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Aqueous extraction of proteins from microalgae: Effect of different cell disruption methods

Carl Safi^{a,b,*}, Alina Violeta Ursu^c, Céline Laroche^c, Bachar Zebib^{a,b}, Othmane Merah^{a,b}, Pierre-Yves Pontalier^{a,b}, Carlos Vaca-Garcia^{a,b,d}

^a Université de Toulouse, INP-ENSIACET, LCA (Laboratoire de Chimie Agro-industrielle), F-31030 Toulouse, France

^b INRA, UMR 1010 CAI, F-31030 Toulouse, France

^c Université Blaise Pascal, Polytech, Institut Pascal, UMR CNRS 6022, 24, Avenue des Landais 63174 Aubière, France

^d King Abdulaziz University, Jeddah, Saudi Arabia

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ABSTRACT

The microalgal structure has been investigated to evaluate the release of proteins in aqueous media from five microalgae after conducting different cell disruption techniques: manual grinding, ultrasonication, alkaline treatment, and high-pressure treatment. After conducting cell disruption, the protein concentration in water was determined for all the microalgae and the results are discussed within the context of their cell wall structure. It was found that the aqueous media containing most protein concentration followed the order: high-pressure cell disruption > chemical treatment > ultrasonication > manual grinding. Fragile cell-walled microalgae were mostly attacked according to the following order: *Haematococcus pluvialis* < *Nannochloropsis oculata* < *Chlorella vulgaris* < *Porphyridium cruentum* ≤ *Arthrospira platensis*.

1. Introduction

Microalgae were first exploited for their capacity to accumulate proteins and, through time, interest in this biomass took a new course especially during the last two decades with increasing demand for sustainable energy. This biomass proved to be an important source of lipids suitable for biodiesel production. Hence, many of the studies were concentrated on lipid extraction for fuel purposes, neglecting the potential of microalgae to produce proteins and other high-value components [1]. However, until now all studies and estimates confirmed that costs of production of biodiesel from microalgae remain high [2,3] and far from being competitive with fossil fuel. Researchers are therefore turning towards valuing other components present in the microalgae such as proteins, pigments, dyes, sugars, etc.

Extracting the totality of a specific component from microalgae is often prevented by the intrinsic rigidity of its cell wall. To overcome this barrier, an initial operation unit of cell disruption is required to permit complete access to the internal components and facilitate the extraction process. Hence, many cell disruption techniques have been tested to break the cell wall of microalgae such as bead milling [4,5], ultrasonication [6–8], microwave radiation [9], enzymatic treatment [10,11], cell homogenizer [12] and high-pressure cell disruption [13]

to recover different components. The efficiency of cell disruption was usually evaluated by extracting a single component especially lipids before and after applying the treatment or by microscopic observation. To our knowledge, studies of microalgal proteins have been focused on: evaluating the nitrogen to protein conversion factor [14–18]; finding the best method to analyze proteins and differentiate between soluble and non-soluble proteins [19]; and analyzing the behavior of proteins at the air/water interface [20].

Therefore, the present study focuses on evaluating the effect of different cell disruption techniques on protein extractability in water of five different microalgae having different cell wall macrostructures. Namely, the Cyanobacterium *Arthrospira platensis*, which has a relatively fragile cell wall, composed mainly of murein and no cellulose [21,22]. The Chlorophycean *Chlorella vulgaris* and the Eustigmatophyceae *Nannochloropsis oculata*, which have a cell wall mainly composed of cellulose and hemicelluloses [23]. Another Chlorophycean *Haematococcus pluvialis* has a thick trilaminar cell wall composed of cellulose and sporopollenin [12,24,25]. The composition of its cell wall, similar to that of spores, makes this microalga less permeable and extremely resistant to mechanical treatments [26]. Finally, the Rodophythe *Porphyridium cruentum*, which lacks a true cell wall, but instead is encapsulated by a layer of sulfurized polysaccharides [27–32].

In addition, the microalgae selected in this study have a cytoplasm containing soluble proteins, and they all have a chloroplast except for *A. platensis*, which instead has thylakoids bundles circling the peripheral part of the cytoplasm with their associated structures, the phycobilisomes

* Corresponding author at: Université de Toulouse, INP-ENSIACET, LCA (Laboratoire de Chimie Agro-industrielle), F-31030 Toulouse, France. Tel.: +33 6 50 45 29 65.

