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Influence of culture medium on growth and protein production by *Haematococcus pluvialis*

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ABSTRACT. Proteins from microalgal biomass have become promising raw materials in several industrial segments. To evaluate growth performance and protein production under different nutritional conditions, *Haematococcus pluvialis* was grown in different culture media: BBM, RM, BG-11, and KM2. The cultures were inoculated at 10^5 cells mL^{-1}, and submitted to a temperature of 24°C, in a continous photoperiod and irradiance of 40 µmol photons m^{-2} s^{-1}. The highest cell density was observed when *H. pluvialis* was maintained in BG-11 medium (142 ± 30 × 10^4 cells mL^{-1}), but no statistical difference was observed when comparing the results with those obtained when culturing this microalga in BBM (101 ± 14 × 10^4 cells mL^{-1}) and RM (105 ± 5 × 10^4 cells mL^{-1}) media. Also, the lowest cell density was found when cultivating *H. pluvialis* in KM2 medium (57 ± 9 × 10^4 cells mL^{-1}), and there was no statistical difference for doubling time, growth rate and specific growth rate results between treatments. In addition, higher protein contents in *H. pluvialis* were reported for RM, BG-11, and KM2 culture media at 55.1 ± 5.6, 49.3 ± 3.6 and 58.4 ± 2.8%, respectively; and lower protein content was found using the BBM medium (31.1 ± 2.9%). The highest cell density and biomass were achieved at greater nitrogen availability and a higher nitrogen to phosphorus ratio. The results suggest that *H. pluvialis* is a potential species for protein production, and that BG-11 is the most suitable medium for growing this microalga as it allowed the achievement of highest biomass production and protein content among the media evaluated.

Keywords: biomass; mixotrophic; nutrients; photoautotrophic.

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Introduction

Microalgae are photosynthetic organisms present in humid, freshwater, estuarine, and marine environments that assimilate nutrients and can be used to produce biomass rich in carbohydrates, lipids and proteins - which often have high biological value (Batista, Gouveia, Bandarra, Franco, & Raymundo, 2013). These microorganisms offer a wide range of applications, processes and products, such as: wastewater treatment (Oliveira et al., 2020), human nutrition (Sathasivam, Radhakrishnan, Hashem, & Abd_Allah, 2019), production of pharmaceuticals (Dantas et al., 2019), feed for aquatic organisms (Abreu et al., 2019), pigment production (Tramontin, Kildegaard, Sudarsan, & Borodina, 2019) and biofuels production (Rizman, Mujtaba, Memom, Lee, & Rashid, 2018).

Among several microalgae studied, *Haematococcus pluvialis* (Chlamydomonadales, Chlorophyta) is a freshwater microalga species with a complex life cycle, comprised of a mobile, a sessile and an encysted form (aplanospore – Zhao et al., 2015; Reinecke, Castillo-Flores, Boussiba, & Zarka, 2018). *H. pluvialis* is worldwide known for its high levels of astaxanthin, a carotenoid with strong antioxidant activity that is produced by this microalga when under stress (Machado Jr. et al., 2016). Also, when this microalga is in its vegetative form, it is capable of producing a high protein content. According to Grewe and Griehl (2012) and Shah, Liang, Cheng, and Daroch (2016), *H. pluvialis* can reach a protein content between 29 and 45% (w w^{-1}) in its cells. Proteins derived from microalgae are commonly used in human food and animal feed (Becker, 2007; Koyande et al., 2019), or as a high-value food additive, such as bioactive peptides, which have nutraceutical importance (Soto-Sierra, Stoykova, & Nikolov, 2018). However, functional aspects related to *H. pluvialis* proteins have still not been widely studied (Ba, Ursu, Laroche, & Djelveh, 2016).

The concentration of biochemical compounds such as proteins in microalgae is influenced by several physical and chemical factors, such as culture medium, light and temperature (Demirel, Yilmaz, Ozdemir, & Dalay, 2018). The culture media differ in terms of sources and concentrations of nutrients, mainly nitrogen (N) and phosphorus (P), which are important to metabolism and cell growth (Kim, Park, Cho, & Hwang, 2013; Cuellar-Bermudez et al., 2017). Ammonia, nitrite and nitrate are the most common nitrogen sources reported in culture media and have different effects on the absorption rate, cell growth and biochemical composition of microalgae - in all these forms, nitrogen is linked to synthesis of amino acids and lipids (Ahmad & Hellebust, 1984). Phosphorus is converted mainly to nucleic acids by storing chemical energy (mainly in the form of carbohydrates and adenosine triphosphate) and actively participates in mechanisms of protein regulation (via phosphorylation – Fábregas, Domínguez, Regueiro, Maseda, & Otero, 2000; Tocquin, Fratamico, & Franck, 2012; Beuckels, SmoLders, & Muylaert, 2015). In addition, the culture media can be differentiated by the presence of organic carbon, used for cultivation in heterotrophic or mixotrophic nutritional modes (Kim et al., 2013).

Therefore, this study aimed to determine the influence of different culture media on growth performance and protein production by the microalga *H. pluvialis*.

Material and methods

Growth conditions and experimental design

H. pluvialis was obtained from the Laboratório de Produção de Alimento Vivo at the Universidade Federal Rural de Pernambuco and experimental cultivations were conducted using the following culture media: Bold's Basal Medium - BBM (Bold, 1949), BG-11 (Stanier, Kunisawa, Mandel, & Cohen-Bazire, 1971), Rudic Medium - RM (Rudic & Dudnícenco, 2000) and KM2 Medium (Tripathi, Sarada, Rao, & Ravishankar, 1999) with three independent replicates. Stocking solutions used in the culture media were prepared using distilled water and autoclaved at 121°C, 1 atm for 20 min., and the culture media compositions are shown in Table 1. Sodium nitrate ($NaNO_3$) was the main N source in the BBM, BG-11 and RM culture media, while in KM2, L-asparagine was the main source. Dipotassium phosphate (K_2HPO_4) and monopotassium phosphate (KH_2PO_4) were the P sources in the BBM, BG-11 and RM media, while KM2 medium had no phosphorus source. Furthermore, only the KM2 medium contained an organic carbon source - sodium acetate.

Table 1. Nutrient concentrations (mmoL L^{-1}) in the culture media (BBM, RM, BG-11 and KM2) used in *Haematococcus pluvialis* experimental cultivation.

