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Extraction of soluble proteins by bead milling from *Tetraselmis chui* in two different physiological states

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Highlights

Two *T.chui* batches had different morphologie: vegetative (A), motile-like cells (B). The motile-like cells from batch B were more fragile than those from batch A. The bead-milling released more proteins from fragile cells than from resistant ones. Distinguishable proteins were released, confirming Physiological state differences. The extraction performances were directly related to the cell physiology.

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Abstract

Tetraselmis is an interesting microalga for food products because of its high protein content and surface productivity as well as the possibility to scale up the culture easily. However, this specie is yet understudied compared to other algae such as *Chlorella*. Different batches of *Tetraselmis chui* were received from the same supplier, with different morphological properties, probably associated with a difference in physiological state: one batch contained circular and dividing cells and the other flagellated cells. The batches were characterized biochemically in terms of protein, carbohydrate and ash content and the rupturability was tested to characterize their behavior to cell disruption. One batch was found way more fragile than the other. The bead milling of the two batches showed also striking differences in terms of cell disruption kinetic, as well as dry matter repartition after centrifugation and protein recovery yields within the supernatant. Nearly no differences in protein yield were observed after bead milling in comparison to freeze-thawing for the resistant batch, with a final yield of 11% of the initial proteins. However, a clear protein release was observed for the fragile batch along the milling residence time, with a final protein yield of 32%. The protein released were characterized by SDS PAGE and zymography and differences were observed, confirming the difference in metabolism. Several physicochemical modifications were applied to the cell lysate to increase the yields using pH, temperature and dilution. This latest modification was the most promising. The results underlined that a difference in physiological state of *Tetraselmis chui* had a significant impact on the following biorefinery, affecting directly the cell disruption and the protein recovery yields. The valorization of the whole microalgae thus necessitates the finest coupling between upstream and downstream.

Keywords: *Tetraselmis chui* Bead-milling Soluble protein Microalgae
Biorefinery Physiological states

1. Introduction

Microalgae are an interesting resource of bio-based products for different industries such as biomaterials, pharmaceuticals, feed and food [1]. Their high protein content and interesting amino acid profile, as well as their high surface productivity, with no use of arable land, make them particularly interesting for food and feed products [2]. However, different hurdles hinder a wider food consumption of microalgae such as undesirable smell, colour and taste, limiting their incorporation at a high percentage into food products [3].

Tetraselmis is a very promising marine specie with a fast growth rate and a halotolerance, allowing easier culture media recycling [4]. The food valorization of *Tetraselmis* is also interesting since it is already approved for food consumption as a lyophilized powder in the EU and has a protein content between 30 to 50%_{DW}. Moreover, beyond proteins, other interesting molecules can be valorized: lipids for feed [5] or biofuel [6,7], and carbohydrates, such as starch as functional food ingredients or bulk chemicals [8].

Biorefinery is a way to increase product value by improving functionality, digestibility and to limit undesirable colour and cost-effectiveness of algal biomass [9]. Pigments are mostly hydrophobic and part of the proteins are water soluble, hence a good way to produce extracts enriched in proteins and purify them from pigments is to improve protein solubility and separate them from solid cell debris. Different conditions can enhance protein water solubility such as extreme pH, that aims at increasing the charge of the proteins if the pH is far from the isoelectric point [10]. Alkaline pH is often used, as it is a classical mean to obtain protein isolate from plant seeds, the most common being soy bean. Other ways to increase protein solubility can involve temperature or dilution, which have been found effective on *Chlorella vulgaris* and *Nannochloropsis* [10,11].

Since some microalgae possess tough cell wall that needs to be overcome to extract proteins, hence algal biorefinery often begins with cell disruption. Different techniques are used, some physical such as high pressure homogenization, bead milling, and others non physical such as enzymatic cell wall hydrolysis [12]. While enzymatic hydrolysis is mild, it requires cell wall knowledge to be optimized. High pressure homogenization is a very efficient technique, but it is harsh and can generate very fine debris, complicating the following purification steps such as membrane filtration [13]. By contrast, bead milling possesses more parameters that can be tuned to modify its mildness [14], while being scalable, explaining why this process is widely studied for microalgae disruption [15–18].

Cell disruption of *Tetraselmis* by bead milling (BM) to extract proteins has been studied by several authors on different species (mainly *T. impellucida*, *T. suecica*) as seen in table 1. The protein recovery yield in the supernatant after BM and centrifugation were distributed in 2 ranges, one with mass protein yields ranging from 12% to 21% of total proteins [19–22], and a second with a mass protein yield of 41%. Despite having similar biomass composition and process conditions, the yield showed significant differences, for example 21% with Schwenzfeier et al. [19] and 41% with Teuling et al. [23]. The bead diameters have shown little effect on *Tetraselmis* cell disruption and protein release kinetic, as well as on protein and carbohydrate recovery yields, on the contrary of what have been obtained on *C. vulgaris* and *N. oleoabundans* [20].

The modification of physicochemical conditions can enhance protein extraction and have been effective on other algae. For example, pH can be used to increase protein charge, increasing the repulsion between proteins, and have been successfully modified to increase protein extraction in *Chlorella* [11,24] and *Nannochloropsis* [10]. However, it can also denature proteins, decrease protein functionality and affect negatively other coproducts, hence a pH of 10 was selected as the maximum. Temperature has also been found to be an important factor to release proteins of a cell lysate, possibly because of an enhanced diffusion rate [10].

The objective of this work was to evaluate the performances of BM for the recovery of soluble proteins from two different batches of frozen *Tetraselmis chui* produced at industrial scale, characterized by significant physiological differences. The first step was to evaluate the rupturability of the two batches based on a method described by Spiden et al. [25]. The second was the characterization of the kinetic of

cell disruption and protein release by bead milling. In the last one, the physicochemical conditions (pH, temperature, ionic strength) were modified to increase protein release.

Table 1 Articles on bead milling of *Tetraselmis* for protein extraction. ¹ YTZ (Yttrium Stabilized-Zirconia beads)

Bead milling and centrifugation parameters	Physicochemical conditions	Protein yield	Protein content in the initial biomass	Article
0.3 L chamber 65% V/V 0.4-0.6 mm beads YTZ ¹ beads DM suspension: 120 g _{DM} .kg ⁻¹ Centrifugation: 40 000g, 30 min 4°C	Dilution with Tris HCl pH 8 EDTA DTT MgCl ₂	21% (calculation method not detailed)	36% Determined by amino acid analysis Frozen paste <i>T.impellucida</i>	Schwenzfeier 2011
0.075 L chamber 65% V/V 0.3 mm 0.4 mm 0.65 mm 1 mm YTZ beads DM suspension: 90 g _{DM} .kg ⁻¹ Centrifugation: 20 000g 20 min	Dilution of the paste with phosphate-buffered saline (PBS) at pH 7	17.4% 21.5% 15.7% 21.1% Concentration ratio between the biomass and the supernatant.	43% 29% 37% 41% Colorimetric Fresh paste <i>T.suecica</i>	Postma 2017
0.3 L chamber 65% V/V 0.4-0.6 mm beads YTZ ¹ beads DM suspension: 120 g _{DM} .kg ⁻¹ Centrifugation: 70 000g, 30 min 4°C	Dilution of the paste with PBS buffer at a final pH of 8	41%	36% ± 2% Amino acid analysis Frozen paste <i>T.impellucida</i>	Teuling 2017
0.075 L chamber 65% V/V 0.4 mm YTZ beads DM suspension: 100 g _{DM} .kg ⁻¹ Centrifugation: 20 000 g, 30 min, 20°C	Dilution of the paste with PBS at a final pH of 7	12%	37% Colorimetric Fresh paste <i>T.suecica</i>	Garcia 2018
0.075 L chamber 65% V/V 0.4 mm YTZ beads DM suspension: 100 g _{DM} .kg ⁻¹ Centrifugation: 14 000 rpm, 30 min, 20°C	Dilution with distilled water to pH 6.5	22.5%	54% Colorimetric Fresh paste <i>T.suecica</i>	Suarez Garcia 2018

