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ENHANCING BIOMASS PRODUCTIVITY AND PHOTOSYNTHETIC PIGMENTS CONTENT OF AN EGYPTIAN *ANABAENA AMBIGUA* BLUE-GREEN ALGA ISOLATE

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ABSTRACT

Maximizing biomass and pigments productivities in microalgae are important objectives to achieve in order for algal culturing to become economically feasible. This study, thus, aims to evaluate the effects of type of culture as well as altering the composition of $BG11_0$ medium on enhancing biomass, chlorophyll-a and total carotenoids of an Egyptian Anabaena ambigua blue-green isolate. For this purpose, the isolate was subjected to different nutrient $BG11_0$ medium concentration (1X and 4X), different types of culturing (batch and Semi-continuous culture), adding nitrogen source (7 mM $NaNO_3$) or inorganic carbon source (50 mg $CaCO_3$ per liter), as well as subjecting it to moderate salinity stress (50 mM NaCl). Overall, growth curves revealed a significant stimulation of growth under $BG11_0$ medium 4X concentration together with $NaNO_3$ and $CaCO_3$ treatments. Results revealed that the best biomass production at 21 days culture age (late exponential phase) was obtained using $BG11_0$ medium 4X concentration (ca. 2050 mg L^{-1}) followed by $CaCO_3$ treatment (ca. 1400 mg L^{-1}). Using semi-continuous culture (ca. 1499 mg L^{-1}) also proved to give significantly more biomass when compared to batch culturing method. Also, using 50 mM NaCl did not significantly affect biomass, indicating the possibility of culturing this isolate with brackish or diluted sea water. Similar results were obtained at 28 days culture age (stationary phase). Regarding pigment contents and productivities, the use of 50 mM NaCl significantly increased both chlorophyll-a and total carotenoids at 21 and 28 days culture age. Moreover, $NaNO_3$ and $CaCO_3$ treatments proved to be beneficial to enhance chlorophyll-a and total carotenoids, respectively. Nevertheless, the type of culturing (batch or semi-continuous) seemed to have very little effect on pigments contents and productivities. Future studies involving other media components and their combination will increase our knowledge on the possible physiological treatments to enhance biomass of microalgae in a sustainable way.

KEYWORDS: Anabaena, Cyanobacteria, Microalgae, Biomass, Chlorophyll, Carotenoids

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Original Article

INTRODUCTION

Microalgae are highly abundant microorganisms living in aquatic ecosystems of plant earth. They are excellent sources of biological products of high value that have many industrial and health benefits. These products include lipids, polyunsaturated fatty acids (PUFAs), proteins, minerals, vitamins, pigments, and many others of great potential. In contrast to higher plants and crops, microalgae can be cultivated in seashores or lands unsuitable for agriculture, posing no direct competition or threat to food and/or feed cultivation. Consequently, microalgae are an innovative and dynamic area in biotechnology where microalgal biomass is attracting attention

to meet an increasing worldwide demand (Borowitzka 2013; Christaki et al. 2013). Several edible microalgae are already commercialized and have biotechnological uses, including green algae (Chlorophyta), red algae (Rhodophyta), and blue-green algae (Cyanobacteria) (Mulders et al. 2014). Blue-green algae, for example, are a widely diverse group of microalgae, both morphologically and genetically, producing a wide range of natural products with multiple industrial applications such as biofuel production of (e.g. biodiesel, ethanol, and butanol) or food, food additives, and single-cell protein. Blue-green algae can be commercially grown in large scale (i.e. mass culturing) using natural sunlight, making their cultivation significantly economic. Furthermore, they fix carbon dioxide and many have the ability to also fix atmospheric nitrogen, making the natural products they produce sustainable. Also, many species of blue-green algae grow in seawater or are salt tolerant, giving a considerable advantage in reducing the use freshwater in their culture. In addition, growing blue-green algae under extreme conditions prevents infections by other organisms allowing a stable long-term cultivation (Sharma et al. 2013).

An important condition for microalgae culture to be economically feasible, is for them to produce high biomass using the lowest (and/or cheapest) possible inputs. Biomass is a term given to any organic, non-fossil material of biological origin. If the biomass is derived from terrestrial and aquatic environments resources, it is called “phytomass”. Biomass is categorized into primary, secondary, and tertiary. Primary biomass is a direct product of photosynthesis harvested and/or collected from the field (or forest) where it is produced. This category includes grains, grasses, wood crops, and crop residues. Secondary biomass consists of residues and by-product from food, feed, fiber, wood, manures, and materials processing plants (e.g. sawdust, dairy byproducts, etc.). Tertiary biomass sources comprises residues and wastes of post-consuming such as fats, greases, oil, wood debris from construction sites, packaging wastes, and municipal solid wastes. Microalgae belongs to the first category of biomass and, recently, a very important research line is dedicated to investigate the means of increasing algal biomass using economic and sustainable methods (Sharma et al. 2013).

