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Article

Evaluation of Cell Rupture Techniques on the Extraction of Proteins from Microalgae *Tetradesmus obliquus*

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Abstract: The high protein content of several microalgal species makes them attractive and unconventional for applications in the food and pharmaceutical industries. Due to microalgae's robust cell wall, cell rupture is necessary to improve the extraction of their intracellular proteins. Thus, choosing a suitable cell-breaking treatment before protein extraction is a vital downstream processing step. Additionally, it is necessary to use an effective technique for monitoring and measuring the impact of rupture treatments on microalgal cell walls. In our study, *Tetradesmus obliquus* cells were disrupted by three different mechanical rupture methods: high-pressure homogenization (HPH), ultrasound (US), and ball milling (BM). The ruptured biomass cells were counted, and soluble proteins were extracted and quantified. The cell counting technique did not detect differences between intact and damaged cells after BM treatment because the dye (erythrosine B) did not permeate the microalgae biomass accurately. The US treatment promoted the highest yield of total protein extraction (19.95%). The yields of protein extraction of *T. obliquus* with HPH (15.68%) and BM (14.11%) were significantly (p value ≤ 0.05) lower than that achieved in the US treatment. The increase in the number of extraction steps increased the protein recovery. Thus, the content of extracted soluble protein was a reliable indirect index for a quantitative comparison of the cell disruption level.

Keywords: ball milling; high-pressure homogenization; ultrasonication; cell counting

1. Introduction

Microalgae are a group of highly diverse organisms with morphological, reproductive, physiological, and ecological characteristics. Microalgae biomass can be applied in the cosmetics industry, wastewater treatment, energy production, and human and animal nutrition. In the food segment, microalgae have gained interest because they are an unconventional source of proteins, presenting the advantages of not being dependent on the climate and propensity for growth in any region [1]. Microalgae can be produced in areas unsuitable for growing traditional crops due to their high capacity to adapt to the environment, not competing with food production sites [2].

Microalgal biomass contains metabolites for industrial use, such as proteins, lipids, vitamins, enzymes, pigments, mineral salts, antioxidants, and antibiotics. Additionally, short- and long-term feeding tests have demonstrated that some microalgae are safe for humans [3–7].

The high protein content of microalgae makes them a potentially attractive and unconventional source of sustainable green protein and bioactive peptides for human nutrition, animal feed, and aquaculture. Depending on the species, microalgal protein content can reach 60% to 70% [8]. Proteins extracted from microalgae have nutritional quality compared to proteins of plant origin [2]. Additionally, their composition of essential amino acids is in the quantities recommended by the Food and Agriculture Organization/FAO [1].

Approximately 70% of microalgal proteins are intracellularly located and adhere to the cell wall. Thus, the cells must be ruptured to extract or release these components [9]. Cell disruption, as one of the first downstream stages for microalgae biotechnological platforms, promotes the permeabilization or complete breakage of the membrane and wall of cells, allowing solvent access to intracellular biomolecules [10]. Consequently, selecting a suitable cell-breaking treatment before protein extraction is crucial for downstream processing.

Mechanical cell rupture methods, such as high-pressure homogenization, ball milling, and sonication, have been used [11–14] because they provide good cell wall disintegration efficiency and do not use chemical reagents. Since the downstream steps represent a large part of the operational costs to the production chain, cell lysis technologies must be low-cost and energy-efficient, improving product quality and yield [10,15]. Moreover, an effective technique is necessary for monitoring and measuring the impact of treatment on microalgal cell disruption. Accordingly, this study aimed (i) to investigate the effect of three different disruption methods, high-pressure homogenization, ball milling, and ultrasound, on *Tetraedesmus obliquus* cells, (ii) to identify the majority of protein in the protein-rich extracts, and (iii) to evaluate the color parameters of the protein-rich extracts. These protein-rich extracts are useful for studying protein techno-functionalities. *Tetraedesmus* spp. is among microalgae, a Brazilian native species abundant in aquatic environments. They present a sizeable morphological variation within each species, show a high growth rate, and contain 30% to 40% protein on a dry-mass basis [8,13,16].

2. Materials and Methods

2.1. *Tetraedesmus obliquus* biomass processing

The *Tetraedesmus obliquus* strain was previously isolated from freshwater reservoirs and cultivated in BG11 culture medium [17,18]. The biomass was separated from the growing medium, concentrated to 6 mass %, frozen, and stored at -10 °C [19]. The microalgal biomass was a gentle gift from the Biofuels Laboratory at the Universidade Federal de Viçosa, Brazil. The frozen biomass was thawed for fractionation, washed with deionized water, filtered in an organza fabric filter medium to remove impurities, packed in containers, and frozen (GE, Brazil) at -80 °C. The frozen biomass was freeze-dried (LS 3000, Terroni, Brazil), giving rise to dry biomass, reaching a final moisture content of 8 mass %. The dry biomass was then sheared through a 59 mm stainless steel sieve (Tyler 28). The resulting fine powder, with standardized granulometry, was refrigerated at 8 °C until use.

2.2. Composition of the *Tetraedesmus obliquus* microalgae

The macronutrient composition of dried *Tetraedesmus obliquus* biomass was characterized by moisture [20], ashes [20], lipids with the Folch method [21], and protein with the Kjeldahl method [20] and a nitrogen factor of 5.89 [22]. The carbohydrate content was calculated by difference (Carbohydrate% = 100% – ashes% - lipids% - proteins%).

