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Aquatic Science and Technology
ISSN 2168-9148
2022, Vol. 10, No. 1

Evaluate the Effect of Using Source of Nitrogen (Soaked Brown Lentils) on Chemical Composition for Marine Microalgae *Nannochlorpsis oceanica*

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Received: June 1, 2021 Accepted: July 21, 2021 Published: September 8, 2021

doi:10.52941/ast.v10i1.19 URL: https://doi.org/10.52941/ast.v10i1.19

Abstract

Microalgae culture media must be efficient, give high growth, meet micro-requirement, and be available. The effect of different levels of brown lentil infusion and use at [25, 50 and 75%] levels on the chemical composition (protein, carbohydrates, fatty acids, and amino acids) in *N. oceanic* was evaluated. Compared to the standard F/2 Guillard. The obtained results indicated that the chemical components of *N. oceanica* were affected by these levels. The highest protein and carbohydrate content and the highest EAA content (55.92%) were obtained using OB3 medium (75% SBL) compared to the control group (100% F/2). The highest biomass production was obtained in OB3 medium. The highest TSFA and USFA were recorded for *N. oceanica* by the OB3 mean.

The present study recommended that it is possible to use microalgae grown on OB3 and OB2 medium as a lipid and protein inducer in aquaculture.

Keywords: Amino acids, Fatty acids, *Nannochloropsis oceanica*, Proximate composition

1. Introduction

Microalgae are a large group of autotrophic eukaryotic organisms that play important roles in marine and freshwater ecosystems (Zhu y. Donford NT., 2013; Piggott *et al*., 2015). Microalgae with high growth rates in various cultural conditions, microalgae, exploit some chemical components in many fields, including biotechnology, food science, and aquaculture (Templeton

& Lewins, 2015). Microalgae are a source of many important elements in biomedicine and balanced nutrients but also in technology. In addition to their natural use in aquaculture, microalgae are used directly in feed for larvae and young aquaculture (Sarker *et al.*, 2016). Knowledge of other aspects is needed in order to increase aquaculture production to generate new, high-quality species of microalgae and use microalgae species as feed sources (Hemaiswarya *et al.*, 2011). Microalgae help improve the traditional nutritional value of foods and promote the growth and development of various food products (Tokuşoglu & nal, 2003).

The chemical composition of microalgae may vary with cultural conditions and age (Carvalho *et al.*, 2009). Diverse cultures influence the types of microalgae that have been studied for the purpose of understanding and knowing their potential for use in aquaculture (Grobbelaar, 2010). Among the various nutritional factors, nitrogen is one of the most important nutrients for growth, as it is a component of all structural and functional proteins such as peptides, enzymes, chlorophyll, energy transport molecules and genetic material in algal cells (Cai *et al.*, 2013). The concentration of nitrogen in the culture medium significantly affects both the cell growth rate and the biochemical compositions of microalgae (Wang *et al.*, 2013), and several studies have shown that when nitrogen is limited in the culture medium, the cell growth rate of microalgae slows down and increases their fat or carbohydrate content, which reduces protein synthesis (Ho *et al.*, 2014). Most microalgae are able to use different forms of nitrogen, including sources of nitrate, nitrite, ammonium, and organic nitrogen such as urea (Baker, 1994); Each nitrogen source is first converted to the ammonium form and assimilated into amino acids through a variety of pathways (Cai *et al.*, 2013).

Demand is increasing for algal lipids which can be produced by biosynthesis of active lipids using appropriate nutrients as well as by improved harvesting strategies that lead to cell/biomass recovery.

Various physical and chemical conditions such as temperature, stress, light intensity, culture time, organic carbon and inorganic nutrients including iron (Fe), phosphorous (P), nitrogen (N), manganese (Mn), zinc (Zn), sulfur (S), cobalt (Co), and others, influence the growth and lipid accumulation of many types of microalgae (Bajpai *et al.*, 2014).

At the level of industrial production of marine hatcheries, the improvement of the efficient culture medium for microalgae species for food culture is absolutely necessary. The nutrient media for microalgae should be quick to prepare, less expensive, produce high growth, and meet the quality and quantity of microalgae in terms of chemical composition. Although F / 2 Guillard medium is the most common method of culturing *Nanochloropsis* in marine hatcheries, the F / 2 medium has some drawbacks, such as difficulties in preparation and may in some cases be costly.

