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Full Length Research Paper

Studies on the growth behavior of *Chlorella, Haematococcus* and *Scenedesmus* sp. in culture media with different concentrations of sodium bicarbonate and carbon dioxide gas

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Growth studies were conducted on green algae *Chlorella, Scenedesmus* and *Haematococcus* strains in batch mode cultures. In this study, the effect of sodium bicarbonate salt ($NaHCO_3$) and carbon dioxide (CO_2) gas as carbon source on microalgal cultures were investigated. For this purpose, growth response of the aforementioned three strains under varying concentrations of $NaHCO_3$ (15, 30, 45, 60 and 75 mg/L,) and CO_2 gas (7929, 4758 and 4400 mg/L,) were investigated. The best growth response showed by *chlorella* strain was observed at 75 mg/L (ppm) bicarbonate (% increase in biomass=82.6mg/L/day for 12 days) and 4758 mg/L CO_2 gas concentration (189.1 mg/L/day for 7 days). While *Haematococcus* strain showed its best growth in 30 ppm bicarbonate (72.9 mg/L/day for 17 days) and 4758 mg/L CO_2 gas (134.1 mg/L for 7 days), the *Scenedesmus* strain showed its best growth in 45 ppm bicarbonate (30.9 mg/L/day for 17 days) and 4758 mg/L CO_2 gas (103.8 mg/L for 7 days). All the strains showed good growth when CO_2 gas was supplied in terms of increase in cell number, biomass and lipid content compared to bicarbonate utilization as carbon source, except *Haematococcus* strain which fail to grow when high concentration of CO_2 gas (7929 ppm) was supplied. Out of the three strains, it was *Chlorella sp.* which showed highest growth rate and lipid content when CO_2 gas was supplied, (specific growth=0.704; 189.1% increase in biomass, g/L/day and 1.015 doubling/day, 31% lipid content in terms of dry cell weight).

Key words: Microalgae, bicarbonate, biomass, lipid.

INTRODUCTION

Biological carbon IV oxide (CO_2) mitigation has attracted much attention in terms of CO_2 fixation through photosynthesis as it leads to production of biomass energy (Kondili and Kaldellis, 2007), (Ragauskas et al., 2006), (de Morais and Costa, 2007). However, the potential for increased CO_2 capture in agriculture by plants has been estimated to contribute only 3-6% of fossil fuel emissions (Skjanes et al., 2007), largely due to the slow growth rates of conventional terrestrial plants. On the other hand, microalgae a group of fast growing unicellular or simple multicellular micro organism has the ability to fix CO_2 while capturing solar energy with an efficiency of 10 to 50 times greater than that of terrestrial plants and higher biomass production compared to energy crops (Wang et al., 2008).

The main environmental factors influencing microalgal growth and chemical composition are light, nutrients, temperature and pH (Rousch et al., 2003). Carbon source is an essential factor for microalgal growth (Wen and Chen, 2003). Generally, the carbon source for microalgae in culture condition is CO_2 from atmosphere which is naturally present at approximately 300 ppm. Microalgae has higher growth rate and CO_2 fixing ability

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Abbreviations: CA, Carbonic anhydrase; **DIC,** dissolved inorganic carbon.

Table 1. CO_2 Tolerance of various species

Species	Known maximum CO_2 concentration (%)	Reference
Cyanidium caldanum	100	Seckbach et al., 1971
Scenedesmus sp.	80	Hanagta et al.,1992
Chlorococcum littorale	60	Kodama et al., 1993
Synechococcus elongatus	60	Miyairi, 1997
Euglena gracilis	45	Nakano et al., 1996
Chlorella sp.	40	Hanagta et al., 1992
Eudorina sp.	20	Hanagta et al., 1992
Dunaliella tertiolecta	15	Nagase et al., 1998
Nannochloris sp.	15	Yoshihara et al., 1996
Chlamydomonas sp.	15	Miura et al., 1993
Tetraselmis sp.	14	Matsumoto et al., 1995

Source, Mark E. Huntley (University of Hawaii) and Donald G. Redalje (University of Southern Mississippi).

and it could completely recycle CO_2 as again CO_2 is converted to chemical energy via photosynthesis, which can be converted to fuels using existing technologies.

Microalgae can fix CO_2 from different sources which can be categorized as (i) CO_2 from atmosphere, (ii) CO_2 from industrial flue gases, and (iii) fixed CO_2 in the form of soluble carbonates ($NaHCO_3/Na_2CO_3$). CO_2 could be directly fed into microalgae culture for biofixation but sometimes it is difficult to obtain a stable and consistent supply of CO_2 unless the location of microalgae cultivation system is very close to a factory or power plant. So one of the alternative way to prepare a large amount of inorganic carbon source for microalgal growth is to capture the CO_2 emitted form industries by alkali absorption and stored it in the liquid as HCO_3^- and CO_3^{2-} ions (Wang et al., 2008). In this way, it would be easier and more convenient to supply carbon source for phototropic growth of microalgae. Also, since bicarbonate and carbonate are much more soluble than CO_2 the problem with the low solubility and low retention time of CO_2 in the medium could be avoided. However the metabolic efficiency and resulting microalgae composition of using CO_2 and bicarbonate /carbonate as carbon source could be different from species to species (de Morais and Costa, 2007). It was found that the solubility of CO_2 gas is 1.45 g/L at 25°C, 100 K Pa. When CO_2 dissolve in water, three inorganic carbon species are produced, namely CO_2 (aq), bicarbonate and carbonate ions. The equilibrium concentrations of the various carbonate species in aqueous solution are controlled by the pH of the solution. More specifically, at a pH below about 4.5, the carbonate species will consist entirely of carbonic acid (H_2CO_3). As the pH is increased to a value of about 8.5, the carbonate species will consist entirely of bicarbonate (HCO_3^-) and as the pH is raised above 8.5, the predominant carbonate species will be carbonate (CO_3^{2-}) (Huber et al., 1999).

