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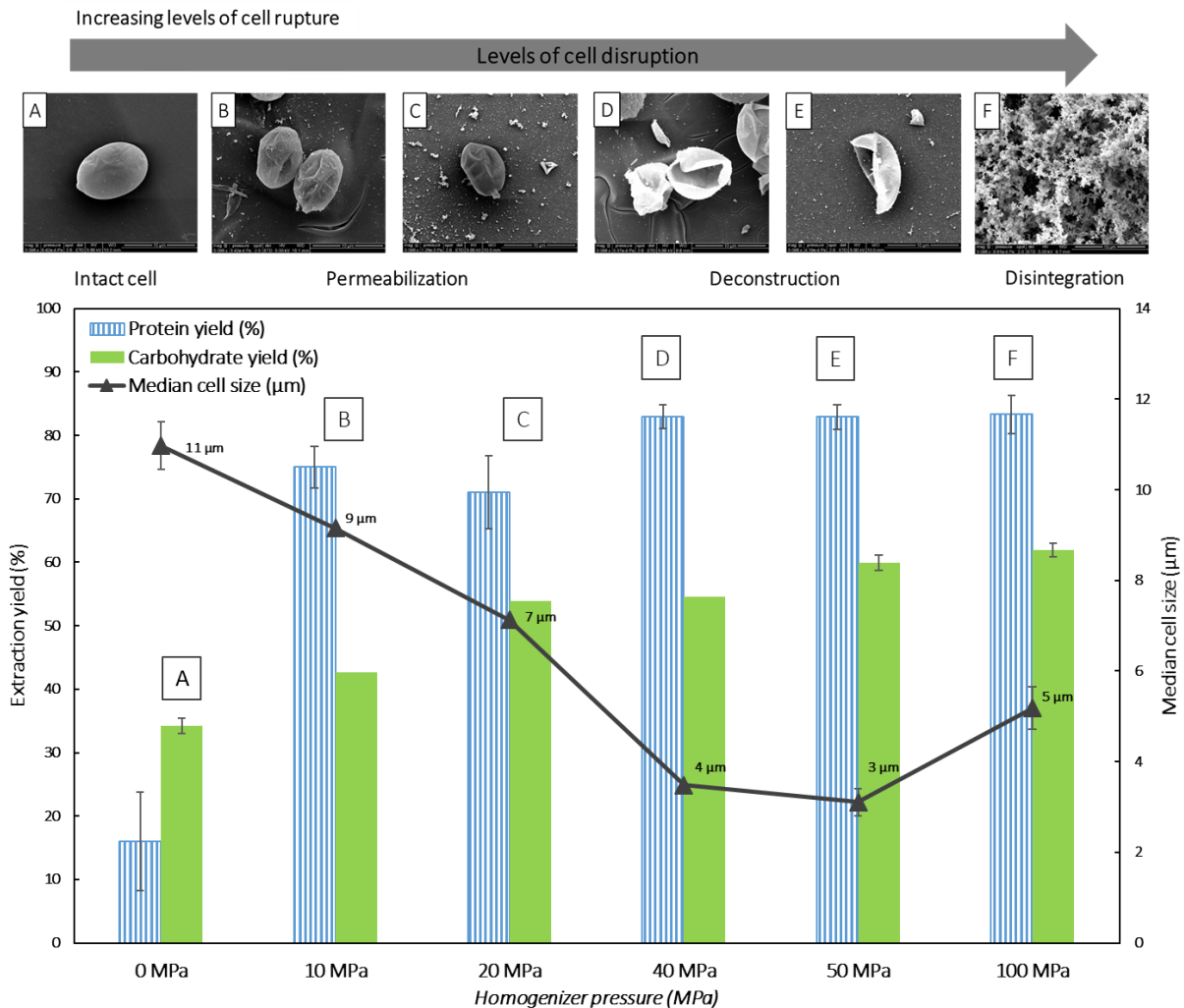
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Graphical Abstract



***Tetraselmis suecica* biofilm cell destruction by high-pressure homogenization for protein extraction**

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Abstract

The efficiency of cell destruction and extraction of intracellular contents by a high-pressure homogenizer was investigated on the *Tetraselmis suecica* microalgae to determine the possibility of integrating it into a biorefinery unit. Different process parameters relating to the use of a high-pressure homogenizer and biomass conditioning were tested. The parameter that has the greatest effect on cell destruction is pressure. From 40 MPa the maximum extraction yields of total proteins (80 %) and total carbohydrates (60 %) are reached and beyond 50 MPa, excessive cell destruction occurs, leading to the formation of aggregates. One single pass is sufficient to recover the maximal protein yield. Extraction in an alkaline solvent does not have a significant positive effect. Finally, it is possible to work in a wide range of biomass concentrations (from 10 g.L⁻¹ to 100 g.L⁻¹) and with fresh or after freeze-thaw cycles without significantly altering the yields.

