EFFECTS OF DIFFERENT NITROGEN CONCENTRATIONS ON THE GROWTH AND NUTRITIONAL PROFILE OF *Chlorella* sp.

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Roll No.: 0118/01

Registration No.: 588

Session: 2018-2019

A thesis submitted in the partial fulfillment of the requirements for the degree of Master of Science in Department of Fishing and Post-Harvest Technology, CVASU

Department of Fishing and Post-Harvest Technology, CVASU

Faculty of Fisheries

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JUNE 2020

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June 2020

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

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JUNE 2020

Dedicated

To my

Beloved parents

Acknowledgement

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June 2020

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ABSTRACT

The study was conducted to evaluate the growth performance and proximate compositions of *Chlorella* sp. cultured in different $NaNO_3$ concentrations. *Chlorella* sp. cultured at 8 g/L nitrogen concentration of $NaNO_3$ had significantly ($p < 0.05$) higher growth performance concerning the cell density (6.33×10^6 cells m/L) and optical density (0.461) compared to those culture at 12 g/L, 16 g/L, 20 g/L, and 24 g/L N, respectively. Additionally, significantly ($p < 0.05$) higher protein content (31.90% dry weight) had seen at 8 g/L N concentration compared to all other culture at different concentrations of $NaNO_3$. Furthermore, significantly ($p < 0.05$) higher carbohydrate content (20.07% dry weight) and lipid content (23.47% dry weight) were observed in *Chlorella* sp. cultured at 8 g/L N concentration in comparison to those grown on other concentrations of $NaNO_3$. Increasing and decreasing the nitrogen concentrations below or above 8 g/L N had a remarkable effect on the growth performance and biochemical composition of *Chlorella* sp.

Key Words: Nitrogen concentrations, *Chlorella* sp., microalgae, growth, and biochemical composition.

CHAPTER-1

INTRODUCTION

CHAPTER-1

INTRODUCTION

Microalgae are photosynthetic single-celled algae or phytoplankton which utilize light energy plus carbon dioxide, with higher photosynthetic efficiency compared to plants for the production of biomass (Converti et al., 2009). Hundreds of species have been tested for feeds, yet a very limited number (presumably less than twenty) was being extensively used in aquaculture (Das et al., 2012). Microalgae are a good source of valuable compounds, because of the strong demand of humans for natural, safe, eco-friendly, and renewable products (Gigova and Marinova, 2016).

Micro algae perhaps an excellent source of carbon compounds, which might be utilized in biofuels, cosmetics, pharmaceuticals, and health supplements. Also, microalgae have an implementation in wastewater treatment, and atmospheric CO_2 mitigation (Khan et al., 2018). They produce a wide range of bio-products for example proteins, carbohydrates, fatty acids, vitamins, minerals and pigments, polysaccharides, hydrocarbons, antibiotics, and other metabolites (Altin et al., 2018).

Microalgae are also a potential originator for biofuel production due to their inflated oil content and rapid biomass productivity (Sirakov et al., 2015). Microalgae must have some key features to be efficacious as aquaculture species (Das et al., 2012). Only microalgae with high overall value were used in aquaculture.

Chlorella sp. is one of the most promising species of microalgae. It is a cosmopolitan genus with small globular cells (about 2-10 mm diameter) existing in both aquatic and terrestrial habitats. The *Chlorella* strains have high-temperature endurance (some strains can grow between 15 and 40 ℃). *Chlorella* has been used as a model microorganism for studying the photosynthetic apparatus and carbon assimilation for a long time because of its simple cell cycle, high growth rate, and having photosynthetic and metabolic pathways similar to higher plants. The strains of *Chlorella* grow phototrophically in an inorganic medium as well as in mixotrophic and heterotrophic conditions (e.g. with the inclusion of acetic acid and glucose). Currently, phototrophic production of *Chlorella* is carried out in the open pond, inclined cascades, or semi-closed tubular photo-bioreactors since its excessive growth rate hinders contamination by other microalgae with a total annual production of

about 5000 metric tons. *Chlorella* is the mass cultured eukaryotic alga since it is widely utilized as a health food and feed supplement, as well as in the cosmetics and pharmaceutical industry. It comprises proteins (up to 60% of dry weight), lipids (12–15%), polysaccharides (10–15%), unsaturated fatty acids, and carotenoids (mostly lutein), as well as some immune-stimulators, vitamins, and minerals. It is essential to record that *Chlorella* also can synthesize larger amounts of storage compounds (neutral lipids or polysaccharides) under stress conditions, e.g. under high irradiance with nutrient insufficiency, creating it a promising source for biofuel production (Masojídek and Torzillo, 2014).

The production of biomass requires well-defined conditions. The leading factors that affect the culture of microalgae comprise environmental (temperature, light, pH, salinity, and nutrient supply) biological parameters, physical (agitation) conditions, species of algae, and the shape of culture canister. These above-mentioned parameters affect the biomass productivity, photosynthetic activity, the physiological and biochemical composition of microalgae (Tebbani et al., 2014).

Nitrogen represents a crucial macronutrient for microalgae cultivation which modulates the metabolism and inevitably, the growth and biochemical composition of microalgae (Zarrinmehr et al., 2020). Nitrogen is a critical constituent and a component of high-value biological macromolecules, such as proteins, chlorophylls, and DNA, execute a rudimentary role in microalgae cultivation. Change of nitrogen concentration can affect growth rate, also protein, lipid, and carbohydrate synthesis in microalgae (Pancha et al., 2014).

Many research have confirmed that lack of nitrogen changes biochemical composition, for instance, carbohydrate and protein, fatty acids composition, pigments, and lipid content, as well as photosynthetic activity of microalgae (Fan et al., 2014).

As mentioned before, the growth and nutrient content of microalgae is affected and influenced by culturing media and even a little changes of media would affect the growth and proximate composition of the *Chlorella* sp. either by raising the value or reducing it and caused the benefits of the microalgae no longer can be obtained by the organisms, so the current study will find the best and low-cost media which can out-turn in higher growth and proximate composition of *Chlorella* sp.

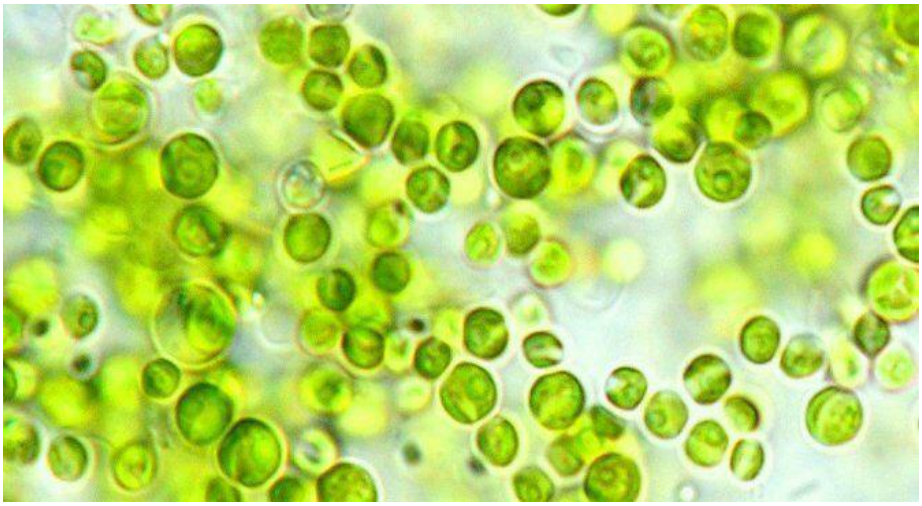


Plate 1: *Chlorella* sp.