A noticeable characteristic of microalgae is their color thanks to their pigments content which are part of their photosynthetic system. These pigments are grouped into three classes: carotenoids, chlorophylls, and phycobiliproteins. Pigments from microalgae are currently in high demand by the market as renewable natural colorants (which are strong dyes even at very low levels) for foods and feeds, and provide several health benefits at the same time. These pigments also have important applications in the pharmacy such as bioindicators and as biochemical tracers. Furthermore, many uses have been described for the cosmetic industry as well such as in their use in production skin creams to stimulate collagen synthesis (Gouveia et al. 2008). Chlorophyll is a green pigment that is abundantly present in nature because it is a crucial component of photosynthesis, and can be easily extracted from the microalgal biomass. Nowadays, there is an increasing interest in commercially producing chlorophyll as a natural pigment in the food and feed industries, and cosmeceuticals (Hosikian et al. 2010). For example, chlorophylls are used in coloring of marmalade, candy sweets and soft drinks. Moreover, it is also registered in the European Union under E-number E-140 (García Sartal et al. 2012). Regarding its medicinal use, chlorophylls have shown to play possible beneficial roles in boosting health and preventing diseases. In processed vegetable food and following ingestion by humans, chlorophyll is converted into pheophytin, pyropheophytin and pheophorbide. These components show antimutagenic effect and may play a role as anticancer agent. (Chernomorsky et al. 1999). Another commercially valuable group of microalgal pigments are carotenoids, which are yellow to red colored isoprenoid molecules. Carotenoids are required in the diet of humans and animals due to their incapability of synthesizing them in their bodies (Latowski et al. 2014). Certain carotenoids such as β-carotene and astaxanthin, have strong antioxidant activity, counteracting the detrimental effects of free radicals by

protecting from peroxidation of lipids and by scavenging reactive oxygen species (ROS) produced under photooxidation (Pangestuti and Kim 2011). Overall, dietary intake of carotenoid antioxidants derived from microalgae has shown the ability to protect organisms against various chronic disorders including cancer, atherosclerosis, and diabetes (Cadoret et al. 2012). Furthermore, the effect of these antioxidants was positive against coronary disease, ischemic brain development, liver, metabolic syndromes, gastrointestinal maladies (Riccioni et al. 2011). Furthermore, some neurodegenerative diseases such as Alzheimer disease and Parkinson disease responded positively as well (Gouveia 2014; Martins et al. 2014).

In order to reducing production costs of microalgae biomass as well as chlorophyll-a and total carotenoids contents, it is mandatory to improve microalgal growth rate, nutrient use efficiency, and reduce the costs of construction operation, and maintenance of bioreactors. Although it is difficult to increase cell growth rates and productivities in photoautotrophic cultures, many species and strains of microalgae respond positively to growth under heterotrophic conditions using, for example, various organic carbon sources. When compared with photoautotrophic cultures, heterotrophic and mixotrophic conditions show higher cellular biomass production, with consequent reduction in the cost of downstream processing. Consequently, heterotrophic cultures have a high potential in reducing the cost of microalgal biomass production. The aim of this work is, thus, to examine different mixotrophic growth conditions effects, by altering the nutrient media composition and method of culture, on increasing biomass, chlorophyll and carotenoid contents of an Egyptian blue-green microalga isolate of *Anabaena ambigua*. To achieve this goal, we subjected the microalgal isolate to different altered BG11 medium compositions (BG11 medium 4X concentration), with additional inorganic carbon source (calcium carbonate), additional nitrate source, salinity stress (50 mM NaCl). The effect of culture method, batch or semi-continuous, was investigated as well.

MATERIALS AND METHODS

The present study was carried out during January-April 2016 in the Plant Physiology division, Department of Agricultural Botany, Faculty of Agriculture, Cairo University, Egypt.

Microalgal Isolate

In the this study, a filamentous heterocystous isolate of cyanobacteria; *Anabaena ambigua* was obtained from Department of Agricultural Microbiology, Soils, Water and Environment Research Institute, Agricultural Research Center (ARC), Giza, Egypt.

Culture Medium and Growth Conditions

The cyanobacterium *Anabaena ambigua* was cultured in nitrogen-free BG-11 medium ($BG11_0$) according to Allen and Stanier (1968) at pH 7.2, temperature 27 ± 2 $^{\circ}$C and continuous light intensity of 100 μE m^{-2} s^{-1} provided by a white fluorescent lamp. All chemicals used in this study were of analytical grade from Sigma Aldrich (St. Louis, MI, USA).

