Nitrogen Starvation Effect Versus its Excess on the Performance of *Arthrospira maxima* in Zarrouk’s Medium

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**ABSTRACT**

Providing a new cheap medium in terms of biomass and pigment production from *Arthrospira maxima* is an important issue. In order to determine the effect of nitrogen starvation versus its excess, urea was used as a cheap nitrogen source in two different modes (alternative and additive) at four different concentrations (0, 1.25, 2.5, 5 g L\(^{-1}\)). It was indicated that an alternative method is better than additive method due to accelerate the ammonia synthesis (NH\(_2\)) and pH changes in the period of the growth that is directly related to biomass and pigments production. Moreover, the intensive production of biomass concentration, PC, APC, \(C_a\), and \(C_{car}\) content were 1.403, 0.074, 0.093, 10.72, and 3.17 mg L\(^{-1}\) with nitrogen starvation medium. Urea considered being one of the inhibition sources of biomass growth due to the formation of NH\(_2\). Analyzing final results by general factorial design concluded that omitting nitrogen sources had the potential possibility for growing *A. maxima* in order to reduce the production costs in large-scale cultivation, which yields better performance than cost-effective Zarrouk’s medium.

**NOMENCLATURE**

| Greek Symbols | Description |
|---------------|-------------|
| \(\mu_{max}\) | Maximum specific growth rate (d\(^{-1}\)) |

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| Symbols | Description |
|---------|-------------|
| A | Absorbance at different wavelength |
| APC | Allophyrocyanin |
| \(C_a\) | Chlorophyll-a |
| \(C_b\) | Chlorophyll-b |
| \(C_{car}\) | Total carotenoids |
| \(C_t\) | Biomass concentration at time \(t\) (gL\(^{-1}\)) |
| \(C_0\) | Biomass concentration at time \(t_0\) (gL\(^{-1}\)) |
| mM | Molar concentration |
| M | Addition method of urea |
| \(N_t\) | Total quantity of added nitrogen (g) |
| \(K\) | Mean daily division rate (division d\(^{-1}\)) |
| \(P\) | Productivity (gL d\(^{-1}\)) |
| \(OD\) | Optical density at time \(t\) |
| \(OD_0\) | Optical density at time \(t_0\) |
| \(R\) | Correlation coefficient |
| \(DT\) | Doubling time (d) |
| \(U\) | Urea |
| \(V\) | Total volume of cultivation (L) |
| \(X_n\) | Maximum dry weight (gL\(^{-1}\)) |
| \(X_i\) | Initial cell dry weight (gL\(^{-1}\)) |
| \(V_{sci}\) | Nitrogen-cell conversion (g(g)) |

**1. INTRODUCTION**

Algae are as biological sources with a broad variety of applications [1]. Interest in the applications of natural colors has increased due to their non-toxic, non-allergic, and antimicrobial effects [2]. The natural pigments are not only colorful but also have physiological activity compared with the synthetic colorants [3]. The ability of natural pigments extraction from microalga has been receiving more attention recently [4, 5]. Currently,
Spirulina sp. is one of the most widely available microalgae sources that contain a higher value of natural pigments like chlorophyll, carotenoids, and C-phycocyanin [6]. A. maxima as a photosynthetic, filamentous, spiral-shaped, multicellular, and blue-green Cyanobacterium was consumed as a food in some counties such as Mexico since 400 years ago [7]. The biomass production rate of Spirulina is mainly dependent on nutrient availability, temperature, and light [8]. The effect of various environmental conditions on biomass and pigment production of A. maxima were reported in the literature [9]. Furthermore, different mediums were successfully used for the microalgae growth like Zarrouk [10], modified Zarrouk [11], CFTRI, PJPM [12], Bangladesh medium [13], and Tofu wastewater [14]. Nutrient limitation is one of the major promising strategies to change and control the microalgae cell cycle and the biochemical production of lipid and other pigments [15]. The essential nutrients are an organic or inorganic carbon source as well as nitrogen, phosphorus, and iron in the course of alga growth [16]. Nitrogen is one of the remarkable factors which has an impact on both growth and pigment production of microalgae [17]. Sodium nitrate is a common nitrogen source used in the spirulina cultivation. Moreover, the feasibility of replacement of basic nitrogen sources of medium with low-cost alternatives such as urea, ammonium sulfate, and ammonium chloride in S. platensis cultivation was already considered [18–20].