1.1 Aims and objectives of the study:

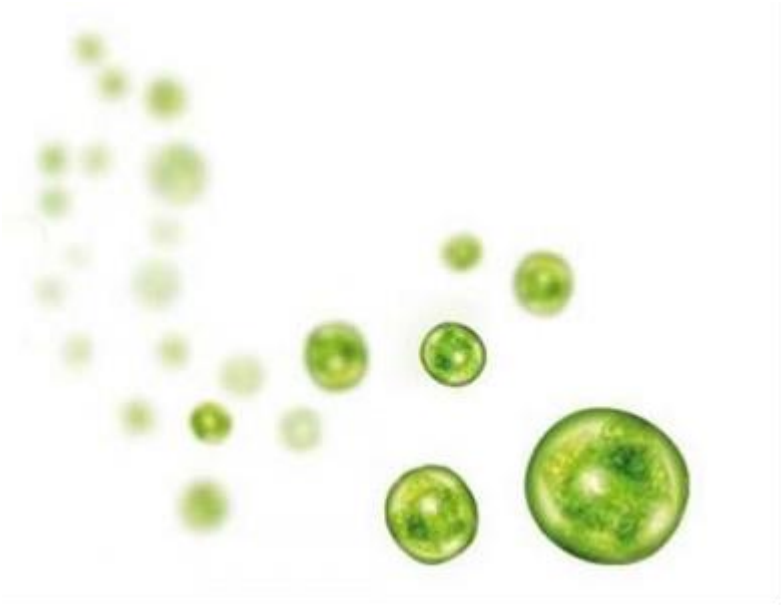
- **Aim**
  - ✓ To compare the growth performance of *Chlorella* sp. cultured at different nitrogen concentrations.
- **Objectives**
  - ✓ To evaluate the proximate composition of *Chlorella* sp. cultured at different nitrogen concentrations.

1.2 Hypothesis

- It is hypothesized that increasing nitrogen concentration in the culture media will either increase or decrease the growth rate of *Chlorella* sp.
- It is hypothesized that increasing nitrogen concentration in the culture media will either enhance or diminish the biochemical composition of *Chlorella* sp.

1.3 Research questions

- Does the increasing nitrogen concentration increase the growth rate of *Chlorella* sp. or decrease the growth rate?
- Does the increasing nitrogen concentration intensify the biochemical composition of *Chlorella* sp. or reduce the biochemical composition?



CHAPTER-2

REVIEW OF LITERATURE

CHAPTER-2

REVIEW OF LITERATURE

Studies regarding the growth and biochemical composition are considered as important tools for understanding the culture strategies of microalgae. Evaluation of the growth conditions at different concentrations of nitrogen in media can give valuable information regarding the biochemical composition of microalgae. This chapter is about the review of sources of nitrogen, nitrogen stress, effects of different concentrations of nitrogen and lipid on cell growth of *Chlorella* sp. The following information was collected to design the present research and validation of the new findings.

2.1 Lipids in microalgae

Wu and Miao (2014) observed that nitrogen impediment was a credible way for stimulating lipids intensification, converting fatty acid compositions, and meliorating biodiesel properties of microalgae.

Zarrinmehr et al. (2020) reported that polyunsaturated fatty acids elevated five-fold under adequate nitrogen concentrations (72 mg/L), in comparison to nitrogen deprivation. On the contrary, the concentration of saturated fatty acids, 75.79% under nitrogen distress, was higher than in the case of nitrogen sufficiency, 36.63%. The highest protein, lipid, and carbohydrate productivities observed were 326.2, 264.6, and 156.7 mg/L under nitrogen concentrations of 0, 72, and 72 mg/L, respectively.

2.2 Source of nitrogen

Simsek and Cetin (2019) concluded that the proportion of lipid and valuable metabolites in *Chlorella vulgaris* grown in media with suitable nitrogen sources can be significantly inflated.

El-Sheekh et al. (2004) studied the growth and biochemical composition of *Scenedesmus obliquus* and *Chlorella kessleri* under different nitrogen sources. Urea stimulated the growth, chlorophyll a, dry weight, and protein content. On the other hand, ammonium-chloride induced the highest carbohydrate content as compared with nitrate, which also increased the total lipids.

2.3 Nitrogen stress

Pancha et al. (2014) investigated the consequences of nitrogen limitation along with subsequent nitrogen starvation on morphological and biochemical changes in *Scenedesmus* sp. CCNM 1077. This respective study opened up that the nitrogen limitation and subsequent nitrogen starvation conditions notably decreases the photosynthetic activity along with crude protein content in the organism, where dry cell weight and biomass productivity remain largely unchanged up to nitrate concentration of about 30.87 mg/L and 3 days nitrate limitation condition. The stress of nitrate was found to possess a significant impact on cell morphology and complete abolition of nitrate from the growth medium resulted in the highest lipid (27.93%) and carbohydrate content (45.74%), making it a promising feedstock for biodiesel and bio-ethanol production.

Wu and Miao (2014) found that the impact of nitrogen limitation on protein content is species-dependent. *Chlorella pyrenoidosa* cultured with nitrate had higher linoleic and linolenic (>54%), resulting in higher DU (>118%) and higher IV (>133 g I2 100g-1) and lower CN values (<44), that is prone to produce biodiesels with poorer ignition properties and oxidation stability.

Shen et al. (2016) studied the consequences of nitrogen insufficiency and different phosphorus supply levels on *Chlorella vulgaris* for producing biodiesel. They found *C. vulgaris* ability to assimilate acetate and accumulate fatty acids concurrently. The highest FAME (fatty acid methyl ester) content (56%) of algae was obtained in nitrogen-deficient media. Charismatically, the production of fatty acids under nitrogen macerated conditions was three times greater than that under nitrogen abundant conditions. Furthermore, the conversion yields of acetate absorbed to fatty acids produced (chemical oxygen demand (COD)-based) in nitrogen-deficient media were too three times higher than those in nitrogen sufficient media.

According to Tam and Wong (1996), stated the final protein content (µg/ml) and the protein value (per cell basis) are generally inflated, when the nitrogen concentration in the medium increased.

Zhu et al. (2014) studied the changes in the metabolism of starch and lipid biosynthesis in the microalgae *Chlorella zofingiensis* under nitrogen deficiency compared to nitrogen abundant conditions. The study revealed that in nitrogen

abounding medium *C. zonfingiensis* exhibit rapid growth and kept stable chlorophyll content, while grievous retardation of cell growth and a prompt degradation of chlorophyll occurred under nitrogen exhaustion.

According to Paes et al. (2016), the chemical profile of microalgae generated mainly by the culture conditions can fluctuate. They studied the consequences of nitrogen deficiency on nutrient uptake, growth, and gross chemical composition of *Chlorella* sp. In absence of nitrogen in culture media, the congregations of all substances varied throughout the growth of *Chlorella* sp., except lipid.

2.4 Effects of nitrogen on lipid production of cells

Singhasuwan et al. (2015) reported different agitation speeds and in media with various initial carbon-to-nitrogen ratios cultivated *Chlorella* sp. TISTR 8990 heterotrophically. They found that the production of the biomass, its total fatty acid content, and the composition of the fatty acids were influenced by the C/N ratio, but not by agitation speed in the range studied.

