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Gamma Radiation as a Pretreatment for Co-extraction of Lipids and Astaxanthin in *Haematococcus pluvialis*

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Abstract

The study aimed to apply gamma radiation as a cell pretreatment method for lipid extraction and for obtaining astaxanthin from residual biomass of *Haematococcus pluvialis*. Factor 1 of the two-factor experimental design was represented by cell pretreatment methods: biomass with chloroform:methanol under ultrasound (BCMU), biomass with chloroform:methanol under γ radiation (BCMR), dry biomass under γ radiation (DBR), and control (without pretreatment). Factor 2 considered the vegetative and cystic phases. Cultivation was performed in a mixotrophic system, and biomass was collected in both phases, centrifuged, lyophilized, and submitted to cell pretreatment and lipid extraction. Lipid content and FAMEs were evaluated comparing pretreatment methods and life cycle phases. Total lipid content was higher with the BCMR method in the vegetative (18% DW) and cystic (14% DW) phases. Gamma radiation combined with organic solvent was more efficient for increasing lipid yield, and DBR had a lipid yield similar to BCMU. FAME content differed between phases and pretreatments for most fatty acids, mainly C16:0, C16:1, C18:1*n*9c, C18:2*n*6c, C18:2*n*6t, and C18:3*n*3. The predominance of saturated or low unsaturated fatty acids makes *H. pluvialis*, in both phases, suitable for biofuel production. The preservation of astaxanthin from residual cystic biomass was observed when submitted to the DBR method, with a concentration similar to the raw biomass (1.5% DW). Gamma radiation in dry biomass has an antioxidant effect. Therefore, the lipid extraction method preceded by gamma irradiation was efficient for vegetative and cystic cells of *H. pluvialis* and contributed to the preservation of astaxanthin from residual cystic biomass.

Keywords Algal biomass · Cell pretreatment · Ultrasound · Fatty acids · Biofuel · Carotenoid

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Introduction

The microalga *Haematococcus pluvialis* has attracted great interest over the years due to its cellular content, being commonly cultivated for the extraction of the carotenoid astaxanthin [1]. This carotenoid is widely used in the pharmaceutical, nutraceutical, cosmetics, and food industries, as it has antioxidant, anti-inflammatory, antitumor, antidiabetic, and immunomodulatory properties. Moreover, it is also used in aquaculture, both for pigmentation and to improve the immune response and zootechnical performance of shrimp and fish [2–5].

In addition to astaxanthin, which represents about 4% of the cellular content in the cystic phase, this microalga also has a high concentration of lipids, varying according to the cultivation mode and the life cycle phase (vegetative or cystic) [6]. The increase in lipid accumulation occurs during the formation of aplanospores, simultaneously with the

production of astaxanthin, and is induced by stress factors such as nutrient deprivation in the culture medium [7]. The high neutral lipid content and the adequate fatty acid profile make this species a potential source for nutraceuticals, aquafeed, and biofuel [8].

In the current scenario, biofuels produced from microalgae are considered a promising alternative for the production of sustainable energy, given the need to reduce the use of fossil fuels, as well as carbon emissions [9]. Compared with terrestrial cultures, microalgae have higher biomass productivity, rapid growth, and low need for water renewal in their cultivation. Furthermore, they require the use of smaller areas for production, and cultivation does not require the use of contaminants (pesticides or herbicides), only sunlight, water, and nutrients [10, 11]. However, having the ability to produce large amounts of lipids and having an ideal fatty acid profile for biofuel production depends on the species, cultivation conditions, season, and stage of the microalgae's life cycle [12, 13].

Moreover, the viability of producing lipids from microalgae also depends on the extraction method used, which should provide good yield and cost-effectiveness, in addition to maintaining the integrity of the lipids, being essential for the production process and to ensure oil quality [7]. On a commercial scale, the most used extraction method, which is considered fast and with low lipid degradation, is solvent extraction. In this method, the solvents used must be of low toxicity, pure, immiscible in water, and selective [14]. Also, in order to facilitate the contact between the lipids and the solvent and to promote high yield, time reduction and low cost, biomass pretreatment methods, which can be physical (mechanical or thermal) and chemical, are necessary [15].

The determination of the cell pretreatment and lipid extraction methods to be used must consider the structure of the microalgal cell wall. *H. pluvialis* has a cell wall formed by cellulose and algaenan, present in the vegetative phase like a no acetolysis-resistant material, while in the cystic phase (aplanospores), it consists of a three-layered algaenan sheath and two polysaccharide layers, having a thicker and highly resistant structure against mechanical and chemical actions [16, 17]. Thus, the pretreatment of microalgae biomass in extraction processes with gamma radiation (γ), the most energetic form of electromagnetic radiation, has the potential to improve the yield and quality of the biomolecules obtained, as it acts in the modification of the structures of the polysaccharides that are present in the cell wall [18].

