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Study of the inhibitory effect of the media culture parameters and cell population to increase the biomass production of *Dunaliella tertiolecta*

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Abstract

Microalgal growth curve, after the exponential phase, shows a stationary phase where algal biomass production is inhibited and remains constant by some factors such as nutrient depletion and intrinsic behavior. The present study is concerned with evaluating effects of the culture medium condition and intrinsic behavior on biomass production by *Dunaliella tertiolecta*. To this end, effect of pH, nutrient concentration, CO_2 ($NaHCO_3$) concentration, possible secreted substances, and cell density on the biomass production by *D. tertiolecta* were investigated. The results showed that biomass yield can be significantly affected by pH ($p<0.01$) and nutrient ($p<0.05$). In a combination of pH and nutrient, the biomass was found to be more influenced by the pH. The results showed a significant interaction between nutrient and $NaHCO_3$ ($p<0.05$), suggesting that CO_2 concentration may limit biomass production only when sufficient nutrients are available. Nutrients concentration and biomass production showed a direct correlation ($p<0.05$). The rate of reaching the maximum biomass was showed to be increased in higher nutrients concentration; however, the maximum point could not to be affected. The existence of secreted compounds with inhibitory effect on the growth was not observed. The inhibitory effect of the cell density on biomass production can not be confirmed.

Key Words: biomass limitation, cell density, *Dunaliella tertiolecta*, intrinsic behavior Media condition.

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Introduction

Algae are multipurpose biomass feedstock for different industries such as food, medicine, feed, and biofuel production (1). Achieving to the highest biomass is important in all these industries (2). *Dunaliella* is one of the most studied unicellular green microalgae for mass culture and is commercially important for β-carotene and biofuel production (3, 4).

Although different factors can be considered in increasing biomass, it is mainly affected by algal intrinsic behavior, environmental conditions (5), and media culture properties (6, 7). Media culture properties include the fresh media properties and their changes during the algal growth (8, 9). These changes are simultaneous and are caused due to the growth, senescence, and cell death (10). The major changes that occur in the media are pH shift (pH of the media may sometimes reach 11 in *Dunaliella* culture), nutrition and CO_2 consumption, cell population increasing, and secreted substances. These changes can limit growth by interfering in nutrient uptake (8), decreasing photosynthesis carbon source (11), and probably by controlling self-cell density (7). Many factors were investigated by researchers in order to increase algal biomass.

Fabregas *et al*. (12) examined the effect of different nutrient concentrations on mass culture of *Dunaliella tertiolecta* in order to obtain a maximum biomass production. They obtained the maximum cellular densities between 12.45×10^6 and 14.23×10^6 cells/ml with 4, 8 and 16 mM of $NaNO_3$. Tang et al. (13) studied the effect of light source, light intensity, CO_2 concentration, and photoperiod on the growth and oil content of *Dunaliella tertiolecta*, suggesting that light intensity increases algal growth. Yet, they observed similar growth rates for 2%, 4%, and 6% CO_2 concentrations. Effects of the of CO_2 aeration on the biomass production and lipid accumulation of *Nannochloropsis oculata* in a semicontinuous culture were investigated by Chiu et al. (14). The maximal biomass was reported 0.480 g/L/day with 2% CO_2 aeration.

Studying different CO_2 concentration and pH values on the *Chlamydomonas reinhardtii* growth showes that the optimal pH for *C. reinhardtii* is 7.5 (15). An injection of air and a moderate amount of CO_2 promote algae growth; however, excess CO_2 inhibits algae growth, due to a significant decrease in pH. Light intensity and photoperiod have important roles in maximum biomass production of *D.bardawil* (16). The most biomass production is obtained in 2Klux and at a photoperiod 16/8 light dark cycle.

