The influence of different *media* and light intensity for the production of *Haematococcus pluvialis* in the green phase: Biomass and biochemical assessment envisioning a good start for the red phase

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1. Introduction

This work is integrated into the PHYTOBLUEFRAC project, which is promoted by the company Phytoalgae, Lda in partnership with the University of Madeira, with EEA Grants funding (PT-INNOVATION-0059). The aim of the project is the study of microalgae for the development of new food supplements, derived from the lipid fraction, rich in carotenoids, fatty acids, and sterols, with high market value and high nutraceutical capacity. Thus, the growing conditions of *Haematococcus pluvialis*, a green eukaryotic microalga, which when subjected to stress produces astaxanthin, a powerful antioxidant, permitted in the EU as a food colourant and as a nutraceutical product with a high market value. The growth conditions were modulated, growth mediums (BBM and RM) and light (1000 - 7000 Lux) to determine the optimal cultivation conditions in the green phase to increase biomass. Also several biochemical parameters were assed to understand the fluctuation of these when the microalgae is subjected to different conditions. The best conditions will be replicated in low cost photobioreactors, to boost productivity. After selecting the optimal conditions for cultivation, assessment of the optimal methodology for an efficient fractionation, using a supercritical CO_2 extractor, extracting carotenoids, fatty acids and sterols, leaving a final residue that its use is intended as a biofertilizer for sustainable agriculture.

2. Aims

- Assess the biomass production and biochemical characterization of *Haematococcus pluvialis* using 2 growth media (BBM and RM) and 5 light intensities (1000, 2500, 4000, 5500 and 7000 Lux);
- Optimize growth conditions for the *H. pluvialis* at the green phase;

3. Methods

Experimental design

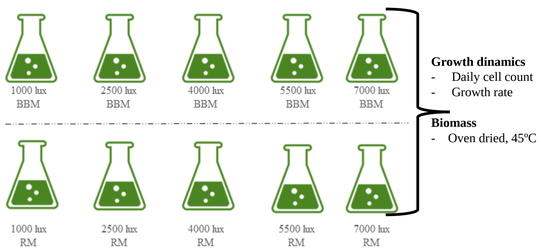

Figure 1 – Experimental design developed to optimize *Haematococcus pluvialis* cultivation parameters in the green phase. Bold's Basal Medium (BBM) and RM with variable exposure to light (1000 - 7000 lux).

➢ Total carotenoids and chlorophyll

$$\text{Chlorophyll } a\,(\mu g.mL^{-1}) = 12.21A_{663} - 2.81A_{646} \qquad \text{Chlorophyll } b\,(\mu g.mL^{-1}) = 20.13A_{646} - 5.03A_{663}$$

$$\text{Carotenoids } (\mu g.mL^{-1}) = \frac{(1000A_{470} - 3.27\text{Chl } a - 104\text{Chl } b)}{198}$$

➢ Protein

Extraction

- Maceration of milled samples with aluminum oxide
- Add NaOH 0,5M
- Ultrasounds 10min 80ºC
- Vortex
- Centrifuge 6000xg 10min

Analysis (Lowry method, 1951)

- 100µL sample + 100µL SDS
- + 1mL Reagent C
- Vortex
- 100µL Folin Ciocalteu reagent (1:1)
- 30 min in dark
- Read at 750nm

➢ Lipids

Bligh & Dyer (1959)

- 50 mg sample + 2mL MeOH + 1mL $CHCl_3$
- 400µL saturated NaCl
- 2 mL $CHCl_3$ + 2 mL H_2O
- Ultrasounds 40 min
- Centrifuge 2935*xg* 10min
- Recover the bottom phase
- Filter
- Dry at 35ºC
- Weight

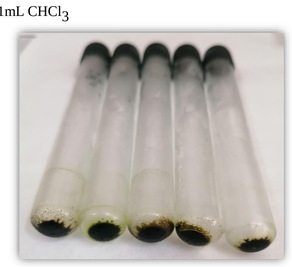

Figure 7 – Lipids extracted from the microalgae biomass.

➢ Total minerals

Figure 2 – Muffle furnace.

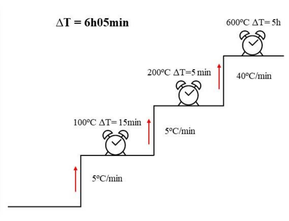

Figure 3 – Incineration ramp.

