% protein, 12-14% lipid and 10-17% carbohydrate, (Becker, 2007). *S. obliquus* contains 31.8 ± 0.01% protein, 42.6 ± 0.01% lipid and 42.6 ± 0.01% carbohydrate content in Bristol media. In case of

BBM, *S. obliquus* can produce about 30.7 ± 0.01% protein and 38.2 ± 0.02% of carbohydrate. *S. bibraianum* contains about 44.7 ± 0.00% protein, 9.4 ± 0.02% lipid in Bristol media (Khatoon et al., 2019).

2.2 Importance of microalgae

Microalgae are potentially rich source of important chemicals with potential application in the feed, food, nutritional, cosmetics, pharmaceuticals, even in fuel industries (Olaizola, 2003) and because of its higher protein content, microalgal biomass is of interest to human nutrition that has been progressively included in diets and "healthy foods".(Koller et al., 2014). The increasing requirements for protein and the high cost of fish meal in the recent years has led to search for new alternatives, as animal and plant sources of protein for the needs of aquaculture (Sirakov et al., 2015) where microalgae are recognized as an economical and sustainable alternative source due to their nutritional quality and potential availability (Badvipour et al., 2016). Due to the appropriateness in their size, high nutritional value, high growth rate, antioxidant property, disease resistance power, popularity of microalgae as aquaculture feed is increasing day by day, not only as basic nutrients but also as a source of pigments to colour the skin on the other (Roy and Pal, 2015). Microalgae are cultivated and use for food, to produce useful compounds, and as biofilters to remove nutrients and other pollutants from wastewaters, in aquaculture (Velichkova et al., 2012). Moreover, microalgae highly utilize in cosmetic and pharmaceutical industry (Sharma et al., 2013) as well as for biofuel production because of their high oil content and rapid biomass production (Hattab and Ghaly, 2014).

The main application of microalgae in aquaculture is connected with their usage for feed purposes and it is estimated that currently 30 percent of the world algal production is used for animal feed (Becker, 2007) but the use in aquaculture is mainly for larval fish, molluscs and crustaceans (FAO, 2009). Indeed, microalgae could be used directly as food for bivalve molluscs (eg. oysters, scallops, clams and mussels) at all development stages, in the juvenile stages of abalone, crustaceans, for larvae of some gastropods and for some fish species in their earliest growth stages (Brown, 2002). Moreover, microalgae used as food for zooplankton indirectly such as for rotifers as it is essential food for some fish larvae. In aquaculture, microalgae are added as a suspension to the organism's environment termed as "Green water" feeding technique, wherein microalgae are grown simultaneously in tanks with larvae

(Brown et al., 1997). The use of algae as an feed additive in aquaculture has received a lot of attention because of the positive effect it has on weight gain, enhanced triglyceride and protein accumulation in muscle, improved resistance to disease, reduce nitrogen output into the environment, increased fish digestibility, physiological activity, starvation tolerance and carcass quality (Becker, 2004).

2.3 Microalgae isolation

Species or strain selection is the first and critical step in bioprospecting of microalgae for any commercial application (Barclay and Apt 2013). Screening of microalgae species involves a series of steps including sample collection, isolation, purification, identification, maintenance and characterization of potential products (Gong and Jiang 2011). Sequencing microalgae taxonomy can be obtained by 18S ribosomal RNA genes for eukaryotic and 16S ribosomal RNA for prokaryotic microalgae (Bellinger and Sigee, 2010) depends on the varieties of microalgae. Although isolation and molecular identification of algae is now a common practice in different regions of the world, very few reports are available for the isolation and molecular identification of microalgae in Bangladesh. In Bangladesh, microalgae isolation is first reported by Tarin et al. (2016) where they isolated and characterized *Chlorella vulgaris* and *Anabaena variabilis* from natural and artificial water bodies of Dhaka University and Khulna to determine their possibility as feedstock and biofuel production. *Pithophora polymorpha* and *Spirogyra maxima* were effectively identified and newly reported microalgae from Bangladesh by analyzing partial 18S rDNA sequences (Alfasane et al., 2019). Recently Islam et al. (2021) characterize four marine microalgae (*Chlorella* sp., *Nannochloropsis* sp., *Tetraselmis* sp. and *Chaetoceros* sp.) on the basis of growth performance, pigments and nutritional composition, isolated from Cox's Bazar coast of Bangladesh. In Bangladesh a very few attention have been paid to the indigenous microalgae and its potentialities especially from the freshwater habitat.

2.4 Growth factors of microalgae

Growth of microalgae is affected by some factors such as length of photoperiod, temperature, pH and light intensity (Wahidin et al., 2013).

2.4.1 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 the 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 that 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.

2.4.2 Temperature

Temperature is the second 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 (up to 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). Optimum temperature of microalgae culture ranged from 20 to 24 ºC.

2.4.3 pH

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

2.4.4 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 and 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 thiamine (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.

2.4.5 Mixing and aeration

Homogenous mixing and aeration is also an important factor for microalgae growth. 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 cell damage (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.

2.5 Microalgal growth

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 and v) decline or death phase. Under suitable condition microalgae show all that phases.

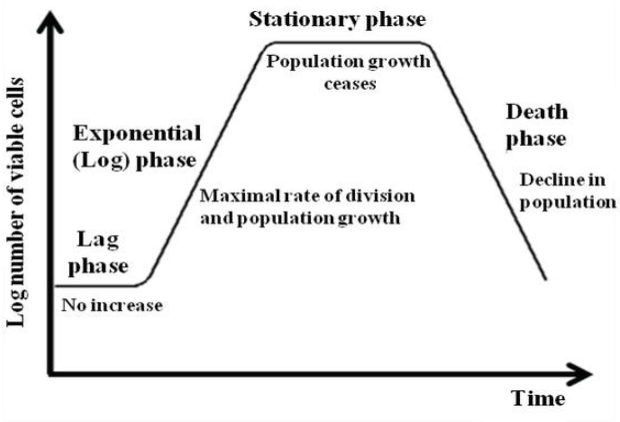


Figure 2.1: Microalgae growth curve (Teresa et al., 2010)

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

Monitoring of cell growth is deliberated as a primitive portion for the usage of microalgae in aquaculture (Santos-Ballardoa et al., 2015). Principally two analytical methods are applied for microalgae growth analysis: cell density determination by counting of cells as cells per milliliter (Godoy-Hernández and Vázquez-Flota, 2006), and spectrophotometric absorbance, by determining the cell suspensions absorbance (Mikschofsky et al., 2009). For microalgae biomass measurement, optical density (OD) determination is an indirect method as it can be directly correlative with the number of cells in the medium (Ribeiro-Rodrigues et al., 2011). A deep knowledge of the behavior of a specific microalgal strain in response to various culture conditions, like nutrient supply, is important for the optimization of microalgal production (Hyka et al., 2013). Several nutritional factors such as nitrogen, phosphorus, carbon and iron are recognized as one of the most important factors influencing the yield of biomass and the lipid accumulation (White et al., 2013). Growth and productivity of microalgae also differ between species to species. Chemical composition and productivity of potentially important indigenous species should be analyzed to assess the nutritional properties of them as they generate a less environmental impact where the system is operating, due to more ecological suitability and high level of adaptability to local environment.

2.6 Microalgal pigments

2.6.1 Chlorophyll

Pigments are considered as one of the most essential product from microalgae (Granado-Lorencio et al., 2009) and chlorophylls, carotenoids, and phycobilins are the significant pigment group found in microalgae (Koller et al., 2014). Chlorophyll is one of the significant bioactive materials that can be extracted from microalgae (Aris et al., 2010) and chlorophyll-a and chlorophyll-b are the two main types of chlorophylls (Cubas et al., 2008). Chlorophyll-a is the main light harvesting pigment that transforms light energy into chemical energy and chlorophyll-b participate indirectly in photosynthesis by converting the light it absorbs into Chlorophyll-a (Nayek et al., 2014). Chlorophyll and its derivatives have antioxidant property, utilized as a natural food coloring agent (Hosikian et al., 2010) and also used widely in pharmaceutical products (Bhagavathy and Sumathi, 2012).

2.6.2 Carotenoid

Carotenoid, this is a natural, fat-soluble, yellow to red pigments, are principally dominant in plants, where they play an important role in photosynthesis in algae and photosynthetic bacteria (Lamers et al., 2012). However, the carotene production is influenced by several factors like salinity, light intensity, nutrient deprivation and temperature. (Kleinegris et al., 2009). The wide use of carotenoids as colorants has been found in natural foods including egg yolk, chicken, and fish. More than 750 carotenoids have been identified; but only a few have been used commercially like astaxanthin, canthaxanthin, lutein, lycopene and β-carotene (Vílchez et al., 2011). The main advantage of the using microalgae as a carrier of carotenoids is their positive impact on human health due to the presence of many other antioxidant compounds. Astaxanthin is synthesized by Chlorophyceae family namely *Chlorella, Chlamydomonas, Dunaliella,* and *Haematococcus* spp., etc. (Pulz and Gross, 2004).

2.6.3 Phycobiliproteins

Phycobiliproteins are a group of proteins with covalently attached linear tetrapyrrole chromophoric groups (Ba´saca-Loya et al., 2009) and most common phycobiliproteins consist of phycoerythrin (PE), phycocyanin (PC), and allophycocyanin (APC) (Wiedenmann, 2008). As phycocyanin protects the photosystems from free radicals and can be able to prevent oxidative damage caused by free radicals, phycocyanin pigment commercially utilized in pharmaceutical industry. Phycocyanin is water soluble, strongly fluorescent blue-green light-harvesting pigment has potent antioxidant properties, specifically found in cyanobacteria exceptional to some algal genera, and belongs to Rhodophyta and Cryptophyta (Mathivanan et al., 2015). Phycobiliproteins used as natural dyes and extensively applied as nutraceuticals and in other biotechnological applications like food, cosmetics, diagnostics and pharmaceutical industries (Becker, 2004).

2.7 Proximate composition of microalgae

Proximate composition of microalgae is generally the percentage composition of some basic constituents like water, protein, fat, carbohydrate and ash (mainly minerals) (Ganguly et al., 2018). In general, the protein content of microalgae constitutes a major portion compared to lipid and carbohydrate (Lavens and Sorgeloos, 1996). The total protein content from microalgae, especially *Chlorella* sp.

is about 43–50% (Phukan et al., 2011). Some algae, particularly blue-green and green algae, contain very high levels of protein, typically 40 to 60% (of dry matter), that can be used as functional food ingredients. Algal proteins possess a high nutritional value in terms of protein content, amino acid quality and nutritional acceptability (Reyes et al., 2012). Lipid content of microalgae is usually in the range of 20–50% of the cell dry weight, and can be as high as 80% under certain conditions **(**Michael and Borowitzka, 2018). Microalgae can overproduce lipids or carotenoids under stress condition such as high salt, high light, or nutrient limitation. For instance, lipid accumulation in *Dunaliella* sp. and *Chlorella vulgaris* was significantly increased under high-salinity stress, reaching 70% and 21.1%, respectively (Singh et al., 2016). Microalgae strains with high oil or lipid content are of great interest in the search for a sustainable feedstock for the production of biodiesel. Compared to other microalgae compounds, carbohydrates have a lesser energy value but they are the preliminary raw component for the synthesis of biofuels via biotechnological conversion (Andreeva et al., 2021; Aytenfisu et al., 2018). The amount of lipids and their proximity or position of double bonds in the carbon chain and carbohydrate content can differ based on the species of microalgae in biomass depends on the type of microalgae and growth conditions (Villarruel-Lopez et al., 2017; Park et al., 2014).

Therefore, the aim of this study is to isolate microalgae from some south-eastern freshwater habitat such as Kaptai Lake, Karnaphuli river and Halda river; as well as to characterize them on the basis of growth, pigments and proximate composition to identify the potential species for different commercial application.

Chapter 3: Materials and Method

3.1 Microalgae sampling site

The freshwater microalgae samples were collected from March to May month from three different sites in Chattogram, Bangladesh including Kaptai Lake, Rangamati ($22^o64'$ N, $92^o19'$ E), Halda River, Chattogram ($22^o51'$ N, $91^o84'$ E) and Karnaphuli River, Chattogram (22°50' N 92°14' E).

3.2 Determination of water quality parameter

The water temperature, pH, dissolved oxygen (DO) and salinity was measured from the surface water by using glass thermometer, a handheld pH meter (pHep-HI98107, HANNA, Romania), dissolve oxygen meter (DO-5509, Lutron) and a handheld ATC refractometer (YEGREN), respectively. All the instruments were calibrated before use. As well as, total ammonium nitrogen (TAN), nitrite–nitrogen (NO2-N) and soluble reactive phosphorous (SRP) was determined by using Parsons et al. (1984) analytical methods.

3.2.1 Determination of total ammonia nitrogen (TAN)

Total ammonia nitrogen was determined according to Parson et al. (1984). Standard stock solution was prepared by weighing 9.343 g of anhydrous grade $(NH_4)_2SO_4$ (dried at 110°C for 1 hr, cooled in desiccator before weighing) and dissolving in 1000 ml deionized water. From the stock solution (1000 mg L^{-1} of total ammonia-nitrogen), a series of standard solutions (0.01, 0.03, 0.05, 0.07, 0.1, 0.3, 0.5 and 1.0 mg L^{-1}) were prepared by mixing with appropriate ratio of deionized water.

Samples and standard solutions (10 ml) were placed in test tube and 0.4 ml of phenol solution (20 g of analytical grade phenol was dissolved in 200 ml of 95% v/v ethyl alcohol and 0.4 ml of sodium nitroprusside (1 g of $Na_2[Fe(CN)_5\ NO]_2H_2O$, dissolved in 200 ml of DDH_2O water) was added in sequence. Finally, 1 ml of oxidizing solution was added and allows cooling at room temperature (20-27°C) for 1 hr. The test tubes were covered with parafilm (the color is stable for 24 hr after the reaction period). The extinction was measured at 640 nm with spectrophotometer (Nano Drop Spectrophotometer, Model-Nanoplus, Germany). Oxidizing solution was prepared by mixing 100 ml of alkaline reagent (dissolve 100 g of sodium citrate and 5 g of sodium

hydroxide in 500 ml of DDH_2O) and 25 ml of sodium hypochlorite solution [commercial hypochlorite (e.g. clorox) which should be about 1.5 N].

3.2.2 Determination of nitrite-nitrogen (NO_2-N)

Nitrite was determined according to Parsons et al. (1984). Standard stock solution was prepared by weighing 4.93 g anhydrous grade $NaNO_2$ (dried at 110°C for 1 hr, cooled in desiccator before weighing) and dissolving in 1000 mL deionized water. From the stock solution (1000 mg L^{-1} of NO_2-N), a series of standard solutions (0.01, 0.03, 0.05, 0.07, 0.1, 0.3, 0.5 and 1.0 mg L^{-1}) were prepared by mixing with deionized water.

Samples and standard solutions (10 ml) were placed in test tube. Then 0.2 ml of sulfanilamide solution (5 g of sulfanilamide was dissolved in a mixture of 50 mL of concentrated hydrochloric acid and dilute to 500 ml with DDH_2O) was added. After more than 2 minutes but less than 10 minutes, 1 ml of NED reagent (0.5 g of the N-(1-napthyl)-ethylenediamine dihydrochloride was dissolved in 500 ml of distilled water) was added and mixed immediately. Between 10 minutes and 2 hr afterwards, the extinction was measured at a wavelength of 543 nm by using the Nano Drop Spectrophotometer (Model-Nanoplus, Germany).

3.2.3 Determination of soluble reactive phosphorous (SRP)

Soluble reactive phosphorous (SRP) was determined according to Parsons et al. (1984). Standard stock solution was prepared by weighing 4.39 g of anhydrous grade potassium dihydrogen phosphate, KH_2PO_4 (dried at 110°C for 1 hr, cooled in dessicator before weighing) and dissolving in 1000 ml deionized water. From the stock solution (1000 mg $L^{-1}$of PO_4-P) a series of standard solutions (0.01, 0.03, 0.05, 0.07, 0.1, 0.3, 0.5 and 1.0 mg L^{-1}) was prepared by mixing with deionized water.

Ten milliliter of samples and standard solutions (10 ml) were placed in test tubes and 1 ml of mixed reagent was added. After 5 minutes and preferably within the first 2-3 hours, the extinction was measured at 885 nm by using Nano Drop Spectrophotometer (Model-Nanoplus, Germany). Mixed reagent was prepared by mixing 100 ml of ammonium molybdate (dissolve 15 g of analytical reagent grade ammonium paramolybdate $(NH_4)6Mo_7O_{24}$ in 500 ml of distilled water), 250 ml sulfuric acid, 100 ml ascorbic acid (dissolve 27 g of ascorbic acid in 500 ml of distilled water) and 50

ml of potassium antimonyl-tartrate solution (dissolve 0.34 g of potassium antimonyl-tartrate (tartar emetic) in 250 ml of water.