Zhu et al. (2015) studied the Cellular biochemical composition of the microalga *Chlorella zofingiensis* under flattering and nitrogen-deficient conditions, with the special insistence on lipid classes and fatty acids allocation. They observed that the algal cells were grown in a nitrogen-free medium (N stress) when contents of lipid and carbohydrate were increased while a reduction in protein content was detected. They also concluded that stress cultivation is essential for lipid quality.

Sudha et al. (2013) investigated the growth and lipid content of *Chlorella marina* and *Dunellialla salina* under the effect of different concentrations of nitrogen sources to enhance the lipid content in microalgae. The study reveals, as the nitrogen concentration in the medium reduced, biomass production was also reduced but the lipid content raised and concluded that microalgae species can be used as the promising aspirant in the industrial production of liquid fuel.

2.5 Cell growth

Agirman and Cetin (2015) observed that nitrogen starvation does not have a significant effect on the cell division of *Chlorella vulgaris*.

Zarrinmehr et al. (2020) studied the growth rate and biochemical composition of *Isochrysis galbana* under the effect of different nitrogen concentrations (0, 36, 72,

144, and 288 mg/L). Cell growth, protein content, and pigments decreased with diminishing nitrogen concentration; where carbohydrates exhibit the highest value, 47%, in case of total nitrogen distress.

2.6 Other factors affecting the growth of microalgae

The energy value, as well as the biochemical composition of algae, was altered rapidly due to aging factors and culture conditions such as pH, temperature, and salinity (Pane *et al.*, 2001).

2.6.1 Temperature

Several authors were in favor of using 25° C as temperature for culturing algae because they discovered that the specific growth rate was optimum at this temperature (Weiss *et al.*, 1985; Molina *et al.*, 1991b; Robert *et al.*, 2001).

2.6.2 pH

Several studies had similar initial pH for the filtered and autoclaved seawater medium. For instance, the initial pH for the seawater was pH 8.0 in the study on the growth and biochemical composition with emphasis on the fatty acids of *Tetraselmis* sp. (Molina *et al.*, 1991a). In another study, the influence of temperature and the initial N:P ratios on *Tetraselmis* sp. growth also used the initial pH 8.0 for seawater (Molina *et al*., 1991b). The initial culture pH was 7.8 for a study on cadmium removal by *Tetraselmis* sp. by Pérez- Rama *et al*. (2002).

2.6.3 Salinity

Fábregas *et al*. (1984) stated that the optimum growth conditions for *Tetraselmis suecica* were within the salinity range of 25 ‰ to 35 ‰. The salinity of the seawater was reduced by adding suitable amounts of distilled water (Fábregas *et al*., 1984).

2.6.4 Light

Generally, the light will stimulate the synthesis of fatty acids, growth, and formation of chloroplast membranes in particular; hence, algal overall lipid composition would reflect these morphological alterations (Guschina and Harwood, 2009). Light has a strong influence on the metabolism of algal lipids and the composition of the lipids (Harwood, 1998).

CHAPTER-3

MATERIALS AND METHOD

CHAPTER 3

MATERIALS AND METHOD

The present investigation was conducted to evaluate the growth performance of *Chlorella* sp. grown at different concentrations of nitrogen in the culture media and also determine the effects of nitrogen concentration on the proximate chemical composition of the respected microalgae. The study was based on laboratory work and data were collected for the interpretation of results. To evaluate the growth response and proximate composition of *Chlorella* sp. the following procedures were followed:

3.1 Culture and maintenance of *Chlorella* sp.

The marine microalgae *Chlorella* sp. was collected from the culture stock of the laboratory of Department of Aquaculture, Chattogram Veterinary and Animal Sciences University. Pure *Chlorella* sp. was cultivated in an Erlenmeyer flask consisting of the Conway culture medium. To maintain healthy and good stocks, the subculture was done every two weeks. Then during the experimental period the stock culture was used.

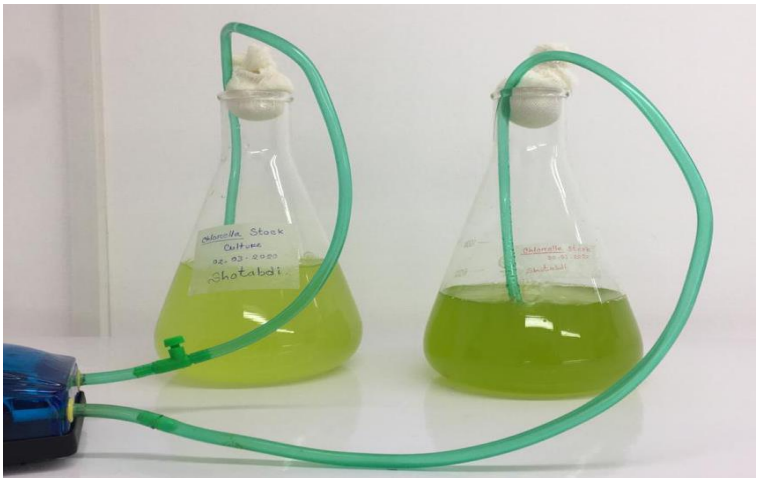


Plate 2: Culture and maintenance of *Chlorella* sp. as stock

3.2 Experiments

The whole experiment for this research project was made up of two parts. In the first part, the growth curve study was followed by the experiment to determine the impact of different nitrogen concentrations on the growth and nutritional profile of the marine microalgae *Chlorella* sp. Both of the experiments were carried out in the Live Feed

Corner and Laboratory of the Aquaculture Department, Faculty of Fisheries at Chattogram Veterinary and Animal Sciences University.

3.3 Purpose of the growth curve experiment

Before starting the main experiment to determine the consequences of different nitrogen concentrations on the growth and nutritional profile in the marine microalgae *Chlorella* sp., it was necessary to carry out the growth curve experiment for *Chlorella* sp. The main purpose of conducting the growth curve experiment on *Chlorella* sp. was to determine the stationary phase of the respective microalgae. This was very important because the knowledge of the stationary phase of *Chlorella* sp. was the fundamental basis of the crucial timing to transfer this microalgae culture grown under normal concentrations of nitrogen ($NaNO_3$) present in Conway medium (100 g/L) to the treatment conditions, whereby the concentrations of nitrogen ($NaNO_3$) present in Conway medium varies depending on the type of treatments. It should be noted that the main minerals stock solution (one of the elements of Conway medium) for 100 g/L $NaNO_3$ was prepared in advance at a concentration of 1,000-fold of the final concentration to use 1 ml of main minerals stock solution per 1,000 ml (1 liter) of filtered and autoclaved seawater respectively (Watanabe, 2005).

3.4 Preparation of culture media

3.4.1 Preparation of natural seawater

As the seawater base, natural seawater was used. Natural seawater of 30 ppt was used which was determined by a hand refractometer (Atago ATC- S/ Mill- E Salinity 0-100%, Japan). By using the vacuum pump filtration unit (Millipore) with 47 mm Ø Whatman® GF/C glass microfiber filter papers the seawater was filtered. After filtration, the seawater was kept in Nalgene® bottles and stored in a cool and dry place before later use.