Few studies were conducted on the cultivation of A. maxima based on the urea (nitrogen starvation) effect according to high production of pigments and low production cost under Mexico city climate [21]. Urea was used as an additive and alternative nitrogen source for investigating the effect of supplemented nitrogen source compared to the nitrogen starvation (as urea) [22–24]. In addition, the incorporation of bicarbonate and nitrate is an important cost-effectiveness technique in large-scale cultivation of Arthrospira [25].

In this research, the responses of A. maxima are studied in terms of nitrogen starvation and urea concentrations on biomass growth, productivity, specific growth rate, and pigment production (including phycocyanin, allophycocyanin, carotenoids, and chlorophylls pigments). Finally, a low-cost effective medium is developed based on inexpensive raw material to be used on a future large-scale production.

2. MATERIALS AND METHODS

2.1. Microorganism and Culture Condition

Arthrospira maxima CIB 79 was provided from the National Polytechnic University (National Polytechnic University, Mexico City, Mexico), which was grown in an axenic batch culture of Zarrouk’s medium. Figure 1 shows a microscopic images of axenic unialga. The stock and test cultures flask carried out at laboratory temperature (28-32 °C) under a white fluorescent light with a photoperiod of 24 h (80µmolm⁻²s⁻¹) and pH control free (8.30-10.30). During the process of growth, the cultures continuously aerated with supporting an air pump [AC-9602 (RESUN, Mexico)] to accelerate the growth process. Urea was consumed at various concentrations (0, 1.25, 2.5, and 5 gL⁻¹) by the medium. The cultivation environment was prepared either with (additive) or without (alternative) basic nitrogen source of Zarrouk’s medium. Then, urea was added to the cultivation at various concentrations. Concentration of the used urea was based on the amount of the standard nitrogen source of Zarrouk’s medium (2.5 gL⁻¹) for comparing the basic Zarrouk’s medium with nitrogen starvation (0 gL⁻¹). In fact, concentration of 1.25 and 5 gL⁻¹ was chosen as a mean and double concentration of basic nitrogen source to study the effect of extra nitrogen source versus nitrogen starvation. Zarrouk medium with following chemicals and composition NaHCO₃ 16.8 gL⁻¹, NaNO₃ 2.5 gL⁻¹, K₂HPO₄ 0.5 gL⁻¹, K₂SO₄ 1.0 gL⁻¹, NaCl 1.0 gL⁻¹, MgSO₄·7H₂O 0.2 gL⁻¹, EDTA·Na₂·2H₂O 0.08 gL⁻¹, CaCl₂·2H₂O 0.04 gL⁻¹, and FeSO₄·2H₂O 0.01 gL⁻¹, micronutrient elements solution (H₂BO₂ 2.86 gL⁻¹, MnCl₂·4H₂O 1.81 gL⁻¹, ZnSO₄·7H₂O 0.222 gL⁻¹, MoO₃ 0.01 gL⁻¹, CoCl₂·6H₂O 0.01 gL⁻¹, CuSO₄·5H₂O 0.079 gL⁻¹) 1.0 mL L⁻¹ was used as a standard medium of Spirulina cultivation. All the chemicals were purchased from Merck and Sigma-Aldrich Company. Treatments were cultivated in a 125 mL flask which were inoculated with 40 mL of A. maxima inoculum an initial concentration of 0.4 OD. The biomass growth and pigment production were recorded once during the maximum 8 days of cultivation. The experiment was performed during 2019-2020 in June.

2.2. Phycobiliproteins and Photosynthetic Analysis

For pigment extraction, the cell walls were ruptured using five freezing and thawing cycles [26]. Thus, the samples were ferozen at -20 °C for 1 h and then thawed at room temperature for 45 min. The samples were centrifuged (with velocity of 14/14 R refrigerated centrifuge, China) at 10000 rpm for 10 min at 4 °C where the pellet was transferred to vials. Then, the supernatant was collected for detecting absorbance of
PC and APC at 620 and 652 nm, respectively. The pellet was picked after centrifugation for photosynthetic pigment measurements like total chlorophylls and carotenoids. Then, it was oven-dried at almost 25 °C for 3 h. Moreover, the pellet was dissolved in 600 µL of an acetone and chloroform mixture (70:30 v/v). Chlorophylls and carotenoids were also quantified at 470, 645, and 662 nm. The pigment extraction was carried out under the dim light condition to avoid its degradation. The pigments concentration of phycobiliproteins (PC and APC), chlorophyll-a (Cₐ), chlorophyll-b (Cₐ), and total carotenoids [C(IX+X)] contents were then calculated using equations (1) to (5) by Bennett and Bogorad [27], Parson and Strickland [28], and Wellburn [29]:

