Chapter -1: Introduction

Global demand for microalgae is increasing day-by-day since it drew attention as a green energy source of biofuel in the next generation. Based on this cue, a range of microalgae species are being cultured in hatcheries for various purposes, including human nutrition, biofuel production, aquaculture nutrition, manufacturer of pharmaceuticals and cosmetics, and biofertilizer production (Muller-Feuga, 2000; Chisti, 2008; Wijesekara et al., 2010; Leckie, 2021). As live feed, microalgae are mostly used in aquaculture food chain for various growth stages of crustacean, bivalve mollusk, some fish species, and zooplankton (Brow, 2002; Mishra and Pabbi, 2004). Microalgae can easily survive in wastewater by absorbing nutrients, which reduces high cultivation cost. This attribute offers eco-friendly, sustainable and energy-efficient biofuel production from microalgae (Muller-Feuga, 2000; Qu et al., 2019). Among microalgae, *Scenedesmus* sp. has an ability to grow well in diverse types of waste water (Toyub et al., 2008; Peng et al., 2017). Having good amount of saturated and unsaturated fatty acids as well as all the essential amino acids, *Scenedesmus* sp. is highly potential for biodiesel and nutraceuticals production (Qu et al., 2019). A better growth performance and biochemical composition of *Scenedesmus obliquus* was found in Bold Basal Media than Bristol and 2.5% waste water media (Toyub et al., 2008; Khatoon et al., 2017). It has also been reported that sodium nitrate has significant impact on the high specific growth rate of microalgae than urea, ammonium carbonate and ammonium chloride (Podevin et al., 2015).

The economic viability of biodiesel produced from microalgae, as well as biomass productivity and lipid yields are important parameters that must be optimized. Alteration of cultivation techniques such as changing the nutritional elements of the growth medium, ambient conditions, and microalgae development phases can increase biomass productivity and composition of biomolecules, particularly fatty acid production in microalgae (Converti et al., 2009; Fan et al., 2014). Recently a lot of works have been done on different microalgae species to enhance its growth performance, lipid production and enrich the nutrient composition (Courchesne et al., 2009; Solovchenko et al., 2013). When a light source and CO_2 are available, nutrient limitation is one of the most effective triggers for increasing lipid accumulation in a single microalgal cell (Li et al., 2011; Han et al., 2013). As a component of high-value biological macromolecules such as proteins, chlorophylls, and DNA, nitrogen is a

crucial macronutrient that affects the metabolism and, as a result, the growth and biochemical composition of microalgae (Fan et al, 2014).

Photosynthetic activity and biochemical composition such as fatty acids, pigment, and protein are notably affected by nitrogen limitation, which is also effective to enhance the lipid content of microalgae (Xin et al., 2010; Pancha et al., 2014). Nitrogen constraints also influence in conversion of free fatty acids into triacylglycerols (TAGs) over time, which is suitable microalgal lipid feedstock for biodiesel production (Nayak et al., 2019). The addition of a limited amount of $NaNO_3$ to the microalgae culture medium on a daily basis improved the growth, biochemical characteristics, and lipid synthesis of *Chlorella* sp. and *Isochrysis galbana* (Zhang and Liu, 2016; Nayak et al., 2019). The effects of different CO_2 and Fe^{3+} ion concentrations on the biomass yield, lipid productivity and accumulation, fatty acid profile, and the level of antioxidant compounds of *Scenedesmus obliquus* were evaluated in N-9 medium and found that enriched fatty acid profile in lower concentrations (El-Baky et al., 2014). Zarrinmehr et al. (2020) found that at sufficient nitrogen concentrations (72 mg/L), polyunsaturated fatty acids (PUFAs) increased five-fold, compared to nitrogen deprivation (34 mg/L), where saturated fatty acids (SFAs) concentrations were higher than nitrogen sufficiency.

Therefore, to improve the growth, nutritional and biochemical composition, it is necessary to standardize the concentration of nutrients, particularly nitrogen for microalgae cultivation. Despite many studies on *Scenedesmus* sp., no research has been done on the effect of different nitrogen concentrations on the growth and biochemical composition of *Scenedesmus* sp. particularly in Bold Basal Media. So, the objectives of this study are:-

I. To examine the effect of different nitrogen concentrations on the growth, productivity, pigment, proximate and fatty acid composition of freshwater *Scenedesmus* sp. cultured in BBM.
II. To improve the nutritional value of *Scenedesmus* sp. by modifying nitrogen concentration.

Chapter- 2: Review of Literature

2.1. Microalage and Their Potentiality:

Microalgae, also known as microphytes, are phytoplanktons that live in both the water column and the sediment and are commonly found in freshwater and marine settings and also are microscopic in nature (Thurman, 1997). They are unicellular species that live alone, in chains, or in groups. Microalgae also create a variety of other commercially significant and important products. They generate vitamins, which increases their value as a source of nutrition for humans and animals (Borowitzka, 1998; Cuellar-Bermudez et al., 2015). They also produce a variety of polysaccharides that are useful in medicine. Chlorophyll, β-carotene and other carotenoids, phycobiliproteins, and astaxanthin are all beneficial and commercially relevant pigments produced by various species (Cuellar-Bermudez et al., 2015). These pigments are important in cancer, neurological disorders, and optical illnesses treatments (Guil-Guerrero et al., 2004; Soletto et al., 2005). Protein is also abundant in microalgae. Their ability to produce critical amino acids boosts their potential as protein-rich diets (Guil-Guerrero et al., 2004). Starch, cellulose, hemicelluloses, and other polysaccharides are produced by microalgae from simple monomeric sugars, primarily glucose. Algal cells are an essential food source due to their high glucose content. They also create and store huge amounts of lipids, which differ by species and are influenced by a variety of circumstances. Glycerol, esterified sugars to various types of fatty acids (12–22 carbon atoms) are the most common lipids found in algal cells (Pulz, 2001; Duong et al., 2015).

Microalgae have been extensively studied around the world as a viable alternative for fossil fuels, as well as for use in leachate remediation, hyper saline wastewater disposal, and as a feedstock for marine life (Krishnan et al., 2015). Furthermore, their production offers a strategy for reducing carbon dioxide emissions, which contribute to climate change. One ton of microalgae are expected to burn 1.83 ton of CO_2 from the atmosphere (Chisti, 2007). Moreover, microalgae-based biofuel is a non-toxic, biodegradable, and renewable resource (Song et al., 2013). Microalgae, on the other hand, are widely used in the cleanup of leachate from landfills (Aravantinou et al., 2013; Richards and Mullins, 2013). In addition, lipid-rich microalgae, particularly in

essential fatty acids, are widely cultivated as a diet for juvenile fish, shellfish, mussel and zooplankton like rotifers (Li et al., 2014).

2.2. Growth Phases of Microalgae:

The lag phase, exponential phase, phase of declining relative growth, stationary phase, and death phase are the five growth phases of microalgae (Farag and Price, 2013). The first phase is the lag phase, during which the cell density increased slowly because the microalgae cell undergoes physiological adaptation of its metabolism, which includes cell division and carbon fixation. The log phase, also known as the exponential phase, is the second phase. The cell density grows as a logarithmic function of time during this phase. The algae species, light intensity, and temperature all influence the specific growth rate.

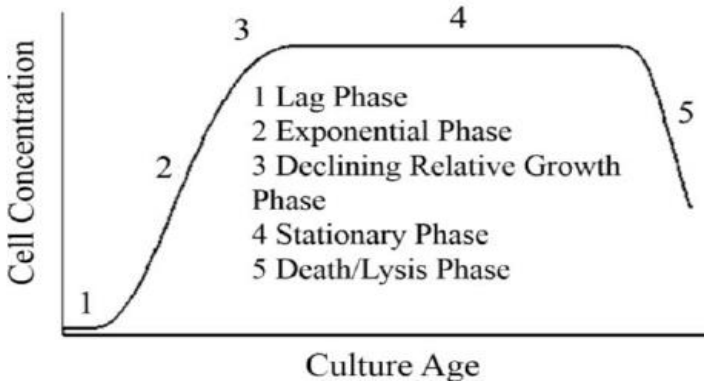


Figure 1. Growth phases of microalgae

The third phase is the declining phase, during which cell division slows and nutrients, light, pH, carbon dioxide, and other physical and chemical variables begin to limit growth. The fourth phase is the stationary phase, during which the cell density remained steady for some time due to the balance between the limiting factor and growth rate. This is the phase with the highest density of microalgae. The death phase is the final stage. At this point, the water quality has deteriorated, nutrient levels have reduced to the point where growth can no longer be sustained, and cell density has rapidly plummeted. The culture has come to an end in this phase.

Microalgae, unlike other energy crops, develop exceedingly quickly and have a high photosynthetic efficiency and lipid content. Despite the fact that microalgae growth is species dependant, they can easily quadruple their biomass in just 24 hours (Moazami et al., 2012; Suzana et al., 2013).

2.3. *Scenedesmus* sp.:

Freshwater green microalgae *Scenedesmus* sp. is colonial and non-motile. This species can exist as a single cell, although it is more commonly found in coenobia of four to eight cells within the parental mother wall (Guiry and Guiry, 2021). A variety of factors influence the genesis of coenobia. At high light intensities and temperatures, a greater proportion of unicellular organisms were detected, suggesting that organisms prefer to be non-colonized at higher growth rates (Guil-Guerrero et al., 2004). Ideal light intensity and nutritional condition help in successful cell growth into larger colonies. However, the large mass limits nutrients uptake and promotes sinking as well as avoidance of grazing predators like zooplankton (Lürling, 1999).

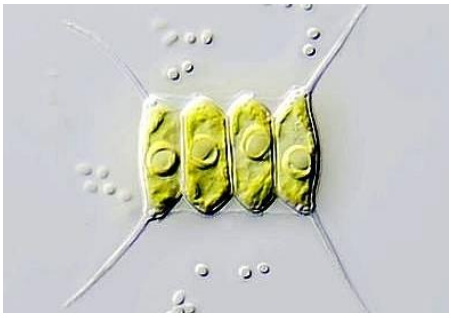


Figure 2. *Scenedesmus* sp.

Taxonomic classification of freshwater microalgae *Scenedesmus* sp. is given below (Guiry and Guiry, 2021):

Division : Chlorophyta

Class : Chlorophyceae

Order : Sphaeropleales

Family : Scenedesmaceae

Genus : *Scenedesmus* (Hegewald, 1977)

Typical mitotic cell division has been occurred in cell growth of *Scenedesmus* sp. (Guiry and Guiry, 2021). The mother cell enlarges during replication and becomes multinucleate after numerous divisions. The cytoplasm is then cleft into uninucleate daughter cells, which often form non-motile autospores. These daughter cells usually

join forces with other daughter cells to create a colony within the original cell wall, which is released later (Pickett-Heaps and Staehelin, 1975).

Scenedesmus quadricauda has a high protein content of 34.69 ± 0.41 % according to proximate analysis of dried microalgal biomass (Nayak et al., 2019). Hence, *S. quadricauda* is a promising potential feedstock for developing novel bioactive chemicals, and its protein-rich biomass may be utilized as animal feed (El-Moneim et al., 2018; Patnaik et al., 2019). *S. quadricauda* also had antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, with minimum inhibitory concentrations of 250 g/ml for both species (Arguelles, 2018). *Scenedesmus* sp. has been studied for its potential application in biodiesel synthesis as it is known to have high biomass productivity and lipid percentage (≥ 12 % dry weight) among green algae (Patnaik and Mallick, 2015). Its heterotrophic biomass and lipid production under optimal conditions is reported to be more efficient than its autotrophic production (da Silva et al., 2009; Shovon and Nirupama, 2009; El-Sheekh et al., 2012). Numerous researches have been conducted to optimize biomass productivity and lipid content of microalgae through altering the proportion of additional nutrients (Zhang and Liu, 2016; Nayak et al., 2019). *Scenedesmus* sp. is more effective in capturing CO_2 than other algae, for example, *Chlorella* sp., *Isochrysis galbana*, *Monoraphidium* sp. etc. (El-Sheekh et al., 2013). Though *Scenedesmus* sp. is capable of producing a wide range of biofuels, including bioethanol, biodiesel, bio-hydrogen, and drop-in fuels, the majority of study has focused on its usage in the manufacture of bio-diesel (Logan and Ronald, 2011). *Scenedesmus* sp., as well as other microalgae like *Chlorella* sp., *Dunaliella* sp., *Chlamydomonas* sp., and *Spirulina* sp., have a high carbohydrate content (>50% dry weight), making them good candidates for bio-ethanol production (John et al., 2011). In one study, *Scenedesmus* sp. was used to yield maximum 0.213 g ethanol /g biomass within 4 hours of fermentation after hydrolysis with 2% sulfuric acid (Ho et al., 2013). *Scenedesmus dimorphus* was found to be more efficient at removing ammonia from agroindustrial wastewater in a cylindrical bioreactor comparing with the efficiency of *Chlorella vulgaris*, while both algae removed phosphorus to the same extent (González et al., 1997).

2.4. Bold Basal Media:

Nitrogen, phosphorus, calcium, potassium, magnesium, microelements, and vitamins are all present in a basic microalgae culture medium. Algae have need of a few prerequisites to grow such as, water, sunlight, carbon, and nutrients like nitrogen and phosphorus. Screening and media optimization are required for low-cost microalgae biofuel before going to big scale, as media composition has a substantial impact on microalgal growth, lipid output, and microalgae biodiesel cost (Yang et al., 2014; Sharma et al., 2015). Because getting nutrients for biomass production from open water or ocean adds significantly to the biomass cost, wastewaters are represented as supplementary of commercial culture media and one of the finest possibilities for sustainable, zero-emission biofuel production (de Carvalho et al., 2019). The influence of several media, such as Blue Green-11(BG-11), Bold Basal medium, Fog's medium, and Basal medium, on microalgae growth and lipid productivity was investigated in a study conducted by Sharma et al. (2015). The Bold basal medium was shown to be the best for biodiesel production, having the highest lipid output (Sharma et al., 2015). For high lipid output of microalgae photoautotrophic cultivation, medium screening and tuning are essential. The quality of the medium used to cultivate microalgae has a significant impact on their growth performance (Devi et al., 2012; Lam and Lee, 2012). The composition of the medium, such as carbon, nitrogen, phosphorus, and some trace elements, has a major impact on cell development and fat accumulation (Liu et al., 2008). Dayananda et al. (2007) cultured *Botryococcus braunii* on BG-11, BBM (Bold Basal medium), and BBM nutrient media, examining how the composition of the culture media affected biomass and lipid output. Sharma et al. (2011) cultivated *Chlorella vulgaris* in Juller's medium, Bold's Basal medium, modified Chu-10 medium, N-8 medium, and Kuhl's medium to see how they affected growth, morphology, and pigmentation. They discovered that Bold's Basal medium was better in terms of growth medium. This medium is quite enriched, and it is used for a lot of green algae cultivation.

2.5. Effect of Different Nutrients on Microalgal Growth and Biochemical Composition:

When microalgae are cultured in changing environmental conditions, such as salinity, pH, and temperature, their biochemical composition can alter all important components like protein, carbohydrates, and lipids of microalgae. Researchers discovered that macronutrients such as nitrogen and phosphorus, as well as their ratios, had a significant impact on the protein, carbohydrate, lipid, fatty acids, chlorophyll-a, and carotenoids content of microalgae (Loureno et al., 1997). The cytoplasmic oil droplets are accumulated in nature under stressful environmental conditions including high temperature, light intensity, and salinity rise (Lynn et al., 2000; Gushina and Harwood, 2009; Solovchenko, 2012). The total lipid concentration and fatty acid profile are more affected by the nutritional composition of the medium in culture, with nitrogen, silicon, and phosphorus playing larger roles (Lopez et al., 2000; Xia et al., 2014). In comparison to the other media, Lynn et al. (2000) found the better algal growth in the medium with higher levels of sodium and trace elements like Fe, Mn, Mo, and Co and lower levels of N and P. These nutrients were also strongly connected with the greatest levels of total lipid (32%), as well as the highest amounts of saturated fatty acids, particularly palmitic acid. peaks in polyunsaturated fatty acids (43.7%), particularly α-linolenic acid (28.4%), were linked to N and P, but the link with K and Mg was stronger. N and P were the most critical elements associated with a high level of monounsaturated fatty acids, followed by K and Mg to a lesser extent.

2.5.1. Phosphorus:

Phosphorus is an essential component for normal algal cell development and proliferation. It is the most important constraint to microalgal growth (Larned, 1998; Raghothama, 2000). Algae contain 1% phosphorous by dry weight (Vymazal, 1988), however that small amount is insufficient for microalgae because all of the supplied phosphorus is not accessible due to their metal ion binding nature. The Calvin-Benson cycle is an important carbon fixation cycle that requires a specific substrate. Due to lack of accessible and needed phosphorus, these substrates could not be synthesized (Barsanti, 2006). Phosphorus deficiency causes lipid deposition in microalgae. Total lipid content in *Scenedesmus* sp. increased from 23 % to 53 % with a reduction in

total phosphorus (as phosphate) concentration of 0.1 to 0.2 mg L^{-1} (Xin et al., 2010). In *Chlamydomonas reinhartdtii*, phosphorus limitation resulted in a decrease in phosphatidylglycerol (PG) (Sato et al., 2000). It is required for normal photosynthesis-2 complex structure-function and cell growth, as well as the maintenance of chlorophyll-protein complex levels. Because of the phosphorus shortage, N-3 PUFA synthesis is also reduced (Reitan et al., 1994). Ge et al. (2018) demonstrated that microalgae cultured in mixotrophic mode with glycerol fed exhibited the highest biomass and lipid productivities. *Scenedesmus* sp. was subjected to phosphorus stress in order to increase lipid synthesis. The addition of 2 mg L^{-1} $NaH_2PO_4.2H_2O$ every 2 days resulted in the maximum lipid production (350 mg L^{-1}) and lipid content (about 41% of dry weight), which were both higher than those in replete phosphorus (Yang et al., 2018). However, other algal species, like *Chlorella zofingiensis* and *Phaeodactylum tricornutum*, showed lipid buildup accompanied by an increase in starch (Breuer et al., 2012).

2.5.2. Trace Metals:

Algal cells contain a large number of trace metals in minute amounts (less than 4 ppm). Manganese (Mn), Iron (Fe), Cobalt (Co), Copper (Cu), Nickel (Ni), and Zinc (Zn), the six trace metals are the most essential for their metabolic processes (Bruland et al., 1991). Microalgal development is also reliant on the availability of trace metals, which are limiting factors. Excess or low concentrations of these can impede growth, hamper photosynthesis, damage the cell membrane, and deplete anti-oxidants. Iron is an essential trace element for photosynthesis and respiration in algae. For nitrogen assimilation, the oxidation reduction reaction process is required; it works as a catalyst in this process. It also acts as a catalyst in a variety of activities, including photosynthesis and electron transport pathways (Abadia, 1986). Because of the lack of iron, the concentration of cellular chlorophyll decreases (Greene et al., 1992). According to Liu et al. (2008), a higher concentration of iron in *Chlorella vulgaris* cultivation was a major element in increasing lipid content. Reduced carotenoid composition has also been seen as a result of iron insufficiency (Kobayashi et al., 1993; van Leeuwe and Stefels, 1998). Liu et al. (2015) suggested that inorganic carbon and magnesium are important for biomass production and ammonia removal by *Scenedesmus* cultured in anaerobic digestion effluent of livestock wastewater.