Keywords (5)

Microalgae, Mechanical and physical processes, Cell lysis, Biorefinery, Green extraction

1. Introduction

Facing a growing demand for proteins (FAO 2020), microalgae have the potential to reduce the dependence on conventional proteins sources in aquaculture (Rahman, 2020). *Tetraselmis suecica* is a green marine microalga, whose cell size is about 10 µm, with a rigid cell wall. *T. suecica* shows good nutritional value as it is rich in proteins and carbohydrates (Pereira et al., 2019) and this makes it a good candidate for alternative protein refining. But these proteins must be produced in a sustainable way and not compete with current food and feed production. Moreover, to produce these proteins in industrial quantities, it is essential to design a biorefinery unit that includes the cultivation and harvesting steps (upstream processing) and the cell destruction steps to extract the molecules of interest (downstream processing) (Chew et al., 2017). Nevertheless, there are still many obstacles to the competitiveness of this industry and there is a need for better control of each step (Zinkoné et al., 2018).

Cell destruction is one of the most expensive steps in downstream processing, accounting for up to 50 % of total production costs compared to only 20-40 % for other industrial processes

(t Lam et al., 2018). Indeed, for most microalgae, the presence of a wall composed of several layers (de Carvalho et al., 2020) represents a major barrier during the cell destruction step, making them resistant to external attacks. To efficiently extract the maximum number of molecules of interest, it is necessary to be able to permeabilize or destroy the microalgae membranes and cell walls (Phong et al., 2018). Additionally, it is important to be able to control the different levels of the cell destruction, depending on the targeted molecules, and to correlate them with the process parameters, in order to be more selective for extracting the molecules of interest. Moreover, it is also important to localize the molecules sought in the cell to adapt the level of cellular destruction (Suarez Garcia et al., 2018).

Several mechanical processes for cell destruction have been described in the literature (Günerken et al., 2015). Among them, High-Pressure Homogenization (HPH) is a process originally used for suspension homogenization but is also employed for cell destruction (Bernaerts et al., 2019). This process consists of pumping the cells into a high-pressure valve. The cells are projected onto an impact ring located in a low-pressure area. This sudden change in pressure leads to cell destruction, caused by shear stress and hydrodynamic cavitation. The energy generated in the implosion zone is responsible for cell lysis. Homogenization is a standard unit operation, with adjustable parameters, such as pressure (generally between 10 MPa and 300 MPa), number of homogenization cycles and initial material concentration of the processed product (Balduyck et al., 2018). There are several types of homogenizers with different valve designs and various configurations depending on the applications (Osorio-Arias et al., 2021). Other parameters, more specific to the nature of the algal biomass used, can influence the efficiency of the process. These parameters include initial biomass concentration, conditioning of the biomass with, for example, pre-treatments like freeze-thaw cycles, freeze-drying or desalting. Many studies have investigated the effects of such parameters on the cell destruction rate of microalgae and on protein extraction efficiency (Papachristou et al., 2020; Safi et al., 2014; Spiden et al., 2013b; Ursu et al., 2014). These studies have been conducted on several microalgal species, depending on the biomass conditioning, its concentration and process parameters, such as pressure and number of passes. More than ten different species of microalgae have been studied in the literature (Cho et al., 2012; Günerken et al., 2015; Magpusao et al., 2022; Nitsos et al., 2020; Zhang et al., 2019). Some studies have also shown that differences can be observed within the same microalgal genus (Grossmann et al., 2018; Safi et al., 2017a, 2017b).

Several methods were used to monitor and measure the efficiency of the cell destruction by laser diffraction, cell count or metabolites recovery (Middelberg, 1995; Spiden et al., 2013a, 2013b). In general, the efficiency of the process on cell destruction seems to be species dependent. *T. suecica* rapidly achieves 100 % cell destruction after only two passes at moderate pressure (40 MPa) (Spiden et al., 2013b).

The overview of literature studies does not show a clear trend but seems to indicate that each microalga reacts differently to cell lysis and that processes need to be adapted to each species. These works concern fresh biomasses, or those having received pre-treatments such as freeze-drying or freezing. The effect of such pre-treatments, which could induce spontaneous cell lysis before HPH, is not considered in the analysis of the results. However, it is interesting to note that for this cell destruction process, the specific energy consumption remains low : 0.42 kWh.kg⁻¹ after 6 passes at 150 MPa for *Nannochloropsis* sp. (Grimi et al., 2014) and 0.02 kWh.kg⁻¹ after 12 passes at 180 MPa for *Nannochloropsis oceanica* (Grossmann et al., 2018).

Few studies have focused on the optimization of homogenization process parameters and the study of their effects on cell destruction. Thus, the main objective of this present study is to

optimize and correlate the HPH and biomass parameters to cell destruction of fresh *Tetraselmis suecica* cultivated as a biofilm. This will be implemented by studying protein and carbohydrate extraction and by tracking the changes in cell morphology, in order to define a cell destruction mechanism.

2. Materials and methods

2.1 Microalga specie: *Tetraselmis suecica* biomass

Tetraselmis suecica was provided by the company, Inalve (Nice, France). It was produced as a biofilm using a patented rotating algal growth system (WO2021180713A1), allowing the harvesting of a concentrated paste, simply by scraping the surface. The dry matter content of the collected *T. suecica* biomass was around 15 %. The fresh biomass was transported respecting a cold chain and stored at 4 °C prior to use. All experiments were carried out within four days of harvesting the biomass, in order to preserve its freshness and to avoid bacterial development.

