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Short communication

Release of hydro-soluble microalgal proteins using mechanical and chemical treatments

Carl Safi [a,b,*], Michael Charton [a,b], Alina Violeta Ursu [c], Céline Laroche [c], Bachar Zebib [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), 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] Center of Excellence for Advanced Materials Research, King Abdulaziz University, Jeddah, Saudi Arabia

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ABSTRACT

In order to release proteins in the aqueous phase, high-pressure homogenization and alkaline treatments were applied to rupture the cell walls of five intensively grown microalgae. Protein characterisation was carried out by analysing the amino acid profiles of both the crude microalgae and the protein extracts, obtained after both types of treatment. The results showed that the proportion of proteins released from microalgae following both treatments was, in descending order: *Porphyridium cruentum* > *Arthrospira platensis* > *Chlorella vulgaris* > *Nannochloropsis oculata* > *Haematococcus pluvialis*, reflecting the increasingly protective, cell walls. Nonetheless, mechanical treatment released more proteins from all the microalgae compared to chemical treatment. The highest yield was for the fragile cell walled *P. cruentum* with 88% hydro-soluble proteins from total proteins, and the lowest from the rigid cell walled *H. pluvialis* with 41%. The proportion of essential and non-essential amino acids in the extract was assessed and compared to the crude microalgae profile. It was higher after alkaline treatment and much higher after high-pressure homogenization. These results suggest that non-essential amino acids are more concentrated actually inside the cells and that different types of proteins are being released by these two treatments.

1. Introduction

In the 9th century AD the Kanem Empire in Chad discovered the benefits of the cyanobacterium *Arthrospira platensis* and used it as food (called *dihé*) for human consumption [1]. Later on in the 14th century AD, the Aztecs harvested the same species from Lake Texcoco and used it to make a sort of cake called *tecuilatl*. They also used these microorganisms as fodder, fertilisers and remedies. Nowadays, additional species are being industrially and profitably marketed worldwide for the same purposes.

The microalgal industry has grown rapidly over the last decade. Primarily, this is due to the capacity of these micro-organisms to produce lipids suitable for the biodiesel industry, and to grow in a wide variety of geographical and environmental locations, thus precluding competition with arable lands as well as intensive deforestation. Therefore, the major part of microalgal studies has concentrated on enhancing this bioenergy production to the detriment of other high-value biomolecules, but forgetting ancient history and the other advantages of these species.

Today the microalgal bioenergy industry is struggling to find a place in the market due to its uncompetitive cost and its overall unsustainable production [2–6] sometimes leaving negative footprints on the environment, and public opinion.

Microalgae were originally considered as an important source of protein, a major fraction of their composition; on a dry weight basis the Cyanobacterium *Arthrospira platensis* is composed of 50–70% proteins [7,8], the Chlorophycea *Chlorella vulgaris* 38–58% [9–11], the Eustigmatophyceae *Nannochloropsis oculata* 22–37% [12], the Chlorophyceae *Haematococcus pluvialis* 45–50% [7], and the Rhodophyta *Porphyridium cruentum* 8–56% protein [13,14]. They have a profile composed of a set of essential and non essential amino acids [10], with relatively similar ratios between species and generally unaffected by growth phase and light conditions [1]. To the best of our knowledge, studies on microalgal proteins have generally either concentrated on finding and proposing the nitrogen to protein conversion factor [10,15–18], in order to avoid incorrect estimations of microalgal total protein content, or focused on determining the best method for protein quantification using colorimetric techniques [19–21]. However, for some species such as the green microalgae *C. vulgaris*, *N. oculata* and *H. pluvialis*, maximising the recovery of proteins requires a unit cell disruption operation to overcome the barrier of their rigid cell wall and release the intracellular biomolecules. Thus, many cell disruption methods were used to break the cell wall of these microalgae, such as

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

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bead milling, ultrasonication, microwaves, enzymatic treatment and high-pressure homogenization [22–26]. Conversely, fragile cell walled microalgae such as *P. cruentum* and *A. platensis* require milder techniques to enhance recovery.

The main objective of this study is to evaluate the effect of two different cell disruption techniques on aqueous phase protein extractability, in five microalgae with different cell wall characteristics, while simultaneously evaluating and comparing the profile of amino-acids subsequent to these two cell disruption methods.

2. Materials and methods

2.1. Microalgae

The selected microalgae were supplied as frozen paste from Alpha Biotech (Asserac, France): the Cyanobacteria *Arthrospira platensis* (strain PCC 8005), two different Chlorophyceae *Chlorella vulgaris* (strain SAG 211-19), and *Haematococcus pluvialis* (unknown strain), one Rhodophyta *Porphyridium centum* (strain UTEX 161), and the Eustigmatophyceae *Nannochloropsis oculata* (unknown strain).

Each microalga was cultivated on a different culture media; Hemerick media was used for *P. cruentum*, Sueoka media for *C. vulgaris*, Basal media for *H. pluvialis*, Conway media for *N. oculata* and Zarrouk media for *A. platensis*. All were grown in batch mode in an indoor tubular Air-Lift PhotoBioReactor (PBR, 10 L) at 25 °C, inoculated from a prior culture in a flat panel Air-Lift PBR (1 L). Culture homogenization was by sterile air injection at the bottom of the PBR. The pH and temperature were recorded using a pH/temperature probe (Mettler Toledo SG 3253 sensor) monitored by LabVIEW acquisition software. The pH was regulated at 7.5 with CO_2 bubbling. Microalgae were harvested during the exponential growth phase, concentrated by centrifugation, and the biomass which contained 20% dry weight, was then frozen.

