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Inorganic Carbon Supplementation to Culture Medium Enhancing Growth Performance and Pigmentation of Freshwater Microalgae *Chlorella ellipsoidea*

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ABSTRACT

The availability of inorganic carbon in the culture media is a limiting factor for the growth of photosynthetic microalgae. However, the expense of supplying carbon dioxide to culture is a significant fraction of total energy usage. Bicarbonate salts, when added to the growth medium, can be an inexpensive inorganic carbon source for microalgae. In this study, *Chlorella ellipsoidea* was used to assess the capability of this species to utilize bicarbonate and determine the impact of this carbon source on growth performance and pigmentation. In a batch experiment, 0.25, 0.5, 1.0, 1.5, and 2.0 g/L of sodium bicarbonate was added to Bold's basal medium. Growth values were compared to those of the control group that received only Bold's basal medium. *C. ellipsoidea* was able to grow in all concentrations of bicarbonate and had a high capacity for biomass production. The exponential growth was increased with the addition of sodium bicarbonate to Bold's basal medium, and it continued on the fifteenth day of culture. Among the culture media, Bold's basal medium with 1.5 g/L sodium bicarbonate had the highest cell density (41.17 ± 0.23 x 105 cells/mL), cell dry weight (24.55 ± 0.12 mg/L), optical density (1.33 ± 0.05), and chlorophyll *a* and chlorophyll *b* contents (6.51 ± 0.09 mg/L and 3.97 ± 0.13 mg/L, respectively). The specific growth rate significantly increased due to the addition of sodium bicarbonate up to 1.5 g/L level. The highest purity (77.04 ± 0.32 %) was found in 1.0 g/L sodium bicarbonate supplementation to Bold's basal medium followed by 73.44 ± 0.32 % purity in 1.5 g/L supplementation. The study revealed that bicarbonate can stimulate algal growth. An appropriate supply of sodium bicarbonate at 1.5 g/L to Bold's basal medium as an inorganic carbon source can be considered an acceptable alternative to carbon dioxide for the production of *C. ellipsoidea.*

INTRODUCTION

Microalgae are a group of fast-growing unicellular or simple multicellular microorganisms with greater photosynthetic efficiency and biomass conversion than terrestrial plants **(Miao & Wu, 2006)**. They are used in numerous commercial applications, including human food, aquafeed supplements, nutraceuticals and biofuel

generation **(Guccione *et al*., 2014; Li *et al*., 2018)**. Microalgae manufacture their food by autotrophic nutrition, which is mostly stored as starch **(Choix *et al*., 2012; Yadala & Cremaschi, 2014)**. In aquatic ecosystems, microalgae are regarded as the most fundamental producers in the food chain **(De Silva *et al*., 2018)**. The potential of microalgae from the genus *Chlorella* to produce important nutritional compounds, such as vital amino acids, polyunsaturated fatty acids, carotene and vitamins, has attracted scientific attention in recent years **(Vadlamani *et al*., 2018; Sampathkumar & Gothandam, 2019)**.

Chlorella ellipsoidea is a fast-growing freshwater microalgae that acts as a vital food supplement for aquatic organisms promoting their growth, reproduction and survival **(Rahman *et al*., 2005; Mandal & Mallick, 2009; Akter *et al*., 2016)**. **Brown *et al*. (1997)** also reported *C. ellipsoidea* as a promising primary producer and a possible source of several nutritive components, such as proteins, carbohydrates, polyunsaturated fatty acids (PUFA), amino acids, vitamins and minerals.

Environmental factors impact microalgal growth and biochemical composition **(Guiheneuf *et al*., 2008)**. Therefore, the culture medium has a significant effect on the growth and quality of microalgae, and Bold's basal medium (BBM) is extensively employed for the cultivation of *Chlorella* sp. **(Xin *et al*., 2010; Ilavarasi *et al*., 2011)**. However, the availability of inorganic carbon sources stimulates phototrophic cell growth and lipid accumulation in both freshwater and marine microalgae **(White *et al*., 2013)**.

The cultivation system is often supplied with inorganic carbon in three ways: (1) as pumping air, (2) and as pumping air with concentrated CO_2 (usually in microalgae production is an approach used to produce a high level of biomass; nevertheless, the overall process may be expensive and inefficient due to CO_2 loss to the atmosphere **(Acien *et al*., 2012; Nunez & Quigg, 2016)**. The CO_2 content of ambient air is quite low (0.04%); hence, a considerable quantity of air is required to aerate the culture, resulting in high energy consumption due to the enormous amount of energy required for pumping **(Markou *et al*., 2014)**. In fact, technological features of capture, compression, transportation, temporary storage issues and gas loss can account for up to fifty percent of the cost of biomass production **(Chi *et al*., 2011; Chisti, 2013)**.