E-mail address: carl.safi@ensiacet.fr (C. Safi).

(containing the phycobiliproteins) present on the surface of the thylakoids like in the chloroplast of *P. cruentum* [21]. Furthermore, the chloroplast also contains soluble proteins and a central pyrenoid, which is a non-membrane, bound organelle composed of RuBisCO.

In this study, proteins released in the aqueous media were evaluated and discussed considering the cell wall macrostructure of each microalga along with the effect of each cell disruption technique used.

2. Materials and methods

2.1. Microalgae

The microalgae selected are the Cyanobacteria *Arthrospira platensis* (strain PCC 8005), two different Chlorophyceae *Chlorella vulgaris* (strain SAG 211–19), and *Haematococcus pluvialis* (unknown strain), one Rhodophyta *Porphyridium cruentum* (strain UTEX 161), and the Eustigmatophyceae *Nannochloropsis oculata* (unknown strain).

Each microalga was cultivated in a different culture medium. Hemerick medium was used for *P. cruentum*, Sueoka medium for *C. vulgaris*, Basal medium for *H. pluvialis*, Conway medium for *N. oculata* and Zarrouk medium for *A. platensis*. All strains were grown in batch mode in a 10 L indoor tubular air-lift photo-bioreactor (PBR at 25 °C [33]) inoculated from a prior culture in a flat panel air-lift PBR (1 L). Culture mixing was achieved by sterile air injection from the bottom of the PBR. The pH and temperature were recorded by a pH/temperature probe (Mettler Toledo SG 3253 sensor), and monitored by the acquisition software LabVIEW. The pH was regulated at 7.5 with CO₂ bubbling. Microalgae were harvested during the exponential growth phase and concentrated by centrifugation, and then supplied as frozen paste from Alpha Biotech (Asserac, France). The biomass concentration of the paste was 20–24% dry weight.

2.2. Reagents

The Lowry kit (a prepared mixture of Lowry reagent, BSA standards and 2 N Folin-Ciocalteu reagents) was from Thermo Scientific. NaOH granules and 37% HCl were purchased from Sigma Aldrich and used as received.

2.3. Microalgae pre-treatment

2.3.1. Freeze-drying

The frozen paste of crude microalga (about 70 g) was directly introduced to a Fisher Bioblock Scientific Alpha 2–4 LD Plus device (Illkirch, France). The pressure was reduced to 0.010 bar and the temperature was further decreased to –80 °C and freeze-drying was conducted under vacuum for 48 h. Dry biomass was stored under anhydrous conditions. Before any disruption treatment, the cells were vigorously rehydrated in distilled water to ensure good homogeneity of the sample.

2.4. Microalgae treatments

2.4.1. Control

Cells (0.5 g) were dispersed for 2 h in 25 mL distilled water and the supernatant was recovered by centrifugation at 10,000 g for 10 min at 20 °C for protein analysis. This treatment was considered as a blank to compare with the other extraction treatments.

2.4.2. High-pressure cell disruptor

A TS Haiva series, 2.2-kW, disrupter from Constant Systems Limited (Northants, UK), was applied, in two passes at a pressure of 2700 bar, to a biomass sample at a concentration of 2% dry weight (0.5 g of dry cells dispersed in 25 mL distilled water).

2.4.3. Ultrasonication

This treatment was carried out using a VC-750HV (20 kHz, 13 mm probe) ultrasonic processor on 0.5 g of dry cells dispersed in 25 mL distilled water. Total treatment time was 30 min in cycles of 5 s of ultrasonication and 15 s of resting time in order to prevent overheating the sample.

2.4.4. Manual grinding

Dry microalgae were manually ground using a mortar for 5 min, and then 0.5 g was dispersed in 25 mL distilled water for 2 h. Samples were taken for protein analysis.

2.4.5. Chemical treatment

Mother solutions were prepared with approximately 500 mL of distilled water and 2 N NaOH was added to adjust the solution to pH 12 for maximum protein solubility. A sample of 0.5 g of freeze-dried biomass was added to 25 mL of mother solution. The mixture was then stirred for 2 h at 40 °C. The separation of the supernatant from the pellet was conducted by centrifugation at 10,000 g for 10 min at 20 °C. The supernatant was then adjusted to pH 3 with 0.1 M HCl in order to precipitate the proteins. The protein isolate was collected after centrifugation at 10,000 g for 10 min at 20 °C and the pellet was neutralized with 0.01 M NaOH [20]. Samples were taken for protein analysis.