This study was designed to evaluate the effects of adding different levels of brown lentil infusion on the biochemical composition of *N. oceanica* and the rate of production of fatty acids and amino acids. Therefore, different media were prepared using different levels to culture *N. oceanica* to replace F / 2 medium to reduce production cost. However, the question is whether *N.*

oceanica cultured at different levels of brown lentil infusion could be effective.

2. Materials and Methods

2.1 Microalgal Strains

Nannochloropsis oceanica strain was from an algae unit of the marine hatchery at the kilo 21 Alexandria - Egypt.

N. oceanica were kept Institute of Oceanography and Fisheries (NIOF), Egypt and cultured under controlled conditions of temperature (22± 2C˚), salinity (35 ± 2 ppt).

F/2 medium (Guillard and Rhyter, 1962), with continuous ventilation and 8:16 h of light, was used in three replicates. Dry weight (CDW) and chemical composition of algal cells (10 days after culturing) were determined. And dry weight (CDW) was determined, according to (Abomohra, *et al*., 2013).

2.2 Experimental Design

The F/2 medium contained (mg. L^{-1}) $NaNO_3$, 75; $NaH_2PO_4.H_2O$, 5; Na_2 EDTA. H_2O, 4.16; $FeC1_3.6H_20$, 3.15; $CuSO_4.5H_20$, 0.01; $ZnSO_4.7H_2O$, 0.022; $COC1_2.6H_20$, 0.01; $MnC1_2.4H_2O$, 0.18; $Na_2MoO_4.2H_2O$, 0.006; Vitamin B12, 0.0005; Vitamin B1, 0.1; and Bi-tin, 0.0005 (Guillard & Rhyter, 1962).

2.3 Culture Conditions

Use 1.5 liter plastic containers filled with 1 liter sterile saline (35 ± 2) and 4gm

of brown lentils were soaked in 50ml of sterile water for 15 minutes. It was put on the stove for 5 minutes and left to cool. The solution was filtered with filter paper and used at levels as described in (Table 1).

2.4 Estimation of the Biochemical Constituents of N. oceanica

The total protein and carbohydrate content was estimated by (Lowry *et al*., 1951) using Bovine Serum Albumin (BSA) as a standard. (Dubois *et al*., 1956) and quantification of total carbohydrate “phenol-sulfuric acid" using d-glucose µg/ml as a standard.

$$\textbf{Biomass productivity}\ (\text{mg } L^{-1}\ \text{day}^{-1}) = (CDW_L - CDW_E) \times (t_L - t_E)^{-1}.$$

With CDW_E representing the CDW (mg L^{-1}) at the days of early exponential phase (t_E) and CDW_L at the days of late exponential phase (t_L). (Abomohra , *et al.*, 2016).

2.5 Total Lipid Content and Fatty Acids Profile

Total lipids and fatty acids were extracted as described by (Folch et al. 1957) and (Bligh & Dyer, 1959). The fatty acids were determined using methyl ester from total fat according to a procedure (Radwan, 1978).

All analyzes for fatty acid determination were performed on a GS-MS instrument, an HP (Hewlett Packard) 7890 GC equipped with a flame ionization detector. GC terms: Device model: HP (Hewlett Packard) 6890GC, Shaft: HP-INNOWax (polyethylene glycol), 60 m, ID 0.25 mm, film thickness of 0.2 µm. Detector: FID (Flame Ionization Detector). Detector temperature: 250°C. Injector temperature: 220°C, injection volume 3 µl, split ratio 50:1.

2.6 Amino Acids Determination

Amino acids of *N. oceanica* were analyzed by hydrolysis in 6N HCL for 22hrs at 110°C; after hydrolysis, the acid was evaporated in a vacuum oven. The residue of the algal sample was dissolved in 1 ml of sample dilution (diluting buffer) (0.2M, pH 2.2) to complete the sample dissolving. Automatic amino acid analyzer was used for amino acid determination (Dionex ICS3000) (Block, 1948).

