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Enhance Growth and Biochemical Composition of *Nannochloropsis oceanica*, Cultured under Nutrient Limitation, Using Commercial Agricultural Fertilizersm

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Abstract

Microalgae culture media should be economic, allow for high growth, satisfy the needs of microalgal cells and easy to prepare. In this study, we evaluate the effect of different media formula prepared from commercial agricultural fertilizers (CAGF), comparing to F/2 Guillard standard medium as a control medium, on growth (cell density, CD; dry weight, DW and specific growth rate, μ) and biochemical composition (lipid, protein, and carbohydrate) of *Nannochloropsis oceanica*. Comparing to N/P ratio (9.6) and actually quantity (12.36 g/l and 1.29 g/l, respectively) of F/2 standard medium, six N/P ratios (19.2, 9.6, 9.6, 4.8, 3.2 and 1.6) were prepared from Nitric Acid (N-Nt) or Ammonium Sulphate (N-Am), as a nitrogen source, with phosphoric acid (P), as a phosphorus source, for culturing media of *N. oceanica*. The results investigated that some CAGF media achieved significant ($P \leq 0.05$) growth and biochemical composition higher than F/2. Comparing to lipid percentage (30.70 %) of F/2, the lipid percentage of *N. oceanica* cultured on different CAGF media were ranging from 18.40% to 46.12%, depending on nutrient limitation, nitrogen source, N/P ratios and actually atom concentrations. Finally, the use of CAGF constitutes a viable alternative of F/2 medium to reduce the production costs *N. oceanica*, the commonly used in marine hatcheries and also other biotechnological applications.

Keywords: *N. oceanica*; Aquaculture; Lipid; Biodiesel; Agricultural fertilizers; Nitric acid

Introduction

Nannochloropsis is considered the main algal species cultured in marine hatcheries and play an important role in aquaculture development [1]. In the industrial production scale in marine hatcheries, it is very important to optimize a suitable nutrient culture media for culturing this species. The microalgae nutrient media should be prepare easy, economical, achieve high growth and satisfy all the microalgae quality and quantity. Although F/2 Guillard medium is considered the most commonly medium used in culturing of *Nannochloropsis* in marine hatcheries, F/2 Guillard medium have some disadvantages, *like* difficult to prepare and set up for outdoor mass culture and expensive.

Microalgae production cost as a live food produced in marine shellfish and shrimp hatcheries are about 30% of the total seeds production cost. The high cost of microalgae production remains an obstacle for marine hatcheries [2]. Therefore, commercial agricultural fertilizers (CAGF) should be commonly used instead of F/2 culture medium [3]. As aquatic organisms, microalgae need water, salts and CO_2 to grow. The major essential macronutrients are nitrogen (N), phosphorus (P) and silicon (Si, for diatoms only). Some vitamins and micronutrients are also required for algal growth (such as magnesium, sulfur, iron, etc). Among all nutrient elements, nitrogen and phosphorus are the main nutrient limiting the growth, lipid percentage and productivity of microalgae [4]. Many authors noticed that the biochemical composition of microalgae modify as a function of nutrient limitation. However, nutrient limitation may increase the lipid content capacities of some microalgae, by 30-60% of the dry cell weight. Among these factors, nitrogen and phosphorus have strong effects on lipids metabolism in various microalgae [5]. The present study aims to find the economical nutrient medium prepared from CAGF and has the positive effect on growth and lipid content of *Nannochloropsis oceanica*, the main species in marine hatcheries, in regarding to reduce the production cost of marine larvae.

Material and Methods

Microalgal strains

Nannochloropsis oceanica strain was isolated from East-south of the Mediterranean Sea (31° 13′ 48" N, 29° 53′ 12" E), Eastern Harbor of Alexandria, Egypt, and cultured in Microalgae Lab., Invertebrate Aquaculture Lab., Aquaculture Division, Alexandria branch, National Institute of Oceanography and Fisheries (NIOF), Egypt. *N. oceanica* were kept and cultured under controlled conditions of temperature (22 ± 2°C), salinity (35 ± 2 ppt), and illumination (750-3000 Lux /24 h.) using F/2 Guillard and Ryther [6], with continuously aeration.