3.3 Sample collection and concentration

For sample collection, 60 µm mash size plankton net was used where about 40-50L freshwater was filtered through the plankton net and collected in a 300ml sample bottle and maintained at refrigerated condition while transferring to the laboratory. Then the samples were concentrated by centrifuging at 4000 rpm for 5 minutes. After centrifugation supernatant were discarded and the concentrate was used for isolation.

3.4 Determination of microalgal diversity

Filtered raw freshwater samples from each of the sampling site were preserved by adding a few drop of lugol's iodine to determine the microalgal diversity. Then the samples were observed under microscope and microalgal diversity was determined by using phytoplankton identification books such as Belcher and Swale (1976); John et al. (2002) and Bellinger and Sigee (2010).

3.5 Isolation of microalgae

3.5.1 Agar plate preparation and streak plating method

For preparing the agar medium, 1.5 % agar was added to 1 L of BBM and solution was sterilized in an autoclaved at 121°C temperature for 15 min under 150 lbs pressure. Then the agar medium was cooled to about 50°C and vitamin solution was added into it while mixing by gently rotating the flask to ensure mixing of the nutrients and avoid bubble formation. After that, the warm medium was aseptically poured into the sterile petri dish where the agar was at least ½ or ¾ the depth of the petri dish, and agar medium was left to cool and solidify and stored in plastic bags in a refrigerator until further use. After preparation of the agar plate 1-2 drops of the concentrated natural collection was placed near the periphery of the agar. Following aseptic technique, wire loop or hockey stick was used to make parallel streaks of the suspension on the agar. The plates were covered, inverted in position and sealed with parafilm to prevent the petri dish from opening unnecessarily or accidentally while incubating. When growth was visible in the dish, the petri dish was taken out from the incubator and chosen colony was removed by using a sterile fine needle and place in 3-5 drops of liquid medium on a sterile dish and observed under microscope to select the desired colonies that are free from other organisms for further isolation. When the

colony contained multiple microalgae, streaking procedure was repeated again to develop single colony.

3.5.2 Serial dilution

For serial dilution, test tubes were filled with 9 ml of culture medium (BBM) prepared in the last lab. Tubes were labeled as 10^{-1}–10^{-10} to indicate dilution factor. After that, test tubes were put in the biological safety cabinet where test tube cap was removed and its neck was flamed and 1 ml of enrichment sample was added to the test tube (10^{-1}) and mixed gently. Then, 1 ml of this dilution was taken and added to the next tube (10^{-2}) and then mixed gently and this procedure was repeated for the remaining tubes (10^{-3}–10^{-10}). After completing the dilution procedure, test tubes were incubated under controlled temperature and light conditions. After 2–4 weeks, the cultures were examined microscopically by withdrawing a small sample aseptically from each dilution tube where unialgal culture found in one of the higher-dilution tubes, e.g., 10^{-6}–10^{-10}. When the tubes contain two or three different species, capillary pipetting method was applied for isolation of single algal species.

3.5.3 Picking up method (Capillary method)

For capillary picking up method, first a micropipette was prepared from a Pasteur pipette, where pasteur pipette was held in the hottest region of the flame, supported on the left by a hand and on the right by forceps and heated until it soft and pliable condition and when it became soft, the pipette was quickly removed from the flame with a gentle pull to produce a thin tube. The forceps was then relocated to the appropriate region of the thin tube to gently bend the thin area, so that it broke and formed a micropipette. A large drop of algal suspension growing by the dilution method was placed in the center of a glass slide. 4-6 drops of prepared BBM was placed in another slide. By using an inverted microscope to observe the cells, the desired single cell from the large drop of the algal suspension was transferred to one of the drops of liquid media. This process was repeated by pipetting the desired algal unit from liquid medium drop number one to two and continued until only one single algal unit was found in the last drop of liquid medium without any foreign substance. The single algal unit was then transferred into the eppendrof containing liquid media and incubated at the desired environmental condition.

3.6 Morphological identification of microalgae:

Morphological identification of isolated microalgae was done microscopically at 40X magnification and based on the morphological characteristics using phytoplankton identification books (Belcher and Swale, 1976; John et al., 2002 and Bellinger and Sigee, 2010).

3.7 Preparation of BBM

For the preparation of BBM, distill water was used as a source of freshwater. Standard BBM was used for the culture of microalgae. To prepare 1 L of BBM, first 10 mL of each stock solution (1-6), 1 mL of stock solution (7, 8 and 10) and 0.7 ml of boric acid solution (9) was added into 940 mL of dH_2O. Finally, 0.5 ml of filter sterilized vitamin solution (11) was poured into it. Table 3.1 showed the different constituents of BBM.

Table 3.1 Constituents of Bold Basal Medium

Name of chemicals	**Quantity**
1. Potassium dihydro phosphate (KH_2PO_4)	8.75 g/500ml
2. Calcium chloride dehydrate ($CaCl_2$. $2H_2O$)	1.25g/500ml
3. Magnesium sulfate hydrate ($MgSO_4$. $7H_2O$)	3.75g/500ml
4. Sodium nitrate ($NaNO_3$)	12.5g/500ml
5. Dipotassium phosphate (K_2PO_4)	3.75g/500ml
6. Sodium chloride (NaCl)	1.25g/500ml
7. $Na_2EDTA.2H_2O$	10g/L
Potassium hydroxide (KOH)	6.2g/L
8. Iron (2) sulfate 7-hydrate ($FeSO_4.7H_2O$)	4.98g/L
Sulphuric acid (H_2SO_4)	1ml/L
9. Boric acid (H_3BO_3)	5.75g/500ml
10. Trace metal solution	g/L
Boric acid (H_3BO_3)	2.86g
Manganese (2) chloride tetrahydrate ($MnCl_2$. $7H_2O$)	1.81g
Zinc sulfate hydrate ($ZnSO_4$. $7H_2O$)	0.222g
Sodium molybdate dihydrate ($Na_2MoO_4.2H_2O$)	0.390g
Copper (2) sulfate pentahydrate ($CuSO_4$. $5H_2O$)	0.079g
Cobalt nitrate hexahydrate ($Co(NO_3)_2$. $6H_2O$)	0.0494g

11. Vitamins			0.5ml
Thiamine HCl	200mg	To 950 ml of dH_2O	
Biotin	1g/L	1ml from each of the two primary stocks was added then and made to 1L	
Cyanocobalamin	1g/L		

3.8 Determination of growth curve

The isolated microalgae were cultivated using the BBM (Stein, 1980). Cultures were grown at a temperature of 24±1°C in a 350 mL culture volume of a sterile 500 mL borosilicate Erlenmeyer flask for each species with three replicates where 2% pure culture stocks were added in each flask. Microalgae cultures were maintained at 24 hour light condition at 150 μEm-$2s^{-1}$ intensity with continuous gentle aeration at a rate of 4.53±0.53 mg/L. The experiment was continued until the death phase and finally completed the growth curve depending on cell density (cells.ml^{-1}) and optical density (absorbance).

3.8.1 Determination of cell density

Microalgae cell count was carried out every day by using a Neubauer hemacytometer (0.0025 mm^2, 0.1 mm deep chambers, Assistent, Germany). Before use, hemacytometer was cleaned with distilled water to make sure it is free from dust, lint and grease. To facilitate counting, Lugol's iodine was added to microalgae sample for fixation and staining. Then small drop of properly mixed sample was placed into the counting chamber and the cells were allowed to settle 3-5 minutes for better counting. Under the magnification of 40X, microalgae cells were counted for both chambers of the hemacytometer. The cells were counted by using the following formulae (Lavens and Sorgeloos, 1996):

$$\text{Cell count (cell/ml) for 25 squares} = \frac{\text{Total number of cells counted}}{50\times4} \times 10^6$$

Where 50 = the squares of the 2 hemacytometer chambers and 4 x 10^{-6} = the volume of samples over the small square areas, that were equivalent to 0.004 mm^3 (0.2 mm x 0.2 mm x 0.1 mm), expressed in cm^3 (ml).

3.8.2 Determination of maximum absorbance (optical density)

Optical density (OD) was measured every day for the growth curve analysis. BBM without any microalgae cells were used as blank. Maximum absorbance value for each microalga was used to perform the growth curve by OD. Maximum absorbance were measured at the wavelength of 430 nm for *Nephrocytium* sp., 450 nm for *Nannochloropsis* sp. and *Selenastrum* sp., 428 nm for *Monoraphidium* sp., 426 nm for *Sphaerocystis* sp., 362 nm for *Pectinodesmus* sp., 363 nm for *Ankistrodesmus* sp. and 630 nm for *Scenedesmus* sp. as those wavelengths gave maximum absorbance when the culture samples were scanned between 300 to 700 nm, using a spectrophotometer (Nano Drop Spectrophotometer, Model-Nanoplus, Germany).

3.9 Experimental design for pigment and proximate composition determination

In large sterile 2L borosilicate Erlenmeyer flasks, having 1.7 L pure BBM were used for this experiment. Each of the microalgae species was cultured to maintain similar environmental condition (Temperature: 24±1°C; Light: 150 μEm-2s-1 intensity) until stationary phase. From the fresh cultured sample, carotenoid and chlorophyll were analyzed at the end of their exponential phase. As phycobiliprotein and proximate composition analysis required dry biomass, all the cultures were harvested at the end of their exponential phase by centrifugation (Hitachi* High-speed Refrigerated Centrifuge, himac CR 21g-II, China) depending on the growth curve experiment, and dried at 40°C temperature by using hot air oven and finally preserved at a refrigerator (4°C) for further use.

3.10 Determination of biomass

Biomass determination is prerequisite for productivity analysis. Biomass were determined by filtering of 1ml microalgae sample from each replication of individual microalgae through a pre-weighted (after marking of filter paper rinsed with 10ml distill water and dried at 100°C for 4 hour in hot air oven) glass microfiber filter paper, which was further rinsed with 10ml distill water for three times. Then the filter paper with biomass was oven dried at 100°C for 4 hour. After that, final weight of filter paper was taken followed by 15 min of desiccation and dry biomass was calculated according to Ratha et al. (2016).

3.11 Determination of productivity

Biomass productivity, areal productivity and lipid productivity were calculated according to Mercado et al. (2020) and Sukkrom et al. (2016). Productivity data was calculated at the end of the exponential phase. Specific growth rates (SGR) and cell doublings per day were calculated according to Daniel and Srivastava (2016) and cell duplication time was calculated according to Chiu et al. (2008).

3.11.1 Volumetric productivity

Following equation was used to calculate volumetric productivity:

$$\text{Volumetric productivity/VP (mg L}^{-1}\text{ day}^{-1}\text{)} = \frac{(X_2 - X_1)}{(t_2 - t_1)}$$

Where, X_1 and X_2 were the biomass concentrations (mg L^{-1}) on days t_1 (start of study) and t_2 (end of the study).

3.11.2 Areal productivity

Following equation was used to calculate areal productivity:

$$\text{AP (mg cm}^{-2}\text{ day}^{-1}\text{)} = \frac{VP \times V}{A}$$

Where, VP = volumetric productivity, V = total volume of the culture, A = surface area occupied ground.

3.11.3 Lipid productivity analysis

Lipid productivity (LP) was calculated by using the following equation:

$$\text{LP (mg L}^{-1}\text{ day}^{-1}\text{)} = \frac{\text{Biomass productivity (mg } L^{-1}\ day^{-1}\text{) x (\% lipid)}}{100}$$

3.11.4 Specific growth rates (SGR)

SGR was calculated using the formula as follows:

$$\text{SGR (r)} = \frac{\ln Nt - \ln No}{\Delta t}$$

Where, Nt is the final cell count and No is the initial cell count; Δt is the number of culture days.

3.11.5 Cell duplication time

Cell duplication time was calculated using the following formula:

Cell duplication time (td)= 0.693/μ

3.11.6 Cell doublings per day

Cell doublings per day was calculated using the following formula:

Cell doublings per day (K) = $\frac{\ln Nn - \ln Ni}{\ln 2(tn - ti)}$

Where, Nn is the final cell count and Ni is the initial cell count; tn is the final time in days and ti is the initial time in days.

3.12 Determination of pigments

3.12.1 Extraction of microalgae for chlorophyll determination

For the extraction of microalgae, first 1ml of $MgCO_3$ solution (after proper shaking) was filtered through the filter paper (47 mm Ø Whatman® GF/C glass microfiber filter papers) by using filtering apparatus, so that most of the area of filter was covered. Then, 10 ml of each algae sample was filtered and the edges which were not coated with residue were trimmed away. After that, the filter paper was fold and place into 15 ml centrifuge tube where the middle of the filter is facing downward. Into the centrifuge tube, 2 ml of 90% acetone was added and grind for 1 minute, again 8ml of 90% acetone was added and grind for 30 sec. Then, the sample was refrigerated in the dark for 1 hour. After 1 hour, sample was centrifuged at 3000 rpm for 10 minutes and transferred the acetone extract into another centrifuge tube and centrifuged at low speed (500 rpm) for 5 minutes. Lastly, the absorbance of acetone extract was measured against 90% acetone as blank.

3.12.2 Determination of chlorophyll

Chlorophyll concentration was determined according to Jenkins (1982). The clean extract was transferred to a 1cm cuvette and OD was measured at 750, 664, 647 and 630 nm wavelength. OD at 664, 647, and 630 nm were used for chlorophyll determination where, OD at 750 nm was used as turbidity correction factor and subtracted from each of the pigments OD values before using them in the equations. Concentrations of chlorophyll-a and b in the extract was calculated by inserting the corrected optical densities in following equations (Jeffrey and Humphrey, 1975):

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

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

Where: C_a and C_b= concentrations of chlorophyll-a and b, mg/L, and OD664, OD647, and OD630 = corrected optical densities (with a 1 cm light path) at the respective wavelengths. After the determination of the concentrations of pigments in the extract, the amount of pigments per unit volume was calculated as follows:

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

3.12.3 Determination of carotenoids

1 mL aliquot of the algal suspension from each culture was taken at their stationary phase and centrifuged at 1000×g for 5 min. Then, the obtained pellet was extracted with 3 mL of ethanol: hexane 2:1 (v/v). After that, 2 mL of water and 4 mL hexane (Sigma, USA) were added to the mixture, shaken vigorously and centrifuged again at 1000×g for 5 min. Finally, absorbance of the separated hexane layer was determined using spectrophotometer at a wavelength of 450 nm. The amount of extracted carotene from the samples in micrograms was determined by multiplying the absorbance ($A_{450)}$ with 25.2 (Shaish, 1992).

3.12.4 Determination of phycobiliproteins

As phycobiliproteins estimation required dried biomass, the cultures were harvested by centrifugation at 6000 rpm for 5 min and the harvested pellets were dried at 40°C for overnight. 40 mg of dried powder was then soaked in 10 mL phosphate buffer (pH 7.0; 0.1 M), mixed properly using vortex mixture, and then stored for 24 hour at 4°C. After that, the samples were centrifuged at 6000 rpm for 10 min. Finally the supernatant was collected and spectrophotometric (Nano Drop Spectrophotometer, Model-Nanoplus, Germany) absorbance was measured against the phosphate buffer solution as blank at the wavelength of 562, 615, 652 and 720 nm where 720 nm measured the absorbance of the cellular debris.

The amount of phycocyanin (PC), and allophycocyanin (APC) in the sample was calculated from the absorbance according to Bennett and Bogorad (1973) and phycoerythrin (PE) was calculated according to Siegelman and Kycia (1978).

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

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

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

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

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

Where, V= solvent volume, DB= dried biomass

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

3.13 Determination of proximate compositions

3.13.1 Protein determination

Protein was determined according to Lowry et al. (1951). For that, 5 mg of each dried microalgal biomass was taken and prepared 25 mL well mixed (tissue homogenizer) microalgae solution by using distilled water. From that 25 mL, 0.5 mL solution was taken from each type of samples, and 0.5 mL of 1N NaOH was added and kept in hot water bath (100°C) for 5 min. After that, the samples were put into cold water bath. After 10 min of cooling, 2.5 mL of mixed reagent (50 mL of Reactive 2 (2g of Na_2CO3 in 100 mL of 0.1 NaOH) and 1 mL of Reactive 1 (1% NP tartrate), mixed) was added in each types of sample. After proper mixing by using vortex mixture, 0.5 mL of folin reagent was added into every sample. Then, the samples were kept in dark place for 30 min and spectrophotometric absorptions were measured at 750 nm wavelength. 2000 µg/L of standard (albumin) stock solution was prepared to produce a calibration graph, from which series of standards at various dilutions (20 µg/L, 40 µg/L, 80 µg/L, 100 µg/L and 200 µg/L) were also prepared and protein analysis procedure was applied as described above. From the obtained absorbance a standard graph was plotted according to the standard results and protein content was determined for each type of samples accordingly.

