



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 gL⁻¹). It was indicated that an alternative method is better than additive method due to accelerate the ammonia synthesis (NH₃) 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_(x+c) content were 1.403, 0.074, 0.093, 10.72, and 3.17 mgL⁻¹ with nitrogen starvation medium. Urea considered being one of the inhibition sources of biomass growth due to the formation of NH₃. 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.

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NOMENCLATURE

A	Absorbance at different wavelength	PC	Phycocyanin
APC	Allophycocyanin	OD _t	Optical density at time t
C _a	Chlorophyll-a	OD ₀	Optical density at time t ₀
C _b	Chlorophyll-b	R	Correlation coefficient
C _(x+c)	Total carotenoids	DT	Doubling time (d)
C _t	Biomass concentration at time t (gL ⁻¹)	U	Urea
C ₀	Biomass concentration at time t ₀ (gL ⁻¹)	V	Total volume of cultivation (L)
mM	Molar concentration	X _m	Maximum dry weight (gL ⁻¹)
M	Addition method of urea	X _i	Initial cell dry weight (gL ⁻¹)
N _t	Total quantity of added nitrogen (g)	Y _{X/N}	Nitrogen-cell conversion (gg ⁻¹)
K	Mean daily division rate (division d ⁻¹)	Greek Symbols	
P	Productivity (gL ⁻¹ d ⁻¹)	μ _{max}	Maximum specific growth rate (d ⁻¹)

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,

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Spirulina sp. is one of the most widely available microalga sources that contain a higher value of natural pigments like chlorophyll, carotenoids, and C-phycoerythrin [6]. *A. maxima* as a photosynthetic, filamentous, spiral-shaped, multicellular, and blue-green *Cyanobacterium* was consumed as a food in some countries 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, JPJM [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 phycoerythrin, allophycoerythrin, 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 ($80\mu\text{molm}^{-2}\text{s}^{-1}$) 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 NaHCO_3 16.8 gL^{-1} , NaNO_3 2.5 gL^{-1} , K_2HPO_4 0.5 gL^{-1} , K_2SO_4 1.0 gL^{-1} , NaCl 1.0 gL^{-1} , $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ 0.2 gL^{-1} , $\text{EDTA-Na}_2\cdot 2\text{H}_2\text{O}$ 0.08 gL^{-1} , $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ 0.04 gL^{-1} , and $\text{FeSO}_4\cdot 2\text{H}_2\text{O}$ 0.01 gL^{-1} , micronutrient elements solution (H_3BO_3 2.86 gL^{-1} , $\text{MnCl}_2\cdot 4\text{H}_2\text{O}$ 1.81 gL^{-1} , $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$ 0.222 gL^{-1} , MoO_3 0.01 gL^{-1} , $\text{CoCl}_2\cdot 6\text{H}_2\text{O}$ 0.01 gL^{-1} , $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$ 0.079 gL^{-1}) 1.0 mLL^{-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 in 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 frozen 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

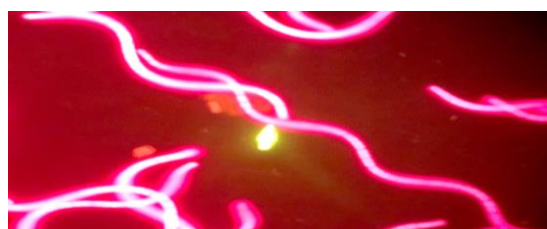


Figure 1. Microscopic images of axenic unialga

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 (C_a), chlorophyll-b (C_b), and total carotenoids [$C_{(X+C)}$] contents were then calculated using equations (1) to (5) by Bennett and Bogorad [27], Parson and Strickland [28], and Wellburn [29]:

$$PC \text{ (g/L}^{-1}\text{)} = (A_{620} - 0.474 A_{652})/5.34 \quad (1)$$

$$APC \text{ (g/L}^{-1}\text{)} = (A_{652} - 0.208 A_{620})/5.09 \quad (2)$$

$$C_a \text{ (mg/L}^{-1}\text{)} = 11.24 \times A_{662} - 2.04 \times A_{645} \quad (3)$$

$$C_b \text{ (mg/L}^{-1}\text{)} = 20.13 \times A_{645} - 4.19 \times A_{662} \quad (4)$$

$$C_{(X+C)} \text{ (mg/L}^{-1}\text{)} = ((1000 \times A_{470}) - (1.09C_a - 63.14C_b))/214 \quad (5)$$