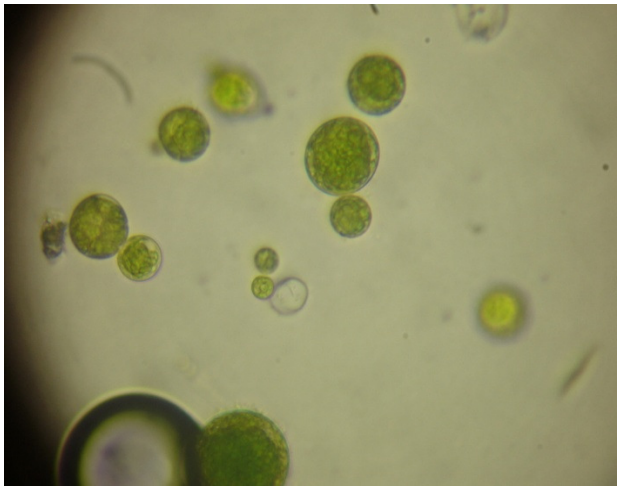
A number of microalgal species have been shown to be able to utilize carbonate such as $NaHCO_3$ and Na_2CO_3 for cell growth (Huertas et al., 2000), (Ginzburg, 1993), (Merrette et al., 1996). Some microalgal species have high extracellular carbonic anhydrase (CA) activities (Huertas et al., 2000), which is responsible for the conversion of carbonate to free CO_2 to facilitate CO_2 assimilation. In addition, the direct uptake of bicarbonate by an active transport system has been found in several species (Colman and Rotatore, 1995). It was also observed previously that $NaHCO_3$ is a better carbon source for most of the microalgal strains than using Na_2CO_3. Adoption of carbonate utilizing strains for CO_2 fixation could be advantageous in many aspects: (i) CO_2 released in night time from industrial facilities could be converted to carbonate salts and stored for conversion in day time, (ii) as only a limited number of microalgal species thieve in media containing high concentration of carbonate salts, so species control become simple (that is, preventing contamination), and (iii) most of microalgal species have high pH optima (in the range of 9.0-11) further simplifying species control (Table 1).

Microalgae have CA on their cell surface and can utilize bicarbonate as well as CO_2. Kinetic study revealed that most of the bicarbonate is utilized after this ion is converted to CO_2 via CA located on the cell surface. Therefore, the actual molecular species which crosses the plasma lemma is mostly free CO_2. The apparent Km (CO_2) values for photosynthesis in most microalgae grown in ordinary air (low CO_2 cells) are as low as in terrestrial C_4 plants, although the algal cells fix CO_2 via C_3 pathway. In contrast the apparent Km (CO_2) values in cells grown on CO_2 enriched air (high CO_2 cells) are as high as those in the terrestrial C_3 plants. This indicates that efficiency of dissolved inorganic carbon (DIC) utilization for photosynthesis in low CO_2 cells is very high. The activity of CA in low CO_2 cells is higher than that in high CO_2 cells (Aizawa and Miyachi, 1986).

This study was aimed at determining the effect of elevated $NaHCO_3$ and CO_2 gas concentration under different pH conditions on green algae *Chlorella,*

Table 2. The composition of BG11 media

Major element	Composition
$NaNO_3$	1.5 g
K_2HPO_4	0.04 g
$MgSO_4 \cdot 7H_2O$	0.075 g
$CaCl_2 \cdot 2H_2O$	0.036 g
Citric acid	0.006 g
Ferric ammonium citrate	0.006 g
EDTA (disodium salt)	0.001 g
$NaCO_3$	0.02 g
Trace metal mix A5	1.0 ml



Photograph 1. *Haematococcus* strain under 40X magnification.

Haematococcus and *Scenedesmus.* The response was monitored in terms of changes in biomass, cell count, cell densities and lipid content.

MATERIALS AND METHODS

In our study, three microalgae strains namely *Chlorella, Scenedesmus* and *Haematococcus* were considered, all the aforementioned strains were isolated from north eastern region of India, Assam, out of which chlorella is the fastest growing microalgae. The aforementioned strains were explored in terms of growth study in air, different concentration of $NaHCO_3$ salt, and different concentration of CO_2 gas.

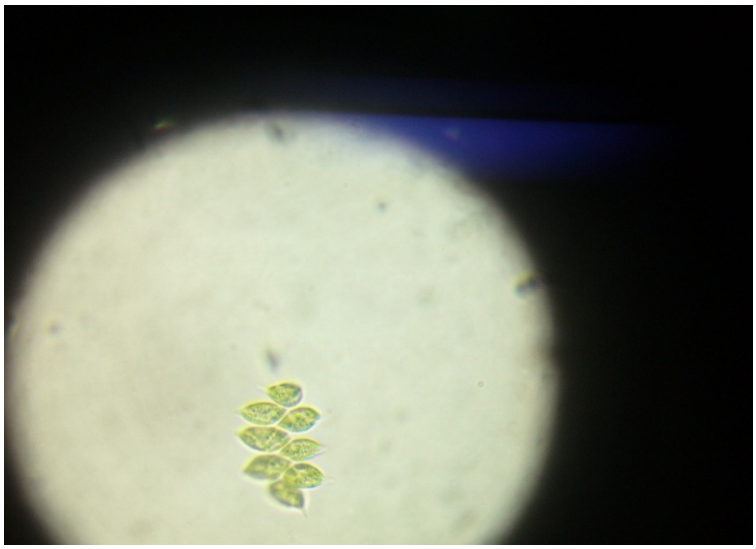
CO_2 is one of the critical factors for photosynthesis in microalgae along with light water and nutrients. Attempts were made to blow CO2 in the media but there is loss of CO_2 in the air because the solubility of CO_2 in media is very low (Wieler et al., 1940). For that CO2 gas is supplied periodically to the cultures by maintaining the pH of the cultures (a particular CO_2 concentration has a particular pH value, so a standard curve was plotted using titration method; data not shown). Carbonate salts have higher solubility than CO_2, exmple: Na_2CO_3 has a solubility of 29.4/100 g of water at 25°C (Kobe and Sheety, 1948).

In our study, $NaHCO_3$ and CO_2 gas is used as a source of inorganic carbon for photosynthesis of three strains. Adding bicarbonate does not require power consumption for bubbling CO_2 gas in the aqueous phase and can minimize the carbon loss to the atmosphere by saturating the bicarbonate concentration in an appropriate pH range for algae culture as well as can be used for photosynthesis in algae (Aizawa and Miyachi, 1986).