In addition to the above mentioned factors, it seems that cell – cell communication and cell density, as bacterial quorum sensing, can act as a limiting factor in algal growth. There are a few reports that show algae may mimic quorum sensing to control self - monitoring of their cell population (17, 18). Also, research into effects of medium factors on *Dunaliella tertiolecta* biomass production is limited. Therefore, in this study the media culture parameters, existence of secreted substances, and cell density were considered in order to determine which of these factors exert more inhibitory effect on the biomass production (cell population) by *Dunaliella tertiolecta*.

Materials and methods

Algae culture

Dunaliella tertiolecta (UTEX LB999) was obtained from the University of Texas at Austin. The algae were cultured in 100 mL Erlenmeyer flask at 25 ± 1 °C. Modified Johnson media (19) was used as media containing of 2 g/L

KNO_3, 0.1 g/L KH_2PO_4, 5 g/L $MgSO_4$, 0.1 g/L $CaCl_2$, 3.7 mg/L of $Na_2EDTA.2H_2O$, 1.1 mg/L of $FeCl_3.6H_2O$, 1.33 mg/L of $MnCl_2.{}_4H_2O$, 0.14 mg/L $ZnCl_2$, 0.024 mg/L of $CoCl_2.{}_6H_2O$, 1.2 mg/L $(NH_4)Mo_7O_{24}.H_2O$, 0.08 mg/L $CuCl_2$. The media was enriched by 1 g/L $NaHCO_3$ as CO_2 resource, 1 mM of NaCl as osmolarity, and pH was adjusted on 7. Cultures were illuminated using fluorescent light with 60-80 µ mole photon m^{-2} s^{-1} intensity in a 16 h light/ 8 h dark cycle.

Algae growth

Biomass and cell density was calculated by measuring optical density at 750 nm (20) with a spectrophotometer (Shimadzu UV160A). Cell population was also determined using a hemocytometer. The standard calibration curve of OD: biomass and OD: cell number was determined, where one unit of OD_{750nm} corresponded to 5×10^6 cells, 0.45 g/L fresh weight, and 0.04 g/L dry weight of the biomass, respectively.

Design of experiments

Three experiments were designed as follows. The flowcharts of the experiments 1 and 2 are shown in Fig.1.