Chlorophyll and its derivatives have been investigated as physiologically active dietary components. In vitro, chlorophyll and its derivatives, pheophytins and pheophorbins, have antioxidant and antimutagenic effects (Ferruzzi et al., 2002), and Negishi et al. (1997) demonstrated chlorophyll's antigenotoxic activity in *Drosophila*. The chlorophyll content of microalgae fluctuates in response to physical elements like light intensity, agitation, and temperature, as well as chemical aspects like nutrition availability. Temperatures above 40°C resulted in a decrease in chlorophyll concentration in *Chlorella vulgaris* (Chinnasamy et al., 2009). Increased agitation reduced the quantity of chlorophyll in *Scenedesmus obliquus* due to more uniform light distribution (Martinez et al., 2000). Variations in nutrient availability in vitro can affect chlorophyll concentration in cultures; for example, in *C. vulgaris* cultures, a reduction in magnesium content causes a fall in chlorophyll concentration (Finkle and Appleman, 1953).

2.6. Effect of Nitrogen on Microalgal Growth and Biochemical Composition:

Nitrogen is a critical nutrient for microalgal growth since it is required for the synthesis of amino acids, proteins, coenzymes, enzymes, chloroplast, and other structures (Turpin, 1991). Nitrogen concentrations in microalgae can alter growth rate, as well as protein, lipid, and carbohydrate synthesis (Pancha et al., 2014). Several studies have shown that whereas nitrogen restriction increases fat accumulation, it decreases biomass productivity (El-Kassas, 2013). Many studies have found that nitrogen deficiency alters the biochemical composition of microalgae, including carbohydrate and protein content, pigments and lipid concentration, fatty acid composition, and photosynthetic activity (Solovchenko et al., 2012; Fan et al., 2014). The effect of nitrogen supply on the biochemical composition, lipid classes, and fatty acid profile of the marine microalga *Isochrysis galbana* was investigated in several trials (Fidalgo et al., 1998; Liu et al., 2015).

2.6.1. Proximate Composition:

Microalgae can employ a range of nitrogen sources (such as ammonia, nitrite, nitrate, and urea), while different nitrogen sources can induce the biochemical composition of microalgae (Angles et al., 2017). More crucially, certain microalgal species could convert proteins or peptides to carbohydrates or lipids as energy reserve materials under nitrogen depleted circumstances (Huo et al., 2011). Illman et al. (2000) showed

that 55% carbohydrate content was found in *C. vulgaris* cultured in a depleted nitrogen medium. D'Souza and Kelly (2000) also stated that nitrogen deficiency combined with carbon-di-oxide feeding might increase the carbohydrate content of *Tetraselmis suecica* cells from 10% to 57 %. The limiting of nitrogen availability appears to be an effective strategy of causing the buildup of carbohydrates in microalgae (Ho et al., 2011). However, since the pathways of metabolism are closely linked with the production and depletion of energy-rich compounds (e.g., lipids and carbohydrates), a negative relationship between lipid and carbohydrate yield under various environmental stresses were found in several studies (e.g., nitrogen depletion) (Rismani-Yazdi et al., 2011; Siaut et al., 2011; Ho et al., 2012).

Nitrogen deprivation is an excellent way to boost microalgae lipid content. Pancha et al. (2014) reported that *Scenedesmus* sp. CCNM 1077 cultivated in a nitrogen-free medium had considerably higher lipid content than *Scenedesmus* sp. CCNM 1077 cultured in BG-11 media. According to Jia et al. (2015), the lipid content of *Nannochloropsis oceanica* cultivated under nitrogen-depleted conditions was roughly twice that of a sample cultured in a standard medium. Converti et al. (2009) found that when the nitrogen concentration in the medium was reduced by 75%, the lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* increased from 7.9% to 15.3 % and from 5.9% to 16.4%, respectively. The overall lipid content of *Chlorella* cells dropped as the nitrogen concentration was raised, according to Lv et al. (2010). Qi et al. (2016) showed that once the cells were switched from a nitrogen-rich to a nitrogen-deficient broth, the lipid content of *Chlorella vulgaris* SDEC-3M rapidly rose. Unfortunately, a nitrogen deficiency may result in an insufficient amount of biomass output. Therefore, Yu et al. (2018) previously used a two-step cultivation strategy to encourage *Chlorella* and *Scenedesmus* to produce lipids. In the first phase, each microalgae was cultivated in a medium containing enough nitrogen to enable a high degree of biomass production. To boost the lipid content, the microalgae was harvested and transferred to a nitrogen-free growing medium in the second stage. The technique for algae and water separation has high production costs, which prohibited the method from being used in large-scale biodiesel manufacturing (Sonkar and Mallick, 2017).

2.6.2. Cell Growth:

A fed-batch culture method can help to increase biomass productivity. Han et al. (2013) found that daily addition of a little amount of $NaNO_3$ to the medium enhanced algae growth and lipid production. Furthermore, lutein produced by *Scenedesmus obliquus* can be used as a by-product of lipid production, potentially lowering lipid production costs even more. *Scenedesmus obliquus*, *Chlorella vulgaris*, and *Chlorella minutissima* produced more biomass and lipids when 0.01 g L^{-1} nitrate was added to the medium every three days, according to Sonkar and Mallick (2017). Lower nitrogen concentrations were observed to have a considerable impact on the microalgal cell growth and photosynthetic activity, as well as affecting cell shape.

2.6.3. Pigments:

Chlamydomonas reinhardtii and *Scenedesmus subspicatus* cultures with low nitrogen contents have lower chlorophyll content (Dean et al., 2010). The effects of nitrogen stress and light intensity, in particular, must be thoroughly detailed because these are the circumstances that are known to modify the cellular content of chlorophyll. The amount of chlorophyll changes according on the stage of cultivation, with the amount of chlorophyll being lowered in the stationary phase (Fidalgo et al., 1998). Because chlorophyll is a possible biotechnological product from microalgae, and its content is linked to photosynthesis efficiency and cell growth, it's crucial to know how culture conditions affect chlorophyll production and other cellular consequences.

Carotenoids are vital in the process of photosynthesis with chlorophyll in algae. Green algae also have carotenoids as supplementary pigments, in addition to the photosynthetic pigment chlorophyll. β-carotene, lutein, violaxantin, anteraxantin, zeaxantin, and neoxantin are the primary carotenoid compounds found in green algae (Burtin, 2003). Nicolau et al. (2020) found that N-replete circumstances increase carotenoid production, resulting in a greater antioxidant capability in *Nephroselmis* sp. Indeed, total carotenoids and peroxyl radical scavenging activity were higher in N-replete settings and drastically decreased in N-limited or famine conditions. Individual carotenoids, such as siphonaxanthin, neoxanthin, xanthophyll cycle pigments, and lycopene, followed the same pattern as total carotenoids, however carotene and lutein remained constant independent of nitrogen availability. The N-replete therapy also resulted in greater carotenoid productivities. Carotenoids'

antioxidant activity is linked to their ability to defend against photo-oxidative damage caused by reactive oxygen species (ROS), which are constantly created during photosynthesis and aerobic metabolism (Young and Lowe, 2001; Sandmann, 2019). Furthermore, N deficiency is known to cause reactive oxygen species (ROS) production in microalgae cells (Zhang et al., 2013; Çakmak et al., 2015; Chokshi et al., 2017).

2.6.4. Biochemical Properties:

As a result of N-deficiency, various authors have observed an increase in hydrogen peroxide (Çakmak et al., 2015; Chokshi et al., 2017; Hamid and Sibi, 2018) and lipid peroxidation (Zhang et al., 2013; Fan et al., 2014; Yilancioglu et al., 2014) in microalgae cells. A well-known example of the huge accumulation of carotene in *Dunaliella* spp. exposed to N-starvation combined with high light and high salinity (Ben-Amotz and Avron, 1983; Lamers et al., 2012) is the massive accumulation of carotene in *Dunaliella* spp. exposed to N-starvation combined with high light and high salinity. However, the effect of nitrogen availability on antioxidant components varies by species; therefore enhanced antioxidant activity owing to secondary carotenoids over accumulation under nutritional stress should not be applied universally. Only a few chlorophytes species can accumulate secondary carotenoids (Mulders et al., 2014), and various studies have found that N-deficiency reduces antioxidant activity and primary carotenoids (Solovchenko et al., 2008; Goiris et al., 2015; Ruiz-Domínguez et al., 2015; Aremu, et al., 2016; Zhang et al., 2019).

The content and source of nitrogen, among other nutrients, can influence and change the biochemical makeup of microalgae cells. *Scenedesmus* sp., based on literature reports of biodiesel production from microalgae, is an excellent candidate for use as a feedstock in the manufacture of biofuel and food, due to its ability to thrive in a variety of wastes and high biomass and lipid production capacity (Pancha et al., 2014; Pezzolesi et al., 2019). Growth, cell density, and fatty acid profiles are all substantially induced when nitrogen is scarce (Sukenik and Carmeli, 1990; Navarro-Peraza et al., 2017). Therefore, it was expected that this research will be helpful to enhance the growth, nutritional and biochemical composition of *Scenedesmus* sp. through standardized nitrogen concentration.

Chapter-3: Materials and Methods

3.1. Microalgae Collection and Culture:

The freshwater *Scenedesmus* sp. was collected from 'Live Feed Research Corner', Faculty of Fisheries, Chattogram Veterinary and Animal Sciences University, Chattogram, Bangladesh. The pure *Scenedesmus* sp. was cultured in modified Bold Basal Media (Stein, 1980) under controlled environment at 25.2 ± 0.7°C, 7.72 ± 0.17 pH, 4.53 ± 0.53 mg/L dissolved oxygen by gentle aeration and 150 µE $m^{-2}s^{-1}$ light intensity for 24 h.

3.2. Experimental Design:

Firstly, the sub-culture was run every two weeks in order to maintain a healthy and good stock by using different volumes (100ml, 250ml, and 500ml) of Erlenmeyer flask as to prepare the *Scenedesmus* sp. stock for the main experiment. This research was conducted to explore the effect of different nitrogen concentrations on growth, pigment and proximate composition of *Scenedesmus* sp. comparing with standard nitrogen concentration in Bold Basal Medium (Stein, 1980).

There were five treatments of different nitrogen ($NaNO_3$) concentration as 13 g/L (T1), 19 g/L (T2), 25 g/L (control: considering standard Bold Basal Medium concentration by Stein, 1980), 31 g/L (T3), and 37 g/L (T4) according to 24% up and down from control. This experiment was divided into two parts: firstly, analysis of growth curves and secondly, analysis of pigment and proximate composition. To analyze the growth curve, 2% *Scenedesmus* sp. seeds (~10^5cells/ml) from pure stock were inoculated in 300 ml culture media of each treatment in a 500 ml conical flask with three replications. Growth parameters such as cell density, optical density, biomass, and chlorophyll (a, b) were measured everyday to explore the stationary phase up to 13 days of the culture period. Afterward, mass culture was continued in a 2L volume Erlenmeyer flask along with 2% *Scenedesmus* sp. seed inoculation till stationary phase for each treatment. Then the harvested microalgae through a centrifuge (Hitachi* High-speed Refrigerated Centrifuge, Himac CR 21g-II) was dried at 40°C temperature using a hot air oven, and subsequently, preserved at refrigerator (4°C) for following pigments, proximate, and fatty acid analysis.

All Erlenmeyer flasks for microalgae culture were autoclaved and also covered with sterilized cotton balls, aeration pipes were sterilized through chlorination treatment;

total culture and sampling were done following precaution to avoid contamination with other microalgae species and protozoa in *Scenedesmus* sp. cultures. The experiment was completed in Disease and Microbiology Laboratory, Faculty of Fisheries, Chattogram Veterinary and Animal Sciences University.

3.2.1. Culture Media Preparation:

Distilled water was autoclaved at 121°C temperature and 15 lbs/ $inch^2$ pressure for 15 minutes, the sterilized water was stored at room temperature. Culture media was prepared along with sterilized distilled water and modified Bold Basal Media (Stein, 1980) according to the following labeled constituents.

Table 1. Preparation of Bold Basal media (Stein, 1980):

Sl No.	**Stock**	**Stock solution**		**ml/L (For culture media preparation)**
1.	KH_2PO_4	8.75g/500ml		10ml
2.	$CaCl_2.2H_2O$	1.25g/500ml		10ml
3.	$MgSO_4.7H_2O$	3.75g/500ml		10ml
4.	$NaNO_3$	12.5g/500ml (25g/L)		10ml
5.	K_2HPO_4	3.75g/500ml		10ml
6.	NaCl	1.25G/500ml		10ml
7.	Na_2 EDTA. $2H_2O$	10g	Per litre	1ml
	KOH	6.2g		
8.	$FeSO_4.7H_2O$	4.98g	Per litre	1ml
	H_2SO_4	1ml		
9.	H_3BO_3	5.75g/500ml		0.7ml
10.	**Trace metal solution**			
	H_3BO_3	2.86g	Per litre	1ml
	$MnCl_2$. $7H_2O$	1.81g		
	$ZnSO_4$. $7H_2O$	0.222g		
	Na_2MoO_4. $2H_2O$	0.390g		
	$CuSO_4$. $5H_2O$	0.079g		
	$Co(NO_3)_2$. $6H_2O$	0.049g		
11.	**Vitamin solution**			
	Thiamine, Vit B_1	0.20g	Per 100ml	0.5ml
	Cyanocobalamine	0.01g		

3.3. Determination of Growth Curve:

Data of the growth curves were determined prior to the start of mass culture to determine the data of pigments, and proximate composition. A total of 300 ml of culture volume was maintained in a sterile 500 ml borosilicate Erlenmeyer flask for each treatment with three replications. The culture was continued up to death phase. Gentle aeration was provided for 24 hrs which offered 4.53 ± 0.53 mg/L dissolved oxygen. Growth curve was determined on basis of cell density (cells/ml), optical density (absorbance), biomass (g/L) and chlorophyll (mg/m^3).

3.3.1. Determination of Cell Density:

Microalgal cells were counted every day with a Hemacytometer throughout the growth curve experiment according to Lavens and Sorgeloos (1996). The hemacytometer and its cover slip (Bright- line improved Neubauer hemacytometer, 0.0025 mm^2, 0.1 mm deep chambers, Assistent, Germany) were cleaned using Milli-Q water (Millipore Corporation) prior to the fill up of the chambers with culture samples. Cells were counted for both chambers of the hemacytometer under an electronic microscope with 40x magnification; Lugol's iodine was added to culture aliquots for fixation and staining to facilitate counting. The formula to calculate the cells is as the following:

$$\text{Cell count calculation (cells/ml) for 25 squares} = \frac{\text{Total number of cells counted}}{50 \times 4} \times 10^6$$

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

3.3.2. Determination of Optical Density:

Daily absorbance change was measured using a NanoDrop Spectrophotometer (NanoPlus, WAVE ANALYTICS D-82362 Weilheim, Germany) at 600 nm wavelength where treatment wise culture media was used as blank.

3.3.3. Determination of Biomass:

Biomass was estimated through slight modification of procedure described by Ratha et al. (2016). After rinsing filter papers (47 mm Whatman® GF/C) with 10 ml distilled water and drying for 4 h at 100°C in a Hot Air Oven (LNO- 150, LABNICS Equipment, USA), subsequently 15-20 minutes desiccation, filter papers were weighed. Vacuum filtration was used to filter 1.0 ml microalgae sample from each culture through pre-weighed filter paper. The filter paper with biomass was then

rinsed three times with 10 mL distilled water before being oven-dried for another four hours at 100°C, followed by 15-20 minutes of desiccation. Further filter paper with biomass was weighed. The dry biomass concentration was then estimated by dividing the difference between the pre and post-filtration weights of the dried filter paper by the filtered volume.

3.4. Specific Growth Rate and Cell Doubling Time Estimation:

The Specific Growth Rate, μ (day^{-1}) and cell doubling time of cultured microalgae was calculated according to Xu et al. (2016).

$$SGR, \mu\ (day^{-1}) = \ln (C_2 - C_1)/ (t_2 - t_1)$$

Where,

$C_2 - C_1$ = Difference in cells/ ml between the end and beginning of the experiment;

$t_2 - t_1$ = Time interval between final and initial days

From the above equation, the cell doubling time of the culture was calculated as days required for duplication using the following formula:

$$t_d\ (day) = 0.693/ SGR$$

3.5. Productivity Estimation:

For each treatment, volumetric (Green et al., 1995), areal (Ugwu et al., 2008), and lipid (Benemann and Tillett, 1987) productivities were estimated using dry biomass and percentage of lipid at the stationary phase of microalgae cultures.

3.5.1. Volumetric Productivity:

Volumetric productivity (VP) indicates the average daily productivity of a culture based on dry weight. Following equation was used to calculate the volumetric productivity:

$$VP\ (gL^{-1}\ day^{-1}) = (X_n - X_0)/ N$$

Where, X_n = Final biomass (gL^{-1}), X_0 = Initial Biomass (gL^{-1}) and N = Culture days

3.5.2. Areal Productivity:

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

$$AP\ (g\ cm^{-2} day^{-1}) = (VP \times V)/A$$

Where, VP = Volumetric Productivity ($g\ L^{-1}\ day^{-1}$), V = Total volume of the culture (L), A = surface area occupied ground (cm^2).

3.5.3. Lipid Productivity:

Lipid productivity (LP) is the amount of lipids produced by microalgae in a day during stationary phase. The lipid productivity was calculated using lipid content (Folch et al., 1957; Bligh and Dyer, 1959) and volumetric productivity at the stationary phase. Following equation was used to calculate the lipid productivity:

$$LP\ (mg\ L^{-1}\ day^{-1}) = VP \times (\%\ lipid / 100)$$

Where, VP = Volumetric productivity of the culture, % lipid = Percentage of lipid content as dry weight basis.

3.6. Determination of Pigments:

Change in chlorophyll a, b (mg/L), carotenoid (mg/L) and phycobiliprotein (mg/g) of *Scenedesmus* sp. due to different nitrogen concentrations' treatment was determined in this experiment.

3.6.1. Determination of Chlorophyll a, b:

3.6.1.1. Microalgae Extraction:

Microalgae sample was extracted according to Jenkins (1982) for chlorophyll determination. For extraction, at first 1 ml of 1% $MgCO_3$ suspension was pipetted after shaking over placed filter (47 mm Ø Whatman® GF/C glass microfiber filter papers). Then vacuum was applied to remove liquid from the filter paper. 1 ml of microalgae sample from the culture had been transferred to the filter. After that the millipore filter was removed and trimmed away the edges which were not coated with residue. Then the filter paper was crumpled into cone shape and placed into 15 ml centrifuge tube making sure the middle of the filter was facing down. Adding 5 ml of 90% acetone, the filter paper was submerged for 2 minutes and then grinded for 1 minute by tissue homogenizer. Further, 5 ml of 90% acetone was added in that mixture and grinded for 30 seconds. Centrifuge tubes were wrapped with aluminum foil and refrigerated in the dark for 1 hour. Thereafter the acetone extracts were centrifuged at 2000 to 3000 rpm for 10 minutes. The clear supernatant was decanted into another 15 ml centrifuge tubes and centrifuged at low speed (300-500 rpm) for 5 minutes. This acetone extract was ready for spectrophotometric measurement in order to determine chlorophyll.