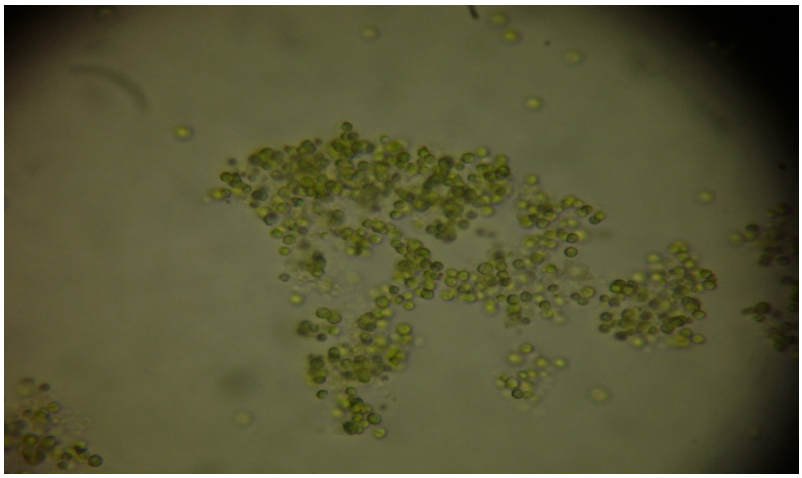
Preparation of medium

BG11 culture media was selected and prepared for the growth of microalgae strains (without adding carbon source). The composition of BG11 media is shown in Table 2, aforementioned strains were isolated from north eastern region of Assam, out of which chlorella is the fastest growing microalgae. In our study, three microalgae strains namely *Chlorella, Scenedesmus* and *Haematococcus* were considered.Control media are prepared having normal BG11 for each of the three strains. Again five + three culture flasks each having different concentration of bicarbonate salts and gaseous CO_2 were prepared separately for each of the three strains. For this purpose, $NaHCO_3$ salt in 15, 30, 45, 60 and 75 mg/L (1 mg/L=1 ppm) were freshly weighed and added to each of the flasks. For bicarbonate study, before inoculation the pH of the each flasks were adjusted to 7.5 with 0.1N HCl and 0.1 N NaOH with the help of L1 120 pH meter, Elico India. About 150 ml of media were distributed to each of the flasks including the blank and all are inoculated with 20 ml of inoculums (cell densities of inoculums; *Chlorella*=$188.48X10^4$, *Haematococcus*=$30.4X10^4$, *Scenedesmus*=$27.2X10^4$) (Photographs 1, 2 and 3). The optical density (O.D) of each the flasks were measured at 680 nm of wavelength at regular interval of time (24 h) with the help of systronics spectrophotometer-104. The strains were checked for 17 days growth period in varying bicarbonate concentration except 12 days for *chlorella* strain (12 days for chlorella as it has a faster growth rate than the other two).

Gaseous CO_2 is also supplied to cultures in variable amounts (7929, 4758 and 4400 mg/L). Firstly, the pH of the cultures were elevated to 10.5, then CO_2 gas is supplied till the pH falls to 5.5, 7 and 8 so that mentioned amount of CO_2 concentration can be reached. It was calculated previously, when we lowered the pH from 10.5 to 5.5 by adding CO_2 gas, the amount of CO_2 gas dissolved is 7929 mg/L, hence a standard curve was plotted using pH and CO_2 concentration using titration method. As CO_2 gas has a poor solubility so there is addition of CO_2 gas periodically by checking the pH of the cultures. Percentage biomass increase/day, cell count and O.D. readings were taken daily. All the strains were checked for 7 days growth period in varying amount of CO_2 gas.



Photograph 2. Scenedesmus strain under 40X magnification.



Photograph 3. Chlorella strain under 40X magnification.
Source, All appendixes were taken at Environmental Biotech Lab. Department of Biotechnology, Gauhati University ,Guwahti Assam.

Light condition

Light intensity is a very important factor for microalgae cultures as requirements of microalgae are relatively low in comparison to higher plants. For our experiment, fluorescent lamps were used as repeated for growth of all the cultures. The temperature was adjusted to 25°C for all the flasks.

Analytical method

Direct microscopic cell count by Neubour haemocytometer was performed using microscope (Labomed). O.Ds of microalgae cultures were measured at regular interval of time (24 h) by absorbance at 680 nm with the help of spectrophotometer (Systronics). The spectrophotometer was blanked every time with each medium, respectively. At the end of the experiment, all the culture flasks were centrifuged and filtered and dry weights of pellets were measured (80°C for 3 h) to study the increase in biomass, cell count and lipid content.

Determination of specific growth rate

Specific growth rate is a measure of number of generations (the number of doublings) that occur per unit of time in an exponentially growing culture. The exponential (straight line) phase of growth was carefully determined and specific growth rate was obtained using following equation (Guillard and Ryther, 1962).

$$\mu = \ln (N_t/N_o)/T_t\text{-}T_o$$

Where, N_t is the no of cells at the end of log phase; N_o is the no of cells at the start of log phase; T_t is the final day of log phase and T_o