\[
PC (\text{gL}^{-1}) = (A_{620} - 0.474 A_{652})/5.34 \tag{1}
\]

\[
APC (\text{gL}^{-1}) = (A_{652} - 0.208 A_{620})/5.09 \tag{2}
\]

\[
C_a (\text{mg}L^{-1}) = 11.24 \times A_{662} - 2.04 \times A_{645} \tag{3}
\]

\[
C_b (\text{mg}L^{-1}) = 20.13 \times A_{645} - 4.19 \times A_{662} \tag{4}
\]

\[
C_{(IX+X)} (\text{mgL}^{-1}) = (\{1000 \times A_{470}\} - (1.09 C_a - 63.14 C_b))/214 \tag{5}
\]

A corresponds to the measuring absorbance in the desired wavelengths (470, 620, 645, 652 and 662 nm), PC, APC, Cₐ, Cₐ and C(IX+X), are phycocyanin, allophycocyaninchlorophyll-a, chlorophyll-b, and Carotenoid, respectively.

### 2. 3. Analytical and Statistical Analysis

The biomass concentration (cell dry weight) was determined by measuring optical density at 674 nm during the sampling time. Optical density [by three replicates (OD)] was recorded by a spectrophotometer (Thermo Scientific, Multitask Go, England). This was used for the pigments estimation (PC and APC), as well. LEGEND MICRO 17 spectrophotometer (Thermo Scientific model, Germany) was applied for spectrophotometric measurement of chlorophylls and carotenoids. Moreover, the maximum specific growth rate (µmax), doubling time (DT), and productivity (P) were determined from the maximum biomass concentration at the end of each run when the algal cultures were in exponential phase by applying the following equations [30]:

\[
\mu_{\text{max}} (\text{d}^{-1}) = \frac{\ln(C_2) - \ln(C_0)}{t_{\text{max}} - t_0} \tag{6}
\]

\[
P (\text{gL}^{-1} \text{d}^{-1}) = \frac{C_t - C_0}{t - t_0} \tag{7}
\]

where, C_t and C_0 are the biomass concentrations at time of t and t_0.

The mean daily division rate (K, division d⁻¹) was estimated using the following equation [31]:

\[K (\text{division d}^{-1}) = \frac{3.3}{st} \times \log OD_1 - \log OD_0 \tag{8}\]

where, t is cultivation days, OD_1 is the optical density at time of t, and OD_0 is the optical density at time of t_0.[11]

In the present study, a general full factorial design was used to obtain all possible ways of levels for two chosen independent variables as shown in Table 1. Moreover, an experimental design was carried out to examine the correlations. Endpoint values of PC, APC, C_a, and C(IX+X) were analyzed by two-way ANOVAs. The values in all figures represent the mean and error ± SEM of three replicates.

Multiple comparisons, Levene and Tukey tests of ANOVA, was used to determine whether the mean of a dependent variable is the same in unrelated groups using MiniTab 19. On the other hand, the biomass growth data was employed to evaluate the normality test using Anderson-Darling (AD), Ryan-Joiner (RJ), and Kolmogorov–Smirnov (KS) model before the analysis.

### 3. RESULTS AND DISCUSSION

#### 3. 1. Estimation of A.maxima Biomass Concentration

The dried biomass concentration of A. maxima was evaluated by measuring the optical density at 674 nm using a calibration curve. The measured biomass concentration linearly related to the optical density that was described by the equation of Y(gL⁻¹)=0.58X-0.0201 (with R²=0.997). where, Y and X are biomass concentration and optical density, respectively.