Treatments

The physiological effect of semi-continuous culturing system, 4 times increased media concentration (4X), sodium nitrate (7 mM), calcium carbonate as inorganic carbon (50 mg/l), sodium chloride (50 mM) were investigated. The cyanobacterium cultures were inoculated with an inoculum, with an optical density of 0.2 at 678 nm, in flasks of 1000

ml capacity containing 700 ml of nitrogen-free BG-11 liquid medium. All inoculated flasks were maintained at 27 ± 2 °C under continuous illumination of white fluorescent for 28 days. The growth curve for biomass accumulation and cell increment in term of cell concentration were detected at 3 day intervals while both of chlorophyll and carotenoids production were evaluated at 21 and 28 days of culture age. All treatments were replicated three times.

Measurement of Algae Optical Density

An increase in optical density (O.D), percent transmission for a growing cell suspension, was determined at 3 day intervals along the incubation period according to Sorokin (1973) using spectrophotometer at 678 nm. The increase in turbidity of the algal suspension is measured as follows:

$T = I / I_o$

Where;

I = the transmission of the sample in percent of the transmission of the blank.

I_o = latter adjusted to read 100 %.

Transmission, T, is then converted into optical density:

$O.D = \log (I_o / I)$.

Measurement of Algae Biomass

Biomass yield (mg L^{-1}) of *A. ambigua* was carried out at 3 day intervals along the incubation period as adopted by Sorokin (1973) with some modifications. A sample was withdrawn and centrifuged at 4000 rpm for 10 min. After that, the algal pellets were transferred into a pre-weighed glass dish. Samples were dried in an oven at 65-70 °C until two successive weightings of the dish, done at intervals, give a constant weight. Weighting on sensitive balance was done after cooling in a desiccator overnight. Biomass weight of algal cells was determined for each replicate by subtracting the obtained weight of dried sample from the glass dish's weight and expressed per unit volume. Biomass productivity (mg L^{-1} d^{-1}) was calculated by multiplying biomass yield (mg L^{-1}) by specific growth rate (d^{-1}).

Specific Growth Rate, Divisions per day and Generation Time

Specific growth rate (μ), divisions per day and generation time (*T*g) were calculated from standard curves according to Levasseur et al. (1993) using the following equations:

$$\mu = \frac{\ln(N_2/N_1)}{t_2 - t_1}$$

$$\text{Divisions day}^{-1} = \frac{\mu}{\ln 2}$$

$$T_g = \frac{1}{\text{Divisions day}^{-1}}$$

Where

μ is the specific growth rate in d^{-1}

***N*2** and ***N*1** (in g) are the biomass at times ***t*1** and ***t*2** (in d) in exponential phase of growth.

Chlorophyll-a and Total Carotenoids Pigments Analysis

Both of chlorophyll a and carotenoid pigments were determined according to Burnison (1980) using dimethyl sulfoxide (DMSO) and 90 % acetone with some modifications. A representative aliquot of algae was withdrawn into falcon tube and 1 ml of 0.01 % $MgCO_3$ solution was added. Algal samples where centrifuged at 4000 rpm for 10 min where supernatant medium was decanted carefully. Samples were washed with distilled water and centrifuged again at 4000 rpm for 10 min. Then, distilled water was decanted carefully and algal cells were resuspended into 4 ml DMSO. Algal cells were homogenized at 1000 rpm for 1 min and incubated in water bath at 65 ^oC for 10 min. Afterwards, 6 ml of 90 % acetone were added to the DMSO-extracted algal cells and mixed very well. Extracted algal samples were kept in a refrigerator for 24- 48 hr in dark conditions. Extracted samples were centrifuged at 5000 rpm for 10 min where the supernatants were transferred into new falcon tubes. Finally, absorbance of extracted pigments was measured using spectrophotometer at 630, 647, 664 and 691 nm for chlorophyll-a and at 468 nm for carotenoids. Concentration (µg/ml) of chlorophyll a and carotenoids were calculated according to Ritchie (2008) and Davies (1976), respectively. Finally results were expressed in µg/mg dry weight of algal sample by multiplying by the solvent volume divided by sample dry weight. Chlorophyll-a or total carotenoids productivities (µg mg^{-1} d^{-1}) were calculated by multiplying chlorophyll-a or total carotenoids contents (µg mg^{-1}) by the specific growth rate (d^{-1}).

Statistical Analysis

Differences among means were tested by a one-way analysis of variance (ANOVA) followed by Duncan post hoc test. Differences were considered statistically significant when $P<0.05$ in all analyses. Graphs and statistical analysis were carried out using Microsoft Office Excel 2007 and IBM SPSS Statistics V. 20, respectively.