2.3. Separation and identification of proteins from the microalgae *Tetraedesmus obliquus*

- Molecular mass profile

The molecular mass distribution of *T. obliquus* proteins was determined by SDS-PAGE methodology. Microalgal biomass samples containing known amounts of proteins were kept at 25.0

°C under stirring overnight. The suspensions were processed and subjected to polyacrylamide gel electrophoresis using a 10% gel (0.75 mm) composed of a separation gel (10% acrylamide) and a stacking gel (5% acrylamide) in a 7 x 10 x 0.1 cm Mini-Protean III tank (Bio-Rad, USA). The microalgal biomass was initially suspended for solubilization in 1 mL of 25 mM Tris-HCl buffer (pH 7.5), and biomass suspensions were prepared with concentrations of 20, 40, and 50 µg of protein. Then, each biomass suspension was (a) mixed with buffer composed of 10% glycerol, 5% β-mercaptoethanol, 2.3% SDS, and Tris-HCl pH 6.8, (b) heated at 95 °C for 5 min, (c) centrifuged (Centrifuge 5430, Eppendorf, Germany), and (d) cooled to room temperature (± 25 °C). A 21 µL aliquot of each suspension was transferred into the gel channels. The molecular mark (5 µL) used was the prestained standards Precision Plus ProteinTM (Bio-Rad, USA) with molecular masses ranging from 10 to 200 kDa.

- Identification by mass spectrometry (MALDI-TOF)

Regions of the gel containing the protein bands with greater intensity and better separation were manually cut into cubes of approximately 1.0 mm³. The selected protein bands and a blank were processed with the in-gel digestion technique. Then, the peptide molecular masses were identified using the Shevchenko et al. [23] methodology. This protocol included the removal of Coomassie G-250 brilliant blue dye, digestion with the enzyme trypsin, and extraction and concentration of the peptides. Subsequently, 1 µL of the peptide samples was mixed with 3 µL of the α-cyano-4-hydroxycinnamic acid matrix (5 µg/mL in 50% acetonitrile and 0.1% TFA) in the well of the steel plate (MTP Anchor Chip TM 600/384 TF, Bruker Daltonics®). MALDI-TOF-MS (laser ionization mass spectrometry) was used to analyze the molecular masses of the peptides originating from protein digestion. External calibration was performed with peptide calibration standard II (Bruker Daltonics®, USA) containing the peptides bradykinin (757 Da), angiotensin II (1046 Da), angiotensin I (1296 Da), P substance (1347 Da), bombesin (1619 Da), ACTH clip 1-17 (2093 Da), ACTH clip 18-39 (2465 Da), and somatostatin (3147 Da). The spectrograms were stored to identify the peptides by comparing their molar masses with those deposited in the SwissProt database using MASCOT software (www.matrixscience.com).

2.3. Biomass cell disruption

Three different disruption methods were evaluated:

- Mechanical disruption by ball mill: The freeze-dried biomass was mechanically disrupted in a ball mill (model MR350, Tecnal Equipamentos Científicos, Brazil) using 10 g of biomass batches for 25 min, following Vieira et al. [16]. The disrupted biomass was kept at 20 °C until use.
- Mechanical disruption by ultrasound: The freeze-dried biomass was mechanically disrupted by ultrasound after resuspension in distilled water (5.0% w/v), following Silva et al. [24] with adaptations. The suspensions were mechanically stirred (IKA, RW20 digital, Germany) at 25.0 °C overnight and disrupted in a tip sonicator (Sonics, VCX 500, USA) at 20 kHz frequency and 98% amplitude for 6 min of ultrasonication. The cells were disrupted under cooling in an ice bath to avoid overheating the system. After disruption, cell suspensions were frozen, freeze-dried (Terroni, LS 3000, Brazil), and stored at 20 °C until use.
- Mechanical disruption by high-pressure homogenization: The microalgal biomass was suspended in distilled water (1.5% w/v) and processed in a homogenizer (Alitec, A100, Brazil) at 350 bar, according to Shene et al. [25]. The number of passes of the suspensions in the homogenizer was 25, and the suspension was cooled to avoid compound degradation due to the temperature increase. The homogenized samples were collected, frozen, freeze-dried (Terroni, LS 3000, Brazil), and stored at 20 °C until use.

2.4. Cell disruption indicators

- Evaluation of the cell disruption level through cell counting

A 100 μ L volume of the working solution was added to 100 μ L of the disrupted cell suspension to count ruptured biomass cells, according to Gminski et al. [26]. The working solution was prepared by diluting 0.1 mL of erythrosine B solution in 5 mL of potassium phosphate buffer. Erythrosine B solution was prepared using an aqueous solution of 10 g/L erythrosine B and a potassium phosphate buffer solution formed with 43.9 g/L dipotassium phosphate (K_2HPO_4) and 15.52 g/L monopotassium phosphate (KH_2PO_4).

The working solution and ruptured biomass cells were added, submitted to a mechanical stirrer (IKA, RW20 digital, Germany), and rested for 10-15 minutes. Samples were removed and poured using a pipette, placed on a standard Neubauer hemocytometer (10 μ L), and observed under an optical microscope (BX53, Olympus Corporation, Japan) at 100x objectives for cell counting. Intact cells appeared green and/or magenta, while dead or damaged cells were stained red. The cells were counted in five quadrants in the Neubauer camera. The cell break degree (% of cells) of the disrupted biomass represents the level of physical destruction of cells and was calculated according to Equation 1.

$$\text{Percentage of rupture cells (\%)} = \left(\frac{\text{number of damage cells}}{\text{number of total cells}} \right) \cdot 100 \quad (1)$$

- Evaluation of the cell disruption level through the amount of extracted soluble protein

The effect of microalgal cell disruption on protein extraction was evaluated using the amount of the total hydrosoluble protein extracted [3]. The disrupted biomass in each treatment was suspended in distilled water at a concentration of 10.0% w/v and then magnetically stirred for 24 h. The pH of the microalgal suspension was adjusted to 10, and the suspension was magnetically stirred (MA 502/D, Marconi, Brazil) at 1500 rpm for 2 h at 25 °C. The mixture was centrifuged at 5000 \times g for 5 min (centrifuge 5430, Eppendorf, Germany), and the supernatant with soluble proteins was collected. For a better protein extraction yield, the residual biomass (pellet) was resuspended in deionized water, and the pH was adjusted again to 10. This extraction procedure was repeated, resulting in 3 sequential extractions.