2.5. Lowry method

After every disruption treatment, the liquid/solid separation was conducted by centrifugation at 10,000 g for 10 min at room temperature and the supernatant was analyzed by the Lowry method. [34]

A calibration curve was prepared using bovine standard albumin at a concentration range of 0 to 1500 µg mL⁻¹. In order to measure the protein content, 0.2 mL of each standard or samples containing the crude protein extract was withdrawn and then 1 mL of modified Lowry reagent was added to each sample. Each sample was then vortexed and incubated for 10 min. After incubation, 100 µL of Folin-Ciocalteu Reagent (1 N) was added and again vortexed and incubated for 30 min. The blue color solution was then measured at 750 nm with a UV-1800 Shimadzu spectrophotometer.

2.6. Elemental analysis

Total nitrogen was evaluated by using a Perkin Elmer 2400 series II elemental analyzer. Microalgal samples (2 mg) were placed in tin capsules and heated at 925 °C, using pure oxygen as the combustion gas and pure helium as the carrier gas, and the nitrogen concentration was evaluated. For all the previous analyses, three experiments were conducted separately with all the microalgae.

2.7. Confocal laser scanning microscopy

Cells were observed with an SP2-AOBS confocal laser-scanning microscope from Leica microsystems (Nanterre-France). The fluorochrome calcofluor white that binds to the cell wall was added to the samples. When excited at 488 nm, the cells are identified as light blue colored.

2.8. Statistical analysis

Three experiments were conducted separately on all microalgae and their protein extract. Statistical analyses were carried out on Microsoft Excel 2011 and Statgraphics Sigma Express. ANOVA test was carried out and measurements of three replicates for each sample were reproducible for ± 5% of the respective mean values.

Table 1

Protein and nitrogen content for each microalga based on three replicates for three experiments \pm SD ($n = 9$).

| Microalga | Total nitrogen (%dw ⁻¹) | Total proteins (% dw ⁻¹) |
|---------------------|-------------------------------------|--------------------------------------|
| <i>H. phuvialis</i> | 8.27 \pm 0.08 | 51.7 \pm 0.43 |
| <i>N. oculata</i> | 7.41 \pm 0.40 | 46.5 \pm 2.14 |
| <i>C. vulgaris</i> | 7.81 \pm 0.18 | 49.6 \pm 1.04 |
| <i>A. platensis</i> | 8.53 \pm 0.20 | 53.5 \pm 1.10 |
| <i>P. cruentum</i> | 9.04 \pm 0.70 | 57.3 \pm 3.84 |

3. Results

The total protein in crude microalgae was determined by obtaining total nitrogen through elemental analysis and converting it into protein percentage using the conversion factor found for each crude microalga in the study conducted by Safi et al. [18]. In all cases, the total protein content was high, ranging from 46 to 57% dw (Table 1).

The fraction of soluble proteins released in water after each cell disruption technique is presented in Fig. 1. The fraction of soluble protein in the total protein present in the microalgae was also evaluated and all these results are given in Table 2.

In this work, four cell disruption techniques are compared, along with a control in distilled water, in order to evaluate protein exiting by diffusion through the membranes and walls into water media. The recovery yield in the control ranges from 6.5% dw with *H. phuvialis* to 25% dw with *P. cruentum*. The latter alga is considered as fragile and the former as resistant to disruption. Among the tested techniques, high-pressure cell disruption was the best technique for all the microalgae, with a recovery yield of 41% to 90% dw. Moreover, the lowest protein concentration for all microalgae was obtained in the water control and through manual grinding, especially for rigid cell walled microalgae. A relative difference was noticed in the concentration of protein released between the microalgae with fragile and rigid cell walls. *P. cruentum* released the most compared to *A. platensis*. After ultrasonication a minor increase in protein concentration was noticeable for the green microalgae, especially for *C. vulgaris*, and a more important increase was observed for the *A. platensis* and *P. cruentum*. Furthermore, the chemical treatment showed a significant increase of protein released (*N. oculata* and *C. vulgaris* statistically released the same protein concentration) (Fig. 1).

In order to better interpret these results, microscopic observation was carried out. The laser scanning confocal microscopic images presented in Fig. 2 showed that in the cases of *P. cruentum* and *A. platensis* a total disruption of the cell wall occurred after high pressure cell

disruption. On the other hand, for *C. vulgaris*, *N. oculata* and *H. phuvialis*, only a majority of cells were completely disrupted, while a few cells maintained their globular form.