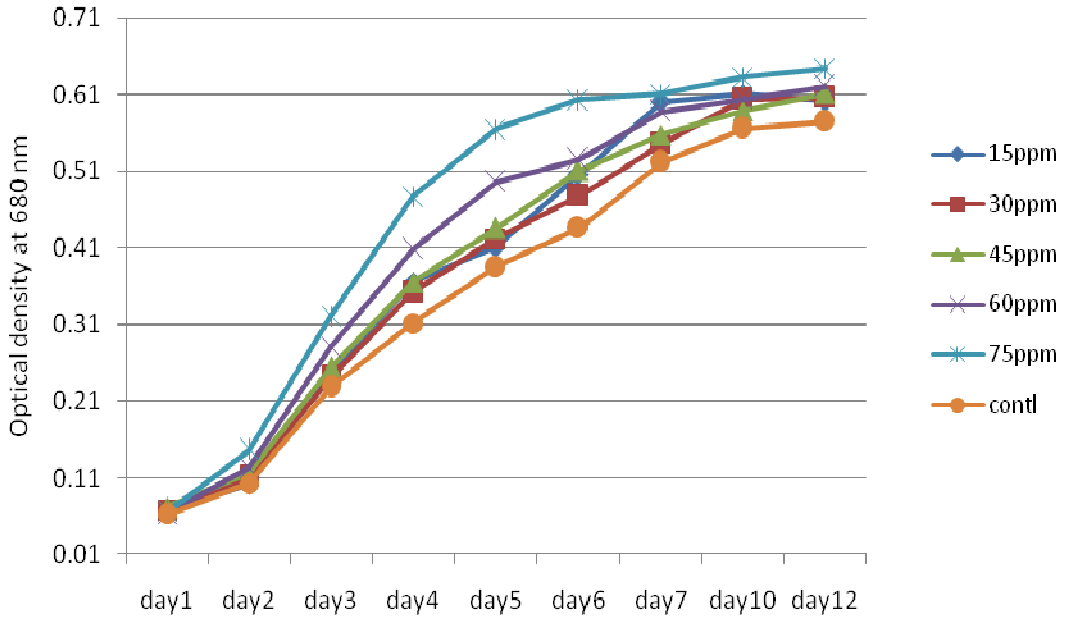


Figure 1. Growth response of *Chlorella* sp. (at 680 nm) under different levels of sodium bicarbonate ($NaHCO_3$) salt concentration.

is the starting day of log phase.

If T expressed in days from the growth rate (μ) can be converted to division or doublings per day (k) by dividing (μ) by the natural log of 2(0.6931).

$$K=\mu/0.6931$$

The time required to achieve a doubling of the number of viable cells is termed as doubling time (T_t) which is calculated by the following formula.

$$T_t= 0.6931/\mu$$

Determination of total lipid

Microalgal lipid extraction was done by Bligh and Dyer method. For that microalgal biomass were collected by centrifuging the cells at 4000 Xg for 10 min. The cells were washed with distilled water, lyophilized and weighed. The known amount of biomass (100 mg) was then homogenized with chloroform: methanol 1:2 at 35°C. Extract was centrifuged for 7 min at 10000 Xg and supernatant was collected in a separating funnel. The residue was further homogenized with chloroform and again centrifuged (10000 Xg) to collect the supernatant. Now 0.9% sodium chloride (NaCl) solution was added to the filtrate and washed, lower layer of chloroform was separated and treated with anhydrous Na_2SO_4 to remove the traces of water. The lipid content was determined gravimetrically and expressed as dry weight % after evaporating the chloroform (Bligh and Dyer, 1959).

RESULTS AND DISCUSSION

This study confirms that all the three strains namely *Chlorella, Scenedesmus*, and *Haematococcus* could grow well in modified media composition that is, in higher bicarbonate concentration and CO_2 gas. Maximum growth recorded in case of *Chlorella* strain is in 75 ppm of bicarbonate, which is equivalent to 1191 ppm of CO_2 (Figure 1; Tables 3 and 4). It was found earlier that 15.3 ppm of bicarbonate is equivalent to 243 ppm of CO_2 (Jeong et al., 2003), whereas in case of *Haematococcus* and *Scenedesmus* strains, showed their best growth in 30 and 45 ppm of bicarbonate concentration, respectively (equivalent to 476 and 714 ppm of CO_2, respectively) (Tables 3 and 4). Although, it was observed that *Haematococcus* strain showed cell coagulation when high CO_2 gas (7929 ppm) was supplied (Figure 2). *Scenedesmus* strain also tends to coagulate a little bit at high CO_2 concentration (7929 ppm) but it continues to grow slowly (Figure 3). Out of the three strains, it was chlorella which showed its best growth in high bicarbonate concentration (75 ppm bicarbonate~ 1191ppm CO_2) and all the three strains showed maximum growth in 4758 ppm of CO_2 gas) (Figures 4, 5 and 6).

From the above study, it was found that the lipid content of all the strains increased when they were grown in media supplemented with bicarbonate salt and carbon dioxide gas. Addition of bicarbonate at 75 ppm in case of *Chlorella*, 30 ppm in case of *Haematococcus* and 45 ppm in case of *Scenedesmus* strain showed highest accumulation of lipid (18, 15 and 14% of dry cell weight), along with highest growth response (Figures 7 and 8). Also, addition of gaseous CO_2 at 4758 ppm to the cultures of *Chlorella, Haematococcus* and *Scenedesmus* strains

Table 3. Specific growth rate and doubling/day calculated for *Chlorella, Haematococcus* and *Scenedesmus* sp. under different levels of sodium bicarbonate salt ($NaHCO_3$) concentration-

HCO_3 concentration	*Chlorella* sp.		*Haematococcus* sp.		*Scenedesmus* sp.	
	Specific growth	Doubling/day	Specific growth	Doubling/day	Specific growth	Doubling/day
15 ppm	0.394	0.568	0.381	0.511	0.09946	0.143
30 ppm	0.378	0.545	0.384	0.554	0.1138	0.164
45 ppm	0.362	0.522	0.247	0.357	0.1217	0.175
60 ppm	0.372	0.546	0.27	0.391	0.1025	0.147
75 ppm	0.396	0.571	0.283	0.409	0.09246	0.133
cntrl	0.309	0.445	0.17	0.246	0.0458	0.066

Table 4. Specific growth rate and doubling/day calculated for *Chlorella, Haematococcus* and *Scenedesmus* sp. under different gaseous CO_2 level.