The soluble protein content in each extraction procedure was quantified by Lowry's methodology [27] using bovine serum albumin (fraction V powder, Sigma-Aldrich, USA) to prepare the standard curve. The percentage of protein recovery from the biomass, or the protein yield, was determined as described by [28] using Equation 2. All extractions were performed in triplicate.

$$\text{Protein Extraction Efficiency (\%)} = \frac{C \times V}{P_i} \times 100\% \quad (2)$$

where C is the hydrosoluble protein concentration (mg/mL) in each extract, and V is the final volume of the protein extract (mL). P_i is the protein content of the microalgal freeze-dried feedstock (mg), which was disrupted with base hydrolysis [16].

The mass of the concentrate of the total extracted protein was determined by gravimetry after freeze-drying (L101, Liobras, Brazil). All extractions were performed in triplicate.

The protein content (%) of each freeze-dried extract was calculated by Equation 3.

$$\text{Protein Content (\%)} = \frac{\text{Mass of protein recovered (g)}}{\text{Mass of freeze-dried biomass (g)}} \times 100\% \quad (3)$$

The color parameters of each freeze-dried extract were determined on a CR-10 colorimeter (Konica Minolta, Japan). The color was evaluated by the CIELab color space method, determining the yellowness (b), redness (a), and lightness (L^*) values.

The freeze-dried extracts were evaluated by scanning electron microscopy (SEM). The samples were covered with gold (15 nm thick) (Quorum, Q150RS, UK) (Berger et al. 2016) and analyzed under an LEO 1430 VP scanning electron microscope (Carl Zeiss, UK) at 20 kV.

2.5. Statistical Analysis

The results of the microalgal cell disruption indicators were submitted to analysis of variance (ANOVA), and the Tukey test determined significant differences ($p < 0.05$) between treatments. The results are represented as the means \pm standard deviations, and the measurements were performed in triplicate.

3. Results

The proximate composition (w/w) of the *T. obliquus* biomass was $40.29 \pm 0.24\%$ proteins, $18.33 \pm 0.03\%$ ashes, $7.87 \pm 0.10\%$ moisture, $4.23 \pm 0.21\%$ lipids, and 29.28% carbohydrates.

The results of the electrophoretic analysis of proteins in homogenized samples of *T. obliquus* in SDS-PAGE gel are shown in Figure 1.

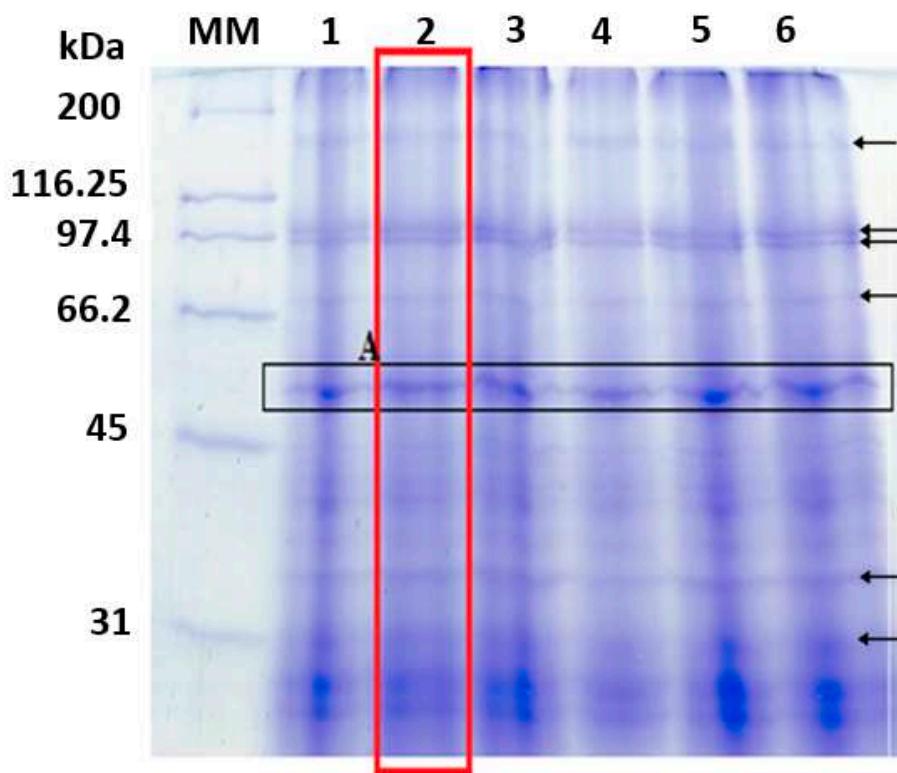


Figure 1. Electrophoretic analysis of *T. obliquus* protein extract (PEXT) using 10% polyacrylamide gel, stained with Coomassie brilliant blue G-250. The samples were MM (marker for molecular mass); band 1 (20 μ g of PEXT); band 2 (40 μ g of PEXT); band 3 (50 μ g of PEXT); band 4 (20 μ g of PEXT - repeat); band 5 (40 μ g of PEXT - repeat); and band 6 (50 μ g of PEXT - repeat).

The extracts with different protein concentrations presented peptide bands with molecular masses varying between 31 and 200 kDa. All samples showed an equal number of bands, but some bands were clearer. The best separation between the bands occurred with the sample from line 2 (40 μ g of protein extract), and the bands with the highest intensity were located in the molecular mass range between 45 and 66.2 kDa.

Among the proteins expressed in the electrophoretic analysis, the protein in letter A of Figure 1 shows the highest intensity and best separation. The corresponding band was cut from the gel and digested with the trypsin enzyme as described in the identification methodology. After tryptic digestion, the molecular mass of the peptides was determined using an Ultraflex III MALDI TOF-TOF mass spectrometer (Bruker Daltonics). This analysis provides the spectrum shown in Figure 2 with the mass/charge ratio of the peptides from the letter A protein.

