IJBPAS, January, 2022, 11(1): 111-123

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

# EFFECT OF PH SPECIFIC CONDITIONS ON THE GROWTH OF *Chlorococcum humicola* (Nägeli) RABENHORST: A GROWTH STANDARDIZATION STUDY ON AN EMERGING ALGA IN BIOPRODUCTION AND BIOREMEDIATION

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**Received 8th March 2021; Revised 6th April 2021; Accepted 6th May 2021; Available online 1st Jan. 2022**

https://doi.org/10.31032/IJBPAS/2022/11.1.5812

## ABSTRACT

The species *Chlorococcum humicola* had been reported to be useful for a mirage range of purposes including as arenewable source of biofuels, bioproducts and as a potential source of important bioactive compounds. However, cell multiplication and growth of the species in liquid culture media was found to be slow. It has been reported that pH is one of the key determining factor in the successful multiplication and growth of almost all micro algal species in both culture as well as natural conditions. Keeping this in mind, the present study was carried out with the objective to determine the optimum pH which favoured the maximum multiplication and growth of *Chlorococcum humicola* in liquid Bold Basal medium. In order to achieve this objective, the multiplication and growth performance of the species was evaluated under different conditions of pH (pH 3-10) for a period of 8 weeks or 56 days (data collected weekly). During the weekly experimental analysis, culture media characteristics like conductivity, temperature, pH and the cultured species multiplication and growth responses like cell count, cell size, turbidity and biomass were analysed. Experimentation was conducted in triplicates with a control at each specific initial pH. It has been found that the optimum pH of Bold Basal medium favouring maximum growth of *Chlorococcum humicola* was that of pH 8.

**Keywords: *Chlorococcum humicola*, pH, Bold Basal Medium**

## INTRODUCTION

Algae, one of the oldest life forms on Earth had been known to constitute almost 50% of the world's oxygen supply. Several characteristics of algae like higher photosynthetic efficiency, shorter life span and rapid multiplication rates, high oil contents etc. makes them good candidates that could replace the synthetically made and conventional resources **[1]**. Both macroalgae and microalgae could potentially be used as a source of natural bioactive constituents, in the production of biofuels, biodiesel and other bio-products that are environmental friendly. Algal species had been scientifically proven to contained useful constituents that provide nutritional quality which can be of great importance and values if added to supplementary foods products **[2]**. In addition, the biomass produced by algal species contained high protein contents and could be a potential source of important proteins in future researches and studies **[3]**. Apart from proteins, photosynthetic green algae also contain significant levels of useful lipids and carbohydrates that could be used in foods and fuels industries. Further, Chiu *et al*., (2015), showed that algae had the ability to degrade heavy metals and toxic pollutants present in water bodies hence, they could also be used in the remediation of such heavy metals pollutants in any aquatic ecosystems **[4]**. By using the carbon fixation process, algae had also been known to possess the much needed ability to reduce carbon dioxide gas from the atmosphere **[5]**.

*Chlorococcum humicola* (Nägeli) Rabenhorst, is a species of algae that belongs to the class Chlorophyta. Over the years, this species had been found to possess numerous advantageous properties such as in the production of renewable sources of biodiesels and bio-products, as a biocontrol species in the remediation of polluted water bodies and in waste water treatments **[6]**. However, cell multiplication and growth of the species in liquid culture media was found to be slow. Hence, optimization and growth standardization study on the species is much needed to ensure maximum response and rapid growth.

Modern day biomedical and pharmaceutical industries are continuously searching for easily available low cost biomaterials for the production of bio resources in a sustainable manner **[7]**. In this quest to find such biomaterials, algae had been identified to be the most promising candidates. However, the sole success of these industries and sectors solely depends on the ability of algae to produce sufficient quantities of biomass. Keeping this in mind, numerous attempts had been made to develop working protocols that would

enable these sectors to mass produce algal biomass. However, this is not an easy task since both macroalgae and microalgae growth and multiplication rates can vary according to their species characteristics and depending on the prevailing physicochemical parameters. Several physicochemical factors like pH, temperature, salinity, nitrate content, phosphate content, the level of carbon and the presence of trace metals like Zn, Pb, Mg etc. had been found to have a determining influence on the photosynthetic efficiency as well as the growth of both macroalgal and microalgal species **[8, 9, 10]**. Hence, knowledge of appropriate optimization of physicochemical parameters such as these, and the effect of individual parameters could enable us to maximized the production of both macroalgal and microalgal biomass. Manipulation and optimization of culture media in order to achieve maximum growth and multiplication of macroalgal and microalgal species had been performed in numerous studies over the years **[11, 12, 13]**. However, the effects of individual physico-chemical parameters like pH, temperature etc. on growth and multiplication of algae seems to vary according to species and their characteristics.