RESULTS

This study evaluated the effects of method of culture (batch or semi-continuous) as well as altering the composition of $BG11_0$ medium (4X medium concentration, 7mM $NaNO_3$, 50 mg $CaCO_3$, and 50 mM NaCl) on enhancing biomass, chlorophyll-a and total carotenoids of an Egyptian *Anabaena ambigua* blue-green isolate under controlled conditions (Figure 1).

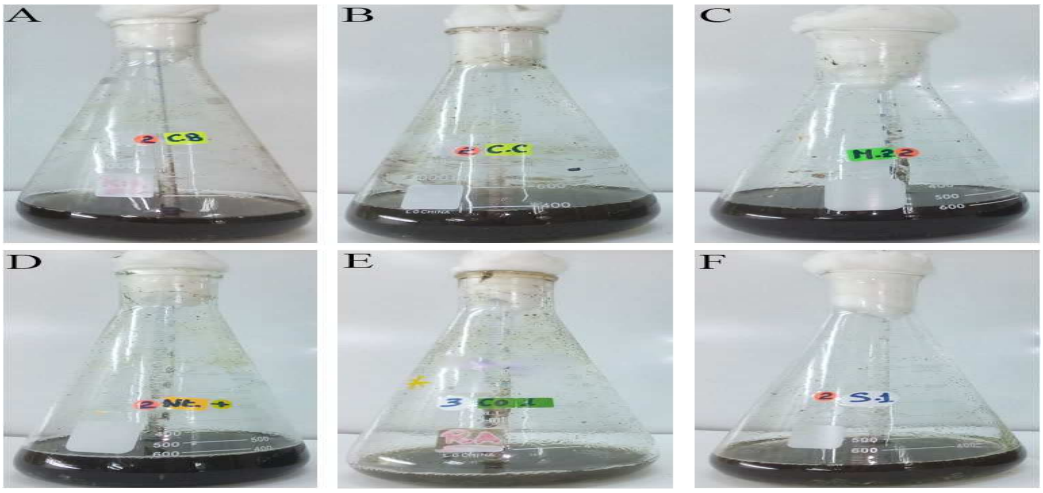

Figure 1: *Anabaena Ambigua* Grown under A) $BG11_0$ Batch Culture, B) $BG11_0$ Semi-Continuous

Culture, C) $BG11_0$ 4X Concentration, D) BG11 + 7 mM $NaNO_3$, E) BG11 + 50 mg $CaCO_3$, and F) BG11 + 50 mM NaCl

Growth Curves

Growth curve phases of *A. ambigua* were studied by measuring optical density of different treatments at 678 nm, taking samples every 3 days along the incubation period. Results illustrated in Figure 2 revealed that the six different media compositions exhibited different growth curve characteristics.

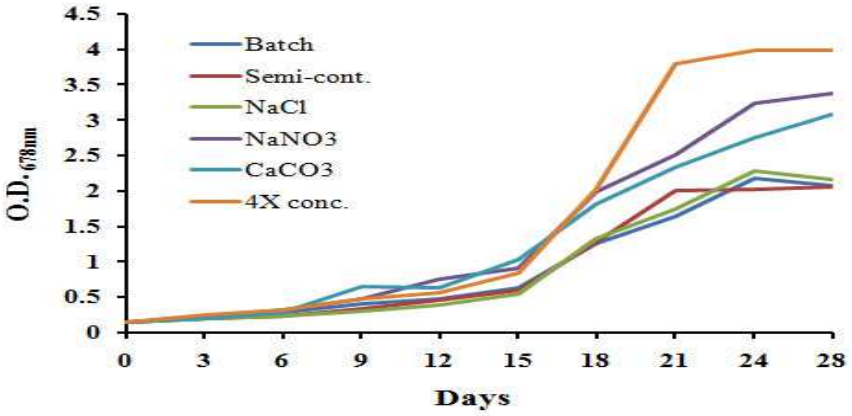

Figure 2: Growth Curves of *A. Ambigua* Grown under the Six different Media Compositions (100 µE m^{-2} s^{-1}, 27 ºC)

Four characteristic phases of the growth curve were recognized; the lag phase started immediately from the inoculation time up to 6 days of culture age. The acceleration phase started from 6 days of incubation period up to 15 days of incubation with very little difference among all treatments. On the other hand, differences among treatments became apparent during the exponential phase (15 up to 24 days of culture age) and during the stationary phase (21 up to 28 days of culture age) as well. For these, growth under $BG11_0$ medium 4X concentration as well $NaNO_3$ and $CaCO_3$ treatments positively and stimulated *A. ambigua* isolate's growth compared to the remaining treatments.