Experimental design

Depending on the atomic mass weight (g/mol) and total quantity (g/l) of N and P that used in F/2 Guillard medium stock solution, different atomic mass weight (g/mol) and quantity (g/l) of N and P were prepared from Nitric Acid (N-Nt) or Ammonium sulphate (N-Am), as nitrogen source, in combination with phosphoric acid (P), as phosphorus source. The compositions and preparing cost of F/2 standard Guillard medium and other CAGF media were presented in Table 1. In addition to nitrogen and phosphorus sources, vitamin B_1 and B_{12} (in form of local human pharmacy ampoules, commercially named Tri-B, produced by The Nile Co. for Pharmaceuticals and Chemical Industries, Cairo,

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Chemical composition and production cost of CAGF media, comparing to F/2 standard medium											
F/2 Standard Medium (control medium)				Nitric Acid-Nitrogen-Based-Media (N-Nt)				Ammonium Sulphate-Nitrogen-Based-Media (N-Am)			
Nutrient source	Ionic Form	Media Stock solution (g/l)	Cost of 1Ton culture ($)	Nutrient Source	Ionic Form	Media Stock solution (g/l)	Cost of 1Ton culture ($)	Nutrient source	Ionic Form	Media Stock solution (g/l)	Cost of 1Ton culture ($)
N	$NaNo_3$	75	3.85	N-Nt100	HNO_3	54.54	0.0245	N-Am100	$(NH_4)_2SO_4$	57.14	0.019
P	$NaH_2PO_4.H_2O$	5	0.0257	P100	H_3PO_4	2.6	0.0052	P100	H_3PO_4	2.6	0.0052
Vitamin	B_1	0.2	0.0088	Vitamin*	B_1	100	0.0777	Vitamin*	B_1	100	0.0777
	B_{12}	1	0.044	(µg/l)	B_{12}	1000		(µg/l)	B_{12}	1000	
	Biotin	0.1	0.0044	-				-			
T.M**	$FeCl_2$	3.15	0.0231								
	Na_2EDTA	4.36	0.0486								
	$CuSO_4.5H_2O$	0.0098	0.0001								
	$ZnSO_4.7H_2O$	0.022	0.0001								
	$CoCl_2.6H_2O$	0.01	0.0018								
	$MnCl_2.4H_2O$	0.18	0.002								
	$Na_2MoO_4.2H_2O$	0.0063	0.0013								
Final Cost			4.0099	0.1074				0.1019			

Table 1: Chemical composition and production cost of CAGF media, comparing to F/2 standard medium.

Egypt) were added in the concentration of 100 µg/l and 1000 µg/l, respectively. All treatments were conducted without aeration in conical flasks 250 ml filled with 100 ml of culture medium (three replicates for each treatment medium) under controlled conditions of temperature (21 ± 1°C), salinity (35ppt), and continuous illumination (1000 Lux/24 h). The samples for growth parameters and biochemical analysis were taken at late exponential phase. All experiments were ended when the growth rate had reached to the death phase.

Tested Parameters

Growth parameters: The growth was assessed by determination of algal cell density (CD) and dry weight (DW). CD was determined firstly by haemocytometer and then celebrated with spectrophotometer (CECIL 2010, double beam) as standard curve by measuring the optical densities (at 540 nm, according to Rocha et al.) [7]. This standard curve was subsequently used to calculate the algal cell density and dry weight. Dry weight (DW) of samples was firstly determined according to Olguin et al. [8] and subsequently was calculate according to the recorded standard curve. Specific growth rate μ was calculated according to Levasseur et al. [9] as the following:

$$\mu\left(\mathrm{div/day}\right) = \mathbf{ln}\ \left(\mathrm{X_2/X_1}\right) / \left(\mathrm{t_2\text{-}t_1}\right)$$

Where: X_1=cell concentration at time t_1, X_2=cell concentration at the time t_2

Biochemical Composition Analysis: 10 ml of culture samples of each treatment medium (three replicates) were centrifuged (3000 rpm/20 min.) and preserved frozen at -20°C until analysis. Total lipids were determined according to Bligh and Dayer [10]. Total proteins were extracted according to Rauch [11] and determined according to Hartree [12]. Total carbohydrates were extracted according to Myklestad and Haug [13] and determined according to Dubois et al. [14].