3.6.1.2. Determination of Chlorophyll a and b (Dichromatic method):

The clean extract was measured absorbance at 750, 664, 647, and 630 nm wavelengths, where OD at 750 nm was used as a turbidity correction factor and subtracted from the other wavelengths' absorbance. The concentrations of chlorophyll

a and b in the acetone extract were calculated by inserting the corrected optical densities in the following equations (Jeffrey and Humphrey, 1975):

$$C_a\,(mg/L) = 11.85\,(OD\ 664) - 1.54\,(OD\ 647) - 0.08\,(OD\ 630)$$

$$C_b\,(mg/L) = 21.03\,(OD\ 647) - 5.43\,(OD664) - 2.66\,(OD\ 630)$$

Where, C_a and C_b = concentrations of chlorophyll a and b, respectively, mg/L, and OD 664, OD 647, and OD 630 = turbidity corrected optical densities (with a 1cm light path) at the respective wavelengths.

After that, the amount of pigment per unit volume was calculated as follows:

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

3.6.2. Determination of Carotenoid:

At the stationary phase of each culture 1.0 ml microalgae sample was centrifuged at 1000 x g for 5 minutes to obtain pellet. Afterwards, the pellet was extracted with 3 ml 2:1 of ethanol: hexane (v/v). Then the pellet with the solvent was shaken vigorously and centrifuged again at 1000 x g for 5 minutes. Thus, the hexane layer was separated and its absorbance was determined spectrophotometrically at the wavelength of 450 nm. The amount of extracted carotenoids from the samples in micrograms was determined by multiplying the absorbance (A_{450}) with 25.2 (Khatoon et al., 2020).

3.6.3. Determination of Phycobiliproteins:

3.6.3.1. Phycobiliproteins Extraction:

Scenedesmus sp. powder (40 mg) was soaked in 10 ml phosphate buffer solution (pH 7.0; 0.1M), vortex mixed thoroughly, and stored at 4°C for 24 h. After centrifuging at 6000 x g for 10 minutes, the supernatant was collected and absorbance was measured using a NanoDrop Spectrophotometer (NanoPlus, WAVE ANALYTICS D-82362 Weilheim, Germany) at wavelengths of 562, 615, and 652 nm, with phosphate buffer as a blank (Siegelman and Kycia, 1978).

3.6.3.2. Spectrophotometric Estimation of Phycobiliproteins:

The amount of phycocyanin, phycoerythrin and allophycocyanin in the sample was determined using the extinction coefficients and the following formulae (Siegelman and Kycia, 1978):

$$\text{Phycocyanin (mg/ml)} = \{A_{615} - (0.474 \times A_{652})\}/\ 5.34$$

$$\text{Allophycocyanin (mg/ml)} = \{A_{652} - (0.208 \times A_{615})\}/\ 5.09$$

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

The following formulas were used to compute total phycocyanin, phycoerythrin, and allophycocyanin (mg/g) (Silveira et al., 2007):

$$\text{Phycobiliprotein (mg/g)} = (\text{Pigment concentration} \times V) / DB$$

Here, V= Solvent volume, DB= Dried biomass

The sum of the phycocyanin, phycoerythrin, and allophycocyanin contents in dried microalgae biomass was used to compute total phycobiliproteins (mg/g).

3.7. Determination of Proximate Composition:

3.7.1. Protein Determination:

Protein was determined by spectrophotometric method according to Lowry et al. (1951) with few alterations. Each sample contained 5.0 mg of oven-dried microalgae biomass, which was used to make a 25 ml well mixed (by tissue homogenizer) solution with distilled water. For each sample, 0.5 ml of the 25 ml solution was taken and 50 ml of Reactive 2 (20 g of Na_2CO_3 in 100 ml of 0.1N NaOH) and 1 ml of Reactive 1 (1 % NP tartrate) were combined with the microalgae solution. After that, 0.5 ml of the mixture was mixed with 0.5 ml of 1N NaOH and maintained at 100°C for 5 minutes in a hot water bath. The samples were then chilled in a cold water bath before being added 2.5 ml of the produced mixed reagent and maintained for 10 minutes. Following that, 0.5 ml of Folin reagent was added to the mixture and then kept in a dark place for 30 minutes. At a wavelength of 750 nm, the absorbance of the mixture was measured using a NanoDrop Spectrophotometer (NanoPlus, WAVE ANALYTICS D-82362 Weilheim, Germany). To create a calibration graph, a stock solution of 2000 µg/L of standard (albumin) was created, and a series of standards (20 µg/L, 40 µg/L, 80 µg/L, 100 µg/L, and 200 µg/L) were made from the stock solution. For the standard series, the same procedures as for protein analysis were used; a calibration line was constructed according to absorbance, and the protein content of each sample was estimated appropriately.

3.7.2. Lipid Determination:

Lipid was determined according to Bligh and Dyer (1959) and Folch et al. (1957). Each sample's aluminum dish was labeled and weighed as the starting weight. Then, with distilled water, 50 mg of each sample was placed in a centrifuge tube and diluted into a 5x volume. The material was then homogenized with 3 ml 2:1 methanol: chloroform (v/v) using a tissue homogenizer. After that, all of the tubes were centrifuged for 4 minutes at 1000 rpm at 4°C, and the supernatants were pipetted into clean tubes and placed in ice. Then 3ml (1:2) methanol: chloroform (v/v) was mixed

homogeneously with the sample once more. The tubes were then centrifuged again using the previously described method, and the supernatants were transferred to the prior tubes of supernatants. 1.5 ml of 0.9 % NaCl was vortex mixed into this combined supernatant, and the tubes were stored at 4°C for 1 h. The tubes were centrifuged at 1000 x g for 10 minutes at 4°C after 1 h. The top layer of methanol and chloroform was discarded, while the bottom layer was transferred to the aluminum dish that had been prepared previously. The solvent was then evaporated in a hot air oven at 40°C (LNO- 150, LABNICS Equipment, USA). The aluminum dishes were then weighed to get the final weight. Finally, the initial weight was subtracted from the final weight to get the lipid weight in the samples. Lipid content was calculated by the following formula:

Lipid (% dry weight) = lipid weight/sample weight

3.7.3. Carbohydrate Determination:

Carbohydrate was determined according to Dubois et al. (1956). A 5 mg oven-dried biomass sample was used to make a 25 ml well mixed (tissue homogenizer) solution with distilled water for each sample. After that, 1.0 ml of the 25 ml solution was taken for each sample, followed by 1ml of 5 % phenol solution and 5.0 ml of sulfuric acid. The samples were then placed in a cold water bath for a period of time. When the mixture become cool, absorbance of the solution was taken at 488 nm wavelength using NanoDrop Spectrophotometer (NanoPlus, WAVE ANALYTICS D-82362 Weilheim, Germany) to estimate carbohydrate. To build a calibration graph, a 1000 µg/L standard (glucose) stock solution was prepared, and then a series of standards at various dilutions (20 µg/L, 40 µg/L, 60 µg/L, 100 µg/L, and 140 µg/L) were made from the stock solution. For the standard series, the same procedures as for carbohydrate analysis were used; a standard graph was constructed based on the standard values obtained from the absorbance, and the carbohydrate content for each sample was calculated appropriately.

3.8. Fatty Acid Analysis:

Fatty acids were determined by “Two steps transesterification (2TE)” method after slight modification according to Griffiths et al. (2010). At first, lipid was extracted by Digital Soxhlet Apparatus (FOOD ALYTRD40) after mixing 70 ml diethyl ether into 500 mg microalgae powder. After extraction, solvent was removed at 60°C by Hot Air Oven. 1.5 ml methanolic NaOH was added into lipid extract and mixed at 80°C by sonicator for 5 minutes. After cooling at room temperature, 2.0 ml BF3 methanol

was added into the mixture and sonicated for 30 minutes at 80°C. After cooling tubes at room temperature, 1.0 ml isooctane and 5.0 ml saturated NaCl was mixed through well shaking. The fatty-acid methyl-esters (FAMEs) containing upper organic layer was transferred to a new tube and 1.0 ml sample was taken into vial for analysis of fatty acid methyl-esters by Gas Chromatography Mass Spectrophotometry (GC-2020plus, SHIMADZU, Japan). A capillary column (length 30 m, internal diameter 0.25 mm, film thickness 0.15 μm, phase ratio 250) was used to separate FAME. Helium was used as the carrier gas, with a flow rate of 1.42 ml/min. The temperature program for the column was as follows: 180° to 280°C at 5°C/min then held at 280°C. By comparing retention time to a standard, FAMEs were identified (FAME mix C8-C24; Sigma- Aldrich; Germany).

3.9. Statistical Analysis:

MS Excel was used to determine the mean of three replications, standard deviation and standard error. IBM SPSS (v. 26.0) was used for statistical analysis. Significant difference between the treatments in terms of growth parameters, productivity, pigments, and proximate composition was evaluated with One Way ANOVA. Duncan test was applied to determine the homogenous subsets (alpha = 0.05).

Chapter-4: Result

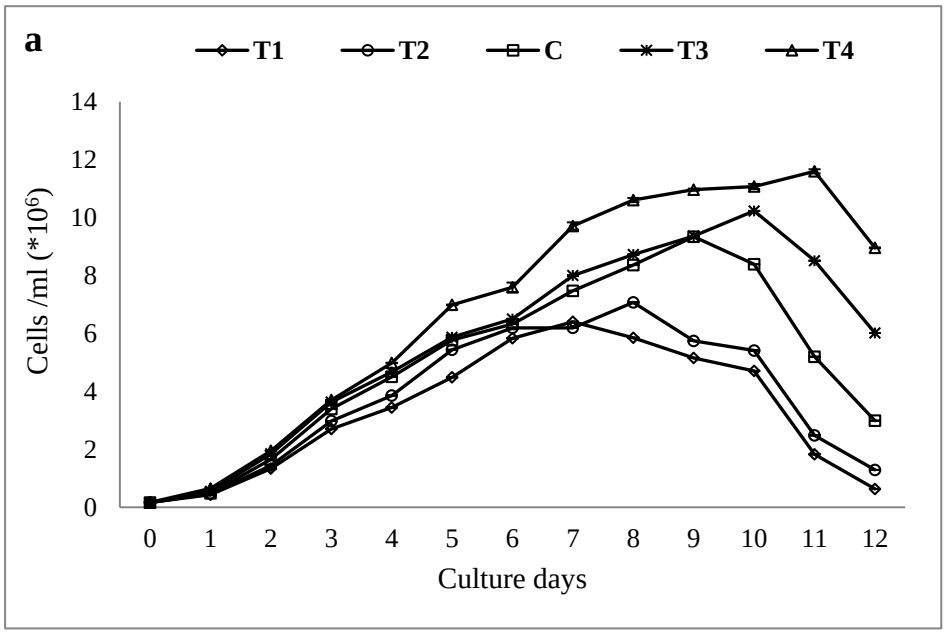
In this study, highest concentration (37 g/L) of $NaNO_3$ resulted maximum cell density (11.6×10^6 cells/ml), biomass productivity (0.58g/L), total chlorophyll, protein (23.58% dry weight) and carbohydrate (27.26% dry weight) significantly ($p<0.05$) compared to control and lower concentration. On the contrary, highest lipid (20.92% dry weight), specific growth rate ($0.521\pm0.01 day^{-1}$), monounsaturated fatty acids (28.53% of total fatty acids) and polyunsaturated fatty acids (67.37% of total fatty acids) were found in the lowest concentration of $NaNO_3$ (13 g/L). In case of carotenoid and total phycobiliprotein content, significantly ($p<0.05$) higher value of 6.43 mg/L and 6.14 mg/g, respectively were produced in control (25 g/L $NaNO_3$) compared to other concentration of $NaNO_3$. Significantly ($p<0.05$) highest value of total saturated fatty acids was found (56.51% of total fatty acids) in 31 g/L concentration of $NaNO_3$.

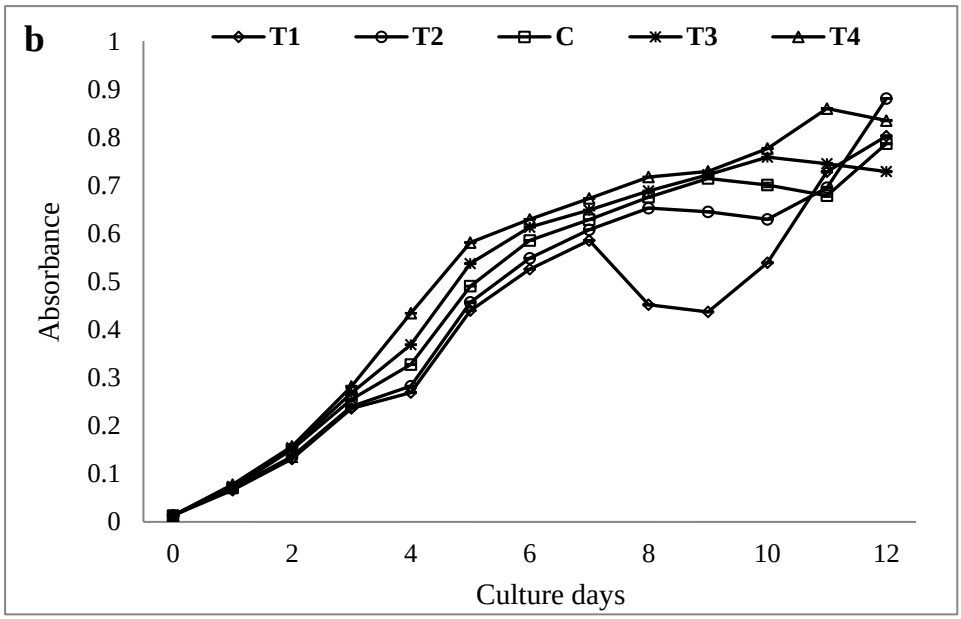
4.1. Growth Curve Analysis:

The effect of different nitrogen concentration on growth of *Scenedesmus* sp. has been shown clearly in Figure 3 based on the growth parameters cell density, optical density, biomass, chlorophyll a and b, which varied significantly ($p<0.05$) from day-1 to day-12, whereas there were no significant difference in day-0 ($p>0.05$). Lag phase remained for day-0 and exponential phase continued from day-1 till stationary phases that varied significantly ($p<0.05$) among the treatments. In case of five treatments stationary phases were found on day-7, 8, 9, 10, 11 respectably in T1 (13g/L $NaNO_3$), T2 (19g/L $NaNO_3$), C (25g/L $NaNO_3$), T3 (31g/L $NaNO_3$), and T4 (37g/L $NaNO_3$), according to all growth parameters. Figure 3(a) shows that the highest cell density 1.16×10^7 cells/ml was obtained under T4 and the lowest cell density 6.41×10^6 cells/ml was obtained under T1 at their stationary phase among all other treatments. On the other hand, C and T3 resulted significantly higher cell density consecutively 9.34×10^6 and 1.02×10^7 cells/ml than T2 (7.08×10^6 cells/ml). Initial cell density was around 1.65×10^5cells/ml for all the treatments.

In Figure 3(b), optical density had been shown in a gradual increment of absorbance 0.585, 0.653, 0.714, 0.758 and 0.835 at the stationary phases in T1, T2, C, T3 and T4, respectively. Biomass has also varied significantly ($p<0.05$) according to nitrogen concentration [Figure 3(c)], where the highest amount of biomass (0.58 g/L) was

produced under T4 having highest nitrogen concentration (37 g/L $NaNO_3$). At the stationary phases, 0.33, 0.38, 0.44, and 0.52 g/L biomass were obtained consecutively from T1, T2, C and T3. So, there was positive relationship between nitrogen concentration and algal biomass of *Scenedesmus* sp.





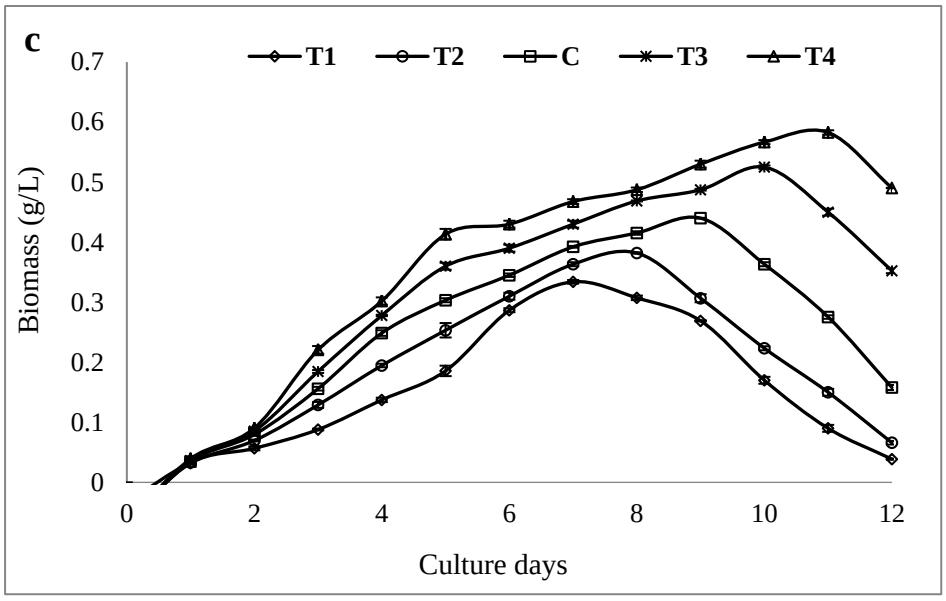


Figure 3. (a) Cell density (cells/ml x 10^6), (b) optical density (Abs) and (c) biomass (g/L dry weight) of freshwater *Scenedesmus* sp. cultured in different nitrogen concentrations. Values are mean ±SE.

4.2. Pigment Analysis:

4.2.1. Chlorophyll a, b:

Significant change in chlorophyll a and b ($p<0.05$) has also been shown in Figure 4 (a, b) in different treatments; moreover, values of chlorophyll a and b had significant strong correlation ($R^2= 0.97$; $p<0.05$) between them. A regular increase in chlorophyll production was observed with increasing $NaNO_3$ concentration among treatments due to continuous cell growth. Maximum chlorophyll a and b was found in 37g/L $NaNO_3$ (T4) treatment, whether minimum chlorophyll was produced in 13g/L $NaNO_3$ (T1) treatment. Chlorophyll a values were sequentially 0.67, 0.72, 0.83, 0.91, and 1.10 mg/L and chlorophyll b values were 0.25, 0.28, 0.33, 0.42, and 0.49 mg/L at the stationary phase of T1, T2, C, T3, and T4, respectively. Among these growth parameters, optical density (R^2 = 0.95), biomass (R^2 = 0.96), chlorophyll a (R^2 = 0.95), and chlorophyll b (R^2 = 0.94), there have highly significant correlation ($p<0.05$) with cell density for all the treatments.