In order to remove some of the salts and the extracellular matrix composing the biofilm, a protocol for desalting the biomass was developed. Before each experiment, the biofilm was first diluted with a saline solution (sodium chloride) and then centrifuged for 20 minutes at 4000 x g, in order to avoid osmotic shocks and to preserve the structural integrity of the cells. The desalted biofilm (pellet) was then rediluted with distilled water to the desired final concentration (10 g.L⁻¹, 50 g.L⁻¹ or 100 g.L⁻¹) for each experiment. Finally, the freeze-thaw method was also studied by simply freezing the biomass at -20 °C for several weeks and thawing it at 4 °C for 24 hours, and applying the desalting and dilution protocol to a desired final concentration of 100 g.L⁻¹.

2.2 *Tetraselmis suecica* biomass characterization

The initial biomass composition was determined through various analyses. Moisture content was determined by drying in an oven at 103 °C for 24 hours (Sluiter, 2008a). The ash fraction was measured by calcination at 550 °C for 12 hours (Sluiter, 2008b). Total proteins were determined by an elemental analysis method using the conversion factor N = 6.25 (Schwenzfeier et al., 2011). Total carbohydrates were measured by the Dubois method (Dubois et al., 1956). The last two methods are detailed in section 2.4.4. Gravimetric quantification of lipids was performed by Soxhlet extraction according to the Bligh and Dyer method (Bligh et Dyer, 1959). Briefly, 5g of dried biomass was transferred to a 25 × 80 mm cellulose thimble and placed into a Soxhlet extraction chamber with 500 mL of a solvent mixture of methanol and chloroform (70 : 30, v/v), where it was heated under reflux at a temperature close to boiling point of the solvent for 8 h (18–22 cycles/h). Then the solvent mixture was evaporated and the extracted residue was weighed gravimetrically.

All chemicals (sulfuric acid, sodium hydroxide, phenol and sugar standards (glucose)) were purchased from Sigma Aldrich Chimie (Saint-Quentin, France) and used as received.

2.3 High-pressure homogeneization set-up

2.3.1 Non-mechanical cell lysis

Non-mechanical water and alkaline extractions were performed and used as controls for this study (Phong et al., 2018). Extraction was performed by placing the 100 g.L⁻¹ suspension in a water or alkaline solution at pH 12 with or without a temperature regulation held constant at 40 °C and by gentle mechanical stirring at 300 rpm for 30 min.

2.3.2 High-pressure homogenizer treatment

The high-pressure homogenizer experiments were carried out using a two-stage lab homogenizer PandaPlus 2000 from GEA (Niro Soavi, Palma, Italy) with a pressure range from 0 MPa to 200 MPa and a flow rate of 9 L.h⁻¹. In this study, only the first stage was used. All experiments were performed at room temperature (approximately 20 °C) without any cooling system. The temperature at the HPH outlet never exceeded 40 °C after one pass at the highest pressure used (150 MPa).

100 mL of each suspension at various concentrations (10 g.L⁻¹, 50 g.L⁻¹ or 100 g.L⁻¹) were pumped into the HPH and homogenized at various pressure levels, 0, 10, 20, 40, 50, 100 and 150 MPa (eq. 0, 100, 200, 400, 500, 1000 and 1500 Bar respectively).

To study the effect of HPH under alkaline conditions, the 100 g.L⁻¹ pH suspension was adjusted to 12 using a 2M sodium hydroxide solution.

The influence of the number of passes (1, 2 or 3 passes) was also studied only for the 100 g.L⁻¹ suspensions. Between each pass through the HPH, the suspension was manually cooled to room temperature using an ice water bath.

Before and after each treatment, suspensions were characterized and then centrifuged at 8000 x g for 20 minutes to collect the supernatant for biochemical analysis. For suspensions at 100 g.L⁻¹ and treated at 10 MPa and 50 MPa, the residue obtained after the centrifugation step was re-suspended at 100 g.L⁻¹ with deionized water and re-submitted for the next pass to the HPH treatment at pressures of 10 MPa and 50 MPa respectively.

For the pressure of 0, 50 and 100 MPa, each experiment was repeated at least three times to ensure the repeatability. Data are expressed as mean ± standard deviation. The error bars, shown on the figures, correspond to the standard deviations.

2.4 Cell destruction characterization

2.4.1 Cell size analysis

The cell size analysis of the entire microalgae suspension before and after cell destruction treatment was determined by laser diffraction using a Malvern Mastersizer 3000 (Malvern Panalytical, UK) apparatus equipped with a HydroMV accessory for liquid samples. A refractive index of 1.45 and an absorption index of 0.100 were used, corresponding to the values for microalgal cells.

2.4.2 Optical microscopy and SEM analysis

The samples were analyzed by scanning electron microscopy (SEM) and light microscopy before and after cell destruction treatment, in order to observe cell morphology.

For SEM investigations, cells were immediately fixed in polylysine buffer and maintained at 4 °C until the sample was prepared for observation. SEM images of *Tetraselmis* suspension were acquired at 5096 fold magnification at 5 kV accelerating voltage with a QUANTA 250 FEG microscope (FEI company, France).

For optical microscopy, immediately after the cell destruction treatment, a 1:10 suspension dilution was prepared and a 20 μ L sample was placed on a glass slide with a cover slip. Observation was performed using a Nikon SMZ 1500 at x1000 fold magnification and the images were captured by a Nikon Eclipse E600 camera.