2. Materials and methods

2.1 Biomass

Tetraselmis chui was grown autotrophically in photobioreactor in Necton facility (Portugal), under solar conditions. The biomass was harvested by centrifugation to obtain a paste at 20%_{DM} and frozen immediately. Two biomass batches were received from the producer and called batch A, produced in December 2019 and batch B, produced in May 2020. They were characterized by microscopy and biochemically in terms of proteins, carbohydrates and ashes, following protocols in the analysis sub section.

2.2 Rupturability measurement

The rupturability of *Tetraselmis* was assessed following the protocol described by Spiden et al [25] by High-Pressure Homogenization (HPH). The biomass was at first diluted at 10 g_{DW}.kg⁻¹ with deionized water containing 30 g.L⁻¹ of NaCl to avoid an osmotic stress. A high pressure homogenizer (TS Series 2.2 kW fabricated by Constant Systems Limited (Northants, UK)) was used. Each sample was sent to the HPH with one pass per tested pressure. The pressure allowing 50% of cell disruption was called the P₅₀ and used for the empirical comparison of strains rupturability [25,26] or physiological states.

2.3 Bead milling

Disruption experiments were conducted in a horizontal 0.6 L bead mill (Dyno-mill multi lab from Willy A Bachofen AG (Muttens, Switzerland)). Algae suspensions at a dry matter concentration of around 60 g_{DM}.kg⁻¹ were prepared by diluting the biomass paste with distilled water. The resulting suspensions had a pH of 8.3 for the batch A and 8.7 for the batch B. The temperature of the suspension was maintained below 25°C with an external cooler jacket connected to the milling chamber. Yttrium-stabilized Zirconium beads with a density of 6 g.cm⁻³ and a diameter of 0.5-0.7 mm were selected. A filling ratio of 80%, a rotational speed of 14 m.s⁻¹ and a flow rate of 200 mL.min⁻¹ were applied according to the results of Montalescot et al. and Suarez Garcia et al. [14,26].

The bead millings were performed in pendulum mode until a residence time of 4.2 min.

2.4 Macerations

Physicochemical modifications were performed on the cell lysate after 4.2 min of residence time in the bead milling, in order to improve protein extraction. Four conditions were tested after cell disruption of batch A biomass: 1) a control with the lysate at 60 g_{DM}.kg⁻¹ at 20°C and native pH (8.3); 2) a diluted condition, with the lysate diluted at 30 g_{DM}.kg⁻¹, using deionized water at 20°C and native pH; 3) a diluted condition at 30 g_{DM}.kg⁻¹ at 40°C and native pH; 4) a diluted condition at 30 g_{DM}.kg⁻¹ at 40°C and pH 10.

Three conditions were tested after cell disruption of the biomass from the batch B: 1) a control with the lysate at 60 g_{DM}.kg⁻¹ at 20°C and native pH (8.7); 2) a diluted condition, with the lysate diluted at 30 g_{DM}.kg⁻¹, using deionized water at 20°C and native pH and finally 3), a diluted condition, with the lysate diluted at 30 g_{DM}.kg⁻¹, using deionized water at 20°C and pH 10.

The maceration has been performed in beakers with an operating volume of 300 mL under agitation. The pH was corrected with a solution of 1M NaOH and the samples were collected at initial time, 30 minutes, 1 hour and 1.5 hour.

2.5 Centrifugation

Centrifugation was conducted in a Sorvall Lynx 6,000 centrifuge (Thermo Scientific) at a relative centrifugal force of 5,000g for 4 min at 20°C in order to simulate an industrial centrifuge and to acquire data for a potential scale-up [11,27].

2.6 Analytical methods

Cell disruptions were characterized as described by Zinkoné et al. [28]. Briefly, cell counting was performed using image analysis with ImageJ software. The images were taken with a Carl Zeiss optical microscope equipped with an Axioscope A1 camera, after fixation with 20µmol of Lugol's iodine per mL of sample to enhance the contrast. A sufficient number of pictures (>20) were taken to ensure statistical representability.

Particle size analysis of cell lysates and supernatants were performed by laser diffraction using a Mastersizer 3,000 (Malvern Panalytical, USA) to monitor cell comminution along the bead milling. The samples were dispersed in distilled water and particle size distributions were obtained using a refraction index of 1.45 and an absorption index of 0.05. Proteins and dry matter quantified in the supernatant were considered as "total" after centrifugation at 5,000g during 4 min and "soluble" after centrifugation of the 5,000g supernatant at 11,000g during 10 min, both at 20°C. The proteins in the supernatant were quantified by the bicinchoninic assay (BCA) with bovine serum albumin (BSA) as a standard. Carbohydrates were quantified by the Dubois method with glucose as standard. The dry matter was measured by weighing the sample before and after drying for 48h at 105°C [11]. Following dry matter analysis, ashes were quantified by burning the remaining dried biomass at 600°C for 16 hours [29].

The protein composition was characterized by SDS-PAGE with Mini-Protean TGX Precast gels gradient 4-20% using Mini-Protean Tetra Cell system (Bio-Rad Life Science, France). The concentration of supernatants of batch A and B Tetraselmis were normalized at 2g/L. All reagents are from Bio-Rad Life Science, France. All samples were diluted with 2x Laemmli sample buffer in reducing conditions with 2-mercaptoethanol 5%. The migration buffer contained 25 mM Tris, 192 mM glycine, 0.1% SDS according to Laemmli protocol [30]. The samples were heated at 100°C for 5 min. Each protein sample was then loaded on a sample well (20 µg of protein/well) of the gradient gel 4-20% polyacrylamide gel electrophoresis. Molecular weight (MW) protein markers from MW 10 to 250 kDa (Precision Plus Protein Standards, All Blue, Bio-Rad Life Science, France) and from MW 14.4 to 116 kDa (unstained Molecular weight marker, Euromedex, Souffelweyersheim, France) were used for MW calibration. The migration was carried out under standard conditions at 200 V for 45 min. The gel was stained with Coomassie Brilliant Blue G-250 staining solution for 2h with gentle agitation on platform rocker according to Lawrence and Besir [31]. The gel was rinsed with distilled water before scanning on a flatbed scanner (Image Scanner iii, GE Healthcare Europe, Velizy-Villacoublay, France).