#### 3. 2. Biomass and Pigment Production

The results were examined in two groups with urea as an additive source and with it as an alternative source. Therefore, a standard cultivation medium was performed to analyze the best results. Addition of urea in basic nitrogen source of Zarrouk’s medium was made according to the method proposed by Chouhan and coworkers to obtain the influence of the additional amount of nitrogen source on biomass growth and pigment production compared to Zarrouk’s medium and nitrogen starvation [32]. The biomass growth curve in the case of additive nitrogen source was graphed according to Figure 2 (a and b). According to these figures,

| Factors | Levels | Values |
|---------|--------|--------|
| U¹ | 4 | 0, 1.25, 2.5, 5 |
| M² | 2 | 0, 1 |

¹: concentration of urea, ²: addition method
significant changes were not observed in the biomass growth in the medium with urea at various concentrations. Similar increment in biomass growth at various concentrations of urea was found for the first four days of cultivation, as well. Obviously, a large difference in biomass growth and cellular growing between medium with nitrogen starvations and urea was shown during six days of cultivation. However, the exponential phase of biomass growth started at the optical density of 0.43 for medium with urea but, the cell number of standard Zarrouk’s medium increased. Furthermore, the medium with urea as a nitrogen source reached exponential phase of the growth within four days of cultivation. Biomass concentration decreased with increasing the concentration of urea after the fourth day. In fact, urea did not yield the highest biomass dry weight when an additive nitrogen source was added. The best growth in biomass composition occurred on the medium with limited amount of urea.

In the second group, urea was substituted with basic nitrogen source of Zarrouk’s medium according to a method proposed by Madkour and his coworkers. \cite{33}. The influence of nitrogen starvation (0 gL$^{-1}$), replacing high concentration of basic sodium nitrate of Zarrouk’s medium with the mean amount of urea (1.25 gL$^{-1}$), was investigated. It was also compared with urea at the same amount of sodium nitrate of Zarrouk’s medium (2.5 gL$^{-1}$) and with pure urea as a cheap nitrogen source instead of sodium nitrate (5 gL$^{-1}$) on the biomass growth and pigments production of microalgae. Figure 3 displays the effect of alternative nitrogen source on optical density and biomass growth. According to Figure 3 (a and b), biomass and cellular growth reached the exponential phase with the urea concentration increment which was maximal at the lowest concentration of urea (0 gL$^{-1}$). In this group, the experiment was stopped due to reaching the exponential phase in microalgae growth in eight day. Furthermore, the biomass concentration was about 1.115 gL$^{-1}$ at 0 gL$^{-1}$ concentration of urea in this group while it was almost 1.26 times higher than that of the observed with 0 gL$^{-1}$ concentration of urea in the additive group. In the current study, it was observed that addition of urea in the medium caused a reduction in biomass growth. Thus, the biomass composition grew when the medium was without nitrogen source.

Then, an original seed culture divided into two or more equal new cells according to Table 2 \cite{34}. According to this table, the mean daily diversion rate was higher than that of a sample containing 0 gL$^{-1}$ urea. Furthermore, the highest value was found for Zarrouk’s medium without nitrogen source (sodium nitrate and urea).

Figure 4 shows analysis of the growth parameters in the various experiments. The effect of additive and alternative nitrogen sources during six days of cultivation was shown in this figure. According to this figure, a further increase of urea concentration from 0 to 5 gL$^{-1}$ will decrease $\mu_{\text{max}}$ and P when urea is added as an additive nitrogen source. Furthermore, $\mu_{\text{max}}$ was higher than that of it in the standard Zarrouk’s medium when urea was added as an additive nitrogen source. Moreover, it was more than that of it in a medium with 1.25 gL$^{-1}$ urea, more than that of it in a medium containing 2.5 gL$^{-1}$ urea and more than that of it in a medium containing 5 gL$^{-1}$ urea, respectively. atmospheric nitrogen amount supplied by
In the present study, an experimental design is carried out to examine the correlations between the variables that affect biomass and pigment production. Table 3 shows normal distribution of biomass growth, and large difference in mean value. It may be due to sampling variance which may decrease by the urea concentration. The data follow a normal distribution if p-value is greater than 0.05 in Anderson-Darling test.