Comparisons between the means: The comparison between the means of the tested parameters (CD, DW and µ, lipid, protein and carbohydrates) of F/2 Guillard medium, as a control medium, and each treatment medium prepared from CAGF were measured by the following equation:

$$M\% = [(\mathrm{T\text{-}F})/\mathrm{F}] \times 100$$

Where M% is the increase or decrease (±) of the value percentage of tested parameter, T: mean value of recorded treatment prepared from CAGF, and F: mean value of recorded F/2 Guillard medium.

Statistical analysis

Statistical analysis was performed using analysis of variance (ANOVA). Differences among means were considered significant at $p<0.05$ multiple range of post hoc comparisons were performed using the least significant difference (LSD) to resolve the differences among the means of replication according to of Duncan, (1955) [15] using SPSS (2007) [16].

Results

The effect of different nutrient medium prepared from CAGF, comparing to F/2 medium, on the growth and biochemical composition of *N. oceanica* were shown in Table 2. The results investigated that some CAGF media achieved significant ($P \leq 0.05$) growth and biochemical composition higher than F/2 while other CAGF media achieved significant ($P \leq 0.05$) growth and biochemical composition lower than F/2, depends on nutrient limitations, nitrogen source, nitrogen and phosphorus ratios and atomic concentrations.

Growth

Comparing to CD (6.87 10^7 cell/ml), DW (0.827 g/l) and µ (0.663 division/day) of *N. oceanica* cultured on F/2 medium, the highest significant differences ($P \leq 0.05$) of CD (9.351 10^7 cell/ml, M% 36) and DW (0.9067 g/l, M% 10) was observed by treatment medium N-Nt100+P100, while the lowest significant ($P \leq 0.05$) CD (4.41 10^7 cell/ml, M% -36) and DW (0.748 g/l, M% -9) were achieved by treatment N-Nt50+P300.On the other hand, N-Nt100+P100 achieved the highest significant differences ($P \leq 0.05$) of µ (0.6637 division/day, M% -1), comparing to F/2 medium (0.6548 division/day). It is worth mentioning that F/2 and N-Nt100+P100 have the same actually quantity of N and P (12.36 g/l and 1.29 g/l, respectively) and also the same N/P ratio (9.6).

Although N-Nt50+P50 and N-Am50+P50 have the same N/P ratio of F/2 and N-Nt100+P100 (9.6), but in half actually quantity of N and P, these treatments achieved the lowest µ (0.211 division/day, M% -68 and 0.196 division/day, M% -70, respectively). Also, some treatments media (with different N/P ratios and actually quantity of N and P) were achieved the lowest significant ($P \leq 0.05$) µ, like N-Nt50+P100 (0.256

division/day, M% -61), N-Am50+P100 (0.234 division/day, M% -65), N-Nt100+P300 (0.228 division/day, M% -66) and N-Am100+P300 (0.201 division/day, M% -70), as shown in Table 2.

Biochemical composition

Total lipid observed in *N. oceanica* cultured on F/2 medium was 30.70%. Treatment N-Am100+P300 (which achieved the lowest significant μ) achieved the highest significant ($P \leq 0.05$) total lipid (46.12%, M% 59), followed by N-Nt50+P100 (42.84%, M% 40), N-Am50+P100 (39.21%, M% 28), N-Am100+P50 (37.21%, M% 21), N-Nt100+P300 (36.00%, M% 17) and N-Am50+P50 (32.01%, M% 4), while the lowest significant total lipid was observed by N-Nt50+P300 (18.4%, M% -40), followed by N-Nt100+P100 (19.72%, M% -36, which achieved the highest significant μ), as shown in Table 2.

Total protein observed in *N. oceanica* cultured on F/2 medium was 14.46%. Only three treatment media, based on ammonium sulphate, through all experimented media were achieved total protein significantly ($P \leq 0.05$) lower than F/2 control, these three media were N-Am100+P100 (10.64%, M% -26), N-Am50+P50 (10.27%, M% -29) and N-Am50+P100 (12.93%, M% -11). On the other hand, the highest significant ($P \leq 0.05$) protein was achieved by N-Nt100+P300 (28.46%, M% 97) and N-Am100+P50 (28.4%, M% 96).