Bicarbonate salts could be employed as an alternate carbon source for algal production **(Chi *et al*., 2013; 2014)**. These salts are more soluble in water than carbon dioxide (i.e., sodium bicarbonate ($NaHCO_3$) solubility > 90 g/L at 25 °C); hence, their use efficiency is anticipated to be greater than that of CO_2 (**Chi *et al*., 2014; Markou *et al*., 2014)**. The $NaHCO_3$ has been shown to increase growth and lipid accumulation in some microalgae **(Gardner *et al*., 2013; White *et al*., 2013; Peng *et al*., 2014)** and could be a more cost-effective and environmentally friendly alternative **(Kim *et al*., 2014; De Farias Silva *et al*., 2016)**. Accordingly, the addition of $NaHCO_3$ to culture media improved inorganic carbon uptake for microalgal cell proliferation and maximized *Chlorella* sp. production **(Mokashi *et al*., 2016)**. In this regard, evaluating the

development and photosynthetic efficiency of the green microalga *C. ellipsoida* cultivated at various bicarbonate concentrations was the main goal of the current investigation.

MATERIALS AND METHODS

Culture of *C. ellipsoidea* and experimental conditions

C. ellipsoidea maintenance cultures were grown in 250 mL Erlenmeyer shake flasks containing Bold's basal medium (BBM) at 135 rpm mixing (Temp. 24 ± 1.0 °C) and under a constant illumination of 29 μmol photons/m^2/s (5x.OSRAM L 18W/965 Biolux fluorescent lamps). The light intensity was measured with a lux meter (DeltaOHM HD 9221).

Experimental cultures of *C. ellipsoidea* were grown in BBM (control) and BBM, supplemented with varying doses of $NaHCO_3$ (0.25 g/L, 0.5 g/L, 1.0 g/L, 1.5 g/L, and 2.0 g/L) (**Srinivasan *et al.*, 2018**). As a supply of bicarbonate, Sigma-Aldrich (St. Louis, Missouri, United States) analytical grade $NaHCO_3$ was utilized. The BBM for the maintenance cultures and the control was prepared in MilliQ- water containing the following components (mg/L): $NaNO_3$ 250.0, $MgSO_4.7H_2O$ 75.0, NaCl 25.0, K_2HPO_4 75.0, KH_2PO_4 175.0, $CaCl_2.2H_2O$ 25.0, $ZnSO_4.7H_2O$ 8.80, $MnCl_2.4H_2O$ 1.50, MoO_3 0.70, $CuSO_4.5H_2O$ 1.60 (**Bernstein *et al.*, 2014**).

C. ellipsoidea was grown in a batch procedure in a conical flask containing 1.0 L of growth medium. Throughout the experiment, there were no additional CO_2 insufflations beyond what was already present in the flushing air. The light source was a cool white LED (T5 15W 6400K, 80 mol m-2s-1), with continuous illumination light (LED in the red and blue spectra, 200 mol photons/m^2/s). The medium was autoclaved after the pH was brought down to 7.5 by adding 1 M HCl. An air pump (ACO-003), a non-sterile disposable syringe filter (Millex Syringe Filter, Nylon, 0.45 m pore size) and an aquarium air curtain were used to provide the sterile air. Over the duration of the 18-day experiment, samples were taken every three days.

Estimation of growth parameters

Using an upgraded Neubauer rule hemocytometer and a light microscope (ZEISS PrimoStar), the cell density of *C. ellipsoidea* was calculated using the following mathematical expression **(Clesceri *et al.*, 1989)**:

Number of cells/mL suspension = Mean number of *C. ellipsoidea* cells/square × Dilution factor ×10^4

Dry cell weight (DCW) was measured using cellulose acetate filters of 0.45μm (Whatman®). To eliminate any moisture from the filters, they were pre-dried for 10 minutes at 105°C. The biomass was filtered and dried at 105°C for two hours before being weighed, and its dry weight was determined using the following formula **(Clesceri *et al.*, 1989)**:

W= [(FFW-IFW) / Volume of the sample filtered (mL)] x 100
Where, W= Dry cell weight (g/L); FFW= Final filter weight (g) and IFW= Initial filter weight (g).

The sample of *C. ellipsoidea* grown under different treatments was placed in a cuvette, and its optical density (OD) at 620 nm was measured with a spectrophotometer (DR 5000™, UV-Vis).

The purity of culture media was assessed using a hemocytometer. If the *C. ellipsoidea* count was X and the other microalgae was Y, then the sum was (X + Y) = A. Therefore, the percentage of *C. ellipsoidea* purity was (X / A) × 100.

The formula below was used to calculate the specific growth rate of *C. ellipsoidea*:
$\mu = (\ln (N_t/N_0)) / (T_t-T_0)$
Where, N_0 and N_t are, respectively, the total number of cells at the start of the log phase (T_0) and the end of the log phase (T_t).