Significance level was set at p > 0.15 and p > 0.1 for KS and RJ tests, respectively. The experimental results of degree of freedom and adjusted sum of square for variables and their interactions are presented in Table 4. Figure 6 shows the Pareto and residual normal probability plots of the variables and their interactions.
Figure 5. Effect of urea concentration on PC (a), APC (b), chlorophyll-a (c) and C_{X+C} (d) contents with time. The dashed line represents urea as an additional source and solid line represents urea as an additive source. (red line: 0 gL^{-1}, green line: 1.25 gL^{-1}, blue line: 2.5 gL^{-1}, black line: 5 gL^{-1})

According to Figure 6, a variable which is greater than baseline (1.746) will affect the target.

TABLE 3. Means, StDev and p-values in the KS, RJ and AD tests

| Urea con (gL^{-1}) | Add mode | Mean | StDev | KS   | RJ | AD | AD p-value |
|-------------------|----------|------|-------|------|----|----|------------|
| 0 (Zarrouk’s medium) | Add      | 0.659 | 0.160 | 0.191 | 0.997 | 0.220 | 0.733      |
| 1.25               | Add      | 0.576 | 0.148 | 0.211 | 0.978 | 0.218 | 0.614      |
| 2.5                | Add      | 0.583 | 0.151 | 0.236 | 0.979 | 0.219 | 0.612      |
| 5                  | Add      | 0.562 | 0.129 | 0.304 | 0.945 | 0.323 | 0.309      |
| 0 (Nitrogen starvation medium) | Add | 0.873 | 0.428 | 0.146 | 0.997 | 0.143 | 0.922      |
| 1.25               | Add      | 0.694 | 0.241 | 0.284 | 0.929 | 0.405 | 0.206      |
| 2.5                | Add      | 0.712 | 0.206 | 0.281 | 0.945 | 0.378 | 0.248      |
| 5                  | Add      | 0.672 | 0.246 | 0.249 | 0.924 | 0.403 | 0.209      |

TABLE 4. Degree of freedom and adjusted sum of square in regression analysis

| Sources             | DF | ADJ SS |
|---------------------|----|--------|
| U                   | 3  | 0.564  |
| M                   | 1  | 0.585  |
| U&M interaction     | 3  | 0.256  |
| Error               | 16 | 0.017  |
| Total               | 23 | 1.424  |

U: Urea concentration, M: Addition method, DF: Degree of freedom

Furthermore, U and M are most important than the interaction between them. The residual values were marked in normal probability plot of each response and the fittest red-line crossing these points. The p-value data show individual effects of variables and their interactions, p-values with a low probability (p < 0.1) indicate a very high significance for the corresponding coefficients. The effects of addition methods for urea (M) and almost urea concentration (U) statistically to be significant in the applied range. On the other hand, the carotenoid value and the combination effect of UM interactions did not have a statistical significance in the applied range. The interaction of variables for the concentration of zero showed a lower statistical significance for the responses whereas p-value amount increased by increasing the urea concentration.

As shown in Table 5, urea addition as an alternative source was the best method. The positive terms of coefficient factor have a synergistic effect on the response while the negative terms have an antagonistic effect on it. This means that yield increases by increasing the variables. As shown in Table 6, urea at the maximum concentration has the highest reducing effect on producing C_a while nitrogen starvation medium has the
largest coefficient effect (due to having a positive sign) in biomass and pigment production. According to Table 7, \( R^2 \) value for biomass (98.77%) indicates the best fitting for the proposed model.

The accuracy of total carotenoids significantly decreased. This may be due to low amount of its concentration in *Spirulina*. The mathematical models for the responses as function of two variables were investigated as Equations (9) to (13):

**Biomass amount**
\[
\text{Biomass amount} = 0.80958 + 0.2604 U^{0.00} - 0.0596 U^{1.25} - 0.0446 U^{2.50} + 0.1771 U^{5.00} + 0.15625 M^{0.00} - 0.0496 U^{0.00} U&M^{0.00,0} - 0.0446 U&M^{0.00,1} - 0.0829 U&M^{2.50,0} + 0.0829 U&M^{2.50,1} + 0.0446 U&M^{5.00,0} + 0.0446 U&M^{5.00,1}.
\]