3.4.2 Preparation of Conway medium

The direct use of natural seawater alone is not feasible in culturing algae because the yield is normally quite low for culture maintenance and laboratory experiments (Harrison and Berges, 2005). Therefore, nutritional enrichments were necessary to boost algal yield. Conway medium was essential as acceptable enrichment for marine microalgae and was comprised of main minerals stock solution, trace metals solution,

vitamin B (thiamine), and vitamin B_{12} (cyanocobalamin) solutions. All the solutions were formulated in different reagent bottles. By convention, per 1000 ml of filtered seawater was combined with 1 ml of main minerals stock solution and 0.5 ml of trace metals solution. The pH 8.0 ± 0.2 was maintained and then autoclaved at 121 °C for 15 minutes (Hirayama Hi- Clave). The mixture was eventually permitted to cool for 1-2 days. Succeding, 0.1 mL of vitamin B (thiamine) and 0.1 mL of vitamin B_{12} (cyanocobalamin) were added to the solution. Therefore, this developed the culture media with Conway medium.

Plate 3: Preparation of main minerals stock solution (100 g/L $NaNO_3$), trace metals solution, vitamin solutions

3.4.3 Main minerals stock solution

The beginning of preparing the main minerals stock solution was to weigh all of the required chemicals accurately using the analytical balance (AND, GR- 200). The name of the weighed chemicals were sodium nitrate (100 g), sodium orthophosphate (20 g), boric acid (33.4 g), sodium EDTA (45 g), manganese chloride (0.36 g), and ferric chloride (1.3 g). The recipe utilize was from James, 1996 with a slight change by using sodium nitrate instead of potassium nitrate. Then, half of a volumetric flask (1000 ml) was loaded with fresh Milli-Q water (Millipore Corp.). After that, the first chemical, sodium nitrate, was mixed into the Milli-Q water and was dissolved by

continuous stirring (Harmony Hotplate Stirrer, HTS- 1003). When it was dissolved completely, the second chemical, sodium orthophosphate, was included in the solution and dissolved by continuous stirring. The aforementioned step was repeated for all the chemicals in particular order till all of them were included in the solution. Hence, the solution was combined to a final volume of 1000 ml with fresh Milli-Q water.

3.4.4 Trace metals solution

For the preparation of the solution, every required chemical was weighed accurately with the analytical balance (AND, GR- 200). Chemicals weighed were zinc chloride (4.2 g), cobalt chloride (4.0 g), ammonium molybdate (1.8 g), and copper sulfate (4.0 g). The recipe was originated from James, 1996. Half of the volumetric flask (1000 ml) was loaded with fresh Milli-Q water (Millipore Corp.). Then, the first chemical, zinc chloride, was included in the Milli-Q water and was dissolved by continuous stirring (Harmony Hotplate Stirrer, HTS- 1003). While it was completely dissolved, the second chemical, cobalt chloride, was incorporated into the solution and was dissolved by continuous stirring too. The aforementioned method was repeated for all the rest remaining chemicals in a particular order until all of them were included in the solution. Afterward, the solution was diluted to a final volume of 1000 ml with fresh Milli-Q water. Finally, the trace metals solution was kept in a Schott- Duran® bottle and stored in a refrigerator (Samsung SilverNano) before later utilization.

3.4.5 Vitamin B (thiamine) and vitamin B_{12} (cyanocobalamin) solutions

At first, half of the volumetric flask (50 ml) was loaded with fresh Milli-Q water (Millipore Corp.). Then, 100 mg of vitamin B (thiamine) was weighed with the analytical balance (AND, GR- 200). The formula utilized was taken from James, 1996. Next, the vitamin B (thiamine) was mixed into the water, dissolved, and then the solution was diluted to a final volume of 50 ml with fresh Milli- Q water. In the end, the vitamin B (thiamine) solution was stored in a tight Schott- Duran® bottle and kept in a refrigerator (Samsung SilverNano) before later utilization.

The method of preparing vitamin B12 (cyanocobalamin) was completely the same as the procedure mentioned to prepare vitamin B (thiamine) solution except that 5 mg of vitamin B12 (cyanocobalamin) was used instead. The formula that was utilized was adapted from James, 1996. Both of the vitamin B (thiamine) and vitamin B12

(cyanocobalamin) solutions were sterilized by filtration as they were heat-labile components and this procedure was suitable and simple for small-volume liquids (Kawachi and Noël, 2005). The two vitamin solutions were sterilized by filtration individually in a laminar flow cabinet operating a sterile, non-pyrogenic 0.20 µm Sartorius MiniSart® single-use filter unit which was fastened to the terminal end of a nonpyrogenic, 10 ml Terumo® syringe.

Plate 4: Main minerals stock solution (100 g/L $NaNO_3$), trace metals solution, vitamin solutions

3.5 Main minerals stock solutions with modified $NaNO_3$ concentrations

One and all the solution was kept and tightly- capped in a reagent bottle and stored in a refrigerator (Samsung SilverNano) till later use. The modified main minerals stock solution with discrete N concentrations was prepared where nitrate ($NaNO_3$) was used as the major source of nitrogen. Thus, the main minerals stock solution was developed with five individual treatments shown in detail in Table 1, respectively. In the end, the main minerals stock solution was kept in a Schott- Duran® bottle and stored in a refrigerator (Samsung SilverNano) before later utilization.

Table 1: Different concentrations of nitrate ($NaNO_3$) with the corresponding N concentrations of each treatment

Treatments	**NaNO3 conc. (g/L)**	**Corresponding N conc. (g/L)**
Treatment 1 (T1)	50	8
Treatment 2 (T2)	75	12
Control Treatment 3 (T3)	100	16
Treatment 4 (T4)	125	20
Treatment 5 (T5)	150	24

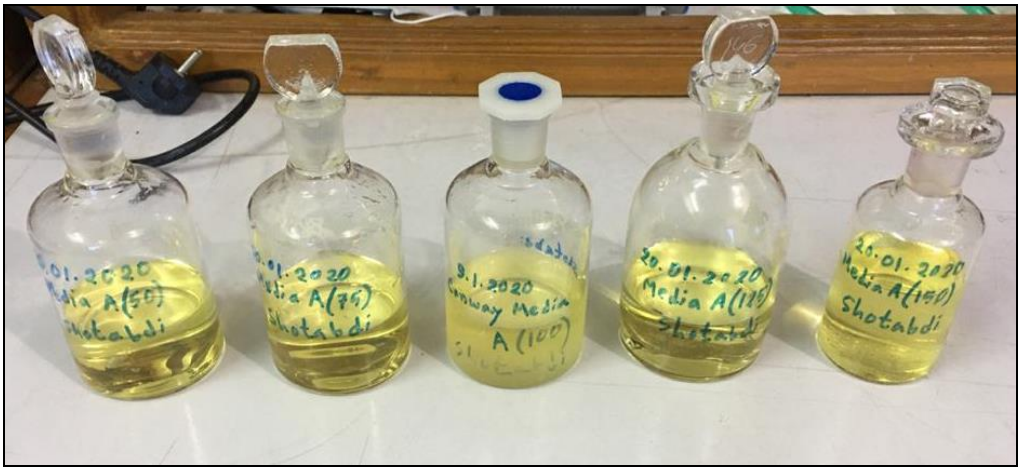


Plate 5: Main minerals stock solution with 50 g/L, 75 g/L, 100 g/L (Control), 125 g/L and 150 g/L concentration of Sodium nitrate ($NaNO_3$)

3.6 Experimental design for growth curve experiment

The culture volume for *Chlorella* sp. in the growth curve experiment was 300 ml that was taken in sterile borosilicate Erlenmeyer flasks (500 ml). The initial composition inside each culture flask was Conway culture medium (270 ml) and *Chlorella* sp. culture (30 ml). There were duplicated cultures of *Chlorella* sp. for carrying out the analysis. The growth curve experiment was conducted until the stationary phase of *Chlorella* sp. was obtained based on the patterns of growth of the constructed growth curve. Both of the *Chlorella* sp. cultures were maintained near to 25 ± 2 °C in the

vicinity of light intensity of 2000 lux applying cool fluorescent light. The photoperiod was 24:0 h and the cultures were aerated continuously with natural sterile air using an air pump.