Estimation of pigment content

Chlorophyll *a* was determined through spectrophotometric analysis. For this, a 10mL- culture sample that had been filtered through 25mm Whatman® GF/F filters was mashed with a glass rod, blended with 10mL of 100 % redistilled acetone, and then left overnight in the refrigerator. Following homogenization, the materials were centrifuged at 4000rpm for 10 minutes. The supernatant was separated, and the absorbance of the light green supernatant was measured at three wavelengths **(Akter *et al.*, 2019)** using a spectrophotometer ((BK-UV1800, BIOBASE). The formula shown below was then used to calculate Chlorophyll *a* **(Clesceri *et al.*, 1989)**:
Chlorophyll *a* (mg/L) = 11.85(OD 664) - 1.54(OD 647) - 0.08(OD 630)

The chlorophyll *b* concentration was measured in triplicate in accordance with Fathi *et al.* (2013). Briefly, 5 ml of *C. ellipsoidea* culture was collected and centrifuged for 10 minutes at 4000 rcf at 15 °C. After discarding the supernatant, the algal culture was centrifuged once again to get rid of any remaining salt. The algal pellet was then placed in a tube containing 5 ml of an 80% acetone solution and left overnight. The tube was then centrifuged at 4,000 rcf at 15 °C for 10 min and placed in a UV-Vis spectrophotometer (BK-UV1800, BIOBASE) to measure the light absorbance at 412, 431, 460, and 480 nm while using 100% acetone as a blank. Lastly, the following formula was used to determine the amount of chlorophyll *b*:
Chlorophyll *b* (mg/L) =−0.171 (A412)−0.230(A431)+ 11.871(A460)− 13.248(A480)

Determination of physico-chemical properties of the culture media

Every three days, physico-chemical parameters were monitored, such as

temperature (Hach hq40d multi-analyzer, USA), dissolved oxygen (Hach hq40d multi-analyzer, USA), pH (SensIONTM+ PH3) and light intensity (LX-9621, China).

Statistical analysis

One-way analysis of variance (ANOVA) and Tukey's post-hoc test for honestly significant difference (HSD) were used to statistically examine all the data. The significance threshold was fixed at $P< 0.05$. The data were reported as means with a standard deviation (SD), and IBM SPSS Statistics V21 was used for the statistical analysis.

RESULTS AND DISCUSSION

Physico-chemical properties

Microalgal growth is greatly influenced by environmental variables such as light, temperature, dissolved oxygen and pH **(Giardono *et al*., 2005; Cho *et al*., 2007; Khoeyi *et al*., 2012)**. In this study, the growth performance of *C. ellipsoidea* was significantly affected by the various environmental conditions of culture media. In the present investigation, the temperature was fluctuated between 26.8 ± 0.2°C and 28.4 ± 0.2°C (Table 1). The highest temperature was recorded on the 15th day of culture for treatment T_3, whereas the lowest was recorded on the 3rd day for treatment T_5. The optimal temperature range for the growths of *Chlorella* sp. is 25 °C to 30 °C **(Rahman *et al*., 2005; Converti *et al*., 2009)**. *C. ellipsoidea* flourished at temperatures between 26.5 to 28.5 °C **(Alam *et al*., 2003; Toyub *et al*., 2007)**. Therefore, the temperature in this study fell within the optimal range for the cultivation of *C. ellipsoidea*.

Table 1. Physico-chemical parameters of culture medium under different $NaHCO_3$ supplementations to BBM