2.7 Statistical Analysis

Statistical analysis was performed using analysis of the one way (ANOVA) was used to test the effects of urea on chemical composition of microalgae. Duncan.

One- way ANOVA was used to match the mean differences by the Statistical Package for the Social Sciences SPSS (2007). As such, the differences were small to be significant at $p<0.05$.

Table 1. Design of the experiment used to grow *Nannochloropsis oceanica*

	Control (CO)	OB1	OB2	OB3
F/2	100	---	---	--
F/2	---	0.75	0.50	0.25
Soaked Brown Lentils (SBL)	----	0.25	0.50	0.75

3. Results

Nannochloropsis oceanica was cultured at different concentrations as shown in Table (1) in the early stationary stage, from which samples were harvested for chemical composition analysis after the late stationary stage (10 days). Cell dry weight and chemical composition were examined. The presented results indicated that there was no significant difference in dry weight (CDW) between the media containing different levels of mixture and control.

Table (2) showed significant differences in the chemical composition of algae between the different levels. The highest percentages of protein and carbohydrates for dry weight (22.79% ± 0.03 and 21.82% ± 0.03, respectively) were shown by OB3 medium (75% SBL and 25% F/2) compared to control and other treatments. The highest total fat content (37.64% ± 0.03) was shown by the mean F/2 relative to the other treatments.

3.1 Biomass Productivity

The data obtained in Table (2) showed significant differences in the biomass productivity of *N. oceanica* species between the different treatments. The highest percentage of dry weight (121.47 ± 0.03 and 109.49 ± 0.02 (mg L-1 day-1, respectively) was obtained by medium OB3 (75% SBL and 25% F/2) and medium OB2 (50% SBL). and 50% F/2 when compared to the control group and other treatments.

Table 2. Chemical composition (percent based on dry weight) mg/g dry weight of *N. oceanica* at different levels of soaked brown lentil (SBL) medium harvested after 10 days of incubation**.**

Medium	CDW ($g\ L^{-1}$)	Protein (%CDW)	Carbohydrate (%CDW)	Lipid (%CDW)	Biomass productivity ($mg\ L^{-1}\ day^{-1}$)
CO	0.82 ± 00^{b}	19.46 ± 0.02^{d}	18.36 ± 0.02^{c}	37.64 ± 0.03^{a}	94.87 ± 0.02^{d}
OB1	0.82 ± 00^{bb}	19.76 ± 0.02^{c}	19.84 ± 0.02^{d}	36.72 ± 0.03^{b}	105.28 ± 0.02^{c}
OB2	0.83 ± 00^{a}	21.54 ± 0.02^{b}	20.63 ± 0.02^{b}	36.44 ± 0.02^{c}	109.49 ± 0.02^{b}
OB3	0.83 ± 00^{aa}	22.79 ± 0.03^{a}	21.82 ± 0.03^{a}	34.72±0.03d	121.47 ± 0.03^{a}

Data are statistically analyzed using ONE-WAY ANOVA. Significant result is obtained at P= 0.05

3.2 The Fatty Acids Analysis

The fatty acid profile of *N. oceanica* was presented in Table 3. The data showed that there was no change in the fatty acid profile between the different milieus. In contrast, there is a noticeable change in the content of each individual fatty acid between the different media. The most abundant saturated fatty acid was palmitic acid (C16:0), which scored the highest value (27.62%) in MF3 medium compared to other media. Palmitic acid is followed by myristic acid (C14:0).

In addition, oleic acid (C18:1) was the most prevalent monounsaturated fatty acid in all treatments, as it scored the highest values and was (25.46%) with OB3 medium, which means that the oleic acid content increased with the increase in lentil soaked medium. Palmitoleic acid (C16:1) also showed an increase of (24.21%) in mean OB2. Moreover, linoleic acid (C18:2) was the most common polyunsaturated fatty acid in all treatments, as the data showed that the highest value of this fatty acid (16.45%) was recorded using OB3 medium, and eicosapentaenoic acid (EPA) was the second is polyunsaturated fatty acids, which recorded the highest percentage (4.33%) in the OB3 medium. Similarly, docosahexaenoic acid (DHA) was the third polyunsaturated fatty acid that scored the highest value (12.83%) with the mean OB3.