CO_2 concentration	*Chlorella* sp.		*Haematococcus* sp.		*Scenedesmus* sp.	
	Specific growth	Doubling/day	Specific growth	Doubling/day	Specific growth	Doubling/day
7929ppm	0.502	0.724	0.602	0.869	0.642	0.926
4758ppm	0.704	1.01	0.664	0.959	0.673	0.971
4400ppm	0.545	0.545	0.662	0.956	0.551	0.794
cntrl	0.133	0.133	0.483	0.693	0.337	0.486

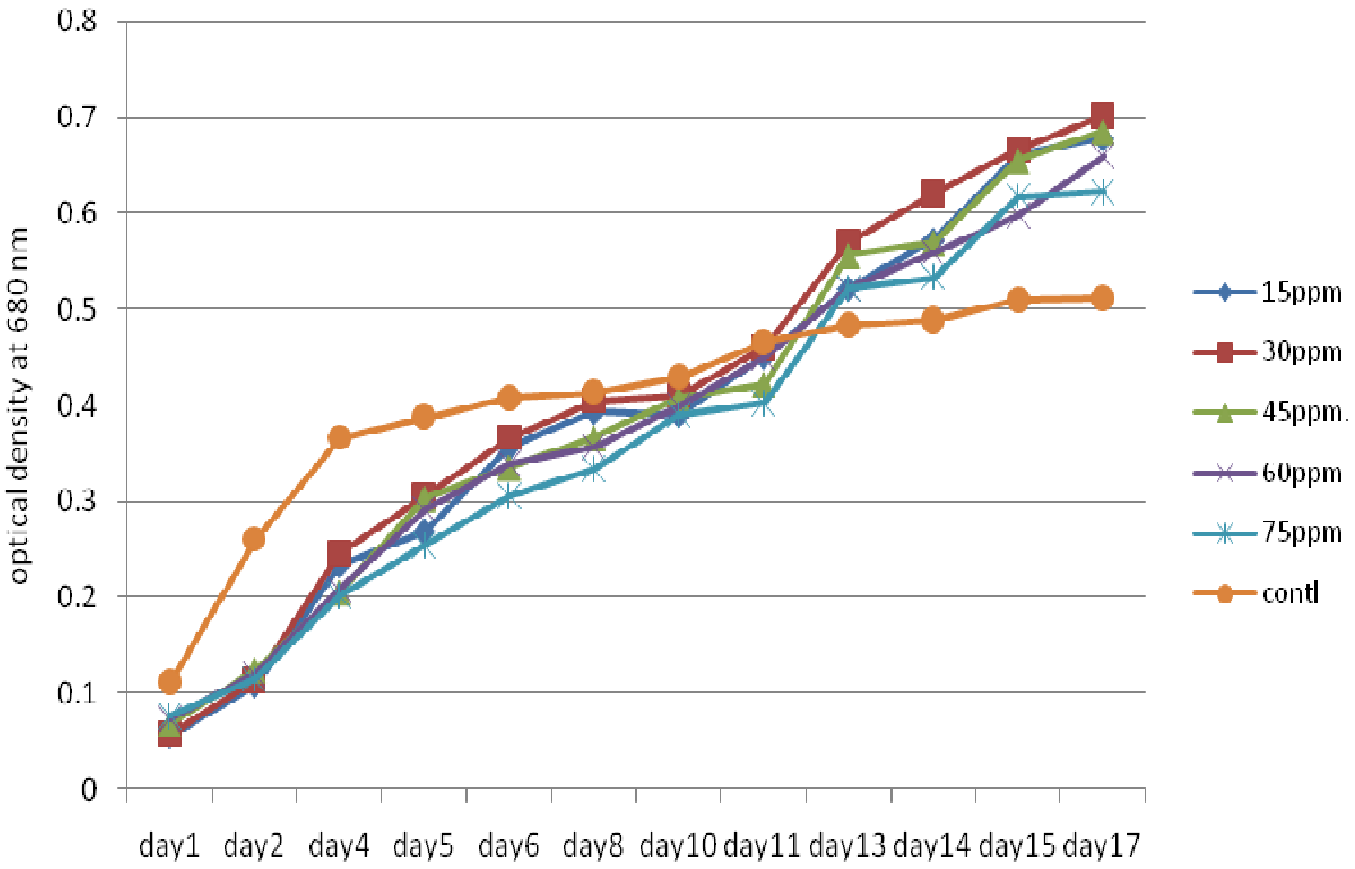


Figure 2. Growth response of *Haematococcus* sp. (at 680 nm*)* under different levels of sodium bicarbonate ($NaHCO_3$) salt concentration.

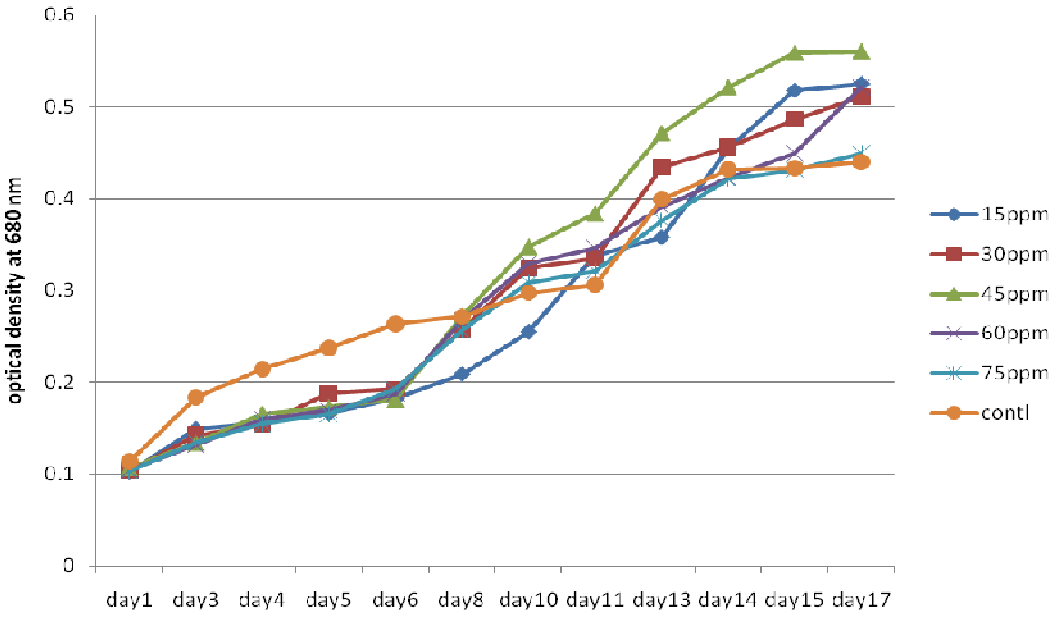


Figure 3. Growth response of *Scenedesmus* sp. (at 680 nm). under different levels of sodium bicarbonate ($NaHCO_3$) salt concentration.