γ radiation is an effective technique for biomass pretreatment, and when combined with others (e.g., chemical and physical), an increase in the efficiency of the entire process is observed [19]. Gamma rays act on the degradation of polysaccharides, such as cellulose, lignin, alginate, carrageenan, and laminarin, which can cause rupture or depolymerization of the cell wall structure [18]. Irradiation is considered to be a promising alternative for biomass pretreatment [18, 19]. The advantages of this process include high energy efficiency, less energy requirement, easy handling, selectivity, mild temperature, short reaction time, low environmental impact, low capital investment, and few hazardous processes [20, 21].

The energy efficiency and cost-effectiveness of cell disruption and lipid extraction are the main challenges in the commercialization of microalgae biofuels, as the costs and energy of this process exceed the current price of crude oil. As a result, microalgae biofuels become less attractive compared to fossil fuels, leading to the need for further research in these areas [15]. In addition, feasibility in microalgae biofuel production can be achieved by using high-value-added coproducts from waste biomass after oil extraction, applying the concept of biorefinery [8, 22]. Residual biomass can be used to generate energy or other liquid and gaseous fuels [23], as well as in the food, nutraceutical, and pharmaceutical industry, as it has a wide variety of biomolecules, such as proteins, carbohydrates, vitamins, pigments, and antioxidants, which can guarantee the sustainability of biofuel production from microalgae [24, 25].

Therefore, further research in these areas focused on techniques that improve lipid extraction and biomass conversion into commercial chemicals and energy are essential to achieve a sustainable economy and increase the viability of microalgae-based biofuels. Thus, the aim of the present study was to apply gamma radiation as a method of cell pretreatment for lipid extraction and to obtain astaxanthin in the residual cystic biomass of the microalga *H. pluvialis*.

Materials and Methods

Experimental Design

A bifactorial design was developed, with factor 1 being represented by the cell pretreatment methods, biomass with chloroform:methanol under ultrasound (BCMU), biomass with chloroform:methanol under γ radiation (BCMR), dry biomass under γ radiation (DBR), and control (without pretreatment), while factor 2 considered the two phases of the microalgae life cycle: vegetative and cystic. The interactions between the factors represented 8 combinations, in triplicate, with a total of 24 experimental runs.

Microalgal Strain and Cultivation

H. pluvialis was obtained from the Live Food Production Laboratory, at the Department of Fisheries and Aquaculture of the Federal Rural University of Pernambuco. This microalga was grown in fresh water that was previously treated with sodium hypochlorite at 3 ppm for 24 h (with aeration), filtered (40 µm) and autoclaved (120 °C for 30 min), and then enriched with

the modified Provasoli culture medium [26] at pH 7.0, presenting in mg L^{-1}: 15 $C_3H_7Na_2O_6P$, 1.98 $C_2H_3NaO_2$, 2.0 yeast extract, 105 $NaNO_3$, 0.075 $ZnCl_2$, 0.0015 $CoCl_2.2H_2O$, 3.0 H_3BO_3, 24.9 EDTA-$Na_2.2H_2O$, 0.15 $FeCl_3.6H_2O$, 10.6 $Fe(NH_4)_2(SO_4)_2.6\ H_2O$, and 0.6 $MnCl_2.4H_2O$.

Cultures were developed in mixotrophic and semi-continuous modes, with microalgae being inoculated at an initial density of 5×10^4 cells mL^{-1}, starting from 40 mL tubes to 20 L flasks. The cultures were maintained with constant aeration, temperature of 22 ± 1 °C, full photoperiod, and light intensity of 40 µmol photons m^{-2} s^{-1}. Microalgal growth was monitored daily, using an optical microscope (magnification of 400×) with the aid of a Neubauer chamber. Upon reaching the stationary phase of growth, the vegetative biomass was harvested. For the production of cystic biomass, on the 12th day of cultivation (stationary phase), light intensity was increased (100 µmol photons m^{-2} s^{-1}) to induce astaxanthin synthesis.

Biomass Harvesting and Drying

Cultures were harvested in the vegetative (zoospores) and cystic phase (aplanospores) by centrifugation (KC5 KINDLY, China) at 3500 × *g* for 15 min (22 °C). The wet biomass was stored at − 80 °C (SANYO MDF U33V) for 24 h and then freeze-dried at high vacuum and low temperature (ALPHA 1–4 LD PLUS) for 48 h [27]. Finally, the biomass was weighed in an analytical balance to determine dry biomass.

Cell Pretreatment

Cell pretreatment with ultrasound (USC-1400A UNIQUE, Brazil) was carried out in 0.7 g of lyophilized biomass homogenized with 14 mL of chloroform:methanol solvent ratio of 1:1 (v/v) at a frequency of 40 kHz and power of 135 W (two cycles of 15 min).

Gamma irradiation was performed at the Department of Nuclear Energy of the Federal University of Pernambuco, using a cobalt-60 gamma-irradiator (Gammacell 220 Excel MDS Nordion) operated at a dose rate of 1.87 kGy/h. The applied dose level was 5.0 kGy, and two methods were applied: radiation in dry biomass (DBR) and radiation in biomass with chloroform:methanol (1:1 v/v) (BCMR).