Carbohydrates observed in *N. oceanica* cultured on F/2 medium was achieved the lowest significant ($P \leq 0.05$) carbohydrate percentage (8.33%), comparing to all nutrient media prepared from CAGF. The highest significant ($P \leq 0.05$) carbohydrate was achieved by N-Nt50+P50 (26.50%, M% 218, which achieved the same N/P ration (9.6) of F/2 but in half N and P quantity), as shown in Table 2.

Discussions

The production cost of microalgae used as live food in marine hatcheries is nearly 30% of the total cost of the industrial seed production [17], however, if stable and economical microalgae production can be developed, the production cost of marine seed will decrease. Our aim is to develop the medium culture of *N. oceanica* (the most important species used in marine hatcheries) by using CAGF to replace f/2 medium, in regarding to reduce the production cost of marine seeds production. But the question is dose *N. oceanica* cultured on CAGF achieved the biochemical composition (like lipid, protein and carbohydrate) and growth (like cell density, dry weight and specific growth rate) like those of cultured on F/2 Guillard medium?

Although F/2 Guillard medium is wildly used in marine aquaculture for microalgae culture more than fifty years, now days, because the different applications of microalgae in biotechnological fields, the F/2 Guillard medium has many disadvantages. However, the effect of nonconventional media prepared from CAGF on microalgae growth [2,18,19] and biochemical composition [20,21] were investigated.

Our results investigated that some CAGF formulas achieved significant ($P \leq 0.05$) growth and biochemical composition higher than F/2, whereas other formulas achieved significant growth and

Growth and biochemical composition of *N. oceanica* cultured on CAGF media, comparing to F/2 standard medium						
Media Abbreviations/ Treatments	**Growth Parameters***			**Biochemical composition (% of dw)**		
	CD (10^7 cell/ml)	**DW (g/l)**	**μ (Division/day)**	**Lipid (% dw)**	**Protein (% dw)**	**Carbohydrate (% dw)**
F/2 Control	6.87 ± 0.034^{d}	0.827 ± 0.001^{d}	0.663 ± 0.014^{a}	30.70 ± 4.57^{cdef}	14.46 ± 1.80^{de}	8.33 ± 1.04^{d}
N-Nt100+P100	9.35 ± 0.087^{a}	0.906 ± 0.002	0.654 ± 0.012^{a}	19.72 ± 4.72^{g}	22.61 ± 2.59^{abc}	14.69 ± 6.50^{bcd}
M%	(36)	(10)	(-1)	(-36)	(56)	(76)
N-Nt50+P50	5.73 ± 0.069^{fg}	0.791 ± 0.002^{g}	0.211 ± 0.093^{c}	24.70 ± 1.23^{efg}	22.53 ± 2.25^{abc}	26.50 ± 4.54^{a}
M%	(-17)	(-4)	(-68)	(-20)	(56)	(218)
N-Nt50+P100	5.88 ± 0.10^{f}	0.795 ± 0.003^{f}	0.256 ± 0.031^{c}	42.84 ± 2.55^{ab}	14.63 ± 3.27^{de}	14.80 ± 2.57^{bcd}
M%	(-14)	(-4)	(-61)	(40)	(1)	(78)
N-Nt50+P300	4.41 ± 0.05^{h}	0.748 ± 0.001^{h}	0.308 ± 0.080^{bc}	18.40 ± 1.97^{g}	26.18 ± 2.47^{ab}	9.84 ± 1.63^{cd}
M%	(-36)	(-9)	(-54)	(-40)	(81)	(18)
N-Nt100+P50	7.62 ± 0.08^{c}	0.851 ± 0.002^{c}	0.319 ± 0.051^{bc}	22.09 ± 5.33^{fg}	16.80 ± 4.06^{cde}	9.64 ± 1.03^{cd}
M%	(11)	(3)	(-52)	(-28)	(16)	(16)
N-Nt100+P300	5.62 ± 0.15^{fg}	0.787 ± 0.005^{fg}	0.228 ± 0.044^{c}	36.00 ± 3.80^{bc}	28.46 ± 3.38^{a}	18.07 ± 1.88^{b}
M%	(-18)	(-5)	(-66)	(17)	(97)	(117)
N-Am100+P100	8.28 ± 0.05^{b}	0.872 ± 0.001^{b}	0.390 ± 0.042^{b}	30.77 ± 1.58^{cdef}	10.64 ± 1.02^{e}	16.87 ± 1.74^{bc}
M%	(21)	(5)	(-41)	(0.2)	(-26)	(103)
N-Am50+P50	5.51 ± 0.06^{g}	0.783 ± 0.002^{g}	0.196 ± 0.057^{c}	32.01 ± 3.69^{cde}	10.27 ± 3.38^{e}	15.03 ± 5.15^{bcd}
M%	(-20)	(-5)	(-70)	(4)	(-29)	(80)
N-Am50+P100	6.51 ± 0.19^{e}	0.815 ± 0.006^{e}	0.234 ± 0.007^{c}	39.21 ± 5.26^{abc}	12.93 ± 2.14^{e}	13.59 ± 3.61^{bcd}
M%	(-5)	(-1)	(-65)	(28)	(-11)	(63)
N-Am50+P300	6.53 ± 0.19^{e}	0.816 ± 0.006^{e}	0.277 ± 0.062^{bc}	30.22 ± 2.35^{def}	15.05 ± 2.81^{de}	12.93 ± 2.67^{bcd}
M%	(-5)	(-1)	(-58)	(-2)	(4)	(55)
N-Am100+P50	7.58 ± 0.20^{c}	0.850 ± 0.006^{c}	0.323 ± 0.064^{bc}	37.21 ± 3.99^{bc}	28.40 ± 3.49^{a}	15.72 ± 2.18^{bcd}
M%	(10)	(3)	(-51)	(21)	(96)	(89)
N-Am100+P300	6.39 ± 0.06^{e}	0.812 ± 0.002^{e}	0.201 ± 0.052^{c}	46.12 ± 3.65^{a}	20.25 ± 2.47^{bcd}	16.79 ± 3.54^{bc}
M%	(-7)	(-2)	(-70)	(50)	(40)	(102)