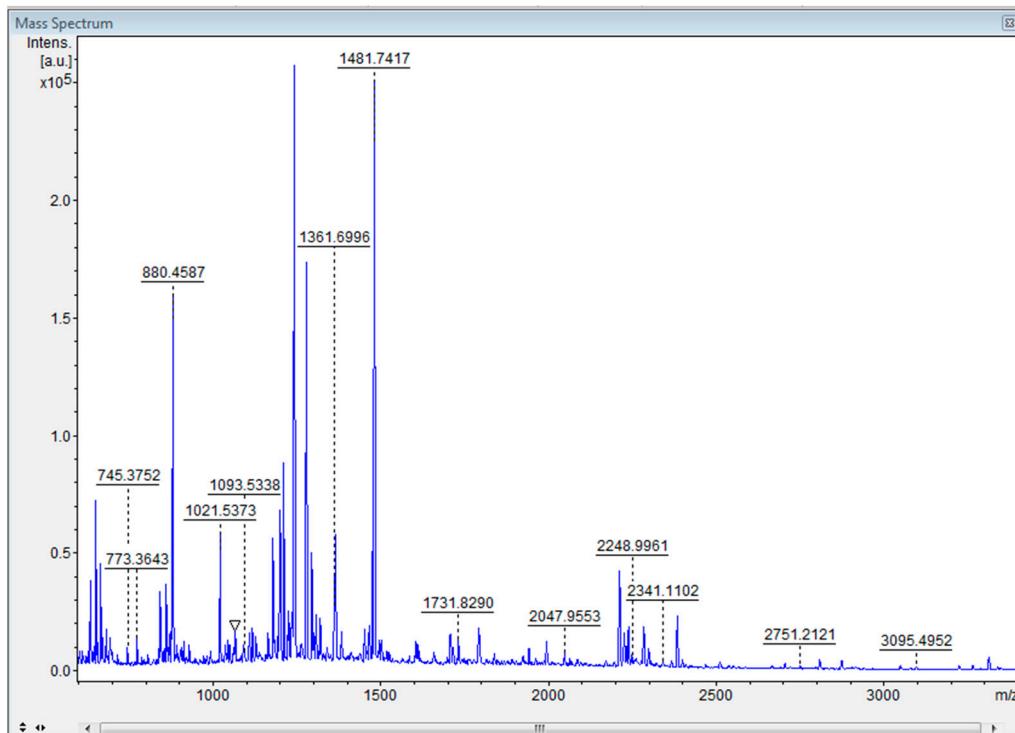


Figure 2. MALDI-TOF-MS1: Mass spectrogram obtained after tryptic digestion of band A.

The large enzyme ribulose bisphosphate carboxylase (RuBisCO), with a molecular mass of 52.574 kg/mol, was identified after comparing the masses of peptides arising from protein band digestion with the molecular masses of peptides from protein cleavage available in the SwissProt database using MASCOT software.

Figure 3 indicates a greater homology (score = 80) of the microalgae *Tetraedesmus obliquus* and the microalgae *Acutodesmus obliquos*, which belong to the same genus as the organism studied, *Tetraedesmus sp.* [29].

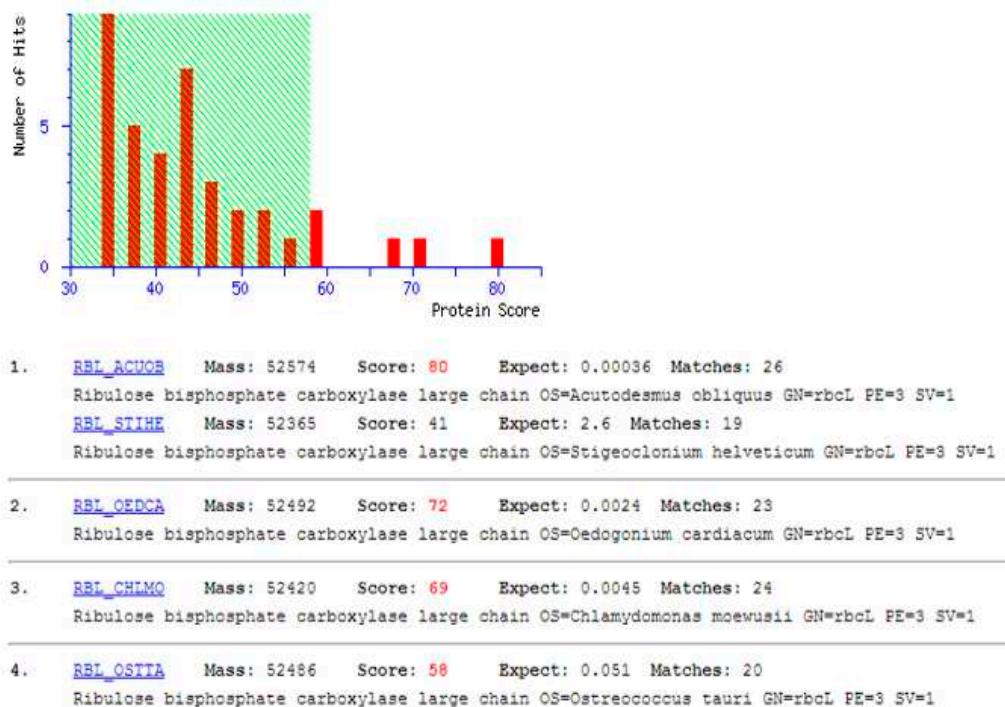


Figure 3. Results of the SwissProt database (MASCOT software) concerning the studied *Tetraedesmus obliquus* protein.

Figure 4 (A-D) shows the morphological photomicrographs of the microalgae cell after mechanical disruption, in which Figure 4A represents the control sample, i.e., the microalgae cells without mechanical disruptions. The control sample showed major cells with a green intact ellipsoidal appearance. Ball mill samples presented grouped cells with a major green color, making it impossible to count the individual cells. High-pressure homogenization showed green and red cells, reaching $78.51 \pm 1.97\%$ of ruptured cells, while ultrasound samples showed the highest number of disrupted cells ($80.17 \pm 0.54\%$).