A corresponds to the measuring absorbance in the desired wavelengths (470, 620, 645, 652 and 662 nm), PC, APC, C_a , C_b and $C_{(X+C)}$, are phycocyanin, allophycocyanin, chlorophyll-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 replications (OD)] was recorded by a spectrophotometer (Thermo Scientific, Multiskan 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 (μ_{max}), 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]:

$$\mu_{max} \text{ (d}^{-1}\text{)} = \frac{\ln(C_t) - \ln(C_0)}{t - t_0}, DT \text{ (d)} = \frac{0.693}{\mu} \quad (6)$$

$$P \text{ (g/L}^{-1}\text{d}^{-1}\text{)} = \frac{C_t - C_0}{t - t_0} \quad (7)$$

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]:

$$K \text{ (division } d^{-1}\text{)} = \frac{3.3}{st} \times \log OD_t - \log OD_0 \quad (8)$$

where, t is cultivation days, OD_t 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 $C_{(X+C)}$ were analyzed by two-way ANOVAs. The values in all figures represent the mean and error \pm 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 MiniTab19. 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 $Y \text{ (g/L}^{-1}\text{)} = 0.58X - 0.0201$ (with $R^2 = 0.997$). 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,

TABLE 1. 2⁴ general factorial design

Factors	Levels	Values
U ¹	4	0, 1.25, 2.5, 5
M ²	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 stravations 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. [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 microalga. Figure 3 displays the effect of alternative nitrogen source on optical density and biomass growth. According to Figure 3 (a and b),

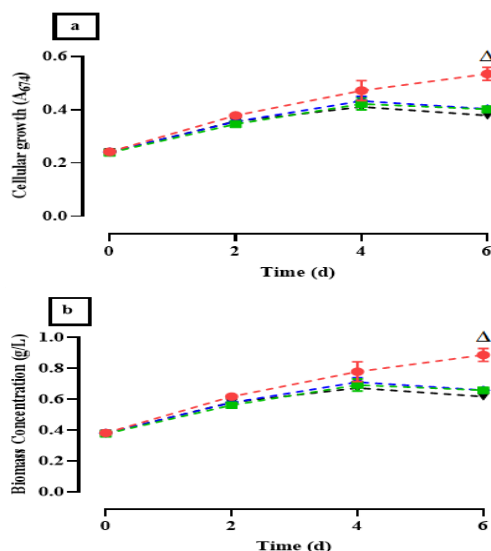


Figure 2. Effect of additive nitrogen source on cellular growth (a), biomass concentration (b) (●: 0 gL^{-1} , ■: 1.25 gL^{-1} , ◆: 2.5 gL^{-1} , ▼: 5 gL^{-1}). Δ symbol displays the best value of medium with standard Zarrouk's medium

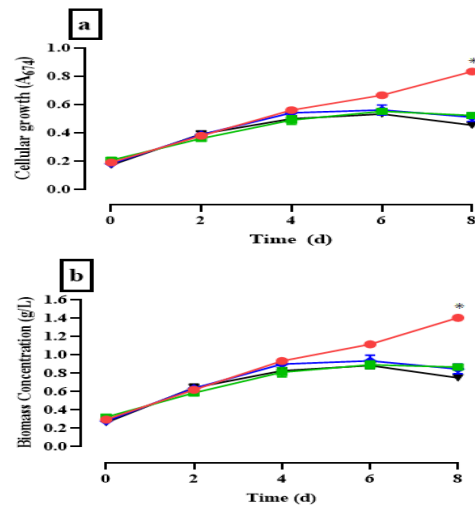


Figure 3. Effect of alternative nitrogen source on cellular growth (a), biomass concentration (b) (●: 0 gL^{-1} , ■: 1.25 gL^{-1} , ◆: 2.5 gL^{-1} , ▼: 5 gL^{-1}). * symbol displays the best value of medium with standard Zarrouk's medium

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 [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 μ_{max} and P when urea is added as an additive nitrogen source. Furthermore, μ_{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