Compound	BBM	RM	BG-11	KM2
$NaNO_3$	29.414	35.296	176.482	-
$C_4H_8N_2O_3$	-	-	-	30.650
KH_2PO_4	12.860	0.1470	-	-
K_2HPO_4	0.4305	0.4592	0.2296	-
CH_3COONa	-	-	-	242.090
$CaCl_2$. 2 H_2O	0.1700	0.3979	0.2449	0.1360
$MgSO_4$.7 H_2O	0.3043	0.0041	0.3043	-
NaCl	0.4278	0.3422	-	-
KOH	0.5525	-	-	-
EDTA	0.1711	0.0257	0.0034	-
$FeSO_4$. 7 H_2O	0.0180	-	-	0.0360
H_3BO_3	0.1844	0.0049	0.0463	0.0010[a]
$ZnSO_4$. 7 H_2O	0.0049	0.0003	0.0008	0.0001[a]
$NaMoO_4$. 2 H_2O	0.0008	-	0.0016	0.00005[a]
$MnCl_2$. 4 H_2O	0.0010	10.106	0.0091	0.001[a]
$CuSO_4$. 5 H_2O	0.0008	0.0003	0.0003	0.0001[a]
$C_6H_8O_7$	-	-	0.0312	-
$C_6H_{11}FeNO_7$	-	-	0.0226	-
Na_2CO_3	-	-	0.1887	-
$MnSO_4$. H2O	-	0.0099	-	-
$(NH_4)_6Mo_7O_{24}$. $4H_2O$	-	0.0002	-	-
$Co(NO_3)_2$. $6H_2O$	0.0004	0.0014	0.0003	0.00008[a]
NH_4VO_3	-	-	-	0.00001[a]
$FeCl_3$. $6H_2O$	-	0.0629	-	-

[a]Trace element concentration according to Renstrøm, Borch, Skulberg, and Liaaen-Jensen (1981).

Concentrations of N and P, and the respective N:P ratio, are given in Table 2. The highest N concentration was found in the BG-11 (17.7 mmol L^{-1}) medium, followed by KM2 (6.1 mmol L^{-1}), RM (3.4 mmol L^{-1}) and BBM (2.8 mmol L^{-1}). The highest P concentration was found in the BBM medium (1.7 mmol L^{-1}), while RM (0.6 mmol L^{-1}) and BG-11 (0.2 mmol L^{-1}) media had lower concentrations.

Experimental cultures were carried out in 2 L Erlenmeyer glass bottles with filtered (0.2 µm) and autoclaved (121°C, 1 atm for 20 min.) freshwater; all culture media were enriched with B-complex vitamins: thiamine (B1), pyridoxine (B6) and cyanocobalamin, at 0.2 mL L^{-1}. *H. pluvialis* cells were inoculated at the initial concentration of 10^5 cells mL^{-1}. The cultures were maintained at a temperature of approximately 24°C, with constant aeration, under an irradiance of 40 µmol photons m^{-2} s^{-1}, in a continuous photoperiod, and were kept until the beggining of the stationary phase of the growth curve.

Table 2. Nitrogen and phosphorus contents (mmol L^{-1}) of the culture media (BBM, RM, BG-11 and KM2) used in *Haematococcus pluvialis* cultivation.

Nutrients	BBM	RM	BG-11	KM2
Nitrogen	2.8	3.4	17.7	6.1
Phosphorus	1.7	0.6	0.2	0
N:P ratio	1.7:1	5.7:1	77.3:1	0

Analysis

To evaluate growth in the vegetative phase, 2 mL samples were taken daily and immediately fixed in formaldehyde (2%) for quantification using a hemocytometer and a binocular optical microscope (OLYMPUS CH30). With this data, the following parameters were calculated: maximum cell density (MCD); growth rate (K, Equation 1), which represents the number of cell divisions per day performed during the total culturing time, expressed as 'division d^{-1}'; specific growth rate (µ, Equation 2), which represents the cell growth rate during the exponential phase of the growth curve as a function of time, expressed as 'd $^{-1}$'; and doubling time, which corresponds to the time required for doubling the initial density, expressed as 'd division^{-1}' (DT, Equation 3 – Stein, 1973). At the end of the cultivation period, growth curves (cell density x cultivation time in days) for *H. pluvialis* were plotted for each culture medium.

$$K\ (\text{division}\ d^{-1}) = \frac{3.322}{(t\text{-}t0) \times \text{Log}\ (D/D0)} \quad (1)$$

where:
t = last incubation day (days);
t_0 = beginning incubation day (days);
D = final cell density (cells mL^{-1});
D_0 = beginning cell density (cells mL^{-1}).

$$\mu\ (d^{-1}) = \frac{\ln\ (N(t)\text{-}N0)}{(t\text{-}t0)} \quad (2)$$

where:
N(t)= number of cells at the end of the exponential phase (cells mL^{-1});
N_0 = number of cells at the beginning of the exponential phase (cells mL^{-1});
t = end of exponential phase (days);
t_0 = beginning of exponential phase (days).

$$DT\ (\text{d division}^{-1}) = \frac{1}{K} \quad (3)$$

where:
K= growth rate; DT= doubling time.

Biomass harvesting and drying

At the end of the cultivation period, the biomass produced was harvested by centrifugation at 3500 × g for 10 min. and then washed three times with distilled water to remove residues from the culture medium (Santana et al., 2017), followed by new centrifugations at the same conditions. Samples were frozen for 24 h at -80°C (SANYO MDF U33V) and then freeze-dried (ALPHA 1-4 LD PLUS) for 48 hours. Finally, the biomass was weighed in an analytical balance (0.001 g) to determine the biomass (g L^{-1}) and biomass yield (g L^{-1} d^{-1}).

Protein analysis

From the dry biomass, the total protein content (TP, %) was determined according to the micro-Kjeldahl method (Association of Official Agricultural Chemists [AOAC], 2012), Equation 4. The nitrogen conversion factor (NCF) used was 6.25, according to Safi et al. (2013) for *H. pluvialis*.

$$\% \text{ TP} = \frac{\text{VA} \times \text{VB} \times 0.14 \times \text{NCF}}{\text{dry biomass (mg)}} \text{ x } 6.25 \quad (4)$$

where:
VA = volume of standardized hydrochloric acid (0.1N) used for sample titration;
VB = volume of standardized hydrochloric acid used in control sample titration;
NCF = correction factor (0.97) of hydrochloric acid solution.

Protein and productivity

The protein production (Equation 5) was calculated using the biomass (g L^{-1}) and the total protein content (TP, %):

Protein production (g L^{-1}) = dry biomass x TP (5)

Protein production rate (PPR, g L^{-1} d^{-1}) was calculated using Equation (6), which uses the values of dry biomass (g), total protein (TP, %), and the total days of cultivation for each treatment:

$$\text{Protein production rate (g L}^{-1}\text{ d}^{-1}\text{)} = \frac{\text{dry biomass x TP}}{\text{Volume x cultivation time}} \quad (6)$$

Statistical analysis

All data are presented as mean ± standard deviation (n = 3). Normality and homogeneity were analyzed using Shapiro-Wilk and Cochran's tests, respectively. Then, the data were submitted to a one-way analysis of variance (ANOVA) to verify the statistical differences for the variables of growth and protein production among the treatments, followed by a Tukey's test ($p < 0.05$) when necessary. Data referring to growth variables (MCD, DT, μ and K) were transformed from log (x+1). For non-normal data, the Kruskal-Wallis test was applied. All analyses were performed using the R software version 3.4 (R Core Team, 2018; Gross & Ligges, 2015; Wickham, 2022). The logistic growth curve was performed using Curve Expert 1.4 software (Hyams, 2010).