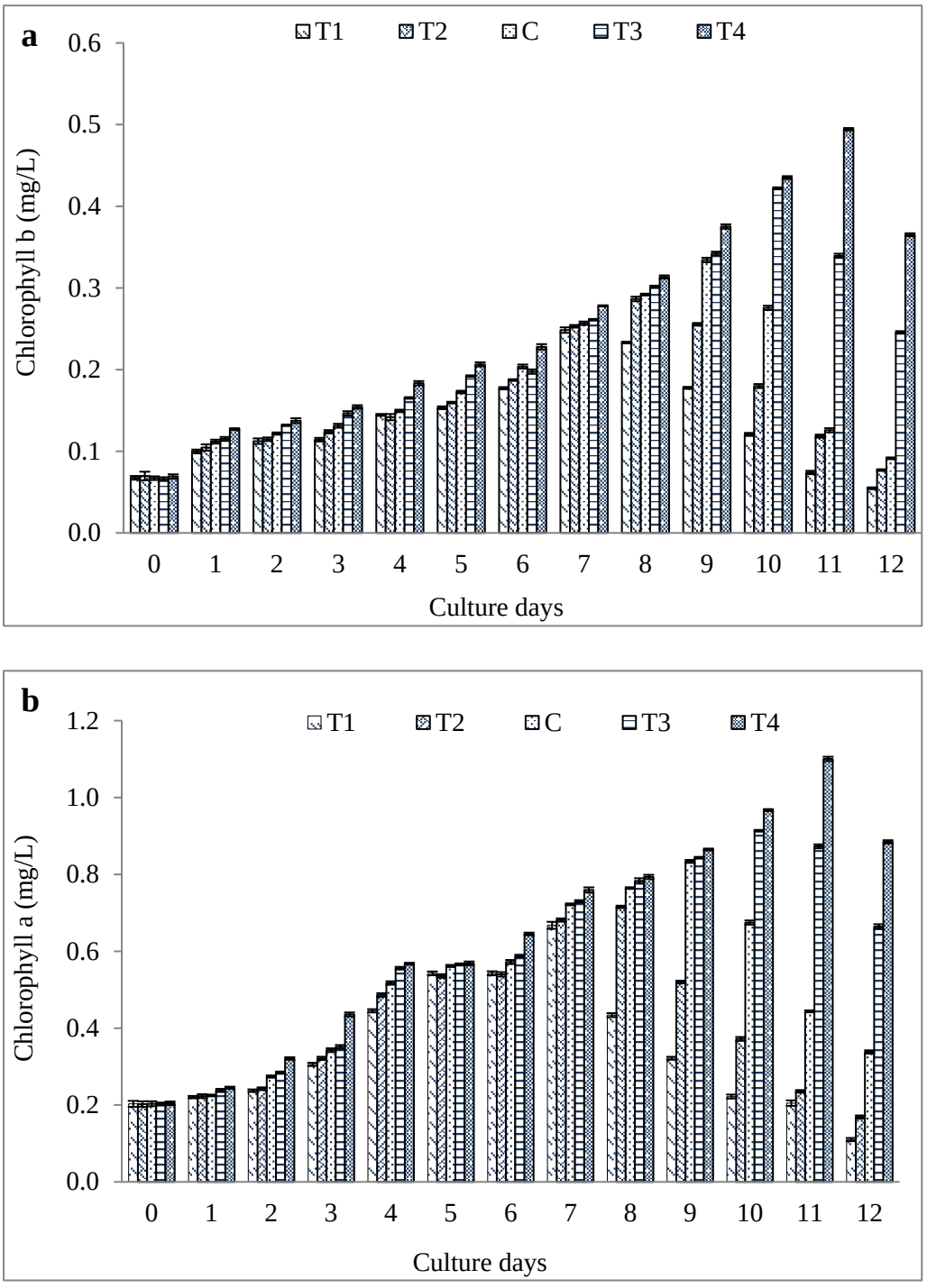


Figure 4. (a) Chlorophyll a content (mg/L) and (b) Chlorophyll b content (mg/L) of freshwater *Scenedesmus* sp. cultured in different treatments of nitrogen concentrations. Values are mean ±SE.

4.2.2. Carotenoid:

Carotenoid content also varied significantly ($p<0.05$) where highest amount of carotenoid (6.43 mg/L) was produced under control treatment and then 4.96 mg/L carotenoid was produced under T4 (Figure 5). The lowest carotenoid content was obtained 3.81 mg/L under T2, the rest treatments T1 and T3 produced 4.75 and 4.71 mg/L carotenoid. There is highly significant difference between C and T2 ($p<0.05$); on the other hand, no significant difference has been found between C and T4 even with T1 ($p>0.05$).

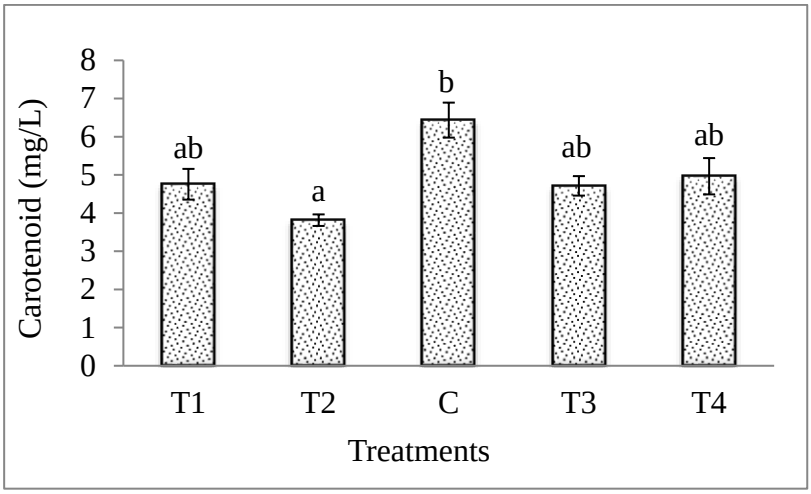


Figure 5. Carotenoid content (mg/L) of freshwater *Scenedesmus* sp. cultured in different nitrogen concentrations. Values are mean ±SE. Different letters with the values indicate significant ($p<0.05$) differences among the treatments.

4.2.3. Phycobiliprotiens:

The mean values of phycobiliprotien, phycocyanin, phycoerythrin and alophycocyanin are significantly different ($p<0.05$) among all the treatments in Table 2. Total phycobiliprotien was the highest 6.14 mg/g in control and the lowest 2.79 mg/g in T4. Significantly higher amount of phycobiliprotien, 5.91 mg/g was produced in T1 than T3 (4.55 mg/g) and T2 (3.95 mg/g). But there is no significant difference between C and T1 ($p>0.05$) in case of total phycobiliprotien, phycocyanin and alophycocyanin. In T1, phycocyanin and alophycocyanin were in the highest amount such as 1.656 and 3.145 mg/g, but phycoerythrin was obtained in the highest amount 1.82 mg/g in control. In T4, phycobiliprotien, phycocyanin, alophycocyanin and

phycoerythrin were produced in comparatively lower amount than other treatments. Although total phycobiliprotien is higher in C than T1 and other treatments, only phycoerythrin is higher in C than T1 (1.108 mg/g) and on the other hand, phycocyanin (1.54 mg/g) and alophycocyanin (2.77 mg/g) are lower in C than T1. Total phycobiliprotien, phycocyanin, phycoerythrin and alophycocyanin were higher in T3 (4.554, 1.211, 0.84, and 2.503 mg/g, respectively) compared to T2 (3.947, 1.187, 0.777, and 1.984 mg/g, respectively).

Table 2: Phycocyanin, alophycocyanin, phycoerythrin and total phycobiliprotein content (mg/g) of freshwater microalgae *Scenedesmus* sp. cultured in different nitrogen concentrations. Values are mean ± SE. Different letters with the values indicate significant ($p<0.05$) differences among the treatments.

Treatments	Phycocyanin (mg/g)	Alophycocyanin (mg/g)	Phycoerythrin (mg/g)	Total Phycobiliprotein (mg/g)
T1	1.656 ±0.016^{d}	3.145 ±0.049^{c}	1.108 ± 0.079^{b}	5.909 ±0.014^{c}
T2	1.187 ±0.096^{b}	1.984 ±0.009^{a}	0.777 ±0.023^{ab}	3.947 ±0.081^{b}
C	1.544 ±0.055^{cd}	2.771 ±0.003^{bc}	1.82 ±0.149^{c}	6.136 ±0.201^{c}
T3	1.211 ±0.062^{bc}	2.503 ±0.097^{b}	0.84 ±0.067^{ab}	4.554 ±0.226^{b}
T4	0.681 ± 0.05^{a}	1.622 ± 0.122^{a}	0.492 ±0.055^{a}	2.795 ±0.227^{a}

4.3. Productivity Assessment:

Change in productivity, specific growth rate and cell doubling time of *Scenedesmus* sp. under different treatments has been shown in Table 3. Volumetric and areal productivity among the treatments are not significantly different ($p>0.05$), but the lipid productivity, SGR and cell doubling time are significantly different among the treatments ($p<0.05$). T3 and T4 resulted similar and highest volumetric productivity, where lowest (0.046 gL^{-1} day^{-1}) volumetric productivity was obtained in T2. Areal productivity was the highest (5.41 mg $cm^{-2}day^{-1}$) in T4, and the lowest (4.6 mg $cm^{-2}day^{-1}$) in T2 and 5.38, 5.07, 5.01 mg $cm^{-2}day^{-1}$ in T3, C, T1 respectively. Lipid productivity was highest 10.559 mg L^{-1} day^{-1} in very low nitrogen concentration (T1) and lowest 6.712 mg $L^{-1}day^{-1}$ in control. In treatment T4, T3, and T2, lipid productivity was higher (8.58, 7.96, 6.89 mg L^{-1} day^{-1}) than treatment C. There was significant difference between C and T1 ($p<0.05$).

Higher SGR values were obtained in T1 (0.521 day^{-1}) than sequentially T2, C, T3, and T4 (0.469, 0.451, 0.411, 0.385 day^{-1}, respectively). There were significant difference between C and T1, T3, T4 except T2 ($p>0.05$). Cell doubling time is higher in T4 (1.8 day) than other treatments; apart from this there was no significant difference between C and T2 ($P>0.05$). There were medium negative correlation ($R^2= 0.12$) between SGR and volumetric as well as areal productivity ($p> 0.05$).

Table 3. Change in productivity of *Scenedesmus* sp. cultured in different nitrogen concentrations. Values are mean ± SE. Different letters with the values indicate significant ($p<0.05$) differences among the treatments.

Parameters	T1	T2	C	T3	T4
Volumetric Productivity ($gL^{-1}day^{-1}$)	0.05 ±0.01[a]	0.046 ±0.01[a]	0.051 ±0.01[a]	0.054 ±0.01[a]	0.054 ±0.01[a]
Areal Productivity ($mgcm^{-2}day^{-1}$)	5.01 ±0.77[a]	4.62 ±0.66[a]	5.07 ±0.59[a]	5.38 ±0.49[a]	5.41 ±0.52[a]
Lipid Productivity ($mgL^{-1}day^{-1}$)	10.56 ±2.36[b]	6.89 ±0.76[a]	6.71 ±0.65[a]	7.96 ±0.68[ab]	8.58 ±0.89[ab]
Specific Growth Rate, μ (day^{-1})	0.52 ±0.01[d]	0.47 ±0.01[c]	0.45 ±0.01[c]	0.41 ±0.01[b]	0.39 ±0.01[a]
Cell doubling time, td (day)	1.33 ±0.03[a]	1.48 ±0.01[b]	1.54 ±0.05[b]	1.69 ±0.03[c]	1.80 ±0.03[d]

4.4. Proximate Composition Analysis:

Figure 6 represents the change of protein, lipid and carbohydrate content of freshwater microalgae *Scenedesmus* sp. for all the treatments T1, T2, C, T3 and T4. The highest protein and carbohydrate content (23.17 and 27.26 % dry weight, respectively) were produced in T4 and the highest lipid content (20.92% dry weight) was obtained in T1. There has highly significant difference in protein, lipid and carbohydrate of *Scenedesmus* sp. according to different nitrogen concentrations ($p<0.05$). Higher nitrogenous treatments resulted higher protein percentage. The protein content was 23.17, 22.47, 22.29, 21.96% and 19.66% dry weight sequentially in T4, T3, C, T2 and T1. Highest lipid percentage (20.92% dry weight) was obtained in T1, on the other side; lowest (13.27% dry weight) was in control treatment. Lipid

percentage was significantly higher ($p<0.05$) in T4 (15.87% dry weight) than T2 (14.98% dry weight) and T3 (14.81% dry weight). In case of lipid production, there remains highly significant difference between C and T1 as well as have also significant difference between C and T4 ($p<0.05$). There is also significant difference between T1 and T4 ($p<0.05$), but no significant difference have been found between C and T2 as well as T3 ($p>0.05$).

The amount of carbohydrate was produced 21.38% dry weight in C; whereas 27.26 and 24.37% dry weight carbohydrate was produced in T4 and T3 where nitrogen concentration was higher in culture media. 17.48% and 22.70% dry weight carbohydrate were produced in T1 and T2 respectively. Carbohydrate was produced more under treatment successively T4, T3, T2 than C.

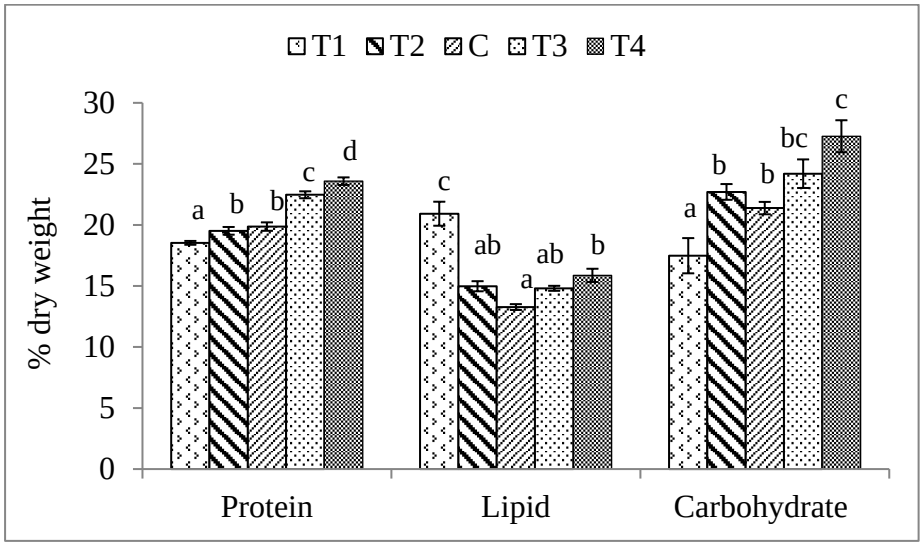


Figure 6. Proximate composition (% dry weight) of freshwater *Scenedesmus* sp. cultured in different nitrogen concentrations. Values are mean ±SE. Different letters with the values indicate significant ($p<0.05$) differences among the treatments.

4.5. Fatty Acid Analysis:

The result of fatty acid methyl esters (FAMEs) composition analysis with GCMS is given in following Table 4. There were 21 compounds identified as fatty acid methyl esters consisting of 9 saturated fatty acids, 5 monounsaturated fatty acids and 7 poly unsaturated fatty acids.

Table 4: Fatty acids content (% of the total fatty acids) of *Scenedesmus* sp. cultured in different nitrogen concentrations. Values are mean ± SE.

Carbon	Fatty acid	T1	T2	C	T3	T4
Saturated fatty acids						
C8:0	Octanoic acid	0.011± 0.01	0.08 ± 0.00	0.01± 0.01	0.018 ± 0.02	0.019 ± 0.01
C10:0	Decanoic acid	0.008 ± 0.00	0.01 ± 0.01	0.002 ± 0.00	0.002 ± 0.00	0.002 ± 0.00
C12:0	Lauric acid	0.18 ± 0.04	1.13 ± 0.2	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00
C13:0	Tridecanoic acid	0.18 ± 0.04	0.87 ± 0.11	0.04 ± 0.01	0.16 ± 0.06	0.03 ± 0.01
C14:0	Myristic acid	0.51 ± 0.08	0.01± 0.00	0.012 ± 0.01	0.06 ± 0.00	0.07 ± 0.01
C16:0	Palmitic acid	3.04 ± 0.19	3.54 ± 0.8	8 ± 0.70	7.3 ± 0.27	7.2 ± 0.42
C18:0	Stearic acid	1.91 ± 0.32	7.2 ± 10	4.72 ± 1.02	5.75 ± 0.08	5.4 ± 0.40
C20:0	Arachidic acid	1.33 ± 0.30	5.7 ± 0.73	12.84 ± 1.22	11.96 ± 1.37	4.32 ± 0.25
C17:0	Heptadeca-noic acid	0.172 ± 0.03	0.07 ± 0.02	0.04 ± 0.02	0.1 ± 0.01	0.08 ± 0.00
C21:0	Heneicosa-noic acid	1.131 ± 0.07	11 ± 1.00	21 ± 1.04	31.14 ± 0.08	25.58 ± 0.60
C22:0	Behenic acid	0.22 ± 0.11	-----	0.2 ± 0.10	0.1 ± 0.05	0.16 ± 0.01
Monounsaturated fatty acids						
C16:1	Palmitoleic acid	1.68 ± 0.07	7.68 ± 0.20	18.78 ± 0.63	13.27± 0.5	18.32 ± 0.78
C18:1	Oleic acid	2.1 ± 0.80	1.32 ± 0.30	0.16 ± 0.06	2 ± 0.40	0.014 ± 0.00
C20:1	cis-11-Eicosenoic acid	0.28 ± 0.01	0.11± 0.2	0.014 ± 0.00	0.01± 0.02	0.016 ± 0.01
C22:1	Erucic acid	24.48 ± 2.06	0.134 ± 0.01	3.34 ± 0.44	4.82 ± 0.21	7.63 ± 0.70
C24:1	Nervonic acid	0.002 ± 0.00	0.02 ± 0.01	0.01 ± 0.00	0.013 ± 0.0	0.004 ± 0.00
Polyunsaturated fatty acids						
C18:2n-6	Linoleic acid	62.23 ± 0.09	54.81±2.70	15.91± 0.94	13.87± 0.004	18.13 ± 0.84
C20:3n-6	Eicosatrienoic acid	1.54 ± 0.104	0.024 ±0.02	2.41± 0.11	1.16 ± 0.64	2.05 ± 0.19
C20:4n-6	Arachidonic acid	0.04 ± 0.02	0.2 ± 0.07	0.08 ± 0.04	0.044 ±0.001	0.62 ± 0.13
C18:3n-3	Linolenic acid	1.4 ± 0.37	5.52 ± 0.42	8.81± 0.55	7.49 ± 0.03	6.3 ± 0.01
C20:5n-3	Eicosapenta-	1.45 ± 0.00	0.37 ± 0.02	3.6 ± 0.65	3 ± 0.34	3.89 ± 0.17

	noic acid					
C22:5n-3	Docosa-pentaenoic acid	0.007 ± 0.00	0.28 ± 0.06	0.09 ±0.005	0.045 ± 0.02	0.094 ± 0.03
C22:6n-3	Docosa-hexaenoic acid	0.69 ± 0.13	0.06 ± 0.01	-----	-----	-----

Figure 7 shows that poly unsaturated fatty acids (PUFAs) percentage and n-6 fatty acids percentage were highest in T1 as 67.37 ± 0.72% and 63.81 ± 0.21% of total fatty acids, followed by T2 which were 61.27 ± 3.29% and 55.03 ± 2.78% of total fatty acids respectively. The lowest PUFAs and n-6 fatty acids percentage were found under T3, but T4 resulted comparatively higher than C.

Highly unsaturated fatty acids (HUFAs) were highest (6.67 ± 0.52% of total fatty acids) in T4 and lowest (0.94 ± 0.18% of total fatty acids) in T2. There was significant difference among the treatments for n-3 fatty acids ($p<0.05$) which was highest 12.5 ± 1.2% of total fatty acids in control treatment, on the other side, lowest 3.6 ± 0.5% of total fatty acids in T1. Mono unsaturated fatty acids (MUFAs) were maximum 28.53 ± 2.94% of total fatty acids under T1, whereas minimum 9.27 ± 0.54% of total fatty acids under T2. In T4 (26 ± 1.45% of total fatty acids), MUFAs percentage was comparatively higher than C (22.31 ± 1.14% of total fatty acids) and T3 (20.1 ± 1.19% of total fatty acids). The highest value of total saturated fatty acids (SAFA) was found 56.51 ± 1.92% of total fatty acids in T3; contrarily lowest concentration was 8.68 ± 1.19% of total fatty acids in T1.