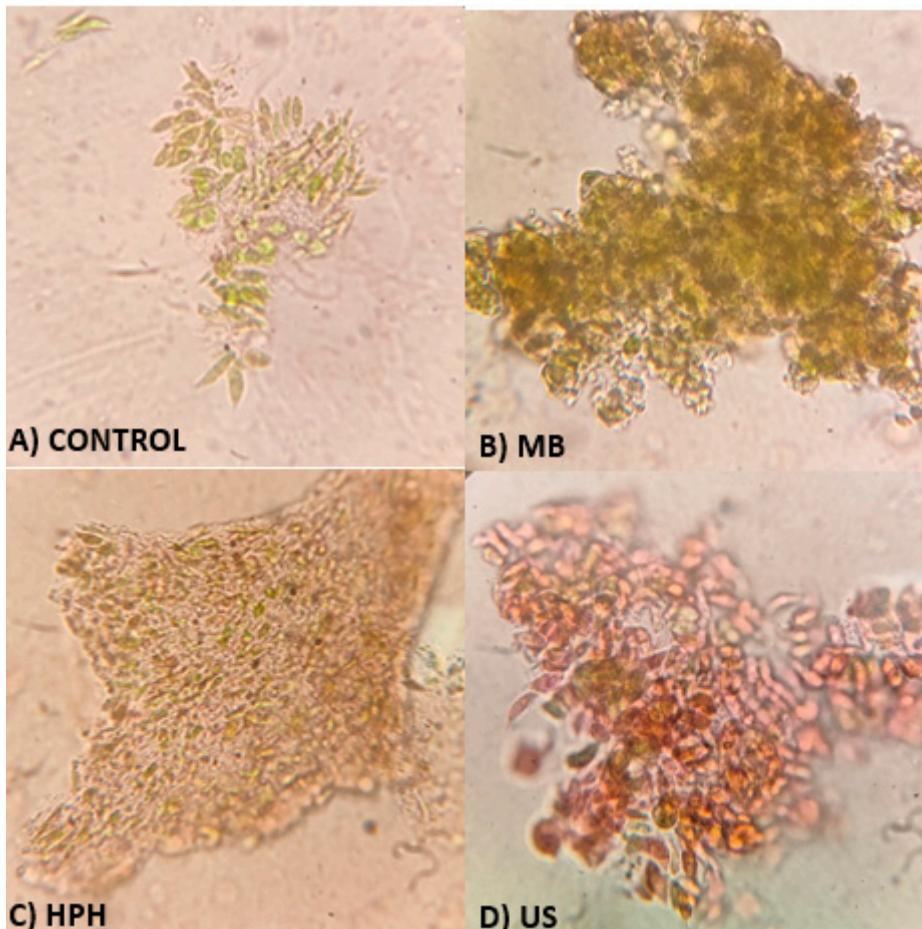


Figure 4. Optical microscopy images of ruptured *T. obliquus* cells at 100X magnification. (A) Control sample (reference: without application of treatment for cell disruption). The samples were treated with (B) a ball mill (BM), (C) high-pressure homogenization (HPH), and (D) ultrasound (US). Intact cells appeared green and/or magenta, while dead or damaged cells were stained red.

Figure 5 shows the protein recovery in each extraction for the ultrasound (US), high-pressure homogenization (HPH), and ball mill (BM) treatments. The highest value of total protein extraction efficiency was obtained for US disruption (19.95%), while HPH and BM showed similar protein recoveries of 15.68% and 14.11%, respectively, with no significant difference between them (p value ≤ 0.05). The main protein separation occurred in the first extraction stage. It was highest for the US treatment (16.02%), and HPH and BM showed similar protein separation levels of 10.87% and 10.37%, respectively. In the second extraction, 2.47% to 3.22% of proteins were separated from the biomasses, while in the third extraction, 0.77% to 2.09% of proteins were separated from the biomasses.

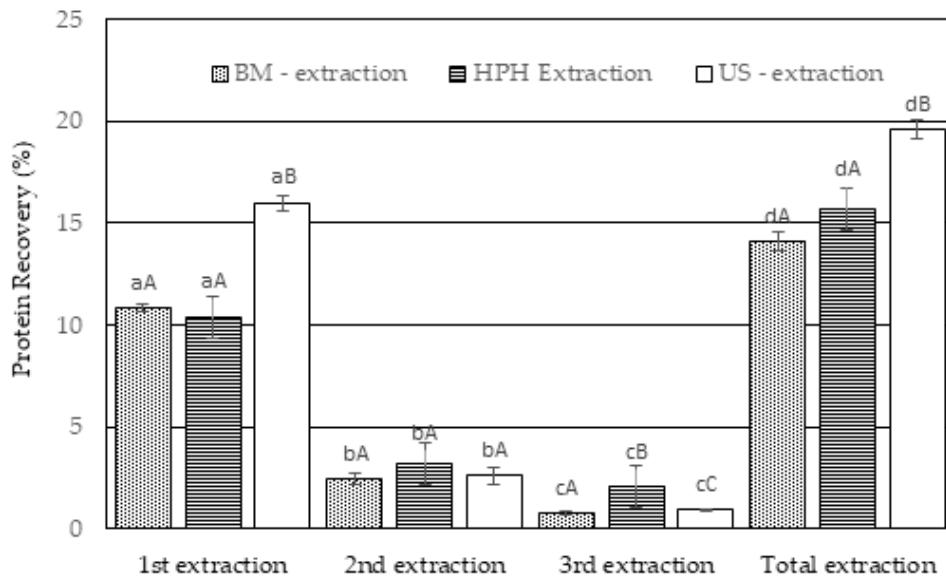


Figure 5. Protein amount recovered (Equation (3)) after *T. obliquus* cell breaking as a percentage of the total protein content. Disruption treatments of biomass: ball mill (BM), high-pressure homogenization (HPH), and ultrasound (US). Different letters indicate a significant difference ($p < 0.05$). Capital letters indicate differences among cell disruption treatments in the same extraction step. Lowercase letters indicate the difference between each extraction step and the total extraction for the same cell disruption treatment. The results are expressed as the mean, and bars indicate the standard deviation ($n = 3$).