The protease identification was conducted by zymogram. The protease identification was performed by 10% zymogram which 1% gelatin was incorporated into the gel providing a substrate for proteases. Samples were diluted 50% v/v with a sample buffer in non-reducing conditions and incubated 10min at 25°C. Then 10µg of sample was loaded in each well and the electrophoresis was run using Tris Glycine SDS buffer migration at 35mA for 1h30min. Molecular weight (MW) protein markers from MW 10 to 250 kDa (Precision Plus Protein Standards, All Blue, Bio-Rad Life Science, France) was used for calibration. After migration, sample proteases must be renatured by placing the gel in renaturing solution containing 2.5% Triton X100 three times for 15min and rinsed two times for 10min with MilliQ water. The gel was incubated for 16h at 37°C with the development solution phosphate buffer 50mM, pH7.8. Then the gel was stained with Coomassie Brilliant Blue R-250 0.5% in ethano/acetic acid/water, v/v, 30/5/65 for 2h at room temperature. The gel was placed in destaining solution ethanol/acetic acid / water until clear bands appeared against the blue background and finally the gel was scanned.

2.7 Calculation methods

Mass compound recovery yield (Y) was defined as the ratio between the mass of the considered molecules in the supernatant, for example proteins ($m_{p,Sup}$), and the mass of the same molecule in the lysate, ($m_{p,Lysate}$):

$$Y_p = \frac{m_{p,Sup}}{m_{p,Lysate}} = \frac{C_{p,Sup} \times V_{Sup}}{C_{p,Lysate} \times V_{Lysate}}$$

with the supernatant protein concentration $C_{p,Sup}$, the volume of supernatant V_{sup} , the lysate protein concentration $C_{p,lysate}$, the lysate volume V_{Lysate} .

The efficiency of the separation of the supernatant from the lysate during the centrifugation is defined by the volume ratio ϕ between the supernatant and the lysate suspension $\phi = \frac{V_{Sup}}{V_{Lysate}}$. This ratio directly affects the final yield, as demonstrated by Liu et al, with a ratio of $74\% \pm 7\%$ when centrifuging a suspension at $30 \text{ g}_{DW} \cdot \text{kg}^{-1}$ and only $35\% \pm 5\%$ when centrifuging at $90 \text{ g}_{DW} \cdot \text{kg}^{-1}$ [11].

2.8. Statistical analysis

The experiments in this study were carried out in triplicate for rupturability, and in duplicate for bead milling with each identified batch of harvested biomass, due to the limitation of biomass quantity for each batch with exactly the same properties, and the biomass quantity needed to perform the following separation and analysis. The physico-chemical modifications were then limited to one. All the values resulting from the samples analysis (dry weight, ashes, proteins and carbohydrates) were based on triplicate measurements. The standard errors (SE) were calculated as the standard deviations of the triplicate. The combined standard error of any value y was calculated by Equation 1 for additions or 2 for multiplications or divisions of two variables X_1 and X_2 :

$$\text{If } y = X_1 + X_2, SE_y = \sqrt{SE_{X_1}^2 + SE_{X_2}^2} \quad \text{Equation 1}$$

$$\text{If } y = X_1^{\pm 1} \times X_2^{\pm 1}, SE_y = y \cdot \sqrt{\left[\frac{SE_{X_1}}{X_1}\right]^2 + \left[\frac{SE_{X_2}}{X_2}\right]^2} \quad \text{Equation 2}$$

3. Results and discussions

3.1 Biochemical characterization

The paste belonging to the two different batches (A and B) has been analyzed in terms of proteins, carbohydrates and ashes as a function of the paste dry matter, as shown in figure 1. The ash content is higher in batch A than in batch B. The percentage of ashes in batch B (23%) is similar to what was found by Teuling et al. on an industrial paste of *T. impellucida*, with an ash content of 17%, among which 11.8% of ashes being considered as intracellular ashes [23]. However, ash content is significantly superior to the 8% found on laboratory paste of *T. suecica* [21,22].

The percentage of proteins was between 28 and 34%, corresponding to the description in literature.

The carbohydrate content (13-16%) is in agreement with the 5 – 25% found in the literature [32]. Such values are generally associated to biomasses which are not under Nitrogen starvation, where carbohydrate content can reach up to 50%, mainly as starch [33,34].

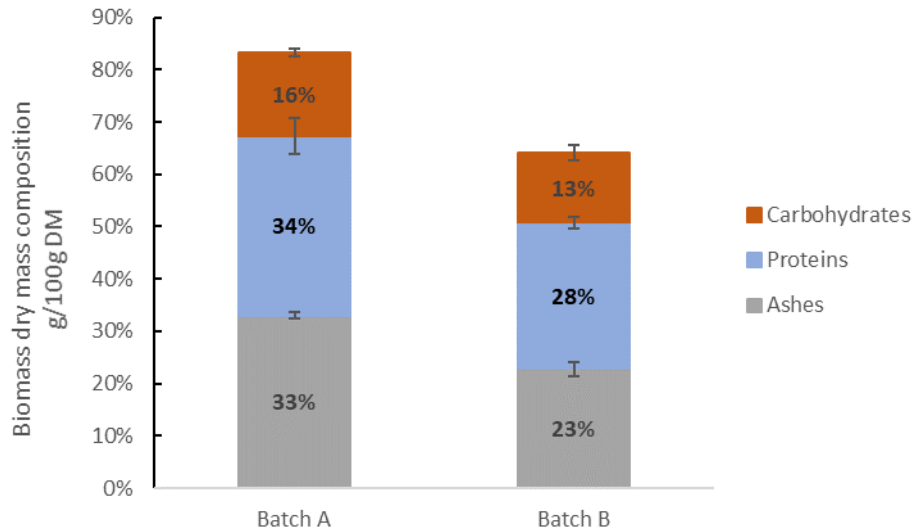


Figure 1 Biomass composition of batch A and batch B pastes in percentage of the dry matter (quantifications performed in triplicates).

3.2 Physiological characterization

The cells were observed by microscopy, as shown in figure 2. A great proportion of the cells in batch A were in cell division ($\approx 80\%$), shown by 2 protoplasts in one cell (figure 2 A1 and A2) while the others were circular and more voluminous than batch B cells. The cells in batch B presented a compressed shape with the presence of a depression near the apex sometimes visible and a single large chloroplast containing one pyrenoid (figure 2 B1 and B2). Some cells were dividing ($\approx 1\%$) but unlike batch A, it was not the majority.

No flagella can be found in batch A. Flexible translucent filamentous objects are present in the liquid phase of batch B, having a length close to the algae, and being absent in batch A. It is highly possible that these objects are flagella that have been detached due to the upstream process (harvesting, freezing etc.) since flagella have been found to have a length similar to *T.chui* cells, according to Norris et al. [35].

Tetraselmis shows different biological characteristics that may influence biorefinery. In our case, batch A differs from batch B in size, morphology and composition. Cells from the genus *Tetraselmis* are commonly represented as quadriflagellate individual cells, with a compressed shape and a single large chloroplast, containing one pyrenoid. However, the presence of a circular shape has recently been identified in *T.chui* by Lu et al. [36] who showed different cellular aspects after a culture in autotrophy and a culture in mixotrophy. Their cells grown under mixotrophic condition look like the cells of batch A, with a circular shape, while their cells grown in autotrophic condition, look like the cells from batch B. However, in the case of batch A and batch B, *T. chui* cells were both cultivated in autotrophic mode.