Earlier studies had reported that pH is one of the key determining physico-chemical parameter that strongly influence the growth and metabolism of algal species **[14-17]**. In soil crust, alkaline pH has been reported to be the best hydrogen ions concentration levels for the growth of *Chlorococcum* species **[18]**. However, this had been done only for those species of *Chlorococcum* that were abundant in soil and not those found in water bodies which are more beneficial in term of water phycoremediation. In this light, this investigative study had been carried out with the sole objective to find out the optimum pH that yielded maximum growth, multiplication and survival rates of *Chlorococcum humicola* cells cultured in Bold Basal medium.

## MATERIALS AND METHODS

Algal samples were collected from various lentic and lotic fresh water ecosystems of Meghalaya, India, during May-June, 2019. Micro algal samples were collected from the surface of water body with the help of a phytoplankton net and from the peripheral regions with the help of scalpel and toothbrush from different substrata like stones, rocks, pebbles, dead leaves and sediments. The algal sample were preserved in 4% formaldehyde solution and brought to the laboratory for further study. Algal sample were observed under a Trinocular microscope and photographed (using Delphi-X observer series microscope). Taxonomic classification up

to species level were carried out with the help of standard books and monographs **[19-25]**.

Taxonomy was updated using the online database, Algae Base [World-wide electronic publication (www.algaebase. Org)] **[26]**. Pure cultures of *Chlorococcum humicola* was achieved after a series of sub culturing in Bolds Basal medium **[27]**, these were then photographed accordingly.

Treatment sets were maintained with 150 ml each of BB medium taken in 100 ml conical flasks. Another sets for biomass determination was done with 1L BB medium. The pH of culture medium contained in each conical flask was adjusted to a specific pH, ranging from 3.0 to 10, with a gradation of 1 using NaOH (0.5 N) and HCl (0.05 N). To each conical flask, after adjusting to the required pH, 5.0 ml of pure culture of *Chlorococcum humicola* was added. Two control sets were maintained, of which one was worked on the first day of the treatment and the other one was kept for further observation. All the sets were kept at illumination during day time. The pH, temperature and conductivity associated with the culture medium and the growth parameters associated with the micro algal species like cell count, cell size, turbidity and biomass were monitored, periodically. Every week after observation, the altered pH was readjusted. Monitoring of the treatment sets were carried out for a period of 8 weeks or 56 days. The entire experimentation was repeated thrice and the average values were reported.

**RESULTS AND DISCUSSION**

After performing the above experimental investigation, it has been found that mean cell counts throughout the eight weeks of study was maximum (12.02 x $10^4$ cells/ml) at pH8 and minimum (6.60 x $10^4$ cells/ml) at pH7. On the second week during the treatment period, it has been observed that there was a formation of precipitate at pH 10. Similarly, precipitate formation was also observed at pH 9 on the fourth week during the treatment period. In addition it has been found that pre adjusted pH tends to move towards the neutral range i.e. pre-adjusted alkaline pH dropped while pre-adjusted acidic pH rises **(Table1, Figure 4)**. Earlier studies had shown that precipitate formation always occur if the hydrogen ion concentration of the medium is extremely alkaline (>pH 9) **[28]**. Further, it has been found that micro algal photosynthesis help raise the concentration of hydrogen ions (pH) of the medium where they are cultured **[29, 30]**. In addition to the process of photosynthesis, variation in the concentration of hydrogen ions (pH) in the culture medium has also been found to be the results of metabolic secretion and the responses of algae to

nutrients that are present in that culture medium **[31]**.

Further, turbidity median value after eight weeks of investigative study was found to be maximum (11.77 NTU) at pH 8 and minimum (4.66 NTU) at pH 3. Temperature recordings of the culture media revealed that in all the experimental sets, the parameter was highest on the first week (31°C) decreases and gradually became constant from the 3rd week to the 8th week (25°C) **(Table 2, Figure 4)**. Earlier studies had shown that micro algae of fresh water origin can tolerate temperature of up to 40°C. However, It has been observed that a temperature raging between 25-35°C is very ideal for the growth and multiplication of these algae **[32]**.