3.6.1 Measurement of parameters for growth curve experiment

Analysis for the determination of absorbance readings at the wavelength of 580 nm (UV-1601 UV- Visible Spectrophotometer SHIMADZU), cell counts (Bright-line improved Neubauer hemacytometer, 0.1 mm chambers deep, Assistant, Germany), and biomass (dry weight basis) for *Chlorella* sp. were carried out daily throughout the growth curve experiment.

3.6.2 Determination of optical density (OD)

The growths of the cultures were monitored daily throughout the growth curve experiment by determining the optical density of the culture aliquots using a spectrophotometer (UV-1601 UV- Visible Spectrophotometer SHIMADZU). The culture medium for *Chlorella* sp. was employed as the blanks. The wavelength of 580 nm was taken for the absorbance readings.

3.6.3 Determination of cell density

Growth and densities of the culture were also monitored daily during the growth curve experiment by resolution of cell density of the culture aliquots. The haemacytometer (Bright-line improved Neubauer hemacytometer, 0.1 mm deep chambers, Assistant, Germany) and its coverslip (0.0025 mm^2) were rinsed with Milli-Q water (Millipore Corp.) before filling up of the chambers accompanied by culture samples. Uniformity of cell distribution was monitored under low power magnification (4X and 10X) of the microscope (Nikon E600). Cells of both chambers of the haemacytometer under magnification of 40X were counted.

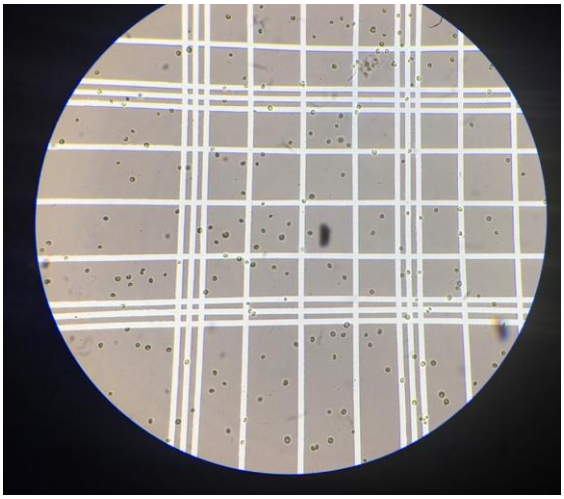


Plate 6: Counting cell under the microscope using haemacytometer

The formulae to count the cells are as mentioned below:

Cell density calculation (cells/ml) for 5 squares = Total no. of cells / (10 x 4 x 10^{-6})

Here, 10 stands in for the 10 squares of the 2 hemacytometer chambers, and 4 x 10^{-6} stand-in for the volume of samples over the small square areas which were equivalent to 0.004 mm^3 (0.2 mm x 0.2 mm x 0.1 mm) indicated in cm^3 (ml).

3.7 Experimental design

The experiment was done in 2 L glass flasks with three replicates. Different concentration of nitrogen in Sodium nitrate ($NaN0_3$) was used in this experiment. Before studying the effects of nitrogen concentrations on the growth rate and nutritional profile, growth curve analysis was conducted because it is important to know the more appropriate phase (exponential or stationary) to inoculate *Chlorella* sp. in 1.5 L Conway media at different nitrogen concentrations. There were five treatments varied by decreasing and increasing the concentration of nitrogen of Conway medium. There were three replicates for each treatment. *Chlorella* sp. was cultivated in 1.5 L Conway medium with different concentrations of nitrogen ($NaN0_3$) which were 50, 75, 100 (control), 125, and 150 g/L. All the cultures were maintained in a test chamber near to 24 ± 1 °C in the vicinity of light intensity of 150 μE m^{-2} s^{-1} applying cool fluorescent light with supplying CO_2 to the cultures and 24 hours continuous illumination. The preliminary cell density was $1X10^3$ cells/ml for all treatments.

Growth parameter such as cell density, optical density in the culture was measured every day. Cells were harvested one day before the stationary phase where cells are matured and nutrient and cells are balanced by centrifugation at 8000 rpm as long as 10 min followed by rinsing twice with sterilized distilled water. The collected biomass was freeze-dried and kept at -20° C for proximate composition analysis.

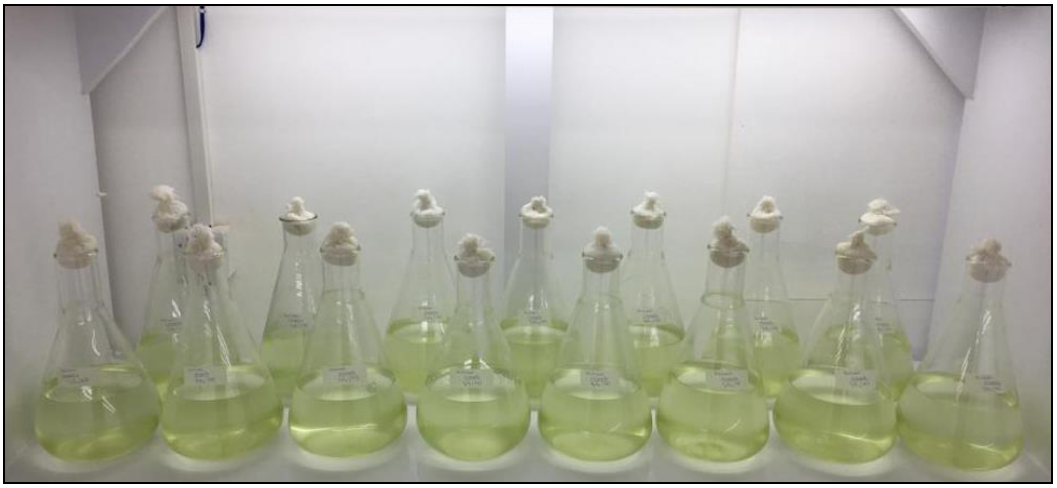


Plate 7: Experiment setup

3.8 Analysis of protein, lipid, and carbohydrate

Protein was determined according to Lowry et al. (1951). For each sample, 25 mL well mixed samples were prepared by using 5mg dried biomass with distilled water. 0.5 mL from 25 ml solution was taken for each sample for protein analysis. 1 mL Reactive 1 mixed with 50 mL of Reactive 2. Then, 0.5 mL sample and 0.5 mL 0f 1N NaOH was mixed and placed in a hot water for 5 minutes bath at 100 ºC. Subsequently, the samples were cooled in a cold-water bath, and 2.5 ml of the prepared mixed reagent was added 10 minutes after cooling. After that, 0.5 mL of Falin reagent was added to the mixed reagent, and then kept in a dark place for 30 minutes. The absorbance of the mixed solution was measured using spectrophotometer at 750 nm wavelength. To develop a calibration graph, 2000 µg/L of standard (albumin) stock solution was prepared, and a series of standards were prepared (20 µg/L, 40 µg/L, 80 µg/L, 100 µg/L and 200 µg/L) from the stock solution. The same procedures as described for protein analysis were applied for the standard series; a calibration line was plotted according to the absorbance, and the protein composition for each sample was determined accordingly.