$NaHCO_3$ (g/L)	Parameter	Sampling time (day)						
		Initial	3	6	9	12	15	18
0	Temperature (°C)	26.9±0.13	26.9 ± 0.21	26.9 ± 0.30	27.3 ± 0.35	27.8 ± 0.43	27.7 ± 0.16	28.0 ± 0.26
	DO (mg/L)	4.80 ± 0.12	4.83 ± 0.11	4.86 ± 0.09	4.93 ± 0.16	5.20 ± 0.11	5.00 ± 0.13	4.79 ± 0.16
	pH	7.50 ± 0.00	7.59 ± 0.21	7.64 ± 0.11	7.81 ± 0.13	7.98 ± 0.13	8.17 ± 0.14	8.26 ± 0.09
0.25	Temperature (°C)	26.9 ± 0.33	27.1 ± 0.21	26.9 ± 0.30	27.5 ± 0.35	27.8 ± 0.33	27.2 ± 0.16	27.1 ± 0.25
	DO (mg/L)	4.72 ± 0.12	4.73 ± 0.11	4.89 ± 0.13	4.83 ± 0.09	4.79 ± 0.11	4.92 ± 0.03	4.99 ± 0.16
	pH	7.50 ± 0.00	7.67 ± 0.21	7.60 ± 0.31	7.82 ± 0.13	8.01 ± 0.17	8.16 ± 0.17	8.26 ± 0.21
0.50	Temperature (°C)	26.9 ± 0.03	27.2 ± 0.21	27.6 ± 0.30	27.2 ± 0.35	27.8 ± 0.63	27.8 ± 0.16	28.4 ± 0.21
	DO (mg/L)	4.79 ± 0.18	4.83 ± 0.11	5.00 ± 0.23	4.63 ± 0.16	5.29 ± 0.11	4.92 ± 0.16	4.98 ± 0.15
	pH	7.50 ± 0.00	7.58± 0.22	7.72 ± 0.13	7.83 ± 0.22	7.98 ± 0.01	8.19 ± 0.14	8.24 ± 0.06
1.00	Temperature (°C)	27.1 ± 0.23	27.1 ± 0.21	27.3 ± 0.30	27.7 ± 0.34	28.0 ± 0.63	27.7 ± 0.17	28.1 ± 0.26
	DO (mg/L)	4.80 ± 0.12	4.73 ± 0.11	4.76 ± 0.13	4.73 ± 0.26	5.28 ± 0.11	4.92 ± 0.33	4.98 ± 0.16
	pH	7.50 ± 0.00	7.59 ± 0.23	7.80 ± 0.16	7.85 ± 0.17	7.92 ± 0.21	8.11 ± 0.04	8.25 ± 0.16
1.50	Temperature (°C)	27.0 ± 0.23	26.8 ± 0.20	27.8 ± 0.30	27.8± 0.35	27.6 ± 0.6	27.9 ± 0.2	28.3 ± 0.3
	DO (mg/L)	4.83 ± 0.12	4.86 ± 0.11	5.10 ± 0.14	4.63 ± 0.09	5.62 ± 0.18	4.68 ± 0.07	4.86 ± 0.16
	pH	7.50 ± 0.00	7.59 ± 0.12	7.69 ± 0.15	7.89 ± 0.12	7.91 ± 0.11	8.10 ± 0.14	8.24 ± 0.15
2.00	Temperature (°C)	27.2 ± 0.2	27.3 ± 0.2	27.6 ± 0.3	28.0 ± 0.4	27.9 ± 0.3	28.0 ± 0.16	28.2 ± 0.3
	DO (mg/L)	4.90 ± 0.13	4.71 ± 0.21	4.91 ± 0.33	4.73 ± 0.16	5.85 ± 0.13	4.72 ± 0.17	4.76 ± 0.15
	pH	7.50 ± 0.00	7.56 ± 0.21	7.68 ± 0.11	7.86 ± 0.19	8.02 ± 0.31	8.15 ± 0.11	8.29 ± 0.14

Note: Values are expressed as mean ± SD, n = 3.

DO varied between 4.63 & 5.85mg/ l in all treatments, with no significant changes over time. This happened because in this investigation, the cultures were

regularly aerated. Some studies reported that the highest growth of *C. ellipsoidea* was observed at DO more than 4.5mg/ L in BBM medium, which is compatible with the findings of the present study **(Karmaker *et al.*, 2001; Rahman *et al.*, 2005)**. Light intensity recorded in this experiment varied between 2170 and 2310 lux/m^2/s, which was within the optimal range of *C. ellipsoidea*, for which an optimal range of 2000 to 2500 lux/m^2/s has been described **(Mondal *et al.*, 2005; Rahman *et al.*, 2005)**. At the beginning of the experiment, the pH was adjusted to 7.50 for all treatments, and it rose steadily as the culture time progressed. However, there were no significant variations in pH between treatments on a given day, likely due to the use of bicarbonate ions in the medium for algae photosynthesis and development **(Jaysanker & Valsala, 2008; Gardner *et al.*, 2013)**. However, the pH in all treatments throughout the study period was also within the optimal range for microalgae **(Fathi *et al.*, 2013; Fatemeh & Mohsen, 2016)**. This experiment revealed that the physical and chemical conditions of culture media in all treatments were optimal for *C. ellipsoidea* cultivation.

Effect of $NaHCO_3$ supplementation on cell density

In all treatments, cell density rose gradually until the 15^{th} day, when it declined. At the end of the 15-day exponential phase, the addition of $NaHCO_3$ to culture media significantly increased the cell density of *C. ellipsoidea*, which varied from 21.08 ± 0.28 to 41.17 ± 0.23 (× 10^5 cells/mL) compared to 19.08 ± 0.30 (×10^5 cells/mL) in the control. **Rahman *et al.* (2005)** conducted an experiment in which the maximum cell density for *C. ellipsoidea* cultivation ranged from 36.2 to 43.8 (× 10^5 cells/mL). **Karmaker *et al.* (2001)** observed a similar range for the cell density of *C. ellipsoidea* in another investigation, ranging from 33.6 to 68.28 (× 10^5 cells/mL). As shown in Fig. (1), the addition of 1.5g/ L $NaHCO_3$ to BBM considerably increased ($P< 0.05$) the cell density on day 15; however, increasing the $NaHCO_3$ level further decreased the cell density. This decrease in cell density was caused by the fact that adding bicarbonate with higher concentrations results in an increase in Na^+ ions in the growth medium, which most freshwater organisms cannot tolerate **(Chen & Jiang, 2009; Chi *et al.*, 2014)**. *Chlorella vulgaris* had the best bicarbonate tolerance at 1.0g/ L, whereas *Nannochloropsis salina*, a marine microalga, developed more quickly and had higher cell densities in cultures that contained $NaHCO_3$ at 5.0g/ L **(Pal *et al.*, 2011; Mokashi *et al.* 2016)**. In the present study, *C. ellipsoidea* responded optimum to 1.5g/ L $NaHCO_3$, and further addition (2.0 g/L) resulted in lower cell densities, comparable to the findings of **Jayasankar and Valsala (2008)** for *C. salina*.