2.4.3 Supernatant analysis

Elemental analysis

The total nitrogen of the dried sample was evaluated using a PerkinElmer 2400 series II elemental analyser. The percentage of nitrogen was evaluated and converted into the protein percentage using the conversion factor of 6.25.

Total carbohydrates quantification

The Dubois colorimetric assay was used to determine the polysaccharide content in each supernatant dried sample, using glucose as a standard solution. The absorbance was determined at 490 nm using a BMG-LabtechSpectrostar-Nano spectrophotometer (BMG LABTECH SARL, Champigny s/Marne, France). Briefly, 10 mg of dry sample was hydrolyzed for 1 hour at 100 °C with sulphuric acid. 200 μ L of the hydrolysate was then mixed with 200 μ L of phenol (5 % w/w) and 1 mL of sulphuric acid and incubated for 1h at 100 °C.

The extraction yields of each compound (proteins and carbohydrates) were then calculated in relation to their initial composition in the biomass.

3. Results & discussion

3.1. *Tetraselmis suecica* proximate composition and cell destruction by non mechanical methods

The composition of *Tetraselmis suecica*, grown as a biofilm, is specified in Table 1. This strain contains 33 % proteins, and this value is identical to other values reported for more conventional growing methods such as planktonic cultures (Pereira et al., 2019), which makes it an interesting microalga for protein refining. The carbohydrate content is 22.5 %, which is in the range of values obtained for other biomass of *T. suecica* (Reyimu and Özçimen, 2017; Tulli et al., 2012). This study identified the total lipid content to be 15 %. This correlates with data found in the literature: the lipid content can range from 8 to 50 % (Lo et al., 2022; Moheimani, 2013; Pugkaew et al., 2019), and depends on various factors, such as biomass cultivation and environmental conditions (Russell et al., 2022). Finally, 24.5 % of the biomass is composed by ashes, a value related to the accumulation of minerals and salts present in the composition of the culture medium (Bernaerts et al., 2018).

Figure 1(a) represents the cell size distribution of *T. suecica* cells after various non-mechanical lysis treatments. These non-mechanical treatments consist of a mild stirring and the use of water at room temperature or with a thermal treatment at 40 °C or a simple stirring with sodium hydroxide at pH 12. After 30 minutes' treatment, only a slight shift of the curves to the left and a small decrease in the proportion of aggregates of approximately 60 μ m was observed, but no difference in the size distribution of the populations was noticed compared to the untreated *T. suecica* suspension. A bimodal distribution is visible whatever the conditions, with two peaks

centered at approximately 60 μm and 13 μm . Only the treatment with sodium hydroxide causes a third population to appear very slightly, centered at 2 μm , suggesting a beginning of cell lysis.

This tendency is confirmed by SEM observations in Figure 1(b). SEM image “A” shows the morphology of *T. suecica* cells before treatment. Round and spherical cells can be observed, with a size of 10 μm , characteristic of this specie. The SEM images corresponding to the cells after the application of the thermal treatment (Figure 1(b) SEM image “B”) or the use of sodium hydroxide (Figure 1(b) SEM image “C”), show that the cells have the same initial aspect and that they do not seem to be damaged or lysed. As it is well known, the presence of a resistant cell wall prevents cell lysis and the release of intracellular contents in most of the microalgae. The cell wall of *T. suecica* is described as being composed by a complex network of sugars and proteins. This network contains mainly galactose, xylose, rhamnose, mannose and arabinose as well as rare sugars such as Kdo (3-deoxy-d-manno-oct-2-ulosonic acid) and Dha (3-deoxy-lyxo-2-heptulosaric acid) (Kermanshahi-pour et al., 2014). All these compounds form a strong and relatively rigid scale structure, which makes the extraction of intracellular compounds difficult and explains these results. Therefore, a simple dilution in water or sodium hydroxide is not enough to cause a strong shock to induce cell wall damages and proteins diffusion. The destruction of *T. suecica* cells therefore requires a higher energy input, which can be induced by mechanical destruction methods with stronger stress forces.

3.2. Effects of different parameters of the high-pressure homogenizer on *Tetraselmis suecica* cell destruction

3.2.1. Pressure levels effects and optimization of cell destruction

To study the cell destruction by high-pressure homogenizer, pressures ranging from 0 to 150 MPa were applied on a 100 $\text{g}\cdot\text{L}^{-1}$ *T. suecica* suspension. Figure 2(a) and 2(b) show the cell size distribution before and after HPH treatment at pressures below and above 50 MPa respectively. No difference was observed in the size distribution between non-treated and 0 MPa treated suspensions. For both, a bimodal distribution is observed, with two peaks centered at 9 μm for intact cells and at 59 μm for cell agglomerates. For lower pressure than 50 MPa (Figure 2(a)), a trimodal distribution is observed. The application of a minimum pressure of 10 MPa initially disperses the 59 μm cell agglomerates without lysing them, thus increasing the proportion of intact cells of the population centered at 9 μm and inducing the initiation of cell lysis with the appearance of two new populations centered at 0.7 and 3 μm . Moreover, the increase in pressure is positively correlated with the increase in populations corresponding to lysed cells (3 and 0.7 μm) and the decrease of intact cells. However, higher homogenization pressures, 100 MPa and 150 MPa (Figure 2(b)) lead to an excessive cell destruction and to a significant agglomeration of the generated cellular debris. Indeed, for 100 MPa, the size distribution shifts to the right and the distribution becomes bimodal with the disappearance of the population at 3 μm in favor of a bigger population. For 150 MPa, a larger increase in the cell size is observed with a new size population centered at 110 μm .