TABLE 2. Mean daily division for *A. maxima* at different concentrations of urea

Time (D)	Urea C (gL ⁻¹)	Biomass ^a	K _{alt} ^a	Biomass ^b	K _{add} ^b
2	0	0.619	0.026	0.625	0.072
	1.25	0.641	0	0.56	0
	2.5	0.635	0.065	0.569	0
	5	0.641	0.093	0.58	0
4	0	0.933	0.514	0.752	0.328
	1.25	0.827	0.431	0.699	0.328
	2.5	0.899	0.523	0.749	0.334
	5	0.827	0.516	0.694	0.307
6	0	1.115	0.624	0.737	0.389
	1.25	0.884	0.545	0.644	0.4
	2.5	0.935	0.606	0.634	0.389
	5	0.884	0.615	0.6	0.383
8	0	1.404	0.688	-	-
	1.25	0.75	0.57	-	-
	2.5	0.846	0.623	-	-
	5	0.75	0.624	-	-

Urea C: Urea Concentration, ^a: urea was added as an alternative source, ^b: urea was added as an additive source. Concentration of 0 gL⁻¹ in additive group pointed at Zarrouk's medium and concentration of 0 gL⁻¹ in alternative group pointed at nitrogen starvation medium.

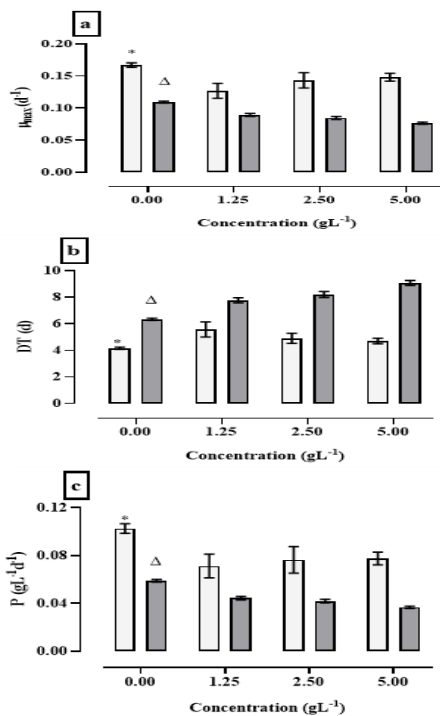


Figure 4. Changes in growth parameters when urea was added as an additional (dark gray bar) and alternative (light gray bar) sources during six days of cultivation. (a) Maximum specific growth rate (μ_{max}), (b) doubling time (DT), and (c) productivity (P). Δ displays the standard Zarrouk's medium. * displays the best value of medium with nitrogen starvation. The horizontal axis (X-axis) shows numeric values of urea concentration.

the aeration source. However, a dramatical enhancement was observed for μ_{max} (0.195 d⁻¹) and P (0.139 gL⁻¹d⁻¹) after 8 days of cultivation in medium with nitrogen starvation but, DT decreased to 3.54 day. Furthermore, the results revealed that second-best result for biomass growth and pigment production was obtained from the treatment with 2.5 gL⁻¹ of urea concentration when urea was added as an alternative nitrogen source. μ_{max} , DT, and P of this treatment (2.5 gL⁻¹) was 0.143d⁻¹, 4.90 day, and 0.076gL⁻¹d⁻¹, respectively. The best result for μ_{max} , DT, and P were found from the group with urea as an alternative source in all concentrations and also their amounts were highest in the medium with nitrogen starvation compared to the medium either with urea or with the mixture of sodium nitrate and urea. The μ_{max} and P were assessed to be approximately double in the medium with nitrogen starvation (0.195 d⁻¹, 0.139 gL⁻¹d⁻¹) compared to the medium with urea starvation (standard Zarrouk's medium, 0.109 d⁻¹, 0.060 gL⁻¹d⁻¹).