Lipid Extraction

Lipid extractions were performed adapting the methodology developed by Bligh and Dyer [28], after cell pretreatment, for biomass in the vegetative and cystic phases. After pretreatment of the dried biomass with radiation (DBR), chloroform:methanol (1:1 v/v) was added and vortexed for 2 min. Then, all samples were submitted to the other stages of lipid extraction. Initially, they were centrifuged at 11,200 × *g* for 15 min; the supernatant was collected; 5.25 mL of Milli-Q water was added, vortexed for 2 min, and centrifuged at 11,200 × *g* for 5 min. The mixtures were transferred into a separatory funnel and stayed for 1 h. The lipid fraction was collected in previously weighed glass tubes, and the solvent was evaporated using a laminar flow hood. After that, the tubes were weighed for the calculation of lipid yield (% DW dry weight) and then stored at − 80 °C protected from light.

Fatty Acid Methyl Ester (FAME) Transesterification and Analysis

Transesterification was performed in aliquot of the lipids (1 mL) using a solution of 0.5 mL of potassium hydroxide in methanol (0.5 mol L^{-1}) and vortex-mixed for 2 min. After that, FAMEs were extracted with 2 mL n-hexane, vortex-mixed for 2 min, and centrifuged at 2000 × *g* for 6 min [29]. Finally, the supernatant was filtered with a 0.22 µm PTFE filter and stored at − 20 °C overnight.

FAMEs were analyzed by gas chromatography using a GC/FID (flame ionization detector), GC-2010Plus, equipped with AOC-20i autoinjector (Shimadzu, Kyoto, Japan). GC analysis was performed in a ZB-5HT capillary column with an initial column temperature of 150 °C (3 min) and a heating rate of 4 °C/min up to 280 °C and then kept at 280 °C for 15 min. The injector and detector temperatures were 250 °C and 300 °C, respectively. Helium was used as the carrier gas (30 mL/min), and the injection volume was 1 µL with a split ratio of 1:100. Chromatographic peaks were identified by comparing retention times with standard certificate material (Supelco FAME Mix C8–C24, Bellefonte, PA, USA). Three replicates of each FAME analysis were performed.

Astaxanthin Extraction, Analysis, and Quantification

The determination of astaxanthin followed the methodology of Dong et al. [30], in which 5 mg of residual cystic biomass from lipid extraction with different pretreatment methods and 5 mg of lyophilized crude biomass (control) were treated with 1 mL of HCl (4 M) and placed in an oven at 70 °C for 2 min and then cooled and centrifuged at 5000 × *g* for 5 min. The HCl-treated sample was washed twice with distilled water, centrifuged at 5000 × g for 5 min, resuspended in 1 mL of 90% acetone, and subjected to an ultrasonic bath on ice for 10 min. Subsequently, the sample was centrifuged again at 3500 × g for 5 min, and the obtained extract was stored in amber vials for quantification in UPLC-MS.

Chromatography was performed in an Acquity H-Class (Waters™, USA) Ultra Performance Liquid Chromatograph (UPLC), using a 2.1 × 100 mm HSS T3 column and a

particle size of 1.8 µm. The mobile phases used consisted of acetonitrile solution containing 0.1% formic acid (eluent A), methanolic solution containing 0.1% formic acid (eluent B), and ethyl acetate solution containing 0.1% formic acid (eluent C), which were pumped at a flow rate of 0.37 mL min^{-1}; elution was performed in isocratic mode (10% A/50% B/40% C) held for 5 min. Ten microliters of sample was injected, and the column and autoinjector temperatures were maintained at 40 and 10 °C, respectively. The UPLC system was coupled to a SQ Detector 2 single quadrupole mass spectrometer (Waters™, USA). The capillary voltage was 1.5 kV, the cone voltage was 50 V, and the desolvation temperature was 350 °C, with a source gas flow of 650 L h^{-1}. Data acquisition was performed in selected ion recording (SIR) mode, seeking the mass of astaxanthin (597.35 Da), in positive ionization. The acquisition of chromatograms and mass spectra was performed using MassLynx™ software (Waters™, USA).

A calibration curve was used with an astaxanthin commercial standard with 97% purity (SML0982 Sigma, St. Louis, MS, USA) to quantify samples. The entire procedure was performed in triplicate and protected from light.

Statistical Analysis

All data are presented as mean ± standard deviation ($n = 3$). A two-way ANOVA was used to determine the effect of algal phase and cell pretreatment and their interaction on lipid yield and FAMEs, after confirming normality (Shapiro–Wilk's test) and homoscedasticity (Bartlett's test). The values for some FAMEs were transformed using Box–Cox transformation to correct for non-normality and heterogeneous variances. Tukey's test was used when differences between factors and treatments were detected by ANOVA ($p < 0.05$). Astaxanthin yield data did not obtain normal distribution and were submitted to the Kruskal–Wallis test ($p < 0.05$). Statistical analyses were performed using the R Core Team [31].