In addition, microscopic observations (Figure 2(c)) confirm the size data and show that one pass at 0 MPa does not damage the cells; however, at 100 MPa, intact cells are no longer visible, and they are completely disintegrated. Several previous studies have identified a phenomenon of increased cell size, attributed to the agglomeration of debris, generated by excessive pressures for the microalga *Tetraselmis suecica* (Spiden et al., 2013b). Reagglomeration occurs between cellular debris, when debris reach a significant degree of micronization, and the release of intracellular contents. This phenomenon was observed for *T. suecica* from pressures higher than 60 MPa (Magpusao et al., 2021). While these studies showed that additional treatment

eventually fragments the agglomerates, our study showed the opposite. Debris reagglomeration is accentuated at 150 MPa, with the appearance of two new population sizes centered at 19 μm and 100 μm , significantly larger than the cell size in the untreated suspension.

Figure 3 shows the evolution of cell micronization by SEM observations, the change in median cell size, as well as the extraction yields of proteins and sugars as a function of applied pressure. For pressures of 10 MPa and 20 MPa, the cells have a deflated, damaged appearance and appear to be permeabilized. For pressures of 40 MPa and 50 MPa, the cells are deconstructed, bursted and are micronized into small debris with a median size of approximately 4 μm . Finally, at 100 MPa, the cells are completely micronized and disintegrated in small debris that cannot be separated from each other, forming agglomerates with a median size of 5 μm . Despite the fact that the mechanisms of cell destruction by HPH have not yet been fully elucidated it is possible to distinguish several levels of cell destruction during homogenization. In parallel, it was observed that the yields of proteins and carbohydrates are not totally correlated with the progress of cell destruction. Indeed, from the lowest pressure, 10 MPa, the maximum yield of proteins (about 80 % at 100 MPa) is almost reached. The same observation can be made for the extraction yield of carbohydrates. Increasing the pressure from 10 MPa to 50 MPa or 100 MPa does not result in significantly more metabolites being extracted. These results correlate with previous studies showing fragmentation and complete disintegration of *Tetraselmis* sp. cells for pressures lower than 100 MPa (Spiden et al., 2013b; Magpusao et al., 2021). Lysis of *T. suecica* is possible at low pressures, between 10 MPa and 50 MPa, thus avoiding the use of pressures leading to reagglomeration of debris. High pressures could have negative consequences on the efficiency of the rest of the biorefinery process during clarification steps for example and because the maximum possible extraction yields after 1 pass are already obtained, while limiting energy consumption.

3.2.2. Effect of multiple homogenization cycles on the cell destruction

The influence of the number of successive passes on the cell lysis of *T. suecica* and on the protein extraction yields was also studied for pressures ranging from 0 MPa to 100 MPa. This parameter is considered crucial, as studies have shown that some microalgae require several treatment cycles to achieve total cell destruction and good protein yields. In the study of Elain et al. (2020) regarding the cyanobacteria *Arthrospira platensis*, four passes at 50 MPa were necessary to reduce the cell size to 0.2 μm indicating a complete cell destruction. Also, Ferreira et al. (2022), showed that only 75 % of cell destruction was obtained for *Tetrademus obliquus* after three passes at 60 MPa, demonstrating a moderate resistance of the microalgal cell walls to a mechanical force. Moreover, two works on *T. suecica* were conducted by Magpusao et al., 2021 and Safi et al., 2014 to analyze the effect of pressure on cells using HPH after only one single pass.

This present study showed that extraction yields for proteins or carbohydrates do not increase after one pass at 40 MPa. Therefore, the objective of this study was to see if the succession of multiple HPH passes could solubilize a larger amount of protein.

Figure 4(a) and 4(b) show that for two different pressures, at 10 MPa and 50 MPa, a single pass is sufficient to lyse the cells. Indeed, from the first pass, a trimodal distribution appears corresponding to intact cells at 9 μm , and two populations of cellular debris at 0.7 μm and 3 μm . Increasing the number of passes does not increase the proportion of cell debris. Similarly, Figure 4(c) shows that at 10 MPa an extraction maximum is reached after one single pass. For higher pressures, at 50 MPa or 100 MPa, the protein content remains constant after 1, 2 or 3 passes. According to these results, a single pass at 50 MPa seems to be sufficient and optimal

for releasing up to 90 % of the total proteins. It should be noted that the extraction rate of total proteins never reaches 100 %, no matter what the extraction conditions used (number of passes, maximum pressure). This loss of proteins can be explained in several ways. Proteins are dispersed in various forms in the cell, and a certain proportion of them may be poorly soluble. Their solubility may be affected by their function and if they contain ramifications, such as polysaccharides for example (Golovanov et al., 2004). This may cause them to migrate into the pellet during the clarification step of the suspension after centrifugation (Liu et al., 2021).