In fact, nitrogen did not play as an essential role in maintaining high μ_{max} (0.167 d⁻¹) and P (0.103 gL⁻¹d⁻¹) in the lowest DT (4.16 d) among the nutrients required for *A. maxima* growth and the

As shown in Figure 5 (a and b), the amount of PC and APC increased with nitrogen starvation compared to the medium urea as an additive and alternative sources. PC (0.0734 mgL⁻¹), APC (0.093 mgL⁻¹), C_a (10.717 mgL⁻¹), and C_(X+C) (3.171 mgL⁻¹) contents in the medium with nitrogen starvation were almost higher than that of it in the control group with a basic amount of nitrogen. According to Figure 5 (c and d), the amounts of C_(X+C) and C_a increased when the medium was without a nitrogen source. Furthermore, the amounts of PC and APC were decreased by increasing urea concentration (and biomass production). Moreover, the PC and APC contents almost were at the same value for 1.25 and 2.5 gL⁻¹ urea concentration when this was added as an additive nitrogen source. The medium with 2.5 gL⁻¹ urea as an alternative source slightly increased biomass growth and pigment content.

3. 4. Statistical Analysis and Experimental Design

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 increment. 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.

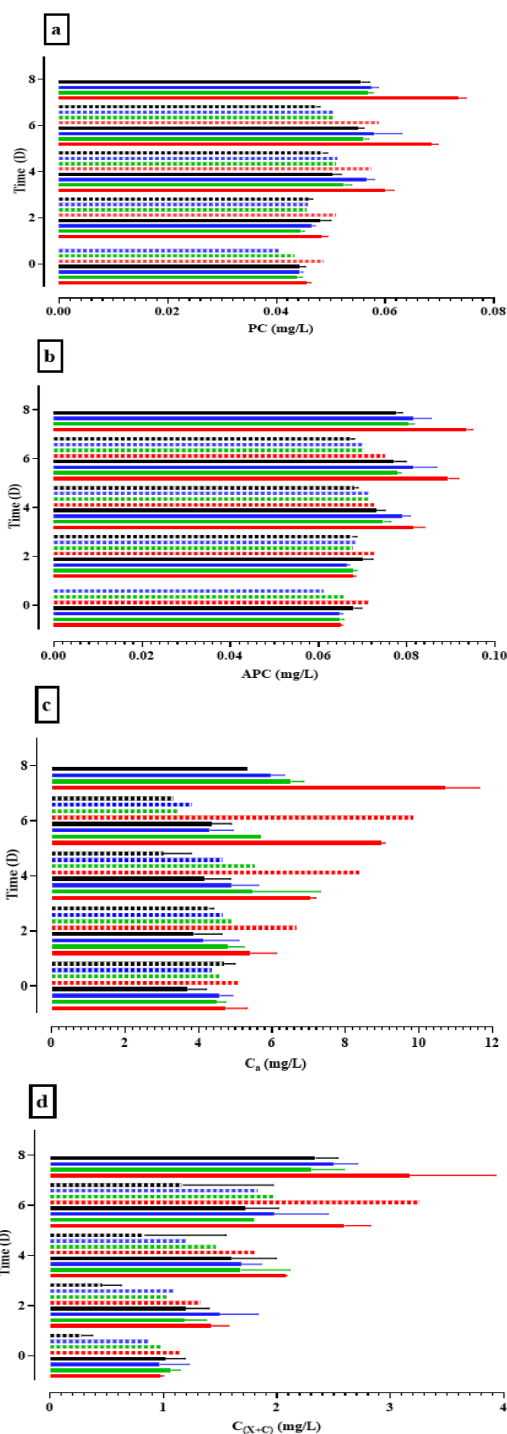


Figure 5. Effect of urea concentration on PC (a), APC (b), chlorophyll-a (c) and $C_{(x-c)}$ (d) contents with time. The dashed line represents urea as an additional source and solid line represents urea as an additive source. (red line: 0 gL^{-1} , green line: 1.25 gL^{-1} , blue line: 2.5 gL^{-1} , 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)		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		0.583	0.151	0.236	0.979	0.219	0.612
5		0.562	0.129	0.304	0.945	0.323	0.309
0 (Nitrogen starvation medium)		0.873	0.428	0.146	0.997	0.143	0.922
1.25	Alt	0.694	0.241	0.284	0.929	0.405	0.206
2.5		0.712	0.206	0.281	0.945	0.378	0.248
5		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