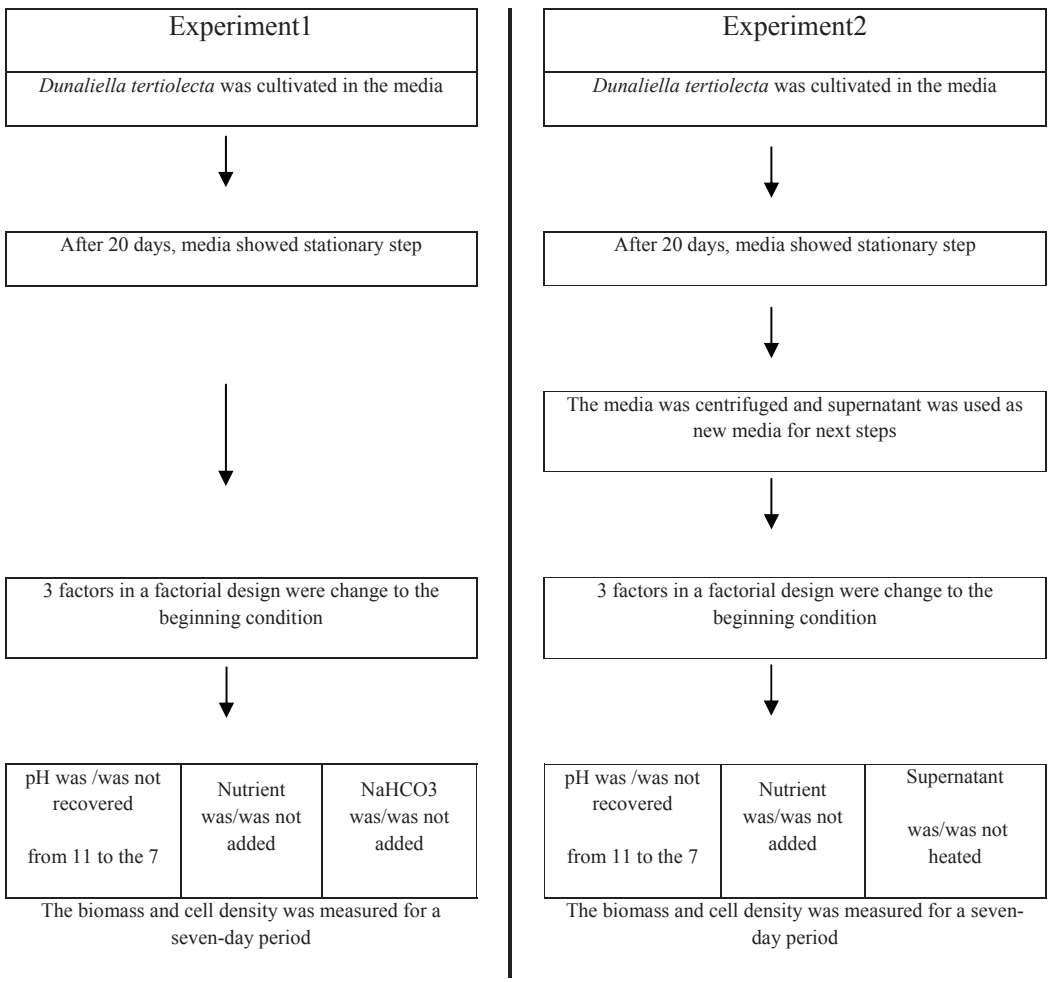

Figure 1. The flowchart of the experiments.

Experiment 1 (inhibitory effect of the media parameters)

This experiment was conducted to recognize which factors in the media may limit *Dunaliella tertiolecta* growth. To this end, *D. tertiolecta* was cultivated in modified Johnson media (pH 7) supplemented with 1 g/l $NaHCO_3$ at 25˚C for 20 days. When the alga reached the stationary phase, then pH value was readjusted from 8.6 to the beginning of the experiment to 7. The nutrient level and $NaHCO_3$ concentration were also exactly readjusted according to the beginning of the experiment. The controls were kept unchanged. This experiment was carried out in a completely randomized block design in a factorial matrix (Table1). Cell density and biomass was monitored for seven days after the 20^{th} day of incubation.

Experiement 2 (inhibitory effect of the secreted substance)

In a randomized complete block design, this experiment was designed to evaluate the possibility of the existence of substances that may be secreted by algae to the media and control the cell population. *Dunaliella tertiolecta* was cultivated in the modified Johnson media after 20 days when algae showed the stationary phase; media was centrifuged and the obtained supernatant was used as a fresh media for reculturing the algae. It was expected that this media was containing substances secreted by the *Dunaliella* cells that may inhibit the biomass production. Before the reculturing, three factors (according to Table 1) were changed as follow:

1. pH was readjusted to 7,
2. nutrient supplement was provided, and
3. the supernatant was heated for deactivation of the possible growth inhibitor substances in the medium.

Table1: the factors and their levels considered for the experiemts

Table1: the factors and their levels considered for each experiment

factor	explain	Sign of level1 used in the text	Sign of level2 used in the text
pH	pH of the media was(level2)/was not(level1) recovered to the 7 by adding sterile HCl	**no-recovered-pH**	**recovered-pH**
Nutrient	New nutrient was(level2)/was not(level1) added. added-nutrient samples, was received new sterile nutrients with the same concentration in a fresh media.	**no-added-nutrient**	**added-nutrient**
$NaHCO_3$	CO_2 concentration was(level2)/was not(level1) received 1 g/L by adding sterile $NaHCO_3$	**no-added-CO_2**	**added-CO_2**
Heat	After 20 days, cultures media was centrifuged and obtained supernatant was used as media for new algal inoculation. Before new inculcation, supernatant was(level2)/was not(level1) heated. The heated samples were kept in boiling for 5 minutes.	**no-heated**	**heated**

