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Growth maximization and carbon dioxide assimilation studies of *Protococcus* species grown under culture conditions using Bolds Basal medium.

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Keywords: *Protococcus*, carbon sequestration, pH, micro algae.

Abstract

Protococcus sp. normally experiences slow growth in Bolds Basal (BB) medium. This is hindering the process of their mass multiplication for specific purposes under laboratory conditions. As pH influences the growth of micro algal members under culture conditions, an attempt has been carried out to assess the optimum pH favouring maximum growth and multiplication of *Protococcus* species in BB medium. For experimentation, cultures were monitored under laboratory conditions using BB medium, kept at varying pH ranging from 3 to 12 with an interval of 0.5 for a period of 7 days. Upon analyzing the major growth parameters like cell count, turbidity and biomass, maximum growth of the species was noticed at pH 10. An attempt has also been carried out to assess the efficiency of *Protococcus* species in assimilating carbon dioxide. Pure cultures of *Protococcus* were introduced to three glass tanks of size 18x18x24 cm, containing 5litres of BB medium, each with a pH of 6.6. The first set was retained as control and the second and third one as aerated and CO_2 treated set respectively, with the later connected to a continuous supply of CO_2 maintained at a flow rate of 9-12 bubbles/ minute. The changes in pH, Dissolved Oxygen and Free carbon dioxide content of the medium together with cell count, cell size and biomass content of the algal members were worked out at an interval of 3 and 6 hours respectively for a period of 48 hours. The algal species exhibited higher rate of DO production, cell count and biomass content in CO_2 treated set, indicating their effectiveness as promising candidates for CO_2assimilation.

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Introduction

Microalgae are a heterogeneous group of microorganisms seen in freshwater, estuarine and marine environments, with rapid multiplication rate, higher photosynthetic efficiency and biomass generation compared to energy crops. Their abundance in heterogeneous habitats, minimal nutrient requirements and being rich source of various bio compounds, makes them interesting candidates for research and developmental purposes in many countries.

The growth requirements of micro algal members differ considerably. Also most of the members which are abundant and virulent under natural conditions, seem to be slow growing under culture conditions. Moreover few micro-algal members tend to dominate in culture systems, in spite of the desired microalgae present in the inoculum (Goldman, 1979).As production of biomass of ideal species in required quantities in short duration seems to be significant in determining the selectivity of a species for specific purpose, adequate studies need to be carried out for maximizing the growth of micro algal members under heterogeneous conditions.

Optimization of factors such as pH, light and temperature; nutrients like nitrate, phosphate and trace metals, mixing regimes, salinity etc. are found to be maximizing the biomass of micro algal members under culture conditions (Mata *et al.,* 2010). Among them, hydrogen ion concentration (pH) is a significant one as the optimum and tolerance range of pH tends to be species specific. Moreover one of the major challenges in micro algal culturing is

contamination from unwanted species (Das *et al.,* 2011) and by adjusting the culture pH, the elimination of such contaminant species can be achieved.

On account of the adverse impacts due to increased greenhouse gas emissions in the atmosphere, many research and development efforts are undertaken to facilitate the reduction of CO_2concentrations. Among them, bio sequestration is noted to be an ideal option for minimizing carbon in both terrestrial and aquatic ecosystems. The ability of microalgae to absorb and assimilate CO_2 suggests an attractive alternative for CO_2 sequestration in aquatic systems (Yun *et al.,* 1997) and recently, enough of research and development activities are getting oriented in this direction.

Thus the present study is undertaken to assess the optimum range of pH maximizing the growth of *Protococcus* species through pH specific modification of Bolds Basal Medium and also to assess the efficiency of the species in carbon dioxide absorption and assimilation.

Materials and methods

The micro algal samples were collected from heterogeneous aquatic environments surrounding Calicut University and pure cultures of *Protococcus* species were isolated and maintained in the laboratory in Bolds Basal medium (Nichols and Bold, 1965).

pH specific growth enhancement studies

Bolds Basal medium (50 ml each) was taken in conical flasks (triplets) and the pH of respective medium was adjusted specifically from 3to12, with a gradation of 0.5 using 0.5 N NaOH and 0.05 N HCl. After adjusting for the required pH, 5 ml each of pure cultures of *Protococcus* species was added to all the treatment sets. A control set was also maintained with original Bolds Basal medium having an original pH of 6.6.All the sets were kept at illumination during day time. The growth performances of microalgae in the cultures were monitored through attributes like cell count, cell size, turbidity and biomass. Similarly, pH, conductivity and resistivity of the culture medium were also monitored daily. Every day after observation, the altered pH was readjusted. Monitoring of the entire treatment set was carried out for a period of 1 week.