*CD: cell density (10^7 cell/ml); DW: dry weight (g/l); μ: Specific growth rate (Division/day); CAGF: commercial agriculture fertilizer, N-Nt and N-Am: Nutrient media prepared from nitric acid and ammonium sulfate, respectively, as nitrogen source in different concentrations. 50, 100 and 300: The half, the same and the triple fold of really nitrogen (N-Nt or N-Am) and phosphorus (P) amount exist in F/2 medium, M%: the means of increase or decrease of the value percentage (± %) of tested medium, comparing to F/2 Guillard, as a standard medium.

Table 2: Growth and biochemical composition of *N. oceanica* cultured on CAGF media, comparing to F/2 standard medium.

biochemical composition lower than F/2. These significant differences may be due to N/P ratios, concentrations and sources. However, to optimize the production of *N. oceanica* for aquaculture purposes in marine hatcheries, CAGF should be used with advantages of reduced cost media, high productivity and easy to prepare of culture medium. Our suggestions were in agreement with Guzman-Murillo et al. who suggested that, CAGF media may be used to improve the biochemical composition of microalgae for the purposes of aquaculture, production of bioactive materials and biotechnology. Bae and Hur, (2011) found that the growth of *N. oceanica* cultured on fertilizer medium was similar to that of *N. oceanica* cultured in F/2 medium. On the other hand, our results disagree with Simental and Sanchez-Saavedra (2003) [22] who pointed that, comparing to F/2 medium, the using of liquid CAGF did not achieve any significant differences in cell concentration and growth rate of *Navicula incerta, Nitzschia thermalis* and *Nitzschia laevis*. This disagree may be due to the experiment conditions, N/P ratios, concentrations and sources

In F/2 medium, the nitrogen (in form of sodium nitrate) and phosphorus (in form of sodium hydrogen orthophosphate.) concentrations in medium stock solution were 12.36 g/l and 1.29 g/l, respectively, and 0.0124 g/l and 0.0013 g/l in microalgae culture solution, respectively, with ratio N/P (9.6). Hsieh and Wu [23] reported that nitrogen sources were strongly affecting microalgae quality and quantity.