2.2. Chemicals

The Lowry kit ((prepared mixture of Lowry reagent plus bovine standard albumin (BSA) standards and 2 N Folin–Ciocalteu reagent)) was purchased from Thermo Fisher Scientific. NaOH and HCl 37% were purchased from Sigma Aldrich and used as received.

2.3. High-pressure cell disruptor

A "TS Haiva series, 2.2-kW" homogenizer from Constant Systems Limited (Northants, UK), was used. For each experiment, a biomass concentration of 2% dry weight (0.5 g of freeze dried cells dispersed in 25 mL distilled water) was passed through the machine twice at a pressure of 2700 bar.

2.4. Alkaline treatment

Mother solutions were prepared with approximately 500 mL of ultrapure water and some drops of 2 N NaOH to adjust to pH 12. A sample of 1 g of freeze-dried biomass was added to 50 mL of mother solution and the mixture heated at 40 °C with stirring for 1 h. Separation of the solid–liquid mixture was conducted by centrifugation at 5000 *g* for 10 min. Samples of the supernatant were taken for protein analysis by the Lowry colorimetric method and for amino acid analysis.

2.5. Lowry method

The procedure involves reacting proteins with cupric sulphate and tartrate in an alkaline solution, leading to the formation of tetradentate copper protein complexes. The addition of the Folin–Ciocalteu reagent leads to the oxidation of the peptide bonds by forming molybdenum blue with the copper ions. Therefore, a calibration curve was prepared using a BSA concentration range from 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 were taken, and then 1 mL of modified Lowry reagent was added to each sample, which was then vortexed and incubated for exactly 10 min at room temperature. After incubation, 0.1 mL of Folin–Ciocalteu Reagent (1 N) was added and the sample again vortexed and incubated for exactly 30 min at room temperature. The blue colour solution absorbance was then measured at 750 nm with a UV-1800 Shimadzu spectrophotometer, previously zeroed with a blank sample containing all the reagents minus the extract.

2.6. Elemental analysis

The total nitrogen was evaluated by LCC (Laboratoire de Chimie de Coordination, Toulouse-France) using a PerkinElmer 2400 series II elemental analyser. The samples of 2 mg were placed in thin capsules and then heated to 925 °C using pure oxygen as the combustion gas, and pure helium as the carrier gas. The percentage nitrogen was evaluated and converted into protein percentage using the conversion factors obtained for each microalga in another study [10].

2.7. Amino acid analysis

The biomass amino acid composition was determined using a well known standard method (Moore and Stein 1948). The samples were hydrolysed with 6 N HCl at 103 °C for 24 h., and the hydrolysed material was then adjusted to pH 2.2 with 6 N NaOH and stabilised with a pH 2.2 citrate buffer solution. The final solution was then filtered over a 0.45 μm PTFE membrane to remove any residual solids remaining in the solution. The analysis was performed using a Biochrom Ltd 32 + (Cambridge, UK) amino acid analyser, equipped with a high pressure PEEK "column + pre-column" (size, 200 × 4.6 mm) packed with Ultropac cation exchange resin containing sodium. The separation of

Table 1
Different protein contents in crude microalgae calculated according to the following equation: Proportion of hydro-soluble protein in total protein for different microalgae.

				Chemical treatment		High-pressure homogenization	
Microalgae	N_{EA}[a] (%)	NTP[b] (%)	P_{TOTAL}	P_{Lowry}[c] (%)	$\frac{P_{Lowry}}{P_{TOTAL}} \times 100(\%)$	P_{Lowry}[c] (%)	$\frac{P_{Lowry}}{P_{TOTAL}} \times 100(\%)$
P. cruentum	9.18 ± 0.61	6.34	58.29 ± 3.78	44.34 ± 0.97	76.07 ± 1.48	51.60 ± 2.45	88.52 ± 1.17
A. platensis	8.76 ± 0.16	6.27	54.92 ± 1.10	37.19 ± 2.67	67.72 ± 1.64	41.75 ± 2.82	76.02 ± 0.75
C. vulgaris	7.98 ± 0.16	6.35	50.67 ± 1.02	21.50 ± 0.34	42.43 ± 0.52	26.18 ± 3.99	51.68 ± 2.03
N. oculata	7.83 ± 0.31	6.28	49.17 ± 2.13	15.52 ± 0.42	31.56 ± 1.06	24.34 ± 0.58	49.50 ± 1.51
H. pluvialis	8.30 ± 0.04	6.25	51.87 ± 0.43	14.23 ± 0.69	27.43 ± 0.49	21.23 ± 3.66	40.93 ± 1.97

P_{TOTAL}: Total protein in microalgae = $N_{EA} \times NTP$.
[a] N_{EA}: Total nitrogen % (d.w) obtained by elemental analysis.
[b] NTP: Nitrogen-to-protein conversion factors of Safi et al. (2012b) for each microalga.
[c] P_{Lowry}: Hydro-soluble protein % (d.w) at pH 12 and 40 °C and by high-pressure homogenization calculated using the Lowry method.