Table 1 shows the results for protein mass yield (%) (g of protein of powder extract/100 g of dried microalgae), % of protein of extracts (g of protein of powder extract/100 g of extract), the visual appearance of protein extracts, and colorimetric parameters of protein extracts. The mass yields of the US, HPH, and BM treatments were 20.8%, 17.1%, and 16.1%, respectively. All samples showed lightness with a yellow tone (b+) and red traces (a+). The protein extract obtained from the HPH treatment showed the highest color parameters. Its lightness and yellow color can also be observed in visual appearance images.

Table 1. Protein mass yield % (g of protein of powder extract/100 g of dried microalgae), % protein of extracts (g of protein of powder extract/100 g of extract), color parameters for protein extracts: L* denotes lightness and ranges from 0 (black) and 100 (white), a(+) red, a(-) green, b(+) yellow, and b(-) blue; and visual appearance.

Characteristics	Ball mill	High-pressure homogenization	Ultrasound
Protein mass yield (g/100 g)	16.1 ± 1.3^a	17.1 ± 1.4^b	20.8 ± 0.8^a
% Protein content (g/100 g)	29.7 ± 1.2^a	29.1 ± 0.6^a	30.7 ± 0.4^b
L*	46.4 ± 0.46^a	52.0 ± 0.76^c	46.1 ± 0.26^a
A	4.73 ± 0.11^a	5.53 ± 0.17^c	4.98 ± 0.096^b
B	13.6 ± 0.40^a	17.6 ± 0.30^c	11.8 ± 0.29^b
Visual appearance			

Means followed by the same letters in the same line do not differ according to the Tukey test at 5% probability.

Figure 6 shows the microstructure of microalgae protein powders on SEM analysis. The samples showed a variety of particle types, including irregular particles, elongated particles, and oversized particle agglomerates sticking to each other. Pores were not observed on the particle surface. BM protein powder (Figures 6A and 6D) showed sheet-like structures with large particle sizes, irregular geometry shapes, and particle sizes larger than those of HPH (Figures 6B and 6E) and US (Figures 6C and 6F) protein powders.

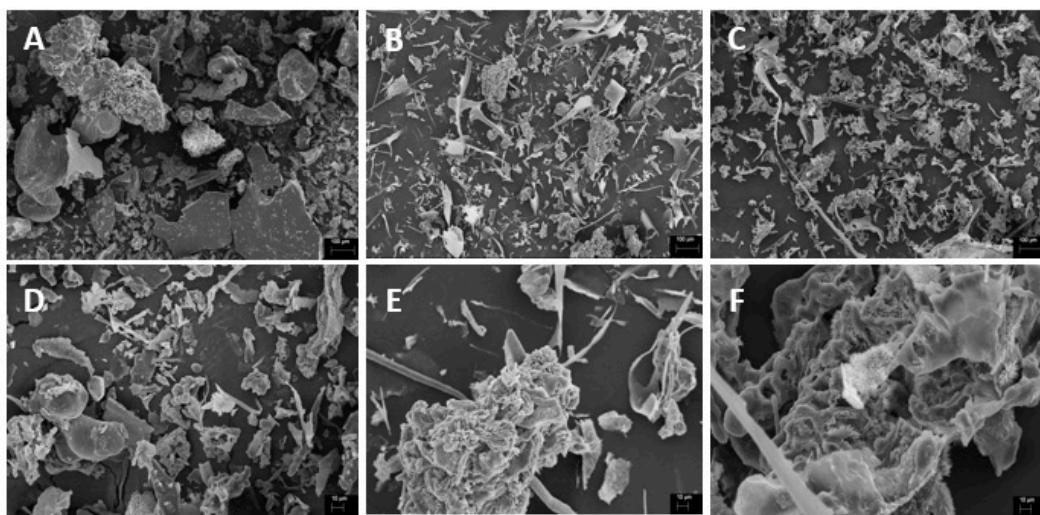


Figure 6. Scanning electron micrographs for protein extracts obtained by mechanical disruption by ball mill (BM) treatments with magnifications of 200 \times (A) and 1000 \times (D); high-pressure homogenization (HPH) with magnifications of 200 \times (B) and 1000 \times (E); ultrasound (US) with magnifications of 200 \times (C) and 1000 \times (F).

4. Discussion

4.1. Composition of *T. obliquus* biomass

The microalgal biomass showed a high content of proteins ($40.29 \pm 0.24\%$ w/w), agreeing with previous literature data for *Tetraselmis spp.* [13,19,24,30]. Thus, *T. obliquus* biomass is a strategic ingredient for the food industry, both in nutritional terms [3] and as a raw material for obtaining protein isolates and concentrates [24,30].

The high ash content (18.33 ± 0.03 w/w) is probably due to the absorption by the organisms of substantial amounts of minerals available in culture media [17]. Since minerals can somewhat negatively affect the applicability of biomass and protein concentrates, the mineral content may be reduced. A suggestion is adding washing steps into biomass production to solubilize salts, especially phosphates and calcium [31].

The observed low lipid content ($4.23 \pm 0.21\%$), compared with reported literature for *T. obliquus* lipid contents [13,16,30], is probably due to the culture media composition and harvest conditions [13]. Carbohydrates were the second major component in biomass (29.28%), including fiber and glycogen. The total carbohydrate content was obtained using the difference between 100% and the other compound contents (proteins, moisture, ash, and lipids); thus, this value considers the sampling error found in other analyses.

4.2. Identification of *T. obliquus* protein extract

The electrophoretic bands with higher intensities corresponded to proteins with molecular masses between 45 and 66.2 kDa (Figure 1). The greatest intensity was close to 60 kDa. Bands with lower intensity were also observed, as indicated by the arrows in Figure 1, corresponding to molecular masses of 147.61 kDa, 126.53 kDa, 106.52 kDa, 102.72 kDa, and 83.34 kDa. The bands for molecular masses greater than 200 kDa represent protein aggregates. The diversity in protein

composition can be explained by the fact that microalgae do not accumulate distinct proteins as an N source [32].