Biomass and Specific Growth Rate

To further investigate the detected differences in the growth curves, we statistically analyzed biomass production under six treatments at 21 and 28 days of culture age. Data in Table 1 show the biomass yield and specific growth rate of the *A. ambigua* isolate after being incubated with the six different media compositions.

Table 1: Biomass (mg L^{-1}), Specific Growth rate (d^{-1}), Divisions per day and Generation Time (T_g) of *A. ambigua* Grown under the Six Different Media Compositions (100 µE m^{-2} s^{-1}, 27 ºC)

	Biomass 21 d (mg L^{-1})	Biomass 28 d (mg L^{-1})	µ (d^{-1})	Div. d^{-1}	T_g (d)
$BG11_0$ (Batch Culture)	566 ± 33^{cd}	833 ± 33^{d}	0.049 ± 0.008^{b}	0.07 ± 0.012^{b}	14.43 ± 2.5^{a}
$BG11_0$ (Semi-continuous Culture)	1499 ± 166^{b}	1700 ± 33^{b}	0.095 ± 0.077^{ab}	0.14 ± 0.111^{ab}	20.99 ± 16.9^{a}
$BG11_0$ (4X Concentration)	2050 ± 150^{a}	3000 ± 100^{a}	0.204 ± 0.018^{a}	0.29 ± 0.027^{a}	3.42 ± 0.31^{a}
BG11 (+$NaNO_3$)	899 ± 33^{c}	1033 ± 33^{c}	0.066 ± 0.003^{b}	0.09 ± 0.005^{b}	10.49 ± 0.49^{a}
$BG11_0$ (+$CaCO_3$)	1400 ± 0^{b}	1600 ± 0^{b}	0.079 ± 0.000^{b}	0.12 ± 0.000^{b}	8.67 ± 0.00^{a}
$BG11_0$ (+NaCl)	533 ± 66^{d}	666 ± 66^{d}	0.082 ± 0.002^{b}	0.12 ± 0.003^{b}	8.43 ± 0.19^{a}

Each value represents the mean ± standard error of 3 replicates. Means with identical letters in the same column

are not significantly different ($P > 0.05$) according to Duncan test.

Results revealed that the increment in biomass yield considerably varied depending upon media concentration, nitrate supplementation and carbon source addition as well. As indicated in Table 1, obtained results revealed that highest biomass production at 21 days culture age (late exponential phase) was obtained using $BG11_0$ medium 4X concentration (*ca.* 2050 mg L^{-1}) followed by $CaCO_3$ treatment (*ca.* 1400 mg L^{-1}) and $NaNO_3$ (*ca.* 899 mg L^{-1}). Using semi-continuous culture (*ca.* 1499 mg L^{-1}) also proved to give significantly more biomass when compared to batch culturing method. Similar results of biomass yield at 28 days culture age (stationary phase) were obtained showing same effects of treatments at 21 days culture age.

Biomass Productivity

Figure 3 shows data of biomass productivity of *A. ambigua* under the different six treatments. The obtained results illustrated that biomass productivity of *A. ambigua* increased with time from 21 to 28 days of incubation in all treatments. Culture of 28 days age, thus, responded better to an increased incubation time compared to that of 21 day old, a finding observed with all applied treatments.

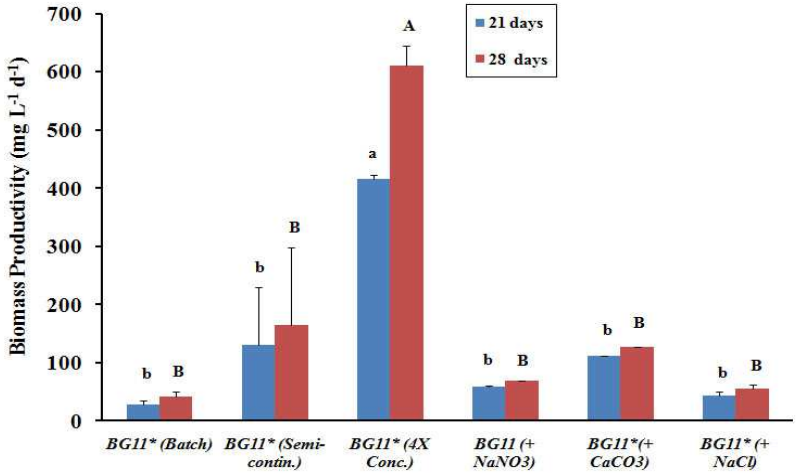

Figure 3: Biomass Productivity (mg L^{-1} d^{-1}) of *A. Ambigua* Grown under the Six Different Media Compositions (100 µE m^{-2} s^{-1}, 27 ºC). Each Value Represents the Mean ± Standard Error of 3 Replicates. Means with Identical Letters (Small Letters for 21 Days and Capital Letters for 28 Days) are Not Significantly Different (P > 0.05) According to Duncan Test