Results and discussion

Growth parameters

The largest MCD was found in BG-11 (142.13 ± 29.52 × 10^4 cells mL^{-1}), but there was no statistical difference in this medium when compared with the results obtained in BBM (101.42 ± 14.68 × 10^4 cells mL^{-1}) and RM (104.83 ± 5.34 × 10^4 cells mL^{-1}). On the other hand, there was a significant statistical difference between the culture media regarding KM2 (56.67 ± 9.09 × 10^4 cells mL^{-1}), which had the lowest MCD, as indicated in Table 3. Also, there was no statistical difference regarding TD, K and μ results between treatments. Significant difference ($p < 0.05$) was observed for biomass at the end of the cultivations, and was higher with the BG-11 medium (0.639 ± 0.07 g L^{-1}). As regards to biomass yield, no significant difference ($p > 0.05$) was reported between the culture media, and it ranged from 0.04 ± 0.01 to 0.048 ± 0.01 g L^{-1} d^{-1}.

Table 3. Growth parameters of the microalga *Haematococcus pluvialis* cultivated in different culture media.

Parameter	Culture medium			
	BBM	RM	BG-11	KM2
MCD (× 10^4 cells mL^{-1})	101.42±14.68[a]	104.83±5.34[a]	142.13±29.52[a]	56.67±9.09[b]
K* (division d^{-1})	0.30±0.02	0.31±0.01	0.27±0.02	0.31±0.03
DT (d $division^{-1}$)	3.31±0.20	3.25±0.07	3.68±0.29	3.24±0.33
M* (d $^{-1}$)	0.44±0.01	0.45±0.01	0.36±0.02	0.46±0.03
dMCD	11	11	14	8
Biomass (g L^{-1})	0.437±0.08[b]	0.455±0.03[b]	0.639±0.07[a]	0.382±0.06[b]
Biomass yield (g L^{-1} d^{-1})	0.04±0.01	0.041±0.00	0.046±0.00	0.048±0.01

Data presented as mean ± standard deviation (*n* = 3). Different letters in the same line indicate significant differences among treatments using Tukey's Test ($p < 0.05$). *Non-normal data evaluated with the Kruskal-Wallis Test. Maximum cell density (MCD); Growth rate (K); Doubling time (DT); Specific growth rate (μ); day of maximum cell density (dMCD).

In the cultivation of microalgae there are factors that are crucial for the proper growth of the species. The adequacy of environmental factors such as light intensity, pH and temperature, as well as the selection of a culture medium with an adequate balance of nutrients, enhance biomass production and yield in the extraction of bioproducts (Imamoglu, Sukan, & Dalay, 2007; Zhuang et al., 2018). In the production of *H. pluvialis*, and other microalgae species, efficient cultivation procedures during the vegetative phase are extremely important for the efficient production of biomass (Shah et al., 2016).

Analyzing the difference between autotrophic and mixotrophic media in the growth of *H. pluvialis*, our study reported a higher cell density in a medium without an organic carbon source (BG-11, BBM and RM), while lower cell density was observed in a mixotrophic medium (KM2). Cell density results in our study were higher than those reported for *H. pluvialis* by Tripathi et al. (1999). These authors have observed higher cell density in the KM2 medium (42×10^4 cells mL^{-1}) than in other culture media (BBM, Z8, KM1, MM1 and MM2), but they used a low irradiance (20.25 µmoL photons m^2 s^{-1}) when compared with this work (40 µmol photons m^{-2} s^{-1}). The influence of irradiance on growth parameters was examined by Sipaúba-Tavares, Berchielli-Morais, and Scardoeli-Truzzi (2015) when culturing *H. pluvialis* using fertilizer NPK and WC with the addition of a macrophyte (*Eichhornia crassipes*) extract. These authors reported an increase in K-value by increasing irradiance from 20 µmol photons m^2 s^{-1} (0.16 division d^{-1}) to 60 µmoL photons m^2 s^{-1} (0.22 division d^{-1}). Regarding the biomass produced, our results were similar to those reported by Dalay, Imamoglu, and Demirel (2007), which reached 0.64 g L^{-1} also using the BG-11 media but with higher irradiance (75 µmol photons m^{-2} s^{-1}) in 12 days of culturing. These results indicate that an irradiance of 40 µmol photons m^{-2} s^{-1} would be sufficient to reach approximately 0.6 g L^{-1} of *H. pluvialis* biomass. Fábregas et al. (2000) evaluating the effect of different irradiance (8.6, 40, 78 and 177 µmol photons m^{-2} s^{-1}) have reported the highest cell density for *H. pluvialis* using 40 µmol photons m^{-2} s^{-1}.

Colusse, Duarte, Carvalho, and Noseda (2019), using BG-11, WC and CHU, obtained 31.3±1.52 x 10^4 cells mL^{-1}, K-value of 0.3 divisions d^{-1}, DT of 3.28, biomass of 0.45±0.04 g L^{-1}, biomass yield of 0.022 g L^{-1} d^{-1}, and final pH of 8.85±0.14, in 22 days of cultivation, under a regime of 12:12 photoperiod at 100 µmol m^{-2} s^{-1} in experimental units of 1.6 L using BG-11. As observed in our study, good results were obtained when growing this microalga in BG-11 medium. On the other hand, Imamoglu et al. (2007), also cultivating *H. pluvialis*, obtained the highest cell concentration in RM culture medium (9.50 x 10^5 cells mL^{-1} in 40 µmol photons m^{-2} s^{-1}) after 10 days of cultivation at 25°C with a pH below 8 by adding CO_2 (1.5% v v^{-1}).

According to Pereira and Otero (2020), temperature significantly affects the growth of *H. pluvialis* in its green phase. Cultures maintained in OHM medium and carried out in 1 L cylindrical photobioreactors, under an irradiance of 100 µmol photons m^{-2} s^{-1}, 12:12 photoperiod (light/dark), with constant aeration, and kept with a pH between 7.0 and 7.5, achieved better results between 20 to 25°C, with approximately 1 division d^{-1}, presenting problems with cell motility when kept at a higher temperature of 30°C. Similarly, Fan, Vonshak, and Boussiba (1994), when cultivating *H. pluvialis* in modified BG-11 in 500 mL flasks, under 130 µmol photons m^{-2} s^{-1} irradiance, and in different temperatures (20, 22, 25, 28, 31 and 33°C), observed an increase in the specific growth rate in cultivation between temperatures from 20 to 28°C, with better results between 25 to 28°C (µ = 0.053-0.054 $hour^{-1}$ and DT 12-13 hour), which was confirmed by the evaluation of cell number and chlorophyll concentration. Still, they also noted that lower temperatures of 20 and 22°C appear to reduce protein and chlorophyll synthesis.

Through the analysis of growth curves, similar growth for *H. pluvialis* were found in the BBM and RM media, with a short lag phase of growth in the first three days of cultivation; in these media, the stationary growth phase was reached after 11 days. In the KM2 medium, *H. pluvialis* had a different growth curve, reaching the stationary growth phase in8 days. The slowest growth was observed in BG-11, which had the highest exponential phase compared to other culture media, reaching the stationary phase after 14 days of cultivation (Figure 1).