Micrometric investigation of the cell sizes of *Chlorococcum humicola* revealed that smaller cell size occurred at alkaline pH (8-10) while bigger cell size was observed from those experimental sets with a pre-adjusted acidic pH (3-6). Colony formation of cells of *C. humicola* was observed at higher pre-adjusted pH (8-10) while at lower pH level, cells of this microalga are mainly solitary. The cell size of *C.humicola* at pH7 was very identical to those present in the control set with a non-adjusted initial hydrogen ions level of 6.82. Without readjustment, the pH of the control set was also monitored weekly and had been found that pH of this set increased from 6.82 to 7.32. Earlier studies had shown that in extreme conditions (such as those associated with pH), micro algae in general undergone a stage of dormancy particularly to ensure their survival when conditions become favourable in a later stage **[33]**. When it comes to micrometric cell sizes investigation, the general observation of a decreased and increased cell sizes in alkaline and acidic pH is an interesting note owing to the fact that algae response differently to varying level of hydrogen ions concentration (pH). These increased or decreased in sizes could potentially be a survival strategy of microalgae to cope with the existing extreme or unfavourable conditions.

Assessment of conductivity of the culture media showed that it was maximum (4851 µS) at pH 8 and minimum (466 µS) at pH 3 (mean value across 8 weeks). Conductivity in acidic pH (especially at pH 3 and 4) decreases from the 1st to the 8th week, while at alkaline pH (8-10), conductivity increases form the 1st to the 8th week.

Biomass productivity assessment throughout the experimental sets showed that heaviest (0.198 g dry weight/L) biomass was recorded at pH8 and lightest (0.011 g dry weight/L) biomass at pH 3. Since there was the formation of precipitate at pH 9 and pH 10, the weight of biomass recorded at these two pH levels though

significantly high was more or less neglected **(Table 3, Figure 4)**.

Elevated hydrogen ions concentration (pH) has been found to have adverse effects on the growth and multiplication of algae mainly due to the altered nutrient uptake that occur in these organisms in the alkaline conditions **[34, 35, 36]**. Similarly, the growth and proliferation of microalgae also suffers a devastating blow and that undesirable outcomes of growth may arise when the hydrogen ions concentration (pH) declined and becomes extremely acidic **[37]**. Since precipitate formation occurs at higher pH values, the culture medium/microenvironment in which algae were grown became stressful and there is a reported limitation of nutrients. All these combined makes growth and multiplication of microalgae in higher pH difficult **[38, 39]**. Biochemical reactions that occur in cells of micro algal origin had been found to decrease significantly with increased concentration of hydrogen ions (pH) in surrounding microenvironment. Reduction in the rate of biochemical reactions together with the changes in cell membrane morphology/properties due to elevating pH in microenvironment has also been found to affect the growth and proliferation of microalgae in culture **[40]**. However, this is not a universal inference owing to the fact that certain groups of algae prefer an acidic environment while others favour alkaline pH for their growth and multiplication.