Results and Discussion

Lipid Content According to Cell Pretreatment and Life Cycle Phases

The total lipid content varied significantly ($p < 0.05$) according to the phase of the life cycle of *H. pluvialis*, with an average yield of 15 ± 0.03% DW for the vegetative phase (zoospores) and 10 ± 0.02% DW for the cystic phase (aplanospores). It also differed significantly ($p < 0.05$) based on the applied extraction method, where BCMR resulted in higher lipid yield, 18.4 ± 1.1% for vegetative, and 13.6 ± 0.6% for cystic phase (Fig. 1). There was no significant difference ($p > 0.05$) in the interaction between phases and cell pretreatments.

In the vegetative phase, the lipid content can reach up to 25%, varying according to the culture conditions: temperature, pH, light intensity, culture medium, and culture system [7, 32]. In the present study, the average lipid yield of 15% can be attributed to culture conditions, such as low light

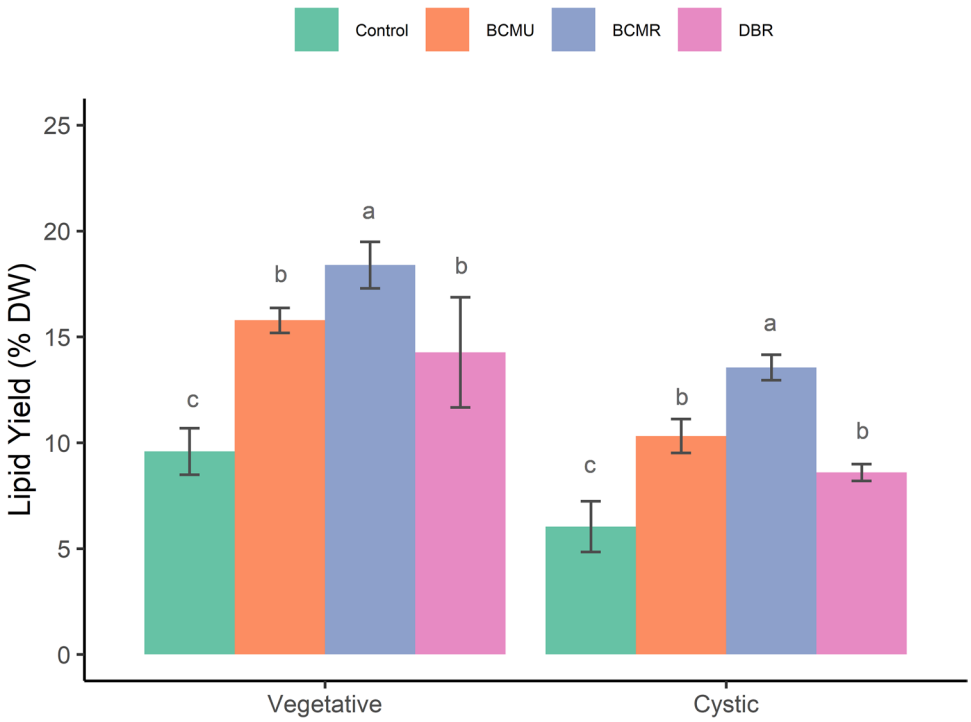

Fig. 1 Lipid yield using different cell pretreatment methods in the two phases of the life cycle of *H. pluvialis* (mean values between pretreatments in the same phase with different letters differ significantly by Tukey's test. Control, without pretreatment; BCMU, biomass with chloroform:methanol under ultrasound; BCMR, biomass with chloroform:methanol under γ radiation; DBR, dry biomass under γ radiation)

intensity (40 µmol photons m^{-2} s^{-1}) and no CO_2 insertion, differing from the results of higher lipid content found in the literature, in which the luminosity varies between 100 and 300 µmol photons m^{-2} s^{-1} [32]. When considering the extraction methods used, these were suitable for the morphological characteristics of the species, especially regarding the composition of the cell wall [33]. In this phase, zoospores have a cell wall formed by cellulose — biosynthesized during the formation of the primary wall — and by residues of algaenan, a biopolymer formed by dicarboxylic acids, alcohols, and fatty acids (C_{22}–C_{26}), resistant to various chemical and enzymatic treatments [34]. These characteristics reveal the need for physical cellular pretreatment followed by the use of chemical agents, such as those used in this study.

In the cystic phase, the lipid content of aplanospores was lower than that of vegetative cells (Fig. 1) and had lower concentrations than in other culture systems, where they reach up to 37% DW [6]. The cell wall of the aplanospores is composed of a trilaminar algaenan sheath, below which is found a secondary layer composed largely of cellulose and mannose in a homogeneous arrangement, in addition to the tertiary layer with heterogeneously arranged cellulose and mannose [17]. This cell wall is thicker (2–3×) than vegetative cells and highly resistant to mechanical and chemical attacks, thus decreasing the bioavailability of accumulated compounds [35].