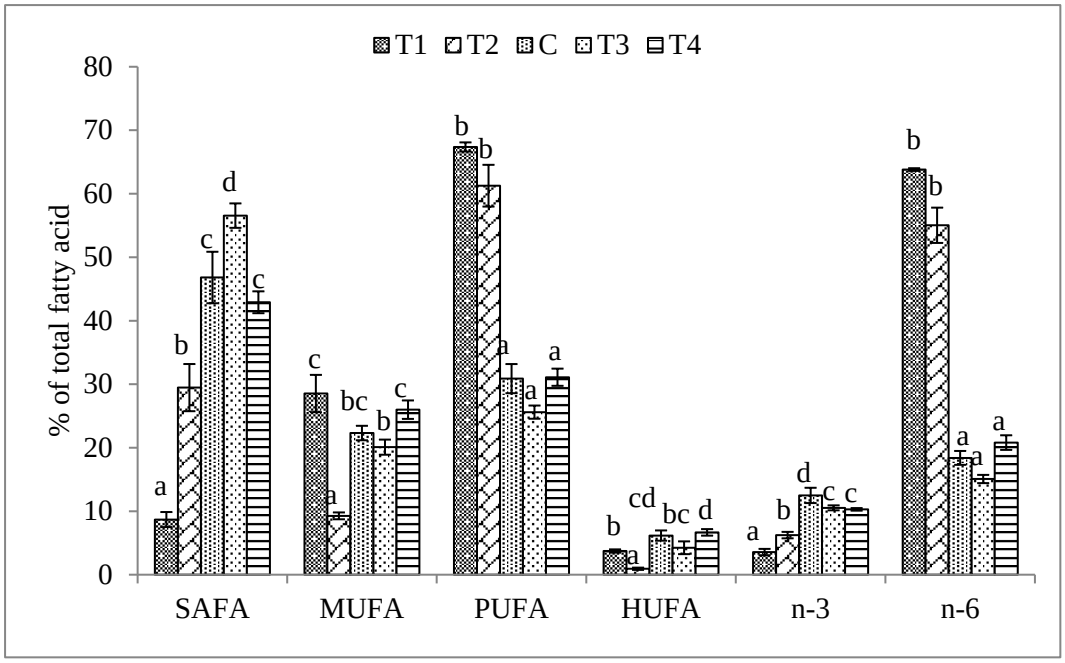


Figure 7. Fatty acids' composition (% total fatty acids) of freshwater *Scenedesmus* sp. cultured in different nitrogen concentrations. Values are mean ±SE. Different letters with the values indicate significant ($p<0.05$) differences among the treatments. *{SAFA- Saturated Fatty Acids, MUFA- Mono Unsaturated Fatty Acids, PUFA- Poly Unsaturated Fatty Acids, HUFA- Highly Unsaturated Fatty Acids, n3- Omega 3 Fatty Acids, n6- Omega 6 Fatty Acids.}

Chapter- 5: Discussion

5.1. Microalgal Cell Growth:

Different $NaNO_3$ concentrations in the Bold Basal Media had a significant effect on microalgae growth, biochemical properties as well as culture duration. Nitrogen's effect on the growth of *Scenedesmus* sp. has been shown clearly in Figure 3 and Figure 4 based on growth parameters such as cell density, optical density, biomass, chlorophyll a and b. In case of five treatments such as 13 g/L $NaNO_3$ (T1), 19 g/L $NaNO_3$ (T2), 25 g/L (control), 31 g/L $NaNO_3$ (T3), and 37 g/L $NaNO_3$ (T4), stationary phases were found on day-7,8,9,10, 11, respectively according to all growth parameters.

Figure 3a showed that the significantly ($p<0.05$) highest cell density 1.16×10^7 cells/ml was obtained under T4 among all other treatments and the lowest cell density 6.41×10^6 cells/ml was obtained under T1 at their stationary phase. Zarrinmehr et al. (2020) reported that minimum and maximum cell concentration of *Isochrysis galbana* were achieved in 36 and 144 mg/L $NaNO_3$ respectively in Walne's medium. *Scenedesmus obliquus* growth was promoted by daily addition of 2 mg N L^{-1} of CH_4N_2O in M-11 culture medium yielded the highest cell density 1.7×10^7 cells mL^{-1} after 20^{th} day of culture period (An et al., 2020). It can be justified as higher nitrogen concentration promotes the algal biomass production, but lower nitrogen concentration has remarkable effect on retarding microalgae growth (Li et al., 2013; Zarrinmehr et al., 2020).

A regular change in optical density over the cultivation period till stationary phases for all the treatments has been shown in Figure 3b, where minimum 0.585 ± 0.001 absorbance was observed in T1 and maximum 0.835 ± 0.001 absorbance in T4 on stationary phases day-7 and day-11 respectively. Biomass had also varied significantly ($p<0.05$) according to the nitrogen concentration (Figure 3c). The lowest (0.33 ± 0.003 g/L) biomass was produced in T1 on day-7 and highest (0.58 ± 0.004 g/L) in T4 on day-11. So, increasing concentration of $NaNO_3$ had positive effect on increasing cell growth. Similar to this result, Yang et al. (2008) reported that biomass production in *Chlamydomonas reinhardtii* was subdued up to 31.7% under nitrogen

deficiency. Nitrogen limited conditions of microalgae culture has led to a significant decrease in the cell density, biomass productivity and chlorophyll synthesis (Dean et al., 2010; Nigam et al., 2011).

5.2. Pigments:

5.2.1. Chlorophyll a, b:

A gradual rise was observed in chlorophyll production from the lower nitrogenous treatment to the higher nitrogenous treatment due to continuous increment of cell density in Figure 4 (a, b). Chlorophyll a values were amplified successively 0.66 ± 0.009 to 1.1 ± 0.005 mg/L and chlorophyll b values were 0.24 ± 0.003 to 0.49 ± 0.001 mg/L from minimum to maximum nitrogenous treatment. Zarrinmehr et al. (2020) also found that chlorophyll a and chlorophyll b reduced with diminishing nitrogen concentration from 144 to 0 mg/L in *Isochrysis galbana*. According to Xie et al. (2017), decreasing nitrogen concentration (3 mmol/L from 5mmol/L $NaNO_3$) was a restriction factor for *Cholrella vulgaris* growth and chlorophyll production. Zhang and Liu (2016) observed that total chlorophyll content in *I. galbana* decreased from 5.9 to 3.9 mg/L under nitrogen deprivation (32 g/L to 12 g/L $NaNO_3$). Nitrogen starvation lessens efficiency of photo system II activity and hampers the photosynthetic pigment synthesis as a restriction factor (Ferreira et al., 2015; Zhang and Liu, 2016).

5.2.2. Carotenoid:

The highest amount of carotenoid was produced in control treatment and lowest carotenoid content was obtained in T2. 4.75 ± 0.4 mg/L carotenoid was produced under the least nitrogen concentration 13 g/L $NaNO_3$ which is considerably higher amount as nitrogen limitation influences carotenoid production in the cell (Juneja et al., 2013). Similar to the result of recent study, Zarrinmehr et al., (2020) also found that the minimum and maximum total carotenoid content were achieved in 36 and 72 mg/L nitrogen, as 1 and 1.21 mg/L, respectively; furthermore, lower carotenoid concentration 0.9 mg/L was obtained in 144 mg/L nitrogen. Change in nitrogen concentration from standard concentration of Bold Basal Media may cause stress on cell metabolism which interferes on carotenoid accumulation (Juneja et al., 2013). On the contrary, Menegol et al. (2017) reported that 1.5-fold higher carotenoid content of the biomass (2.47 mg g^{-1}) was resulted in *Heterochlorella luteoviridis* culture with the highest nitrogen concentration (60 mgL^{-1} $N\text{-}NO_3$) than standard medium (12 mgL^{-1} $N\text{-}NO_3$). As *Heterochlorella luteoviridis* was cultured in photobioreactor at 28°C in f/2

medium, but freshwater *Scenedesmus* sp. was cultured at 25°C in Bold Basal medium. So, this difference in culture condition may affect the carotenoid production in microalgae.

5.2.3. Phycobiliproteins:

Total phycobiliprotein was significantly ($p<0.05$) higher in control treatment and the lowest in T4 (Table 2). Almost similar amount of phycobiliprotein was recorded in T1 and C which is contradictory with nitrogen deficiency led to the decline of photosynthetic performance by reducing production of photosynthetic pigments such as chlorophyll and phycobiliproteins (Zhao et al., 2017). As phycobiliprotein is an accessory pigment for light harvesting in photosynthesis, lower chlorophyll production and photosynthetic activity tends to increase in phycobiliprotein in T1 compared to standard nitrogen concentration. Phycocyanin and alophycocyanin were highest in T1, but highest amount of phycoerythrin was obtained in control treatment. Shifting nitrogen concentration from standard concentration may interfere in enzymatic activity of pigment production and photosynthesis (Juneja et al., 2013).

5.3. Productivity:

Volumetric and areal productivity among the treatments are not significantly different ($p>0.05$), but the lipid productivity, SGR and cell doubling time have significant difference among the treatments ($p<0.05$) (Table 3). Volumetric and areal productivity were highest in high nitrogen concentration T4. The findings of the current result was supported by Zhu et al. (2014) who demonstrated that maximum biomass productivity was obtained in higher nitrogen concentration. Lipid productivity was highest in very low nitrogen concentration (T1) and lowest in control.

Nitrogen stress has positive response in case of lipid production, as change in standard nitrogen concentration may cause hectic culture environment that result in higher lipid production rate. Compared to adequate nitrogenous medium, two fold lipids were produced in nitrogen deficient culture conditions (Pruvost et al., 2011; Jia et al., 2015). Maximum specific growth rate was obtained in least nitrogen concentration, T1 and minimum in highest nitrogen concentration, T4. Cell doubling time was reduced in lower nitrogen concentration and increased with the higher nitrogen concentration. Table 2 shows positive relation between specific growth rate and cell

doubling time as lower cell doubling time aided in higher specific growth rate. Like the present result, Kendırlıoglu et al. (2015); Saha et al. (2013) stated that cell growth rate was greater in cultures subjected to lower nitrogen concentration.

5.4. Proximate Composition:

Figure 6 presents the effect of gradient concentration of nitrogen on the protein, lipid and carbohydrate content of *Scenedesmus* sp. There was highly significant difference in protein, lipid and carbohydrate content of *Scenedesmus* sp. according to nitrogen stress ($p<0.05$). Gradual increment of protein production was found from lower nitrogenous treatment to higher nitrogenous treatment. Maximum protein content 23.58 ± 0.31 % dry weight was produced under T4 and minimum 18.53 ± 0.16 % dry weight was produced under T1. Compared to the control treatment, lipid percentage was significantly higher in nitrogen stress treatments (T1, T4, T2 and T3) as alteration of nitrogen concentration from standard concentration creates stress condition (Chu et al., 2013).

Protein percentage increased gradually according to increment of nitrogen concentration as nitrogen is a major component for the biosynthesis of nitrogenous organic compounds like protein, chlorophyll and nucleic acids (Saha et al., 2003; Simionato et al., 2013). Menegol et al. (2017) attained 45% higher protein content (138 mg g^{-1}) in *Heterochlorella luteoviridis* cultivated with 60 mg L^{-1} $N\text{-}NO_3$ nitrogen concentration than 12 mg L^{-1} $N\text{-}NO_3$. Jia et al. (2015) reported that two fold lipid was produced by nitrogen deficient culture condition than nitrogen sufficient medium.

As storage products lipid and carbohydrate accumulation in microalgae is caused by nitrogen starvation, which is also accompanied by degradation of cellular nitrogenous compounds, such as nucleic acid, proteins, and photosynthetic pigments, Ch-a, Ch-b, and carotenoids (Pruvost et al., 2011). Due to lack of sufficient nitrogen concentration in microalgae culture, most of the carbon fixed in photosynthesis is used in the lipid synthesis instead of protein. This change in lipid metabolic pathway influences the accumulation of neutral lipid including triacylglycerides (TAGs) in the cytoplasm of microalgae as a source of carbon and energy (Yang et al., 2008; Li et al., 2012; Chu et al., 2013).

The highest 27.26 ± 1.31% dry weight carbohydrate was produced under T4 and lowest 17.48 ± 1.44 % dry weight in T1. More carbohydrate was produced under cultivation with higher nitrogen concentration and gradual decrease was found in

lower nitrogen concentration except T2. A previous study also found equivalent outcome with this present study that is lower carbohydrate yield (18.75 ± 0.59%) in *Tetradesmus obliquus* was obtained after 14 days at nitrogen stress condition, compared to the control (22.04 ± 0.61%). Nitrogen stress condition induced the accumulation of carbohydrate at early stage but started to reduce on day 4 when the carbon shifted towards lipid production (Nadzir et al., 2021). Li et al. (2011) stated that, after 2 days of nitrogen stress condition, the photosynthetically assimilated carbon flux of *Pseudochlorococcum* sp. was turned into fatty acid and neutral lipid synthesis in lieu of carbohydrate synthesis.

5.5. Fatty Acid Composition:

The lowest PUFAs and n-6 fatty acids percentage were found under T3, but T4 resulted comparatively higher than control treatment. Highly unsaturated fatty acids (HUFAs) were highest in T4 and lowest in T2. There was significant difference among the treatments for n-3 fatty acids ($p<0.05$) which was highest in control treatment, on the other side, lowest in T1. Mono unsaturated fatty acids (MUFAs) were maximum in T1, whereas minimum in T2. The highest value of total saturated fatty acids (SAFA) was found in T3; contrarily lowest concentration was in T1. Higher nitrogen concentration compared to standard concentration increased production of saturated fatty acids which are potential for biofuel production.

In support of the present study, Kudahettige et al. (2018) also found that the relative percentage of unsaturated fatty acids were higher in *S. dimorphus* than saturated fatty acids in the culture media of lowest nitrogen content. Cheng and He (2014) reported that microalgae growing under nutrient stress conditions could change its metabolic strategies and biochemical composition. Zarrinmehr et al., (2020); Shen et al., (2016) support the results of this study regarding increasing of C18:1 as well as decreasing of C18:3 amount under nitrogen deficiency. Poly unsaturated fatty acids PUFA content of *I. galbana* decreased into 41.48% of total fatty acids in sufficient nutrient concentration (Foo et al., 2017). Microalgae growing under nutrient stress conditions could change its metabolic strategies and biochemical composition (Juneja et al., 2013).

Chapter-6: Conclusions

In this experiment, the response of *Scenedesmus* sp. under different nitrogen concentration was evaluated compared to standard nitrogen concentration of Bold Basal Media on the basis of cell growth, photosynthetic pigment chlorophyll and phycobilliprotein, carotenoid, proximate composition and fatty acid composition. Higher nitrogen concentration had greater impact on increment of biomass productivity, photosynthetic chlorophyll concentration, protein and carbohydrate percentage, 37 g/L $NaNO_3$ concentration resulted in highest value. Although lower nitrogen concentration had significant effect on enhancing lipid, monounsaturated and polyunsaturated fatty acid percentage, as 13 g/L $NaNO_3$ caused in highest value. The results of this study will be helpful to boost the production and improve the nutraceuticals as well as pharmaceutical attributes of the freshwater microalgae *Scenedesmus* sp. through mass cultivation with appropriate nitrogen concentration. Fish farm, microalgae cultivator, fish hatchery, fish feed industry, pharmaceutical industry, and microalgae researchers will be benefited from this dataset. Based on this dataset, it is possible to improve the nutritional profile of the fish diet formulated by *Scenedesmus* sp. and explore the antimicrobial properties to develop drug. Therefore, it can be concluded that this result will enrich the potentiality of *Scenedesmus* sp. in aquaculture industry, biofuel production worldwide and pave the way for future research as well.

Chapter- 7: Recommendation and Future perspectives

The purpose of this study was to observe the effect of different nitrogen concentration on cell growth, photosynthetic pigment chlorophyll and phycobilliprotein, carotenoid, proximate composition and fatty acid composition of *Scenedesmus* sp. Although a qualitative approach was followed to explore the objective of the research, there some limitation which can be alleviated through following recommendations:

- Preparation of amino acids' profile.
- Specification of *Scenedesmus* sp. by molecular identification before and after culture to detect the molecular change in the species.
- Trial of outdoor culture to assess the potentiality of commercial mass culture.
- Determination of nitrogen uptake rate to show a relation with proximate and biochemical composition, which also will help to optimize the nitrogen concentration.
- Assessment of antioxidant properties of microalgae under different treatments.

However, future perspective of this study may include the followings:

- Detection of antioxidants and antibacterial properties of *Scenedesmus* sp. cultured under different nitrogen concentrations as biochemical properties are changed significantly known from this research.
- Mass culture of *Scenedesmus* sp. for nutritious product development for fish, animal and human being.
- Feeding trial over fish through zooplankton fed by *Scenedesmus* sp. cultured with optimized nitrogen concentration.
- Assessment of cost effectiveness for commercial culture practice of *Scenedesmus* sp. and fish culture.

References

Abadia J. 1986. Function of iron in chloroplasts. Journal of Plant Nutrition. 9 (3-7): 609-646.

Angles E, Jaouen P, Pruvost J, Marchal L. 2017. Wet lipid extraction from the microalga *Nannochloropsis* sp.: Disruption, physiological effects and solvent screening. Algal Research. 21 (4): 27-34.

An M, Gao L, Zhao W, Chen W, Li M. 2020. Effects of nitrogen forms and supply mode on lipid. Energies. 13 (3): 691-697.

Aravantinou AF, Marios A, Theodorakopoulos MID. 2013. Phytoremediation potential of bioenergy plants. Bioresource Technology. 147 (3): 124- 130.

Aremu AO, Masondo NA, Molnár Z, Stirk WA, Ördög V, Staden JV. 2016. Changes in phytochemical content and pharmacological activities of three *Chlorella* strains grown in different nitrogen conditions. Journal of Applied Phycology. 28 (1): 149-159.

Arguelles E. 2018. Proximate analysis, antibacterial activity, total phenolic content and antioxidant capacity of a green microalga *Scenedesmus quadricauda* (Turpin) Brébisson. Asian Journal of Microbiology, Biotechnology and Environmental Sciences. 20 (1): 150-158.

Barsanti PGL. 2006. Algae: Anatomy, Biochemistry, and Biotechnology. 2nd ed.

Ben-Amotz A, Avron M. 1983. On the factors which determine massive β-Carotene accumulation in the halotolerant alga *Dunaliella bardawil*. Plant Physiology. 72 (3): 593-597.

Benemann JR, Tillett DM. 1987. Microalgae lipid production. Energy from biomass and waste XI. Conference Proceeding, Institute of Gas Technology.

Bligh EG, Dyer WJ. 1959. A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology. 37 (8): 911-917.

Borowitzka MA. 1998. Vitamins and fine chemicals from microalgae. In: Borowitzka MA, Borowitzka LJ, editors. Microalgal Biotechnology. Cambridge University Press, Cambridge. pp. 153-196.

Breuer G, Lamers PP, Martens DE, Draaisma RB, Wijffels RH. 2012. The impact of nitrogen starvation on the dynamics of triacylglycerol accumulation in nine microalgae strains. Bioresource Technology. 124: 217-226.

Brow MR. 2002. Nutritional value of microalgae for aquculture. In: Avances en NutriciónAcuícola VI. Memorias del VI Simposium Internacional de NutriciónAcuícola. Cruz-Suárez LE, Ricque-Marie D, Tapia-Salazar M, Gaxiola-Cortés MG, Simoes N, editors. Cancún, Quintana Roo, México.