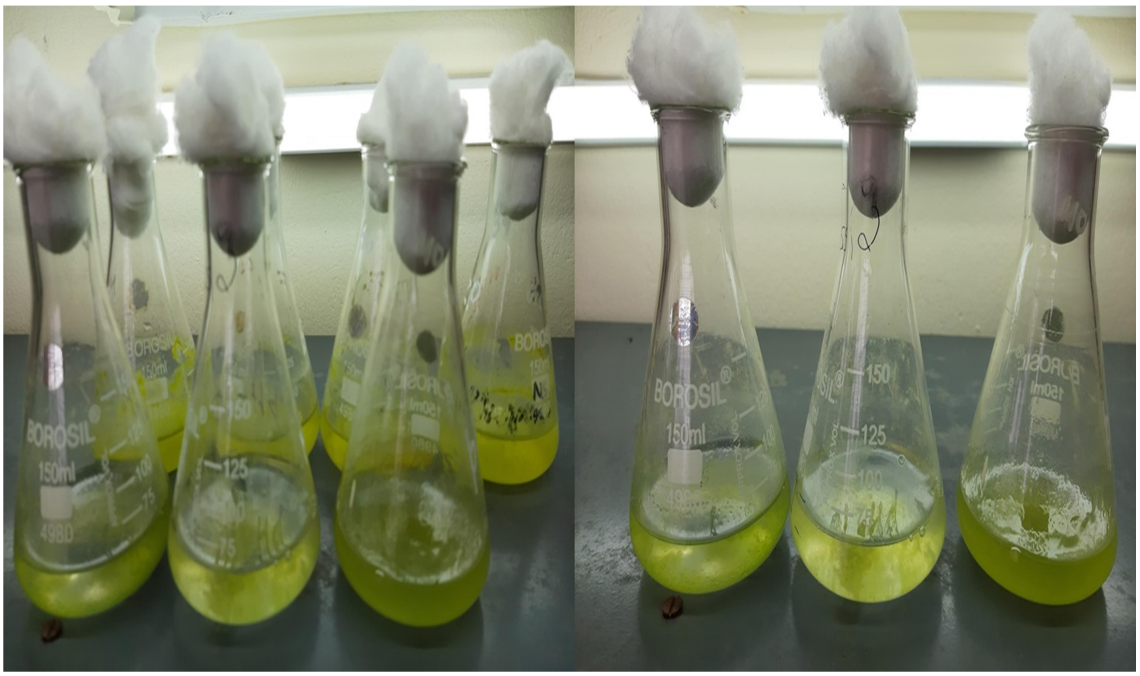


**Figure 1:** ***Chlorococcum humicola cells*** **culture in Bold Basal Medium**

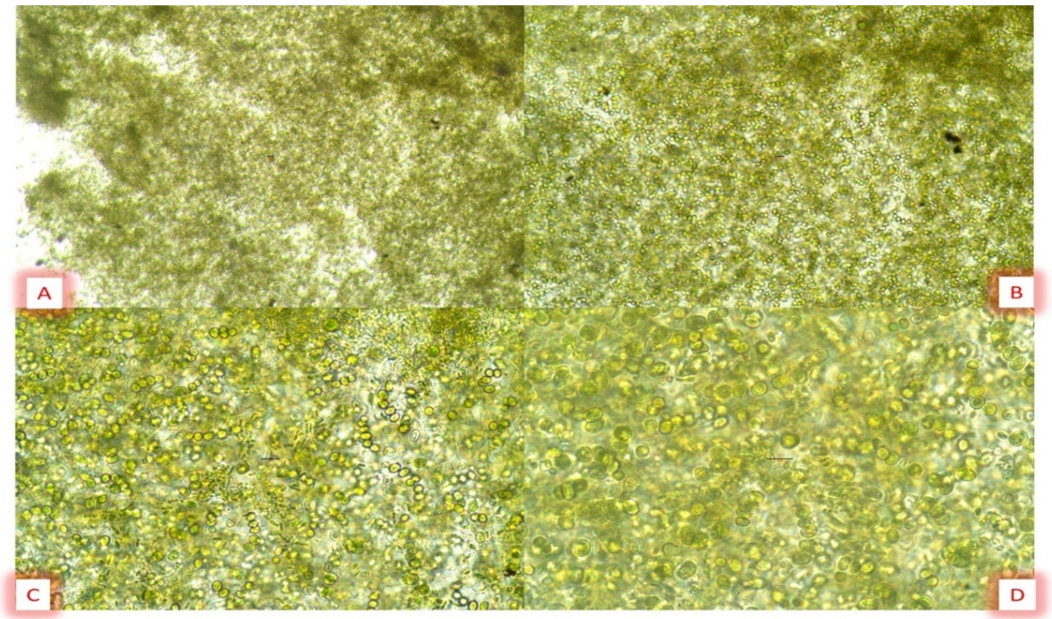


**Figure 2: *Chlorococcum humicola* cells under different magnifications A: 10X; B: 20X; C:40X; D:60X (pH 8: 1st Week)**

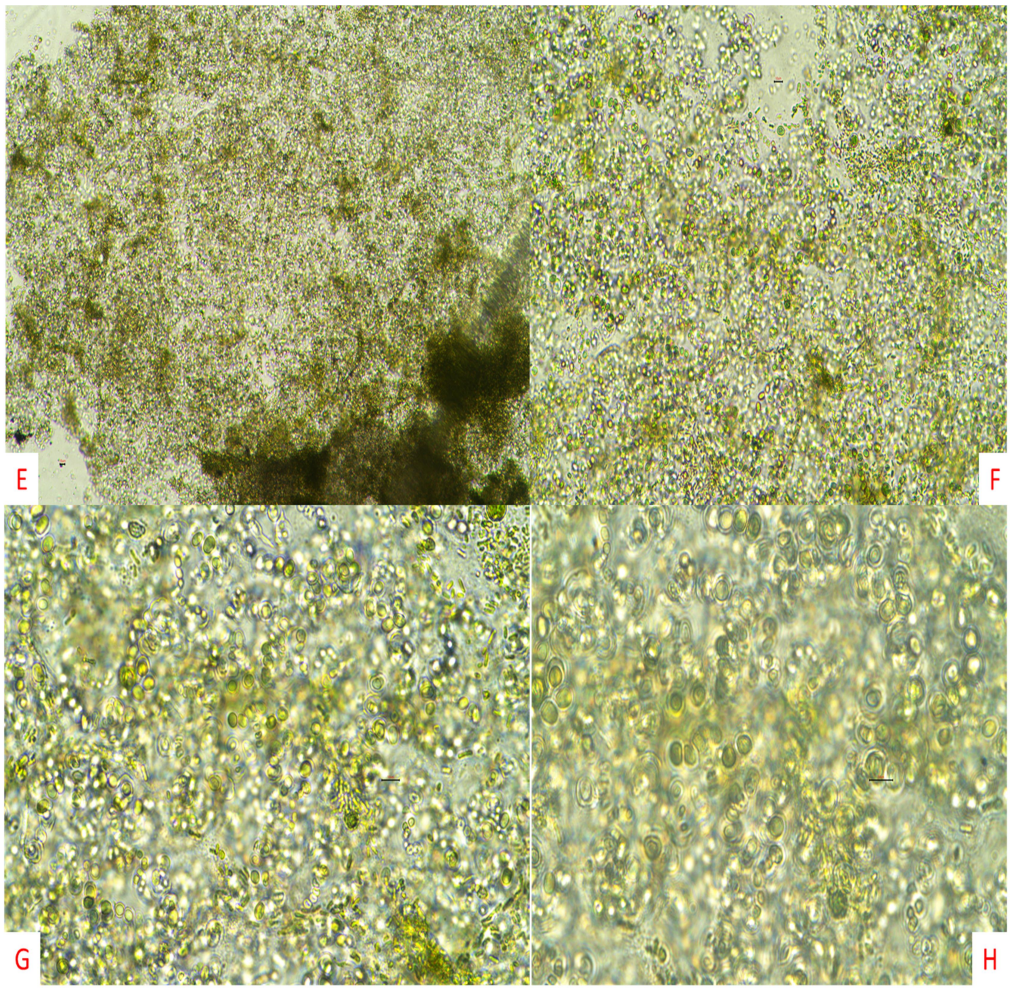


**Figure 3: *Chlorococcum humicola* cells under different magnifications E: 10X; F: 20X; G: 40X; H: 60X (pH 8: 8th week)**

**Table 1: Cell count difference in cultures of *Chlorococcum humicola***

| Experimental sets | 1st week | 2nd week | 3rd week | 4th week | 5th week | 6th week | 7th week | 8th week | Median values (x10$^4$ cells/ml) |