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

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

3.9 Data analysis

The data were analyzed by one-way analysis of variance (ANOVA) comparing the different concentrations of N accompanied by a significance level of 95% ($p < 0.05$) along with the Tukey multiple comparisons test (where applicable). Graphical representation of the growth specifications and biochemical compositions (% dry weight) for different treatments were analyzed by Origin V8, Microsoft Office 2016, SPSS software, respectively. All the outcomes have been represented as average ± standard deviation.

CHAPTER-4

RESULTS

CHAPTER 4

RESULTS

The results section represents the findings of an investigation in a systematic, logical, concise, and comprehensive manner. This section presents detailed information of the present research work about the effects of different nitrogen concentrations on the growth and nutritional profile of *Chlorella* sp.

4.1 Growth of microalgae under different nitrogen concentrations

The cell density (cells mL^{-1}) and optical density (580 nm) of autochthonous marine microalgae *Chlorella* sp. cultivated in a controlled environment in response to five different N concentrations for $NaNO_3$ are shown in Figure 1. The result shows that *Chlorella* sp. cultivated under different N concentrations had extended their stationary phase almost on the same day. However, no significant differences ($p > 0.05$) were observed among T1 (50 g/L), T2 (75 g/L) and T3 (100 g/L). The initial cell density (Cells/mL) remarkably increased after 7 days of cultivation in all cultures with different nitrogen concentrations. The minimal (5.225×10^6 cells/mL) cell concentration achieved in T4 (125 g/L) was significant to ($p < 0.05$) the control T3 (100 g/L) which exhibit the cell density of 6.25×10^6 cells/mL (Fig. 1a). But maximum (6.333×10^6 cells/mL) cell concentration achieved in T1 (50 g/L) was not significant ($p > 0.05$) to the control T3 (100 g/L) which showed the cell density of 6.25×10^6 cells/mL (Fig. 1a). Moreover, the commensurate N concentrations of the growth medium for each treatment had significantly ($p < 0.05$) different, regarding nitrate ($NaNO_3$) (Table 1).1a

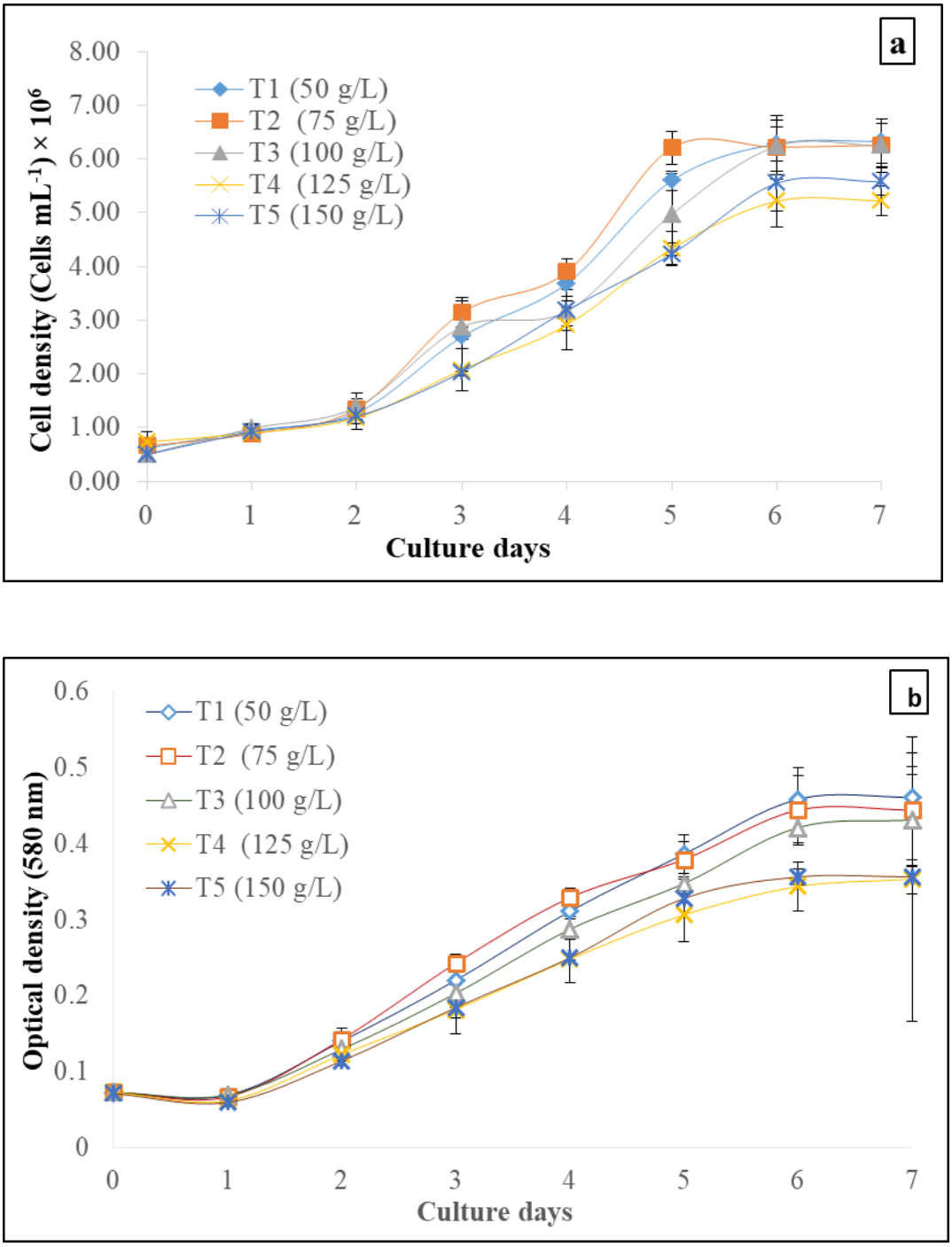


Figure 1: Effects of different N concentrations on (a) cell density (cells/mL), (b) optical density (580 nm) of *Chlorella* sp. in five different $NaNO_3$ concentrations (Values are average ± standard deviation).

Maximum density had found in T1 (50 g/L) (Fig. 1a) with the cell density of 6.333×10^6 cells/mL, which was not significant ($p > 0.05$) regarding control T3 (100 g/L). *Chlorella* sp. showed lower cell density in T4 (125 g/L) comprised of 5.225×10^6 cells/mL which was significant ($p < 0.05$) regarding control T3 (100 g/L).

The results also revealed that the increased N concentrations in the culture media had significantly ($p < 0.05$) complement the growth performance, where *Chlorella* sp. reached the stationary phase at day 7 relatively with low growth performance ($p > 0.05$).

An indistinguishable vogue had observed for optical density (OD) measurements by absorbance reading (580 nm) (Fig. 1b). The lowest optical density 0.352 (comprising 20 g/L N), and the maximum optical density 0.461 (comprising 8 g/L N) were recorded, regarding $NaNO_3$, was significant ($p < 0.05$) to each other, respectively.

4.2 Effects of different nitrogen concentrations on proximate composition

Variation in nitrogen concentrations in the growth medium of *Chlorella* sp. had a significant ($p < 0.05$) impact on protein, carbohydrate, and lipid content, concerning nitrate ($NaNO_3$) being a source of N (Fig. 2a, 2b, and 2c).