3.13.2 Carbohydrate determination

Carbohydrate was determined according to Dubois et al. (1956). 5 mg of each freeze dried biomass was taken and prepared 25 mL well mixed (tissue homogenizer) microalgae solution by using distilled water. From that 25 mL, 1 mL solution was taken from each type of samples, and 1 mL 5% phenol and 5 mL of sulfuric acid were poured into it maintaining every 30 sec reaction period. After that, the samples were

put in the cold water bath. After cooling, spectrophotometric absorptions were taken for each types of solution at 488 nm wavelength to estimate carbohydrate. 1000 µg/L of standard (glucose) stock solution was prepared to produce a calibration graph, from which, a series of standards at various dilutions (20 µg/L, 40 µg/L, 60 µg/L, 100 µg/L and 140 µg/L) were also prepared and carbohydrate analysis procedure was applied as described above. From the obtained absorbance a standard graph was plotted according to the standard results and carbohydrate content was determined for each type of samples accordingly.

3.13.3 Lipid determination

Lipid was determined according to Bligh and Dyer (1959), and Folch et al. (1957). Aluminum dishes were labeled and weighted as initial weight for each types of sample. In a centrifuge tube, 50 mg of each sample was taken and diluted into 5x volume using distilled water. Then, 3 mL methanol: chloroform (2:1, v/v) was added and mixed (tissue homogenizer) properly. After that, all the tubes were centrifuged at 1000 rpm for 4 min at 4°C. After centrifugation, the supernatants were transferred into clean tubes by pasteur pipette, and placed them in ice. In the sample tubes, again 3 mL of methanol: chloroform (2:1, v/v) was mixed (tissue homogenizer) properly and centrifuged again at the same conditions, and the supernatants were transferred to the previous supernatants tubes. In the combined supernatants, 1.5 mL of 0.9% NaCl was poured and mixed well by using vortex mixture and kept in the refrigerator for 1 hr at 4°C temperature. Then the tubes were centrifuged at 1000 rpm for 10 min at 4°C temperature that results in two separate layer from which the upper layer of methanol and chloroform was discarded, while, the lower layer was transferred in previously prepared aluminum dish. After that, the solvent was evaporated at 60°C using hot air oven. Afterwards, the aluminum dishes were weighed to get the final weight and lipid weight in the samples was determined by subtracting initial weight from the final weight.

3.14 Statistical analysis

Mean and standard error (SE) of mean were calculated using MS excel. When assumptions were met, all statistical analyses regarding the growth parameters, proximate composition, and pigments content were performed by using the IBM SPSS (v. 26.0). Descriptive statistics were performed for different treatments;

thereafter, a test for homogeneity of variance was done. The collected data were analyzed using a one-way analysis of variance (ANOVA). Significant differences amongst treatments were analyzed using Tukey HSD multiple comparison tests at 95% confidence interval level.

Chapter 4: Result

4.1 Water quality parameters of the sampling sites

The physicochemical parameter of collected water from different sites was shown in Table 4.1 where differences in physical and chemical parameters were observed among different sites.

Table 4.1: Water quality parameters of the sample water collected from different freshwater sites:

Parameters	**Halda river**	**Karnaphuli river**	**Kaptai lake**
Temperature (˚C)	26.4	28.2	30
DO (mg/L)	4.2	7.7	6.33
pH	8.9	8.0	8.4
Total Ammonia nitrogen (TAN) (mg/L)	0.03	0.02	0.03
Soluble reactive phosphate (mg/L)	0.16	0.04	0.09
Nitrite-Nitrogen (mg/L)	0.12	0.06	0.04

4.2 Microalgal diversity in the sampling sites

Microalgal diversity of collected water from different sites was shown in Table 4.2 and Figure 4.1 where *Cyclotella* sp. is dominant in both Halda river and Karnaphuli river. Moreover, *Staurastrum* sp., *Ceratium* sp., *Microcystis* sp. and *Chroococcus* sp. were dominant in both Kaptai lake and Karnaphuli river.

Table 4.2. Microalgal diversity in different sampling sites where "*" represent the presence of species and "***" represent the most dominant species in the sampling site.

Microalgae species	**Halda river**	**Kaptai lake**	**Karnaphuli river**
Nannochloropsis sp.	***		
Pinnularia sp.	*		
Cryptomonas sp.	*		
Navicula sp.	*		*
Monoraphidium sp.	*		
Thalassiosira sp.	*	*	*

Cyclotella sp.	***	*	***
Synedra sp.	*		
Nitzschia sp.	*		*
Oedogonium sp.		*	
Coelastrum sp.		*	
Staurastrum sp.		***	***
Sphaerocystis sp.		*	
Cosmarium sp.		***	
Ceratium sp.		***	***
Anabaena sp.		*	*
Tribonema sp.		*	
Pediastrum sp.		*	*
Microcystis sp.		***	***
Dinobryon sp.		***	*
Tetraedron sp.		*	***
Chroococcus sp.		***	***
Guinardia sp.			*
Spirogyra sp.			*
Epithemia sp.			*
Gomphonema sp.			*
Nephrocytium sp.			*
Monoraphidium sp.			*
Pectinodesmus sp.			*
Selenastrum sp.		*	
Ankistrodesmus sp.		*	
Scenedesmus sp.		*	

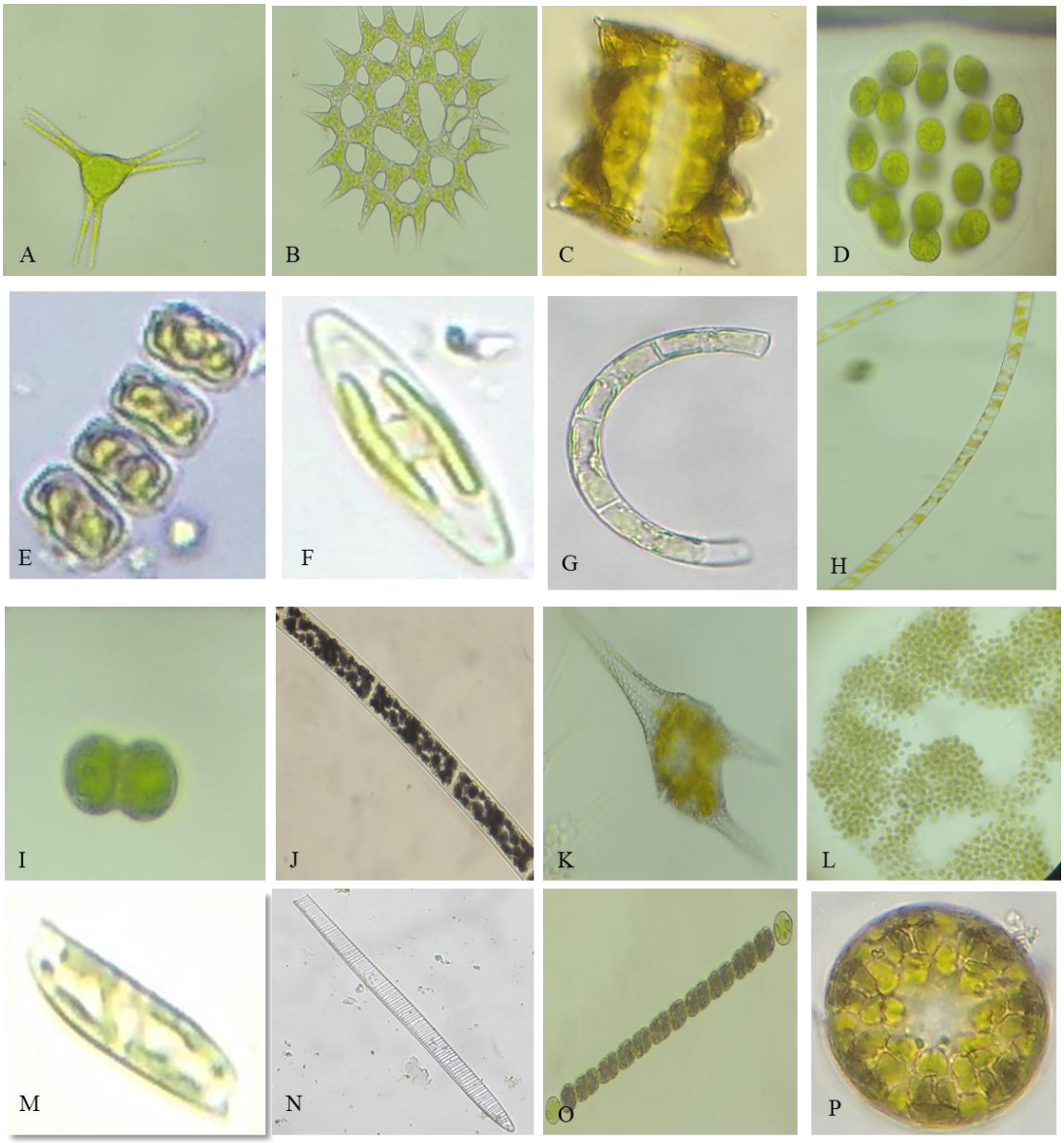


Figure 4.1: Light microscopic pictures of commonly found microalgae, *Staurastrum* sp. (A), *Pediastrum* sp. (B), *Cyclotella* sp. (C), *Coelastrum* sp. (D), *Thalassiosira* sp. (E), *Navicula* sp. (F), *Guinardia* sp. (G), *Oedogonium* sp. (H), *Chroococcus* sp. (I), *Spirogyra* sp. (J), *Ceratium* sp. (K), *Microcystis* sp. (L), *Pinnularia* sp. (M), *Synedra* sp. (N), *Anabaena* sp. (O) and *Cyclotella* sp. (P).

4.3 Characterization of isolated microalgae

Eight species of microalgae were isolated in this study (Table 4.3 and Figure 4.2). According to John and Whitton (2002), isolated microalgae were *Nephrocytium* sp. (A), *Nannochloropsis* sp. (B), *Monoraphidium* sp. (C), *Sphaerocystis* sp. (D), *Selenastrum* sp. (E), *Pectinodesmus* sp. (F), *Ankistrodesmus* sp. (G) and *Scenedesmus* sp. (H) of which the largest one is *Pectinodesmus* sp. (32µm) and the smallest one is

Nannochloropsis sp. (3.3µm). Among the eight microalgae, *Scenedesmus* sp., *Selenastrum* sp., *Sphaerocystis* sp. and *Ankistrodesmus* sp. were isolated from the Kaptai lake, where *Nephrocytium* sp., *Monoraphidium* sp. and *Pectinodesmus* sp. from Karnaphuli river and *Nannochloropsis* sp. from Halda river.

Table 4.3: Characteristics of isolated microalgae

Microalgae species	**Characteristics**
Scenedesmus sp.	➢ Flat colonies of 2, 4 or 8 elongated cells arranged in a row ➢ Species have spines at the corners of the colonies ➢ Single cells are 8-10 µm long; colonies are up to 30 µm long
Selenastrum sp.	➢ Single-celled species, 8 to 10 µm in length ➢ Cells are half-moon shaped or so curved as to form an almost complete circle, gradually narrowing to acute aspices
Nannochloropsis sp.	➢ *Nannochloropsis* are small, round or ovoid and nonmotile ➢ Have a diameter of about 2.5 to 3.3 micrometers
Nephrocytium sp.	➢ Coenobia of 4, 8 or 16 irregularly or spirally arranged cells and often retained prior to release within a thickened and well-defined gelatinizing mother cell wall, usually surrounded by a mucilaginous envelop ➢ Single cells are 8 to 10 µm in length and 6 to 8 µm in width ➢ Cells are ovoid, elongate ovoid, ellipsoidal and with obtuse apices or spindle-shaped with almost acute aspices, asymmetrical or distinctly curved
Monoraphidium sp.	➢ Cells are free living, without a specialized organ, if attached, more or less spindle-shaped, straignt, curved or spirally twisted, gradually or abruptly narrowing to acute aspices ➢ Cells are 20 to 22 µm in length and 2 to 4 µm in width ➢ Thin-walled and without a mucilaginous envelope ➢ Chloroplasts parietal, single and without a visible pyrenoid when examined with the light microscope
Sphaerocystis sp.	➢ Colonies are microscopic, consisting of 4, 8 or 16(32) regularly arranged cells within a spherical to cllipsoidal and homogeneous mucilaginous envelope

	➢ Single cells are about 5 to 10 µm in diameter, where coenobia of 4 to 8 cells range from 15 to 20 µm in diameter ➢ Cells are spherical with walls thin and smooth ➢ Chloroplast is parietal, cup-shaped, with a single pyrenoid
Ankistrodesmus sp.	➢ Cells are usually very elongate, narrowly spindle-shaped to cylindrical ➢ Straight, gradually or abruptly narrowing to acute apices ➢ Single cells are about 20 to 25 µm in length and 2.5 to 3.3 µm in width ➢ Chloroplast is parietal, single and covering most of cell with or without a pyrenooid
Pectinodesmus sp.	➢ Straight to slightly bent microscopic colony, consisting of 2 to 8 regularly arranged cell about 3.2 to 32 µm in length ➢ Single cells are about 1.5 to 15.32 µm in length and 0.5 to 5. 32 µm in width

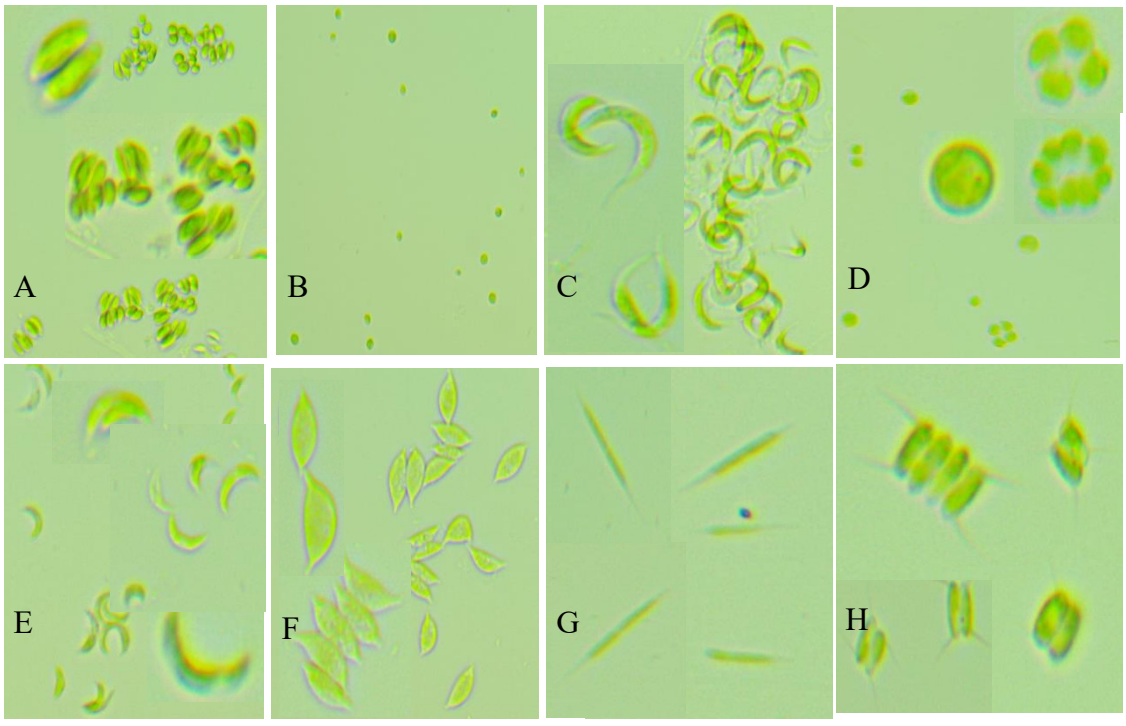
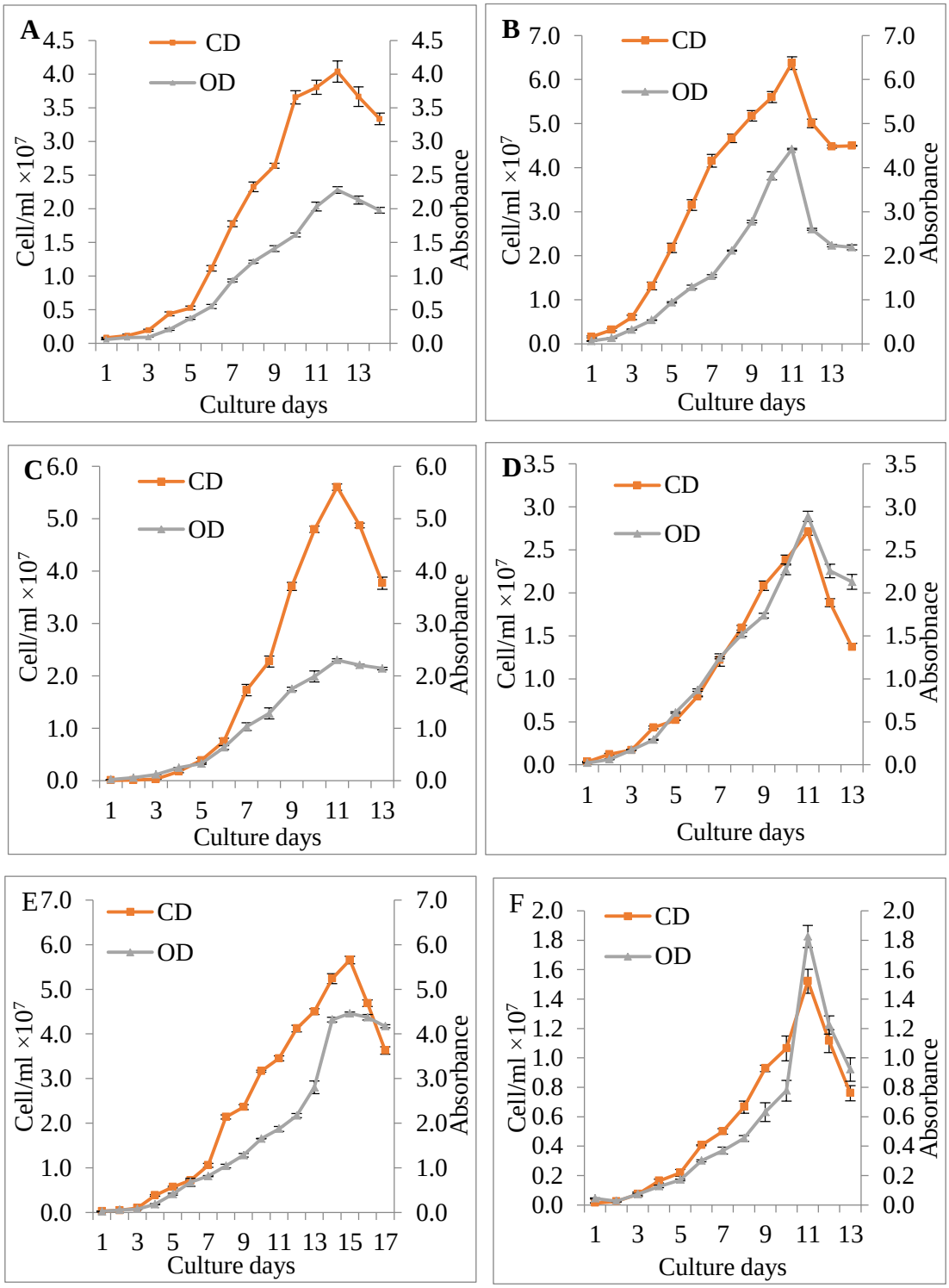