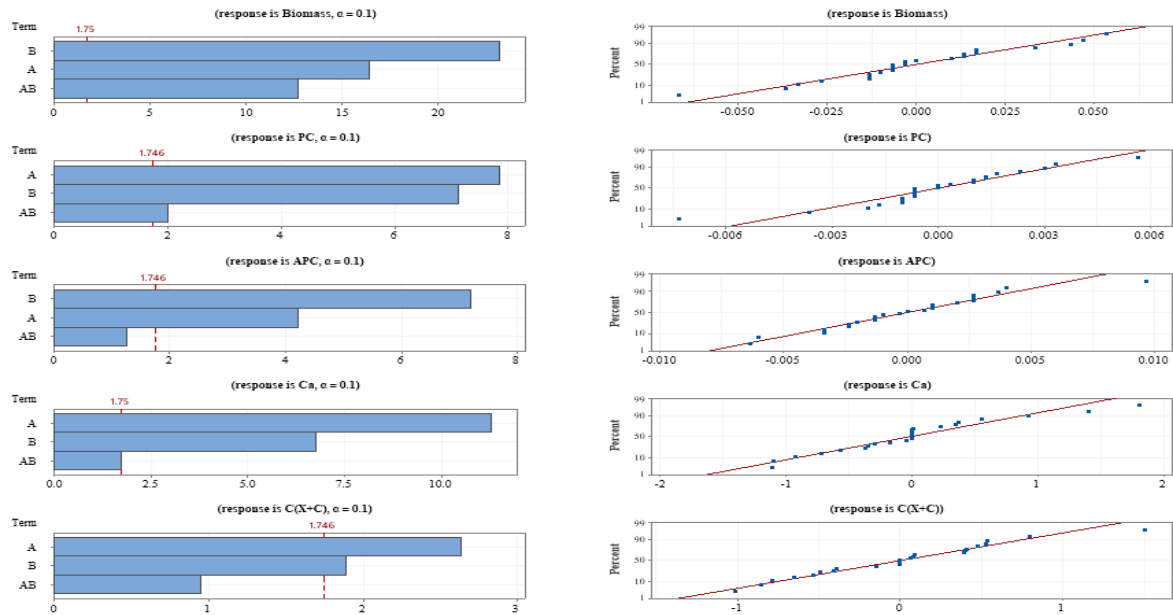


Figure 6. Pareto chart for the standardized effects and residual normal probability plot of each response (factor A, B, AB indicate U, M and interaction of U and M, respectively)

largest coefficient effect (due to having a positive sign) in biomass and pigment production. According to Table 7, R² 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):

$$\begin{aligned} \text{Biomass amount} = & 0.80958 + 0.2604 U_{0,00} - 0.0596 \\ & U_{1,25} - 0.0646 U_{2,50} - 0.1363 U_{5,+} + 0.15625 M_0 - 0.15625 \\ & M_{1,+} + 0.1771 U \& M_{0,00,0} - 0.1771 U \& M_{0,00,1} - 0.0496 \\ & U \& M_{1,25,0,+} + 0.0496 U \& M_{1,25,1,-} - 0.0446 U \& M_{2,50,0,+} \\ & + 0.0446 U \& M_{2,50,1,-} - 0.0829 U \& M_{5,00,0} + 0.0829 \\ & U \& M_{5,00,1s} \end{aligned} \quad (9)$$

$$\begin{aligned} \text{PC amount} = & 0.056125 + 0.01004 U_{0,00} - 0.00229 \\ & U_{1,25} - 0.00262 U_{2,50} - 0.00513 U_{5,00,+} + 0.004375 \\ & M_0 - 0.004375 M_{1,+} + 0.00312 U \& M_{0,00,0} - 0.00312 \\ & U \& M_{0,00,1} - 0.00121 U \& M_{1,25,0,+} + 0.00121 \\ & U \& M_{1,25,10,00154} U \& M_{2,50,0,+} + 0.00154 \\ & U \& M_{2,50,1,-} - 0.00037 U \& M_{5,00,0,+} - 0.00037 \\ & U \& M_{5,00,1} \end{aligned} \quad (10)$$

$$\begin{aligned} \text{APC amount} = & 0.076458 + 0.00788 U_{0,00} - 0.00229 \\ & U_{1,25} - 0.00229 U_{2,50} - 0.00329 U_{5,00,+} \\ & 0.006042 M_0 - 0.006042 M_{1,+} + 0.00296 U \& M_{0,00,0} - \\ & 0.00296 U \& M_{0,00,1} - 0.00121 U \& M_{1,25,0,+} \\ & + 0.00121 U \& M_{1,25,1,-} - 0.00188 U \& M_{2,50,0} + \\ & 0.00188 U \& M_{2,50,1} + 0.00012 U \& M_{5,00,0} - \\ & 0.00012 U \& M_{5,00,1} \end{aligned} \quad (11)$$