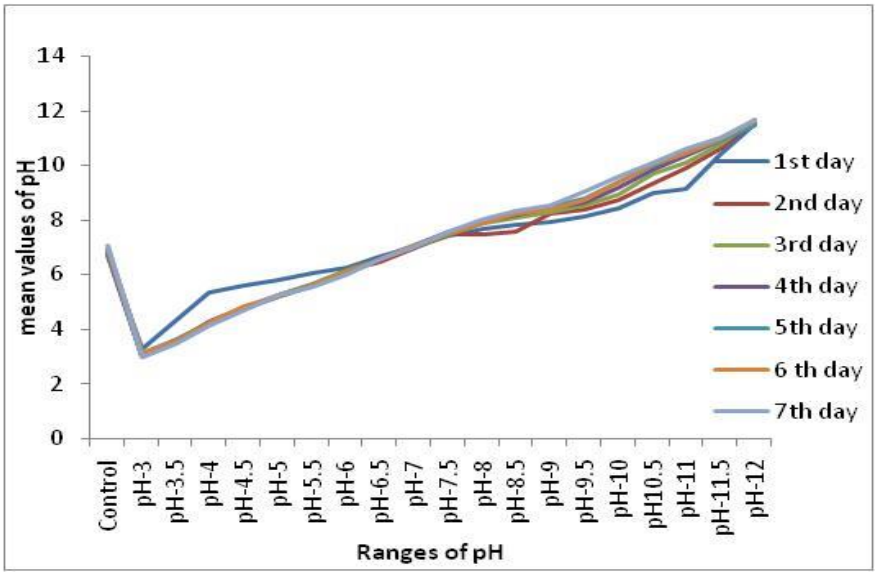
Carbon assimilation studies

For fulfilling this objective, 12litres of Bolds Basal medium was prepared (pH 6.6) and to this, pure cultures of *Protococcus* was added and kept for incubation for a period of 24hours. They were then transferred to three glass tanks, (5 litres each) with a size of 18x18x24 cm. The first glass tank containing the culture was maintained as such and was treated as Control. To the second culture tank, provision for bubbling air has been attached and has been treated as the aerated set. To the third tank, carbon dioxide from a cylinder has been bubbled out at regular intervals and was retained as CO_2 treated set. The experiment was initiated at 6 am on the first day and continued for 48 hours. The control was kept idle and through the second and third culture tank, air and CO_2 has been bubbled out respectively at a constant flow rate (6 – 9 bubbles per minute) from 6am to 6 pm every. All the three sets were kept at illumination during the day time. pH, Dissolved Oxygen and Free carbon dioxide content of respective culture was monitored at an interval of 3 hours, whereas cell count, cell size and biomass were worked out at an interval of 6 hours. Sampling and monitoring of the cultures were carried out for a period of 48 hours.

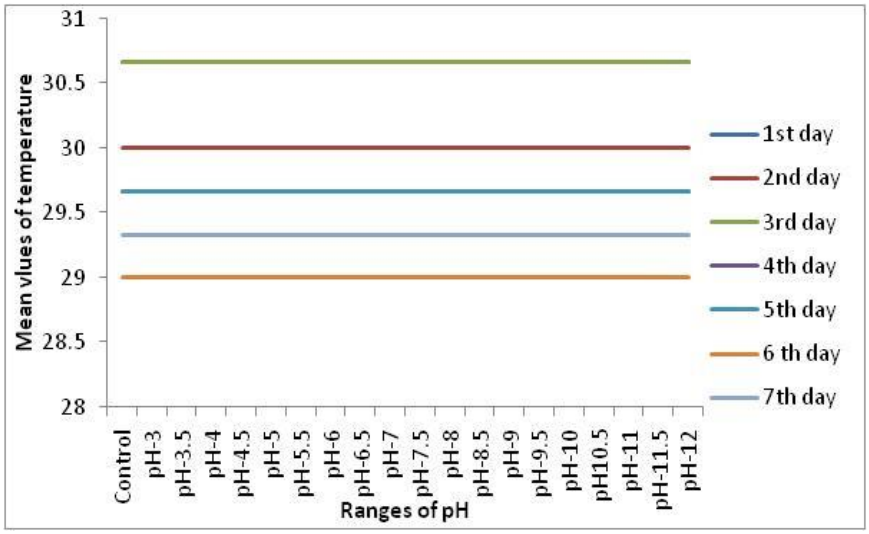
Results and discussion

Changes in pH, temperature, conductivity, resistivity, cell count, cell size, turbidity and biomass associated with *Protococcus* in response to different ranges of pH are represented in Figures 1a – h.