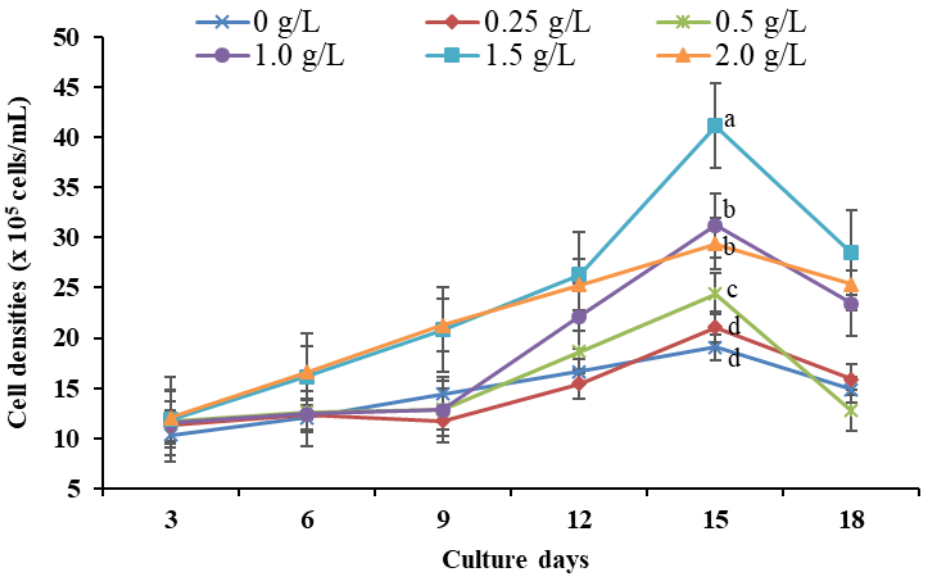

Fig. 1. Cell densities (×10^5 cells/mL) in *C. ellipsoidea* control (BBM) and bicarbonate enriched cultures ($NaHCO_3$ 0.25, 0.5, 1.0, 1.5 and 2.0 g/L). Error bars represent SD (n = 3). The values with the same letter were not significantly different ($P< 0.05$, ANOVA, Tukey's HSD).

Effect of $NaHCO_3$ supplementation on dry cell weight (DCW)

Among the treatments, $NaHCO_3$ supplemented media demonstrated superior DCW than the medium without supplementation (control medium) that are shown in Table (2). At the end of the 15 days exponential phase, the minimum average DCW was 19.34 ± 0.10mg/ L (control, BBM). In contrast, the maximum value was 24.55 ± 0.12 mg/L when 1.5g/ L $NaHCO_3$ was added, which was followed by 1.00, 2.00, 0.50 and 0.25 g/L $NaHCO_3$, respectively. In all the treatments, except control, DCW was increased with the addition of $NaHCO_3$ (up to 1.5g/ L) to the culture medium and tended to decrease with supplementation of 2.0 g/L $NaHCO_3$. In addition, lower $NaHCO_3$ supplementation concentrations (0.25 and 0.5g/ L) resulted in decreased DCW throughout the culture.

Table 2. Dry cell weight (mg/L) of *C. ellipsoidea* cultured under different $NaHCO_2$ supplementations to BBM

$NaHCO_3$ (g/L)	Culture period (days)					
	3	6	9	12	15	18
0	2.88 ± 0.03^{e}	6.36 ± 0.10^{f}	11.85 ± 0.07^{d}	15.67 ± 0.11^{e}	19.34 ± 0.10^{e}	17.60 ± 0.11^{f}
0.25	2.95 ± 0.07^{d}	7.43 ± 0.11^{e}	11.93 ± 0.10^{c}	16.59 ± 0.12^{d}	21.07 ± 0.13^{d}	19.74 ± 0.12^{e}
0.50	3.12 ± 0.04^{c}	7.75 ± 0.09^{d}	11.34 ± 0.11^{e}	17.06 ± 0.06^{c}	22.04 ± 0.11^{c}	20.10 ± 0.14^{d}
1.00	3.33 ± 0.05^{b}	8.02 ± 0.10^{c}	11.77 ± 0.10^{d}	17.17 ± 0.12^{b}	23.30 ± 0.05^{b}	20.48 ± 0.10^{c}
1.50	3.41 ± 0.04^{a}	9.42 ± 0.12^{a}	14.16 ± 0.11^{a}	18.12 ± 0.13^{a}	24.55 ± 0.12^{a}	22.56 ± 0.10^{a}
2.00	3.32 ± 0.03^{b}	8.73 ± 0.11^{b}	12.56 ± 0.12^{b}	17.23 ± 0.10^{b}	23.24 ± 0.11^{b}	22.13 ± 0.12^{b}

Note: Values are expressed as mean ± SD, n = 3. Values in each column that have different superscripts are statistically different ($P< 0.05$).