The protein content exhibits significant ($p < 0.05$) changes of different vogue for nitrate ($NaNO_3$), concerning sufficient or deficient N concentrations (Fig. 2a). Inside the growth medium of *Chlorella* sp. provided with 8 g/L N, protein content (% dry weight) exhibited the highest percentage which was about 31.90% dry weight were recorded from T1 (50 g/L) was significant ($p < 0.05$) to the control treatment T3 (100 g/L) which showed the protein content about 24.94% dry weight (comprising 16 g/L N). Increment of N concentrations in the culture medium significantly ($p < 0.05$) decreased the protein content (% dry weight). The lowest protein content of 21.98% dry weight (comprising 24 g/L N) was observed in T5 (150 g/L) which was not significant ($p > 0.05$) regarding the control T3 (100 g/L).

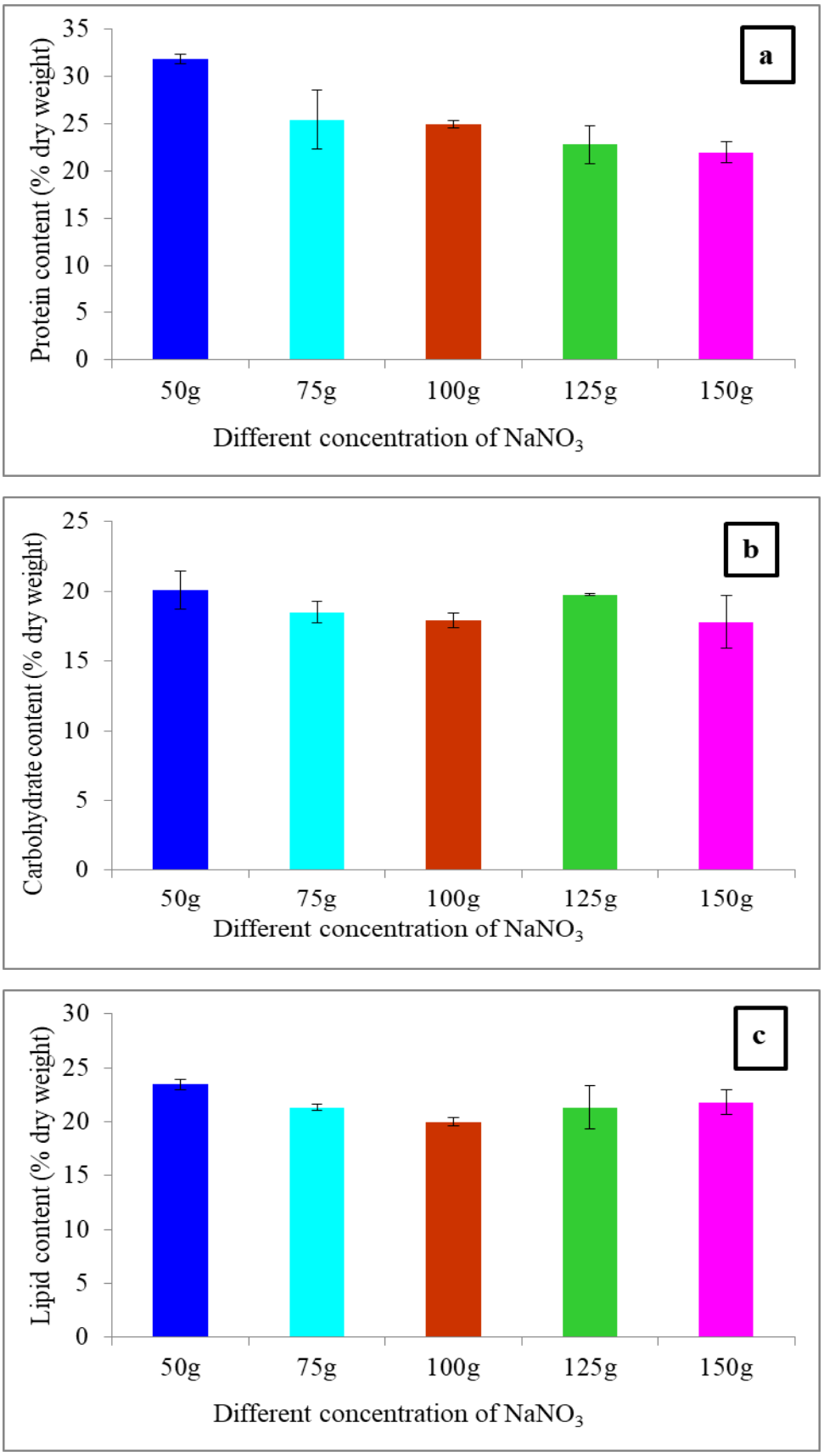
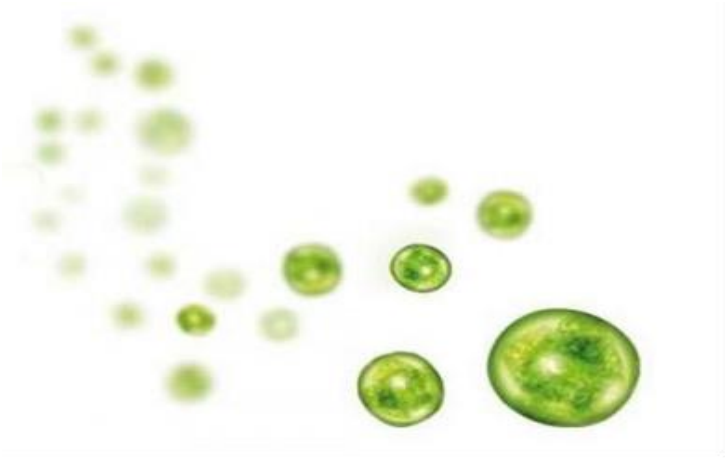


Figure 2: Proximate composition (2a. protein, 2b. carbohydrate, and 2c. lipid) (% dry weight) of *Chlorella* sp. under different concentrations of nitrate $NaNO_3$ (Values are average ± standard deviation).

The carbohydrate content exhibits significant ($p < 0.05$) substitutions of contrary vogue for nitrate ($NaNO_3$), concerning sufficient or deficient N concentrations (Fig. 2b). The growth medium of *Chlorella* sp. which was supplied with 8 g/L N, carbohydrate content (% dry weight) revealed the highest percentage which was about 20.07% dry weight were recorded from T1 (50 g/L) had no significance ($p > 0.05$) in respect to the control T3 (100 g/L) which showed the carbohydrate content about 17.91% dry weight (comprising 16 g/L N). The lowest carbohydrate content 17.80% dry weight (comprising 24 g/L N) was showed by T5 (150 g/L) which had no significance ($p > 0.05$) in respect to the control T3 (100 g/L). The other treatment T2 (75 g/L) and T4 (125 g/L) had shown the carbohydrate content in percent which were 18.49% and 19.76%, was also not significant ($p > 0.05$) in respect to the control T3 (100 g/L).

The lipid content exhibit a significant ($p < 0.05$) difference of contrary vogue for nitrate ($NaNO_3$), regarding sufficient or deficient N concentrations (Fig. 2c). The growth medium of *Chlorella* sp. that was provided with 8 g/L N, lipid content (% dry weight) exhibit the highest percentage which was about 23.47% dry weight were recorded from T1 (50 g/L) had no significance ($p > 0.05$) regarding the control T3 (100 g/L) which showed the lowest lipid content about 20.00% dry weight (comprising 16 g/L N).