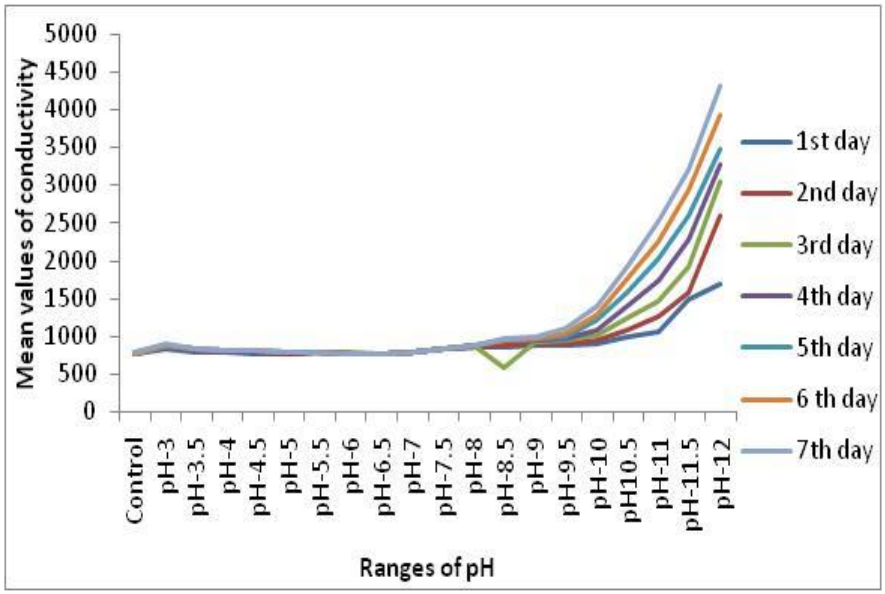
1a- pH



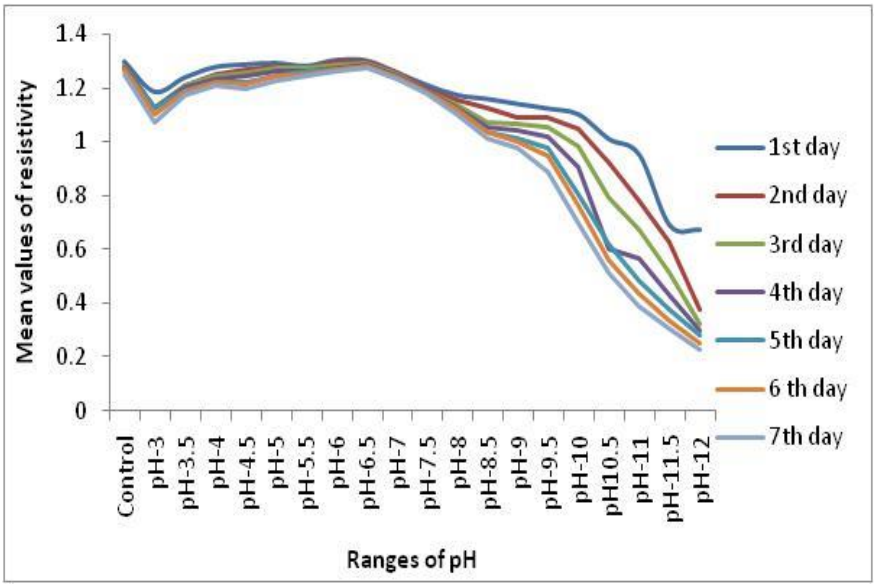
1b-Temperature



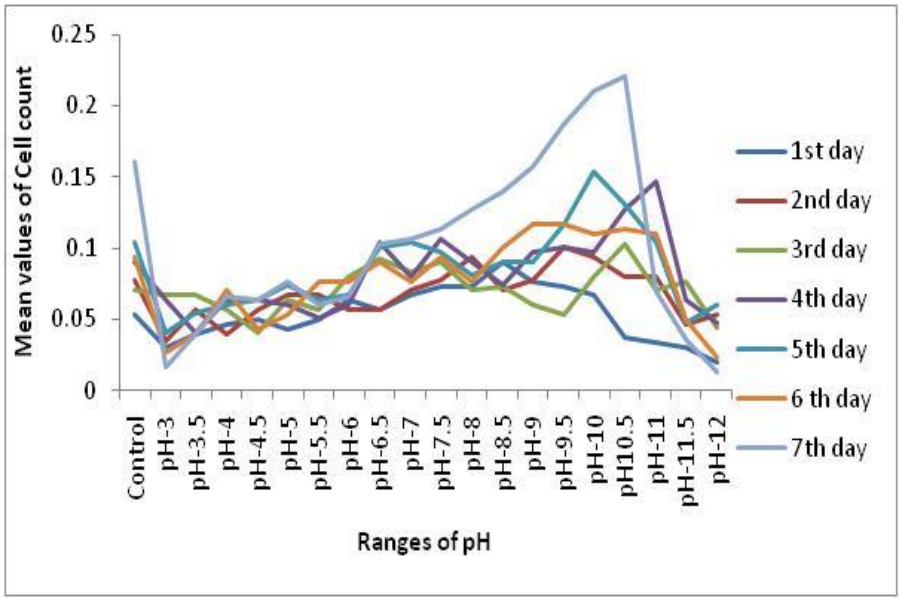
1c-Conductivity



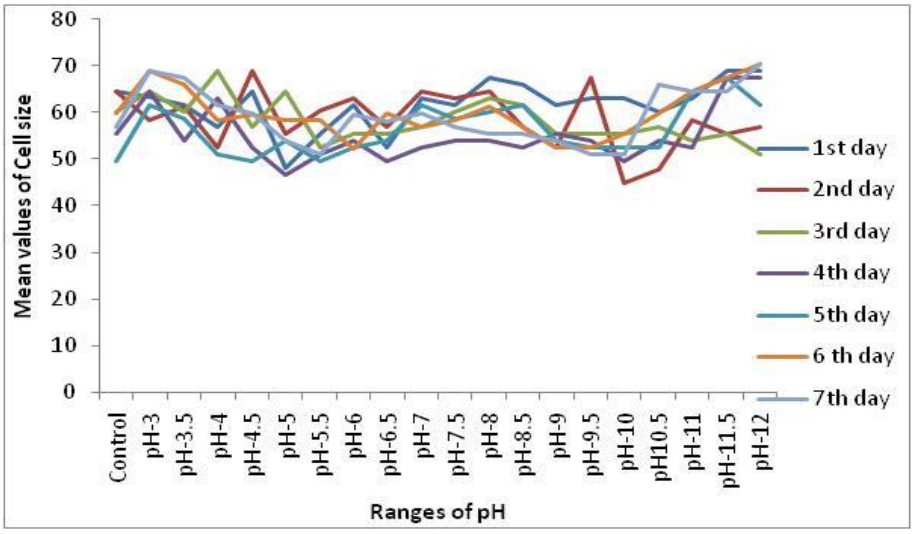
1d-Resistivity



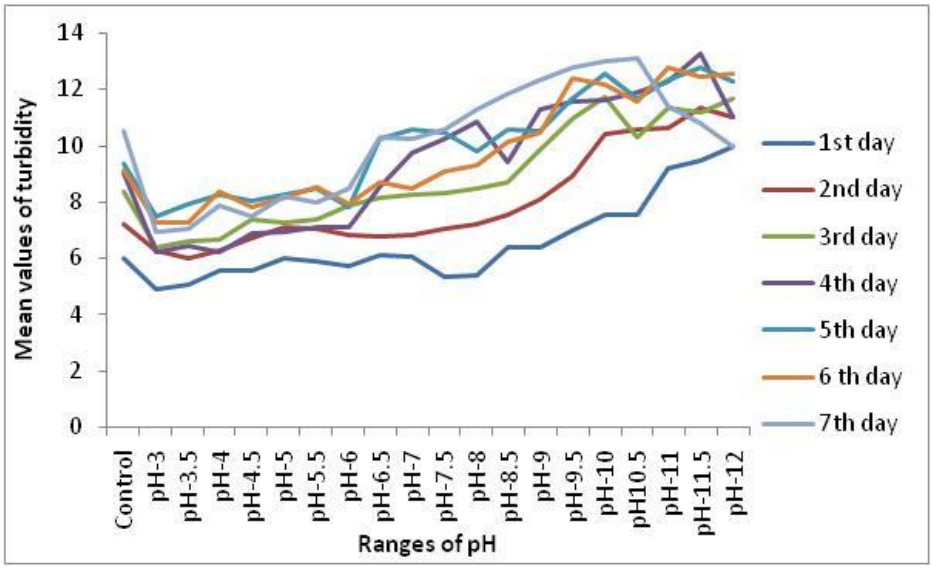
1e-Cell count



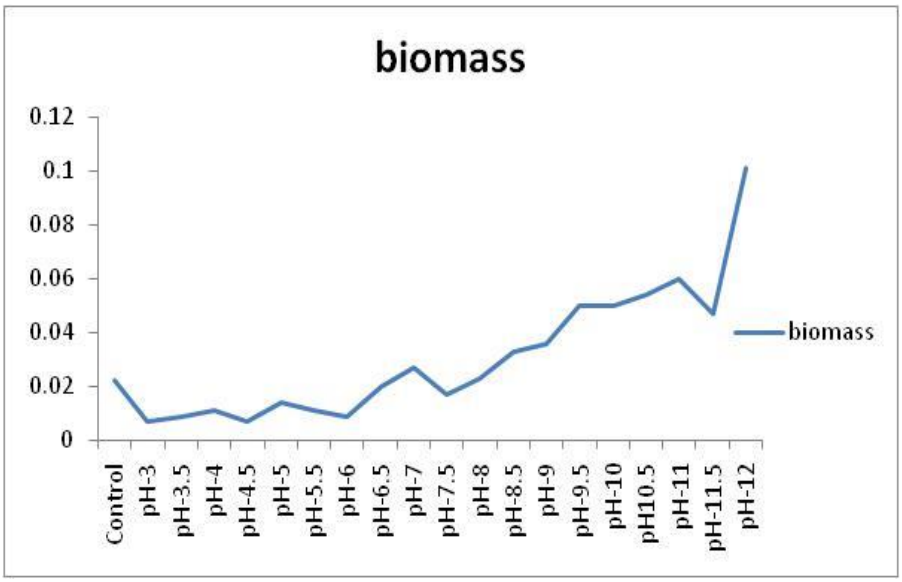
1f-Cell size



1g-Turbidity



1h-Biomass



Figures 1a to 1h, representing changes in pH, temperature, conductivity, resistivity, cell count, cell size, turbidity, and biomass associated with *Protococcus* species in response to different ranges of pH.

In the present study, each range of pH showed a tendency to attain neutral levels. This is evidenced by an increase in values of the pH of samples of the acidic range and a decrease in values at the