Figure 4.2: Light microscopic pictures of isolated microalgae, *Nephrocytium* sp. (A), *Nannochloropsis* sp. (B), *Monoraphidium* sp. (C), *Sphaerocystis* sp. (D), *Selenastrum* sp. (E), *Pectinodesmus* sp. (F), *Ankistrodesmus* sp. (G) and *Scenesdesmus* sp. (H).

4.4 Growth phases of isolated microalgae

In the present study, different microalgae showed different cell concentration, amount of pigment and proximate composition as those can vary from species to species. Figure 4.3 illustrate the cell density and optical density versus the cultivation time of each of the eight species (*Nephrocytium* sp.-A, *Nannochloropsis* sp.-B, *Monoraphidium* sp.-C, *Sphaerocystis* sp.-D, *Selenastrum* sp.-E, *Pectinodesmus* sp.-F, *Ankistrodesmus* sp.-G and *Scenesdesmus* sp.-H) during the cultivation in BBM. Observation results of growth showed that onset of stationary phase (9-14 days) varied among the eight species. Based on the growth curve it is possible to determine the growth phases of those eight microalgae. Based on Figure 4.3 the growth phases are almost same for *Nannochloropsis* sp., *Sphaerocystis* sp. and *Pectinodesmus* sp. that showed the lag phase on days 1 to 3, the exponential phase on days 3 to 11, the stationary phase on days 10 to 11, and finally the phase of death from 11 days. Similarly, *Nephrocytium* sp. showed the lag phase on 1 to 3 days, the exponential phase on 3 to 12 days, the stationary phase on 11 to 13 days and the death phase from 13 days. From 1 to 4 days of lag phase, 4 to 15 days of exponential phase, and 14 to 16 days of stationary phase was observed in *Selenastrum* sp. Along with this, *Monoraphidium* sp. resulted the lag phase, exponential phase, stationary phase and death phase on days 1 to 4, 4 to 11, 10 to 12 and from 12 days, respectively. Moreover, *Ankistrodesmus* sp. showed the lag phase on days 1 to 6, exponential phase on days 6 to 15, stationary phase on days 15 to 16 and death phase from 16 days. Furthermore, in *Scenedesmus* sp. from days 1 to 2, days 2 to 10 and days 9 to 10 was recorded as lag phase, exponential phase and stationary phase, respectively. In the stationary phase, cell density was significantly higher ($p < 0.05$) in *Nannochloropsis* sp. (6.374×107 cells/ml) compared to the other microalgae. But in terms of cell density, no significant difference ($p < 0.05$) was found between *Monoraphidium* sp. and *Selenastrum* sp. Similarly, *Sphaerocystis* sp. and *Pectinodesmus* sp. showed almost similar cell density. Significantly lower ($p < 0.05$) cell density (0.936×107 cells/ml) and optical density (0.714 Abs) was observed in *Scenedesmus* sp.


A
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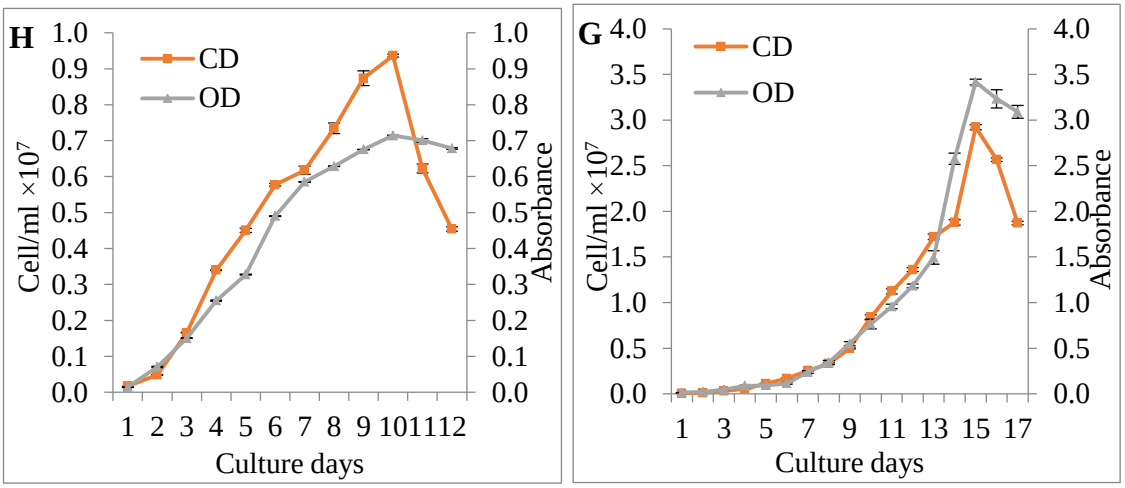


Figure 4.3: Growth curve in terms of cell density (cells/ml×10^7) and optical density (Absorbance) of freshwater microalgae *Nephrocytium* sp. (A), *Nannochloropsis* sp. (B), *Monoraphidium* sp. (C), *Sphaerocystis* sp. (D), *Selenastrum* sp. (E), *Pectinodesmus* sp. (F), *Ankistrodesmus* sp. (G) and *Scenesdesmus* sp. (H). Values are means ± standard error. CD and OD represent cell density, and optical density, respectively.

4.5 Specific growth rate (SGR), cell duplication time, cell doublings per day and cell density on harvest of isolated microalgae

Cell duplication time, cell doublings per day (K) and cell density on harvest varied among those different microalgae (Table 4.4) where significantly highest ($p \leq 0.05$) SGR was detected for *Scenedesmus* sp. and lowest for *Selenastrum* sp. Moreover, *Selenastrum* sp. and *Scenedesmus* sp. showed significantly ($p \leq 0.05$) highest and lowest cell duplication time, respectively. Along with this, *Nannochloropsis* sp. and *Sphaerocystis* sp. showed almost similar cell duplication time. On the other hand, significantly highest ($p \leq 0.05$) amount of cell doublings was detected for *Monoraphidium* sp. while *Selenastrum* sp. resulted the lowest. At the end of the exponential phase, significantly highest and lowest ($p \leq 0.05$) amount of cell density was detected from *Nannochloropsis* sp. and *Scenedesmus* sp. In terms of cell density, no significant difference was detected from *Monoraphidium* sp., *Selenastrum* sp. and *Sphaerocystis* sp., *Ankistrodesmus* sp.

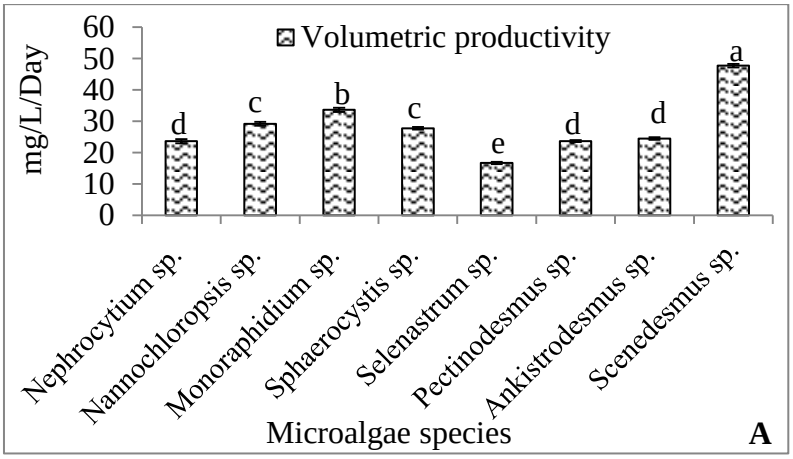
Table 4.4: Cell duplication time (Day), cell doublings per day (K) and cell density on harvest (cells/ml) (mean ± SE) of isolated microalgae. Values with the different letters within each series indicate significant differences ($p < 0.05$) among the species.

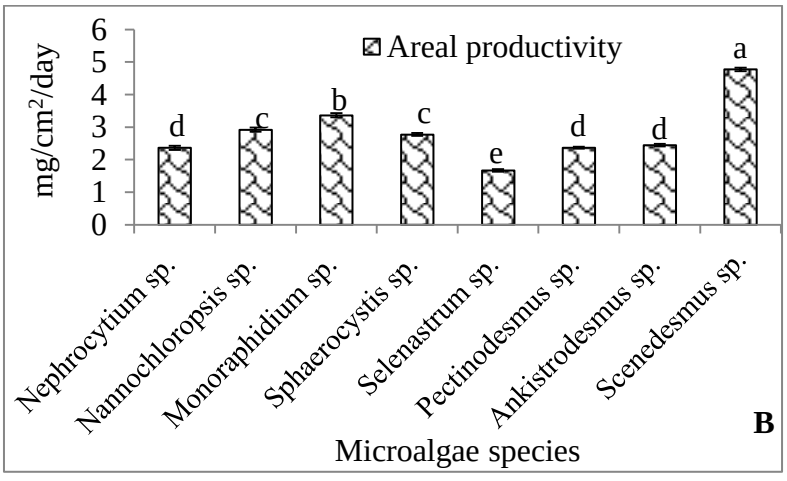
Microalgal Species	**Cell duplication time (Day)**	**Cell doublings per day (K)**	**Cell Density on harvest (cells/ml)**	**SGR(mg/day)**
Nephrocytium sp.	1.156± 0.004^{c}	0.493± 0.008^{d}	4.037×10^{7} ±0.157^{c}	0.60±0.002^{c}
Nannochloropsis sp.	1.042± 0.003^{e}	0.485± 0.020^{d}	6.374×10^{7} ±0.144^{a}	0.665±0.002^{b}
Monoraphidium sp.	1.022± 0.003^{f}	0.910± 0.013^{a}	5.604×10^{7} ±0.061^{b}	0.678±0.002^{b}
Sphaerocystis sp.	1.049± 0.002^{e}	0.574± 0.017^{c}	2.711×10^{7} ±0.042^{d}	0.661±0.001^{b}
Selenastrum sp.	1.555± 0.005^{a}	0.482± 0.008^{d}	5.658×10^{7} ±0.083^{b}	0.446±0.001^{d}
Pectinodesmus sp.	1.073± 0.002^{d}	0.625± 0.014^{bc}	1.521×10^{7} ±0.081^{e}	0.646±0.001^{bc}
Ankistrodesmus sp.	1.395± 0.003^{b}	0.581± 0.005^{c}	2.923×10^{7} ±0.029^{d}	0.497±0.001^{d}
Scenedesmus sp.	0.820± 0.001^{g}	0.644± 0.001^{b}	0.936×10^{7} ±0.004^{f}	0.846±0.001^{a}

4.6 Volumetric, areal and lipid productivity of isolated microalgae

In this study, different freshwater tropical microalgae species were cultured in BBM providing uniform parameters to determine the difference in their productivity. Figure 4.4A showed the variation in volumetric productivity for the eight microalgae species where significantly highest and lowest ($p \leq 0.05$) amount of volumetric productivity was detected for *Scenedesmus* sp. (47.738 ± 0.576 mg/L/Day) and *Selenastrum* sp. (16.680± 0.393 mg/L/Day), respectively. Moreover, *Nephrocytium* sp., *Pectinodesmus* sp. and *Ankistrodesmus* sp. resulted almost similar amount of volumetric productivity (23.628 ± 0.648, 23.656 ± 0.318 and 24.459 ± 0.423 mg/L/Day respectively). The results also showed that areal productivity varied among

those species (Figure 4.4B). The areal productivity varied as 2.363±0.065, 2.922±0.061, 3.366±0.066, 2.774±0.044, 1.668±0.039, 2.366±0.032, 2.446±0.042 and 4.774±0.058 mg/cm^2/day in *Nephrocytium* sp., *Nannochloropsis* sp., *Monoraphidium* sp., *Sphaerocystis* sp., *Selenastrum* sp., *Pectinodesmus* sp. *Ankistrodesmus* sp. and *Scenesdesmus* sp., respectively. Areal productivity was significantly ($p < 0.05$) highest in *Scenesdesmus* sp. and lowest in *Selenastrum* sp. Lipid productivity in various microalgae also differs from species to species which is represented in Figure 4.4C. *Nephrocytium* sp., *Nannochloropsis* sp., *Monoraphidium* sp., *Sphaerocystis* sp., *Selenastrum* sp., *Pectinodesmus* sp., *Ankistrodesmus* sp. and *Scenesdesmus* sp. resulted in about 4.810±0.303, 5.935±0.093, 8.513±0.258, 4.068±0.132, 2.915±0.075, 2.790±0.102, 4.006±0.070 and 6.623±0.260 mg/L/Day of lipid productivity, respectively. Significantly ($p < 0.05$) maximum and minimum amount of lipid productivity were detected from *Monoraphidium* sp. and *Pectinodesmus* sp. Together with this, similar kind of lipid productivity was observed from *Nephrocytium* sp., *Sphaerocystis* sp. and *Ankistrodesmus* sp.





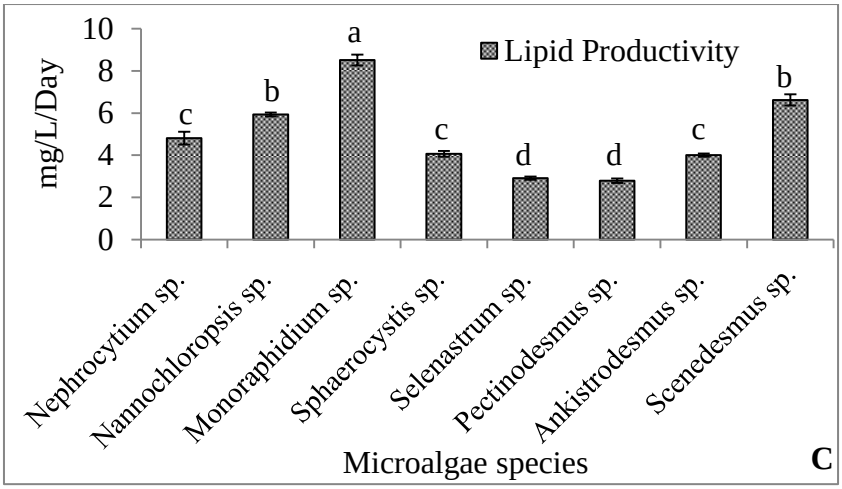


Fig 4.4: Volumetric productivity (A), areal productivity (B) and lipid productivity (C) (mean ± SE) of isolated microalgae. Values with the different letters within each series indicate significant differences ($p < 0.05$) among the species.