CHAPTER-5

DISCUSSION

CHAPTER 5

DISCUSSION

This research work was designed to evaluate the growth performance and proximate composition of *Chlorella* sp. grown in culture medium containing different concentrations of nitrogen. This chapter described the comparison of overall results, the achievement of the study, and problems associated with the research work. Information of previous experience from other studies around the world was taken into consideration to accomplish the respective research.

5.1 Growth of microalgae under different nitrogen concentrations

Being one of the most essential nutrients, nitrogen influences the cell growth of microalgae, and the growth rate of the algal cells is significantly affected by the concentrations of nitrogen in the growth medium (Wu and Miao, 2014). Moreover, the biochemical compositions of microalgae are also significantly affected by the concentrations of nitrogen in the growth medium (Kim et al., 2016; Wang et al., 2013). The current investigation revealed that *Chlorella* sp. having little amount of nitrogen concentration of 8 g/L N exhibit the highest cell density. On the other hand raising concentrations of N in the culture media, lowest density of 5.225×10^6 cells/mL was registered from nitrate (NaNO3), considering 20 g/L N. The aforementioned data have too certainly corresponded to the optical density estimation during the experimental session. The results obtained from the present study is more or less in agreement with those reported by Saha et al., 2013 and Gu et al., 2015 on the growth performance of *Chlorella* sp. grown in culture medium containing different concentrations of nitrogen.

Saha et al. (2013) reported that Chlorella growth is faster in cultures with low nitrogen content. The present study revealed the positive growth performance of *Chlorella* sp. with decreasing concentration of nitrogen.

Similar work by Gu et al. (2015) also revealed that various species such as *Chlorella vulgaris*, *S. acutus*, *N. oleoabundans*, and *Nannochloropsis* sp. had a good growth under nitrogen-insufficient conditions by utilizing their intracellular nitrogen reserves resembling pigment-protein molecules, that's too supports the current findings that are growing cells concentration within low nitrogen concentrations as well. The

current investigation over and above unfolded the decreasing growth performance of *Chlorella* sp. with inflated nitrogen concentration at 24 g/L N for $NaNO_3$.

Furthermore, it is recorded by Razaghi et al., 2014 that excessive rising of the concentrations of nitrogen causes hindrance of other vitamins, trace metals, and nutrients, plus negatively act on the growth of the cell under that condition.

5.2 Effects of different nitrogen concentrations on proximate composition

The microalgal cell growth and biochemical composition differ from species to species in respect of favorable nitrogen concentrations (Kim et al., 2016). In this current research, *Chlorella* sp. within different nitrogen concentrations appreciably gave rise to protein content unto 31.90% dry weight concerning the decrement of nitrogen concentrations in the growth medium.

A similar vogue had observed for carbohydrate concentrations regarding $NaNO_3$. Fernandez-Reiriz et al. (1989) as well documented that production of protein is favored with adequate nitrogen as well as lessened productivity when the nitrogen is insufficient, while production of carbohydrate inflated with the deficient nitrogen concentrations too.

Further research by El-Kassas (2013) also described the similar concept in consideration of *Picochlorum* sp. where carbohydrate content raised by about 21% and 44% supplemented with 50% and 100% NaNO3 within the growth medium, however, the protein content was reduced notably by 54% and 69.7% under the similar concentrations, respectively.

The consequences of preliminary nitrogen concentrations on the growth of microalgae as well as lipid content of microalgae were investigated. In the current research, *Chlorella* sp. under various concentrations of nitrogen significantly influenced lipid content unto 23.47% concerning the decrement of concentrations of nitrogen in the growth medium.

Research by Sudha et al. (2013) studied the effects of different nitrogen concentrations on the growth plus lipid content on *Chlorella marina* and *Dunellialla salina* to enhance the lipid content in microalgae. The study revealed that when the concentration of nitrogen in the medium lessened, production of biomass was also reduced yet the lipid content rises.

CHAPTER-6

CONCLUSION

CHAPTER-6

CONCLUSION

Microalgae are photosynthetic single-celled algae or phytoplankton which utilize light energy plus carbon dioxide, with higher photosynthetic efficiency compared to plants for the production of biomass. Nitrogen is one of the greatest crucial nutrients which act on the biochemical composition and cell growth of microalgae. *Chlorella sp*. is one of the most promising species of microalgae. The response of *Chlorella* sp. under different concentrations of nitrogen was investigated. Deficient and sufficient concentrations of nitrogen influenced distress in *Chlorella* sp. as compared to the control group (100 g/L), production of protein and carbohydrate increased under 50 g/L and decreased under 125 g/L nitrogen. Raising the concentration of nitrogen from 100 to 150 g/L decreased cell concentrations, yet at lower concentrations (50 g/L) of nitrogen, the cell density increased. The findings of this research exhibits that both the growth rate and proximate composition of *Chlorella* sp. can be directed by suitably changing the concentration of nitrogen in the culture medium. Advanced research work should be done to intensify the growth rate and proximate composition by using different nitrogen sources such as- Urea, KNO_3, NH_4NO_3, etc.

CHAPTER-7

RECOMMENDATIONS AND FUTURE PERSPECTIVES

CHAPTER-7

RECOMMENDATIONS AND FUTURE PERSPECTIVES

This research intended was to describe the consequences of different nitrogen concentrations on the growth and biochemical composition of *Chlorella* sp. In the hatchery phase of various aquacultures, microalgae and zooplankton represent an important role being feed for larval and juvenile crustaceans and fish. Fish are important as an export item to the economies of Bangladesh. People prefer good quality fish and this fish can fulfill the nutritional requirements of the human body. Various algae are being reported as a promising source of protein in consideration of both livestock and human consumption. Disseminating the findings of the present study to the fish farmers or people will facilitate to improve live feed production, provide the employment opportunities, poverty reduction, and also production of quality aquaculture fish. So, this study will be directly helpful to the marginal fishermen, fish farmers for large-scale production of quality fish at the lowest cost all year round.

Although a qualitative approach was followed to explore the objective of the research, there are some limitations of the study which can be minimized by following the recommendations:

- ✓ Microalgae should be isolated and identified directly from the sea because authentic samples lead to a concrete result.
- ✓ Different sample collection sites should be followed.
- ✓ Different concentrations of nitrogen should be used for further study.
- ✓ Different sources of nitrogen such as- urea, ammonium nitrate, potassium nitrate, etc. should be taken under consideration.
- ✓ Fresh and properly preserved samples give better output. So, samples should be used as early as possible after collection.
- ✓ For enhancing the nutritional quality of fish and zooplankton, microalgae should be used either as a full or partial enrichment.

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APPENDIX

APPENDIX A

Pictures of laboratory activities

Plate no 8: Laboratory activities

Brief Biography

Brief Biography

Afsana Haque, Daughter of Md. Zahurul Haque and Aleya Haque from Tangail District of Bangladesh. She completed her graduation on B.Sc. Fisheries (Hon's) in 2017 from the Faculty of Fisheries of Chattogram Veterinary and Animal Sciences University (CVASU), Chattogram, Bangladesh with CGPA 3.61 out of 4.00. She has a strong passion for the research of fisheries and her research interests are on the nutritional value of different fish and fish related products, live feed, development of various fish and fish-related products, etc. Now, she is a candidate for the degree of MS in Fishing and Post-Harvest Technology under the Department of Fishing and Post-Harvest Technology, Faculty of Fisheries, Chattogram Veterinary and Animal Sciences University (CVASU).