The large enzyme RuBisCO was identified as the band between 45 and 66.2 kDa with the help of MALDI-TOF MS. MALDI-TOF MS is a mass spectrometric technique widely used in the chemotaxonomy of microorganisms, allowing the analysis of proteins, peptides, and other biomolecules due to its low ionization energy. The chemotaxonomy is mainly based on the differences in the spectral patterns obtained for each organism [34]. Protein identification is considered valid if more than two peptides are matched and the MASCOT software score is greater than, or equal to, the significance threshold ($p < 0.05$) [35].

Among the identified proteins, Figure 3 indicates a greater homology (score = 80) of the microalgae *T. obliquus* and the microalgae *Acutodesmus obliquos* than with other organisms listed in the protein ranking. The microalga *Acutodesmus obliquos* belongs to the same genus as the organism studied, *Tetradesmus* sp. [29].

RuBisCO is present in plastids and chloroplasts of plants, algae, and cyanobacteria. It catalyzes two reactions: the carboxylation of D-ribulose 1,5-bisphosphate, the primary event in carbon dioxide fixation, and the oxidative fragmentation of the pentose substrate in the photorespiration process. Both simultaneous reactions compete for the same active site [33].

4.3. Cell rupture and protein extraction from *T. obliquus*

- Evaluation of the cell disruption level through cell counting

The optical microscopic examination of cells is a common technique to measure the cell breaking level. Figure 4A shows the control sample used in the disruption studies. It comprises cells of *T. obliquus* not subjected to any disruption treatment, also named original *T. obliquus* cells in the present work. The control sample showed an ellipsoidal shape, with a concave or linear fusiform contour and cells grouped in numbers of 4 or 8, the cenobia. Even the control sample showed the presence of cells with a reddish color, suggesting that some of the cells may have been damaged or ruptured. This behavior is probably due to cell disintegration and membrane permeabilization of microalgal species with a rigid cell wall, such as *T. obliquus* [19], during freeze-drying [36].

A ball mill (BM) is a simple method for disrupting the cell walls of different microorganisms [37]. However, Figure 4B indicates that the BM treatment did not effectively disrupt the microalgal cells. The biomass submitted to BM treatment showed strong agglomeration. In the BM treatment, the microalgal biomass was placed inside a closed chamber in the presence of small steel spheres with vigorous agitation. Due to shear force, kinetic energy is transferred to biomass to break the cells. This behavior hinders the dye's permeation in the medium, making differentiating intact and damaged cells impossible. Furthermore, it was not possible to determine the percentage of ruptured cells.

Bunge et al. [38] observed complete cell disruption of the bacterium *Arrhrobacter* sp. in a stirred BM, in which the enzymes were released without any degradation by using small grinding balls. The energy for cell rupture is well utilized when the disintegration rate correlates with a specific energy. Schuller et al. [39] used BM to extract lutein and β -carotene from wet and lyophilized biomass of *Tetraselmis* sp. The break with wet biomass glass beads best responded to the extractive treatments. However, the literature reported promising results for BM mechanical disruption with dried biomass [13,16].

The high-pressure homogenization (HPH) of the microalgal cells revealed significant differences between the treatment and control samples (Figure 4C) and broken fragments (parts with a redder color) of the cell wall. The percentage of ruptured cells reached $78.51 \pm 1.97\%$. In the HPH process, cell disruption is achieved through the high-pressure impact (shear forces) of the accelerated fluid jet on the homogenizer stationary valve surface and hydrodynamic cavitation from the pressure drop-induced shear stress [10]. Thus, HPH is especially suitable for emulsification processes.

Dias et al. [12] used HPH, followed by solvent extraction, to simultaneously extract lipids and carotenoids from *Rhodospiridium toruloides* biomass without any harvesting technique. The combined use of HPH and solvent extraction promoted higher extraction than conventional solvent extraction.

Günerken et al. [10] argue some disadvantages of the HPH process, such as nonselective intracellular compound releases, difficulties in breaking rigid cell walls, generation of superfine cell debris, not a mild technique, and inability to isolate fragile functional compounds. However, among the different cell disruption techniques, HPH is easily scalable.

Figure 4D shows that the ultrasound (US) treatment exhibited the highest number of red cells compared to the BM and HPH treatments, suggesting that US effectively disrupted the microalgal biomass. The percentage of ruptured cells was $80.17 \pm 0.54\%$. Ultrasound is a physical treatment based on bubble cavitation that uses sound waves to propagate pressure fluctuations, induces cavitation, and promotes nonspecific cell-surface barrier disruption [40]. The wall structure and size of the cells are critical factors affecting cell disruption efficacy in ultrasonic processing. Concerning the wall structure, microalgae with cellulose carbohydrate-based cell walls typically show more resistance against ultrasound than cell walls mainly composed of hydroxyproline-rich glycoproteins [41]. According to Do Carmo Cesário et al. [19], the *T. obliquus* cell wall is filled predominantly with fibrous material and has three well-defined layers, making breaking down even more challenging.

Spiden et al. [42] used the cell counting technique to evaluate *Saccharomyces cerevisiae* cell disruption after HPH treatment. According to the authors, an interval between 10 and 30 min was necessary to perform the cell count, depending on the sample volume and the cell concentration. Due to cell debris, reliable automated cell counting is not always possible. These observations show that a study of cell counting is insufficient to understand cell disruption, and an effective technique for monitoring and measuring the impact of treatment on microalgae cell disruption is necessary. Thus, protein extraction yield under different rupture treatments is another parameter to evaluate cell breaking.

- Evaluation of the cell disruption level through the amount of extracted soluble protein

The resistance of cell walls to disruption is a barrier hindering the efficient removal of intracellular components and may interfere with the accuracy of compound quantification [39]. Thus, the more intracellular compounds recovered after cell breaking, the greater the efficiency of the disruption method. According to Safi et al. [43], the amount of protein in the aqueous supernatant was a technique appropriate for evaluating the degree of cell disruption in three species of microalgae: *M. aeruginosa*, *C. pyrenoidosa*, and *C. reinhardtii*.