4.7 Pigments content of isolated microalgae

4.7.1 Chlorophyll

Chlorophyll, carotenoid and different phycoboliproteins content were investigated under this study where chlorophyll-a, chlorophyll-b and carotenoid were recorded as µg/mL and phycobiliprotain content in mg/g.

Different microalgae showed a considerable variation in chlorophyll (Figure 4.5), carotenoid (Figure 4.6) and phycobiliproteins (Table 4.5 and Figure 4.7) content. Significantly highest ($p < 0.05$) amount of chlorophyll-a (11.26±0.06 µg/mL) and chlorophyll-b (2.08±0.07 µg/mL) were recorded in *Monoraphidium* sp. on day-10. On the other hand, *Scenedesmus* sp. showed significantly minimum ($p < 0.05$) amount of chlorophyll-a (0.83±0.00 µg/mL) and b (0.33±0.00 µg/mL) on day-9.

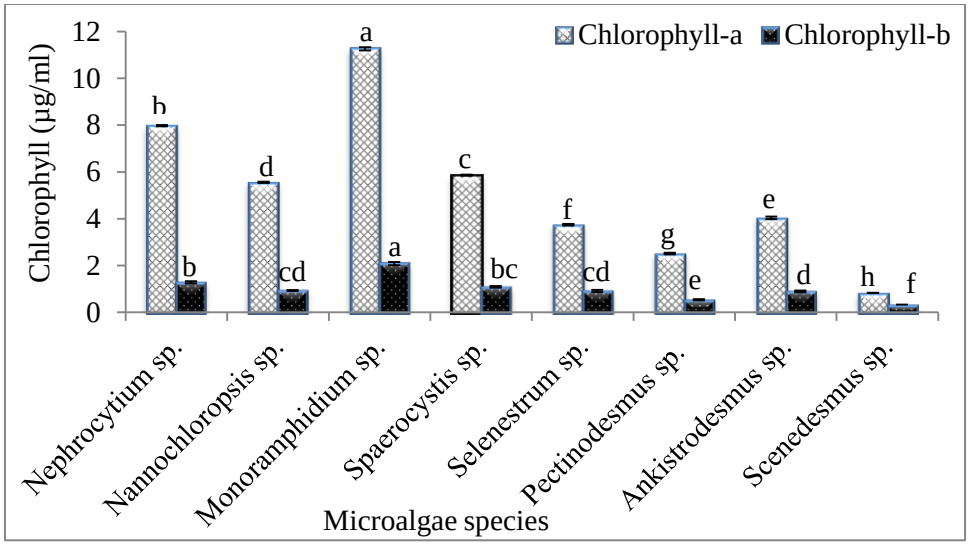


Figure 4.5: Chlorophyll-a and b (means ± SE) of isolated microalgae. Values with the different letters within each series indicate significant differences ($p < 0.05$) among the species.

4.7.2 Carotenoid

Total carotenoid content varied among those different microalgae (Figure 4.6). Significantly ($p < 0.05$) highest and lowest quantity of carotenoid accumulation was detected at day 10 from *Monoraphidium* sp. (8.05±0.07 µg/mL) and *Pectinodesmus* sp. (2.07±0.07 µg/mL), respectively. Moreover, identical amount of total carotenoid was detected from *Sphaerocystis* sp. (6.80±0.12 µg/mL) and *Scenedesmus* sp. (6.30±0.19 µg/mL).

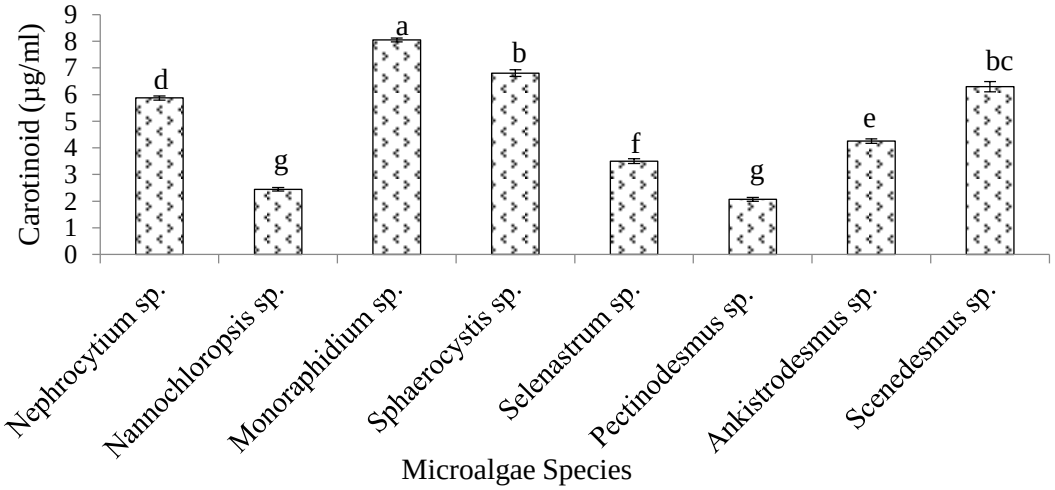


Figure 4.6: Carotenoid content (µg/ml) (mean ± SE) of isolated microalgae. Values with the different letters within each series indicate significant differences ($p < 0.05$) among the species.

4.7.3 Phycobiliproteins

Phycobiliproteins analysis found that, phycocyanine, allophycocyanin and phycoerythrin content were significantly ($p < 0.05$) highest in *Scenedesmus* sp. at day 9 with 1.54±0.03 mg/g, 2.75±0.02 mg/g and 1.81±0.09 mg/g, respectively (Table 4.5). Significantly ($p < 0.05$) minimum and maximum ($p < 0.05$) quantity of total phycobiliprotein content was achieved in *Pectinodesmus* sp. (1.15±0.01 mg/g) and *Scenedesmus* sp. (6.10±0.12 mg/g) at day 10 and day 9, respectively (Figure 4.7).

Table 4.5. Different phycobiliproteins (phycocyanine, allophycocyanin and phycoerythrin) of isolated microalgae. Values are means ± SE. Values with the different letters within each series indicate significant differences ($p < 0.05$) among the species.

Algal Species	Phycocyanine (mg/g)	Allophycocyanin (mg/g)	Phycoerythrin (mg/g)
Nephrocytium sp.	$0.68±0.01^{b}$	$2.27±0.02^{b}$	$0.55±0.01^{bc}$
Nannochloropsis sp.	$0.50±0.00^{d}$	$1.67±0.03^{c}$	$0.48±0.01^{bcd}$
Monoraphidium sp.	$0.42±0.01^{d}$	$1.34±0.01^{f}$	$0.42±0.01^{cde}$
Sphaerocystis sp.	$0.23±0.01^{e}$	$0.90±0.02^{g}$	$0.17±0.00^{f}$
Selenastrum sp.	$0.57±0.00^{c}$	$1.43±0.01^{e}$	$0.62±0.01^{b}$
Pectinodesmus sp.	$0.21±0.01^{e}$	$0.66±0.01^{h}$	$0.29±0.01^{ef}$
Ankistrodesmus sp.	$0.22±0.01^{e}$	$1.52±0.01^{d}$	$0.39±0.01^{de}$
Scenedesmus sp.	$1.54±0.03^{a}$	$2.75±0.02^{a}$	$1.81±0.09^{a}$

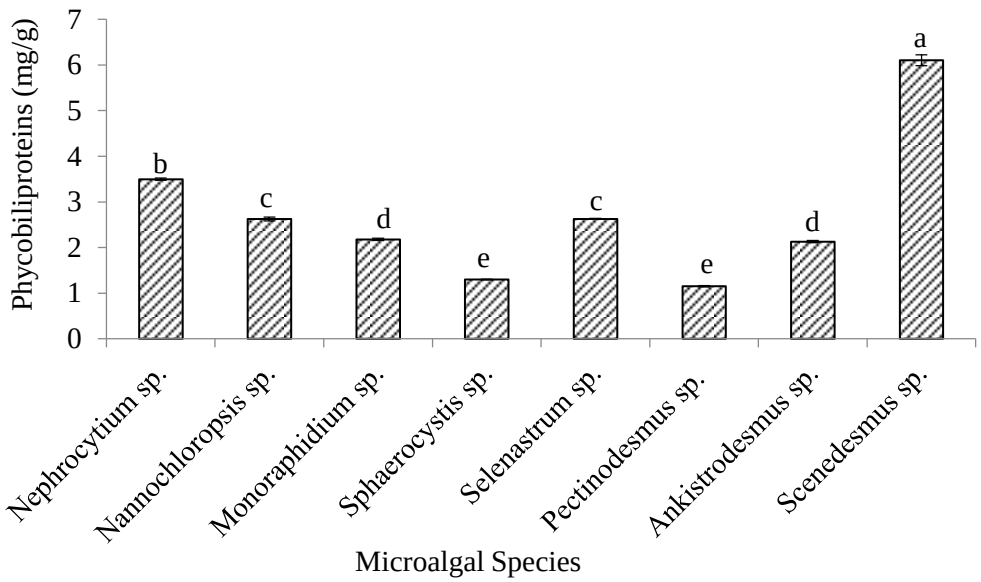


Figure 4.7: Total phycobiliprotein content (mg/g) (mean ± SE) of isolated microalgae. Values with the different letters within each series indicate significant differences ($p < 0.05$) among the species.

4.8 Proximate composition of isolated microalgae

In this study, different freshwater tropical microalgae species were cultured in BBM providing uniform parameters to determine the difference in their biochemical composition. Figure 4.8 shows the variation in protein content for the eight microalgae species ranged from 21.77±2.17 to 34.34±1.22 % dry weight. The percentage of protein was significantly ($p < 0.05$) highest in *Nannochloropsis* sp. and lowest in *Scenedesmus* sp. Among *Nephrocytium* sp., *Nannochloropsis* sp., *Monoraphidium* sp., *Sphaerocystis* sp., *Selenastrum* sp. and *Pectinodesmus* sp. there was no significant ($p < 0.05$) difference in protein content. Moreover, *Ankistrodesmus* sp. and *Scenesdesmus* sp. showed nearly similar percentage of protein content with 24.26±0.78 and 21.77±2.17 % dry weight, respectively. The results showed that biosynthesis of lipid varied among those species (Figure 4.9). The total lipid content varied as 20.32±0.78, 20.33±0.55, 25.28±0.31, 14.69±0.70, 17.47±0.14, 11.79±0.27, 16.40±0.58, 13.86±0.37 % dry weight among *Nephrocytium* sp., *Nannochloropsis* sp., *Monoraphidium* sp., *Sphaerocystis* sp., *Selenastrum* sp., *Pectinodesmus* sp. *Ankistrodesmus* sp. and *Scenesdesmus* sp., respectively. Lipid content was significantly ($p < 0.05$) higher in *Monoraphidium* sp. but did not show any significant difference among *Nephrocytium* sp. and *Nannochloropsis* sp.; *Selenastrum* sp. and *Ankistrodesmus* sp.; *Sphaerocystis* sp. and *Scenesdesmus* sp. Carbohydrate production

in various microalgae also differ from species to species which is represented in Figure 4.10. *Nephrocytium* sp., *Nannochloropsis* sp., *Monoraphidium* sp., *Sphaerocystis* sp., *Selenastrum* sp., *Pectinodesmus* sp., *Ankistrodesmus* sp. and *Scenesdesmus* sp. resulted in 22.79±1.16, 17.80±1.20, 20.78±0.83, 24.78±1.25, 15.17±1.11, 25.23±0.61, 17.71±0.97 and 21.06±0.19 % dry weight of carbohydrate, respectively. Significantly ($p < 0.05$) maximum and minimum amount of carbohydrate were detected in *Pectinodesmus* sp. and *Selenastrum* sp. respectively. Together with this, similar kind of carbohydrate content was detected in *Nephrocytium* sp., *Monoraphidium* sp., *Sphaerocystis* sp., *Pectinodesmus* sp. and *Scenesdesmus* sp.

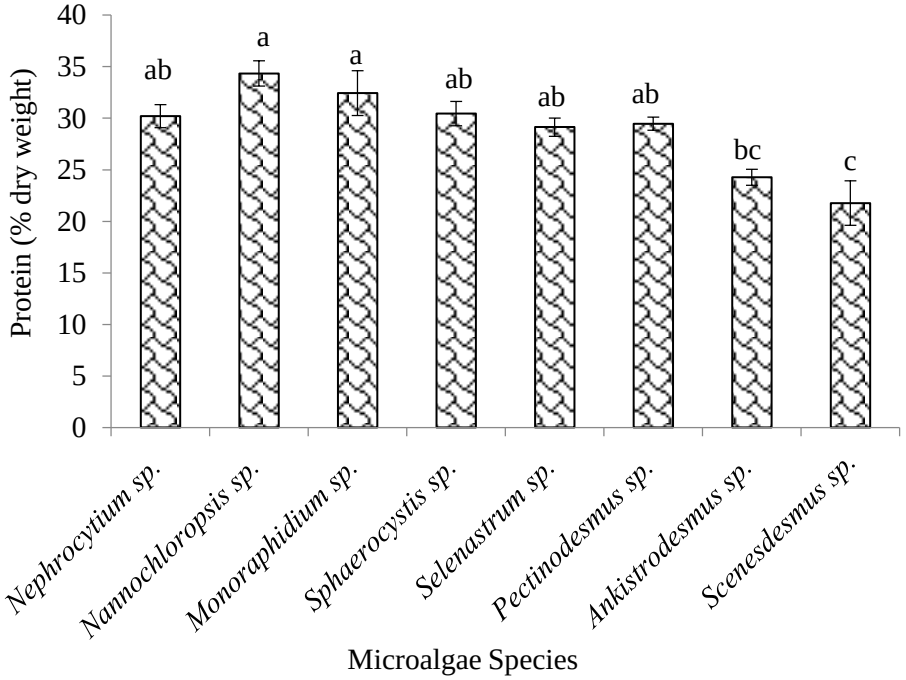


Figure 4.8: Protein content (% dry weight) (mean ± SE) of isolated microalgae. Values with the different letters within each series indicate significant differences ($p < 0.05$) among the species.

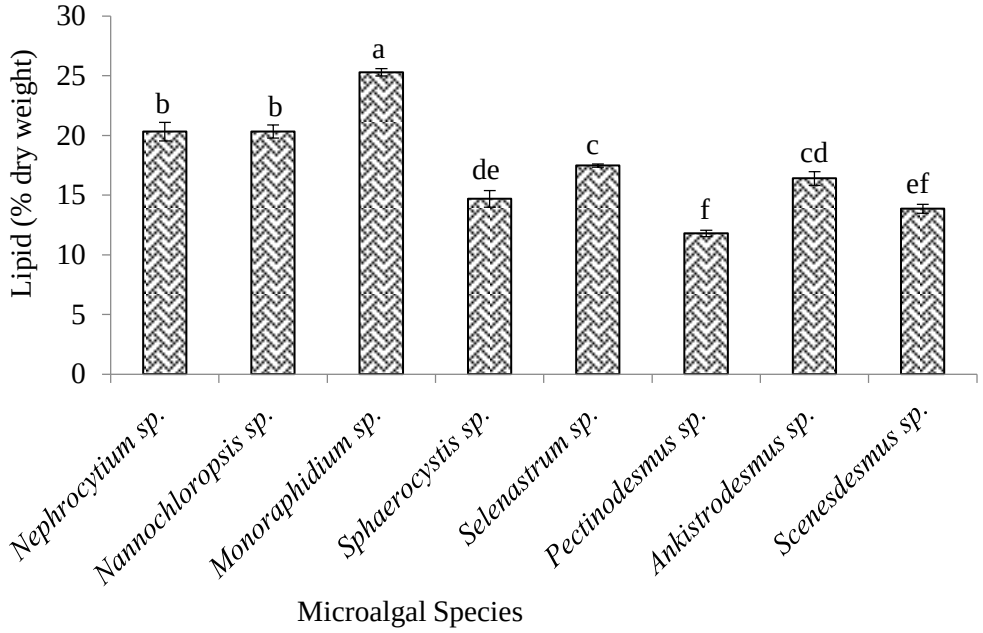


Figure 4.9: Lipid content (% dry weight) (mean ± SE) of isolated microalgae. Values with the different letters within each series indicate significant differences ($p < 0.05$) among the species.