In this investigation, the growth performance of *C. ellipsoidea* under comparable carbon levels is consistent with those of other microalgae species **(Sanchez-Saavedra *et al.*, 1996; Shu *et al.*, 2012; Woodworth *et al.*, 2015)**. According to **Mokashi *et al.* (2016)**, adding 1.0 g/L of $NaHCO_3$ to the growth medium for *Chlorella* sp. resulted in significantly larger biomass ($P< 0.05$). In another study, DCW production of *C. vulgaris* rose with increasing $NaHCO_3$ concentration, reaching a maximum at 1.2g/ L, and a further increase in $NaHCO_3$ concentration inhibited DCW production **(Yeh *et al.*, 2010)**. Cultivation conditions can optimize the algal biomass production rates. Carbon limitations resulting from the poor solubility of CO_2 in water can hinder plant growth **(Giordano *et al.*, 2005; Aishvarya *et al.*, 2012)**. $NaHCO_3$ is more soluble than CO_2 and could serve as an alternative inorganic carbon source for microalgal culture **(Hsueh *et al.*, 2007)**. Accordingly, the addition of 1.5g/ L $NaHCO_3$ to BBM was suitable for *C. ellipsoidea* biomass generation in the present experiment.

Influence of $NaHCO_3$ on optical density, purity, and growth rate

Table (3) summarizes the effects of $NaHCO_3$ supplementation on the optical density, purity and specific growth rate of *C. ellipsoidea*. Optical density (1.33 ± 0.05) and specific growth rate (0.174 ± 0.02 μ/day) were substantially greater in BBM supplemented with 1.5g/ L $NaHCO_3$. However, the highest purity (77.04 ± 0.32%) was achieved with 1.0g/ L $NaHCO_3$, followed by 73.73 ± 0.22% with 1.5g/ L $NaHCO_3$. Control, without $NaHCO_3$, exhibited the lowest growth performance ($P< 0.05$), except for purity, which was the lowest (61.92 ± 0.21%) in treatment with 0.5 g/L $NaHCO_3$ supplementation.

Table 3. Optical density, purity and specific growth rate for *C. ellipsoidea* culture under different $NaHCO_2$ supplementations to culture media

NaHCO3 (g/L)	Optical density	Purity (%)	Specific growth rate (μ/day)
0	0.66 ± 0.01^{e}	67.23 ± 0.16^{d}	0.149 ± 0.01^{b}
0.25	0.71 ± 0.01^{d}	70.55 ± 0.19^{c}	0.157 ± 0.02^{ab}
0.50	0.72 ± 0.01^{d}	61.92 ± 0.21^{e}	0.157 ± 0.01^{ab}
1.00	1.09 ± 0.03^{b}	77.04 ± 0.32^{a}	0.158 ± 0.01^{ab}
1.50	1.33 ± 0.05^{a}	73.73 ± 0.22^{b}	0.174 ± 0.02^{a}
2.00	0.98 ± 0.01^{c}	70.39 ± 0.28^{c}	0.163 ± 0.01^{b}

Note: Values are expressed as mean ± SD, n = 3. Values with different superscripts in each column are significantly different ($P< 0.05$).

The specific growth rate of the microalgae population is another indicator of the increase in biomass, and a time-dependent rise in the specific growth rate was found in the present study. **Mokashi *et al.* (2016)** found the highest specific growth rate (0.653 μ /day) for *Chlorella vulgaris* when 1.0g/ L $NaHCO_3$ was added to the culture medium. However, the highest specific growth rate was found in up to 1.5g/ L $NaHCO_3$ supplementations in the present study, which was comparatively lower than that obtained

by **Mokashi *et al.* (2016)**. These discrepancies may be attributable to differences in species and culture media. The best purity was obtained in the current study when 1.00–1.50g/ L of $NaHCO_3$ was added. **Banerjee *et al.* (2016)** tested with *Chlorella* sp. cultivation in low-cost media and obtained a higher cell density of 29.5 ($\times 10^5$ cells/mL) with 76.6% purity, which is comparable to the purity observed in the current experiment. **Peng *et al.* (2014)** claimed that, sufficient $NaHCO_3$ concentrations caused protozoa to decline in cultures, possibly as a result of increasing osmotic pressure-induced water outflow. These benefits, rapid dose-dependent effects on growth, photosynthetic efficiency, and pigment content, in addition to the sodium bicarbonate's bactericidal and bacteriostatic effects on the culture medium, indicate that this salt can serve as a substitute for gaseous CO_2.