alkaline range, tending towards neutral range. This is indicative of the adaptabilities of micro algal members to survive in modified

environments by altering the pH to optimum levels. The optimum pH for most freshwater microalgal species is found to range from 7 to 9 (Whitacre, 2010).

As a major growth parameter, the maximum cell count was noticed in mid-alkaline range (pH 8.5 to 11). At higher alkaline conditions (pH 11.5 and 12) and in acidic conditions (pH 3 to 4) cell growth was low. Guckert and Cooksey (1990) reported that the alkaline pH increases the flexibility of the cell wall of mother cells, which prevents its rupture and inhibits autospore release and thus increases the time for cell cycle completion. Similarly the acidic conditions can also alter nutrient uptake (Gensemer *et al.,* 1993) or induce metal toxicity and thus affect the algal growth (Anderson and Morel 1978).

The highest cell size was observed in pH 12 and lowest in pH 10. Visviki and Santikul (2000) monitored the effects of hydrogen ions on the growth and ultra structure of *Chlamydomonas applanata* and pointed out that introduction of microalgae to pH 4.4 results in increased pyrenoidal volume and the existing single cells were larger than controls, with smaller chloroplasts, thicker cell walls and larger vacuole. However increase in the cell size cannot be taken as a parameter of growth as the cells with bigger size are normally subjected to division and multiplication. The undivided cells are likely to appear in large size and newly formed cells in small size.