4. Discussion

The goal of the present study was to highlight the release of protein into aqueous media after the application of different cell disruption techniques. The results do not only rely on the mechanical rigidity of the cell wall of each microalga but also on its chemical characteristics. Indeed, having a deep understanding of the macrostructure is necessary in order to evaluate the release of components after any treatment was conducted on the cells. This approach has been considered in a study conducted by Jubeau et al. in order to selectively extract intracellular components such as proteins and phycoerythrin after cell disruption of *P. cruentum* [13]. Moreover, the freeze-drying process that conserves the samples well makes the protein extraction harder for some species [18]. In addition, after freeze-drying the cells are more aggregated, which lowers the contact surface with the extracting solvent, and also could affect the integrity of the cell wall in fragile species [35,36].

Osmosis is the net movement of solvent (water) molecules through a partially permeable membrane into a region of higher solute concentration. Water usually travels through the membrane, the vacuole, the chloroplast, and the mitochondria by diffusing across the phospholipid bilayer via water channels (aquaporins), which are proteins embedded in the cell membrane that regulate the flow of water. Hence, the water only treatment was not considered as a cell disruption technique, but it was carried out in this study as a reference control for the other techniques. Surprisingly, the dispersion of microalgae in water released up to 19–25% of soluble proteins per dry weight (Table 2) from *A. platensis* and *P. cruentum*, coloring the water light blue for the former and light red for the latter. This indicates that water penetrated the cell walls of both microalgae but also succeeded in penetrating the intra-thylakoids space of *A. platensis* and permeated the chloroplast of *P. cruentum* to slightly dissolve the phycobilisomes present on the thylakoid membranes. On the contrary, the osmosis phenomenon was not strongly effective for the green microalgae, which are known to have rigid cell walls that resist water permeating the structure and, thus, releasing only 6–10% proteins (Table 2).

Taking into account the standard deviation of three samples considered for the green microalgae (*C. vulgaris*, *N. oculata* and *H. phuvialis*), all the values of released proteins after water treatment and manual grinding shown in Fig. 1 are statistically equivalent, indicating again the resistance of their cell walls after manual grinding. This was not the case for the *A. platensis* and *P. cruentum* with stronger coloration of water

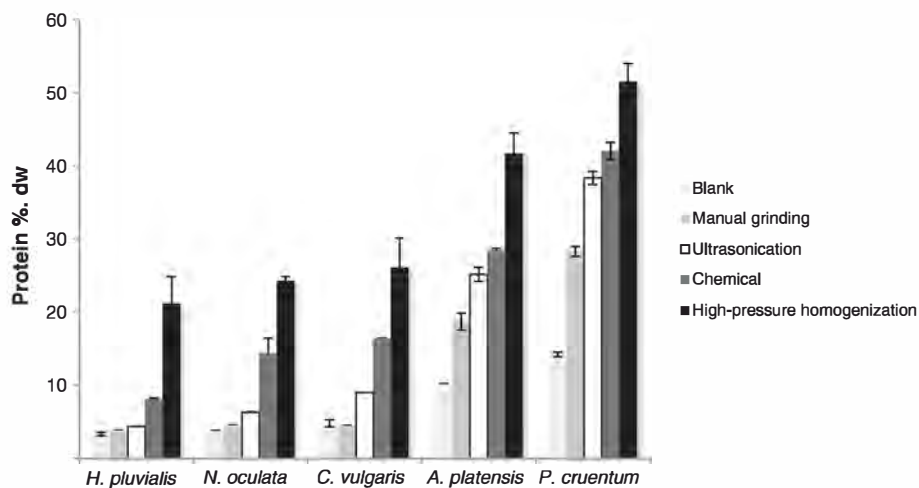


Fig. 1. Soluble protein concentration released in water after each cell disruption technique (\pm SD for three replicates in three experiments ($n = 9$)).