Effects of $NaHCO_3$ supplementation on pigmentation

During the culture period, pigment concentrations (chlorophyll *a*, chlorophyll *b*) of *C. ellipsoidea* increased until the exponential phase (15^{th} day), at which point they began to decline (Table 4). At the end of the exponential phase, chlorophyll *a* (6.51 ± 0.14 mg/L) and chlorophyll *b* (3.97 ± 0.13 mg/L) were substantially higher ($P< 0.05$) in the 1.5g/ L $NaHCO_3$ supplemented culture compared to the control (5.46 ± 0.12 and 3.20 ± 0.11 mg/L, respectively). Some other microalgae recorded an increase in chlorophyll content upon the increase in $NaHCO_3$ concentration in the growing medium **(White *et al.*, 2013)**.

Table 4. Pigment contents of *C. ellipsoidea* cultured under different $NaHCO_3$ supplementations to culture media

$NaHCO_3$ (g/L)	Sampling time (day)					
	3	**6**	**9**	**12**	**15**	**18**
	Chlorophyll *a* (mg/L)					
0	1.22 ± 0.04	2.81 ± 0.07d	4.66 ± 0.08c	4.72 ± 0.08e	5.46 ± 0.12d	5.23 ± 0.09d
0.25	1.28 ± 0.05	3.10 ± 0.07c	4.73 ± 0.07bc	4.62 ± 0.11e	5.76 ± 0.09c	5.41 ± 0.14c
0.50	1.18 ± 0.03	3.23 ± 0.08c	4.92 ± 0.05b	5.24 ± 0.07d	6.01 ± 0.13b	5.60 ± 0.09b
1.00	1.13 ± 0.04	3.63 ± 0.06b	4.73 ± 0.09bc	5.51 ± 0.10c	6.14 ± 0.14b	5.69 ± 0.08b
1.50	1.25 ± 0.09	3.93 ± 0.04a	5.20 ± 0.06a	6.32 ± 0.07a	6.51 ± 0.09a	6.03 ± 0.12a
2.00	1.21± 0.08	3.70 ± 0.09b	5.00 ± 0.08b	5.82 ± 0.09b	6.03 ± 0.11b	5.71 ± 0.13b
	Chlorophyll *b* (mg/L)					
0	0.87 ± 0.03	1.23 ± 0.06c	1.62 ± 0.09c	2.11 ± 0.10d	3.20 ± 0.11d	2.95 ± 0.11c
0.25	0.86 ± 0.05	1.32 ± 0.07bc	1.72 ± 0.09c	2.27 ± 0.07c	3.54 ± 0.08c	3.13 ± 0.07c
0.50	0.95 ± 0.03	1.39 ± 0.04b	1.88 ± 0.11a	2.33 ± 0.07c	3.79 ± 0.09b	3.54 ± 0.06b
1.00	0.93 ± 0.04	1.43 ± 0.09b	1.81 ± 0.08a	2.53 ± 0.10b	3.66 ± 0.12b	3.59 ± 0.08b
1.50	0.99 ± 0.06	1.62 ± 0.06a	1.94 ± 0.07a	2.72 ± 0.09a	3.97 ± 0.13a	3.76 ± 0.11a
2.00	0.94 ± 0.07	1.59 ± 0.08a	1.83 ± 0.09a	2.63 ± 0.06a	3.81 ± 0.08a	3.72 ± 0.07a

Note: Letters within the same column for a specific pigment indicate significant ($P< 0.05$) differences of means within the treatments (n = 3).

Chlorophyll is typically recognized as a reliable and accepted indication of algal biomass **(Wiltshire *et al.*, 1998; Knefelkamp *et al.*, 2007)**. In this study, chlorophyll concentration was taken into account as an indicator of how inorganic carbon (sodium bicarbonate) affects the physiology of *C. ellipsoidea* because carbon reflects the physiological condition of microalgae to chlorophyll ratio. Both the chlorophyll *a* and

chlorophyll *b* contents of cultures were higher in the maximum bicarbonate concentration and decreased with decreasing concentrations. In the presence of 1.0g/ L $NaHCO_3$, chlorophyll biosynthesis was increased in *C. vulgaris* **(Mokashi *et al.*, 2016)**. However, it was 0.5g/ L for the maximum chlorophyll production in *C. salina* **(Jayasankar & Valsala, 2008)**. The addition of bicarbonate to *C. sorokiniana* culture led to a rise in chlorophyll *a* for the duration of the experiment, with the highest levels in cells supplemented with 3g/ L $NaHCO_3$ **(Salbitani *et al.*, 2020)**. The rise in chlorophyll *a* in the experimental cultures may be a result of the increased availability of inorganic carbon in the growth media, which is required for pigment synthesis. Since chlorophyll content is a valid predictor of the physiological status of microalgae **(Srinivasan *et al.*, 2018)**, the high amounts of chlorophyll seen with bicarbonate treatments indicate healthy cells. It can be inferred that the addition of bicarbonate at the concentrations used in this study did not impair the photosynthetic performance of the algal cells since the amount of phaeopigments in plant cells typically increases in response to abiotic stress (pH, temperature, etc.) or with age **(Hortensteiner, 2006; Borghini *et al.*, 2009)**. In the present study, cell density and cell dry weight exhibited a strong positive connection with chlorophyll *a* and chlorophyll *b* pigments across all treatments (Table 5).