Among the six treatments, the $BG11_0$ 4X media concentration treatment recorded the significantly highest biomass productivity of 400 and 600 mg l^{-1} d^{-1} at 21 and 28 days of incubation period, respectively. On the other hand, different types of culturing (batch and Semi-continuous culture), adding nitrogen source (7 mM $NaNO_3$) or inorganic carbon source (50 mg $CaCO_3$ per liter), as well as subjecting *A. ambigua* to moderate salinity stress (50 mM NaCl) recorded a biomass productivity not exceeded 200 mg l^{-1} d^{-1}, and not significantly different from each other.

Chlorophyll-a and Total Carotenoids Content

Results in Table 2 shows chlorophyll-a and total carotenoids contents of the *Anabaena ambigua* isolate after being incubated with the six different media compositions.

Table 2: Chlorophyll-a and Total Carotenoids (μg mg^{-1} Dry Weight) of *A. ambigua* Grown under the Six Different Media Compositions (100 μE m^{-2} s^{-1}, 27 ºC)

	Chlorophyll-a content 21 d (μg mg^{-1})	Chlorophyll-a content 28 d (μg mg^{-1})	Carotenoids content 21 d (μg mg^{-1})	Carotenoids content 28 d (μg mg^{-1})
BG110 (Batch Culture)	7.50 ± 0.13^{b}	8.03 ± 0.28^{c}	1.57 ± 0.04^{b}	1.46 ± 0.10^{c}
BG110 (Semi-continuous Culture)	3.36 ± 0.06^{d}	3.34 ± 0.23^{d}	0.67 ± 0.01^{d}	0.53 ± 0.14^{d}
BG110 (4X Concentration)	3.03 ± 0.07^{d}	3.22 ± 0.04^{d}	0.65 ± 0.02^{d}	0.78 ± 0.02^{d}
BG11 (+ NaNO3)	7.91 ± 0.60^{b}	9.44 ± 0.51^{ab}	1.59 ± 0.07^{b}	1.78 ± 0.04^{b}
BG110 (+ CaCO3)	4.33 ± 0.10^{c}	8.29 ± 0.60^{bc}	0.87 ± 0.02^{c}	1.73 ± 0.10^{b}
BG110 (+ NaCl)	10.45 ± 0.07^{a}	10.13 ± 0.34^{a}	2.09 ± 0.02^{a}	2.37 ± 0.06^{a}

Each value represents the mean ± standard error of 3 replicates. Means with identical letters in the same column are not significantly different ($P > 0.05$) according to Duncan test.

Regarding culture age, the obtained results revealed that chlorophyll-a increased with the incubation period for the 4X media concentration and batch culture in addition to both nitrate and calcium carbonate treatments. On the other hand, semi-continuous and NaCl treatments exhibited a similar values in chlorophyll-a at 21 and 28 days of culture age. As for total carotenoids, results shown in Table 2 indicated an increase in contents with increasing culture age under all treatments except the batch and semi-continuous cultures treatments, where the contents of total carotenoids at 21 and 28 days were similar.

With respect to the effect of six different media compositions, the use of 50 mM NaCl significantly increased both chlorophyll-a and total carotenoids at 21 and 28 days culture age. Moreover, $NaNO_3$ and $CaCO_3$ treatments proved to be beneficial to enhance chlorophyll-a and total carotenoids, respectively. Nevertheless, the type of culturing (batch or semi-continuous) seemed to have very little effect on both pigments contents and productivities.

Chlorophyll-a and Total Carotenoids Productivity

Chlorophyll-a and total carotenoids productivities results, at 21 and 28 days culture age, are illustrated in Figures 4 and 5, respectively. Results revealed that both pigments productivities of *A. ambigua* increased significantly from 21 to 28 days of incubation in the calcium carbonate treatment, while the remaining media treatments showed similar values at both 21 and 28 days of culture age.