Bruland KW, Donat JR, Hutchins DA. 1991. Interactive influences of bioactive trace metals on biological production in oceanic waters. Limnology and Oceanography. 36 (8): 1555-1577.

Burtin P. 2003. Nutritional value of seaweeds. The Electronic Journal of Environmental, Agricultural, and Food Chemistry. 2 (4): 498-503. https://www.researchgate.net/publication/228554296_Nutritional_value_of_seaweeds.

Çakmak ZE, Ölmez TT, Çakmak T, Menemen Y, Tekinay T. 2015. Antioxidant response of *Chlamydomonas reinhardtii* grown under different element regimes. Phycological Research. 63 (3): 202-211.

Cheng D, He Q. 2014. Assessment of environmental stresses for enhanced microalgal biofuel production: an overview. Frontiers in Energy Research. 2 (1): 1-8.

Chinnasamy S, Ramakrishnan B, Bhatnagar A, Das KC. 2009. Biomass production potential of a wastewater alga *Chlorella vulgaris* ARC 1 under elevated levels of CO_2 and temperature. International Journal of Molecular Sciences. 10 (2): 518-532.

Chisti Y. 2007. Biodiesel from microalgae. Biotechnology Advances. 25 (3): 294-306.

Chisti Y. 2008. Biodiesel from microalgae beats bioethanol. Trends in Biotechnology. 26 (3): 126-133.

Chokshi K, Pancha I, Ghosh A, Mishra S. 2017. Nitrogen starvation-induced cellular crosstalk of ROS-scavenging antioxidants and phytohormone enhanced the

biofuel potential of green microalga *Acutodesmus dimorphus*. Biotechnology for Biofuels. 10 (1): 1-12.

Chu FF, Chu PN, Cai PJ, Li WW, Lam PKS, Zeng RJ. 2013. Phosphorus plays an important role in enhancing biodiesel productivity of *Chlorella vulgaris* under nitrogen deficiency. Bioresource Technology. 134 (1): 341-346.

Converti A, Casazza AA, Ortiz EY, Perego P, Borghi MD. 2009. Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production. Chemical Engineering and Processing. 48 (6): 1146-1151.

Courchesne NMD, Parisien A, Wang B, Lan CQ. 2009. Enhancement of lipid production using biochemical, genetic and transcription factor engineering approaches. Journal of Biotechnology. 141 (1-2): 31-41.

Cuellar-Bermudez SP, Aguilar-Hernandez I, Cardenas-Chavez DL, Ornelas-Soto N, Romero-Ogawa MA, Parra-Saldivar R. 2015. Extraction and purification of high-value metabolites from microalgae: essential lipids, astaxanthin and phycobiliproteins. Microbial Biotechnology. 8 (2): 190-209.

da Silva TL, Reis A, Medeiros R, Oliveira AC, Gouveia L. 2009. Oil production towards biofuel from autotrophic microalgae semicontinuous cultivations monitorized by flow cytometry. Applied Biochemistry and Biotechnology. 159 (2): 568-578.

Dayananda C, Sarada R, Rani MU, Shamala TR, Ravishankar GA. 2007. Autotrophic cultivation of *Botryococcus braunii* for the production of hydrocarbons and exopolysaccharide in various media. Biomass and Bioenergy. 31 (1): 87-93.

Dean AP, Sigee DC, Pittman BEJK. 2010. Using FTIR spectroscopy for rapid determination of lipid accumulation in response to nitrogen limitation in freshwater microalgae. Bioresource Technology. 101 (12): 4499-4507.

de Carvalho JC, Sydney EB, Tessari LFA, Soccol CR. 2019, Chapter 2: Culture media for mass production of microalgae. In: Pandey A, Chang JS, Soccol CR, Lee DJ, Chisti Y, editors. Biomass, Biofuels, Biochemicals, Biofuels from Algae. 2nd ed. Elsevier. pp. 33-50.

Devi MP, Subhash GV, Mohan SV. 2012. Heterotrophic cultivation of mixed microalgae for lipid accumulation and wastewater treatment during sequential growth and starvation phase's effect of nutrient supplementation. Renewable Energy. 43: 276-283.

D'Souza FML, Kelly GJ. 2000. Effects of a diet of a nitrogen-limited alga (*Tetraselmis suecica*) on growth, survival and biochemical composition of tiger prawn (*Penaeus semisulcatus*) larvae. Aquaculture. 181 (3-4): 311-329.

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. 1956. Colorimetric method for determination of sugars and related substances. Analytical Chemistry. 28 (3): 350- 356.

Duong VT, Ahmed F, Thomas-Hall SR, Quigley S, Nowak E, Schenk PM. 2015. High protein- and high lipid-producing microalgae from northern Australia as potential feedstock for animal feed and biodiesel. Frontiers in Bioengineering and Biotechnology. 3 (1): 53-58.

El-Baky HHA, El-Baroty GS, Ibrahim EA. 2014. Antiproliferation and antioxidant properties of lipid extracts of the microalgae *Scenedesmus obliquus* grown under stress conditions. Der Pharma Chemica. 14 (3): 24- 34.

El-Kassas HY. 2013. Growth and fatty acid profile of the marine microalga *Picochlorum* sp. grown under nutrient stress conditions. Egyptian Journal of Aquatic Research. 39 (4): 233-239.

El-Moneim A, Afify MR, El Baroty GS, El-Baz FK, El-Baky HHA, Murad SA. 2018. *Scenedesmus obliquus*: Antioxidant and antiviral activity of proteins hydrolyzed by three enzymes. Journal of Genetic Engineering and Biotechnology. 16 (2): 399-408.

El-Sheekh M, Abomohra Ael-F, Hanelt D. 2013. Optimization of biomass and fatty acid productivity of *Scenedesmus obliquus* as a promising microalga for biodiesel production. World Journal of Microbiology and Biotechnology. 29 (5): 915-922.

Fan J, Cui Y, Wan M, Wang W, Li Y. 2014. Lipid accumulation and biosynthesis genes response of the oleaginous *Chlorella pyrenoidosa* under three nutrition stressors. Biotechnology for Biofuels. 7 (1): 14-28.

Farag I, Price K. 2013. Resources conservation in microalgae biodiesel production. International Journal of Engineering and Technical Research. 1 (8): 49-56.

Ferreira VS, Pinto RF, Anna CS. 2015. Low light intensity and nitrogen starvation modulate the chlorophyll content of *Scenedesmus dimorphus*. Journal of Applied Microbiology. 120 (3): 661-670.

Ferruzzi MG, Bohm V, Courtney PD, Schwartz SJ. 2002. Antioxidant and antimutagenic activity of dietary chlorophyll derivatives determined by radical scavenging and bacterial reverse mutagenesis assays. Journal of Food Science. 67 (7): 2589-2595.

Fidalgo JP, Cid A, Torres E, Sukenik A, Herrero C. 1998. Effects of nitrogen source and growth phase on proximate biochemical composition, lipid classes and fatty acid profile of the marine microalga *Isochrysis galbana*. Aquaculture. 166 (1-2): 105-116.

Finkle BJ, Appleman D. 1953. The effect of magnesium concentration on chlorophyll an catalase development in *Chlorella*. Plant Physiology. 28 (4): 652-663.

Folch J, Lees M, Stanley GHS. 1957. A simple method for the isolation and purification of total lipids from animal tissues. The Journal of Biological Chemistry. 226 (1): 497-509.

Foo SC, Yusoff FM, Ismail M, Basri M, Yau SK, Khong NM, Ebrahimi M. 2017. Antioxidant capacities of fucoxanthin-producing algae as influenced by their carotenoid and phenolic contents. Journal of Biotechnology. 241 (1): 175-183.

Ge YH, Duan B, Li CY, Tang Q, Li X, Wei ML, Chen YR, Li JR. 2018. γ-Aminobutyric acid delays senescence of blueberry fruit by regulation of reactive oxygen species metabolism and phenylpropanoid pathway. Scientia Horticulturae. 240 (4): 303-309.

Goiris K, Colen WV, Wilches I, León-Tamariz F, De-Cooman L, Muylaert K. 2015. Impact of nutrient stress on antioxidant production in three species of microalgae. Algal Research. 7 (1): 51-57.

González LE, Cañizares RO, Baena S. 1997. Efficiency of ammonia and phosphorus removal from a Colombian agroindustrial wastewater by the microalgae *Chlorella vulgaris* and *Scenedesmus dimorphus*. Bioresource Technology. 60 (3): 259-262.

Greene RM, Geider RJ, Kolber Z, Falkowski PG. 1992. Iron-induced changes in light harvesting and photochemical energy conversion processes in eukaryotic marine algae. Plant Physiology. 100 (2): 565-575.

Green FB, Lundquist T, Oswald W. 1995. Energetics of advanced integrated wastewater pond systems. Water Science and Technology. 31 (12): 9-20.

Griffiths MJ, Van-Hille RP, Harrison STL. 2010. Selection of direct transesterification as the preferred method for assay of fatty acid content of microalgae. Lipids. 45 (11): 1053-1060.

Guil-Guerrero JL, Navarro-Juarez R, Lopez-Martinez JC, Campra-Madrid P, Rebolloso-Fuentes MM. 2004. Functionnal properties of the biomass of three microalgal species. Journal of Food Engineering. 65 (4): 511-517.

Guiry MD, Guiry GM. 2021. Algae Base. World-wide electronic publication, National University of Ireland, Galway (taxonomic information republished from AlgaeBase with permission of Guiry MD). *Scenedesmus communis* Hegewald E., 1977. http://www. algaebase. org (2019).

Gushina IA, Harwood JL. 2009. Algal lipids and effect on the environment on their biochemistry. In: Arts MT, Brett MT, Kainz M, editors. Lipids in aquatic ecosystems. Springer-Verlag. New York. pp. 1-24.

Hamid S, Sibi G. 2018. Antioxidant system response in green microalga *Chlorococcopsis minuta* against nutrient stress in growth media. Asian Journal of Biological Sciences. 11 (1): 210-216.

Han F, Huang J, Li Y, Wang W, Wan M, Shen G, Wang J. 2013. Enhanced lipid productivity of *Chlorella pyrenoidosa* through the culture strategy of semi-continuous cultivation with nitrogen limitation and pH control by CO_2. Bioresource Technology. 136 (2): 418- 424.

Ho SH, Chen CY, Lee DJ, Chang JS. 2011. Perspectives on microalgal CO_2-emission mitigation systems - A review. Biotechnology Advances. 29 (2): 189-198.

Ho SH, Chen CY, Chang JS. 2012. Effect of light intensity and nitrogen starvation on CO_2 fixation and lipid/carbohydrate production of an indigenous microalga *Scenedesmus obliquus* CNW-N. Bioresource Technology. 113 (2): 244-252.

Ho SH, Li PJ, Liu CC, Chang JS. 2013. Bioprocess development on microalgae-based CO_2 fixation and bioethanol production using *Scenedesmus obliquus* CNW-N. Bioresource Technology. 145 (1):142-149.

Huo YX, Cho KM, Rivera JGL, Monte E, Shen CR, Yan Y, Liao JC. 2011. Conversion of proteins into biofuels by engineering nitrogen flux. Nature Biotechnology. 29 (4): 346-351.

Illman AM, Scragg AH, Shales SW. 2000. Increase in *Chlorella* strains calorific values when grown in low nitrogen medium. Enzyme and Microbial Technology. 27 (8): 631-635.

Jeffrey SW, Humphrey GF. 1975. New spectrophotometric equations for determining chlorphylls a, b, and c, in higher plants, algae and natural phytoplankton. Biochemie und Physiologie der Pflanzen. 167 (2): 191 -194.

Jenkins SH. 1982. Standard methods for the examination of water and wastewater. Water Research. 16 (10): 1495-1496.

Jia J, Han D, Gerken HG, Li Y, Sommerfeld M, Hu Q, Xu J. 2015. Molecular mechanisms for photosynthetic carbon partitioning into storage neutral lipids in *Nannochloropsis oceanica* under nitrogen-depletion conditions. Algal Research. 7 (1): 66-77.

John RP, Anisha GS, Nampoothiri KM, Pandey A. 2011. Micro and macroalgal biomass: A renewable source for bioethanol. Bioresource Technology. 102 (1): 186-193.

Juneja A, Ceballos MR, Murthy SG. 2013. Effects of environmental factors and nutrient availability on the biochemical composition of algae for biofuels production: a review. Energies. 6 (9): 4607-4638.

Kendırlıoglu G, Agırman N, Cetın AK. 2015. The effects of photoperiod on the growth, protein amount and pigment content of *Chlorella vulgaris*. Turkish Journal of Science and Technology. 10 (2): 7-10.

Khatoon H, Rahman N, Suleiman S, Banerjee S, Abol-Munafi A. 2017. Growth and proximate composition of *Scenedesmus obliquus* and *Selenastrum bibraianum* cultured in different media and condition. Proceedings of the National Academy of Sciences, India. Section B: Biological Sciences. 89 (37).

Khatoon H, Yuan GTG, Mahmud AI, Rahman MR. 2020. Growth and carotenoid production of *Dunaliella salina* (Dunal) teodoresco, 1905 cultured at different salinities. Asian Fisheries Science. 33 (1): 207-212.

Kobayashi M, Kakizono T, Nagai S. 1993. Enhanced carotenoid biosynthesis by oxidative stress in acetate-induced cyst cells of a green unicellular alga, *Haematococcus pluvialis*. Applied and Environmental Microbiology. 59 (3): 867-873.

Krishnan V, Uemura Y, Thanh NT, Khalid NA, Osman N, Mansor N. 2015. Three types of marine microalgae and *Nannocholoropsis oculata* cultivation for potential source of biomass production**.** Journal of Physics: Conference Series. 622.

Kudahettige NP, Pickova J, Gentili FG. 2018. Stressing algae for biofuel production : biomass and biochemical composition of *Scenedesmus dimorphus* and *Selenastrum minutum* grown in municipal untreated wastewater. Frontiers in Energy Research. 6 (1): 1-10.

Lamers PP, Janssen M, De-Vos RCH, Bino RJ, Wijffels RH. 2012. Carotenoid and fatty acid metabolism in nitrogen-starved *Dunaliella salina*, a unicellular green microalga. Journal of Biotechnology. 162 (1): 21-27.

Lam MK, Lee KT. 2012. Potential of using organic fertilizer to cultivate *Chlorella vulgaris* for biodiesel production. Applied Energy. 94 (3): 303-308.

Larned ST. 1998. Nitrogen versus phosphorus limited growth and sources of nutrients for coral reef macroalgae. Marine Biology. 132 (3): 409-421.

Lavens P, Sorgeloos P. 1996. Manual on the production and use of live food for aquaculture. Food and Agriculture Organization of the United Nations, Rome.

Leckie E. 2021. Adelaide scientists turn marine microalgae into 'superfoods' to substitute animal proteins. ABC News. Australian Broadcasting Corporation.

Li S, Jilin X, Jiao C, Juanjuan C, Chengxu Z, XiaojunY. 2014. Three types of marine microalgae and *Nannocholoropsis oculata* cultivation for potential source of biomass production. Aquaculture. 53(1): 622-625.

Li T, Wan L, Li A, Zhang C. 2013. Responses in growth, lipid accumulation, and fatty acid composition of four oleaginous microalgae to different nitrogen sources and concentrations. Chinese Journal of Oceanology and Limnology. 31(1): 1306-1314.

Liu Y, Wang F, Chen X, Zhang J, Gao B. 2015. Cellular responses and biodegradation of amoxicillin in *Microcystis aeruginosa* at different nitrogen levels. Ecotoxicology and Environmental Safety. 111 (4): 138-145.

Liu ZY, Wang GC, Zhou BC. 2008. Effect of iron on growth and lipid accumulation in *Chlorella vulgaris*. Bioresource Technology. 99 (11): 4717-4722.

Li Y, Fei X, Deng X. 2012. Novel molecular insights into nitrogen starvation induced triacylglycerols accumulation revealed by differential gene expression analysis in green algae *Micractinium pusillum*. Biomass Bioenergy. 42 (3): 199-211.

Li Y, Han D, Sommerfeld M, Hu Q. 2011. Photosynthetic carbon partitioning and lipid production in the oleaginous microalga *Pseudochlorococcum* sp. (Chlorophyceae) under nitrogen-limited conditions. Bioresource Technology. 102 (1): 123-129.

Logan C, Ronald S. 2011. Production and harvesting of microalgae for wastewater treatment, biofuels, and bioproducts. Biotechnology Advances. 29 (6): 686-702.

Lopez AD, Belarbi EH, Sevilla FJM, Ruiz RJ, Grima EM. 2000. Acyl lipid composition variation related to culture age and nitrogen concentration in

continuous culture of the microalga *Phaeodactylum tricornutum*. Phytochemistry. 54 (5): 461-471.

Loureno SO, Marquez UML, Mancini-Filho J, Barbarino E, Aidar E. 1997. Changes in biochemical profiles of *Tetraselmis gracilis* I. Comparison of two culture media. Aquaculture. 148 (2-3): 153-168.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the folin phenol reagent. Journal of Biological Chemistry. 193 (1): 265-275.

Lürling M. 1999. The smell of water: Grazer-induced colony formation in *Scenedesmus*. Thesis. Agricultural University of Wageningen. https://edepot.wur.nl/197509.

Lv JM, Cheng LH, Xu XH, Zhang L, Chen HL. 2010. Enhanced lipid production of *Chlorella vulgaris* by adjustment of cultivation conditions. Bioresource Technology. 101 (17): 6797-6804.

Lynn SG, Kilham SS, Kreeger DA, Interlandi SJ. 2000. Effect of nutrient availability on the biochemical and elemental stoichiometry in the freshwater diatom *Stephanodiscus minutulus* (Bacillariophyceae). Journal of Phycology. 36 (3): 510-522.

Marttınez ME, Sanchez S, Jimenez JM, Yousfi FE, Munoz L. 2000. Nitrogen and phosphorus removal from urban wastewater by the microalga *Scenedesmus obliquus*. Bioresource Technology. 73 (3): 263-272.

Menegol T, Diprat AB, Rodrigues E, Rech R. 2017. Effect of temperature and nitrogen concentration on biomass composition of *Heterochlorella luteoviridis*. Food Science and Technology. 37 (3): 28-37.

Mishra U, Pabbi S. 2004. Cyanobacteria: a potential biofertilizer for rice. Resonance. 9 (1): 6- 10.

Moazami N, Alireza A, Reza R, Mehrnoush T, Roghieh E, Ali SN. 2012. Large-scale biodiesel production using microalgae biomass of *Nannochloropsis*. Biomass and Bioenergy. 39 (2): 449- 453.

Mulders KJM, Lamers PP, Martens DE, Wijffels RH. 2014. Phototrophic pigment production with microalgae: Biological constraints and opportunities. Journal of Phycology. 50 (2): 229-242.

Muller-Feuga A. 2000. The role of microalgae in aquaculture: situation and trends. Journal of Applied Phycology. 12 (1): 527-534.

Nadzir SM, Yusof N, Nordin N, Kamari A, Yusoff MZM. 2021. Production of lipid and carbohydrate in *Tetradesmus obliquus* UPSI-JRM02 under nitrogen stress condition. Jurnal Teknologi. 83 (2): 27-35.

Navarro-Peraza RS, Piña-Valdez P, Cuevas-Rodriguez EO, Nieves-Soto M, Soto-León S, Contreras-Andrade I, Viveros-García T. 2017. Effects of temperature and nitrogen limitation on growth kinetics, proximate composition and fatty acid profile of *Nannochloropsis* sp. Revista Mexicana de Ingeniera Quimica. 16 (2): 359-369.