These characteristics lead to the need to use more aggressive methods in the pretreatment of cells, such as γ rays, which are the most energetic form of electromagnetic radiation and, therefore, have a greater capacity to penetrate the biomass than other types of radiation [19]. Gamma radiation acts in two ways: directly by interacting with biological molecules, promoting excitation, injury, and splitting of the polymeric structure or indirectly producing free radicals in cells; thus, it can damage or modify cell components, depending on the level of irradiation [20]. The solvent irradiation method (BCMR), in both phases of the microalgae life cycle, was more effective than the dry biomass irradiation (DBR) (Fig. 1). This was due to the high formation of free radicals that occurs in the liquid, consequently generating a greater indirect effect [36].

Gamma radiation is an effective technique for the pretreatment of lignocellulosic biomass, modifying the structure, reducing the degree of polymerization of cellulose, and disrupting the cell wall [37]. The use of radiation can be combined with other physical or chemical methods, allowing to reduce the radiation dose used [19, 38]. In the present study, by using a dose of 5.0 kGy with solvents, a greater efficiency in disrupting the cell wall of vegetative and cystic cells of *H. pluvialis* was found. According to Cheng et al. [39], doses of 0.25–4.0 kGy produce mutant *H. pluvialis* cells with greater biomass production, while 5.0 kGy prevents microalgae growth, as it causes irreversible damage to cells. Comparatively, in other studies, doses of 1.2 and 5.0 kGy were sufficient for the disruption and depolymerization of the cell wall structure of other biomasses [40, 41].

Gamma irradiation alone is an effective technique for biomass pretreatment, and when the irradiation process is combined with other ones (chemical, physical), there is an increase in the efficiency of the entire process [37]. Thus, the use of γ radiation without solvent (DBR) resulted in lipid yield similar to ultrasound with solvent (BCMU), because of the fact that, in dry conditions, there is a decrease in the efficiency of radiation [20].

Ultrasonic vibration has a direct proportion to power, providing physical effects such as the rise of osmoses and diffusion of solutes, in addition to turbulence between surfaces and damage to the cell wall [42]. Ultrasonic treatment must be allied to the characteristics of the cell wall of the species and biomass conditions, and a relatively high amount of energy is usually required due to the attenuation of power within the medium and due to high cell concentration [43]. For example, the maximum lipid yield for the wet *Nannochloropsis* and *Chlorella* paste is reached with powers of 400 and 1000 W, respectively [44, 45]. When ultrasound is applied after biomass harvesting and drying, lower powers can be used, such as 100 to 200 W. In fact, these values can be used for *H. pluvialis* powder, as in this study, where a power of 135 W was used [46, 47]. In addition, a combination with high temperatures and use of solvents results in more intense effects [43]. Dong et al. [30] using ultrasound combined with acetone, after pretreatment with HCl (4 M) at 70 °C, obtained a lipid yield of 33.3 ± 1.1% for *H. pluvialis* in the cystic phase, higher than that achieved with hexane, isopropanol, soybean oil, and chloroform:methanol.

Therefore, considering the morphological characteristics of aplanospores, higher radiation doses would certainly provide greater wall rupture, enabling greater lipid yield. However, it is worth noting that there is a need to verify the vulnerability of the compounds.

Fatty Acid Methyl Esters

The fatty acid profile of *H. pluvialis* was similar for the vegetative and cystic phases, the main ones found being palmitic (C16:0), palmitoleic (C16:1), oleic (C18:1*n*9c), linoleic (C18:2*n*6c), linolelaidic (C18:2*n*6t), and α-linolenic (C18:3*n*3), corroborating with the findings of Bilbao et al. [48]. Regarding the fatty acid content, there was a significant difference between the vegetative and cystic phases for all FAMEs, except for C18:2*n*6c (Table 1). As regards to cell pretreatment methods, only myristic (C14:0), palmitoleic (C16:1), α-linolenic (C18:3*n*3), erucic (C22:1*n*9), and lignoceric (C24:0) did not show significant difference (Table 1).

The level of saturated fatty acids (SFA) was lower than unsaturated (UFA) in all pretreatments and in the two

Table 1 Fatty acid profile (% FAMEs) according to cell pretreatment methods and life cycle phases