Table 2
Concentration of water-soluble protein from total protein released in the aqueous phase after cell disruption.

| Microalga | Protein yield (%) | | | | |
|---------------------|-------------------|-----------------|-----------------|------------|------------------------------|
| | Blank | Manual grinding | Ultrasonication | Chemical | High-pressure homogenization |
| <i>H. pluvialis</i> | 6.5 ± 0.2 | 7.4 ± 0.1 | 8.5 ± 0.0 | 15.8 ± 0.1 | 41.0 ± 3.7 |
| <i>N. oculata</i> | 8.1 ± 0.1 | 9.7 ± 0.0 | 13.5 ± 0.1 | 31.1 ± 2.0 | 52.3 ± 0.6 |
| <i>C. vulgaris</i> | 9.7 ± 0.5 | 9.0 ± 0.1 | 18.1 ± 0.0 | 33.2 ± 0.0 | 52.8 ± 0.6 |
| <i>A. platensis</i> | 19.0 ± 0.1 | 35.0 ± 1.2 | 47.1 ± 0.9 | 53.4 ± 0.2 | 78.0 ± 2.8 |
| <i>P. cruentum</i> | 24.8 ± 0.3 | 49.5 ± 0.7 | 67.0 ± 0.9 | 73.5 ± 1.2 | 90.0 ± 2.4 |

Based on three replicates for three experiments ± SD (n = 9).

Protein yield was calculated according to the following equation: proportion of hydrosoluble protein in total protein = $(P_{Lowry} / N_{ea} \times NTP) \times 100$ (%).

N_{ea} : total nitrogen in initial biomass (% dw) obtained by elemental analysis.

NTP: nitrogen-to-protein conversion factor from Safi et al. [18].

P_{Lowry} : water-soluble protein.

associated with increase in protein concentration, indicating that the internal structure of both microalgae is being further altered, and simultaneously facilitating the penetration of water to dissolve more proteins.

Ultrasonication produces cavitation in cells and facilitates cell disruption; it did not cause any change for *H. pluvialis*, but showed a minor effect on the cell wall of *N. oculata* and *C. vulgaris* by possibly making it difficult for water to extract cytoplasmic proteins without altering the structure of their chloroplast. The concentration of soluble proteins and coloration kept increasing for the fragile cell-walled microalgae by releasing 47–68% dw.

Chemical treatment was a key process that showed increases in protein concentration compared to the other treatments. *P. cruentum* lacks a well-defined cell wall. Since protein solubility is dependent on pH, cell wall characteristics and chemical composition [11], the high pH easily solubilized proteins without any resistance from its pseudo-cell wall. But in the case of the green microalgae, the sodium hydroxide is able to perform a process similar to mercerization, by penetrating the microcrystalline structure of the cellulosic cell walls of the green microalgae [18]. The alkaline solution can also easily dissolve the hemicelluloses attached to cellulose as it has been demonstrated during the refining of lignocellulosic substrates (straw, bran, and wood). In addition, it indicates that this treatment gave more access to the cytoplasmic proteins, and recovered the same concentration of proteins from *N. oculata* and *C. vulgaris* (Fig. 1). However, the sporopollenin contained in the most rigid cell wall (*H. pluvialis*) is known to be extremely resistant to chemical agents [26], which explains the low recovery of soluble proteins. *A. platensis* has a cell wall rich in amino sugars cross-linked with oligopeptide chains. The former are labile in alkaline conditions by deamidation of the *N*-acetylglucosamine and the latter are soluble in alkaline conditions. Therefore, the cell wall becomes permeable allowing the alkaline extraction of proteins [18]. Hence, all these results demonstrate that the chemical action acts in synergy with the mechanical characteristics of the cell wall.

High-pressure cell disruption was the most efficient technique for all microalgae; the concentration of proteins was statistically the same for the green microalgae, with evidence that the majority of the cells were broken while some of them remained intact (Fig. 2). The chloroplast of these species was also partially damaged, as it is revealed by the coloration in light green (chlorophyll) of the aqueous extract. Indeed, chlorophyll is a hydrophobic pigment; its presence in the aqueous phase indicates the formation of micellar structures and it points to a possible alteration of the chloroplast. The other indication is that some cell debris containing the green pigment were extremely reduced in size and did not precipitate in the pellet after centrifugation at 10000 g, leading to a greenish color of the supernatant as it occurred in previous work [7]. Hence, after two passes, water had access to cytoplasmic proteins and partially infiltrated the chloroplast. Therefore, this method released almost half of the proteins present inside the rigid cell-walled microalgae (Table 2), indicating again the resistance of their cell wall. On the other