**PC amount**
\[
\text{PC amount} = 0.056125 + 0.01004 U^{0.00} - 0.00229 U^{1.25} + 0.00262 U^{2.50} + 0.00513 U^{5.00} + 0.004375 M^{0.00} + 0.00312 U&M^{0.00,0} - 0.00312 U&M^{2.50,0} - 0.006042 M^{0.00} + 0.00296 U&M^{0.00,0} - 0.00296 U&M^{0.00,1} - 0.00229 U&M^{1.25,0} + 0.00188 U&M^{1.25,1} + 0.00296 U&M^{1.25,1} - 0.00188 U&M^{5.00,0} + 0.00012 U&M^{5.00,1}.
\]

**APC amount**
\[
\text{APC amount} = 0.076458 + 0.00788 U^{0.00} - 0.00229 U^{1.25} - 0.00229 U^{2.50} - 0.00329 U^{5.00} + 0.006042 M^{0.00} + 0.000296 U&M^{0.00,0} - 0.00239 U&M^{0.00,1} + 0.00188 U&M^{1.25,0} + 0.00296 U&M^{1.25,1} - 0.00188 U&M^{5.00,0} + 0.00012 U&M^{5.00,1}.
\]

**TABLE 5.** p-value of each factor and interaction between \( U \) and \( M \)

| Response | \( U \) | \( M \) | \( U&M \) |
|----------|--------|--------|---------|
| Biomass  | 0.125  | 2.5    | 0       |
| PC       | 0.125  | 1.25   | 0       |
| APC      | 0.125  | 1.25   | 0       |
| Ca       | 0.125  | 1.25   | 0       |
| \( C_{(X+C)} \) | 0.002 | 0.400 | 0.077 |

**TABLE 6.** Analysis of variance of biomass and pigments production

| Sources  | \( U \)         | \( M \)         | \( U&M \)        | Constant |
|----------|-----------------|-----------------|------------------|----------|
| Biomass  | 0.2604          | -0.0596         | -0.646           | 0.15625  |
| PC       | 0.01004         | -0.00229        | -0.00262         | 0.004375 |
| APC      | 0.00788         | -0.00229        | -0.00229         | 0.000694 |
| Ca       | 4.030           | -1.285          | -1.370           | 1.152    |
| \( C_{(X+C)} \) | 0.888 | -0.214 | -0.196 | 0.270 | -0.317 | -0.122 | 0.027 | 2.326 | 6.255 | 0.80958 | 0.05612 | 0.07645 |
### TABLE 7. \(R^2\) values obtained from the 2\(^4\) factorial experiment matrix

| Biomass | PC   | APC  | Ca  | \(C_{a+C}\) |
|---------|------|------|-----|-------------|
| \(R^2\) (%) | 98.77 | 90.46 | 84.32 | 93.73 | 56.21 |

\(C_a\) amount= 6.255 + 4.030 \(U_{0.00}\) - 1.285 \(U_{1.25}\) - 1.370 
\(U_{2.50}\) - 1.375 \(U_{5.00}\) + 1.152 \(M_0\) - 1.152 \(M_1\) - 0.721 
\(UM_{0.00,0}\) + 0.721 \(UM_{0.00,1}\) + 0.391 \(UM_{1.25,0}\) - 0.391 (12) 
\(UM_{1.25,1}\) - 0.088 \(UM_{2.50,0}\) + 0.088 \(UM_{2.50,1}\) + 0.417 
\(UM_{5.00,0}\) - 0.417 \(UM_{5.00,1}\)

\(C_{a+C}\) amount= 2.326 + 0.888 \(U_{0.00}\) - 0.214 \(U_{1.25}\) - 0.196 \(U_{2.50}\) - 0.478 \(U_{5.00}\) + 0.270 \(M_0\) - 0.270 \(M_1\) - 0.317 \(UM_{0.00,0}\) + 0.317 \(UM_{0.00,1}\) - 0.122 \(UM_{1.25,0}\) + 0.122 \(UM_{1.25,1}\) + 0.027 \(UM_{2.50,0}\) + 0.027 \(UM_{2.50,1}\) + 0.412 \(UM_{5.00,0}\) + 0.412 \(UM_{5.00,1}\)