Nayak M, Suh WI, Chang YK, Lee B. 2019. Exploration of two-stage cultivation strategies using nitrogen starvation to maximize the lipid productivity in *Chlorella* sp. HS2. Bioresource Technology. 276 (4): 110-118.

Negishi T, Rai H, Hayatsu H. 1997. Antigenotoxic activity of natural chlorophylls. Mutation Research. 376 (1-2): 97-100.

Nicolau E, Barthelemy V, Schreiber N, Brun P, Lebouvier N. 2020. Effects of nitrogen availability on the antioxidant activity and carotenoid content of the microalgae *Nephroselmis* sp. Marine Drugs. 18 (9): 453–62.

Nigam S, Rai MP, Sharma R. 2011. Effect of nitrogen on growth and lipid content of *Chlorella pyrenoidosa*. American Journal of Biochemistry and Biotechnology. 7 (3): 124-129.

Pancha I, Chokshi K, George B, Ghosh T, Paliwal C, Maurya R, Mishra S. 2014. Nitrogen stress triggered biochemical and morphological changes in the microalgae *Scenedesmus* sp. CCNM 1077. Bioresource Technology. 156 (1): 146- 154.

Patnaik R, Mallick N. 2015. Utilization of *Scenedesmus obliquus* biomass as feedstock for biodiesel and other industrially important co-products: an

integrated paradigm for microalgal biorefinery. Algal Research. 12 (2): 328-336.

Patnaik R, Singh NK, Bagchi SK, Rao PS. 2019. Utilization of *Scenedesmus obliquus* protein as a replacement of the commercially available fish meal under an algal refinery approach. Frontiers in Microbiology. 10 (1): 1-15.

Peng Q, Zhao M, Shen G, Gan X, Li M. 2017. Linear alkylbenzenesulfonate (LAS) promotes sedimentation and lipid accumulation in *Scenedesmus obliquus*. RSC Advances. 7 (15): 9244- 9250.

Pezzolesi L, Mazzotti M, Vanucci S, Pistocchi R. 2019. Assimilation of inorganic nitrogen for scaling up *Desmodesmus communis* (Scenedesmaceae) biomass production. Journal of Applied Phycology. 31 (5): 2833-2844.

Pickett-Heaps JD, Staehelin LA. 1975. The ultra structure of *Scenedesmus* (Chlorophyceae) II cell division and colony formation. Journal of Phycology. 11 (2): 186-202.

Podevin M, De-Francisci D, Holdt SL, Angelidaki I. 2015. Effect of nitrogen source and acclimatization on specific growth rates of microalgae determined by a high-throughput in vivo microplate autofluorescence method. Journal of Applied Phycology. 27 (4): 1415-1423.

Pruvost J, van Vooren G, le Gouic B, Couzinet-Mossion A, Legrand J. 2011. Systematic investigation of biomass and lipid productivity by microalgae in photobioreactors for biodiesel application. Bioresource Technology. 102 (1): 150-158.

Pulz O. 2001. Photobioreactors: production systems for phototrophic microorganisms. Applied Microbiology and Biotechnology. 57 (1): 287-293.

Qi F, Pei H, Ma G, Zhang S, Mu R. 2016. Improving productivity and quality of biodiesel from *Chlorella vulgaris* SDEC-3M through customized process designs. Energy Conversion and Management. 129: 100-107.

Qu Z, Duan P, Cao X, Liu M, Lin L, Li M. 2019. Comparison of monoculture and mixed culture (*Scenedesmus obliquus* and wild algae) for C, N, and P removal and lipid production. Environmental Science and Pollution Research. 26 (20): 20961-20968.

Raghothama KG. 2000. Phosphate transport and signaling. Current Opinion in Plant Biology. 3 (3): 182-187.

Ratha SK, Rao PH, Govindaswamy K, Jaswin RS, Lakshmidevi R, Bhaskar S, Chinnasamy S. 2016. A rapid and reliable method for estimating microalgal biomass using a moisture analyser. Journal of Applied Phycology. 28 (1): 1725-1734.

Reitan KI, Rainuzzo JR, Olsen Y. 1994. Effect of nutrient limitation on fatty acid and lipid content of marine microalgae. Journal of Phycology. 30 (6): 972-979.

Richards RG, Mullins BJ. 2013. Using microalgae for combined lipid production and heavy metal removal from leachate. Ecological Modelling. 249: 59-69.

Rismani-Yazdi H, Haznedaroglu BZ, Bibby K, Peccia J. 2011. Transcriptome sequencing and annotation of the microalgae *Dunaliella tertiolecta*: Pathway description and gene discovery for production of next-generation biofuels. BMC Genomics. 12 (1): 1-17.

Ruiz-Domínguez MC, Vaquero I, Obregón V, de-la-Morena B, Vílchez C, Vega JM. 2015. Lipid accumulation and antioxidant activity in the eukaryotic acidophilic microalga *Coccomyxa* sp. (strain *onubensis*) under nutrient starvation. Journal of Applied Phycology. 27 (3): 1099-1108.

Saha SK, McHugh E, Hayes J, Moane S, Walsh D, Murray P. 2013. Effect of various stress-regulatory factors on biomass and lipid production in microalga *Haematococcus pluvialis*. Bioresource Technology. 128: 118-124.

Saha SK, Uma L, Subramanian G. 2003. Nitrogen stress induced changes in the marine cyanobacterium *Oscillatoria willei* BDU 130511. FEMS Microbiology Ecology. 45 (3): 263-72.

Sandmann G. 2019. Antioxidant protection from UV- and light-stress related to carotenoid structures. Antioxidants. 8 (7): 219-227.

Sato N, Hagio M, Wada H, Tsuzuki M. 2000. Environmental effects on acidic lipids of thylakoid membranes. Biochemical Society Transactions. 28 (6): 912-914.

Sharma AK, Sahoo PK, Singhal S. 2015. Screening and optimization of culture media for *Chlorella* sp. as a raw material for biodiesel production. International Journal of Pharma and Bio Sciences. 6 (3): 251-262.

Sharma R, Singh GP, Sharma VK. 2011. Comparison of different media formulations on growth, morphology and chlorophyll content of green alga, *Chlorella vulgaris*. International Journal of Pharma and Bio Sciences. 2 (2): 509- 516.

Shen XF, Liu JJ, Chauhan AS, Hu H, Ma LL, Lam PK, Zeng RJ. 2016. Combining nitrogen starvation with sufficient phosphorus supply for enhanced biodiesel productivity of *Chlorella vulgaris* fed on acetate. Algal Research. 17 (C): 261- 267.

Shovon M, Nirupama M. 2009. Microalga *Scenedesmus obliquus* as a potential source for biodiesel production. Applied Microbiology and Biotechnology. 84 (2): 281-291.

Siaut M, Cuiné S, Cagnon C, Fessler B, Nguyen M, Carrier P, Beyly A, Beisson F, Triantaphylidès C, Li-Beisson Y, Peltier G. 2011. Oil accumulation in the model green alga *Chlamydomonas reinhardtii*: Characterization, variability between common laboratory strains and relationship with starch reserves. BMC Biotechnology. 11 (1): 1-15.

Siegelman H, Kycia J. 1978. Algal bili-proteins: Handbook of psychological method. Cambridge University Press, Cambridge. p. 71-79.

Silveira ST, Burkert JFM, Costa JAV, Burkert CAV, Kalil SJ. 2007. Optimization of phycocyanin extraction from *Spirulina platensis* using factorial design. Bioresource Technology. 98 (8): 1629-1634.

Simionato D, Block MA, La Rocca N, Jouhet J, Maré- chal E, Finazzi G, Morosinotto T. 2013. The response of *Nannochloropsis gaditana* to nitrogen starvation includes de novo biosynthesis of triacylglycerols, a decrease of chloroplast galactolipids, and reorganization of the photosynthetic apparatus. Eukaryotic Cell. 12 (5): 665-676.

Soletto D, Binaghi L, Lodi A, Carvalho JCM, Converti A. 2005. Batch and fed-batch cultivations of *Spirulina platensis* using ammonium sulphate and urea as nitrogen sources. Aquaculture. 243 (1-4): 217-224.

Solovchenko AE, Khozin-Goldberg I, Didi-Cohen S, Cohen Z, Merzlyak MN. 2008. Effects of light and nitrogen starvation on the content and composition of carotenoids of the green microalga *Parietochloris incisa*. Russian Journal of Plant Physiology. 55 (4): 455-462.

Solovchenko AE. 2012. Physiological role of neutral lipid accumulation in eukaryotic microalgae under stresses. Russian Journal of Plant Physiology. 59 (2): 192-202.

Solovchenko A, Solovchenko O, Khozin-Goldberg I, Didi-Cohen S, Pal D, Cohen Z, Boussiba S. 2013. Probing the effects of high-light stress on pigment and lipid metabolism in nitrogen-starving microalgae by measuring chlorophyll fluorescence transients: studies with a D5 desaturase mutant of *Parietochloris incisa* (Chlorophyta, Trebouxiophyceae). Algal Research. 2 (3): 175-182.

Song M, Haiyan P, Wenrong H, Guixia M. 2013. Evaluation of the potential of 10 microalgal strains for biodiesel production. Bioresource Technology. 8 (1): 141 -245.

Sonkar S, Mallick N. 2017. Development of a single phase nitrate feeding strategy for enhanced lipid productivity from green microalgae for biodiesel production. Environmental Progress and Sustainable Energy. 36 (1): 222-231.

Stein JR, editor. 1980. Handbook of phycological methods: Culture methods and growth measurements. Cambridge University Press, Cambridge. pp. 448.

Sukenik A, Carmeli Y. 1990. Lipid synthesis and fatty acid composition in *Nannochloropsis* sp. (Eustigmatophyceae) grown in a light-dark cycle 1. Journal of Phycology. 26 (3): 463-469.

Suzana W, Ani I, Sitti R, Muhamad S. 2013. The influence of light intensity and photoperiod on the growth and lipid content of microalgae *Nannochloropsis* sp. Bioresource Technology. 129 (C): 7-11.

Thurman HV. 1997. Introductory Oceanography. New Jersey, USA: Prentice Hall College.

Toyub M, Miah M, Habib M, Rahman M. 2008. Growth performance and nutritional value of *Scenedesmus obliquus* cultured in different concentrations of

sweetmeat factory waste media. Bangladesh Journal of Animal Science. 37 (1): 86- 93.

Turpin, DH. 1991. Effects of inorganic N availability on algal photosynthesis and carbon metabolism. Journal of Phycology. 27 (1): 4-20.

Ugwu CU, Aoyagi H, Uchiyama H. 2008. Photobioreactors for mass cultivation of algae. Bioresource Technology. 99 (10): 4021-4028.

van Leeuwe MA, Stefels J. 1998. Effects of iron and light stress on the biochemical composition of *Antarctic Phaecosystis* sp. (Prymnesiophyceae) II. pigment composition. Journal of Phycology. 34 (3): 496-503.

Vymazal J. 1988. The use of periphyton communities for nutrient removal from polluted streams. Hydrobiologia. 166 (3): 225-237.

Wijesekara I, Pangestuti R, Kim SK. 2010. Biological activities and potential health benefits of sulfated polysaccharides derived from marine algae. Carbohydrate Polymers. 84 (1): 14- 21.

Xia L, Yang H, He Q, Hu Ch. 2014. Physiological responses of freshwater oleaginous microalgae *Desmodesmus* sp. NMX451 under nitrogen deficiency and alkaline pH-induced lipid accumulation. Journal of Applied Phycology. 27 (2): 649-659.

Xie T, Xia Y, Zeng Y, Li X, Zhang Y. 2017. Nitrate concentration-shift cultivation to enhance protein content of heterotrophic microalga *Chlorella vulgaris*: over-compensation strategy. Bioresource Technology. 233: 247-255.

Xin L, Hu HY, Ke G, Sun YX. 2010. Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a freshwater microalga *Scenedesmus* sp. Bioresource Technology. 101 (14): 5494- 5500.

Xu Y, Ibrahim MI, Harvey JP. 2016. The influence of photoperiod and light intensity on the growth and photosynthesis of *Dunaliella salina* (chlorophyta) CCAP 19/30. Plant Physiology and Biochemistry. 106: 305-315.

Yang F, Long L, Sun X, Wu H, Li T, Xiang W. 2014. Optimization of medium using response surface methodology for lipid production by *Scenedesmus* sp. Marine Drugs. 12 (3): 1245-1257.

Yang F, Xiang W, Li T, Long L. 2018. Transcriptome analysis for phosphorus starvation-induced lipid accumulation in *Scenedesmus* sp. Scientific Reports. 8 (1): 1-11.

Yang L, Chen J, Qin S, Zeng M, Jiang Y, Hu L, Xiao P, Hao W, Hu Z, Lei A, Wang J. 2008. Growth and lipid accumulation by different nutrients in the microalga *Chlamydomonas reinhardtii*. Biotechnology for Biofuels. 11 (1): 40-48.

Yilancioglu K, Cokol M, Pastirmaci I, Erman B, Cetiner S. 2014. Oxidative stress is a mediator for increased lipid accumulation in a newly isolated *Dunaliella salina* strain. PLoS ONE. 9 (3): e91957.

Young AJ, Lowe GM. 2001. Antioxidant and prooxidant properties of carotenoids. Archives of Biochemistry and Biophysics. 385 (1): 20-27.

Yu Z, Pei H, Jiang L, Hou Q, Nie C, Zhang L. 2018. Phytohormone addition coupled with nitrogen depletion almost tripled the lipid productivities in two algae. Bioresource Technology. 247: 904-914.

Zarrinmehr MJ, Farhadian O, Heyrati FP, Keramat J, Koutra E, Kornaros M, Daneshvar E. 2020. Effect of nitrogen concentration on the growth rate and biochemical composition of the microalga *Isochrysis galbana*. Egyptian Journal of Aquatic Research. 46 (1): 153-158.

Zhang L, Liu J. 2016. Enhanced fatty acid accumulation in *Isochrysis galbana* by inhibition of the mitochondrial alternative oxidase pathway under nitrogen deprivation. Bioresource Technology. 211: 783-786.

Zhang YM, Chen H, He CL, Wang Q. 2013. Nitrogen starvation induced oxidative stress in an oil-producing green alga *Chlorella sorokiniana* C3. PLoS ONE. 8 (7): e69225.

Zhang Y, Wu H, Yuan C, Li T, Li A. 2019. Growth, biochemical composition, and photosynthetic performance of *Scenedesmus acuminatus* during nitrogen starvation and resupply. Journal of Applied Phycology. 31 (5): 2797-2809.

Zhao LS, Li K, Wang QM, Song XY, Su HN, Xie BB, Zhang XY, Huang F, Chen XL, Zhou BC, Zhang YZ. 2017. Nitrogen starvation impacts the

photosynthetic performance of *Porphyridium cruentum* as revealed by chlorophyll a fluorescence. Scientific Reports. 7 (1): 8542-8546.

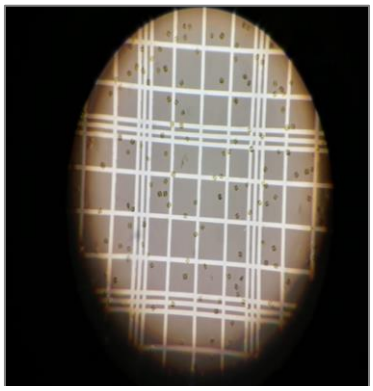
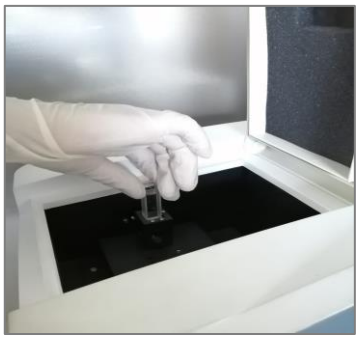
Zhu S, Huang W, Xu J, Wang Z, Xu J, Yuan Z. 2014. Metabolic changes of starch and lipid triggered by nitrogen starvation in the microalgae *Chlorella zofingiensis*. Bioresource Technology. 152: 292-298.

Appendices

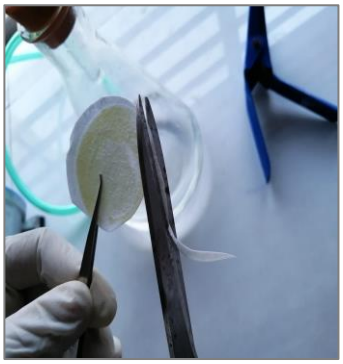
Appendix A: Media Preparation and Stock Culture of Pure *Scenedesmus* sp.

Appendix B: Experiment set up for Growth Curve Analysis

Appendix C: Measurement of Cell Density, Optical Density and Dry Biomass

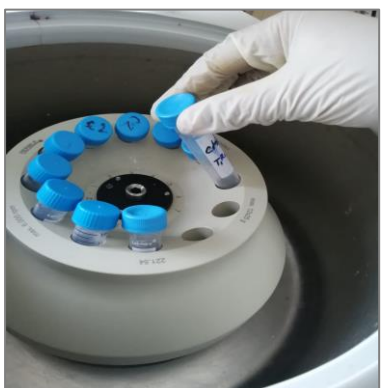





Appendix D: Extraction of Chlorophyll

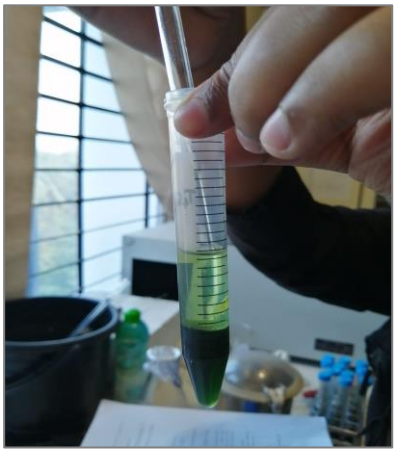



Appendix E: Microalgae Seed Inoculation and Mass Culture

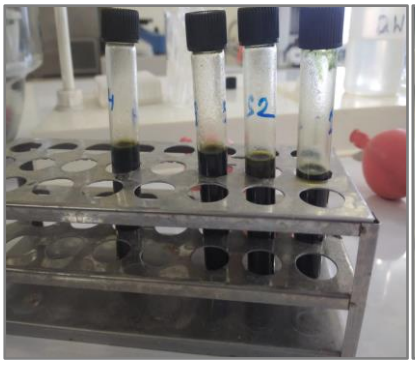
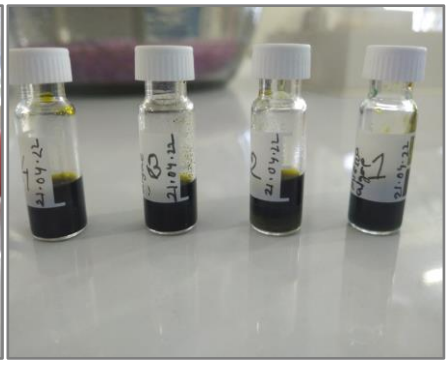

Appendix F: Extraction for Carotenoid and Phycobiliprotein Estimation




Appendix F: Proximate Composition Analysis




Appendix G: Fatty Acid Analysis

Appendix I: One-way Analysis of Variance Examining Cell Density of *Scenedesmus* sp. in Five Treatments