Experiment3 (inhibitory effect of the cell density as intrinsic behavior)

Experiment 3 was carried out in order to understand the inhibitory effect of cell density on limiting biomass production. To this end, three nutrient strength concentrations 1X, 2X, and 3X (where x is the basic concentration of nutrients in modified Jonson's solution as mentioned above) were inoculated with 1×10^6, 2×10^6, and 3×10^6 cells/mL respectively. Biomass and cell density was measured until the stationary phase. For cell population, the percent of control was calculated

as biomass in 7th day /biomass in the beginning. MINITAB v. 6 software was used for design and statistical analysis in all experiments.

Results

Experiment 1 (inhibitory effect of the media parameters)

In this experiment, in a twenty-day cultured medium, three factors (pH, nutrient level, CO_2) were readjusted to the beginning condition. Biomass production was found to be significantly (analysis of variance not shown) affected by pH and nutrient ($p < 0.01$). The time dependence of biomass and pH is shown in Fig. 2. Significant differences ($p>0.05$) of biomass production between the treatments started from the first day after the treatment and reached the maximum (0.95 g/L) in

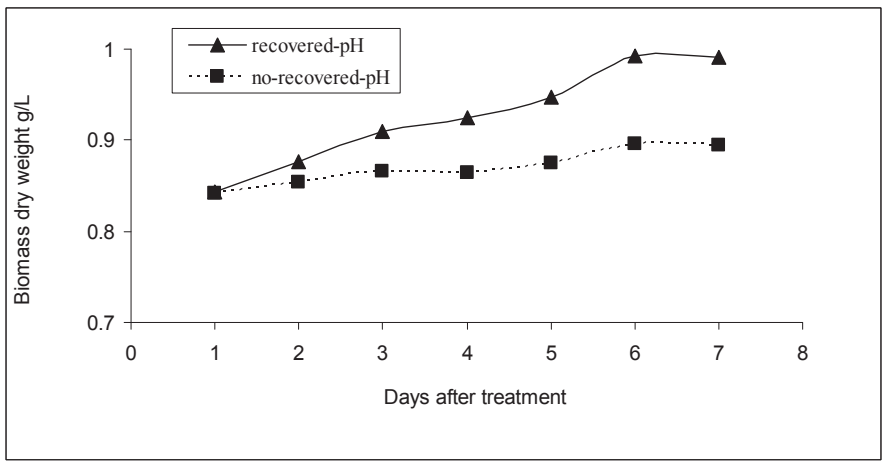

Figure 2. ***Dunaliella tertiolecta*** **biomass production in different pH conditions. Recovered-pH: in 20-day culture, pH recovered to the 7. No-recovered-pH: culture continued without changing in the pH. The values are mean of 3 replications.**