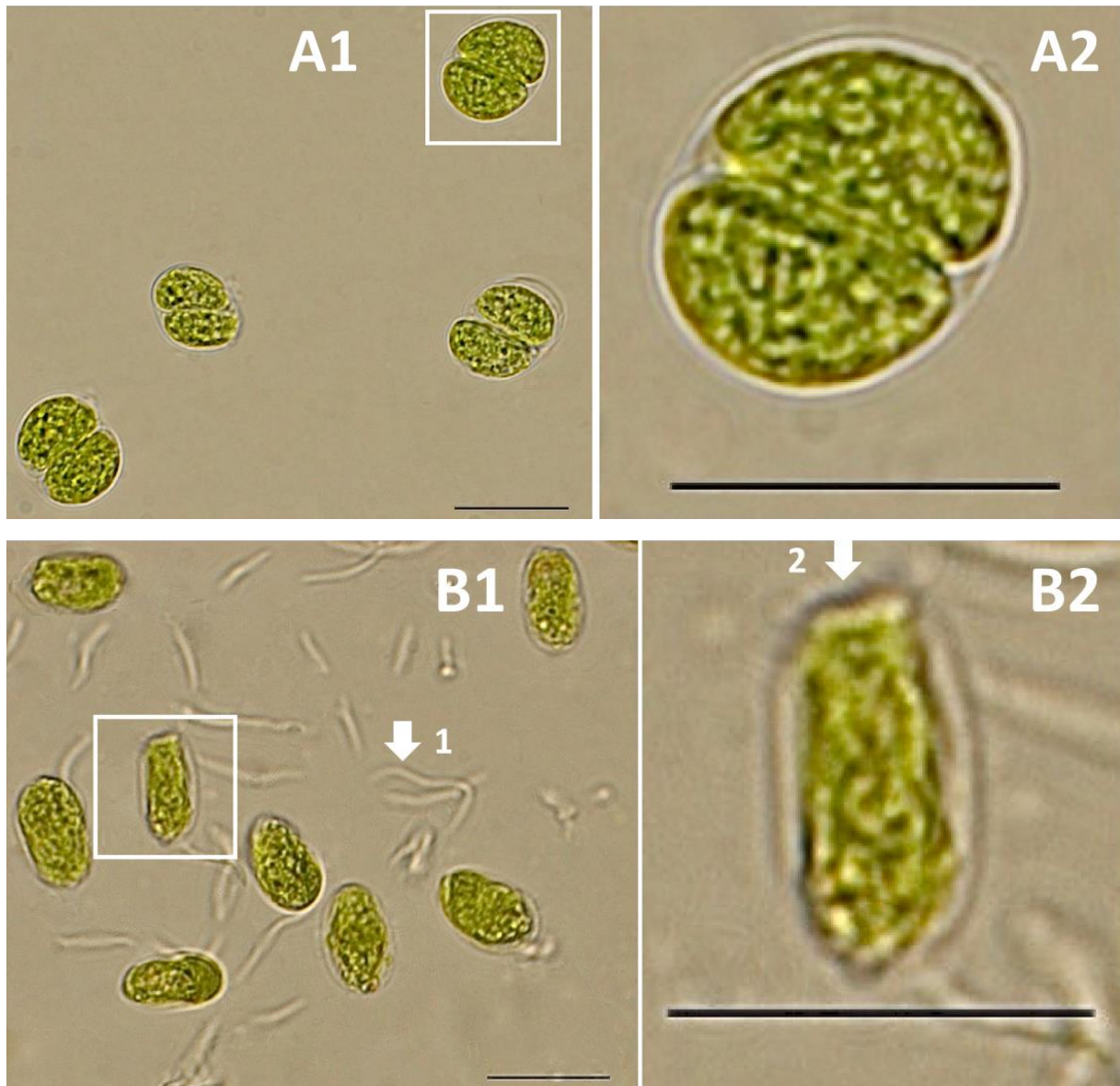


Figure 2 Light microscopic images of *Tetraselmis chui* of batch A cells (A1 and A2) and batch B cells (B1 and B2). The scale bars are 10 μm for all pictures. The first arrow shows objects corresponding to flagella, and the second arrow shows a pit, where flagella are usually inserted.

Phycologists, such as Norris, Hori, and Chihara described different life cycle stages of *Tetraselmis* in the 80's [35] with the presence of at least 3 phases, a flagellate phase i.e. with motile cells, a non-motile vegetative phase in which cells settle and replicate and a phase where the cells become aflagellate cysts. According to the authors, cells are in flagellate form and then undergo a physiological change to become non-motile vegetative cells, also called resting cells, just before to replicate, or in unfavorable conditions. The authors also identified the presence of cysts in very unfavorable conditions. The presence of different life cycle phases in *Tetraselmis* has also been observed by different authors on *T.indica* [37,38] and *T.jejuensis* [39] with circular shape associated with the resting cells.

Cells present in batch B look like motile cells, with the flagella being released in the medium. On the other hand, Cells present in batch A look like non-motile cells, before and during reproduction as shown by Norris, Hori, and Chihara [35]. No images of cyst on *T.chui*, to the authors knowledge, have been yet published. Nevertheless, the cell pictures do not look like cysts present in other algal life cycle, such as *Haematococcus pluvialis* [40]. Moreover, the biochemical composition does not show an accumulation of carbohydrates that could be induced by stress, for example due to Nitrogen and/or Phosphorus starvation. The two physiological state of *Tetraselmis* could also come from a natural

synchronization caused by the light and dark cycle, which has been found to be possible on *Tetraselmis suecica* [41]. The cells can spontaneously synchronize their cell cycle states, as demonstrated for the microalgae *Medakamo hakoa* by Kato et al. 2023 [42]. This could explain the high number of cells in division..

The life cycle in *Tetraselmis* has been poorly described in bioprocess engineering related to culture [4,33,43–46] or biorefinery [20,21,23,47,48]. However, in more intensively studied algae like *Haematococcus pluvialis*, some life cycle stages are known to negatively affect the cell disruption and the extraction of target molecules [49,50]. But some consequences have been noticed and used: a loss of flagella and settling was identified following a nutrient starvation stress [51] or UV C treatment [52], allowing an easier harvesting. Natural settling has been found in the production of *Tetraselmis sp.* CTP4 [44]. It was exploited for low-cost biomass harvesting until pilot scale, reaching a concentration in dry matter 20 times higher than the culture and removing 97% the total biomass without energy input [53] but the life cycle stage was not detailed.

This settling property is interesting for biomass producers for harvesting but the corresponding physiological state may have different effects on cell disruption and compound release which will be deepened in the following paragraphs.

3.3 Rupturability characterization

Mechanical cell resistance is a key parameter to take into account for microalgal and cyanobacterial biorefinery, especially because of their wide variety of cell wall composition [54,55]. For example, *Arthrospira platensis* does not need an intensive cell disruption technique unlike other strains such as *Nannochloropsis* or *Chlorella*.

Cell rupturability was assessed by HPH and a P_{50} between 400 and 1000 bars was obtained for the batch A while batch B's P_{50} was 190 bars \pm 46 bars, much lower than the batch A. Intact cells were still present at 1200 bars with batch A. Despite the uncertainty in cell rupturability of batch A, probably due to difficulties in full cell identification by image analyses, a different behavior was obtained for the different physiological states.

The P_{50} of batch B is close to what Spiden et al. [25] found on *T.suecica*, with a P_{50} of 170 bar. Magpusao et al. [56] observed similar results on *Tetraselmis sp.* Their intact cells looked like batch B cells and were completely disrupted at 300 bars, as for batch B.