	Vegetative				Cystic				Factor analysis		
	Control	BCMU	BCMR	DBR	Control	BCMU	BCMR	DBR	P	C	P × C
C14:0	0.83 ± 0.01	0.68 ± 0.02	0.73 ± 0.02	0.70 ± 0.0	1.23 ± 0.06	1.05 ± 0.02	1.21 ± 0.01	1.10 ± 0.03	***	n.s.	n.s.
C16:0	31.04 ± 0.12	27.67 ± 0.27	28.53 ± 0.27	27.41 ± 1.26	34.48 ± 0.68	31.91 ± 0.52	32.28 ± 0.73	29.83 ± 0.22	***	***	n.s.
C16:1	9.81 ± 0.07	12.82 ± 0.08	12.63 ± 0.27	9.82 ± 0.06	4.37 ± 0.26	5.54 ± 0.09	6.74 ± 0.57	6.70 ± 0.11	***	n.s.	*
C18:0	2.20 ± 0.03	0.93 ± 0.20	0.91 ± 0.09	1.07 ± 0.05	4.81 ± 0.71	1.14 ± 0.05	1.23 ± 0.13	1.30 ± 0.05	***	***	**
C18:1*n*9c	17.94 ± 0.06	19.62 ± 0.23	19.12 ± 0.33	16.12 ± 0.50	19.81 ± 0.43	22.44 ± 0.30	22.40 ± 0.52	22.24 ± 0.69	***	***	***
C18:1*n*9t	4.81 ± 0.22	4.28 ± 0.52	3.94 ± 0.11	4.43 ± 0.12	6.64 ± 0.30	4.46 ± 0.10	4.76 ± 0.14	5.18 ± 0.19	***	***	n.s.
C18:2*n*6c	16.31 ± 0.18	21.75 ± 1.20	23.10 ± 0.41	21.13 ± 1.15	19.75 ± 0.49	23.81 ± 0.28	23.30 ± 0.36	23.36 ± 0.35	*	**	n.s.
C18:2*n*6t	0.93 ± 0.04	1.92 ± 0.23	2.01 ± 0.03	1.27 ± 0.17	1.86 ± 0.08	2.00 ± 0.07	2.57 ± 0.38	2.82 ± 0.06	***	**	***
C18:3*n*3	14.66 ± 0.13	9.68 ± 0.17	8.52 ± 0.08	16.94 ± 3.66	2.64 ± 0.44	1.81 ± 0.18	2.37 ± 0.33	3.99 ± 0.13	***	**	n.s.
C20:0	0.29 ± 0.02	0.15 ± 0.0	0.14 ± 0.0	0.31 ± 0.03	0.78 ± 0.05	0.61 ± 0.07	0.50 ± 0.01	0.64 ± 0.05	***	***	*
C22:0	-	-	-	0.05 ± 0.01	0.41 ± 0.03	0.32 ± 0.02	0.40 ± 0.03	0.49 ± 0.03	***	***	n.s
C22:1*n*9	-	-	-	0.15 ± 0.01	0.05 ± 0.0	0.56 ± 0.05	0.62 ± 0.03	0.71 ± 0.08	***	n.s	n.s
C24:0	-	-	-	0.07 ± 0.03	0.48 ± 0.02	0.45 ± 0.02	0.55 ± 0.03	0.56 ± 0.02	***	n.s	n.s
∑SFA	34.38 ± 0.096	29.38 ± 0.16	30.27 ± 0.23	29.61 ± 1.24	42.18± 1.134	35.48± 0.547	36.18 ± 0.806	33.93± 0.261	***	***	***
∑UFA	64.51 ± 0.085	70.06 ± 0.87	69.33 ± 0.5	69.85 ± 2.01	55.34 ± 1.051	60.62 ± 0.767	62.75 ± 0.757	64.29 ± 1.238	***	***	***
∑FAME N.D.	1.11 ± 0.01	-	-	-	2.48 ± 0.70	3.90 ± 1.32	1.07 ± 0.05	1.08 ± 0.20	n.s	*	n.s

Mean of 3 replicates ± standard deviation. The mean values for the same row were significantly different by two-way ANOVA. *P*, phase (factor 1); *C*, cell pretreatment methods (factor 2), *P* × *C*, interaction between phases and cell pretreatments. ***=$p<0.001$; **=$p<0.01$; *=$p<0.05$; *n.s.*, not significant. C14:0, myristic acid; C16:0, palmitic acid; C16:1, palmitoleic acid; C18:0, stearic acid; C18:1*n*9c, oleic acid; C18:1*n*9t, elaidic acid; C18:2*n*6c, linoleic acid; C18:2*n*6t, linoelaidic acid; C18:3*n*3, α-linolenic acid; C20:0, arachidic acid; C22:0, behenic acid; C22:1*n*9, erucic acid; C24:0, lignoceric acid; *SFA*, saturated fatty acid; *UFA*, unsaturated fatty acid; *FAME N.D.*, no detected fatty acid methyl esters.

phases, and both presented a significant difference between cell pretreatments, phases, and interaction of factors. The highest content of SFA was observed in the cystic phase and in the absence of cell pretreatment (control), followed by BCMR and BCMU (Table 1). The predominance of SFAs is a prerequisite to maintain the properties of biofuels, due to the lower risk of polymerization and greater stability when compared to UFAs [49]. Therefore, the high lipid yield combined with the higher SFA content makes cystic *H. pluvialis* under BCMR suitable for biofuel production.