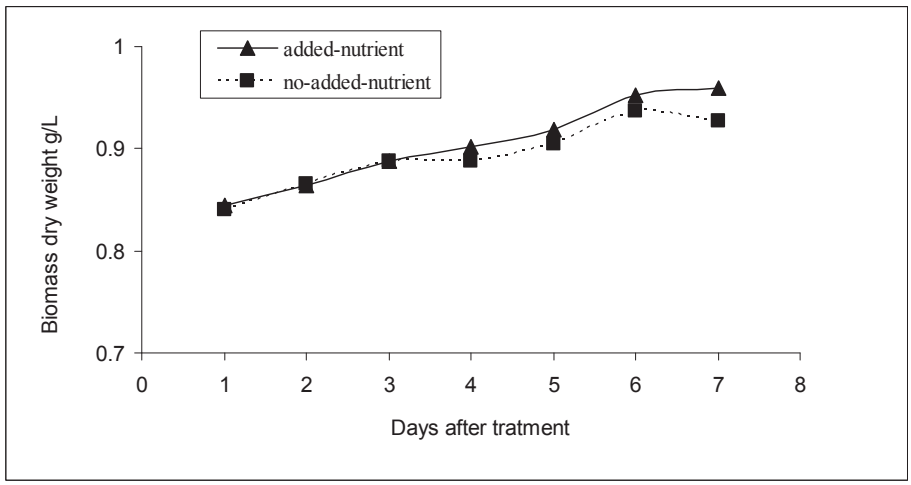

Figure 3. ***Dunaliella tertiolecta*** **biomass production in different nutrient conditions. After 20 days of the culture, the samples were (added-nutrient)/were not (no-added-nutrient) received new sterile nutrients with the same concentration in a fresh media. The values are mean of 3 replications.**

recovered samples in the 7th day. The effect of nutrient on biomass production is shown in Fig. 3. In the added-nutrient samples, more significant biomass production was detected three days after the treatment. The maximum amount of biomass (0.98 g/L) was observed in the 7th day. According to the results shown in Fig.4, difference between the maximum and minimum amount of biomass was more affected by pH treatment than nutrient. The observed difference was 0.6 g/L and 0.1 g/L for pH- and nutrient-treated samples, respectively. The interaction between $NaHCO_3$ and nutrient showed significant effects ($p<0.05$) on biomass production, as shown in Fig. 5. More biomass was obtained in no-added-nutrient samples, when $NaHCO_3$ was not added.

Experiment 2 (inhibitory effect of the secreted substance)

The effect of heating on biomass production is shown in Fig. 6. The amount of biomass in heated samples was found to be significantly less ($p<0.01$) than that of the no-heated after 2 days of incubation. As shown in Fig. 7, a biomass yield of 0.39 g/L and 0.18 g/L was

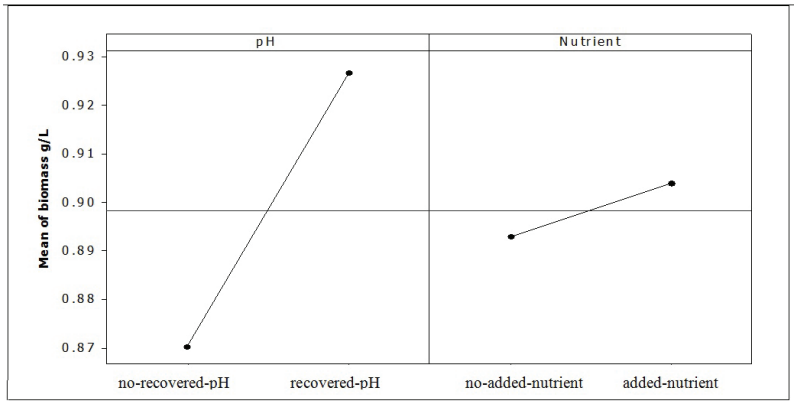

Figure 4. The main effect of pH and nutrient on *Dunaliella tertiolecta* biomass.

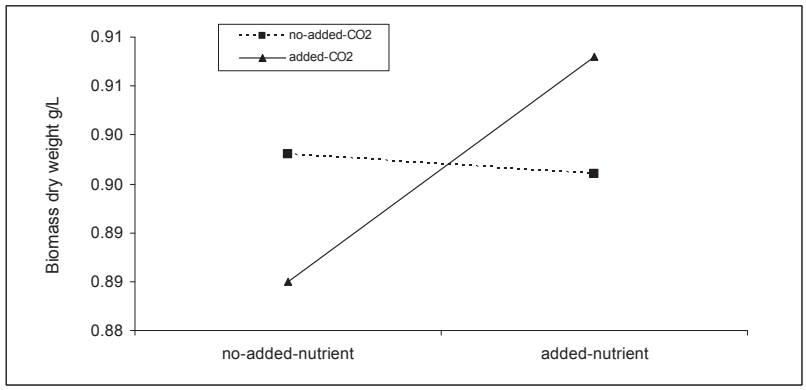

Figure 5. Interaction between nutriment and $NaHCO_3$. After 20 days, CO_2 concentration was (added-CO_2)/was not(no-added-CO_2) added 1 g/L sterile $NaHCO_3$ to the media.