Minasyan (2018) also observed that cultivation of *H. pluvialis* in the BG-11 medium achieved greater density with a more gradual growth rate. Nahidian, Ghanati, Shahbazi, and Soltani (2018), evaluating the development of the four cellular stages of *H. pluvialis* during 35 days of cultivation, also observed a longer growth time in a BG-11 than BBM medium. The different responses in cell density and biomass production might be linked to the nutritional composition of the culture medium, which can influence the development of these organisms (Demirel et al., 2018).

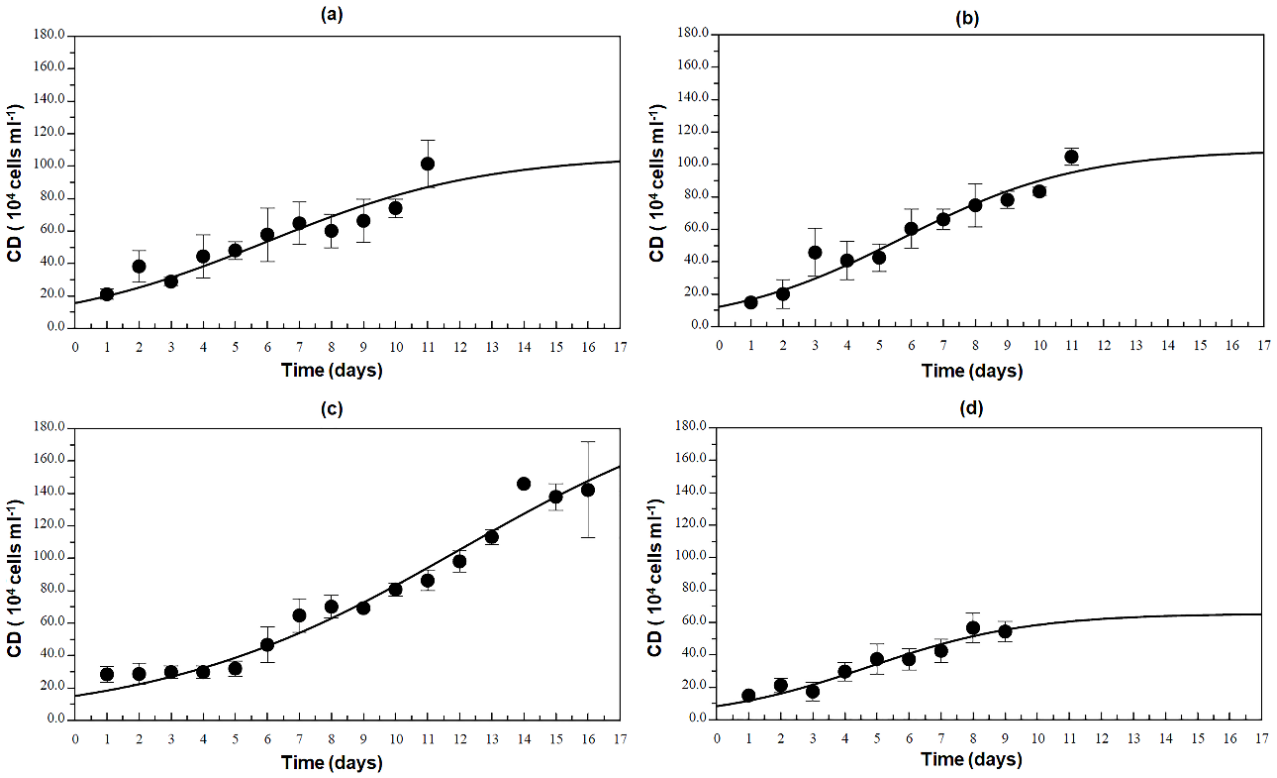

Figure 1. Logistic growth curve of *Haematococcus pluvialis* grown in different culture media (a) BBM, (b) RM, (c) BG-11 and (d) KM2. Bars represent standard deviations (n = 3).

Proteins

Total protein content (%), protein production (g L^{-1}) and protein production rate (g L^{-1} d^{-1}) in *H. pluvialis* cultured under different culture media are shown in Table 4, and it is possible to notice that the culture medium, in fact, influenced the protein content of *H. pluvialis*. A significant difference ($p < 0.05$) in protein content was observed only for BBM (31.11 ± 2.93%), which had the lowest percentage, and similar values were noticed in the other media: RM (55.08 ± 5.6%), KM2 (58.37 ± 2.8%) and BG-11 (49.33 ± 3.64%). As the cultivation time was different for the different culture media, protein production rate also varied significantly between them: the KM2 medium resulted in a production of (0.028 ± 0.00 g L^{-1} d^{-1}), which was similar ($p > 0.05$) to RM (0.023 ± 0.00 g L^{-1} d^{-1}); while the BG-11 (0.02 ± 0.00 g L^{-1} d^{-1}) and BBM (0.012 ± 0.00 g L^{-1} d^{-1}), had lower protein production rate. Higher protein production was found using the BG-11 (0.31 g L^{-1}) and RM (0.25 g L^{-1}) media; the KM2 medium (0.22 g L^{-1}) was similar ($p > 0.05$) to RM, but lower ($p < 0.05$) than BG-11. Still, the lowest protein production was obtained in the BBM (0.13 g L^{-1}).

The supply of proteins obtained from microalgae biomass can be used as feed for aquatic organisms and also as an alternative source of food for humans. Studies have already shown that microalgae can contain similar quality and levels of protein compared to traditional food sources, besides presenting bioactive properties. These properties, in fact, may present important biological activities, such as: antitumor, immunomodulatory, antihypertensive and others (Batista et al., 2013; Koyande et al., 2019). It is worth noting that for a higher protein yield, the careful selection of the culture medium is essential (Baroni, Yap, Webley, Scales, & Martin, 2019; Colusse et al., 2019), as each one has its own formulation, presenting variations in nutrient availability and concentration.

Table 4. Protein production parameters of the microalga *Haematococcus pluvialis* cultured under different culture media (BBM, RM, BG-11 and KM2).

Parameter	Culture medium			
	BBM	RM	BG-11	KM2
TP (%)	31.11±2.93^{b}	55.08±5.6^{a}	49.33±3.64^{a}	58.37±2.8^{a}
Protein production (g L^{-1})	0.13±0.02^{c}	0.25±0.03^{ab}	0.31±0.03^{a}	0.22±0.03^{b}
PPR (g L^{-1} d^{-1})	0.012±0.00^{c}	0.023±0.00^{ab}	0.022±0.00^{bc}	0.028±0.00^{a}

Data presented as mean ± standard deviation (n = 3). Different letters in the same line indicate significant difference among treatments. TP: Total protein content (%). PPR: Protein Production Rate.