Different algal species have been evaluated by this method, with a P_{50} of 1060 bar for *Chlorella sp.* [25], 400 bar for *Porphyridium cruentum* and 1400 bar for *Nannochloropsis oculata* [26].

Compared to other species, *T.chui* seems to be fragile in batch B and moderately resistant in batch A. This difference might be caused by the presence of accumulated concentric cast-off walls in non-motile vegetative phase [35].

3.4 Bead milling

The first bead milling experiment was conducted to assess the optimal residence time. The different liberation kinetics of proteins and dry matter were also evaluated to find a compromise between protein recovery yield and protein purity since the latter can facilitate the protein purification step for the achievement of protein isolates.

3.4.1 Cell disruption

The disruption of *Tetraselmis* cells was very fast, with a disruption rate of more than 70% after 1.4 min residence time for the milling of the batch A and more than 90% for the batch B as seen in figure 3. A

limited difference of cell disruption between the two batches was evaluated by cell counting. However more differences can be seen using complementary analysis.

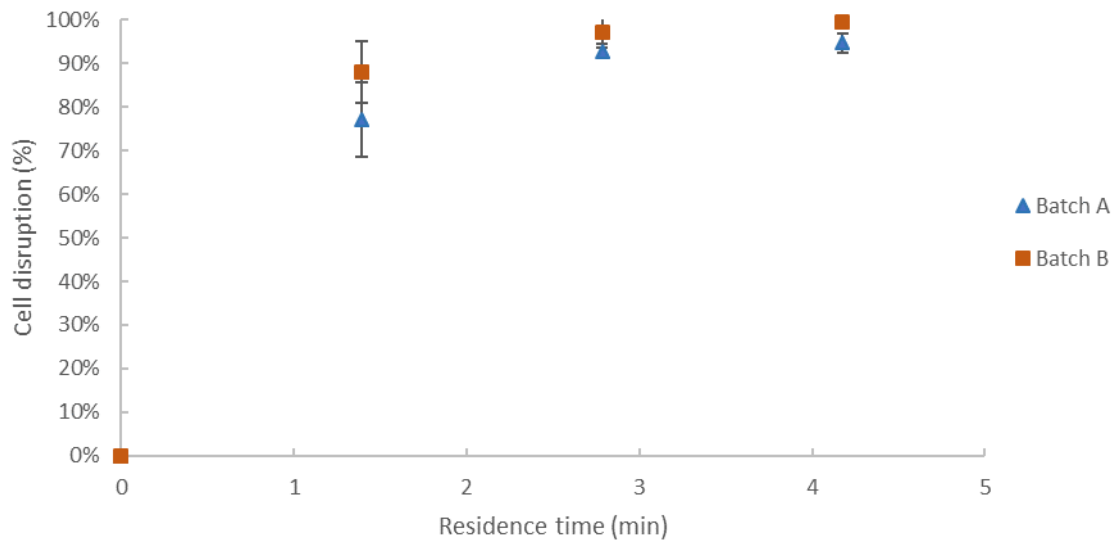


Figure 3 Cell disruption as a function of the milling residence time for the two different batches, (each experiment performed twice, mean value and standard deviations presented).

This result can be compared to particle size distribution (PSD) of cell lysates along bead milling residence time, as shown in figure 4.A1 and B1. During batch A cell disruption, a clear diminution of the maximum peak is seen, from 13.6 μm before milling (original cell diameter), to 10.5 μm after 1.4 min and 7.2 μm after 4.2 min residence time. After 4.2 min residence time, 2 peaks remain, one with a maximum at 7.2 μm , representing big cell debris and one at 0.8 μm , which represent small cell fragments. However, no further diminution in cellular fragment size can be seen after 4.2 min residence time for the batch A cells (results not shown).

The PSD is different for the milling of the batch B as seen in figure 4 B1, with a widening of the bigger cell debris peak along the milling toward smaller sizes, especially visible at 4.2 min. The generation of fine debris is also more important in batch B, compared to batch A. These results show that the cells from batch B are easier to break than the cells from batch A, linking the rupturability results and the bead milling results. The presence of fine debris will have an influence on the purification step since a centrifugation at industrial scale has limited acceleration ($< 10,000g$) and so a limited settling power. Hence, the smaller debris ($< 1\mu\text{m}$) are more difficult to separate with this operation unit [57] and a finer technique has to be used to remove them, such as microfiltration.

The PSD of clarified supernatant after bead milling has been characterized for the two batches and reported in figure 4 A.2 and B.2, respectively. For batch A (figure 4 A.2), the proportion of fine particles increases after 1.4 min of milling residence time and stays constant after. The peak is well defined and is comprised between 0.1 to 1 μm , with a mode at 0.49 μm . A peak around 10 μm , corresponding to few intact cells in the supernatant, is present before milling but disappears after and no particles with a size between 1 to 10 μm are detected after milling. Peaks present around 100 μm are suspected to be rare agglomerates. For batch B (figure 4.B2), a peak of fine particles appears after milling, being wider and less defined than the peak in figure 4.A2. Some particles are present between 1 to 10 μm and their proportion decreases during milling, while the proportion of fine particles increases, showing a further comminution.

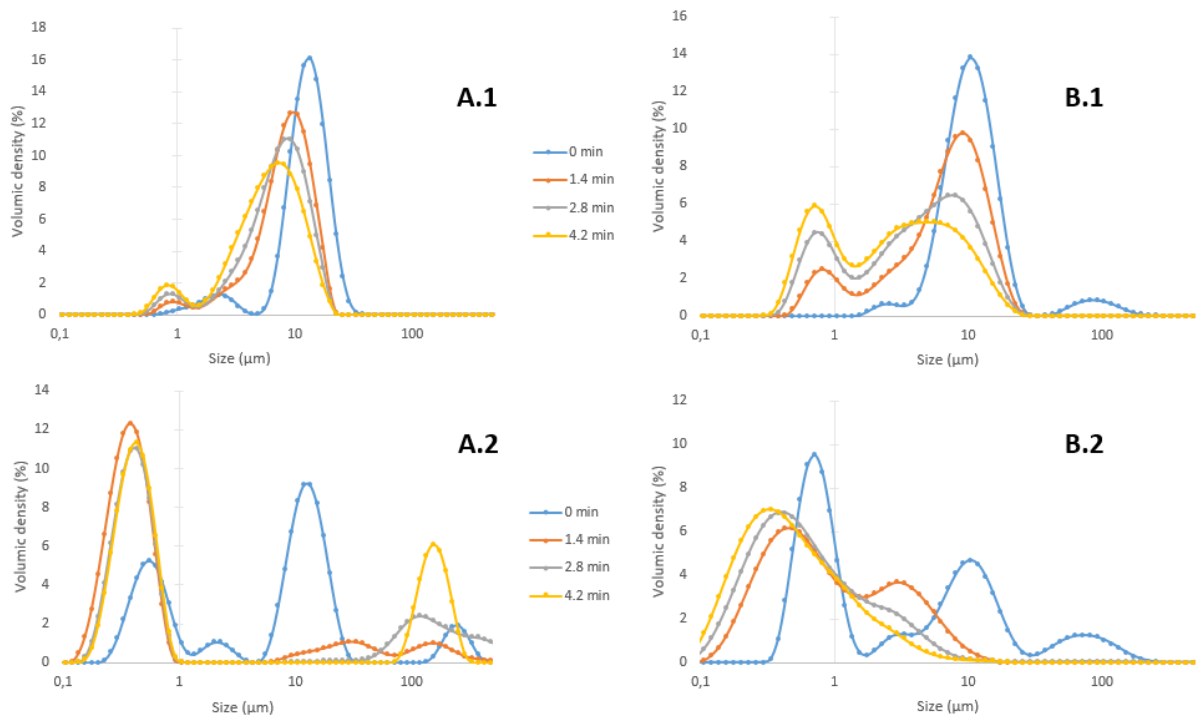


Figure 4 Particle size distribution of cell lysates (A.1 and B.1) and clarified supernatants (A.2 and B.2) as a function of the residence time in the bead mill for batch A and batch B. (measurements performed in triplicates)

3.4.2 Dry matter and protein release

The dry matter released in 5,000 g and 11,000 g supernatants was followed as a function of the milling residence time.