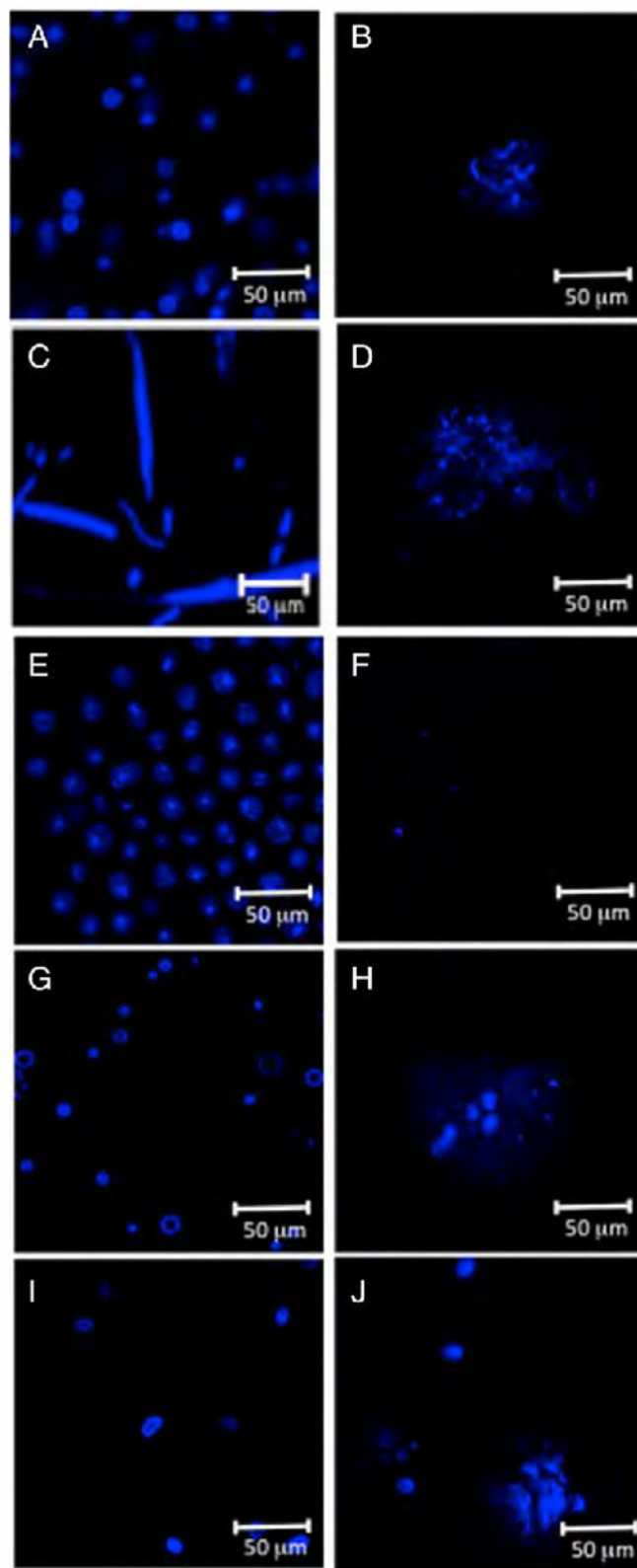


Fig. 2. Confocal laser scanning microscopy of five microalgae before (right) and after (left) high-pressure cell disintegration. (A and B) *C. vulgaris*, (C and D) *A. platensis*, (E and F) *P. cruentum*, (G and H) *H. pluvialis*, (I and J) *N. oculata*.

hand, as expected according to their fragile cell wall (Table 2), *A. platensis* and *P. cruentum* did not show much resistance, and the soluble protein concentration of total protein was 78% dw for the former and 90% dw for the latter (Table 2). Moreover, an important coloration

of the aqueous extract for both microalgae was observed with a pellet having lost its red coloration for *P. cruentum*. This was also supported by microscopic observation, showing that their structure was completely altered (Fig. 2).

The same order of rigidity was obtained in another study [18] that took into account the values of the nitrogen to protein conversion factors before and after protein extraction and then attributed them to the rigidity of the cell walls. This result shows that in order to compare the efficiency of cell disruption technology, it is more accurate to use fragile cell algae such as *P. cruentum*.

The present study shows additional insight into the understanding of the recovery of proteins after different cell disruptions. Hence, among all the techniques used the high-pressure cell disruption was the most efficient but not sufficient to recover more than 50% of the proteins for the green microalgae, indicating that more passes are required to completely disrupt their macrostructure, and thus more energy input will be necessary.