In this regard, Marinho et al. (2021), developing the experimental cultivation of *H. pluvialis* with a focus on astaxanthin production, evaluated the influence of culture media (BBM, MM2, KM1, Provasoli and modified Provasoli) on the biochemical composition of this alga. They observed differences in the concentration of proteins in the biomass between the initial phase (green phase) and final phase (red phase) of the experimental period, between the culture media, with a reduction of 25 to 61% of this content. The lowest protein concentration was observed in KM1, a heterotrophic media (26.2±0.58%), and the highest concentration, unlike what was obtained in our study, was reached in BBM, a photoautotrophic (62.7±3.38%) media. Sipaúba-Tavares et al. (2015), using NPK fertilizer and WC (with the addition of the macrophyte *Eichhornia crassipes* extract), obtained a variation from 30 to 48% of protein, as a function of dry biomass of *H. pluvialis*, and the highest levels were positively associated with N content.

Influence of concentration and nitrogen and phosphorus ratio on growth and protein content

N and P concentrations, as well as N:P ratio (Table 2), were related to growth parameters and protein production in *H. pluvialis* (Figure 2 and 3). The different N:P from the culture media presented significant difference ($p < 0.05$), and it is possible to state that the highest N:P ratio resulted in the highest MCD and protein production (Figure 3). Also, the highest production of biomass and protein (g L^{-1}) was reported in the culture media with the highest N content. As for P content, the lowest cell density was observed in KM2, where this nutrient was absent (Table 3 and 4).

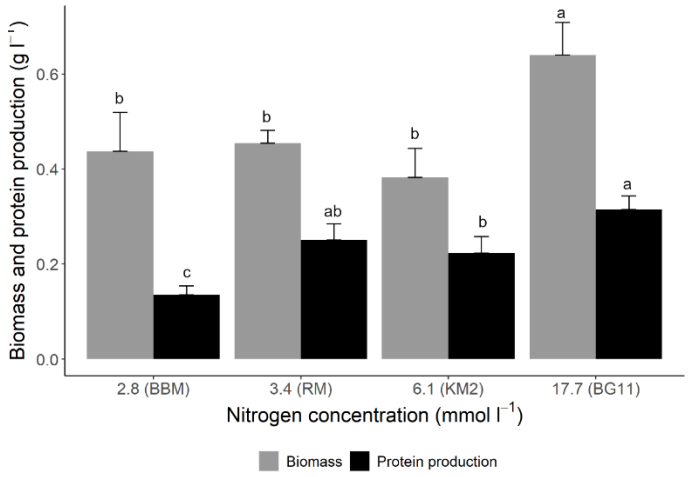

Figure 2. Biomass and protein production (g L^{-1}) of *Haematococcus pluvialis* at increasing nitrogen concentrations in the different culture media (BBM, RM, BG-11, and KM2). Bars represent standard deviations ($n = 3$) and different letters represent significant differences between the treatments.

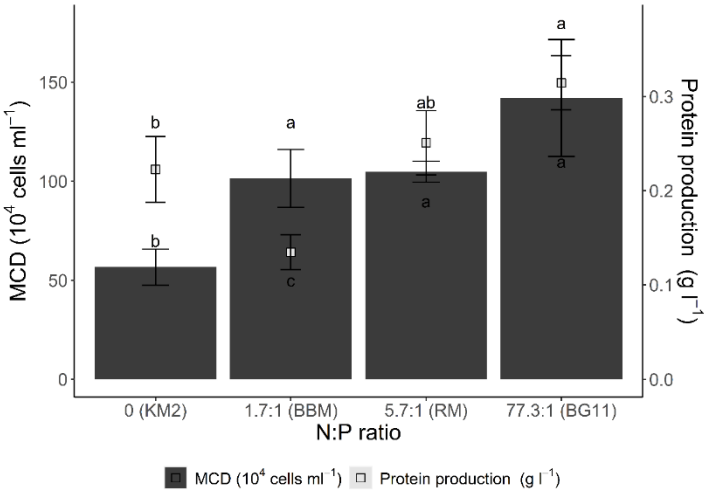

Figure 3. Maximum cell density (MCD) and protein production (g L^{-1}) of *Haematococcus pluvialis* at increasing N:P ratios in the different culture media (BBM, RM, BG-11 and KM2). Bars represent standard deviations ($n = 3$) and different letters represent significant differences between the treatments.

According to Huang, Lou, and Wang (2021), different concentrations of N and P on the BG-11 culture medium can influence the cultivation of *Chlorella vulgaris*. They have reported that N has high influence in the metabolic pathway of algae, in the production of biomass, specific growth rate and in the content of carbohydrates and proteins. Also, the authors have stated that the total protein content in *C. vulgaris* biomass was higher when the N supply increased from 1 to 2 mg L^{-1} and from 3 to 8 mg L^{-1}, and they verified a decrease in carbohydrates content in opposition to the increase of total protein under higher nitrogen concentration in the medium. This can be justified because when under N-replete conditions, the biosynthesis of reserve compounds (such as lipids and carbohydrates) is reduced (Ross et al., 2018). Regarding P, this nutrient is responsible for energy storage and transfer, and is required for the synthesis of cell structures (such as nucleotides and phospholipid membranes) (Tocquin et al., 2012). Therefore, the absence of P in the KM2 culture medium may explain the lowest MCD observed in this culture medium.

A positive relationship between N concentration and protein content has also been verified in a number of studies. According to Ranadheer, Kona, Sreeharsha, and Mohan (2019), the protein content and biomass concentration of *Scenedesmus* sp. was proportional to the nitrate concentration. In addition, Colusse et al. (2019) observed the variation of protein content in *H. pluvialis* when using WC (a culture medium poor in N and P), CHU (a culture medium rich in N) and BG-11culture media, with values of 11, 34, and 41% of dry weight, respectively. The higher protein production in our study was also obtained in BG-11 media, because this media, besides having the highest N concentration, may have stimulated an over-compensation. More specifically, this mechanism occurs when the microalgae are starved of an element and then re-exposed to it, like the inoculum that was in the BBM media (low nitrogen concentration). Thus, nitrogen is absorbed in excess and stored on the protein form (Xie, Xia, Zeng, Li, & Zhang, 2017).

On the other hand, insufficient N can inhibit cell division (Fábregas et al., 2000) or increase the synthesis of reserve compounds (such as lipids and carbohydrates) thereby reducing the protein content (Beuckels et al., 2015). This is probably why the BG-11 medium had both the highest MCD and biomass, because of its higher nitrogen concentration. Nahidian et al. (2018), evaluated different concentrations of N and P in BBM for *H. pluvialis*, and found higher growth rate to be directly proportional to the concentration of these nutrients. The N-depletion can also influence the cell morphology and growth of *Nannochloropsis salina*, *Chlorella* sp., and *H. pluvialis*, which have shown morphological changes and reduced cell multiplication when under this condition (Baroni et al., 2019).