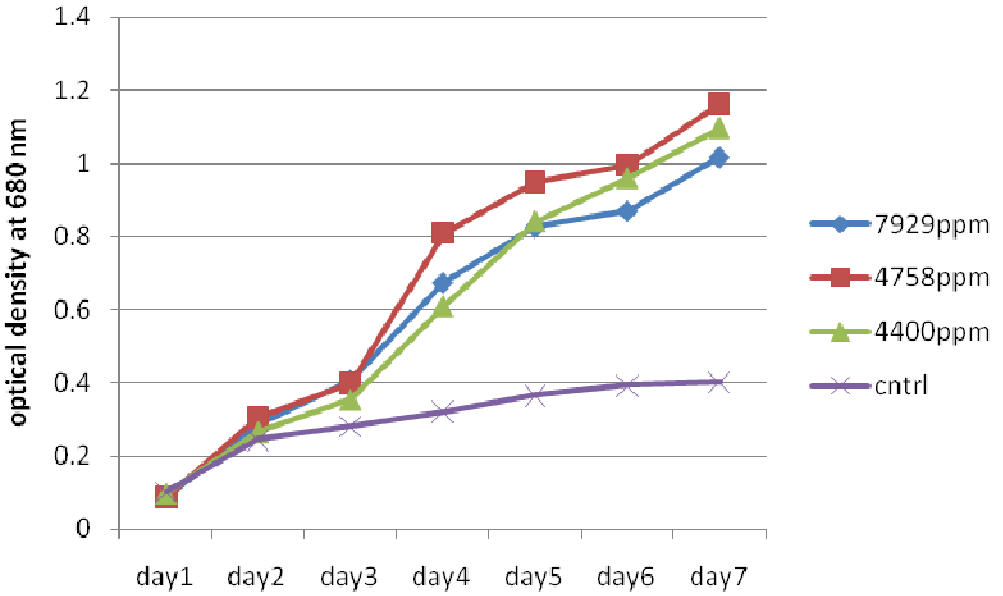


Figure 4. Growth response of the *Chlorella* sp. under different levels of CO_2 gas for 7 days.

enhance total lipid content (22, 17 and 15% of dry cell weight, respectively) (Figures 9 and 10)

Microalgae have considerable biotechnological potential including producing valuable substances for the food additive, cosmetic, biofuel and pharmaceutical industries. A feasible microalgal CO_2- mitigation model can effectively fix CO_2 and also convert biomass to different valuable byproducts (Ono and Cuello, 2006). Recent studies showed that *Scenedesmus* sp. not only, is the promising CO_2-fixating microalga (de Morais and Costa, 2007), (Ho et al., 2010), but also a good microalgal lipid producer. It was found that *Scenedesmus* sp. could covert approximately 15-25% atmospheric CO_2 into biodisel for transportation fuel (Ho et al., 2010), (Mandal and Mallick, 2009) and its biomass could accumulate lutein and other pigments for health food applications

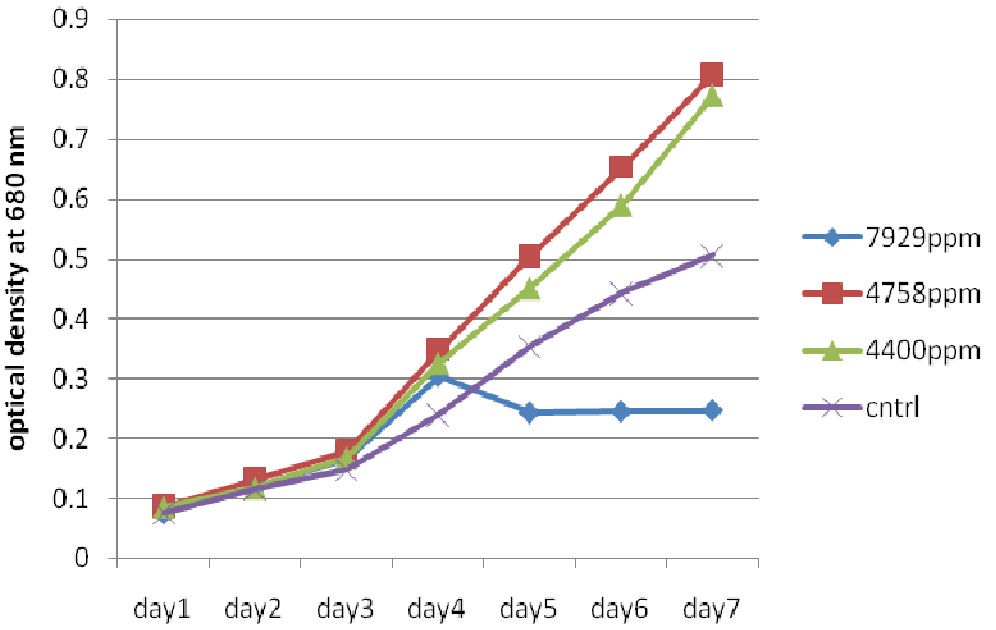


Figure 5. Growth response of the *Haematococcus* sp. under different level of CO_2 gas for 7 days.