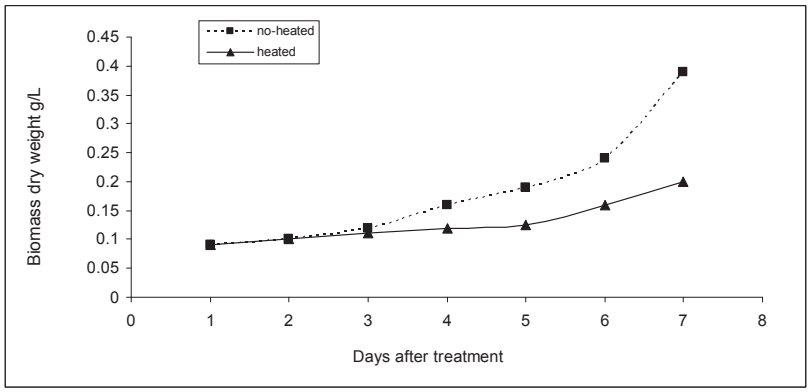

Figure 6. Influence of heating on *Dunaliella tertiolecta* biomass production. A 20-day culture was centrifuged and its supernatant was heated (heated) /was not heated (no-heated) and then used as new culture. The values are mean of 3 replications.

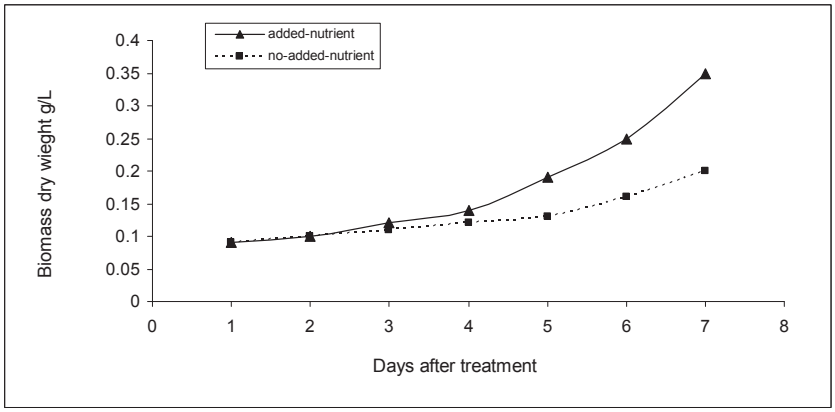

Figure7. The effect of nutrient on *Dunaliella tertiolecta* biomass production in experiment2 condition. A 20-day culture was centrifuged and its supernatant was enriched (added-nutrient) /was not enriched (no- added-nutrient) by nutrient and then used as new culture. The values are mean of 3 replications.

obtained from the added-nutrient and no-added-nutrient samples, respectively. The interaction between the nutrient and pH was found to be significant ($p<0.01$) (Fig. 8). Whereas the no-recovered-pH samples dramatically responded to the nutrient, there was no significant difference between the levels of nutrient in recovered-pH samples.

Experiment 3 (inhibitory effect of the cell density as intrinsic behavior)

From ANOVA results (not shown) it was found that the effect of different nutrient concentration on the rate of biomass and cell population was significant ($p<0.01$). 1X showed the lowest amount of biomass and cell population until the 6th day. Maximum cell ($8.7x10^6$ cells/mL) was obtained in the 7th day in all treatments. The changes of biomass and cell populations over the time are shown in Fig. 9A, B. The most (1.305 g/L) and the lowest (1.136 g/L) biomass was observed in 3X and 1X respectively, while the most growth rate, compared with the control, was obtained in 1X Fig.10 B.