While comparing the mean values of temperature of the culture medium, lower temperature was observed on 6^{th} day, (29±1.41) and high temperature on 1^{st} day (30.6±0.943). Generally cell growth increases with temperature until the temperature optimum was reached and further increase of temperature reduces growth (Dauta *et al.,* 1990). Temperature lower than 16^0C will results in slow growth and higher than 35^0C will have lethal effects (Oligae, 2009). Analysis of data on temperature pertaining to the present study revealed that the temperature prevalent in the culture conditions were almost within the range to support the growth of *Protococcus*. Also at higher alkaline range (pH 12), the conductivity of the medium was higher and in near neutral ranges (pH-6, pH-6.5 and in control), conductivity was lower. The Resistivity was found maximum in pH 6.5 and minimum in pH 12.

In the treatment set, highest turbidity was noticed in pH 10.5 and lowest in pH 3. Though highest turbidity was also noticed in higher alkalinity ranges like pH11.5 and pH 12, this can be attributed to the formation of precipitates, which are evident in the treatment set. In higher alkaline ranges (pH 12), the cell count was low and from the 4^{th} day of treatment, green colour of microalgae changed to white, following a precipitate formation. In the present study, highest biomass (gm) was obtained in pH 10.5 and 11 (0.05 ±0.003 and 0.05 ±0.004 respectively) while biomass in the control set was lowest (0.019±0.002).

For the confirmation of appropriate pH level at which maximum growth of *Protococcus* species occurred, the mean values of the 7 days triplicate data pertaining to major growth parameters like cell count, biomass and turbidity were worked out and compared and the highest growth was noticed at pH 10. Hence for ensuring better biomass production of *Protococcus* species in Bolds Basal medium, a pH of 10 need to be maintained.

In connection with carbon assimilation studies, the results obtained for pH, Dissolved Oxygen and Free CO_2 content of the culture media are given in Table 1a. Also results of cell size, cell count, and biomass content in response to continuous supply of CO_2 are given in Table 1b and1c.

Upon comparison, it has been noticed that all treatment sets with CO_2 supply exhibited pH in slightly acidic range. Sobczuk *et al.,* (2000) stated that at higher CO_2 concentration, the culture pH decreases due to the formation of high amount of bicarbonate buffer. The pH of culture media in CO_2 treated set can be attributed to this reason. It was also noticed that the *Protococcus* species survived in slightly acidic range and showed better growth performances. Also maintenance of culture pH within a range are indicative of their adaptability and CO_2 assimilation capabilities(Menon and Harilal,2016).There are also reports regarding the growth performances of various micro algal members in acidic environments. *Chlorella* is one among them, which exhibit better growth in pH ranging from 4.0–6.0 (Yue and Chen, 2005) and in 4.0 to 7.0 (Ponnuswamy *et al.,* 2014).

The Mean values of DO content was found to be higher in CO_2 treated set, followed by control and aerated set. This can be an indication of their active photosynthetic process in presence of available CO_2 and subsequent release of oxygen. Treatment set fed with continuous supply of CO_2 exhibited higher free CO_2 content in the culture

media which can happen as a result of residual CO_2. Availability of residual free CO_2 can have an influence on the pH of concerned cultures. Goswami et al.(2012) reported that when gaseous CO_2 was added the pH of the culture medium falls very rapidly as CO_2 forms carbonic acid making the medium acidic and intolerable. The acidic range of pH associated with all cultures fed with CO_2 can be attributed to this reason.

Increase in cell count in most cases was considered to be an index of growth (Karampudi,2011). In the present study, mean values of the results of cell count was found to increase in treatment set fed with continuous supply of CO_2, wherein the supply of CO_2 might have accelerated the growth of all micro algal members. Studies by Goswami et al. (2011) also reported increased cell growth of micro algal members due to higher concentration of carbon dioxide supply. A comparative assessment of the mean values of the size of cells from CO_2 treatment set were either higher than their control or aerated set which indicates that *Protococcus* species have positive growth response in accordance with continuous supply of CO_2. Upon comparison of the mean values, it has been noticed that the biomass was higher in CO_2 treated set, followed by aerated set and control, indicating their higher growth potential. Goswami et al. (2011) also proved increased biomass production of microalgae in CO_2treated medium.

Upon generalization of the results, it has been noticed that a pH of 10 is ideal for the mass multiplication of *Protococcus* species. Carbon dioxide assimilation studies of *Protococcus* in Bolds Basal medium having original pH of 6.6 indicated that continuous supply of CO_2 has favored the growth of micro algal members, which are evidenced by high D.O content, cell count and biomass. Since Bolds Basal medium maintained in pH 10 favours maximum growth of *Protococcus*, there can be efforts of carbon dioxide assimilation using BB medium kept at pH 10 instead of 6.6.