The results revealed that the highest percentage of total saturated fatty acids TSFA (38.81%) was obtained by OB2 medium. The present study explained that the highest rate of the total unsaturated fatty acids USFA (68.61%) was detected by OB3 medium, where this percentage is mainly consisting of 34.79% MUFA and 33.82% PUFA. On the other hand, the highest ratio (0.66) between SFA/USFA was achieved by CO. In addition, the highest ratios between n-3/n-6 were 1.12% which exhibited by CO medium and DHA/EPA were 2.50 obtained by OB3 medium

(Table 3).

Table 3. Shows the fatty acid percentages (%) of *N. oceanica* at different levels of medium and the harvest was 10 days after incubation period Soaked brown lentil (SBL)

Fatty acid	CO	OB1	OB2	OB3
C14:0 (Myristic acid)	3.51±0.02^{d}	5.12±0.01^{c}	5.24±0.02^{a}	5.15±0.02^{b}
C15:0 (Pentadecylic acid)	0.54±0.02^{d}	0.82±0.01^{a}	0.79±0.02^{b}	0.76±0.01^{c}
C16:0 (Palmitic acid)	20.27±0.02^{d}	23.16±0.02^{c}	24.21±0.02^{b}	23.32±0.02^{a}
C17:0 (Margaric acid)	0.29±0.02^{d}	0.72±0.01^{a}	0.67±0.02^{c}	0.69±0.02^{b}
C18:0 (Stearic acid)	3.64±0.02^{d}	4.77±0.02^{b}	4.94±0.02^{a}	4.69±0.02^{c}
C21:0 (Heneicosanoic acid)	0.68±0.02^{c}	1.11±0.02^{a}	1.09±0.02^{b}	1.09±0.01^{bb}
C24:0 (Lignoceric acid)	1.41±0.02^{d}	1.45±0.02^{c}	1.87±0.02^{a}	1.58±0.02^{b}
∑Saturated (SFA)	**30.34**	**37.15**	**38.81**	**37.28**
C14:1 (Myristoleic acid)	0.12±0.02^{b}	0.14±0.01^{a}	0.12±0.02^{bb}	0.14±0.01^{aa}
C15:1 (cis-10-pentadecenoic acid)	0.07±0.01^{a}	0.06±0.02^{b}	0.06±0.02^{bb}	0.06±0.01^{bc}
C16:1 (Palitoleic acid)	4.37±0.02^{d}	5.82±0.02^{c}	5.91±0.02^{b}	6.08±0.02^{a}
C17:1 (cis-10-Heptadecenoic acid)	0.46±0.01^{a}	0.37±0.02^{c}	0.39±0.02^{b}	0.33±0.01^{d}
C20:1 (Paullinic acid)	2.15±0.02^{a}	2.11±0.02^{b}	1.91±0.02^{d}	2.07±0.01^{c}
C18:1n9 (Oleic acid)	14.12±0.02^{d}	25.13±0.02^{c}	25.61±0.02^{a}	25.46±0.02^{b}
C22:1 (Erucic acid methyl)	0.53±0.02^{d}	0.71±0.02^{a}	0.68±0.02^{b}	0.65±0.02^{c}
∑Monosaturated (MUFA)	**21.82**	**34.34**	**34.68**	**34.79**
C18:2n6 (Linoleic acid)	10.34±0.02^{b}	16.32±0.02^{c}	16.27±0.02^{d}	16.45±0.02^{a}
C18:3n6 (y-Linoleic acid)	0.19±0.02^{d}	0.21±0.02^{c}	0.24±0.01^{a}	0.22±0.01^{b}
C18:3n3 (α- Linolenic acid)	1.32±0.02^{c}	1.27±0.02^{d}	1.46±0.01^{a}	1.36±0.01^{b}
C20:2n6 (Eicosadienoic acid)	0.75±0.02^{a}	0.59±0.02^{c}	0.57±0.01^{d}	0.63±0.01^{b}
C20:5n-3 (Ecosapentaenoic acid)	3.67±0.01^{d}	4.26±0.02^{b}	4.16±0.02^{c}	4.33±0.02^{a}
C22:6n-3 (Docosahexaenoic acid)	7.64±0.02^{d}	9.81±0.02^{c}	9.91±0.02^{b}	10.83±0.02^{a}
∑Polyunsaturated (PUFA)	**23.91**	**32.46**	**32.61**	**33.82**
∑Usaturated	**45.73**	**66.80**	**67.29**	**68.61**
SFA/MSFA	**1.39**	**1.08**	**1.12**	**1.07**
SFA/PSFA	**1.27**	**1.15**	**1.90**	**1.10**
SFA/USFA	**0.66**	**0.56**	**0.58**	**0.54**
n 3∑	**12.63**	**15.34**	**15.53**	**16.52**
∑n6	**11.28**	**17.12**	**17.08**	**17.30**
∑n3/n6	**1.12**	**0.90**	**0.91**	**0.96**
DHA/EPA	**2.08**	**2.30**	**2.38**	**2.50**