According to Figure 5, the number of extraction steps positively affected protein recovery. The ultrasound treatment resulted in the highest total protein extraction yield (19.95%). This result agrees with the behavior of cell disruption observed by optical microscopy after US treatment, in which most cells were red-colored, and the number of disrupted cells (80.17%) was the highest. Therefore, the efficiency of US treatment is evident in disrupting the cell wall, allowing protein extraction from microalgae cells.

The US treatment for cell disruption is based on the emission of high-frequency wave sounds (up to 15–20 kHz) in liquid. These sound waves create gas bubbles that achieve a critical size, collapsing and releasing large amounts of energy [44]. Cells adjacent to collapsing cavitation bubbles are broken, while cells located farther away from bubble cavitation also experience a smaller local energy flux [45]. Thus, the cell disruption power extends beyond the effect on the cell wall, reaching other microalgae cell organelles.

Furthermore, it is emphasized that acoustic cavitation occurs by increasing the local temperature [44], and this temperature rise might destroy target compounds, especially proteins. As proteins can be used as a techno-functional ingredient in food systems, the protein extraction process must occur without drastic conditions that could make them nonfunctional [9]. González-Fernandez et al. [46] reported temperatures up to 85 °C when 100.7 MJ/kg of energy was supplied to *Scenedesmus* biomass through US treatment for 15 min. The authors suggested that thermal effects might have accounted for cell disruption of *Scenedesmus* biomass. US treatment needs to be optimized to avoid thermal overexposure of biomass; once cell disruption is achieved, more energy is absorbed or scattered by the cell debris [47]. Cooling is usually used to prevent overexposure during or after ultrasound operation.

Recently, Delran et al. [11] verified that a low-frequency ultrasound of 20 kHz was adequate for breaking *Tetraselmis suecica* cells at a power of 120 W and 60 min ultrasonication, allowing the extraction of 90% of the total proteins. Compared with the literature, the low protein extraction (19.95%) observed in our US study could be explained by the intensity of cell rupture. Do Carmo Cesário et al. [19] observed the presence of proteins in the nucleus and cytoplasmic regions of *T. obliquus* by histochemistry tests. Therefore, mechanical treatments should collapse different parts of microalgae cells to achieve protein extraction.

Lupatini et al. [48] studied the US-assisted extraction of algal proteins from *Spirulina platensis*. The authors found that sonication degraded the cell wall entirely or partially, providing a valuable technique to extract proteins and carbohydrates. The optimized percentage of protein extracted was 75.76%. The ultrasound bath conditions used were 30 °C, 37 Hz, and 100% sonication amplitude for 35 min of sonication. Keris-Sem et al. [49] applied US treatment to disintegrate microalgal cells and extract components other than protein. The authors achieved higher protein, carbohydrate, and lipid extraction efficiency using an ultrasonic energy intensity of 0.4 kWh/L. Soto Sierra et al. [50] achieved a maximized yield of *Chlamydomonas reinhardtii* protein using autolysin coupled with sonication.

The results observed in Figure 5 show that the amounts of protein extracted from *T. obliquus* with HPH and BM treatments were lower than that achieved with US treatment.

Katsimichas et al. [51] reported protein extraction from *Chlorella pyrenoidosa* after HPH treatment. The application of 800 bar of pressure and a four-pass treatment caused maximization of protein recovery of 382.0 mg proteins/g dry biomass. In our study, the pressure was lower, at 350 bar. Safi et al. [43] noted HPH as a more efficient cell disruption technique than manual grinding, ultrasonication, and alkaline treatment for extracting proteins from *Nannochloropsis oculata*, *Chlorella vulgaris*, and *Haematococcus pluvialis*, a green microalgae with rigid cell walls. The biomass at 2% dry weight was disrupted by HPH working with two passes at 2700 bar.

In our tests, HPH effectively broke the *T. obliquus* cell wall, as demonstrated by optical microscopy (Figure 4C) and by disrupted counting (78.51%). Additionally, the HPH protein extract showed a remarkable light color (Table 1), showing potential for food ingredient application. Most likely, fewer chlorophyll pigments were extracted in the HPH mechanical treatment. HPH allowed greater protein extraction and was ineffective in breaking other microalgae organelles, suggesting that higher pressures can contribute to greater protein recovery efficiency [43].

Zhang et al. [14] studied the effect of US, HPH, and their combination on the efficiency of biomolecule extraction from *Parachlorella kessleri*. The authors verified that applying a preliminary US treatment with 10% dry matter and a final HPH treatment with 1% dry matter increases extraction efficiency and decreases energy consumption.

Since parts of the cellular fragments came together and formed large aggregates, it was not possible to quantify the disruption effect with ball mill treatment using the cell counting method; however, it was possible to determine the disruption effects through the extraction of soluble proteins. The protein extraction yield of *T. obliquus* with BM treatment was 14.11% (Figure 5), in which the protein showed particles with larger sizes and irregular shapes, including sheet-like structures, as seen in Figure 6. The changes in morphology could significantly affect the functional properties of microalgae protein extract. Despite the low yield found, ball milling and HPH are the preferred methods for industrial-scale microalgae cell disruption [10].

5. Conclusions

The content of extracted soluble protein was a reliable indirect index to measure the level of cell disruption. Optical cell counting was not an optimal indicator to monitor cell disruption, although it is a common technique for determining cell concentration in microbial cultures. Microalgal disruption under different mechanical treatments could be evaluated using the extracted protein content combined with the cell counting technique. Ultrasound treatment performed better in microalgal cell breaking than high-pressure homogenization and ball mill treatments. However, the efficiency of the extraction of protein must be improved. Combining conventional techniques, e.g.,

ultrasonication with high-pressure homogenization, may reduce the energy demand of mechanical disruption methods and provide better protein extraction yield.

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