In the present investigation the growth efficiency of *Protococcus* species in Bolds Basal medium kept at varying levels of pH were assessed. Attempts were also carried out to assess their potential in carbon fixation. pH, Dissolved Oxygen, Free carbon dioxide content, cell count, cell size and biomass of the culture were worked out and monitored for a period of 48 hours.

Upon consolidation of the results, it has been noticed that the cultures maintained in Bolds Basal medium with a pH 10 has facilitated the mass production of *Protococcus* species. Also the continuous supply of carbon dioxide accelerated the dissolve oxygen content, cell count and biomass of *Protococcus* species, indicating their potentialities in carbon assimilation. As maximum growth of *Protococcus* has been noticed at a modified pH of 10, there can be efforts of carbon assimilation at pH 10, instead of 6.6.

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Table 1a.Results on the responses of *Protococcus* sp. to various parameters.

Parameters analyzed	**I day**					**II day**						**III day**		**Mean± SD**
	6 AM	**9 AM**	**12 PM**	**3 PM**	**6 PM**	**12 AM**	**6 AM**	**9 AM**	**12 PM**	**3 PM**	**6 PM**	**12 AM**	**6 AM**	
pH														
Control	6.81	7.08	6.98	6.95	7.06	6.95	6.91	7.09	6.96	7.08	7.11	6.73	6.95	6.98**±** 0.11
Air supply	6.81	6.91	6.84	6.88	7.03	6.85	6.77	6.96	6.86	6.99	7.03	6.87	6.85	6.89± 0.08
CO_2 supply	6.81	6.38	6.05	6.19	6.13	6.25	6.26	6.27	6.26	6.38	6.43	6.34	6.38	6.31± 0.18
Dissolved Oxygen (mg/l)														
Control	5.4	4	5.8	6	6.2	7.4	7	7.6	8	8.8	10.4	8	8.2	7.13± 1.66
Air supply	5.4	3	5.2	6.4	7.6	6. 8	6.6	6.8	8	8.8	10.4	8.6	7.6	6.49± 1.94
CO_2 supply	5.4	4	6.4	7.6	8.8	8	7.2	7.8	9.2	10.8	11.2	9.4	8. 2	7.37± 2.09
Free CO_2 (mg/l)														
Control	48.4	44	48.4	44	39.6	44	35.2	52.8	39.6	39.6	39.6	39.6	35.2	42.31± 5.25
Air supply	48.4	52.6	48.4	48.4	52.8	48.4	52.8	52.8	48.4	48.4	44	48.4	48.4	49.40± 2.62
CO_2 supply	48.4	101.2	145.2	105.6	114.4	96.8	92.4	96.8	101.2	96.8	96.8	88	110	99.51± 21

Table 1b.Results on the responses of *Protococcus* sp. to various parameters.

Parameters analyzed	First day			Second day				Third day		Mean
	6AM	12PM	6PM	12AM	6AM	12PM	6PM	12AM	6AM	
Cell count (x10^{4}cells/ml)										
Control	3	3	3	3	3	3	3	3	3	3.00±0
Air supply	3	2	2	2	3	3	3	3	2	2.56±0.5
CO_2 supply	3	3	3	3	4	4	4	4	4	3.56±0.5
Micrometry (µm)										
Control	55	55	55	55	66	66	66	66	66	61.1±5.8
Air supply	55	55	55	55	55	66	66	66	66	59.9±5.8
CO_2 supply	55	66	66	66	66	77	77	77	77	69.7±7.8

Table 1c.Results on the responses of *Protococcus* sp. to various parameters.

Parameters analyzed	First day			Second day				Third day		Mean
	6AM	12PM	6PM	12AM	6AM	12PM	6PM	12AM	6AM	
Biomass (gm)										
Control	0.0210	0.0241	0.0202	0.0213	0.0255	0.0239	0.0329	0.0327	0.034	0.026±0.1
Air supply	0.0210	0.0214	0.0306	0.0333	0.0258	0.03	0.0365	0.0312	0.0312	0.029±0.1
CO_2 supply	0.0210	0.0286	0.0326	0.0239	0.0268	0.0335	0.0543	0.0321	0.0351	0.032±0.1