Figure 7 shows the mean effect plot for pigment and biomass content versus addition mode and urea concentration. The effect of biomass variation with changing urea concentration in Zarrouk’s medium depicted in Figure 7 (a and b). Figure 7(b) shows Levene’s test of biomass growth versus urea concentration. Levene’s test is used to verify the normality of variances. The Levene’s test statistically showed a significant differences between biomass growth changes and urea concentration (\(P = 0\)). According to Figure 7(c and d), PC and APC were in maximum at the lowest concentration of urea. The pigments content was higher than that of it when urea was added as an alternative source. Furthermore, a low value of F-value (F= 0.91) implies that there is no significant difference between the mean of carotenoids and urea concentrations. However, a higher ratio of F-value (F=131.09) indicates that the variation among groups means (alternative and additive group) are greatly different from each other compared to the variation of the individual observations in each group. The mean value of \(C_a\) reaches a stationary phase by increasing the concentration of urea in alternative and additive nitrogen source from 1.25 to 5 gL\(^{-1}\). This indicates that nitrogen starvation has the highest mean value for both groups of addition mode while it is started to be declined, there after.

This study aims to evaluate the impact of all variables and their interactions on the biomass and pigments production. Moreover, the optimal conditions were also obtained and illustrated in Table 8. For the optimal conditions validation, an experiment was carried out at the same conditions during 8 days of cultivation.

According to the microscopic analysis, the cells breakdown was intensified by increasing the concentration of urea and incubation time. In addition, the cells breakdown was extremely intensified when urea as an additive source was added. The optical density was raised during the first 4 days, due to existing more \(N_2\) in the culture medium when sodium nitrate and urea were

![Figure 7](image_url)
simultaneously added. In contrast, cultures supplemented with urea showed slow growth rate compared to the traditional Zarrouk medium and the medium with an alternative nitrogen source, respectively. Since, urea is as an excellent nitrogen source and can successfully be metabolized by algae, *Spirulina* efficiently utilizes ammonia nitrate [18, 19]. The inhibition effect of urea can slightly be marked due to enzymatic hydrolysis of it by the urease enzyme [35]. Furthermore, the concentration of sodium nitrate in Zarrouk medium (2.5 g L\(^{-1}\)) reduces without losing the productivity as an important cost-saving factor in large-scale mass production of *S. platensis* [36]. Urea concentration increment in the medium (due to binding two amide groups) causes the cell division rate reduction. Cost et al. [24] used urea as a nitrogen source for growing *S. platensis*. They found that the production rate is in minimum when nitrate source of medium is replaced by urea. It also was found that *A. maxima* culture dies in the tanks with a very strong ammonia odor. In fact, nitrogen in culture is naturally reduced by the biological process of nitrification-denitrification [24]. According to Abou-Eleala et al. [37], an aqueous solution of ammonia consists of two forms (NH\(_4^+\) and NH\(_3\)). Their formation depends on culture pH and temperature. NH\(_4^+\) ions were produced when pH value was below 8.75 while NH\(_3\) molecules were produced when pH value was above 9.75 [21]. In addition, the presence of NH\(_3\) increased the toxicity of biomass culture [38]. In our experimental research, it was concluded that a high concentration of urea in culture medium may cause death of *A. maxima* by increasing incubation time. Soletto et al. [39] used urea as an alternative nitrogen source which was more suitable than the classic nitrate-based culture medium for *S. platensis* growth. However, the use of urea in fed-batch Schlosser culture improved the biomass growth and increased the feeding rate of urea from 0.56 mM to 1.7 mM to prevent from the accumulation of ammonia in the medium during the cultivation process [39]. Moreover, urea contains two nitrogen atoms in a molecule with more than 45% of nitrogen while standard Zarrouk’s medium was containing 16% of the nitrogen in sodium nitrate [40]. Abd El-Baky [41] found that nitrogen concentration reduction in *S. platensis* culture decreases phycocyanin, chlorophyll and protein contents due to breaking down the chloroplasts. The concentration of chlorophyll increased when nitrogen was used in the *S. platensis* culture medium, as well [41]. According to the current research, chlorophyll-a content decreased with increasing the urea concentration. Although, nitrogen sources did not play a significant role in chlorophyll-a and carotenoids production but, their contents stayed higher with nitrogen starvation during the cultivation process. However, the other researchers demonstrated that the concentration of sodium nitrate in Zarrouk’s medium was easily replaced by sodium nitrate (0.3 g L\(^{-1}\)) for the *S. platensis* growth. Furthermore, they modified Zarrouk’s medium with an urea substitution [42]. In several researches, nitrogen starvation increased the lipid fraction of some microalgae such as *S. platensis* [43–46]. Rodrigues et al. [35] concluded cyanobacteria initially used the nitrogen source of medium for the cells growth. Then, its excess used for the protein production [19]. This is in good agreement with the results obtained from the current research on the *A. maxima* growth with nitrogen starvation in the medium. de Castro et al. [47] demonstrated that the concentration of sodium nitrate in the Zarrouk’s medium will be reduced by increasing the biomass growth while a higher biomass production depends on the bicarbonate sodiummount. Moreover, a research showed that the highest lipids content and biomass production of *Spirulina platensis* without nitrogen source during 31 days of cultivation may be obtained while according to the present research, nitrogen had no significant effect on the biomass production enhancement. Its reason may be due to the applied range of this research [48].