Table 5. Pearson correlation analysis among the growth parameters and pigment contents under different treatments

$NaHCO_3$ (g/L)	Parameter	Cell density	Cell dry weight	Chlorophyll *a*	Chlorophyll *b*
0	Cell density	1			
	Cell dry weight	0.976**	1		
	Chlorophyll *a*	0.988**	0.964**	1	
	Chlorophyl *b*	0.983**	0.958**	0.996**	1
0.25	Cell density	1			
	Cell dry weight	0.968**	1		
	Chlorophyll *a*	0.970**	0.969**	1	
	Chlorophyll *b*	0.989**	0.943**	0.993**	1
0.50	Cell density	1			
	Cell dry weight	0.975**	1		
	Chlorophyll *a*	0.983**	0.969**	1	
	Chlorophyll *b*	0.978**	0.975**	0.989**	1
1.00	Cell density	1			
	Cell dry weight	0.977**	1		
	Chlorophyll *a*	0.984**	0.970**	1	
	Chlorophyll *b*	0.989**	0.978**	0.993**	1
1.50	Cell density	1			
	Cell dry weight	0.969**	1		
	Chlorophyll *a*	0.975**	0.977**	1	
	Chlorophyll *b*	0.989**	0.968**	0.990**	1
2.00	Cell density	1			
	Cell dry weight	0.976**	1		
	Chlorophyll *a*	0.983**	0.973**	1	
	Chlorophyll *b*	0.973**	0.959**	0.991**	1

Note: Correlation is significant at the 0.01 level (2-tailed). Correlation coefficients (r) are indicated with numeric values. The scale of the correlation coefficient is $0.8 \leq r \leq 1.0$: A very strong positive correlation.

In light of algal growth parameters from the present investigation, it can be concluded that the culture of *C. ellipsoidea* was significantly affected by the addition of inorganic carbon sources such as $NaHCO_3$ to BBM. Furthermore, whereas bicarbonate salts may easily be supplied to algal plants and stored until needed, gaseous CO_2 requires expensive storage and transportation. Bicarbonate's inclusion promotes the creation of cellular material, which leads to maximum productivity and increases inorganic carbon absorption. While, the addition of bicarbonate to microalgae cultures has not always resulted in favorable outcomes, particularly for freshwater species **(Chi *et al*., 2014)**. In reality, introducing bicarbonate causes an increase in Na+ ions in the growth medium, which most freshwater organisms cannot tolerate **(Chi *et al*., 2014)**. A high bicarbonate concentration (160 mM) hindered cell development in *C. vulgaris*, resulting in the creation of many colonial cells **(Li *et al*., 2018)**. According to **De Farias Silva *et al*. (2016)**, an excessive amount of $NaHCO_3$ caused damage to the PSII complex in *Synechococcus* sp., which resulted in salt stress and the formation of ROS (reactive oxygen species) in addition to a decrease in photosynthetic efficiency. Data from the current study indicate that bicarbonate treatment of *C. ellipsoidea* cells did not cause any damage, indicating that the concentrations of up to 1.5g/ L used in this study were below the level that was deemed tolerable.

CONCLUSION

This investigation demonstrated that the growth performance of *C. ellipsoidea* was enhanced by the addition of $NaHCO_3$ during the exponential phase, which lasted until the 15^{th} day of culture and then began to decline on the 18^{th} day. *C. ellipsoidea* grew considerably ($P< 0.05$) faster when 1.5g/ L $NaHCO_3$ was added to BBM than in the control (without $NaHCO_3$); however, further addition of $NaHCO_3$ inhibited algal growth. It is possible that the addition of inorganic carbon $NaHCO_3$ has a beneficial effect on microalgal culture, and that a sufficient supply of carbon sources promote the growth and photosynthetic efficiency of *C. ellipsoidea*. In creating strategies for eco-sustainable microalgae cultivation, the use of $NaHCO_3$ should be regarded as a suitable alternative to the use of gaseous CO_2 that might contribute to the same CO_2 mitigation processes. In addition, a similar trend was noted in the amount of pigment per cell. A further consideration is that the addition of bicarbonate reduces bacterial growth, indicating bacteriostatic and bactericidal action. All these results suggest that the use of bicarbonate in *C. ellipsoidea* culture may be an effective substitute for CO_2 insufflation, both for the intensive development of the culture and for the quick production of potentially beneficial compounds.

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