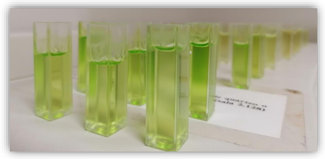

Figure 4 – **Acetone 80% extract** for chlorophyll *a*, *b* and total carotenoids assessement.

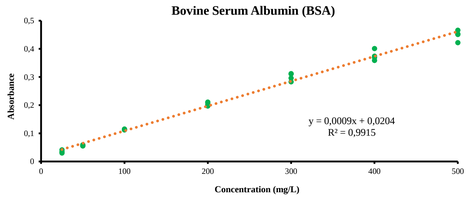

Figure 5 – Lowry calibration curve using BSA as a standard.

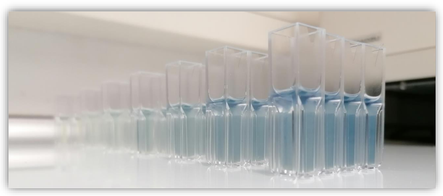

Figure 6 – Samples to read at 750nm using Lowry methodology.

➢ Medium remaining Nitrogen

- 2 mL of sample + 48 mL HCl
- Filter 0.22µm
- Quartz cuvettes
- Read 220 (NO_3^-) and 275nm (interference)

$$NO_3^- = Abs_{220} - (2 \times Abs_{275})$$

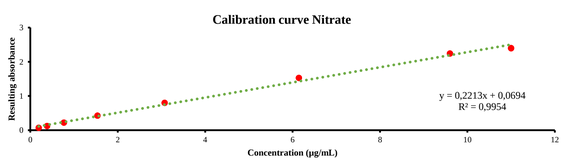

Figure 8 – Calibration curve to quantify the dissolved nitrate in the remaining medium.

4. Results

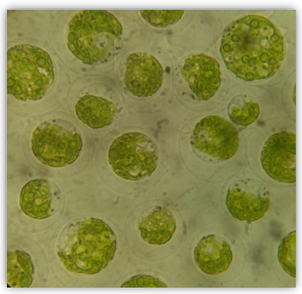

Figure 9 – *Haematococcus pluvialis* green phase – Total magnification 1000x.

Figure 10 – *Haematococcus pluvialis* red phase culture.

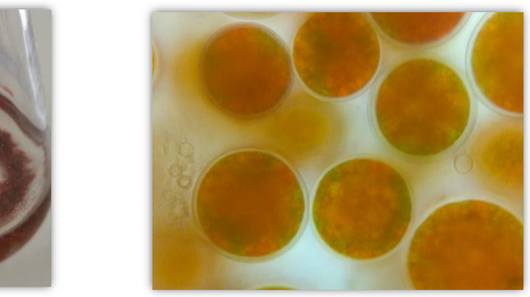

Figure 11 – *Haematococcus pluvialis* red phase – Total magnification 1000x.

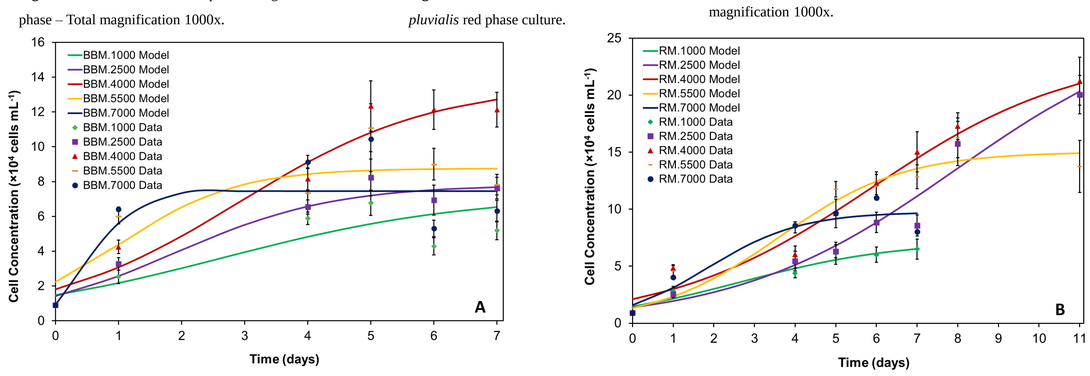

Figure 12 – Comparison of growth data and statistical models of *Haematococcus pluvialis* microalgae cultivated in different conditions. A) BMM with different light conditions; B) RM medium with different light conditions.