In addition to adequate concentrations of N and P, the source, whether organic or inorganic, can also affect the growth and biochemical composition of microalgae (Shanthi, Premalatha, & Anantharaman, 2018). In our study, only the KM2 medium had an organic nitrogen source (L-asparagine), which may have influenced the lowest growth performances and protein production, since nitrate is the N-form that provides greater cell growth (Sarada, Tripathi, & Ravishankar, 2002). A few organic N compounds can be used by some microalgae but these are considered poor sources and their assimilation is strongly dependent on a supply of acetate as a carbon source (Fernández, Llamas, & Gálvan, 2009). On the other hand, the carbon source apparently does not influence protein yield in *H. pluvialis*. As demonstrated by Pang, Fu, Fernandez, and Chen (2019), the addition of D-ribose, sodium acetate or sodium gluconate in a BG-11 medium, under mixotrophic nutritional mode, did not affect protein yield.

Regarding the micronutrients concentration, Fábregas et al. (2000) demonstrated that oligoelements play different roles in cell growth. Zinc, boron, iodine and vanadium were considered non-essential, and higher cell density was obtained in the absence of these elements. Lower variations in concentrations of copper and selenium were not significant, but the growth was higher when the concentrations of magnesium, calcium, chromium, arsenic and manganeseincreased. Micronutrients play roles as cofactors of the cell metabolic pathways and in the regulation and induction of the production of metabolites, such as proteins (Procházková, Brányiková, Zachleder, & Brányik, 2014). The magnesium, in particular, is required by some enzymes such as ATPase, protein kinase and RNA polymerases for their functions (Shaul, 2002; Daneshvar, Santhhosh, Antikainen, & Bhatnagar, 2018). Thus, the absence of magnesium in the KM2 medium may have limited the growth of *H. pluvialis* since this medium corresponded to the lowest cell density reported in the present study. Furthermore, the protein content may vary with iron availability, since the assimilation of inorganic N, such as nitrate, depends on this metal (Raven & Giordano, 2016). In addition, it has been shown that iron in the form of Fe-citrate is better absorbed by *H. pluvialis* (Cai, Li, & Qi, 2009). This element influences the greater accumulation of dry biomass in the green stage and the subsequent accumulation of astaxanthin and lipids in the red stage of this microalga (Zhu et al., 2021). In the present study, BG-11 media was the only media with Fe (NH_4) citrate levels, which have consequently resulted in the highest production of protein and biomass.

Conclusion

To conclude, this study found higher biomass production and protein content using a medium with a higher nitrogen concentration, fact that suggests that the BG-11 culture medium is the most suitable for greater production of biomass and protein from *H. pluvialis*. Still, based on the results obtained, future researches are recommended to further investigate this microalga's bioactive and functional properties.

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References

Abreu, J. L., Brito, L. O., Lima, P. C. M., Silva, S. M. B. C., Severi, W., & Gálvez, A. O. (2019). Effects of addition of *Navicula* sp. (diatom) in different densities to postlarvae of shrimp *Litopenaeus vannamei* reared in a BFT system: growth, survival, productivity and fatty acid profile. *Aquaculture Research, 50*(8), 2231-2239. DOI: https://doi.org/10.1111/are.14104

Ahmad, I., & Hellebust, J. A. (1984). Nitrogen metabolism of the marine microalga *Chlorella autotrophica*. *Plant physiology, 76*(3), 658-663. DOI: https://doi.org/10.1104/pp.76.3.658

Association of Official Agricultural Chemists [AOAC]. (2012). *Official methods of analysis* (19th ed.). Gaithersburg, MD: AOAC.

Ba, F., Ursu, A. V., Laroche, C., & Djelveh, G. (2016). *Haematococcus pluvialis* soluble proteins: Extraction, characterization, concentration/fractionation and emulsifying properties. *Bioresource Technology, 200*, 147-152. DOI: https://doi.org/10.1016/j.biortech.2015.10.012

Baroni, É. G., Yap, K. Y., Webley, P. A., Scales, P. J., & Martin, G. J. O. (2019). The effect of nitrogen depletion on the cell size, shape, density and gravitational settling of *Nannochloropsis salina*, *Chlorella* sp. (marine) and *Haematococcus pluvialis*. *Algal Research, 39*, 101454. DOI: https://doi.org/10.1016/j.algal.2019.101454

Batista, A. P., Gouveia, L., Bandarra, N. M., Franco, J. M., & Raymundo, A. (2013). Comparison of microalgal biomass profiles as novel functional ingredient for food products. *Algal Research, 2*(2), 164-173. DOI: https://doi.org/10.1016/j.algal.2013.01.004

Becker, E. W. (2007). Micro-algae as a source of protein. *Biotechnology Advances, 25*(2), 207-210. DOI: https://doi.org/10.1016/j.biotechadv.2006.11.002

Beuckels, A., Smolders, E., & Muylaert, K. (2015). Nitrogen availability influences phosphorus removal in microalgae-based wastewater treatment. *Water Research, 77*, 98-106. DOI: https://doi.org/10.1016/j.watres.2015.03.018

Bold, H. C. (1949). The morphology of Chlamydomonas chlamydogama, Sp. Nov. *Bulletin of the Torrey Botanical Club, 76*(2), 101-108. DOI: https://doi.org/10.2307/2482218

Cai, M., Li, Z., & Qi, A. (2009). Effects of iron electrovalence and species on growth and astaxanthin production of *Haematococcus pluvialis*. *Chinese Journal of Oceanology and Limnology, 27*(2), 370. DOI: https://doi.org/10.1007/s00343-009-9176-1

Colusse, G. A., Duarte, M. E. R., Carvalho, J. C., & Noseda, M. D. (2019). Media effects on laboratory scale production costs of *Haematococcus pluvialis* biomass. *Bioresource Technology, 7*, 100236. DOI: https://doi.org/10.1016/j.biteb.2019.100236

Cuellar-Bermudez, S. P., Aleman-Nava, G. S., Chandra, R., Garcia-Perez, J. S., Contreras-Ângulo, J. R., Markou, G., ... Parra-Saldivar, R. (2017). Nutrients utilization and contaminants removal. A review of two approaches of algae and cyanobacteria in wastewater. *Algal Research*, *24*(Part B), 438-449. DOI: https://doi.org/10.1016/j.algal.2016.08.018

Dalay, M. C., Imamoglu, E., & Demirel, Z. (2007). Agricultural fertilizers as economical alternative for cultivation of *Haematococcus pluvialis*. *Journal of Microbiology and Biotechnology, 17*(3), 393-397.