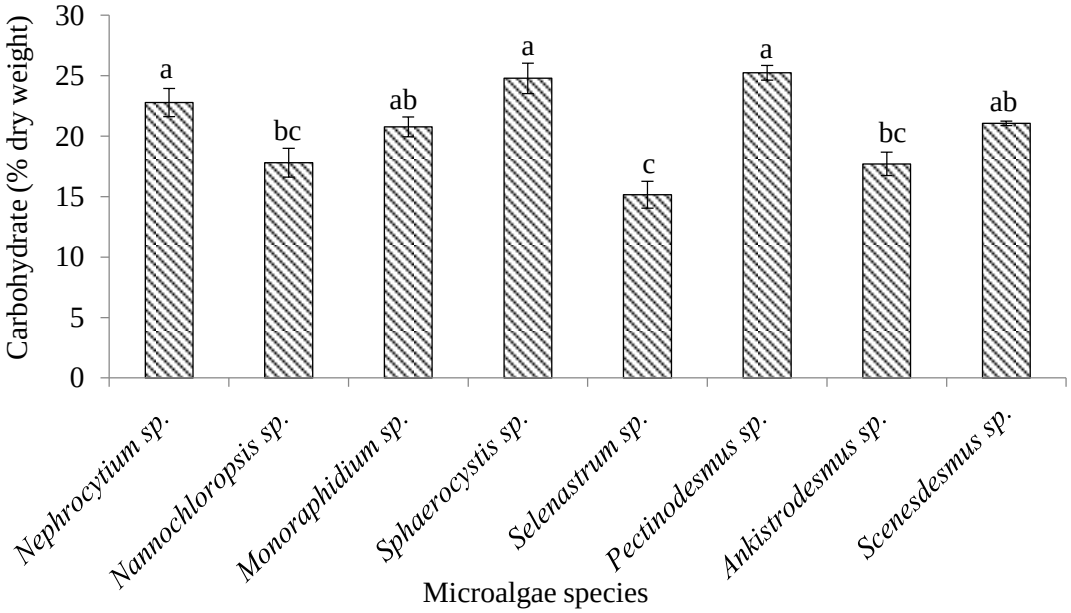


Figure 4.10: Carbohydrate content (% dry weight) (mean ± SE) of isolated microalgae. Values with the different letters within each series indicate significant differences ($p < 0.05$) among the species.

Chapter 5: Discussion

5.1 Water quality parameters of the sampling sites

Macro and micro nutrients availability plays a significant role on the growth and biochemical composition where sufficient amount of nutrients mainly nitrogen, phosphorus is mandatory to achieve optimum growth rates in microalgal cells (Xia et al., 2013). The growth rate reduced when the metabolic requirements and supplied nutrients are not balanced properly (Zarrinmehr, 2019). Some other factors like temperature, light, salinity, pH etc. also play a major rule in growth and biochemical compositions of microalgae (Yeh and Chang, 2012). According to Santhosh and Singh (2007) pH should be between 6.5 and 9.0, which were observed from all sampling site. On the other hand, DO >5 mg/L is essential to support good fish production (Bhatnagar and Singh, 2010), and DO of Karnaphuli River and Kaptai Lake were in optimal range. DO in Halda river was below the optimum range as the daily DO is maximum in mid-afternoon due to photosynthesis and minimum in the early morning due to highest respiration and decomposition than photosynthesis (CWC, 2019). Temperature of the sampling sites was in ideal level where the optimum growth temperature is mostly between 20 and 30˚C for most marine microalgae (Chisti, 2008). Optimum phosphorus concentration for microalgae is between 0.001 g/L to 0.179 g/L (Roopnarain et al., 2014), where TAN concentration must be less than 0.5 mg/L and desirable range of nitrite-nitrogen is 0-1 mg/L (Stone and Thomforde, 2004). In the entire sampling site, nitrogen and phosphorus concentration was in ideal range that is required for plankton growth.

5.2 Characterization of isolated microalgae

In Bangladesh, huge numbers of algal species were reported to occur in freshwater, brackish water and marine habitats (Ahmed et al., 2008) that could be a potential source of feed for aquaculture, biofuel production, pharmaceuticals and nutraceuticals industry. Characterization of isolated microalgae was done according to John et al. (2002) and Belcher and Swale (1976) and isolated microalgae was *Nephrocytium* sp., *Nannochloropsis* sp., *Monoraphidium* sp., *Sphaerocystis* sp., *Selenastrum* sp., *Pectinodesmus* sp., *Ankistrodesmus* sp. and *Scenesdesmus* sp. Along with this, in respect of Nägeli (1849); Komárková-Legnerová (1969); Chodat (1897); Reinsch (1867); Schoch et al. (2020); Corda (1838); Meyen (1829) isolated microalgae:

Nephrocytium sp., *Monoraphidium* sp., *Sphaerocystis* sp., *Selenastrum* sp., *Pectinodesmus* sp., *Ankistrodesmus* sp. and *Scenesdesmus* sp. respectively, belong to the class of chlorophyceae where *Nannochloropsis* sp. belong to Eustigmatophyceae (Hibberd, 1981). As the sampling site were mostly dominated by chlorophyceae microalgae, it was easy to isolate some of them from those water sources.

5.3 Growth phases of isolated microalgae

No previous study has been reported for *Nephrocytium* sp. In terms of cell density, *Ankistrodesmus* sp. showed almost similar result in BBM at 21 ± 1˚C and in 1700 lux (34 µmol m^{-2} s^{-1}) light intensity (Okomoda et al., 2021) which was reported from *Nephrocytium* sp. in the present study and can be concluded as *Nephrocytium* sp. can ideally flourish with BBM. In case of *Nannochloropsis* sp., Ermavitalini et al. (2019) reported almost similar growth pattern (lag, exponential and stationary phase) in *Nannochloropsis* sp. in the combined treatment media of Indole 3-acetic acid (IAA) and 6-Benzyl Amino Purine (BAP). But cell density was higher than the earlier study found 4.877×10^7 cells mL^{-1} in Conway media reported by Khatoon et al. (2014). The differences in the performances observed can be linked to different growth media used in *Nannochloropsis* sp. growth and different strains from different environment. A previous study done by Lin et al. (2019) found slightly declined cells number in *Monoraphidium* sp. (HDMA-20) after 18 days in BG-11 medium, whereas, in the present study highest cell density of *Monoraphidium* sp. was detected after day 11. The findings of the current study suggested that *Monoraphidium* sp. can flourish by using BBM. No previous growth data available on *Sphaerocystis* sp. but Kumari and Singh (2016) found that, a mixed culture of *Chlorella, Anabaena, Euglena, Oocystis* and *Sphaerocystis* sp. in modified BBM showed maximum cell density on days 11. However, in the current study, *Sphaerocystis* sp. solely represented maximum cell density and optical density on day 10. Similarly, *Selenastrum capricornutum* cultured in $NaNO_3$, $MgCl_2$, $CaCl_2$, H_3BO_3, $MnCl_2$, $ZnCl_2$, CoCl, $CuCl_2$, Na_2MoO_4, $FeCl_3$, Na_2EDTA, $MgSO_4$, K_2HPO_4, $NaHCO_3$ containing growth medium shows maximum cell density after 9 days of cultivation in photobioreactor (Pugliese et al., 2020), but in the current study *Selenastrum* sp. resulted highest cell density and optical density on day 14. The same trend of performance in cell density was observed by Rocha et al. (2017) who reported that *Pectinodesmus pectinatus* (BR019) shows maximum cell density at day 10 in G-11 medium but in the present study cell density was higher

which suggested that *Pectinodesmus* sp. productivity can be enhanced by culturing in BBM. Okomoda et al. (2021) reported that *Ankistrodesmus falcatus* showed maximum cell density (39.00 ± 0.58 $\times 10^6$ cells/ ml) at day 8 in BBM but in this study, *Ankistrodesmus* sp. showed highest (2.923± 0.029 $\times 10^7$ cells/ ml) cell density at day 14 in BBM. The observed differences in case of *Selenastrum* sp. and *Ankistrodesmus falcatus* can be justified as, microalgae growth characteristics vary from species to species and impacted by multiple factors, like reactor feature, culture conditions (light, nutrients, temperature, pH, aeration) and the physiological need of the microalgae species (Guedes and Malcata, 2012). Current study is also accompanied with the findings done by Difusa et al. (2015) who found that *Scenedesmus* sp. showed maximum cell density on day 9 at 67.5 µmol $m^{-2}s^{-1}$ light intensity in BG-11 medium but cell densities was high in case of BG-11 medium than the present study. The variations in growth at different media are probably due to the apparent variations in the media's composition (Okomoda et al., 2021). Among the eight species *Nannochloropsis* sp. cell density was the highest followed by *Selenastrum* sp. because smaller size species grow rapidly than the larger ones because of their large surface or volume ratio of smaller sized cells which simplify assimilation of nutrients at comparatively faster rate (Phatarpekar et al., 2000). Moreover, in this study, optical density showed a similar pattern like cell density as they are directly co-related and this direct relation of the cell number and optical density assure the appropriate management of the culture (Nur et al., 2008).

5.4 Specific growth rate (SGR), cell duplication time, cell doublings per day and cell density on harvest of isolated microalgae

In Bangladesh, a very limited attention has been paid to the indigenous microalgae and its potentialities. No previous study has been reported in our country on the growth and productivity analysis of *Nephrocytium* sp., *Nannochloropsis* sp., *Monoraphidium* sp., *Sphaerocystis* sp., *Selenastrum* sp., *Pectinodesmus* sp. *Ankistrodesmus* sp. and *Scenesdesmus* sp. Some world wide data are available regarding the SGR of *Nannochloropsis* sp., *Monoraphidium* sp. *Selenastrum* sp. *Ankistrodesmus* sp. and *Scenesdesmus* sp. Yustinadiar et al. (2020) reported almost similar growth rate in case of *Nannochloropsis* sp. isolated from marine habitat and resulted about a growth rate of 0.25/day in stationary phase and 0.55/day on day 1 at 0.75:0.25 hr flashing light treatment in Walne medium. Moreover, *Scenedesmus* sp.

for 11 days of culture duration resulted 0.51 ± 0.06 day^{-1} and 0.54 ± 0.14 day^{-1} SGR in BG11 medium and 100% dairy wastewater medium, respectively (Mercado et al., 2020). Along with this, Dhup and Dhawan (2014) observed that growth rates of *Monoraphidium* sp. affected by different nitrate concentrations in BG11 medium for a culture period of 15 days where highest SGR was 0.089/day at 0.072 mg/l NO_3^- concentrations. Okomoda et al. (2021) reported about 0.443 ± 0.001 day^{-1} of specific growth rate in *Ankistrodesmus falcatus* in BBM. Previous study done by Maa et al. (2012) reported about 0.25±0.02 day^{-1} of growth rate from *Selenastrum* sp. with 10 days of cultivation time. Therefore, compared with the circumstances stated above, it was found that, locally isolated *Nephrocytium* sp., *Nannochloropsis* sp., *Monoraphidium* sp., *Sphaerocystis* sp., *Selenastrum* sp., *Pectinodesmus* sp. *Ankistrodesmus* sp. and *Scenesdesmus* sp. performed differently in BBM which can be justified as, microalgae growth characteristics vary from species to species and impacted by multiple factors, like reactor feature, culture conditions (light, nutrients, temperature, pH, aeration) and the physiological need of the microalgae species (Guedes and Malcata, 2012).

The microalga *Scenedesmus* sp. showed lower cell duplication time compared to the other microalgae strains. Microalgae growth characteristics vary from species to species. Moreover cell doublings per day was also varied among the species because of the variation in lag phase, log phase and stationary phase of individual species. Due to variation in growth rate which depend not only microalgae species but also culture environments. In different growth phases, microalgae growth rate differ due to the variation in several activity like in lag phase it adapts to the culture condition such as medium, pH, temperature and lighting (Krishnan et al., 2015). And then start to undergo rapid cell division and the cell of the culture will increase gradually in exponential order, as enzymes and metabolites needed for cell division are available here (Prayitno, 2016). After which, stationary phase taken place when the equal rate of the cell division and cell death occur due to depletion of nutrients in the medium (Krishnan et al., 2015). Along with this, *Nannochloropsis* sp. resulted highest cell number on harvest because smaller size species grow rapidly than the larger ones because of their large surface or volume ratio of smaller sized cells which simplify assimilation of nutrients at comparatively faster rate (Phatarpekar et al., 2000).

5.5 Volumetric, areal and lipid productivity of isolated microalgae

The biomass production of microalgae is a mere function of the instantaneous growth rate and algal cell concentration in culture (Daniel and Srivastava. 2016). In the present study, different microalgae showed variation in biomass productivity which can be justified as productivity of microalgae is also influenced by the microalgal strain used and the characteristics of the environment, where it grows (Mercado et al., 2020). Moreover, lipid productivity of microalgae also varied among species as it depends on biochemical composition of microalgae, nutrient content of culture media and different stress factors. Microalgae cells accumulated a larger quantity of lipid when they were subject to unfavorable culture conditions (Lucas-Salas et al., 2013). Though no previous study reported on *Nannochloropsis* sp. culture in freshwater media, but in case of marine *Nannochloropsis* sp. strains, Daniel and Srivastava (2016) detected about1088.44 $gm^{-3}d^{-1}$ mean volumetric productivity, 10.10 $gm^{-2}d^{-1}$ mean areal productivity from 30 mm thickness of thick tubular photobioreactor. Dhup and Dhawan (2014) detected 0.19 g/l/day of lipid productivity from *Monoraphidium* sp. at 0.36 mg/l NO_3 concentration. The maximum lipid productivity of 38.32 mg/L/day was recorded from *Ankistrodesmus* sp. in modified BG11 medium at the dilution rate of 0.16 day (Sukkrom et al., 2016). *Scenedesmus* sp. resulted about 350.81±33.05 mgg^{-1} of lipid productivity in standard BG11 culture medium (Mercado et al., 2020). Based on the outcomes of the current study, *Nannochloropsis* sp., *Monoraphidium* sp., and *Scenesdesmus* sp. can be utilized a potential source of biofuel production as they showed higher lipid productivity.

5.6 Pigments content of isolated microalgae

5.6.1 Chlorophyll

The present study reported higher amount of chlorphyll-a, and b content from *Nephrocytium* sp. Almost similar amount of chlorophyll content was reported by Singh et al. (2017) from *Chlorella vulgaris* cultured in urban waste water medium. Previous study done by Fakhri et al. (2017) reported that, *Nannochloropsis* sp. showed different chlorophyll-a content at different light intensities such as 4.977±0.095 µg/mL at 1500 lux, 6.520±0.049 µg/mL at 3000 lux and 8.304±0.248 µg/mL at 4500 lux in 28°C temperature with photoperiod 24: 0 and continuous aeration in Walne medium. But the present study, *Nannochloropsis* sp. showed lower

chlorophyll-a content than Fakhri et al. (2017) study which due to varieties in the nutrient composition of the culture medium (Oo et al., 2017). In case of *Monoraphidium* sp. (NTAI02 strain), Ilavarasi et al. (2011) reported that the total chlorophyll was 8.587 and 42.91 μgmL^{-1} on 5 and 25^{th} day respectively, in BBM. Similarly, Oo et al. (2017) reported equivalent amount of chlorophyll-a and b content from *Chlorella* sp. at 25±2°C temperature, 25 ppt salinity and 18:6 light/dark cycle photoperiod in Conway medium. On the other hand, chlorophyll-a and b of *Sphaerocystis* sp. was almost similar as *Ankistrodesmus falcatus* in BBM at 702 lux light intensity in a 12 day culture period (Ogbonna et al., 2021). Moreover, chlorophyll content in *Pectinodesmus* sp. in the current study was almost similar with the study done by Donghui et al. (2016) on *Dunaliella tertiolecta* at the N: P of 16:1 during the period of cultivation in the urea containing Tk medium. As no previous study has been done on *Pectinodesmus* sp. pigment content, our study concluded that, *Pectinodesmus* sp. have more chlorophyll-a and b content than *Scenedesmus* sp. reported here. Earlier work by Ogbonna et al. (2021) reported that, chlorophyll-a content of *Ankistrodesmus falcatus* varied under different light intensities and in different growth medium, such as 31 mg/g at 702 lux, 18 mg/g at 1307 lux and 15 mg/g at 1786 lux in BBM, as well as in poultry medium it resulted about 26 mg/g, 22 mg/g and 18 mg/g at 702 lux, 1307 lux and 1786 lux respectively, at 27 ± 2°C temperature. However, finding of the current study reported lower chlorophyll-a content in *Ankistrodesmus falcatus* at 24°C temperature and 150 $\mu E\ m^{-2}s^{-1}$ intensity light condition in BBM. Although light is important for chlorophyll synthesis, very high light intensities inhibit chloroplast development (Wang et al., 2018), may be the reason for observed differences herein. Green microalgae *Scenedesmus obliquus* cultured in medium Zarrouk, medium Schlosser, and Haematococcus Provasolli Medium (PHM) showed that, chlorophyll-a and b content were higher in the PHM than the others, at 25°C temperature with 2500 lux light intensity (Rinanti et al., 2013). But in the present study, *Scenedesmus* sp. showed much higher chlorophyll-a and b content than the previous study reported by Rinanti et al. (2013). Chlorophyll content was increased with the increasing of light intensity (Ogbonna et al., 2021). From the present study, it can be concluded that, *Nephrocytium sp., Nannochloropsis sp., Monoramphidium sp., Spaerocystis sp.* and *Ankistrodesmus sp.* can widely be used as a great source of chlorophyll.