References

- [1] P.M. Foley, E.S. Beach, J.B. Zimmerman, Algae as a source of renewable chemicals: opportunities and challenges, *Green Chem.* 13 (2011) 1399–1405.
- [2] Y. Chisti, Biodiesel from microalgae, *Biotechnol. Adv.* 25 (2007) 294–306.
- [3] L. Rodolfi, G. Chini Zittelli, N. Bassi, G. Padovani, N. Biondi, G. Bonini, et al., Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor, *Biotechnol. Bioeng.* 102 (2009) 100–112.
- [4] J. Doucha, K. Livansky, Influence of processing parameters on disintegration of *Chlorella* cells in various types of homogenizers, *Appl. Microbiol. Biotechnol.* 81 (2008) 431–440.
- [5] J.Y. Lee, C. Yoo, S.Y. Jun, C.Y. Ahn, H.M. Oh, Comparison of several methods for effective lipid extraction from microalgae, *Bioresour. Technol.* 101 (Suppl. 1) (2010) S75–S77.
- [6] T. Furuki, S. Maeda, S. Imajo, T. Hiroi, T. Amaya, T. Hirokawa, et al., Rapid and selective extraction of phycocyanin from *Spirulina platensis* with ultrasonic cell disruption, *J. Appl. Phycol.* 15 (2003) 319–324.
- [7] J.A. Gerde, M. Montalbo-Lomboy, L. Yao, D. Grewell, T. Wang, Evaluation of microalgae cell disruption by ultrasonic treatment, *Bioresour. Technol.* 125 (2012) 175–181.
- [8] L. Gouveia, A.C. Oliveira, Microalgae as a raw material for biofuels production, *J. Ind. Microbiol. Biotechnol.* 36 (2009) 269–274.
- [9] H. Zheng, J. Yin, Z. Gao, H. Huang, X. Ji, C. Dou, Disruption of *Chlorella vulgaris* cells for the release of biodiesel-producing lipids: a comparison of grinding, ultrasonication, bead milling, enzymatic lysis, and microwaves, *Appl. Biochem. Biotechnol.* 164 (2011) 1215–1224.
- [10] J. Fleurence, The enzymatic degradation of algal cell walls: a useful approach for improving protein accessibility? *J. Appl. Phycol.* 11 (1999) 313–314.
- [11] Y.W. Sari, M.E. Bruins, J.P.M. Sanders, Enzyme assisted protein extraction from rapeseed, soybean, and microalgae meals, *Ind. Crop. Prod.* 43 (2013) 78–83.
- [12] M.M. Mendes-Pinto, M.F.J. Raposo, J. Bowen, A.J. Young, R. Morais, Evaluation of different cell disruption processes on encysted cells of *Haematococcus pluvialis*: effects on astaxanthin recovery and implications for bio-availability, *J. Appl. Phycol.* 13 (2001) 19–24.
- [13] S. Jubeau, L. Marchal, J. Pruvost, P. Jaouen, J. Legrand, J. Fleurence, High pressure disruption: a two-step treatment for selective extraction of intracellular components from the microalga *Porphyridium cruentum*, *J. Appl. Phycol.* 25 (2012) 983–989.
- [14] J.A. Gerde, T. Wang, L. Yao, S. Jung, L.A. Johnson, B. Lamsal, Optimizing protein isolation from defatted and non-defatted *Nannochloropsis* microalgae biomass, *Algal Res.* 2 (2013) 145–153.
- [15] C.V. Lopez, C. Garcia Mdel, F.G. Fernandez, C.S. Bustos, Y. Chisti, J.M. Sevilla, Protein measurements of microalgal and cyanobacterial biomass, *Bioresour. Technol.* 101 (2010) 7587–7591.
- [16] S.O. Lourenço, E. Barbarino, P.L. Lavín, U.M. Lanfer Marquez, E. Aidar, Distribution of intracellular nitrogen in marine microalgae: calculation of new nitrogen-to-protein conversion factors, *Eur. J. Phycol.* 39 (2004) 17–32.
- [17] S.O. Lourenço, E. Barbarino, U.M.L. Marquez, E. Aidar, Distribution of intracellular nitrogen in marine microalgae: basis for the calculation of specific nitrogen-to-protein conversion factors, *J. Phycol.* 34 (1998) 798–811.