Our study investigated that in the case of nitric acid, the treatment medium N-Nt100+P100, which has the same N/P ratio and concentrations of F/2 medium, achieved growth (cell density, dry weight and specific growth rate) higher than F/2 and/or ammonium sulphate-nitrogen based media. Until now, there is no recorded data available about using of nitric acid as nitrogen source in medium composition of marine microalga *N. oceanica*. To date, nitrate is a commonly studied as a nitrogen source used to understand nutrient limitation to induce lipid accumulation [24]. In the current study, we compare ammonium sulphate and nitric acid, as CAGF nitrogen sources, with sodium nitrate used in F/2 medium. The results illustrated that nitric acid candidate to be best investigated nitrogen source achieved the highest quantity (cell density, dry weight and growth rate) of *N. oceanica*, while ammonium sulphate candidate to be best investigated nitrogen source achieved high lipid content of *N. oceanica,* depending on (1) N/P ratio, (2) N, P concentrations, and (3) N, P sources. From our results we concluded that *N. oceanica* may be need to a specific N/P ratio, concentration and sources to achieve the highest significant growth and/or biochemical composition. However, the results suggests that in the algal strain *N. oceanica*, the optimal N/P ratio, concentration and sources that achieved the highest growth may be not the optimal to achieve the highest biochemical composition. Finally, from our results we can conclude that to determine the optimal N/P ratio, concentration and sources for each species, we should examine that according to the final purpose of culture. According to literature, *Nannochloropsis* have a wide range of lipid percentage (28% to 68.5%), as shown in Table 3, and dry weight (0.05 to 2.67 g/l), as shown in Table 4, which make *Nannochloropsis* have respect for biotechnological applications, beside its important role as a live food source in marine hatcheries.

The production cost of microalgae culture mediums is another favorable factor should be discussed. Microalgae productivities (lipid and biomass) are the most important key parameters affecting the economic feasibility of microalgae production in marine hatcheries, as well as in the industrial scale [25]. Simental and Sanchez-Saavedra (2003) reported that the production cost of microalgae cultured on

Lipid percentage (%) of *Nannochloropsis*, as reported in the literature		
Species	**Lipid (%)**	**References**
Nannochloropsis sp.	68.5	[27]
Nannochloropsis sp.	60	[28]
Nannochloropsis	31	[29]
Nannochloropsis oceanica	28	[30]
Nannochloropsis oceanica	18.40-46.12	The present study

Table 3: Lipid percentage (%) of *Nannochloropsis*, as reported in the literature [27-30].

Dry weight (g/l) of *Nannochloropsis*, as reported in the literature		
Species	**Dry weight (g/l)**	**References**
Nannochloropsis gaditana	1.18-2.67	[31]
Nannochloropsis oculata	1.59-2.21	[32]
Nannochloropsis oculata	1.2	[33]
Nannochloropsis sp.	0.8-1.4	[34]
Nannochloropsis sp.	0.44-0.66	[35]
Nannochloropsis oceanica	0.74-0.90	The present study

Table 4: Dry weight (g/l) of *Nannochloropsis*, as reported in the literature [31-35].

CAGF is lower than F/2 medium about eight times. According to our conditions, sodium nitrate used in F/2 Guillard medium is much expensive more than CAGF, however, some CAGF media achieved *N. oceanica* quality and quantity higher than F/2 standard medium, with advantage of reduced production cost [26]. Finally, our results reported that the cost of CAGF media (prepared from nitric acid and/or ammonium sulphate in the same N/P ratio and concentration of F/2 standard medium) was about 1/37:1/39 times lower than F/2 culture medium, respectively, as shown in Table 1.

Conclusion

From the present study we can concluded the following:

1. To reduce production cost and enhance quality and quantity of *N. oceanica*, CAGF may be used rather than F/2 medium

2. To increase lipid cell content of *N. oceanica,* CAGF of ammonium sulphate and phosphoric acid must be considered, depending on N/P concentrations and ratio.

3. Microalgae nutrient medium prepared from CAGF, have many advantages more than F/2 standard medium, like (1) easy to prepare, (2) reduce production cost, as well as depending on N/P concentrations and ratio, (3) increase dry weight, (4) increase cell density, and (5) enhance lipid percentage.

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