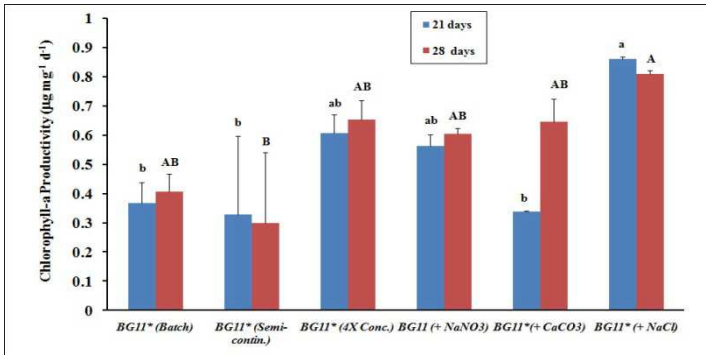

Figure 4: Chlorophyll-a Productivity (μg mg^{-1} d^{-1}) of *A. ambigua* Grown under the Six Different Media Compositions (100 μE m-2 s-1, 27 ºC). Each Value Represents the Mean ± Standard Error of 3 Replicates. Means with Identical Letters (Small Letters for 14 Days and Capital Letters for 21 Days) are Not Significantly Different (P > 0.05) According to Duncan Test

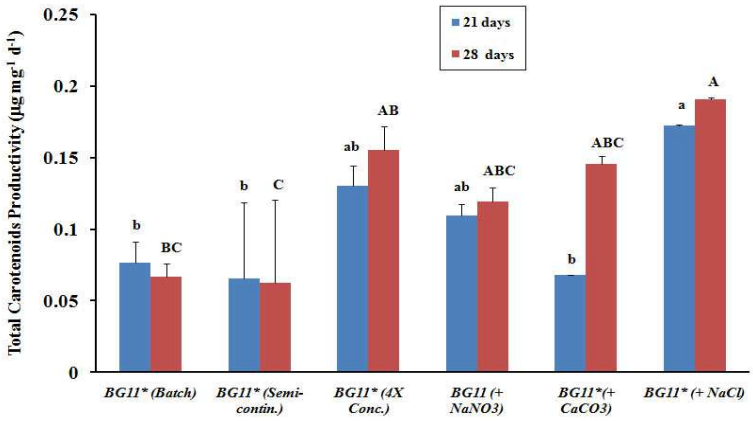

Figure 5: Total Carotenoids Productivity (µg mg^{-1} d^{-1}) of *A. ambigua* Grown under the Six Different Media Compositions (100 µE m^{-2} s^{-1}, 27 ºC). Each Value Represents the Mean ± Standard Error of 3. Replicates Means with Identical Letters (Small Letters for 14 Days and Capital Letters for 21 Days) are Not Significantly Different (P > 0.05) According to Duncan Test

Regarding the effect of different treatments on chlorophyll-a productivity (Figure 4), the NaCl treatment recorded the significantly highest productivity when compared to batch culture, giving value of *ca.* 0.9 µg mg^{-1} d^{-1} at 21 days of culture age. This, however, was not the case at 28 days, were no significant differences were detected between NaCl and batch culture treatments. On the other hand, NaCl treatment showed significantly higher chlorophyll-a productivity when compared with semi-continuous culture method, at both 21 and 28 days of culture age. Regarding $BG11_0$ medium 4X concentration as well $NaNO_3$ and $CaCO_3$ treatments, chlorophyll-a productivities were neither significantly neither different from batch culture nor semi-continuous culture treatments. The abovementioned differences were similarly present among *A. ambigua* total carotenoids productivities subjected to the six treatments, as well (Figure 5). However, in the case of total carotenoids productivity under NaCl treatment, differences were significant with both batch and semi-continuous culture treatments at both 21 and 28 days of culture age.

DISCUSSIONS

The semi-continuous method of culture proved to increase biomass yields compared to batch culture (Table 1) at 21 and 28 days of culture age. This could be due to the frequent replenishing of nutrients essential to growth, that are consumed and becoming deficient in the case of batch culturing. The method of culture, however, had little effect on pigments contents and productivities because, as mentioned earlier, these pigments are enhanced by stress or by additional nitrogen.

It is well documented that nitrogen is an essential component of structural and functional proteins in algae, making approximately 7%–20% of dry weight of cells (Hu 2004). Nitrogen fulfills many important physiological roles in algal cells (Raven and Giordano 2016) and, therefore, its deficiency limits many important functions in algal cells (Li et al. 2008). Among these functions, nitrogen is an essential component of chlorophyll and, thus, photosynthetic productivity of algae, especially in oceans, is often limited by the availability of combined nitrogen (i.e. nitrogen covalently bonded to one or more elements other than nitrogen such as NO_3^-, NH_4^+, and NO_2^-), where its concentration is usually below 10 mmol m^{-3} (Falkowski and Raven 2007). Nevertheless, in our experiments, the presence of recommended concentration of $NaNO_3$ in BG11 medium as formulated by Allen and Stanier (1968) had minor effect on biomass yield (Table 1) and biomass productivity (Figure 3) of *A. ambigua* when compared to control $BG11_0$ of batch