|---|---|---|---|---|---|---|---|---|---|
| Control | 9.50 | 9.90 | 9.30 | 10.10 | 10.50 | 10.60 | 10.60 | 10.90 | 10.17 |
| pH 3 | 8.70 | 8.90 | 8.30 | 8.30 | 7.60 | 6.60 | 6.90 | 7.00 | 6.60 |
| pH 4 | 6.80 | 6.85 | 6.87 | 6.80 | 6.84 | 6.83 | 6.99 | 6.72 | 6.83 |
| pH 5 | 7.20 | 6.88 | 7.33 | 7.38 | 6.98 | 7.25 | 7.28 | 7.32 | 7.20 |
| pH 6 | 6.60 | 6.75 | 6.98 | 7.35 | 7.66 | 7.99 | 8.23 | 8.29 | 7.48 |
| pH 7 | 5.50 | 5.75 | 5.96 | 6.28 | 6.58 | 6.88 | 7.65 | 8.26 | 7.78 |
| pH 8 | 9.80 | 10.58 | 10.98 | 11.26 | 12.25 | 12.95 | 13.85 | 14.52 | 12.02 |
| pH 9 | 7.30 | 7.45 | 7.49 | 7.97 | 8.25 | 8.35 | 8.45 | 9.00 | 8.03 |
| pH 10 | 6.60 | 6.65 | 6.92 | 7.56 | 7.65 | 7.88 | 8.01 | 8.88 | 7.51 |