Figure 4(d) shows that while multiple passes have no effect on protein release, resuspension of the pellet after centrifugation step in water and retreating at 10 MPa or 50 MPa improves yields. Resuspension of the pellet at the same final concentration, 100 g.L⁻¹ in water, acts as a rinse and thus favors a better solubilization and diffusion of the remaining proteins in the medium. The protein extraction yield is improved and tends to reach 100 %.

3.3. Influence of the biomass parameters on biomolecule extraction and cell destruction efficiency

Biomass conditioning and pre-treatment prior to HPH treatment can also affect the efficiency of cell destruction and the extractable yields. The initial biomass state (fresh, frozen or dried biomass) also seems to be able to influence the efficiency of cell destruction and molecule extraction. The literature also shows that the choice of dilution and initial biomass concentration has an impact on the final results. For instance, two studies on the extraction of proteins from frozen *Chlorella vulgaris* biomass, by HPH using the same treatment conditions (2 passes - 270 MPa) result in different extraction yields. Indeed, at 13 g.L⁻¹ (Ursu et al., 2014) the maximal protein yield obtained was 98 % while at 20 g.L⁻¹ (Safi et al., 2014) it was only 25 %. Finally, on fresh *Chlorella vulgaris*, but using two different HPH configurations and two different biomass concentrations, Papachristou et al. (2020) showed that five passes at 150 MPa and at 100 g.L⁻¹ leads to 67 % cell destruction while Yap et al. (2014) showed that 1 pass at 140 MPa and at 3.6 g.L⁻¹ were enough to reach 80 %. Some of these parameters for *T. suecica* were investigated in this study.

3.3.1. Effect of freeze-thaw cycles

It is sometimes necessary to freeze freshly harvested biomass to ensure its preservation or stability over time before processing. However, literature analyses indicate that freeze-thaw cycles can cause cell lysis. Indeed, freezing wet biomass causes the crystallization of intracellular water. The thawing of the biomass then causes these crystals to expand, leading to cell rupture (Pagels et al., 2021).

Figure 5(a) shows the cell size distribution of the native 100 g.L⁻¹ suspension and after one freeze-thaw cycle. The mean cell size after one freeze-thaw cycle remains the same as that of the untreated biofilm, with two size populations centered at 45 µm and 11 µm. However, the volume of intact cells (11 µm) decreases by half after one thawing cycle while the proportion of agglomerates (45 µm) increases, indicating a significant loss of intact cells. The protein yield after one pass at 0 MPa for the frozen biofilm (Figure 5(b)) confirms this hypothesis. The protein content is higher compared to the fresh biofilm, showing that a majority of cells are already permeabilized or damaged to release three times more proteins into the medium.

However, this trend is reversed as soon as low pressure is applied (10 MPa). For each pressure level, protein yields are always lower for frozen biofilm than for fresh biofilm. For the tests discussed in this section, the biomass was frozen, thawed and then desalted before being used. This stage of biomass conditioning may explain this systematic loss of proteins. Indeed, during

thawing, lysis of cells occurred, releasing a certain amount of proteins. These proteins were then lost during the desalting protocol of the biomass. Thus, freezing finally shows a lesser effect on the cell destruction of *T. suecica*, leading to a significant release of proteins. However, it is still necessary to apply pressure to obtain the maximum level of protein extraction.

3.3.2. Effect of solvent dilution on the protein extraction yield and on the cells

Alkaline treatment is a common non-mechanical treatment used for extraction and solubilization of proteins from microalgae (Nitsos et al., 2020). This treatment can lead to a significant increase in extracted protein concentration. Sodium hydroxide causes the hydrolysis of ester and hydrogen bonds between the different components (polysaccharides and non-polysaccharides) of the microalgae cell wall (Phong et al., 2018). Indeed, Suarez Garcia et al. (2018) hypothesized that in *T. suecica* cells, proteins would be divided into three categories: “aqueous proteins, function and structure”. Each pool of proteins has its own properties and sometimes requires solubilization by various solvents (sodium hydroxide, water or detergents for example).

Figure 5(c) shows that sodium hydroxide allows the extraction of more than twice the amount of protein obtained with the water medium. An alkaline solution and a change in pH, therefore leads to a better solubilization of the proteins. However, the benefit effect of sodium hydroxide on yield is no longer observed as soon as pressure is applied (10 MPa or 50 MPa). The protein yields obtained no longer depend on the solvent but only on the pressure level. The maximum protein yield level reaches more than 80 % with water and is slightly lower with sodium hydroxide. Under the conditions of this study, it is therefore not necessary to use sodium hydroxide in addition to the HPH. The use of HPH makes it possible to extract proteins using water, making this process greener and more eco-friendly.