4. CONCLUSION

In this research, biomass and pigment production of *A. maxima* were measured to investigated some correlations between the effect of addition methods and urea concentrations. For this purpose, the experimental design was carried out by the general factorial method and the results were analyzed under urea concentrations in the range of 0-5 g L\(^{-1}\), urea addition methods as an additive, and as an alternative. The results showed that urea concentration was the most effective factor in the biomass and pigments production. Therefore, the biomass and pigments production was disrupted and significantly decreased for urea concentrations over 1.25 g L\(^{-1}\) although urea introduced into the culture medium had no positive effect under the conditions tested in this study. Moreover, urea (from 1.25 to 5 g L\(^{-1}\)) as an additive nitrogen source could limit the intensive growth of microalgae. However, culture cells will breakdown by the urea concentration increment in presence of NH\(_3\) but, this may poison the microalgas. Furthermore, sodium nitrate is one of the most cost-effective sources in *A. maxima* cultivation was used. The effects of binary interactions of parameters on the biomass and pigments
production were investigated by two-way ANOVA, as well. p-value showed that some independent variables such as U&M considerably affected all responses expect the carotenoid pigment which the other factors could control its production. It was concluded that biomass and pigments were highly produced through the alternative method. This may be due to accelerating NH$_3$-N concentration and changing pH during the growth process. The maximum biomass growth (1.403 g L$^{-1}$), the highest specific growth rate (0.167 d$^{-1}$) with minimum doubling time (4.16 d) and maximum biomass productivity (0.103 g L$^{-1}$d$^{-1}$) with nitrogen starvation source were investigated. It is concluded that atmospheric nitrogen can grow microalga up to convert into the pigments. Besides, the sodium nitrate omission will decrease the production costs of large-scale cultivation.

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چکیده
تهیه محیط کشت جدید ارزان قیمت و تولید بیشتر زیستتوده و رنگدانه از ریزجلبک Arthrospira maxima (A. maxima) از اهمیت بسزایی برخوردار است. برای انجام این تحقیق، اوره به عنوان یک منبع نیتروژن ارزان قیمت در دو حالت (جایگزین و افزودنی) و چهار غلظت مختلف (0، 25/1، 5/2، 5 گرم بر لیتر) برای تعیین تاثیر فقر منبع نیتروژن در مقابل میزان مازاد منبع نیتروژنی استفاده شد. مشخص شد که روش افزودن اوره به عنوان جایگزین ارجحتر از روش افزودن مازاد است. که این امر ممکن است به دلیل سرعت بخشیدن به سنتز آمونیاک (NH3) و تغییرات pH در طول رشد باشد که تأثیر مثبتی بر تولید زیستتوده و رنگدانه دارد. تولید زیستتوده، فایکوسیانین، آلوفیکوسیانین، کلروفیل-A و کاروتئنید-403/074/093/097/107/117 میلی گرم بر لیتر از محیط کشت با فقر منبع نیتروژن حاصل گردید. اوره به دلیل تشکیل نیتروژنی (NH3) و امکان رشد ریزتوده محیطی می‌تواند به عنوان یک روش فناوری جدید برای کاهش هزینه‌های تولید زیستتوده به عنوان یک روش مناسب در مقایسه بیشتر دارد چرا که عامل رشد مازادی منبع نیتروژنی می‌تواند به صورت اقتصادی مقرون به صرفه باشد.