**Table 2: Comparison in turbidity difference observed in culture of *Chlorococcum humicola***

| Experimental sets | 1st week | 2nd week | 3rd week | 4th week | 5th week | 6th week | 7th week | 8th week | Median values (NTU) |
|---|---|---|---|---|---|---|---|---|---|
| Control | 6.60 | 6.90 | 7.30 | 7.55 | 7.77 | 7.95 | 8.25 | 8.80 | 7.64 |
| pH 3 | 4.20 | 4.32 | 4.42 | 4.25 | 4.65 | 4.88 | 5.25 | 5.32 | 4.66 |
| pH 4 | 4.80 | 5.25 | 5.46 | 5.67 | 5.23 | 6.21 | 6.21 | 6.23 | 5.63 |
| pH 5 | 4.90 | 5.65 | 5.69 | 5.88 | 6.25 | 6.38 | 7.58 | 7.21 | 6.19 |
| pH 6 | 4.00 | 4.88 | 5.21 | 5.76 | 6.21 | 6.78 | 7.56 | 8.25 | 6.08 |
| pH 7 | 5.20 | 5.88 | 6.35 | 6.95 | 7.46 | 8.54 | 10.32 | 12.40 | 7.88 |
| pH 8 | 6.30 | 7.25 | 8.66 | 9.95 | 10.87 | 14.57 | 16.99 | 19.57 | 11.77 |
| pH 9 | 6.20 | 7.24 | 8.64 | 9.86 | 10.78 | 13.98 | 15.88 | 18.99 | 11.44 |
| pH 10 | 5.90 | 6.98 | 7.68 | 8.74 | 10.11 | 12.98 | 16.23 | 19.01 | 10.95 |

**Table 3: Comparison in biomass difference observed in cultures of *Chlorococcum humicola***

| Experimental sets | Biomass (gm dry weight/L) |
|---|---|
| Control | 0.120 |
| pH 3 | 0.011 |
| pH 4 | 0.013 |
| pH 5 | 0.022 |
| pH 6 | 0.101 |
| pH 7 | 0.122 |
| pH 8 | 0.198 |
| pH 9 | 0.092 |
| pH 10 | 0.089 |

**Table 4: Recorded temperature readings of the culture medium containing *C. humicola* cells throughout the 8 weeks of study**

| Experimental sets | Temperature (°C) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1st week | 2nd week | 3rd week | 4th week | 5th week | 6th week | 7th week | 8th week | Mean value |
| Control | 31 | 27 | 25 | 25 | 25 | 25 | 25 | 25 | 26 |
| pH 3 | 31 | 26 | 25 | 25 | 25 | 25 | 25 | 25 | 25.875 |
| pH 4 | 31 | 27 | 25 | 25 | 25 | 25 | 25 | 25 | 26 |
| pH 5 | 31 | 26 | 25 | 25 | 25 | 25 | 25 | 25 | 25.875 |
| pH 6 | 31 | 26 | 25 | 25 | 25 | 25 | 25 | 25 | 25.875 |
| pH 7 | 31 | 27 | 25 | 25 | 25 | 25 | 25 | 25 | 26 |
| pH 8 | 31 | 28 | 25 | 25 | 25 | 25 | 25 | 25 | 26.125 |
| pH 9 | 31 | 27 | 25 | 25 | 25 | 25 | 25 | 25 | 26 |
| pH 10 | 31 | 28 | 25 | 25 | 25 | 25 | 25 | 25 | 26.125 |

**Table 5: Recorded conductivity readings of the culture medium containing *C. humicola* cells throughout the 8 weeks of study**

| Experimental sets | Conductivity (µS) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1st week | 2nd week | 3rd week | 4th week | 5th week | 6th week | 7th week | 8th week | Mean value |
| Control | 1990 | 1897 | 1898 | 1889 | 1896 | 1911 | 1922 | 1925 | 1916 |
| pH 3 | 463 | 466 | 469 | 469 | 468 | 462 | 464 | 467 | 466 |
| pH 4 | 489 | 491 | 499 | 501 | 500 | 501 | 509 | 504 | 499.25 |
| pH 5 | 501 | 511 | 514 | 517 | 522 | 549 | 569 | 587 | 533.75 |
| pH 6 | 1004 | 1014 | 1042 | 1046 | 1049 | 1044 | 1051 | 1068 | 1039.75 |
| pH 7 | 1998 | 1991 | 1993 | 1999 | 2001 | 2005 | 2011 | 2054 | 2006.5 |
| pH 8 | 4845 | 4847 | 4846 | 4845 | 4851 | 4852 | 4855 | 4867 | 4851 |
| pH 9 | 4782 | 4783 | 4788 | 4798 | 4799 | 4792 | 4756 | 4758 | 4782 |
| pH 10 | 4812 | 4817 | 4819 | 4811 | 4820 | 4822 | 4827 | 4822 | 4818.75 |