5.6.2 Carotenoid

There was no previous study on pigment content of *Nephrocytium* sp. However, *Dunaliella salina* contain similar amount of carotenoid in 0.7M salt concentration in Conway medium reported by Khatoon et al. (2020). Carotenoid contents of *Nannochloropsis* sp. vary at various light condition such as 2.830±0.014 µg/mL at 1500 lux, 3.518±0.018 µg/mL at 3000 lux and 3.892±0.016 µg/mL at 4500 lux in 28 °C temperature with photoperiod 24: 0 and continuously aeration in Walne medium (Fakhri et al., 2017). But in the current study, *Nannochloropsis* sp. resulted lower carotenoid content than Fakhri et al. (2017) detected earlier. Differences in light intensity significantly influenced the total carotenoid contents and increased with the increment of light intensity in *Nannochloropsis* sp. (Fakhri et al., 2017). Another study done by Kaha et al. (2021) reported that, *Monoraphidium* sp. (SP03) had no production of astaxanthin either in control or black light condition after 15 days of cultivation, however, after 30 days of culture, the production of astaxanthin was increased and estimated about 0.476 µg/mL and 0.363 µg/mL in black light and without black light conditions, respectively. Along with this, Sangapillai and Marimuthu (2019) detected almost similar amount of carotenoid content from *Asterarcys quadricellulare* (8.92 ± 0.031 mg/L) in modified BBM medium like as *Monoraphidium* sp., which concluded that it can be considered as a good candidate for the production of carotenoids. *Micractinium* sp. (CCAP IPOME-2) a green microalgae belong to the Chlorophyta phylum like *Sphaerocystis* sp. showed almost similar amount of carotenoid concentrations (6.04 ± 0.03 µg/mL) in BBM (Dharma et al., 2017) as *Sphaerocystis* sp. in the current study. Marzorati et al. (2020) had earlier opined that *Spirulina* sp. contained about 3.5 ± 0.2 $mg.g^{-1}$ of total carotenoid content. However, in the current study, *Selenestrum sp.* showed almost same amount of carotenoid content. Eze et al. (2021) earlier found that, *Desmodesmus subspicatus* (LC172266) resulted about 15.5 ± 0.1 $mg.g^{-1}.cell^{-1}$ carotenoid in the photobioreactor with reflective broth circulation but in the flask culture 2.3 ± 0.1 $mg.g^{-1}$ cells by urea supplementation using BG11 medium. *Pectinodesmus* sp. as a species of Scenedesmaceae family, findings of this study was almost equivalent with the finding of Eze et al. (2021) in flask condition. Moreover, Ogbonna et al. (2021) also found that, *Ankistrodesmus falcatus* showed different carotinoid content in different light condition like 2.2 mg/g at 702 lux, 2.9 mg/g at 1307 lux and 7.8 mg/g

at 1786 lux in BBM at 27 ± 2°C temperatures during their stationary phase on day 15. However, in the present study, carotenoid content differ from the previous study reported by Ogbonna et al. (2021) because the time requirement of cultivating microalgae for carotenoid production differs from one microalga strain to another (Ogbonna et al., 2021). Multiple environmental parameters, like photoperiods, temperature, pH, salinity, irradiances, colored wavelength, nutrient limitation, can have an effect on the production of microalgal pigments (Hemlata and Fatma, 2009). Earlier study reported about 4.9 mg/L carotenoid content in *Scenedesmus almeriensis* at 30°C (Sanchez et al., 2008). Present study reported lower amount of carotenoid content in *Scenedesmus* sp. than Přibyla et al. (2015) who reported about 10.17 ± 0.12 $mgL^{-1}d^{-1}$ of total carotenoid productivity under controlled laboratory conditions and 8.94 ± 0.46 mg $L^{-1}d^{-1}$ in the thin-layer PBR using 1/2 SŠ medium at 500μmol $m^{-2}s^{-1}$ PAR light intensity and at 30 ± 1.0 °C temperature. Which can be justified as, nutrient composition of culture media, temperature and light influenced the growth rate and biochemical composition in microalgae (Varshney et al., 2018). Present study also concluded that, *Monoramphidium* sp., *Spaerocystis* sp., *Scenedesmus* sp., *Nephrocytium* sp. and *Ankistrodesmus* sp. have high carotenoid content and huge potentiality to contribute in human and animal food industry.

5.6.3 Phycobiliproteins

In an earlier study Zuorro et al. (2021) opined that *Oscillatoria* sp. grown in optimized BG-11 media at 28 °C with a light: dark cycle of 12:12 h at 100 μmol $m^{-2}s^{-1}$ for 15 days resulted with values of 15.21, 3.95, and 1.89 (% w/w), phycocyanin, allophycocyanin and phycoerythrin, respectively. Previous study done by Montero-Lobatoa et al. (2020) reported that, in a light-dependent process *Chroococcidiopsis* sp. accumulated phycobiliproteins and produced 204 mg g^{-1}, under 10 μmol photons·m^{-2} ·s^{-1} light intensity, with a relative abundance of 40.9% for phycocyanin, 23.3% for phycoerythrin, and 35.8% for allophycocyanin. Together with this, Arashiro et al. (2020) claimed that *Nostoc* sp., *Arthrospira platensis* and *Porphyridium purpureum* showed maximum 199 mg/g DW, maximum 303 mg/g DW and 93 mg/g total phycobiliproteins in industrial wastewater. However, very lower amount of total phycobilipreotain was recorded from *Nephrocytium* sp., *Nannochloropsis* sp., *Monoramphidium* sp., *Spaerocystis* sp., *Selenestrum* sp., *Pectinodesmus* sp. *Ankistrodesmus* sp., *Scenedesmus* sp. compared to Cyanobacterial strains (Zuorro et

al., 2021; Montero-Lobatoa et al., 2020; Arashiro et al., 2020), as the light harvesting pigments phycobiliprotein commonly found in cyanophyceae and cryptophyceae (Glazer, 1994). On top of that all the microalgae in the present study belong to the Chlorophyceae class, and Chlorophyta (Green microalgae) reported to contain mostly chlorophyll-a, b, β-carotene, prasinoxanthin, siphonaxanthin, astaxanthin (Graham and Wilcox, 2000).

5.7 Proximate composition of isolated microalgae

The present study showed that the protein, lipid and carbohydrate content in *Nephrocytium* sp. was significantly higher in BBM and unanimous with the study done by Renaud et al. (1999) who claimed that in the late-logarithmic growth phase, microalgae hold approximately 30–40 %(w/w) protein, 10–20 %(w/w) lipids and 5–15 %(w/w) carbohydrates. As *Nephrocytium* sp. contain about 20% lipid, it may have potentiality in biofuel production because high oil yield of many microalgae species has been exploited in biofuel production (Hussain et al., 2017). The protein and lipid content in *Nannochloropsis* sp. in the present study is lower than the earlier study reported by Khatoon et al. (2014) where *Nannochloropsis* sp. contained about 44.3%, protein, 32% lipid in 30ppt salinity (dry basis). The biochemical composition of microalgae varies with different medium compositions and under different culture conditions (Chen et al., 2011), hence this could be the reason for the differences. Due to its higher protein and lipid content *Nannochloropsis* sp. can be extensively used as animal feed in aquaculture (Rodolfi et al., 2003). The results of the study was supported by Reyes et al. (2012) who opined that *Monoraphidium* sp. contain nearly 19–35 % lipid, 28–45 % protein and 17–25 % carbohydrates, in relation to the dry weight of biomass. In the present study, lipid content of *Monoraphidium* sp. is higher at stationary phase in BBM than BG11 reported by Dhup and Dhawan (2014). The increase in lipid content usually occurs due to different culture condition (Yoo et al., 2010); this may justify the differences detected herein for the variation in the concentration of nutrients in culture media at different culture days. On the other hand, no previous data has been reported regarding biochemical composition of *Sphaerocystis* sp., while in our study, comparatively higher percentage of protein and lipid content was recorded from *Sphaerocystis* sp. and can be a potential species in feed industry for fish and other animals as Bleakley and Hayes (2017) reported that higher protein containing microalgae can be a potential source for fish and animal

feed industry. Lipid content of *Selenastrum* sp. in the present study was almost same in photobioreactor reported by Pugliese et al. (2020). Another study done by Ma et al. (2012) stated that, *Selenastrum* sp. cultured in 50% BG11 medium supplemented with phosphorus according to aquaculture wastewater quality resulted in 19.2% crude protein content while in the current result, protein content was significantly higher in BBM. This finding of the current study is however suggestive that protein content in *Selenastrum* sp. can ideally flourish with BBM. The protein, lipid and carbohydrate content in *Pectinodesmus* sp. was 15%, 21.27% and 20.78% DW respectively, in BG-11 broth (Samadhiya et al., 2021). However, in our study, protein and carbohydrate content of *Pectinodesmus* sp. was much greater than that reported by Samadhiya et al. (2021) but showed lower lipid content which may vary due to changes in the growth condition or nutrient concentration (Converti et al., 2009). The findings of Okomoda et al. (2021) showed that, *Ankistrodesmus falcatus* cultured in BBM resulted in 46.41±0.57 % protein, 23.22±0.56 % lipid and 32.99±0.26 % carbohydrate content. However, the present study reported comparatively lower amount of protein, lipid and carbohydrate content. Along with this, Becker (2007) reported about 50–56 % protein, 12–14 % lipid and 10–17 % carbohydrate from *Scenedesmus obliqus*. Another study done by Khatoon et al. (2017) reported that, *S. bibraianum* showed 44.7% protein when cultured in Bristol media while *S. obliquus* showed 30.7 ± 0.01% (BBM) and 31.8 ± 0.01% (Bristol) protein, together with, 42.6 ± 0.01% lipid in *S. obliquus* cultured in Bristol and 9.4 ± 0.02% lipid in *S. bibraianum* in Bristol. Moreover, 42.6 ± 0.01% and 38.2 ± 0.02% carbohydrate content was observed respectively in Bristol and BBM in *S. obliquus* (Khatoon et al., 2017). The present study found almost similar lipid content that was observed by Becker (2007) in *Scenedesmus obliqus* but resulted lower amount of protein and carbohydrate content that was reported by Khatoon et al. (2017). The growth rate and biochemical composition of microalgae are highly influenced by environmental factors like light, salinity and nutrient availability (García et al., 2012) and differ from species to species (Khatoon et al., 2017).

Chapter 6: Conclusion

Considering the results achieved from the current study it can be concluded that, SGR of microalgae varied among different growth phases. *Nephrocytium* sp., *Nannochloropsis* sp., *Monoraphidium* sp., *Sphaerocystis* sp., *Selenastrum* sp., and *Ankistrodesmus* sp. grow well in BBM with elevated quantity of protein and lipid content and showed their importance in fish or animal feed industry and fuels production. Moreover, *Scenedesmus* sp. showed lowest cell duplication time and *Monoraphidium* sp. resulted highest cell doublings per day which can be utilized for mass production of microalgae. Along with this, *Monoraphidium* sp. accumulated much higher lipid in a day and can be as a potential species for biodiesel production. Interestingly, *Monoramphidium* sp., *Nephrocytium* sp., *Sphaerocystis* sp., *Nannochloropsis* sp., *Ankistrodesmus* sp. and *Scenedesmus* sp. showed higher chlorophyll and carotenoid content and confirms their potentiality in pigment production and utilization of those microalgae in as natural food coloring agent.

Chapter 7: Recommendations and Future Prospects

Nephrocytium sp., *Nannochloropsis* sp., *Monoraphidium* sp., *Sphaerocystis* sp., *Selenastrum* sp., and *Ankistrodesmus* sp. showed a good quantity of protein but it could not represent the digestibility of microalgal protein as human and fish feed. Besides, this study couldn‘t represent the amino acid composition of microalgae which is required to utilize the microalgae as fish or animal feed. Moreover, fatty acid content of microalgae was not determined in this study which is required to determine the potentiality of microalgae for biofuel production. Along with this, *Monoramphidium* sp., *Nephrocytium* sp., *Sphaerocystis* sp., *Nannochloropsis* sp., *Ankistrodesmus* sp. and *Scenedesmus* sp. showed higher chlorophyll and carotenoid content but pigment (chlorophyll and carotenoid) utilization by fish and other animal required to determine to identify the microalgal pigment potentiality as natural food coloring agent. Therefore, future research attempts may include the followings:-

- Digestibility of isolated microalgae to utilize as fish or animal feed;
- Fatty acid composition of isolated microalgae to determine the potentiality of biodiesel production;
- Amino acid profiling of the isolated microalgae to boost up the feed industry and;
- Pigment utilization capability by fish or other aquatic animal to strengthen the aquaculture industry.

Future prospects

- This study may establish a complete isolation procedure of microalgae
- Will provides a complete profile of eight different freshwater microalgae isolated from south-eastern freshwater habitat of Bangladesh
- Current results can be used as a basis for the manipulation of microalgae for high protein, lipid and pigments production
- May contribute to select strains that possess fast growth, suitable pigments and proximate composition, to boost up the aquaculture, pharmaceuticals and nutraceuticals industry

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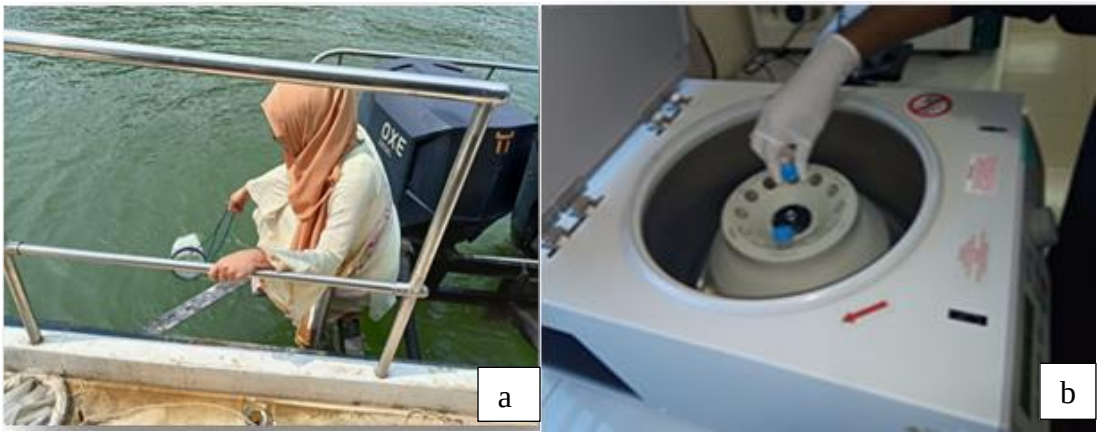
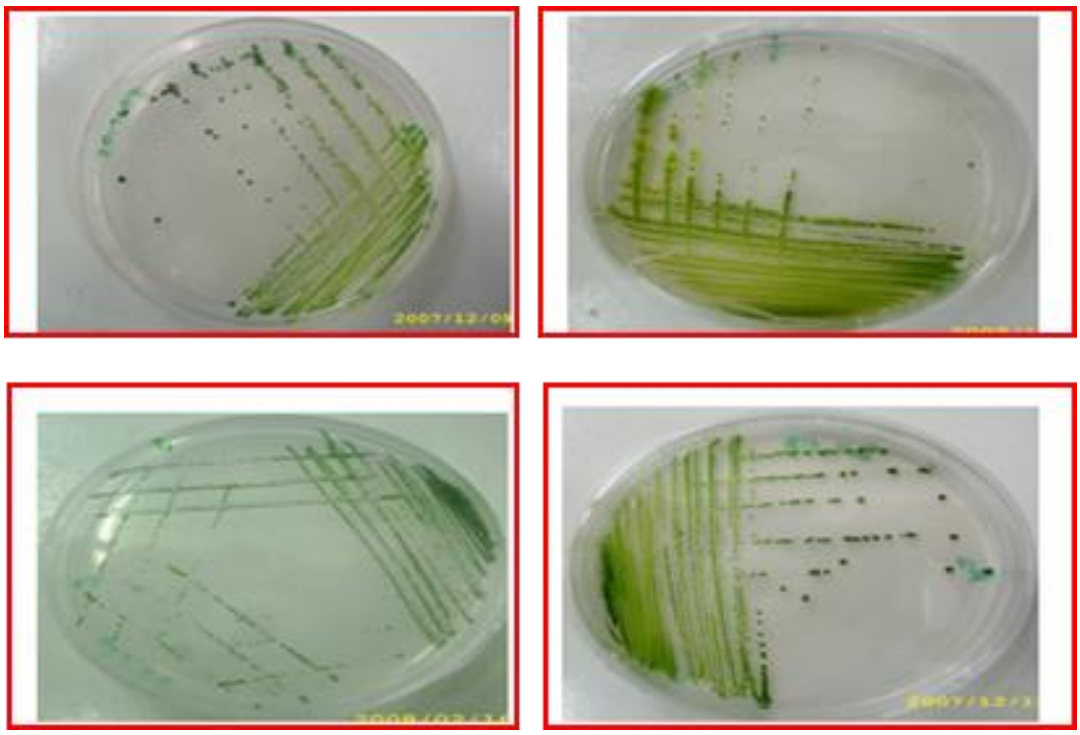
Appendices