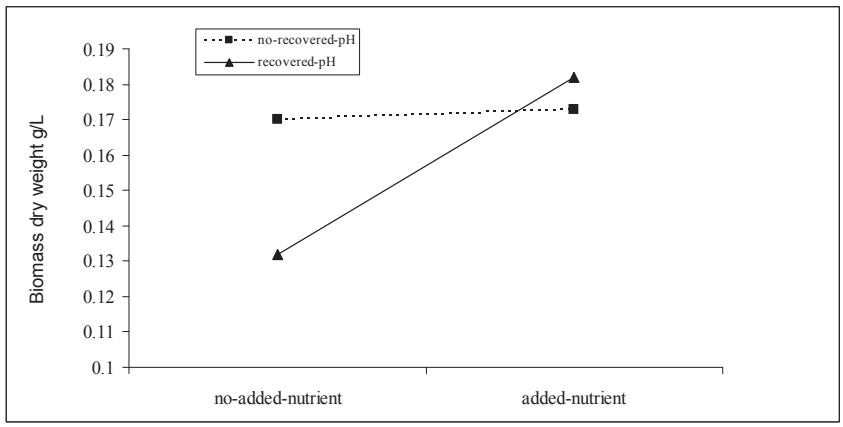

Figure 8. Interaction between nutrient and pH on biomass in experiment2.

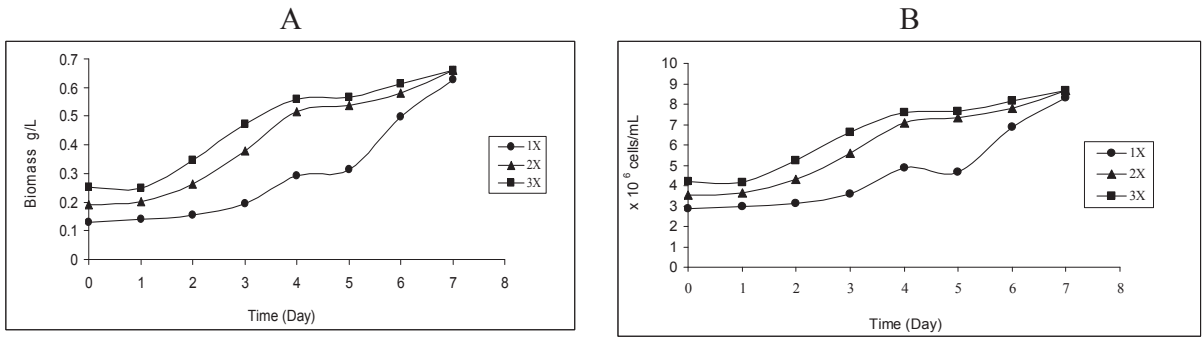

Figure 9. Biomass (A) and cell population (B) of *Dunaliella tertiolecta* over time. X is basic concentration of nutrient in Johnson media as mentioned in the text. 2X and 3X had 2 fold and 3 fold nutrient strength.