3.3.3. Effect of initial biomass concentration on the protein extraction yield

The initial biomass concentration is also a parameter to consider. In particular, it can influence the efficiency of centrifugation and protein recovery as well as the total specific energy consumption of the process (Günerken et al., 2015). Three concentrations, 10 g.L⁻¹, 50 g.L⁻¹ and 100 g.L⁻¹, were studied using a single pass applying a pressure in the range of 0 to 100 MPa. For a pressure of 100 MPa, the distribution of the suspension at 10 g.L⁻¹ shows a higher proportion of cell debris at 0.7 µm and a lower proportion of agglomerates than for the other two concentrations (Figure 6(a)). This higher rate of cell destruction for the lowest suspension concentration could be explained by the effect of dilution. A larger volume of water comes into contact with the cells, causing greater stress, as well as an osmotic shock causing cell lysis. An osmotic gradient can form across the cell membrane when the biomass is resuspended in a medium with a lower salt concentration or with fresh water, which is the case in this study (Halim et al., 2021).

For a concentration of 10 g.L⁻¹ there are fewer cells, the distribution of cellular debris is more homogeneous, explaining the absence of agglomerates for high pressures (Liu et al., 2021). Moreover, Halim et al. (2013) showed that the rupture rate constant for HPH is inversely proportional to the initial suspension concentration. For a low cell concentration, the kinetic energy provided by the HPH is distributed over a small number of cells, each cell moves at a high velocity and experiences more forces when colliding with both the valve seat and the impact ring.

However, the variation of the concentration seems to have less effect on the protein yield than the pressure (Figure 6(b)). Xie et al. (2016) reported that the efficiency of homogenization was

not affected by cell concentration for a specific range of concentrations. They suggested that the release rate of intracellular products depends on the product localization, only allowing to work in a wide range of cell concentration and not to have to carry out a large dilution to work with a HPH. After one pass at 0 MPa, the protein extraction rate is higher for the lowest biomass concentration of 10 g.L⁻¹ compared to concentrations of 50 and 100 g.L⁻¹. Without any mechanical effect provided by HPH, this variation in protein extraction rates can be explained by the increase in the amount of water in relation to the amount of biomass input, which significantly lowers the salinity and causes a greater osmotic shock than for higher biomass amounts. However, this positive effect on yields observed at low concentration disappears as soon as a pressure higher than 0 MPa is applied. Protein extraction yields for 10 g.L⁻¹ increase at 10 MPa and 20 MPa until reaching a plateau at 40 MPa with a final extraction yield of 75 % compared to 85 % for 50 g.L⁻¹ and 100 g.L⁻¹ of biomass. A possible hypothesis to explain these observations is that at lower concentrations the viscosity of the suspension decreases leading to a reduction of the shear effect normally observed during the passage of the biomass in the HPH, which results in less efficient cell destruction.

Conclusion

In this study, various HPH parameters were evaluated on *Tetraselmis suecica* biomass produced as a biofilm. The parameter that has the most influence on the efficiency of cell destruction is pressure. A single pass at medium pressures, 40 MPa, is sufficient to permeabilize the cells and to release more than 80 % of total proteins and 60 % of polysaccharides. Freezing the biomass does not facilitate cell destruction, the cells seem resistant to this pre-treatment. The use of alkaline solvent does not improve extraction yields. The HPH technique enables working in a wide range of cell concentration, making it easy to integrate in an industrial biorefinery design.

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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.

CRedit authorship contribution statement

Pauline Delran: Conceptualization, Investigation, Methodology, Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Christine Frances:** Supervision, Conceptualization, Methodology, Validation, Writing – review & editing. **Freddy Guiheneuf:** Supervision, Resources, Data curation, Writing – review & editing. **Jérôme Peydecastaing:** Investigation, Validation, Writing – review & editing. **Pierre-Yves Pontalier:** Writing – review & editing. **Laurie Barthe:** Project administration, Supervision, Conceptualization, Methodology, Data curation, Validation, Writing – review & editing.

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Tables and Figures

Table 1: Biochemical composition of the raw Tetraselmis suecica, cultivated as biofilm.

Composition	Total proteins	Total carbohydrates	Lipids	Ash	Other extractibles
Content (% DW)	33.5 ± 3.8	22.5 ± 0.3	15 ± 0.5	24.5 ± 2.9	4.5

DW, dry weight

Values are reported as mean ± SD (n = 3)

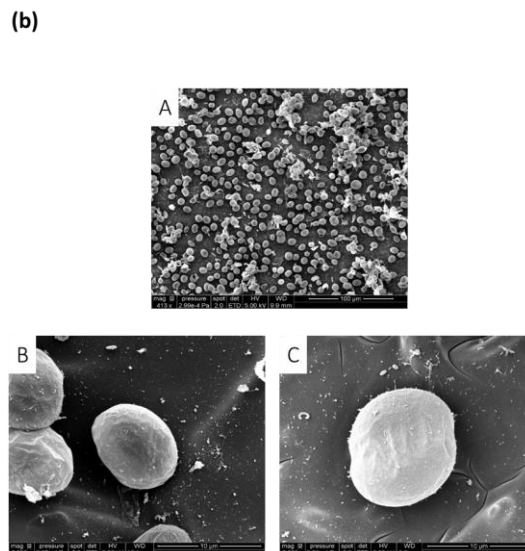
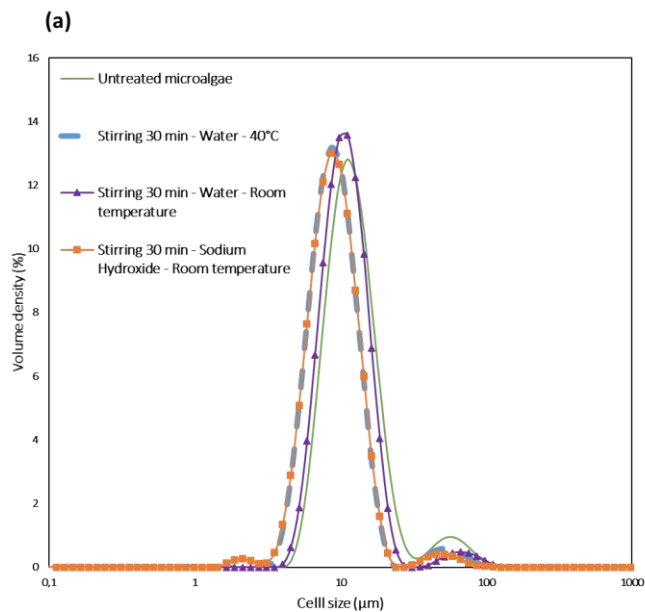