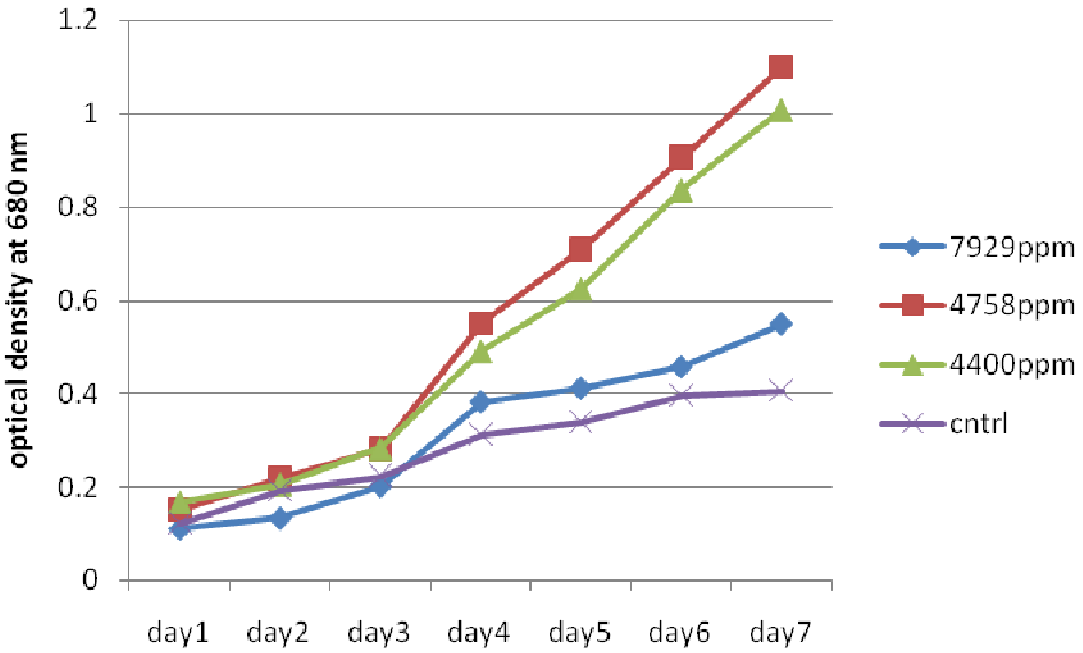


Figure 6. Growth response of the *Scenedesmus* sp. under different level of CO_2 gas for 7 days.

(Ceron et al., 2008). The merit of cultivation of micro-algae, CO_2 mitigation and biofuel production which could be combined in an economically sustainable manner, the feasibility of this strategy could be further enhanced by fixing CO_2 from industrial exhaust gases such as flue gases. CO_2 concentration plays an important role in the increase of lipid productivity (Wang et al., 2008). It was found that at higher CO_2 concentration, growth under normal condition gave higher lipid productivity (Widjaja et al., 2009). All the mentioned three strains namely *Chlorella, Scenedesmus*, and *Haematococcus* were tested thoroughly in terms of their growth in high bicarbonate salt and high CO_2 gas concentration. The study reveals that bicarbonate is an effective carbon

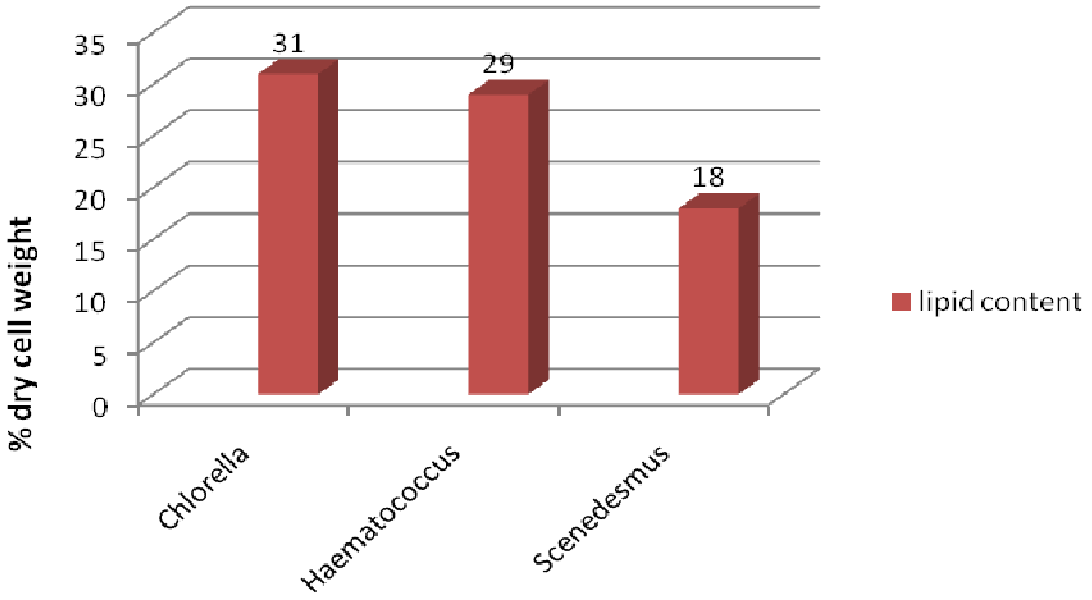


Figure 7. Total lipid content in terms of % dry cell weight in *Chlorella, Haematococcus,* and *Scenedesmus* sp. under 4758 ppm of CO_2 gas.

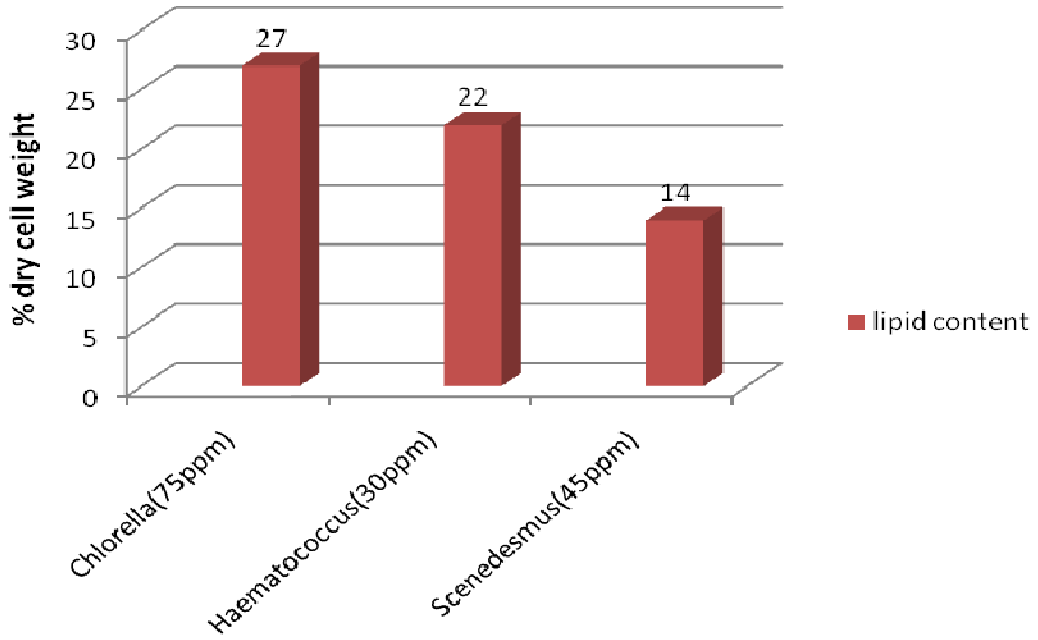


Figure 8. Total lipid content in terms of % dry cell weight in *Chlorella, Haematococcus, Scenedesmus* sp. at 75, 30, 45 ppm of sodium bicarbonate salt ($NaHCO_3$), respectively,(where maximum growth rate observed).