TABLE 5. p-value of each factor and interaction between U&M

Response	P- VALUE						
	U			M			
	0	1.25	2.5	0	1.25	2.5	
Biomass	0	0	0	0	0	0.001	0.002
PC	0	0.047	0.025	0	0.01	0.274	0.167
APC	0	0.135	0.135	0	0.059	0.419	0.216
Ca	0	0	0	0	0.027	0.204	0.771
C _(X+C)	0.002	0.400	0.400	0.077	0.219	0.630	0.916

TABLE 6. Analysis of variance of biomass and pigments production

Sources	U			M	U&M			Constant
	0	1.25	2.5	0	0	1.25	2.5	
Biomass	0.2604	-0.0596	-0.646	0.15625	0.1771	-0.0496	-0.0446	0.80958
PC	0.01004	-0.00229	-0.00262	0.00437	0.00312	-0.00121	-0.00154	0.05612
APC	0.00788	-0.00229	-0.00229	0.00604	0.00296	-0.00121	-0.00188	0.07645
Ca	4.030	-1.285	-1.370	1.152	-0.721	0.391	-0.088	6.255
C _(X+C)	0.888	-0.214	-0.196	0.270	-0.317	-0.122	0.027	2.326

TABLE 7. R² values obtained from the 2⁴ factorial experiment matrix

	Biomass	PC	APC	Ca	C _(X+C)
R ² (%)	98.77	90.46	84.32	93.73	56.21

$$C_a \text{ amount} = 6.255 + 4.030 U_{0,00} - 1.285 U_{1,25} - 1.370 U_{2,50} - 1.375 U_{5,00} + 1.152 M_0 - 1.152 M_1 - 0.721 UM_{0,00,0} + 0.721 UM_{0,00,1} + 0.391 UM_{1,25,0} - 0.391 UM_{1,25,1} - 0.088 UM_{2,50,0} + 0.088 UM_{2,50,1} + 0.417 UM_{5,00,0} - 0.417 UM_{5,00,1} \quad (12)$$

$$C_{(X+C)} \text{ amount} = 2.326 + 0.888 U_{0,00} - 0.214 U_{1,25} - 0.196 U_{2,50} - 0.478 U_{5,00} + 0.270 M_0 - 0.270 M_1 - 0.317 U \& M_{0,00,0} + 0.317 U \& M_{0,00,1} - 0.122 U \& M_{1,25,0} + 0.122 U \& M_{1,25,1} + 0.027 U \& M_{2,50,0} - 0.027 U \& M_{2,50,1} + 0.412 U \& M_{5,00,0} - 0.412 U \& M_{5,00,1} \quad (13)$$

Figure 7 shows the mean effect plot for pigmet 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 N₂ in the culture medium when sodium nitrate and urea were