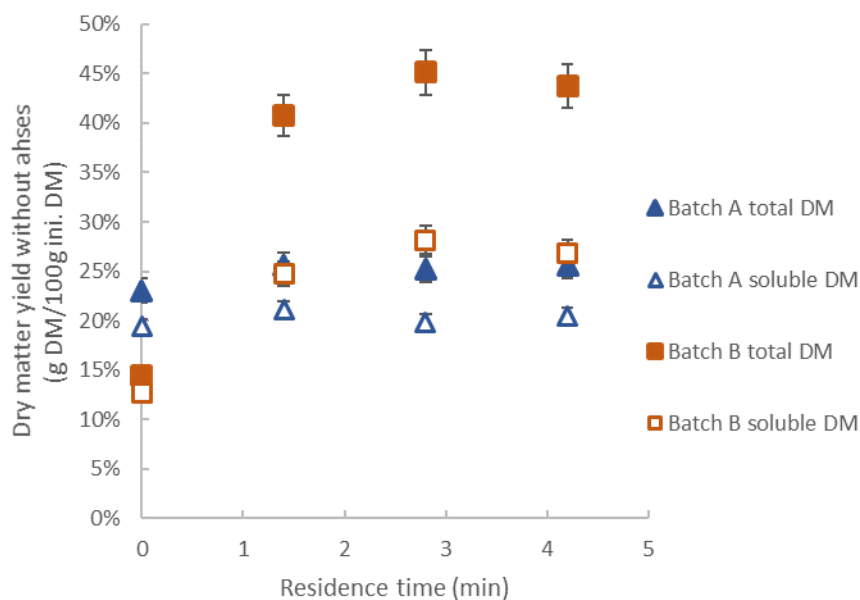


Figure 5 Total and soluble dry matter yields without ashes (ashes being removed to consider only organic compounds) in supernatants as a function of the residence time within the bead mill for the two conditions. (each experiment performed twice, analysis in triplicates, mean value and standard deviations presented).

The objective of cell disruption by bead milling is the comminution of cells to release water-soluble compounds. At the same time, cell fragments are dispersed within the aqueous phase. The separation of particles from the aqueous phase by centrifugation depends on different criteria, such as the diameter and density of the small debris and the centrifugation parameters (relative centrifugal force, time and temperature). Since different PSD were obtained for different lysate batches, it is interesting to evaluate the quality of this separation with the analysis of dry matter repartition within the supernatant for different conditions, as seen in figure 5.

Total dry matter yield without ashes recovered in batch A supernatant increases slightly from 23% after thawing to 26% after 4.2 min of milling residence time. This little difference is also observed on soluble dry matter measured after centrifugation at 11000 g of batch A supernatant with a slight increase from 19 to 20%. The negligible effect of bead milling on the dispersion of dry matter in the liquid phase for batch A cells is very contrasted with the results for batch B. In the supernatant from batch B, total dry matter recovery yield increases from 14% to 44% in supernatants along the milling time. The soluble dry matter yield of batch B is also very different from batch A, with an increases from 13% to 27%.

Total protein recovery yield in supernatant from batch A increases from 9% after thawing to 11% of total proteins after 4.2 min of milling as seen in figure 6. Moreover, no statistical difference is observed in total and soluble protein yields, and no increase of protein yield was observed when milling residence time was increased to 8.2 min (results not shown). On the contrary, the total protein recovery yield in the supernatant, after cell disruption of batch B, increases from 7% of initial proteins after thawing to 48% after only 1.4 min of milling residence time and stabilizes after. The soluble protein recovery yield increases continuously, from 6% to 32% after 4.2 min of milling residence time.

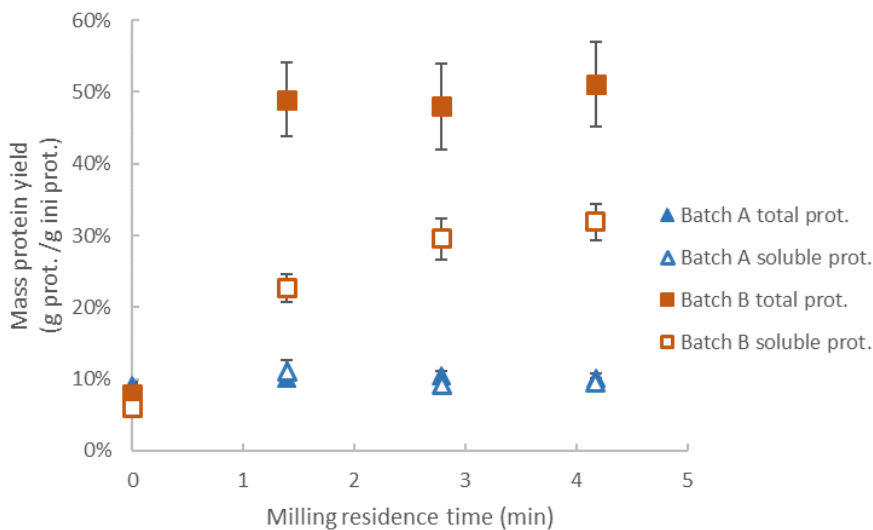


Figure 6 “Total” and “soluble” protein recovery yields in supernatant as a function of the milling residence time for batch A and batch B, (each experiment performed twice, analysis in triplicates, mean value and standard deviations presented).

The differences in the release of proteins and dry matters between batches are probably related to the generation of more fine particles in batch B, more difficult to remove from water than for batch A. As seen in figure 4 A2, milling residence time has little influence on particle size in batch A supernatants, leading to a similarity between “total” and “soluble” proteins versus residence time. However, for batch B, milling residence time induces a comminution of larger cell debris as seen in figure 4 B2, leading to an increase in submicrometric particles in the supernatant. This increase of fine particles, which should contain proteins, increases the “total” protein yield in the supernatant. Since more fine particles are generated, some of them can also be found in the supernatant at 11,000 g, increasing the “soluble” protein yield. Proteins can be associated with fine cell debris containing chlorophyll and being hard to centrifuge, even at high relative centrifugal force [24,58].

The soluble protein yields obtained, 11% for batch A and 32% for batch B are aligned with results presented within literature, as presented in table 1. Most of the yields presented are comprised between 12 to 22% of total proteins [19,21,22], while one study mentions a protein yield of 41% [23]. No data on the physiological state of *Tetraselmis* has been given by the different authors.