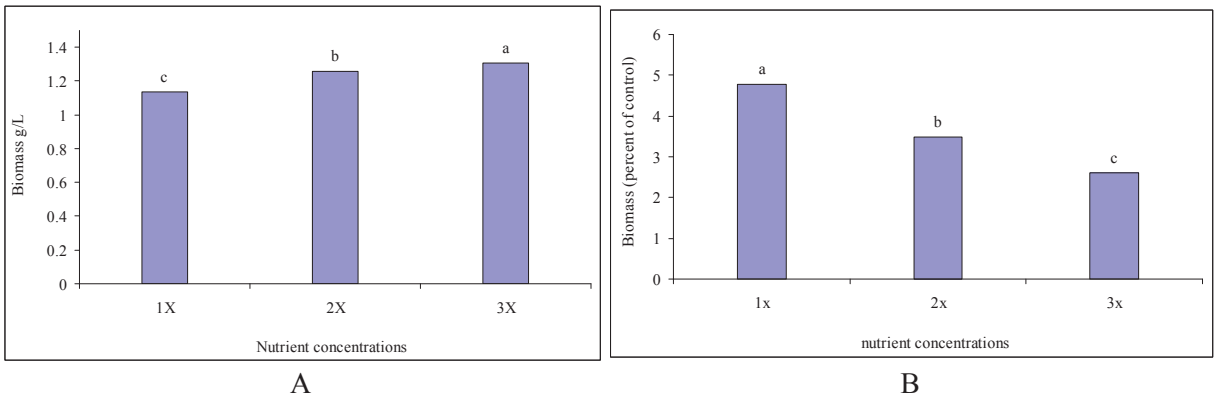

Figure 10. Mean comparison of *Dunaliella tertiolecta* biomass in different nutrient concentration (A) and percent of control (B). X is basic concentration of nutrient in Johnson media as mentioned in the text. 2X and 3X had 2 fold and 3 fold nutrient concentrations. Means with the same letter in a column are not significant at 5% level Percent of control: (biomass in 7th day /biomass in beginning)

Discussion

The results showed that both of pH and nutrient can limit the biomass production; however, pH was found to be more effective. It is known that pH can influence the solubility of nutrients in a media and also nutrients absorption by algae (5). pH became more effective during the last days of experiment. It may be due to the prevention of nutrient uptake by high pH level in no-recovered-pH samples. Considering the main role of CO_2 in photosynthesis (21), the effect of $NaHCO_3$ on increasing the biomass via photosynthesis is expected, as reported in some previous studies (14, 15) but our results showed no significant increase in biomass production in presence of $NaHCO_3$.

Consistent with our results, Tang *et al*. (13) reported a similar growth rates for *Dunaliella tertiolecta* in presence of 2%, 4%, and 6% CO_2. This suggests that 1 g/L of $NaHCO_3$ is enough for photosynthesis during 1-month growth and it may be due to the respiration/ photosynthesis cycle that can replace the consumed CO_2 during photosynthesis (22). Yet a further possible reason includes the ability of algal cell in CO_2 concentrating(23) that helps algal cells grow in a low CO_2 concentration in the medium (11). Our results also showed a significant interaction ($p<0.05$) between $NaHCO_3$ and nutrient, indicating that the presence of nutrient without an efficient photosynthesis could not lead to more growth. This observation is also supported by Solan and Jocob (24). pH monitoring (not shown) showed that pH was increased from 7 to 8.6 during 20 days. Basic pH reduced the solubility of some nutrient.

It can be, therefore, concluded that pH changes the biomass production by affecting the solubility of nutrients in the medium. In industrial applications, it is thus recommended to consider the above-mentioned factors. It is known that the biomass do not exceed a certain maximum of cell density, even if all conditions are kept in optimum. This may be due to the effect of cell population and cell – to –cell communication (7). It has been reported that algae, as bacteria, may use self-monitoring strategy to control their population using molecules secreted by the cell similar to bacteria (25). The results obtained from experiment 2 (Fig. 6) showed that even if such molecules are present in the culture medium, they are not deactivated by heating. The results of experiment 3 suggested that high nutrients may increase the rate of growth, not the maximum peak of biomass. It seems that the cell population may control the maximum cell density and more nutrients may just help achieve this goal rapidly.

As a result, it can be concluded that neither pH nor nutrient nor CO_2 concentration can change the peak point of biomass; however, it can be achieved by the intrinsic behavior of the cells. It seems that identifying and removing this limitation factor can lead to an increase in the maximum point of biomass production. The authors suggest that further studies need to be target the identification of the structure and function of the biomass self-controlling system in *D. tertiolecta*.

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