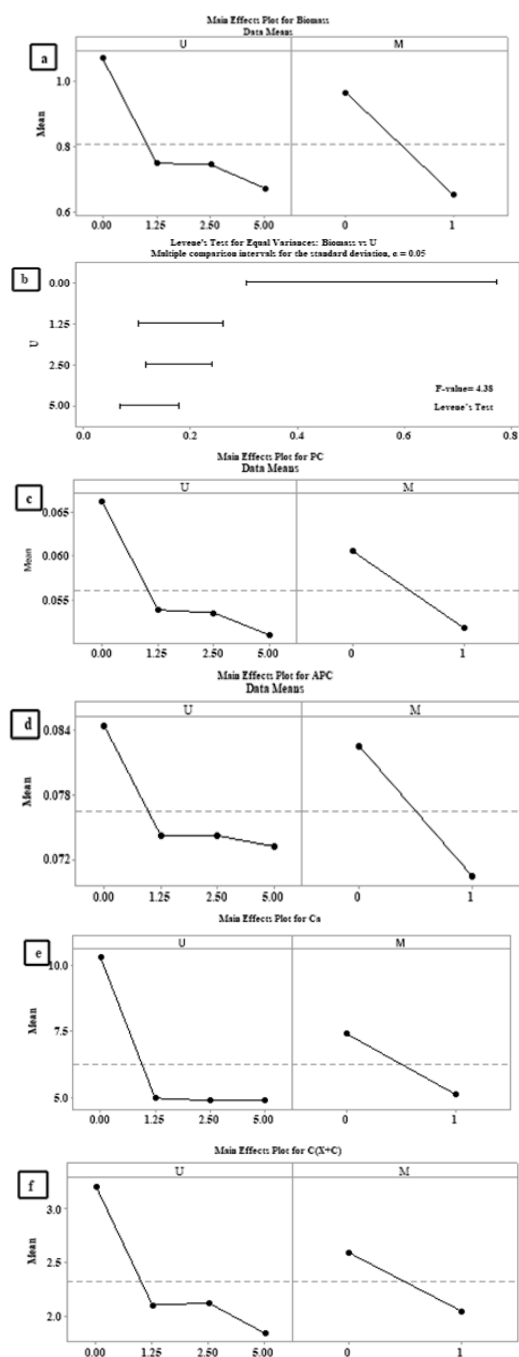


Figure 7. Mean effect plot for biomass growth (a&b), PC (c), APC (d), C_a (e) and $C_{(X+C)}$ (f) content versus addition mode and urea concentration. (0, 1.25, 2.5 and 5 (gL⁻¹) are urea concentration (U) and addition method of urea (M) was at 0 and 1 (gL⁻¹) as alternative and additive source of Zarrouk's medium, respectively

TABLE 8. Optimal conditions and their responses

Cond	U	M	Biomass fit	PC fit	APC fit	C _(X-C) fit	C _a fit
mo	0	0	1.403	0.074	0.094	3.167	10.717
Ex	0	0	1.436	0.078	0.103	3.214	10.689

Cond: Condition; Optimal data based on (mo) the model, (Ex) the experiment (validation)

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 gL⁻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 (NH₄⁺ and NH₃). Their formation depends on culture pH and temperature. NH₄⁺ ions were produced when pH value was below 8.75 while NH₃ molecules were produced when pH value was above 9.75 [21]. In addition, the presence of NH₃ 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 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 gL⁻¹) for the *S. platensis* growth. Furthermore, they modified Zarrouk's medium as 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 sodium amount. 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 investigate 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 gL⁻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 gL⁻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 gL⁻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 NH₃ but, this may poison the microalgae. 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 except 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 NH₃ molecules and changing pH during the growth process. The maximum biomass growth (1.403 gL⁻¹), the highest specific growth rate (0.167 d⁻¹) with minimum doubling time (4.16 d) and maximum biomass productivity (0.103 gL⁻¹d⁻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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Persian Abstract

چکیده

تهیه محیط کشت جدید ارزان قیمت و تولید بیشتر زیست توده 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) r چهار غلظت مختلف (۰، ۱/۲۵، ۲/۵، ۵ گرم بر لیتر) برای تعیین تاثیر فخر منبع نیتروژن در مقابل میزان مازاد منبع نیتروژنی استفاده شد. مشخص شد که روش افزودن اوره به عنوان منبع جایگزین ارجح تر از روش افزودنی مازاد است. که این امر ممکن است به دلیل سرعت بخشیدن به سنتز آمونیاک (NH_3) و تغییرات pH در طول رشد باشد که تقریباً تأثیر مستقیمی بر تولید زیست توده و رنگدانه دارد. علاوه بر این، بیشترین تولید زیست توده، فایکوسیانین، آلفوکوسیانین، کلروفیل-a و کاروتنوئید ۱/۴۰۳، ۰/۰۷۴، ۰/۰۹۳، ۱۰/۷۲ و ۳/۱۷ میلی گرم بر لیتر از محیط کشت با فخر منبع نیتروژنی حاصل گردید. اوره به دلیل تشکیل NH_3 یکی از منابع مهار رشد زیست توده محسوب می شود. تجزیه و تحلیل نتایج نهایی به روش فاکتوریل جزئی اثبات کرد که حذف منابع نیتروژن امکان رشد *A. maxima* را به منظور کاهش هزینه های تولید کشت در مقیاس وسیع دارد که عملکرد بهتری نسبت به محیط اقتصادی مقرون به صرفه زا روک دارد.