3.4.3 Differences between proteins released from batches A and B

The protein profile of the supernatants, before and after bead milling of the different batches of biomass, were analyzed by SDS-PAGE electrophoresis, as seen in figure 7 (1). The presence of protease was characterized by a zymogram (figure 7 (2)).

The electrophoresis profiles of batch A were similar before (0 min) and after bead milling. An important quantity of peptides with a molecular weight under 10 kDa was observed. Intense bands were present between 10 and 15 kDa, one at 25 kDa and one at 45 kDa.

The electrophoresis profiles of the batch B supernatants were different before (0 min) and after bead milling. Before cell disruption, the most intense bands were present at a molecular weight around 12 kDa, and between 20 and 25 kDa. The peptides under 10 kDa were not visible. After bead milling, some peptides appear. The two bands between 20 and 25 kDa are far more intense. Among several new bands, three ones are more intense at 37, 50 and 70 kDa. The corresponding proteins were released massively during cell disruption. The electrophoretic profile of the soluble proteins from the batch B supernatant after 4.2 min of residence time is similar to the profile of the total proteins. No specific band was removed due to stronger centrifugation conditions.

The protein profiles are very different between the two batches. However, some common bands could be attributed to the small subunits (near 20 kDa), and large subunits (between 35 and 50 kDa) of the RuBisCO, a water-soluble enzyme found in the stroma of the chloroplast and in the pyrenoid in Eukaryotes [59]. The subunits of the Rubisco of *Tetraselmis chui* are indeed respectively 22.6 kDa [60] and 40.4 kDa [61]. However, a Western blot using specific antibodies against the RuBisCO should be used to prove it. The RuBisCO was already observed during the bead milling of other strains such as *C.vulgaris*, *T.suecica*, *N.oleoabundans* [20].

Green spots corresponding to the presence of chlorophyll were also present in the electrophoresis gel for the batch B at 1.4 and 4.2 min in high quantity, as well as in the supernatant after 11,000g centrifugation. The presence of chlorophyll in the supernatant after cell disruption of microalgae was largely reported in literature as colloid [24,58]. This can be due to the presence of chlorophyll a-b binding protein.

The presence of protease among the proteins in the supernatant of different extracts was also followed by a zymogram. The zymogram of batch A supernatant showed a band at 150 kDa before and after bead milling. The supernatant of batch B after freeze-thawing showed a high quantity and diversity of proteases. It may testify a high metabolic activity, with different proteases being produced. After bead milling, the overall quantity of proteins increases, hence the sample had to be diluted, thus the relative proportion of proteases decreases. Some proteases still remain visible at 37 kDa and 100-150 kDa.

The presence of proteases in the supernatant of batch A and batch B has been confirmed, before and after bead milling. A part of these enzymes is extracted after the freeze-thawing step. The presence of different proteases confirms the presence of two different physiological states, as already mentioned in the literature. Indeed, proteases, such as autolysins, capable to degrade cell walls, were found to be life cycle stage-specific in *C.reinhardtii* [62,63]. Moreover, the localization of these proteases in the cell and their solubility can change. Their release in the soluble phase was found difficult when the cells were vegetative whereas it was not the case for gametic cells [64].

Thus the difference of proteins between the two batches can also explain why more proteins are released from batch B than from batch A.

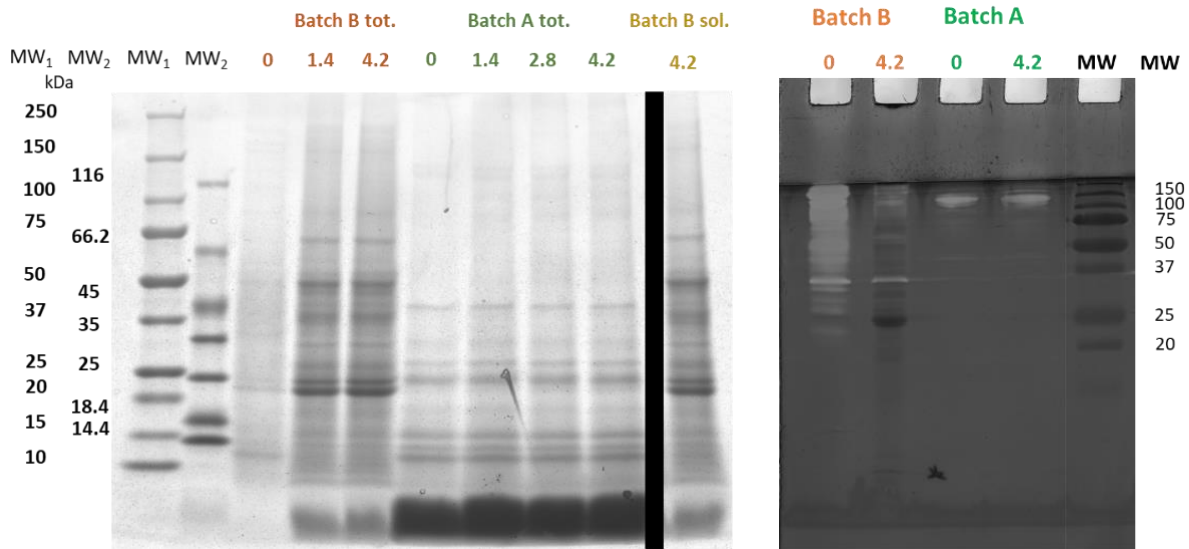


Figure 7: (1) Electrophoresis SDS PAGE in reducing conditions of total (tot.) and soluble (sol.) and (2) zymogram of supernatants after bead milling and centrifugation of *Tetraselmis*, batch A and batch B. The residence time (0 to 4.2 min) in the bead mill is shown above the well, the standards are indicated as MW in kDa.

Considering the reduced protein release for batch A cells, macerations of the lysate after bead milling were carried out, changing different physicochemical conditions: dilution, pH and temperature. Indeed, 90% of the cells being disrupted, the reason for little protein release was most likely to be a biochemical lock. The preselected conditions were also applied to batch B cells, taking into account the previous results, to further improve protein extraction for comparison.

3.4.4 Bead milling: modified conditions with macerations

Physicochemical conditions such as temperature or pH can strongly affect protein extraction [11,58]. Several conditions were tested on the cell lysate after 4.2 min of milling residence time and “total” and “soluble” protein recovery yields obtained in supernatants were measured as a function of the maceration time, between 30 min and 1.5 h. No significant differences were measured between 30 min, 1h and 1.5 h of maceration. Thus, the figure 8 presents the mean protein yields for the different conditions.