source for microalgal growth, as optimum bicarbonate concentration is beneficial for highest biomass production. The findings indicates that CO_2 gas supply can strongly affect the microalgae growth, because CO_2 gas supplied culture flasks showed faster growth rate and quick accumulation of biomass in all the tested three strains which ultimately leading to high lipid production. It was found that at 15.3 ppm of bicarbonate salt which is equivalent to 243 ppm of gaseous CO_2 , *C. vulgaris* strain exhibited fastest growth rate when tested (Jeong et al.,

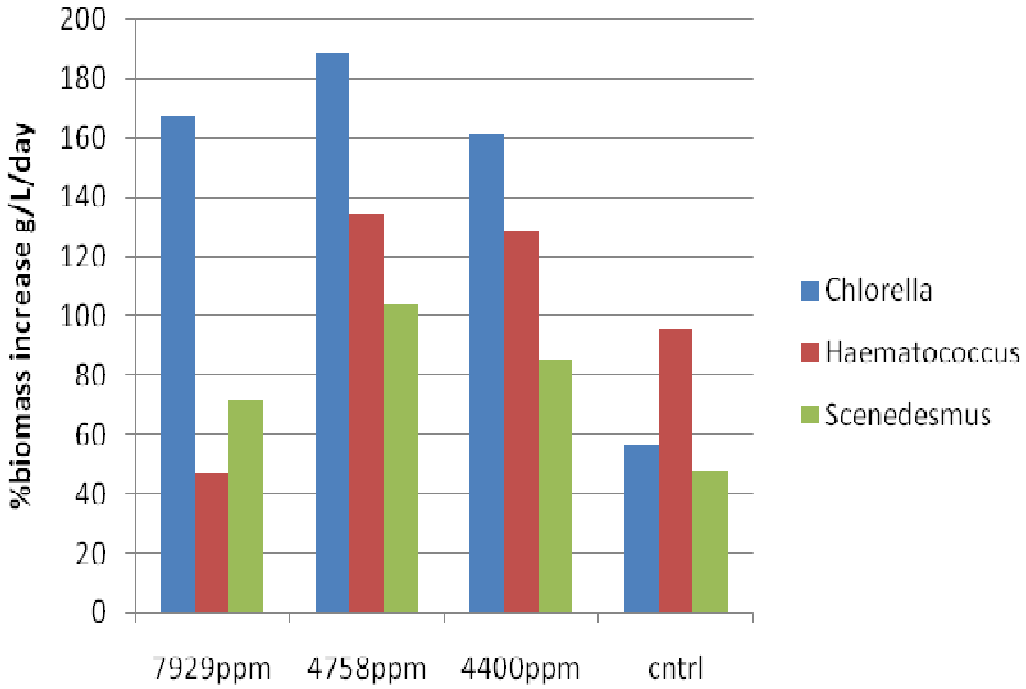


Figure 9. % Increase in biomass in case of *Chlorella, Haematococcus and Scenedesmus sp.* when gaseous CO_2 supplied.

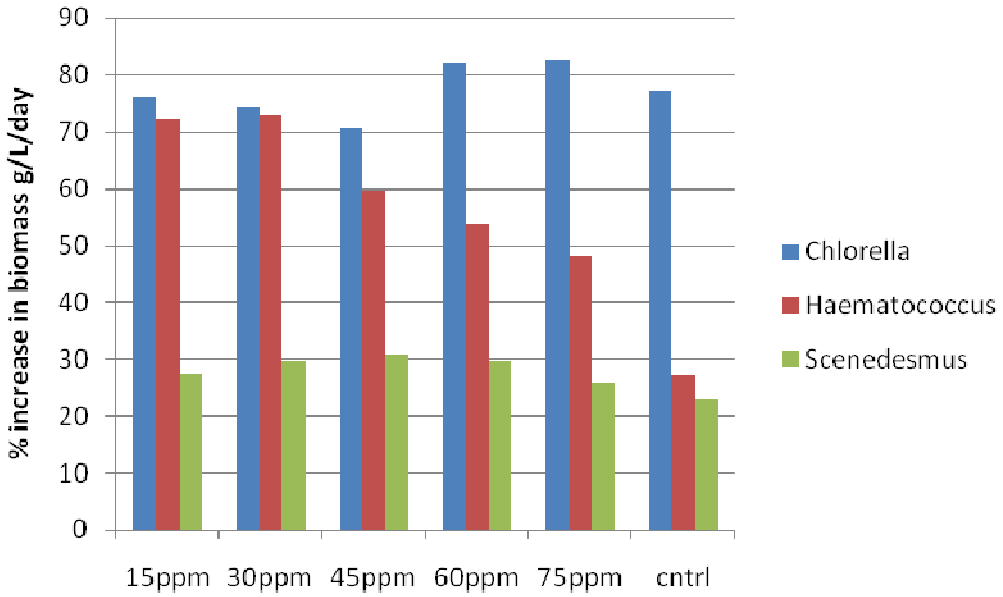


Figure 10. % Increase in biomass in case of *Chlorella Haematococcus* and *Scenedesmus* strains in different sodium bicarbonate salt ($NaHCO_3$) concentration.

2003). In our study, the *Chlorella* sp. performed well in terms of its bicarbonate salt and CO_2 (gas) utilization; it showed its maximum growth at 75 ppm bicarbonate (~1191 ppm CO_2). So, the study proves a great deal of potential for chlorella strain to use in huge outdoor culture systems for mitigating flue gases and Industrial waste gases. *Chlorella* sp. is also a potential candidate for the production of biomass which are used in aquaculture for feeding, nutraceutical food additives and animal feed as it is rich in vitamins. So to develop an effective CO_2

mitigation technology, it is necessary to select an efficient and fast growing microalgae strain which has a good CO_2 fixing efficiency and promising valuable components.

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