3.3 Amino Acids Analysis

The amino acid profiles of different culture media were presented for *N.oceanica* species. There was a clear discrepancy in the content of each individual amino acid between the different media. The results showed that *N. oceanica* recorded the highest percentage of EAA essential amino acids (55.92%) by OB3 medium, while the lowest value was recorded by CO medium (100% F/2). The results showed that the top four EAAs in OB3 medium were lysine (5.81%) phenylalanine (5.93%), histidine (2.61%) and isoleucine (4.03%) (Table 4). And the opposite for non-essential amino acids (NEAA), where the highest percentage of NEAA (50.91%) was detected by CO medium (100% F/2), while the lowest value for NEAA was achieved by OB3 medium. . The three most abundant NEAAs in F/2 medium were aspartate (10.13%), glutamine (11.25%), serine (6.31%) and proline .(%6.31)

Table 4. Amino acids profile (%) in *N. oceanica* at different levels of **Soaked Brown Lentils (SBL)** medium harvested after 10 days incubation period.

	Medium			
Amino acid (AA)%	**CO**	**OB1**	**OB2**	OB3
	Essential amino acids (*EAA*)			
Arginine	5.52±0.02^{c}	5.91±0.02^{b}	5.94±0.02^{a}	5.41±0.02^{d}
Histidine (HIS)	1.84±0.02^{d}	1.95±0.02^{c}	2.23±0.02^{b}	2.61±0.02^{a}
Isoleucine (ILE)	3.74±0.02^{d}	3.83±0.02^{c}	3.91±0.02^{b}	4.03±0.02^{a}
Leucine (LEU)	9.16±0.02^{d}	9.18±0.02^{c}	9.41±0.02^{b}	9.73±0.02^{a}
Lysine (LYS)	4.33±0.02^{d}	4.52±0.02^{c}	4.71±0.02^{b}	5.81±0.01^{a}
Methionine (MET)	4.42±0.01^{d}	4.63±0.02^{c}	4.67±0.02^{b}	4.83±0.02^{a}
Phenylalanine (PHE)	6.51±0.02^{a}	5.03±0.02^{d}	5.87±0.02^{c}	5.93±0.02^{b}
Threonine (THR)	5.64±0.02^{d}	5.89±0.02^{c}	6.47±0.02^{b}	6.91±0.02^{a}
Tryptophan (TRP)	1.96±0.02^{d}	2.45±0.02^{c}	2.75±0.02^{a}	2.73±0.02^{b}
Valine (VAL)	5.97±0.02^{d}	6.27±0.02^{c}	7.37±0.02^{b}	7.93±0.02^{a}
Total EAA	**49.09**	**49.66**	**53.33**	**55.92**
	Non-essential amino acids (*NEAA*)			
Alanine (ALA)	6.66±0.02^{d}	7.63±0.02^{c}	7.66±0.02^{b}	7.81±0.02^{a}
Aspartate (ASP)	10.13±0.02^{a}	8.13±0.02^{b}	7.67±0.02^{c}	6.14±0.02^{d}
Cystine (C-C)	4.45±0.02^{b}	4.63±0.02^{a}	2.55±0.02^{d}	3.81±0.02^{c}
Glutamine (GLU)	11.25±0.02^{b}	10.90±0.02^{c}	10.22±0.02^{d}	11.39±0.02^{a}
Glycine (GLY)	4.35±0.02^{d}	4.51±0.02^{c}	5.66±0.02^{a}	4.71±0.02^{b}
Proline (PRO)	5.25±0.02^{c}	5.64±0.01^{b}	6.43±0.02^{a}	5.21±0.02^{d}
Serine (SER)	6.31±0.02^{b}	6.51±0.02^{a}	4.91±0.02^{c}	3.43±0.02^{d}
Tyrosine (TYR)	2.51±0.02^{a}	2.39±0.02^{b}	1.57±0.02^{d}	1.58±0.02^{c}
Total NEAA	**50.91**	**50.34**	**46.67**	**44.08**