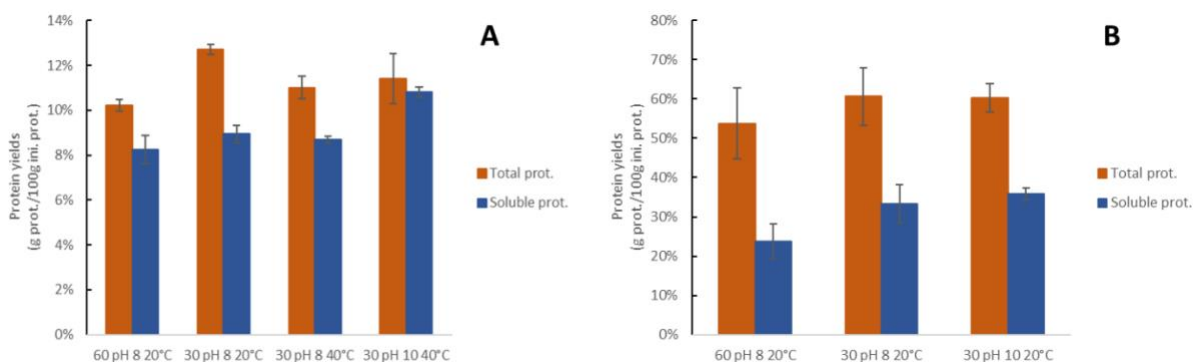


Figure 8 “Total” and “soluble” protein yields for different conditions applied on the final cell lysate of batch A (A) and batch B cells (B) (quantifications performed in triplicates)

A control at 60 $g_{DM}\cdot kg^{-1}$ and pH 8 at 20°C was tested on batch A final cell lysate and compared to the other conditions. The dilution at 30 $g_{DM}\cdot kg^{-1}$ (native pH, 20°C) improved the protein yield for total

proteins, from 10.2% to 12.7%, while the increase was less significant for soluble proteins, from 8.2% to 8.9%. The rise of temperature between 20°C and 40°C had a negative impact on total protein yield and limited impact of soluble proteins at native pH. However, pH 10 and 40°C slightly improved the protein yield on soluble proteins, but with a 2-3% of protein yield increase only.

On batch B, dilution to 30 g_{DM}.kg⁻¹ had an impact on “soluble” protein release compared to the control, with an increase from 24 to 36%. However, the increase in pH from 8.7 to 10 did not improve protein solubility, with no statistical differences observed.

The pH modification did not induce a strong rise in protein recovery yield as expected according to the literature. Its effect on protein solubility is theoretically strong but is function of the protein isoelectric point and solubility. A complementary analysis of the protein solubility extracted from batch A showed that they were 100% soluble at pH above 5. This is coherent with a limited effect of a rise of pH from 8 to 10. Moreover, protein solubility is also strongly affected by ionic strength, as shown by Teuling et al. on *Tetraselmis impellucida*, *Scenedesmus dimorphus*, *Nannochloropsis gaditana* and *Arthrospira maxima* extracts [23]. Protein solubility was affected by ionic strength in all tested extracts, with a higher ionic strength leading to a decrease in protein solubility.

Since *Tetraselmis* is a marine species, the paste is salted, as shown in figure 1, with 33% and 23% of ashes in batch A and batch B paste, respectively. Seawater is mainly composed of NaCl, contributing over 85% of the charges and a part of it is present in the paste. Thus, considering sodium chloride as the main salt in the supernatant, an ionic strength close to 0.3 M can be estimated for batch A supernatant while it is 0.1 M for batch B supernatant. This difference might be significant, according to Teuling et al. results, however, it does not explain entirely the gap between batch A and B protein yields.

Thus, the desalination of the biomass could be a solution to facilitate protein release. However, desalination of the frozen paste at industrial scale necessitates another operation unit such as microfiltration or centrifugation, to recover the desalted biomass in the retentate or the pellet respectively. The medium salts would hence be washed out in the liquid phase, as well as the proteins released after freeze-thawing, inducing a significant protein loss. A washing step after harvesting and before freezing might be a more suitable option, or a culture at lower salinity if the strain allows it.

The increase in protein yield after dilution was already noticed also because the separation between pellet and supernatant was more efficient [11]. Thus, a dilution can be interesting to produce protein extracts, but this conclusion could be mitigated depending on the purification step applied, because a larger volume to be treated would also induce larger costs. If microfiltration is selected to purify soluble white proteins, the dilution can be useful, increasing membrane performances by enhancing permeation, as seen by Liu et al. [11].

4. Different process conditions for different biomass characteristics

The bead milling operation unit increases the yield of soluble protein from 9% after freeze-thawing to 11% after batch A bead milling and no further improvement can be achieved increasing the residence time. Since freeze-thawing is a mandatory operation unit in our case, the biomass being frozen, the CAPEX, the OPEX, and the environmental costs associated with the bead mill in comparison with simple freeze-thawing do not seem reasonable. Moreover, while the dry matter repartition was slightly increased after bead milling, the supernatant after simple freeze-thawing contained less chlorophyll. This was shown by the lightly green color compared to the strong green color obtained in the supernatants after bead milling. The light presence of chlorophyll in the supernatant after freeze-thawing can avoid an additional purification unit if a lightly green product is acceptable. Food products such as bread, with *T.chui* containing low chlorophyll after ethanol-extraction have been seen as more acceptable than using raw disrupted algae [65]. Hence, to produce low color protein extract from *T.chui*, using biomass in the same physiological state as batch A, a simple thawing would be recommended and a final protein yield of 9% can be expected. Bead milling on batch B cells releases a more significant protein quantity than freeze-thawing, so the addition of the operation unit could be useful to produce proteins extracts. However, the recovered protein extract shows a green color thus a purification step

may be needed, depending on the application target. A dilution before centrifugation can be effective but it leads to a higher volume to be purified. However, pH and temperature did not improved significantly the protein yields in the tested conditions.

Defining the characteristics of the biomass that can change is important on one hand to adapt the biorefinery process to it and on the other hand, to identify the culture conditions that can act on these characteristics or not.

5. Conclusion

Two batches of *Tetraselmis chui* paste were received and characterized. They presented difference in aspect, with batch A, having mostly dividing cells or circular cells, while batch B contained compressed cells with a depression at the apex. The differences were attributed to different physiological states of *Tetraselmis*. The rupturability of batch A was achieved at higher pressures compare to batch B. This was aligned with a faster cell disruption by bead milling for batch B compared to batch A.

The differences of dry matter and protein recovery yields in the supernatant after centrifugation between the two batches were strikingly different as well. An increase from only 19 to 20% of initial soluble dry matter without ashes within the supernatant was observed for batch A, while it was 13 to 27% for batch B. The soluble mass protein yields only improved from 9% after thawing, to 11% after milling for batch A, while it increased from 6% to 32% after milling for batch B. The difference of physiological state was confirmed through the supernatant analysis by electrophoresis and zymograms. Physicochemical modifications were applied on the lysate, with an improvement of protein solubility when the extract was diluted by half, but without a significant effect of temperature or pH.

Through these results, it was demonstrated that the microalgal physiological state deeply impacts the biorefinery performances. A better understanding of *Tetraselmis* physiology and its links to the culture process should facilitate the selection of the best harvesting conditions (period, duration etc.) to recover fragile cells and improve biorefining. The coupling of upstream (culture, harvesting) to downstream processes for microalgae valorization is still a challenge. The assessment of the correlation between the physiological state of the biomass and its extraction performances can definitively contribute to unlock the potential of microalgae protein isolates. The characteristics of the target product, mainly driven by consumer demand, will deeply affect the number of operations and the cost.

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Credit authorship contribution statement

Hugo Mear: Conceptualization, Methodology, Investigation, Writing – original draft, Visualization ; **Pascale Gillon:** Conceptualization, Methodology, Investigation, Writing – review & editing, Supervision ; **Imma Gifuni:** Conceptualization, Methodology, Writing – review & editing, Supervision **Laurence Lavenant:** Investigation ; **Alexia Poidevin:** Investigation ; **Estelle Couallier:** Conceptualization, Methodology, Investigation, Writing – review & editing, Supervision, Project administration

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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