Daneshvar, E., Santhosh, C., Antikainen, E., & Bhatnagar, A. (2018). Microalgal growth and nitrate removal efficiency in different cultivation conditions: effect of macro and micronutrients and salinity. *Journal of Environmental Chemical Engineering, 6*(2), 1848-1854. DOI: https://doi.org/10.1016/j.jece.2018.02.033

Dantas, D. M. M., Oliveira, C. Y. B., Costa, R. M. P. B., Carneiro-da-Cunha, M. G., Gálvez, A. O., & Bezerra, R. S. (2019). Evaluation of antioxidant and antibacterial capacity of green microalgae *Scenedesmus subspicatus*. *Food Science and Technology International, 25*(4), 318-326. DOI: https://doi.org/10.1177/1082013218825024

Demirel, Z., Yilmaz, F. F., Ozdemir, G., & Dalay, M. C. (2018). Influence of media and temperature on the growth and the biological activities of *Desmodesmus protuberans* (F.E. Fritsch & M.F. Rich) E. Hegewald. *Turkish Journal of Fisheries and Aquatic Sciences, 18*, 1195-1203. DOI: https://doi.org/10.4194/1303-2712-v18_10_06

Fábregas, J., Domínguez, A., Regueiro, M., Maseda, A., & Otero, A. (2000). Optimization of culture medium for the continuous cultivation of the microalga *Haematococcus pluvialis*. *Applied Microbiology and Biotechnology, 53*(5), 530-535. DOI: https://doi.org/10.1007/s002530051652

Fan, L., Vonshak, A., & Boussiba, S. (1994). Effect of temperature and irradiance on growth of *Haematococcus pluvialis* (Chlorophyceae). *Journal of Phycology, 30*(5), 829-833. DOI: https://doi.org/10.1111/j.0022-3646.1994.00829.x

Fernández, E., Llamas, Á., & Gálvan, A. (2009). Nitrogen assimilation and its regulation. In E. H. Harris, D. B. Stern, & G. B. Witman (Eds.)*, The chlamydomonas sourcebook* (2nd ed., p. 69-113). Cambridge, GB: Academic Press.

Grewe, C. B., & Griehl, C. (2012). The carotenoid astaxanthin from *Haematococcus pluvialis*. In C. Posten, & C. Walter (Eds.)*, Microalgal biotechnology: integration and economy* (p. 129-144). Berlim, DE: De Gruyter.

Gross, J., & Ligges, U. (2015). *Nortest: tests for normality*. Retrieved from https://CRAN.R-project.org/package=nortest

Huang, Y., Lou, L., & Wang, X. C. (2021). Insight into nitrogen and phosphorus coupling effects on mixotrophic *Chlorella vulgaris* growth under stably controlled nutrient conditions. *Science of the Total Environment, 752*, 141747. DOI: https://doi.org/10.1016/j.scitotenv.2020.141747

Hyams, D. G. (2010). *CurveExpert basic*. Retrieved from http://www.curveexpert.net

Imamoglu, E., Sukan, F. V., & Dalay, M. C. (2007). Effect of different culture media and light intensities on growth of *Haematococcus pluvialis*. *International Journal of Natural and Engineering Sciences, 1*(3), 05-09.

Kim, S., Park, J.-e., Cho, Y.-B., & Hwang, S.-J. (2013). Growth rate, organic carbon and nutrient removal rates of *Chlorella sorokiniana* in autotrophic, heterotrophic and mixotrophic conditions. *Bioresource Technology, 144*, 8-13. DOI: https://doi.org/10.1016/j.biortech.2013.06.068

Koyande, A. K., Chew, K. W., Rambabu, K., Tao, Y., Chu, D.-T., & Show, P.-L. (2019). Microalgae: a potential alternative to health supplementation for humans. *Food Science and Human Wellness, 8*(1), 16-24. DOI: https://doi.org/10.1016/j.fshw.2019.03.001

Machado Jr., F. R. S., Trevisol, T. C., Boschetto, D. L., Burkert, J. F. M., Ferreira, S. R. S., Oliveira, J. V., & Burkert, C. A. V. (2016). Technological process for cell disruption, extraction and encapsulation of astaxanthin from *Haematococcus pluvialis*. *Journal of Biotechnology, 218*, 108-114. DOI: https://doi.org/10.1016/j.jbiotec.2015.12.004

Marinho, Y. F., Malafaia, C. B., de Araújo, K. S., Silva, T. D., Santos, A. P. F., Moraes, L. B., & Gálvez, A. O. (2021). Evaluation of the influence of different culture media on growth, life cycle, biochemical composition, and astaxanthin production in *Haematococcus pluvialis*. *Aquaculture International, 29*(2), 757-778. DOI: https://doi.org/10.1007/s10499-021-00655-z

Minasyan, A. (2018). Stimulated growth of green algae *Haematococcus pluvialis* under different environmental conditions. *Annals of Valahia University of Targoviste Geographical Series, 18*(1), 53-61. DOI: https://doi.org/10.2478/avutgs-2018-0006

Nahidian, B., Ghanati, F., Shahbazi, M., & Soltani, N. (2018). Effect of nutrients on the growth and physiological features of newly isolated *Haematococcus pluvialis* TMU1. *Bioresource Technology, 255*, 229-237. DOI: https://doi.org/10.1016/j.biortech.2018.01.130

Oliveira, C. Y. B., Abreu, J. L., Oliveira, C. D. L., Lima, P. C., Gálvez, A. O., & Dantas, D. M. M. (2020). Growth of *Chlorella vulgaris* using wastewater from Nile tilapia (*Oreochromis niloticus*) farming in a low-salinity biofloc system. *Acta Scientiarum. Technology, 42*(1), e46232. DOI: https://doi.org/10.4025/actascitechnol.v42i1.46232

Pang, N., Fu, X., Fernandez, J. S. M., & Chen, S. (2019). Multilevel heuristic LED regime for stimulating Lipid and bioproducts biosynthesis in *Haematococcus pluvialis* under mixotrophic conditions. *Bioresource Technology, 288*, 121525. DOI: https://doi.org/10.1016/j.biortech.2019.121525

Pereira, S., & Otero, A. (2020). *Haematococcus pluvialis* bioprocess optimization: Effect of light quality, temperature and irradiance on growth, pigment content and photosynthetic response. *Algal Research, 51*, 102027. DOI: https://doi.org/10.1016/j.algal.2020.102027

Procházková, G., Brányiková, I., Zachleder, V., & Brányik, T. (2014). Effect of nutrient supply status on biomass composition of eukaryotic green microalgae. *Journal of Applied Phycology, 26*, 1359-1377. DOI: https://doi.org/10.1007/s10811-013-0154-9

R Core Team. (2018). *R: A language and environment for statistical computing*. Vienna, AT: R Foundation for Statistical Computing.

Ranadheer, P., Kona, R., Sreeharsha, V., & Mohan, S. V. (2019). Non-lethal nitrate supplementation enhances photosystem II efficiency in mixotrophic microalgae towards the synthesis of proteins and lipids. *Bioresource Technology, 283*, 373-377. DOI: https://doi.org/10.1016/j.biortech.2019.03.089

Raven, J. A., & Giordano, M. (2016). Combined nitrogen. In M. Borowitzka, J. Beardall, & J. Raven (Eds.)*, The physiology of microalgae. Developments in applied phycology* (p. 143-154). Cham, CH: Springer.