4. Discussions

Improving culture conditions is necessary to raise the efficiency and economic value of microalgae production in the future. New methods of efficient production and farming can be created to improve productivity and reduce costs. For more than 50 years, Guillard F/2 intermediate in marine aquaculture has been known for the cultivation of microalgae, currently, due to the various uses of microalgae in various fields of biotechnology; The F/2 Guillard broker has several drawbacks. Our results achieved that some levels of brown lentil soak achieved significant biochemical components compared to F/2 (control).

The present study showed that addition of medium (SBL) could improve the content of protein, carbohydrate, PUFA, EAA and biomass in *N.oceanica* algae. In protein it was higher than the results (Abugrara, *et al.*, 2019), when used as a nitrogen source in the development of *N.oceanica*, due to the presence of urea, and closer to the results (Ashour, *et al.*, 2019) who used (F/2 at 100%) on the algae *N.oceanica* and higher than (Ashour M. and Abd El-Wahab K., 2017) when using 50-100% nitrogen and phosphorous on the same algae.

As for fat, it was higher than the results of (Ashour *et al.*, 2019 when using an average F/2, and higher than (Ashour M. and Abd El-Wahab K., 2017) when using 50-50% nitrogen and phosphorous, and higher than (Ashour M. and Abd El-Wahab K., 2017 (Zhang, *et al.*, 2016) that used different levels of nitrogen, and was lower than what was achieved (Abugrara, *et al.*, 2020) when using different levels of bicarbonate. Sodium in *N. oculata* was higher than (Ashour, *et al.*, 2019) using an average F/2 on the same algae and lower than (Ashour M. and Abd El-Wahab K., 2017) when N-P was used at a ratio of 50-50. % and less than (Chun W. *et al.*, 2012) when using an average F/2 on the same algae.

Biomass productivity was higher than (Ashour, *et al.*, 2019) using F/2 medium on the same algae, and higher than (Mata, *et al.*, 2010) on *N. oceanica* alga.

The lipid yield was higher than the results (Ashour *et al.*, 2019) when Medium F/2 was used on the same algae. The results were similar to (Aarón Millán *et al.*, 2015) that used nitrate and CO_2 on *N. oculata* and higher than the results (Chun Wan, *et al.*, 2013) on the same alga using different nitrogen sources. This is what was found (Mata *et al.*, 2010) in *N.oculata*.

It showed an increase in polyunsaturated fatty acids, and PUFA scored higher in OB3 than found (Abugrara, *et al.*, 2020), where sodium bicarbonate was used on the same algae, and in DHA was also lower at all levels, and higher than (Ashour, *et al.*, 2019).), who used Medium F/2 on the same algae, and the results (Madhusree *et al.*, 2016) were higher on the same algae where wastewater was used at different levels with some types of media, and were higher than the results of (Jean HB & Sung Bum H ., 2011) when Medium F/2 was used on *Nannochloropsis spp.* , *Nannochloropsis sp* . As well as the highest proportion of essential amino acids, EAA at OB3 level was higher than (Abugrara, *et al.*, 2020), NEAA was lower at this level, and it was higher than (Jean HB & Sung Bum Hu., 2011).

5. Conclusion

In summary, this research showed that it is possible to enhance the growth of biomass. The results indicated that the use of soaked brown lentils is a beneficial source of nitrogenous protein, and the use of 75-25% as a culture medium for *N.oceanica* had a significant impact on the production of chemical compounds. Including protein, carbohydrates, polyunsaturated fatty acids and essential amino acids (especially arginine and leucine) for their high nutritional value and essential for use in aquaculture feed.

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