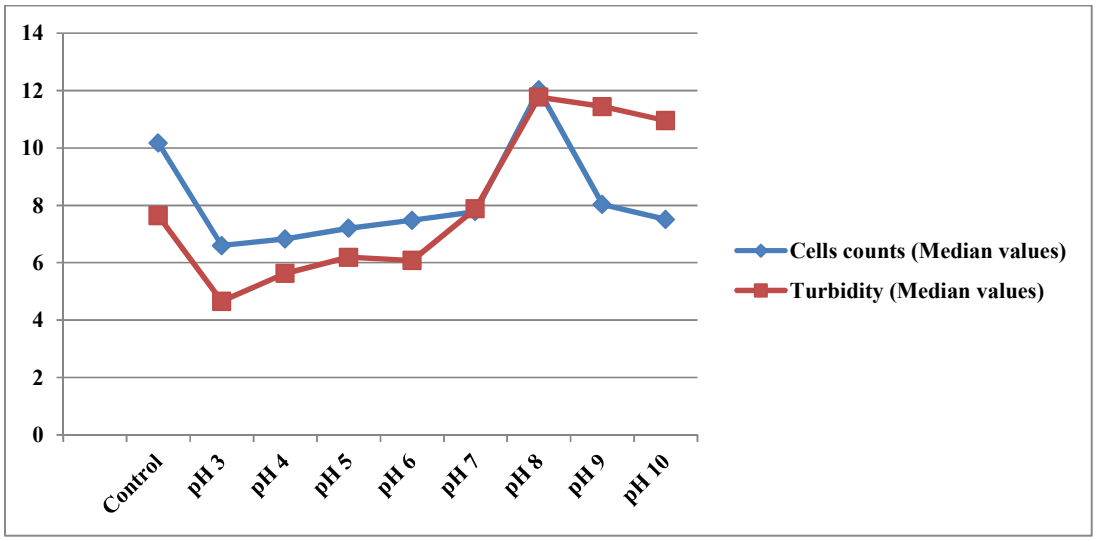


**Figure 4: Graphical representation of the changes in cell counts and turbidity of cultures of *Chlorococcum humicola* throughout the experimental pH sets (Mean values)**