In Fig. 2, the contents of the main fatty acids are represented in boxplot, C16:0 (a), C16:1 (b), C18:1*n*9c (c), C18:2*n*6c (d), C18:2*n*6t (e), and C18:3*n*3 (f), highlighting the differences between cell pretreatment methods and phases. As in other species of Chlorophyceae, in *H. pluvialis*, saturated palmitic (C16:0) and monounsaturated oleic (C18:1n9c) fatty acids are predominant in both the vegetative and cystic phases [50]. BCMR and BCMU pretreatments in the cystic phase reached higher palmitic acid content (Fig. 2a), while the oleic acid content was higher for the cystic phase in all cell pretreatments applied (Fig. 2c). As for polyunsaturated fatty acids (PUFAs), linoleic (Fig. 2d), linoelaidic (Fig. 2e), and linolenic (Fig. 2f) acids were predominant; these have a maximum of three unsaturations and an intermediate chain length, with a maximum of 18 carbons. This predominance of saturated or low unsaturated fatty acids, with medium carbon length, makes this microalga a potential to produce biodiesel that remains as liquid at low temperatures with a high energy content [7, 49]. Furthermore, microalgal biomass can also be used as feedstock for bioethanol or biomethane production after refining of value-added molecules, like astaxanthin [8].

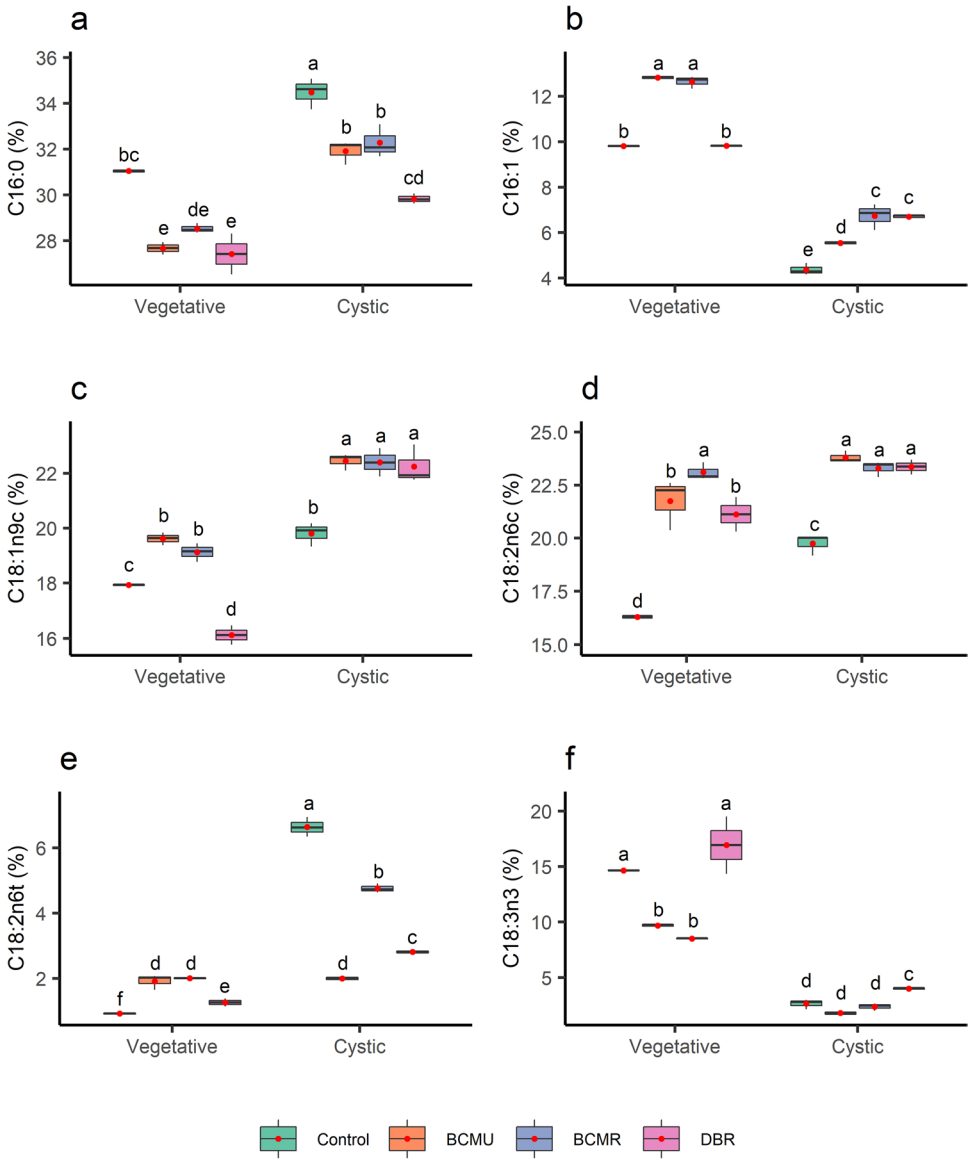

Fig. 2 Boxplot of the main fatty acids found in *H. pluvialis* using different methods of cell pretreatment in the vegetative and cystic phases (different letters indicate significant differences between the means of the factors by Tukey's test)

Astaxanthin in Residual Cystic Biomass

Considering the residual cystic biomass after lipid extraction, conservation of the astaxanthin was observed when using the pretreatment method with gamma radiation in dry biomass (DBR), obtaining an astaxanthin concentration similar to raw biomass — not subjected to cell pretreatment and lipid extraction (control), of approximately 78 mg L^{-1} and 1.55% DW (Fig. 3). This method obtained a lipid yield equivalent to the method in which ultrasound was used and lower than the one using gamma radiation in biomass with solvent (Fig. 1). However, the carotenoid concentration in its residual biomass was higher (Fig. 3). The use of gamma radiation in dry biomass has an antioxidant effect [51], preserving astaxanthin from potential degradation, while the gamma radiation methods in biomass with solvent and ultrasound caused the degradation of this carotenoid.