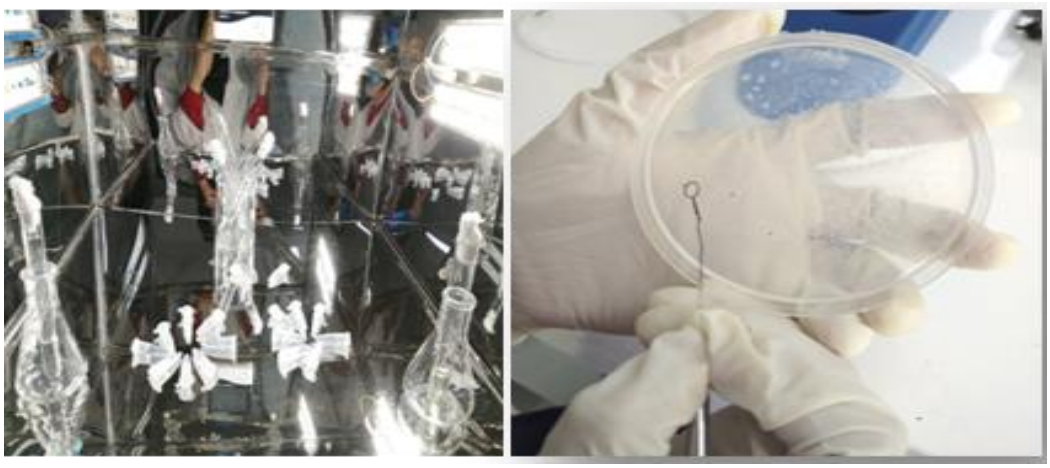
Appendix A: (a) Water sample collection, (b) Sample concentration

Appendix B: Determination of water quality parameter

Appendix C: Agar media preparation



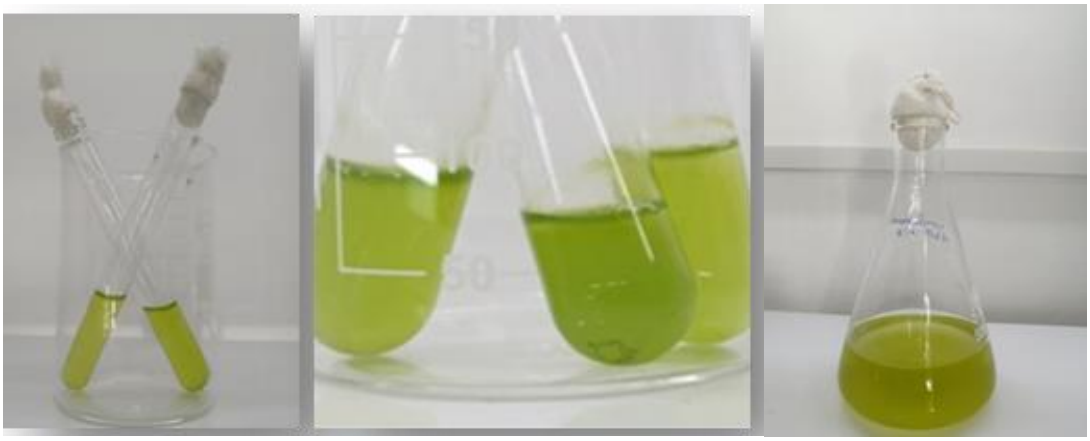
Appendix D: Streaking of water sample in agar plate



Appendix E: Microalgae isolation

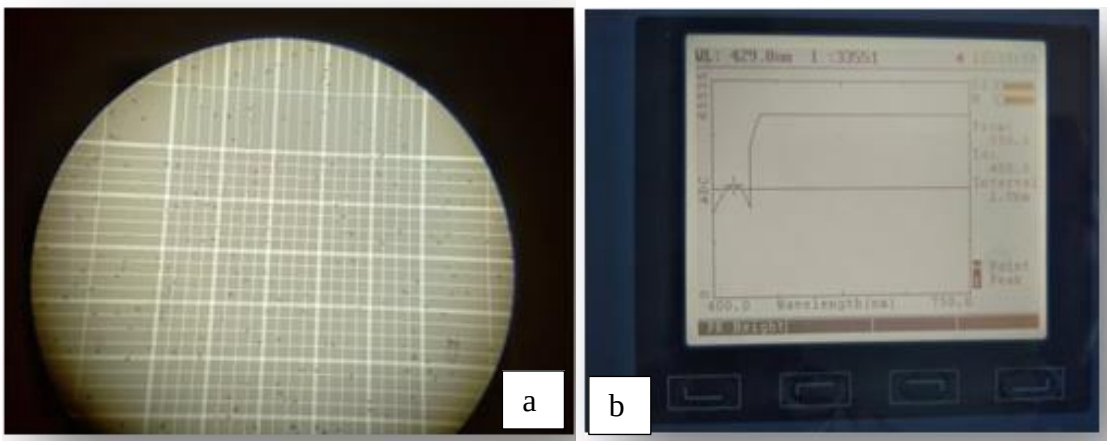
Appendix F: Morphological identification of microalgae

Appendix G: Preparation of culture media



Appendix H: Culture and maintenance of single isolated microalgae

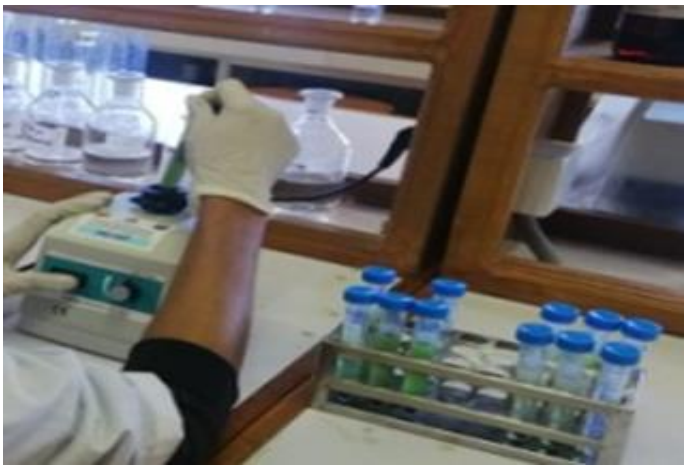
Appendix I: Growth curve determination of isolated microalgae



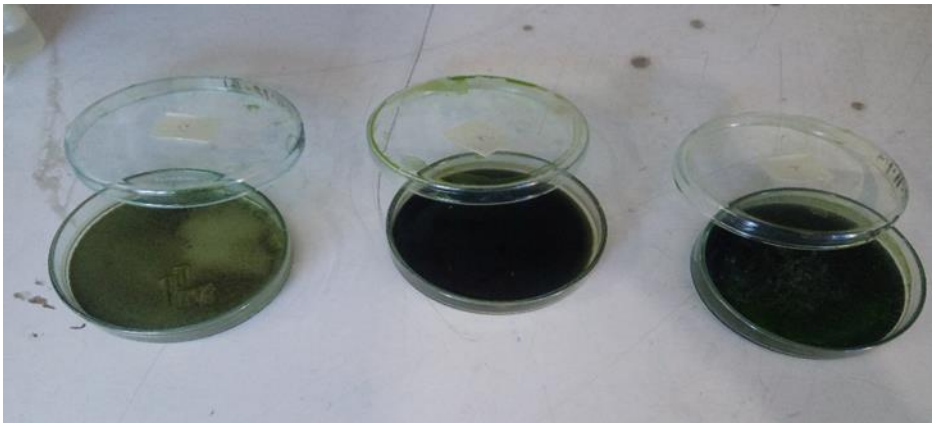
Appendix J: Determination of cell density (a) and optical density (b)

Appendix K: **Determination of chlorophyll content**

Appendix L: Determination of carotenoid content



Appendix M: Determination of phycobiliprotein content



Appendix N: Biomass extraction

Appendix O: Determination of lipid (a) and protein (b) content

Appendix 1: One way analysis of variance examining the difference in cell density on stationary phase of the isolated microalgae.

	Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Cell Density (cells/ml×10^7)	Between Groups	86.848	7	12.407	508.930	.000
	Within Groups	.390	16	.024		
	Total	87.238	23			

Appendix 2: One way analysis of variance examining the chlorophyll-a and b content of isolated microalgae.

	Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Chl-a	Between Groups	224.428	7	32.061	6929.492	.000
	Within Groups	.074	16	.005		
	Total	224.502	23			
Chl-b	Between Groups	5.811	7	.830	173.537	.000
	Within Groups	.077	16	.005		
	Total	5.888	23			

Appendix 3: One way analysis of variance examining the difference in carotenoid content of isolated microalgae.

	Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Carotinoid	Between Groups	98.465	7	14.066	427.172	.000
	Within Groups	.527	16	.033		
	Total	98.992	23			

Appendix 4: One way analysis of variance examining the difference in phycobiliprotein content of isolated microalgae.

	Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Phycocyanine	Between Groups	4.031	7	.576	1013.7	.000
	Within Groups	.009	16	.001		
	Total	4.040	23			
Allophycocyanin	Between Groups	9.807	7	1.401	1965.7	.000
	Within Groups	.011	16	.001		
	Total	9.818	23			
Phycoerythrin	Between Groups	5.528	7	.790	264.3	.000
	Within Groups	.048	16	.003		
	Total	5.575	23			
Total Phycobiliprotein	Between Groups	51.609	7	7.373	1088.3	.000
	Within Groups	.108	16	.007		
	Total	51.717	23			

Appendix 5: One way analysis of variance examining the difference in proximate composition (protein, lipid and carbohydrate) of isolated microalgae.

	Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Protein	Between Groups	356.665	7	50.952	8.84	.000
	Within Groups	92.220	16	5.764		
	Total	448.885	23			
Lipid	Between Groups	394.598	7	56.371	72.6	.000
	Within Groups	12.423	16	.776		
	Total	407.020	23			
Carbohydrate	Between Groups	269.026	7	38.432	13.523	.000
	Within Groups	45.473	16	2.842		
	Total	314.499	23			

Appendix 6: One way analysis of variance examining the difference in volumetric productivity of isolated microalgae.

	Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Volumetric Productivity (mg/L/Day)	Between Groups	1802.554	7	257.51	314.9	.000
	Within Groups	13.082	16	.818		
	Total	1815.636	23			

Appendix 7: One way analysis of variance examining the difference in areal productivity of isolated microalgae.

	Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Areal Productivity ((mg/cm2/day)	Between Groups	18.026	7	2.575	314.943	.000
	Within Groups	.131	16	.008		
	Total	18.156	23			

Appendix 8: One way analysis of variance examining the difference in lipid productivity of isolated microalgae.

	Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Lipid Productivity (mg/L/Day)	Between Groups	80.865	7	11.552	112.72	.000
	Within Groups	1.640	16	.102		
	Total	82.505	23			

Appendix 9: One way analysis of variance examining the difference in cell duplication time of isolated microalgae.

	Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Cell duplication time (Day)	Between Groups	1.129	7	.161	5184.9	.000
	Within Groups	.000	16	.000		
	Total	1.129	23			

Appendix 10: One way analysis of variance examining the difference in Cell doublings per day of isolated microalgae.

	Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Cell doublings per day (K)	Between Groups	.414	7	.059	129.109	.000
	Within Groups	.007	16	.000		
	Total	.422	23			

Appendix 10: One way analysis of variance examining the difference in SGR of isolated microalgae.

	Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
SGR	Between Groups	.312	7	.045	5714.3	.000
	Within Groups	.000	16	.000		
	Total	.312	23			

Brief Biography of the Author

Zannatul Nayma is the daughter of Didarul Haque and Jariatul Zannaat was born and grown up in Chakaria, Cox's Bazar. She has achieved B.Sc. Fisheries (Hons) degree from Faculty of Fisheries, Chattogram Veterinary and Animal Sciences University. Recently she has achieved "Deans Award" for holding 2nd position in her graduation life. She is now a candidate of Master of Science in Department of Aquaculture, Chattogram Veterinary and Animal Sciences University. Out of the three semesters of MS, she has successfully completed her 1st and 2nd semester and obtained 4.00 out of 4.00 in both semesters. She has successfully completed her research work on "Isolation, morphological identification and characterization of indigenous microalgae from different south-eastern freshwater habitat of Bangladesh". She worked as a "Research Assistant" in the project entitled "Isolation and Identification of Marine and Freshwater Indigenous Microalgae from bay of Bangle and Kaptai Lake of Bangladesh" funded by University Grand Commission (UGC) for a period of January 2020 to December 2021. As well as, she worked in "Aquaculture wastewater as a low cost medium for mass production of marine microalgae and it's utilization as feed for culturing sea bass and crab larvae" project funded by Bangladesh Fisheries Research Institute (BFRI). Together with this, from January 2022 to till now she is working as a "Research Assistant" in the project entitled "Isolation and identification of indigenous microalgae from different coastal regions of Bangladesh and its utilization as live feed for aquaculture industry" funded by Krishi Gobeshona Foundation (KGF). She has already published some scientific papers as first author, as well as co-author in some well reputed national and international journals. Her research interest includes microalgae (isolation, identification, mass production and feed formulation, antimicrobial activity of microalgae, pharmaceuticals and nutraceuticals industry of microalgae), zooplankton and seaweed. She is passionate to qualify herself as a competent researcher, and thus to develop the aquaculture sector of Bangladesh.

List of Publications

Publications	Status
a) **Nayma Z.**, Khatoon H., Rahman M.R., Mukta F.A., Sultana R. 2022. Comparative study of growth, pigments and proximate composition of selected indigenous freshwater microalgae isolated from Bangladesh. Journal of Innovation in Applied Research. 5(2): 13-24.	Published
b) **Nayma Z.**, Khatoon H., Rahman M.R., Mukta F.A., Sultana R., Hossain M.N., Iqbal M.Z. 2021. A comparative study on the productivity of selected tropical freshwater microalgae. Bangladesh Journal of Fisheries. 33(2): 255–264.	Published
c) Mukta F.A., Khatoon H., Rahman M.R., Acharjee M.R., Newase S., **Nayma Z.**, Sultana R., Hasan S.J. 2021. Effect of different nitrogen concentration on the growth, proximate and bio hemical composition of freshwater microalgae *Scenedesmus communis*. Journal of Energy and Environmental Sustainability. 11: 36–42.	Published
d) Khatoon H., Penz K.P., Banerjee S., Rahman M.R., Minhaz T.M., Islam Z., Mukta F.A., **Nayma Z.**, Sultana R., Amira K.I. 2021. Immobilized *Tetraselmis* sp. for reducing nitrogenous and phosphorous compounds from aquaculture wastewater. Bioresource Technology. 338: 125529.	Published
e) Khatoon H., Leng M.Y., Rahman M.R., Sarker J., Minhaz T.M., Sultana R., **Nayma Z.**, Mukta F.A. 2021. Efficiency of *Chlorella vulgaris* beads in improving water quality and growth of juvenile siamese fighting fish (*Betta splendens*). Bangladesh Journal of Veterinary and Animal Sciences. 9(1): 74–86.	Published
f) Usha S.Z., Rahman M.R., Sarker J., Hasan S.J., Sultana R., **Nayma Z.**, Mukta F.A., Khatoon H. 2021. Cultivation of *Chlorella vulgaris* in aquaculture wastewater as alternative nutrient source and better treatment process. Bangladesh Journal of Veterinary and Animal Sciences. 9(1): 43–51.	Published
g) Rahman M.R., Islam S.M.R., **Nayma Z.**, Sultana R., Sarker J. 2020. Bio-economic evolution of snakeheads and Indian major carps culture in IMTA system. Bangladesh Journal of Veterinary and Animal Sciences. 8(1): 117–127.	Published
h) Sultana R., Khatoon H., Rahman M.R., Haque M.E., **Nayma Z.**, Mukta F.A. 2022. Potentiality of *Nannochloropsis* Sp. As Partial Dietary Replacement of Fishmeal on Growth, Proximate Composition, Pigment and Breeding Performance in Guppy (*Poecilia Reticulata*). Bioresource Technology Reports.	Accepted