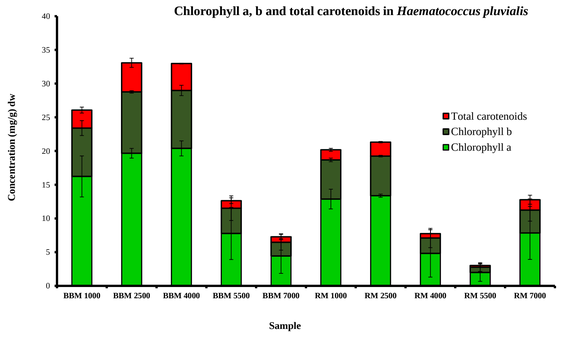

Figure 13 – Assessement of chlorophyll *a*, *b* and total carotenoids in *Haematococcus pluvialis*, 80% acetone extracts in mg/g.

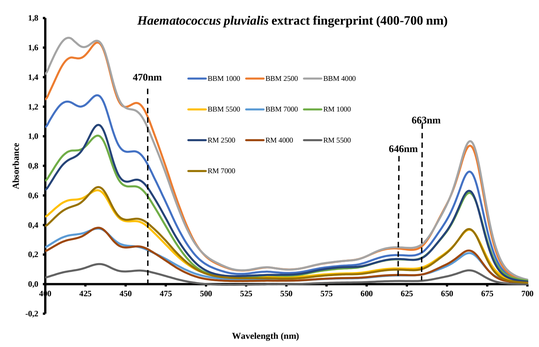

Figure 14 – Spectrophotometer assessment (400-700 nm) of *Haematococcus pluvialis*, 80% acetone extract to produce a fingerprint of the extract absorbance. There are 3 markers set at 470, 646 and 663 nm, the selected wavelengths for chlorophyll *a*, *b* and total carotenoids concentration calculations.

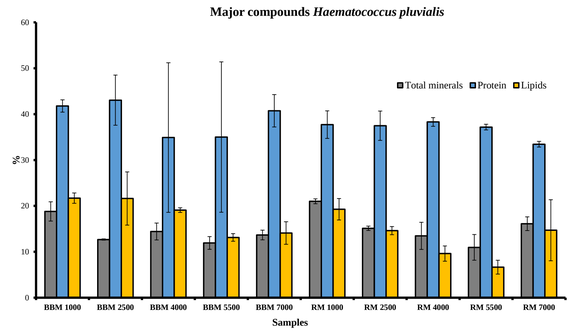

Figure 15 – Assessement of total minerals, protein and lipids in *Haematococcus pluvialis* samples.

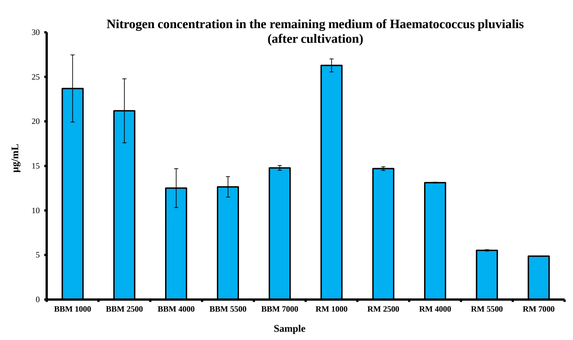

Figure 16 – Nitrogen in the médium after *Haematococcus pluvialis* production.

5. Conclusions

- The concentration of chlorophylls *a*, *b* and carotenoids were higher when using BBM 2500 and 4000 lux;
- *Haematococcus pluvialis* demonstrated a different behaviour in pigments concentration, when varying the cultivation medium;
- Major compounds analysis showed a percentage of about 10% in total minerals for all the samples and the highest concentration was detected in RM 1000 lux;
- Protein average 40% and it was higher using BBM, but with more variation;
- Lipids concentration overcomed 20% using BBM 1000 and 2500 lux.
- The light intensity 4000 lux showed the highest cell concentration using both *media* but RM developed the highest production rate.

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