The action of γ rays in dilute biomass causes high formation of free radicals, which contributed to a higher lipid yield, as well as to the greater performance of astaxanthin as an electron donor, neutralizing free radicals, due to its antioxidant property [2]. Furthermore, this carotenoid is a highly unsaturated molecule that easily decomposes when exposed to heat, light, and oxygen, whose reactions can be caused by the formation of free radicals [52]. Its exposure to ultraviolet light, for example, can result both in cis–trans isomerization and cause the destruction of this molecule under more energetic conditions, such as when exposed to a wavelength smaller than 300 nm and diluted in lipophilic solvents [2]. Gamma radiation has a very short wavelength and is very energetic, so when this method of cell pretreatment is combined with the use of chloroform:methanol in the biomass, it is possible to observe greater degradation of astaxanthin.

The ultrasound method caused the degradation of astaxanthin through the propagation of ultrasonic waves, which involves formation, growth, and collapse of microscopic bubbles, generating high temperatures and mechanical action between the biomass and solvent interfaces [53]. In this technique, the astaxanthin content decreases as the potency and treatment time increase, being able to reduce by 25.1% (100 W), 25.5% (300 W), and 29.4% (600 W) after 6 min [52]. In the present study, there was an 87% reduction after lipid extraction at the power of 135 W for 30 min and subsequent extraction of astaxanthin at the same power for 10 min.

Therefore, using the DBR method, it was possible to extract lipids (~ 8.6%) in the cystic phase and preserve astaxanthin from the residual biomass, demonstrating the importance of using γ radiation for these purposes. In *H. pluvialis*, astaxanthin is the most economically valuable bioproduct, due to its biological properties, being widely used in the pharmaceutical, nutraceutical, cosmetics, and food industries, in addition to aquaculture [1–3]. Thus, promoting the use of residual cystic biomass (from lipid extraction) to obtain the carotenoid offers greater economic viability to the biofuel production process.

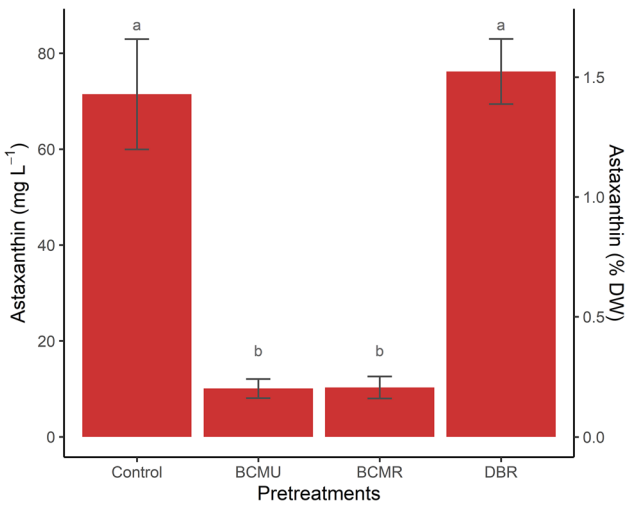

Fig. 3 Astaxanthin concentration in residual cystic biomass of *H. pluvialis* after cell pretreatment and lipid extraction (mean values with distinct superscripts are significantly different by Kruskal–Wallis test. Control, raw biomass; BCMU, residual biomass after ultrasound with chloroform:methanol; BCMR, residual biomass after γ radiation with chloroform:methanol; DBR, residual biomass after γ radiation on dry biomass)

Conclusions

The present study proposed a new and promising method of biomass pretreatment for lipid extraction from *Haematococcus pluvialis* using gamma rays. In this perspective, gamma radiation as a cell pretreatment promoted higher lipid yield of *H. pluvialis* in the vegetative and cystic phases. It is also possible to state that the predominance of saturated or low unsaturated fatty acids makes *H. pluvialis*, in both phases, suitable for biofuel production. In addition, astaxanthin is obtained from residual cystic biomass subjected to gamma radiation in dry biomass as a cell pretreatment of lipid extraction. Therefore, considering the current challenges regarding the economic feasibility of producing biofuels from microalgae, the extraction of high-value coproducts, such as astaxanthin, combined with high lipid yield and adequate lipid profile makes the process more viable.

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Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing Interests The authors declare no competing interests.

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