- [18] C. Safi, M. Charton, O. Pignolet, F. Silvestre, C. Vaca-Garcia, P.-Y. Pontalier, Influence of microalgae cell wall characteristics on protein extractability and determination of nitrogen-to-protein conversion factors, *J. Appl. Phycol.* (2012) 1–7.
- [19] E. Barbarino, S.O. Lourenço, An evaluation of methods for extraction and quantification of protein from marine macro- and microalgae, *J. Appl. Phycol.* 17 (2005) 447–460.
- [20] I.S. Chronakis, A.N. Galatanu, T. Nylander, B. Lindman, The behaviour of protein preparations from blue-green algae (*Spirulina platensis* strain Pacifica) at the air/water interface, *Colloids Surf. A Physicochem. Eng. Asp.* 173 (2000) 181–192.
- [21] R.E. Lee, *Phycology*, 4th ed. New York, Cambridge University Press, Cambridge, England, 2008.
- [22] H.K. Lu, C.C. Hsieh, J.J. Hsu, Y.K. Yang, H.N. Chou, Preventive effects of *Spirulina platensis* on skeletal muscle damage under exercise-induced oxidative stress, *Eur. J. Appl. Physiol.* 98 (2006) 220–226.
- [23] M.F. Payne, R.J. Rippingale, Evaluation of diets for culture of the calanoid copepod *Gladioliferus imparipes*, *Aquaculture* 187 (2000) 85–96.
- [24] C. Aflalo, Y. Meshulam, A. Zarka, S. Boussiba, On the relative efficiency of two- vs. one-stage production of astaxanthin by the green alga *Haematococcus pluvialis*, *Biotechnol. Bioeng.* 98 (2007) 300–305.
- [25] A. Montsant, A. Zarka, S. Boussiba, Presence of a nonhydrolyzable biopolymer in the cell wall of vegetative cells and astaxanthin-rich cysts of *Haematococcus pluvialis* (Chlorophyceae), *Mar. Biotechnol.* (New York, NY) 3 (2001) 515–521.
- [26] C. Hagen, S. Siegmund, W. Braune, Ultrastructural and chemical changes in the cell wall of *Haematococcus pluvialis* (Volvocales, Chlorophyta) during aplanospore formation, *Eur. J. Phycol.* 37 (2002) 217–226.
- [27] M. Adda, J.C. Merchuk, S. Arad, Effect of nitrate on growth and production of cell-wall polysaccharide by the unicellular red alga *Porphyridium*, *Biomass* 10 (1986) 131–140.
- [28] S. Arad, M. Adda, E. Cohen, The potential of production of sulfated polysaccharides from *Porphyridium*, *Plant Soil* 89 (1985) 117–127.
- [29] S.M. Arad, O.D. Friedman, A. Rotem, Effect of nitrogen on polysaccharide production in a *Porphyridium* sp. *Appl. Environ. Microbiol.* 54 (1988) 2411–2414.
- [30] S. Geresch, S. Arad, The extracellular polysaccharides of the red microalgae: chemistry and rheology, *Bioresour. Technol.* 38 (1991) 195–201.
- [31] S. Geresch, A. Mamontov, J. Weinstein, Sulfation of extracellular polysaccharides of red microalgae: preparation, characterization and properties, *J. Biochem. Biophys. Methods* 50 (2002) 179–187.
- [32] T.M. Sobczuk, F.G. Camacho, E.M. Grima, Y. Chisti, Effects of agitation on the microalgae *Phaeodactylum tricornutum* and *Porphyridium cruentum*, *Bioprocess Biosyst. Eng.* 28 (2006) 243–250.
- [33] K. Loubiere, J. Pruvost, F. Aloui, J. Legrand, Investigations in an external-loop airlift photobioreactor with annular light chambers and swirling flow, *Chem. Eng. Res. Des.* 89 (2011) 164–171.
- [34] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, *J. Biol. Chem.* 193 (1951) 265–275.
- [35] B. Cordero, D. Voltolina, Viability of mass algal cultures preserved by freezing and freeze-drying, *Aquac. Eng.* 16 (1997) 205–211.
- [36] N. Rossignol, L. Vandanjon, P. Jaouen, F. Quéménéur, Membrane technology for the continuous separation microalgae/culture medium: compared performances of cross-flow microfiltration and ultrafiltration, *Aquac. Eng.* 20 (1999) 191–208.