culture treatment. This is due to the fact that *A. ambigua* cyanobacterium is capable of diazotrophy, i.e. the production of ammoniacal nitrogen (NH_3 /NH_4) from atmospheric molecular nitrogen (N_2) (Zehr 2011). In the case of cyanobacteria, the limiting factor is usually phosphorus, which is the case in freshwater in contrast to oceans, as higher precipitation of phosphorus in insoluble complexes occurs in freshwater habitats (Falkowski and Raven 2007). In fact, this is corroborated by results of Biomass yield (Table 1) and productivity (Figure 3) which are significantly enhanced with nitrogen free 4X concentrated $BG11_0$ medium treatment, where it contains four-fold concentrations of phosphorus and other nutrients important for growth such as potassium, among others. On the other hand, adding $NaNO_3$ to BG11 medium had a major effect in increasing both chlorophyll-a and total carotenoids contents of *A. ambigua* (Table 2) and productivities (Figures 4 and 5) when compared to control $BG11_0$ of batch culture treatment.

Carbon is another essential element for algae physiology. It is essential for photosynthesis and consequently growth and reproduction. Atmospheric carbon fixed by the algae will have three fates. It will either be used for respiration, as an energy source, or as a raw material in the formation of additional cells (Berman-Frank et al. 1999). Carbon can be utilized in the form of CO_2, carbonate, or bicarbonate for autotrophic growth and in form of acetate or glucose for heterotrophic growth. Therefore, adding an inorganic or organic carbon source to the medium (i.e. mixotrophic growth) may enhance algal growth rates. In the case of our experiments, it was evident that adding of $CaCO_3$ (50 mg L^{-1}) to $BG11_0$ medium significantly shifted growth curve of *A. ambigua* (Figure 2), directly in second place after 4X medium treatment. This holds true also regarding biomass yield (Table 1) and biomass productivity (Figure 3). Nevertheless, this treatment had little effect on chlorophyll-a and total carotenoids contents of *A. ambigua* (Table 2) and productivities (Figures 4 and 5) when compared to control $BG11_0$ of batch culture treatment.

The majority of liquid water on the Earth surface is characterized by rather high amounts of inorganic ions, and with the present problem of climate change and increased world population, freshwater is becoming increasingly scarce and valuable. Therefore, the ability to culture algae in brackish, saline, or diluted sea water is a highly desirable trait to screen for in algae. Salinity is an important factor that alters the physiology and composition of algae. Exposing algae to lower or higher salinity levels than their native habitat levels may change growth rate and alter composition. Some studies reported that salinity may increase algal growth rates (Rao et al. 2007) while other authors reported the complete opposite (Vazquez-Duhalt and Arredondo-Vega 1991). In our experiments, adding 50 mM (equivalent to approximately 1:10 of seawater) to $BG11_0$ medium did not negatively affect (nor enhanced) biomass yield (Table 1) or biomass productivity (Figure 3), when compared to batch culture $BG11_0$ medium. This shows a good adaptability of *A. ambigua* to moderate salt stress, an important beneficial trait as mentioned earlier. Equally important, 50 mM NaCl significantly increased chlorophyll-a and total carotenoids contents of *A. ambigua* (Table 2) over all other treatments. This results is important to investigate in future studies to determine the exact NaCl concentration to use to produce maximum biomass and pigments content.

CONCLUSIONS

This work's objective is especially important because microalgae could be a good source of value-added products such as carotenoids and chlorophyll-a which are natural antioxidants and pigment that can replace the artificial colorants in food and feed as well as cosmetics production and, consequently, having lower risk on human and animal health. Results of the present work indicate that, although biomass yield under semi-continuous culture was significantly higher than batch culturing method at both 21 and 28 days of culture age, the method of culturing showed no effect on biomass productivity.

Increasing $BG11_0$ medium concentration four times (4X treatment) proved very efficient in significantly increasing biomass yield and productivity over all treatments. Also, although adding nitrate or carbonate sources to the $BG11_0$ medium increased biomass yields of *A. ambigua*, these had little effects on biomass productivity. Finally, adding 50 mM NaCl to $BG11_0$ medium did not hinder biomass yield or productivity of *A. ambigua* when compared to normal batch culture medium treatment. Moreover, and more importantly, NaCl significantly increased both chlorophyll-a and total carotenoids pigments contents and productivities over all other treatments. This result should prove as useful practical application giving the possibility to culture this *A. ambigua* isolate, and possibly other members of *Anabaena* genus, with brackish or diluted sea water, with no effect on biomass productivity and with a beneficial effect on enhancing chlorophyll-a and total carotenoids pigments productivities.

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