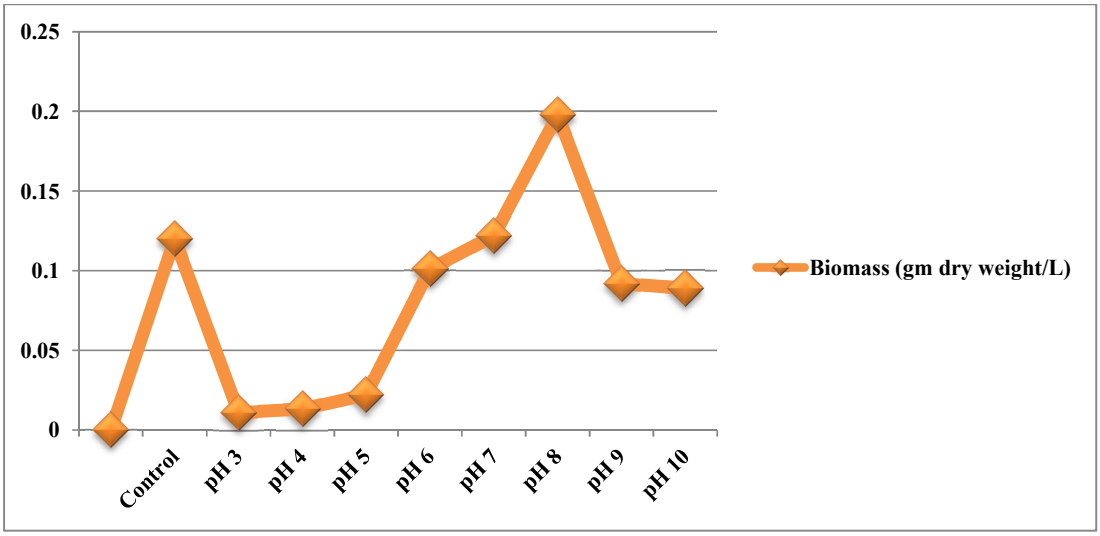


**Figure 5: Graphical representation of the changes in biomass across the different experimental pH sets (Mean values)**

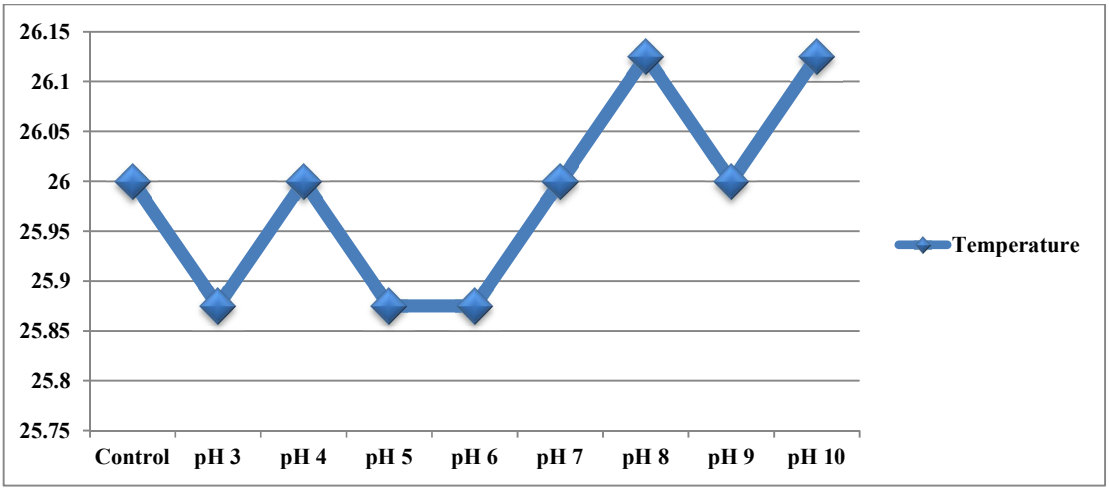


**Figure 6: Graphical representation of the changes in temperature (°C) throughout the 8 weeks of study in all the pH experimental sets (Mean values)**

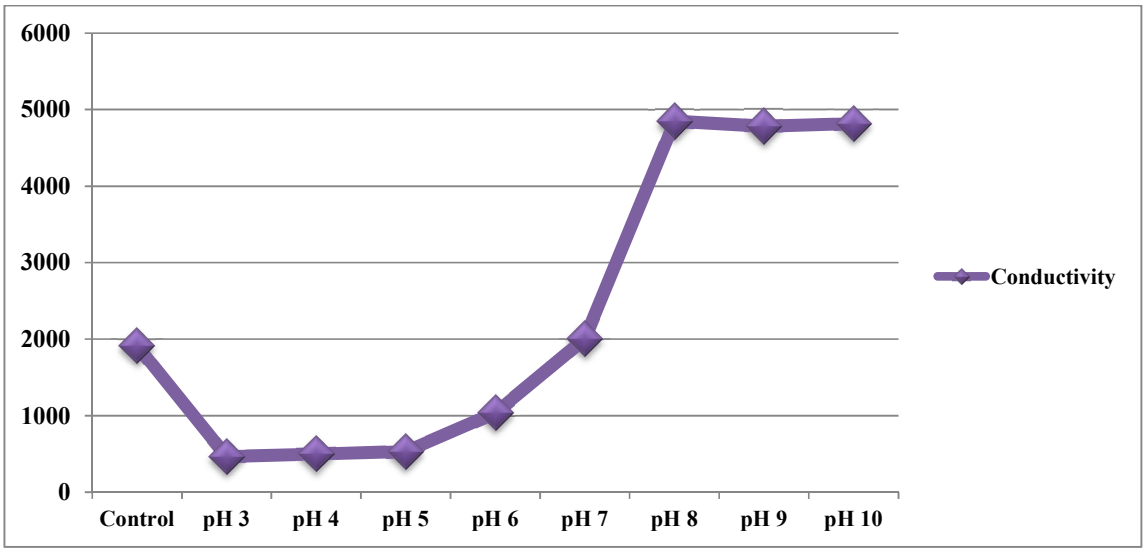


**Figure 7: Graphical representation of the changes in conductivity (µS) of the culture medium throughout the 8 weeks of study in all pH experimental sets (Median values)**

## CONCLUSION

In the present investigative study, an attempt has been made to determine the optimum pH thatfavour the maximum growth and proliferation of cells of *Chlorococcum humicola* (Nägeli) Rabenhorts culture in liquid Bold Basal medium. Experiments were conducted in varying pH levels ranging from pH 3-pH 10. Continuous monitoring and evaluation of the treatments sets were carried out for a period of 8 weeks (56 days). Finally, by analysing the mean values of major growth parameters like cell counts, biomass, turbidity etc., it can be concluded that to ensure maximum production of biomass and rapid multiplication of *Chlorococcum humicola* cells in culture conditions, a pH of 8 can be maintained.

## ACKNOWLEDGEMENT

The authors are thankful to the Head of the Department of Botany, North Eastern Hill University for providing us will all the necessary laboratory facilities which contributed towards the successful completion of this investigative study.

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