ANOVA						
		Sum of Squares	df	Mean Square	F	Sig.
Cell_Day_0	Between Groups	90000000.000	4	22500000.000	.175	.94
	Within Groups	1283333333.33	10	128333333.333		
	Total	1373333333.33	14			
Cell_Day_1	Between Groups	84743333333.3	4	21185833333.33	84.743	.00
	Within Groups	2500000000.00	10	250000000.000		
	Total	87243333333.3	14			
Cell_Day_2	Between Groups	821106666666.6	4	205276666666.6	179.020	.00
	Within Groups	11466666666.66	10	1146666666.667		
	Total	832573333333.3	14			
Cell_Day_3	Between Groups	2222760000000	4	555690000000.0	2525.86	.00
	Within Groups	2200000000.000	10	220000000.000		
	Total	222496000000.0	14			
Cell_Day_4	Between Groups	472997333333.3	4	118249333333.3	8446.38	.00
	Within Groups	1400000000.000	10	140000000.000		
	Total	4731373333333	14			
Cell_Day_5	Between Groups	9715399999999.996	4	2428849999999.999	6175.042	.00
	Within Groups	3933333333.333	10	393333333.333		
	Total	9719333333333	14			
Cell_Day_6	Between Groups	5302973333333	4	1325743333333	43.783	.00
	Within Groups	302800000000.0	10	30280000000.00		
	Total	5605773333333	14			

Cell_Day_7	Between Groups	2405869333333	4	6014673333333	218.398	.00
	Within Groups	275400000000.0	10	27540000000.00		
	Total	2433409333333	14			
Cell_Day_8	Between Groups	3888533333333	4	9721333333333	1350.18	.00
	Within Groups	72000000000.00	10	7200000000.000		
	Total	3895733333333	14			
Cell_Day_9	Between Groups	7669503999999	4	1917375999999	867.591	.00
	Within Groups	221000000000.0	10	22100000000.00		
	Total	7691603999999	14			
Cell_Day_10	Between Groups	9539497333333	4	2384874333333	1368.52	.00
	Within Groups	174266666666.6	10	17426666666.66		
	Total	9556924000000	14			
Cell_Day_11	Between Groups	2041862266666	4	5104655666666	1043.8	.00
	Within Groups	48933333333.33	10	4893333333.333		
	Total	2042351600000	14			
Cell_Day_12	Between Groups	1450078933333	4	3625197333333	9355.3	.00
	Within Groups	38750000000.00	10	3875000000.000		
	Total	1450466433333	14			

Appendix J: One-way Analysis of Variance Examining Optical Density of *Scenedesmus* sp. in Five Treatments

ANOVA						
		Sum of Squares	df	Mean Square	F	Sig.
OD_Day_0	Between Groups	.000	4	.000	.875	.512
	Within	.000	10	.000		

	Groups					
	Total	.000	14			
OD_Day_1	Between Groups	.000	4	.000	4.290	.028
	Within Groups	.000	10	.000		
	Total	.000	14			
OD_Day_2	Between Groups	.002	4	.000	10.255	.001
	Within Groups	.000	10	.000		
	Total	.002	14			
OD_Day_3	Between Groups	.004	4	.001	248.348	.000
	Within Groups	.000	10	.000		
	Total	.004	14			
OD_Day_4	Between Groups	.054	4	.014	4852.226	.000
	Within Groups	.000	10	.000		
	Total	.054	14			
OD_Day_5	Between Groups	.041	4	.010	2186.050	.000
	Within Groups	.000	10	.000		
	Total	.041	14			
OD_Day_6	Between Groups	.023	4	.006	2110.938	.000
	Within Groups	.000	10	.000		
	Total	.023	14			
OD_Day_7	Between Groups	.014	4	.003	1496.171	.000
	Within Groups	.000	10	.000		
	Total	.014	14			
OD_Day_8	Between Groups	.135	4	.034	10807.62	.000
	Within Groups	.000	10	.000		
	Total	.135	14			

OD_Day_9	Between Groups	.183	4	.046	12274.17	.000
	Within Groups	.000	10	.000		
	Total	.183	14			
OD_Day_10	Between Groups	.115	4	.029	1815.332	.000
	Within Groups	.000	10	.000		
	Total	.115	14			
OD_Day_11	Between Groups	.061	4	.015	1770.434	.000
	Within Groups	.000	10	.000		
	Total	.061	14			
OD_Day_12	Between Groups	.038	4	.010	1669.547	.000
	Within Groups	.000	10	.000		
	Total	.038	14			

Appendix K: One-way Analysis of Variance Examining Biomass of *Scenedesmus* sp. in Five Treatments

ANOVA						
		Sum of Squares	df	Mean Square	F	Sig.
B_Day_0	Between Groups	.003	4	.001	.200	.933
	Within Groups	.033	10	.003		
	Total	.036	14			
B_Day_1	Between Groups	.000	4	.000	12.349	.001
	Within Groups	.000	10	.000		
	Total	.000	14			
B_Day_2	Between Groups	.002	4	.001	42.369	.000
	Within	.000	10	.000		

	Groups					
	Total	.002	14			
B_Day_3	Between Groups	.031	4	.008	157.43	.000
	Within Groups	.000	10	.000		
	Total	.032	14			
B_Day_4	Between Groups	.053	4	.013	266.87	.000
	Within Groups	.000	10	.000		
	Total	.053	14			
B_Day_5	Between Groups	.095	4	.024	115.66	.000
	Within Groups	.002	10	.000		
	Total	.097	14			
B_Day_6	Between Groups	.041	4	.010	142.41	.000
	Within Groups	.001	10	.000		
	Total	.042	14			
B_Day_7	Between Groups	.034	4	.008	196.36	.000
	Within Groups	.000	10	.000		
	Total	.034	14			
B_Day_8	Between Groups	.062	4	.016	680.65	.000
	Within Groups	.000	10	.000		
	Total	.062	14			
B_Day_9	Between Groups	.155	4	.039	796.91	.000
	Within Groups	.000	10	.000		
	Total	.156	14			
B_Day_10	Between Groups	.373	4	.093	2070.9	.000
	Within Groups	.000	10	.000		
	Total	.373	14			

B_Day_11	Between Groups	.507	4	.127	1729.9	.000
	Within Groups	.001	10	.000		
	Total	.508	14			
B_Day_12	Between Groups	.453	4	.113	4390.3	.000
	Within Groups	.000	10	.000		
	Total	.453	14			

Appendix L: One-way Analysis of Variance Examining Chlorophyll a of *Scenedesmus* sp. in Five Treatments

ANOVA						
		Sum of Squares	df	Mean Square	F	Sig.
C_a_Day_0	Between Groups	8.487	4	2.122	.018	.999
	Within Groups	1151.713	10	115.171		
	Total	1160.200	14			
C_a_Day_1	Between Groups	1349.916	4	337.479	8.719	.003
	Within Groups	387.073	10	38.707		
	Total	1736.989	14			
Ca_Day_2	Between Groups	13891.611	4	3472.90	113.34	.000
	Within Groups	306.413	10	30.641		
	Total	14198.024	14			
Ca_Day_3	Between Groups	30519.796	4	7629.94	116.24	.000
	Within Groups	656.393	10	65.639		
	Total	31176.189	14			
Ca_Day_4	Between Groups	30461.637	4	7615.40	162.17	.000
	Within Groups	469.580	10	46.958		
	Total	30931.217	14			

Ca_Day_5	Between Groups	2631.591	4	657.898	13.017	.001
	Within Groups	505.407	10	50.541		
	Total	3136.997	14			
Ca_Day_6	Between Groups	21970.583	4	5492.64	80.800	.000
	Within Groups	679.787	10	67.979		
	Total	22650.369	14			
Ca_Day_7	Between Groups	16806.597	4	4201.64	42.357	.000
	Within Groups	991.967	10	99.197		
	Total	17798.564	14			
Ca_Day_8	Between Groups	272497.68	4	68124.4	938.01	.000
	Within Groups	726.260	10	72.626		
	Total	273223.94	14			
Ca_Day_9	Between Groups	715927.63	4	178981.	5519.3	.000
	Within Groups	324.280	10	32.428		
	Total	716251.91	14			
Ca_Day_10	Between Groups	1287791.1	4	32197.1	5712.1	.000
	Within Groups	563.633	10	56.363		
	Total	1288354.7	14			
Ca_Day_11	Between Groups	1903284.3	4	47582.1	6236.3	.000
	Within Groups	762.980	10	76.298		
	Total	1904047.3	14			
Ca_Day_12	Between Groups	1321194.8	4	330298	4990.5	.000
	Within Groups	661.853	10	66.185		
	Total	1321856.6	14			

Appendix M: One-way Analysis of Variance Examining Chlorophyll b of *Scenedesmus* sp. in Five Treatments

ANOVA						
		Sum of Squares	df	Mean Square	F	Sig.
C_b_Day_0	Between Groups	28.509	4	7.127	.237	.911
	Within Groups	300.327	10	30.033		
	Total	328.836	14			
C_b_Day_1	Between Groups	1313.771	4	328.44	15.875	.000
	Within Groups	206.887	10	20.689		
	Total	1520.657	14			
C_b_Day_2	Between Groups	1405.980	4	351.495	20.990	.000
	Within Groups	167.460	10	16.746		
	Total	1573.440	14			
C_b_Day_3	Between Groups	3200.543	4	800.136	49.223	.000
	Within Groups	162.553	10	16.255		
	Total	3363.096	14			
C_b_Day_4	Between Groups	3612.443	4	903.111	58.148	.000
	Within Groups	155.313	10	15.531		
	Total	3767.756	14			
C_b_Day_5	Between Groups	5921.276	4	1480.319	175.574	.000
	Within Groups	84.313	10	8.431		
	Total	6005.589	14			
C_b_Day_6	Between Groups	4431.651	4	1107.913	68.412	.000
	Within Groups	161.947	10	16.195		
	Total	4593.597	14			
C_b_Day_7	Between	1549.760	4	387.440	32.030	.000

	Groups					
	Within Groups	120.960	10	12.096		
	Total	1670.720	14			
C_b_Day_8	Between Groups	11442.553	4	2860.63	290.008	.000
	Within Groups	98.640	10	9.864		
	Total	11541.193	14			
C_b_Day_9	Between Groups	76575.543	4	19143.8	1305.79	.000
	Within Groups	146.607	10	14.661		
	Total	76722.149	14			
C_b_Day_10	Between Groups	238299.451	4	59574.8	4686.26	.000
	Within Groups	127.127	10	12.713		
	Total	238426.577	14			
C_b_Day_11	Between Groups	388363.007	4	97090.7	6964.57	.000
	Within Groups	139.407	10	13.941		
	Total	388502.413	14			
C_b_Day_12	Between Groups	215569.897	4	53892.4	9579.18	.000
	Within Groups	56.260	10	5.626		
	Total	215626.157	14			

Appendix N: Regression Analysis between Cell Density and Optical Density

Model Summary[b]				
Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.977[a]	.954	.951	.021454
a. Predictors: (Constant), Cell Density				
b. Dependent Variable: Optical Density				

ANOVA[a]						
Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	.125	1	.125	270.786	.000[b]
	Residual	.006	13	.000		
	Total	.131	14			
a. Dependent Variable: Optical Density						
b. Predictors: (Constant), Cell Density						

Appendix O: Regression Analysis between Cell Density and Biomass

Model Summary				
Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.979[a]	.958	.955	.019975
a. Predictors: (Constant), Cell Density				

ANOVA[a]						
Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	.119	1	.119	298.521	.000[b]
	Residual	.005	13	.000		
	Total	.124	14			
a. Dependent Variable: Biomass						
b. Predictors: (Constant), Cell Density						

Appendix P: Regression Analysis between Cell Density and Chlorophyll a

Model Summary				
Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.973[a]	.947	.943	38.2256
a. Predictors: (Constant), Cell Density				

ANOVA[a]						
Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	337060.423	1	337060.423	230.675	.000[b]
	Residual	18995.530	13	1461.195		
	Total	356055.953	14			

a. Dependent Variable: Chlorophyll a
b. Predictors: (Constant), Cell Density

Appendix Q: Regression Analysis between Cell Density and Chlorophyll b

Model Summary				
Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.969[a]	.939	.934	23.92845720
a. Predictors: (Constant), Cell Density				

ANOVA[a]						
Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	113839.353	1	113839.353	198.821	.000[b]
	Residual	7443.424	13	572.571		
	Total	121282.777	14			
a. Dependent Variable: Chlorophyll b						
b. Predictors: (Constant), Cell Density						

Appendix R: One-way Analysis of Variance Examining Carotenoid Content of *Scenedesmus* sp. in Five Treatments

ANOVA					
Carotenoid					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	10.766	4	2.692	6.535	.007
Within Groups	4.119	10	.412		
Total	14.885	14			

Appendix S: One-way Analysis of Variance Examining Phycobiliprotein, Phycocyanin, Allophycocyanin and Phycoerythrin Content of *Scenedesmus* sp. in Five Treatments

ANOVA

Phycobiliprotien

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	15.467	4	3.867	64.650	.000
Within Groups	.299	5	.060		
Total	15.766	9			

ANOVA

Phycocyanine

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.162	4	.290	38.769	.001
Within Groups	.037	5	.007		
Total	1.199	9			

ANOVA

Alophycocyanin

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.960	4	.740	69.058	.000
Within Groups	.054	5	.011		
Total	3.014	9			

ANOVA

Phycoerythrin

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.036	4	.509	34.824	.001
Within Groups	.073	5	.015		
Total	2.109	9			

Appendix T: One-way Analysis of Variance Examining Volumetric, Areal and Lipid Productivity, Specific Growth Rate and Cell Doubling Time of *Scenedesmus* sp. in Five Treatments

ANOVA						
		Sum of Squares	df	Mean Square	F	Sig.
Volum Productivity	Between Groups	.000	4	.000	.841	.530
	Within Groups	.000	10	.000		
	Total	.000	14			
Lipid Productivity	Between Groups	29.051	4	7.263	4.654	.022
	Within Groups	15.605	10	1.560		
	Total	44.656	14			
Areal Productivity	Between Groups	.000	4	.000	.841	.530
	Within Groups	.000	10	.000		
	Total	.000	14			
SGR	Between Groups	.033	4	.008	102.8	.000
	Within Groups	.001	10	.000		
	Total	.034	14			
Cell Doubling Time	Between Groups	.402	4	.100	107.4	.000
	Within Groups	.009	10	.001		
	Total	.411	14			
Total Chlorophyll	Between Groups	885537	4	221384	2787	.000
	Within Groups	794.313	10	79.431		
	Total	886331	14			

Appendix U: One-way Analysis of Variance Examining Protein, Lipid and Carbohydrate Content of *Scenedesmus* sp. in Five Treatments

ANOVA					
Protien					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	21.366	4	5.341	8.819	.003
Within Groups	6.056	10	.606		
Total	27.422	14			

ANOVA					
Lipid					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	102.346	4	25.586	27.868	.000
Within Groups	9.181	10	.918		
Total	111.527	14			

ANOVA					
Carbohydrate					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	157.976	4	39.494	6.777	.007
Within Groups	58.273	10	5.827		
Total	216.249	14			

Appendix V: One-way Analysis of Variance Examining Fatty Acids of *Scenedesmus* sp. in Five Treatments

ANOVA						
		Sum of Squares	df	Mean Square	F	Sig.
C8:0	Between Groups	.007	4	.002	54.960	.000
	Within Groups	.000	5	.000		
	Total	.007	9			
C10:0	Between Groups	.000	4	.000	.645	.654
	Within Groups	.000	5	.000		
	Total	.000	9			
C12:0	Between Groups	1.908	4	.477	46.029	.000
	Within Groups	.052	5	.010		
	Total	1.960	9			
C13:0	Between Groups	.966	4	.242	32.316	.001
	Within Groups	.037	5	.007		
	Total	1.003	9			
C14:0	Between Groups	.357	4	.089	30.856	.001
	Within Groups	.014	5	.003		
	Total	.371	9			
C16:1	Between Groups	425.448	4	106.362	192.616	.000
	Within Groups	2.761	5	.552		
	Total	428.209	9			
C16:0	Between Groups	43.310	4	10.828	20.027	.003
	Within Groups	2.703	5	.541		
	Total	46.013	9			

C18:2	Between Groups	3969.892	4	992.473	100.689	.000
	Within Groups	49.284	5	9.857		
	Total	4019.176	9			
C18:1	Between Groups	7.727	4	1.932	5.488	.045
	Within Groups	1.760	5	.352		
	Total	9.487	9			
C18:3	Between Groups	63.037	4	15.759	64.154	.000
	Within Groups	1.228	5	.246		
	Total	64.265	9			
C18:0	Between Groups	30.264	4	7.566	8.369	.019
	Within Groups	4.520	5	.904		
	Total	34.784	9			
C20:0	Between Groups	199.091	4	49.773	30.781	.001
	Within Groups	8.085	5	1.617		
	Total	207.176	9			
C20:4	Between Groups	.490	4	.122	12.714	.008
	Within Groups	.048	5	.010		
	Total	.538	9			
C20:5	Between Groups	17.943	4	4.486	19.697	.003
	Within Groups	1.139	5	.228		
	Total	19.081	9			
C20:3	Between Groups	6.820	4	1.705	9.084	.016
	Within Groups	.939	5	.188		
	Total	7.759	9			
C17:0	Between Groups	.020	4	.005	7.411	.025

	Within Groups	.003	5	.001		
	Total	.023	9			
C21:0	Between Groups	1147.955	4	286.989	312.053	.000
	Within Groups	4.598	5	.920		
	Total	1152.554	9			
C22:6	Between Groups	.404	1	.404	22.984	.041
	Within Groups	.035	2	.018		
	Total	.439	3			
C22:5	Between Groups	.090	4	.023	10.923	.011
	Within Groups	.010	5	.002		
	Total	.100	9			
C22:1	Between Groups	767.963	4	191.991	43.924	.000
	Within Groups	21.855	5	4.371		
	Total	789.817	9			
C20:1	Between Groups	.113	4	.028	137.485	.000
	Within Groups	.001	5	.000		
	Total	.114	9			
23:00	Between Groups	.051	3	.017	1.666	.310
	Within Groups	.041	4	.010		
	Total	.092	7			
C24:1	Between Groups	.000	4	.000	2.561	.165
	Within Groups	.000	5	.000		
	Total	.001	9			

Brief Bio-Data of the Student

Fardous Ara Mukta is the 1st daughter of Md. Mofijul Haque Shah and Amena Begum, was born and grown up in Narayanganj district. She has completed Dakhil from Darunnazat Siddikia Kamil Madrasah, Dhaka in 2013 and HSC from Dr. Mahbubur Rahman Molla College, Dhaka in 2015. She has also achieved her B.Sc. degree in Fisheries from Chattogram Veterinary and Animal Sciences University in 2019. She is now a candidate of Master's degree of the same institute from the Department of Aquaculture. She has expertise on both field and laboratory works. She has done many farm works in Cox's Bazar district and microalgae laboratory research. Internship in Bangladesh's various fisheries related organizations and also in University Malaysia Terrenganu, UMT is her advanced qualification besides academic study. She was trained on freshwater pearl production by Bangladesh Fisheries Research Institute. Her one scientific paper is published in an international journal. She was employed as a research assistant under the project "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" funded by Bangladesh Fisheries Research Institute (BFRI) from January 2020 to till date and project '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 since January 2022. She has a lot of experience on co-curricular activities. She was a organizing secretary at 'Badhon CVASU Unit' (2017-2020), a social welfare's organization and charter member at 'Rotaract Club of CVASU' (2018 – 2020). Her research interest areas include, microalgae, fish breeding, microbiology, fish genetics, bio-floc technology, fish disease, ecology, and advanced aquaculture technologies. She is determined to make her a competent researcher and wants to develop the aquaculture sector of Bangladesh.