Reinecke, D. L., Castillo-Flores, A., Boussiba, S., & Zarka, A. (2018). Polyploid polynuclear consecutive cell-cycle enables large genome-size in *Haematococcus pluvialis*. *Algal Research, 33*, 456-461. DOI: https://doi.org/10.1016/j.algal.2018.06.013

Renstrøm, B., Borch, G., Skulberg, O. M., & Liaaen-Jensen, S. (1981). Optical purity of (3S,3'S)-astaxanthin from *Haematococcus pluvialis*. *Phytochemistry, 20*(11), 2561-2564. DOI: https://doi.org/10.1016/0031-9422(81)83094-4

Rizwan, M., Mujtaba, G., Memom, S. A., Lee, K., & Rashid, N. (2018). Exploring the potential of microalgae for new biotechnology applications and beyond: a review Exploring the potential of microalgae for new biotechnology applications and beyond: a review. *Renewable and Sustainable Energy Review, 92*, 394-404. DOI: https://doi.org/10.1016/j.rser.2018.04.034

Ross, M. E., Davis, K., McColl, R., Stanley, M. S., Day, J. G., & Semião, A. J. C. (2018). Nitrogen uptake by the macro-algae *Cladophora coelothrix* and *Cladophora parriaudii*: Influence on growth, nitrogen preference and biochemical composition. *Algal Research, 30*, 1-10. DOI: https://doi.org/10.1016/j.algal.2017.12.005

Rudic, V., & Dudnícenco, T. (2000). Process for cultivation of green alga *Haematococcus pluvialis* (Flotow). MD Pat ent Nr. A 2000 0154.

Safi, C., Charton, M., Pignolet, O., Silvestre, F., Vaca-Garcia, C., & Pontalier, P.-Y. (2013). Influence of microalgae cell wall characteristics on protein extractability and determination of nitrogen-to-protein conversion factors. *Journal of Applied Phycology, 25*, 523-529. DOI: https://doi.org/10.1007/s10811-012-9886-1

Santana, H., Cereijo, C. R., Teles, V. C., Nascimento, R. C., Fernandes, M. S., Brunale, P., ... Brasil, B. S. A. F. (2017). Microalgae cultivation in sugarcane vinasse: Selection, growth and biochemical characterization. *Bioresource Technology, 228*, 133-140. DOI: https://doi.org/10.1016/j.biortech.2016.12.075

Sarada, R., Tripathi, U., & Ravishankar, G. A. (2002). Influence of stress on astaxanthin production in *Haematococcus pluvialis* grown under different culture conditions. *Process Biochemistry, 37*(6), 623-627. DOI: https://doi.org/10.1016/S0032-9592(01)00246-1

Sathasivam, R., Radhakrishnan, R., Hashem, A., & Abd_Allah, E. F. (2019). Microalgae metabolites: A rich source for food and medicine. *Saudi Journal of Biological Sciences, 26*(4), 709-722. DOI: https://doi.org/10.1016/j.sjbs.2017.11.003

Shah, M. M. R., Liang, Y., Cheng, J. J., & Daroch, M. (2016). Astaxanthin-producing green microalga *Haematococcus pluvialis*: from single cell to high value commercial products. *Frontiers in Plant Science, 7*, 531. DOI: https://doi.org/10.3389/fpls.2016.00531

Shanthi, G., Premalatha, M., & Anantharaman, N. (2018). Effects of L-amino acids as organic nitrogen source on the growth rate, biochemical composition and polyphenol content of *Spirulina platensis*. *Algal Research, 35*, 471-478. DOI: https://doi.org/10.1016/j.algal.2018.09.014

Shaul, O. (2002). Magnesium transport and function in plants: the tip of the iceberg. *Biometals, 15*, 307-321. DOI: https://doi.org/10.1023/A:1016091118585

Sipaúba-Tavares, L. H., Berchielli-Morais, F. A., & Scardoeli-Truzzi, B. (2015). Growth of *Haematococcus pluvialis* Flotow in alternative media. *Brazilian Journal of Biology, 75*(4), 796-803. DOI: https://doi.org/10.1590/1519-6984.23013

Soto-Sierra, L., Stoykova, P., & Nikolov, Z. L. (2018). Extraction and fractionation of microalgae-based protein products. *Algal Research, 36*, 175-192. DOI: https://doi.org/10.1016/j.algal.2018.10.023

Stanier, R. Y., Kunisawa, R., Mandel, M., & Cohen-Bazire, G. (1971). Purification and properties of unicellular blue-green algae (Order *Chroococcales*). *Bacteriological Reviews, 35*(2), 171-205. DOI: https://doi.org/10.1128/br.35.2.171-205.1971

Stein, J. R. (1973). *Handbook of phycological methods: culture methods and growth measurements*. Cambridge, GB: Cambridge University Press.

Tocquin, P., Fratamico, A., & Franck, F. (2012). Screening for a low-cost *Haematococcus pluvialis* medium reveals an unexpected impact of a low N/P ratio on vegetative growth. *Journal of Applied Phycology, 24*, 365-373. DOI: https://doi.org/10.1007/s10811-011-9771-3

Tramontin, L. R. R., Kildegaard, K. R., Sudarsan, S., & Borodina, I. (2019). Enhancement of astaxanthin biosynthesis in oleaginous yeast *Yarrowia lipolytica* via microalgal pathway. *Microorganisms, 7*(10), 472. DOI: https://doi.org/10.3390/microorganisms7100472

Tripathi, U., Sarada, R., Rao, S. R., & Ravishankar, G. A. (1999). Production of astaxanthin in *Haematococcus pluvialis* cultured in various media. *Bioresource Technology, 68*(2), 197-199. DOI: https://doi.org/10.1016/S0960-8524(98)00143-6

Wickham, H. (2022). *Readxl: read excel files*. Retrieved from https://CRAN.R

Xie, T., Xia, Y., Zeng, Y., Li, X., & Zhang, Y. (2017). Nitrate concentration-shift cultivation to enhance protein content of heterotrophic microalga *Chlorella vulgaris*: over-compensation strategy. *Bioresource Technology, 233*, 247-255. DOI: https://doi.org/10.1016/j.biortech.2017.02.099

Zhao, Y., Shang, M., Xu, J.-W., Zhao, P., Li, T., & Yu, X. (2015). Enhanced astaxanthin production from a novel strain of *Haematococcus pluvialis* using fulvic acid. *Process Biochemistry, 50*(12), 2072-2077. DOI: https://doi.org/10.1016/j.procbio.2015.09.004

Zhu, Y., Zhang, Z., Xu, X., Cheng, J., Chen, S., Tian, J., ... Crocker, M. (2021). Simultaneous promotion of photosynthesis and astaxanthin accumulation during two stages of *Haematococcus pluvialis* with ammonium ferric citrate. *Science of the Total Environment, 750*, 141689. DOI: https://doi.org/10.1016/j.scitotenv.2020.141689

Zhuang, L.-L., Yu, D., Zhang, J., Liu, F.-f., Wu, Y., Zhang, T.-Y., ... Hu, H.-Y. (2018). The characteristics and influencing factors of the attached microalgae cultivation: a review. *Renewable and Sustainable Energy Reviews, 94*, 1110-1119. DOI: https://doi.org/10.1016/j.rser.2018.06.006