Figure 1(a): Effect of non-mechanical treatments on the cell size distribution of *Tetraselmis suecica*. Figure 1(b): Scanning electronic microscopy (SEM) of A. untreated cells; B. cells obtained after 30 minutes of mild stirring at 40 °C in water; C. cells obtained after 30 minutes of mild stirring at pH 12 in sodium hydroxide.

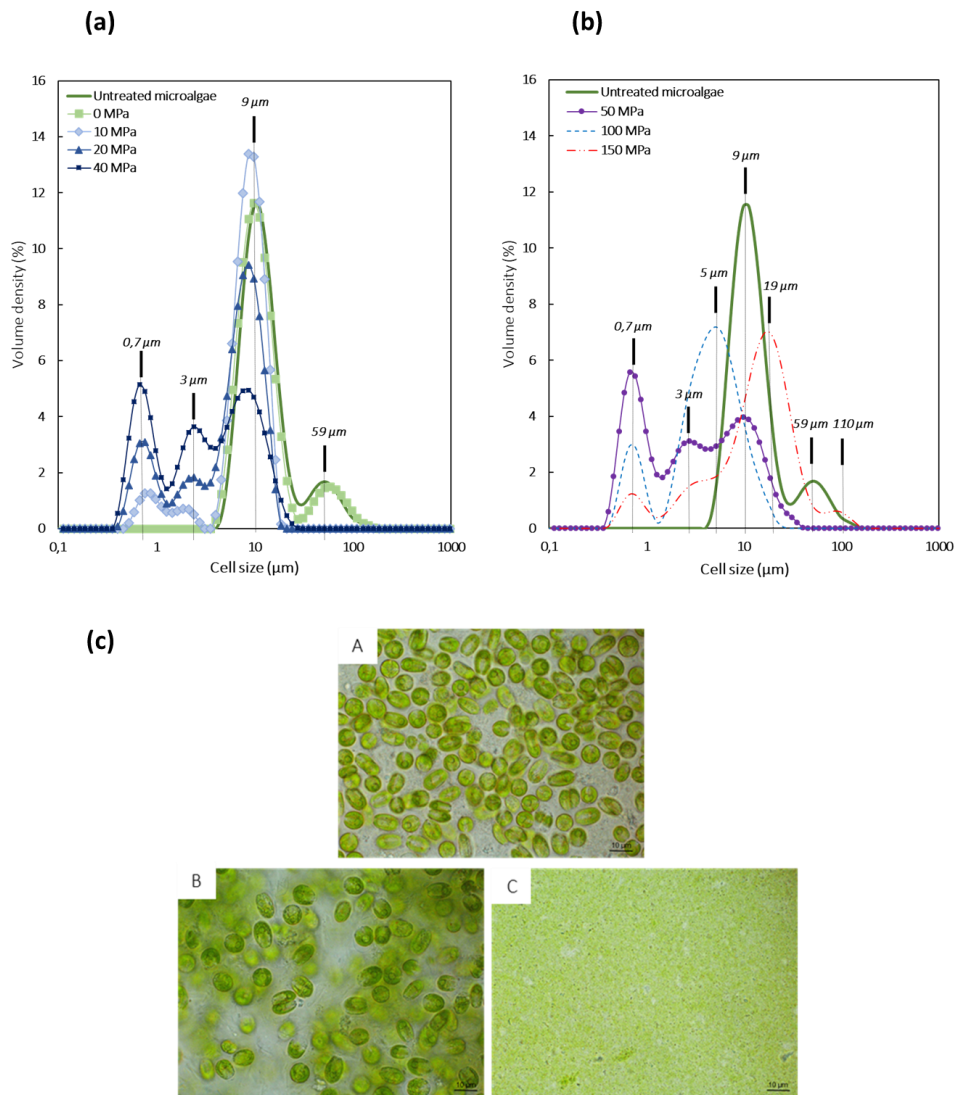


Figure 2: Influence of pressure levels (MPa) for (a). low pressures and (b). high pressures, on the cell size distribution of *Tetraselmis suecica* suspension. Figure 2(c): Microscopic observation at x1000 magnification before and after one cycle of high-pressure homogenization of *Tetraselmis suecica*: A. Microalgae untreated; B. 0 Mpa; C. 100 Mpa.

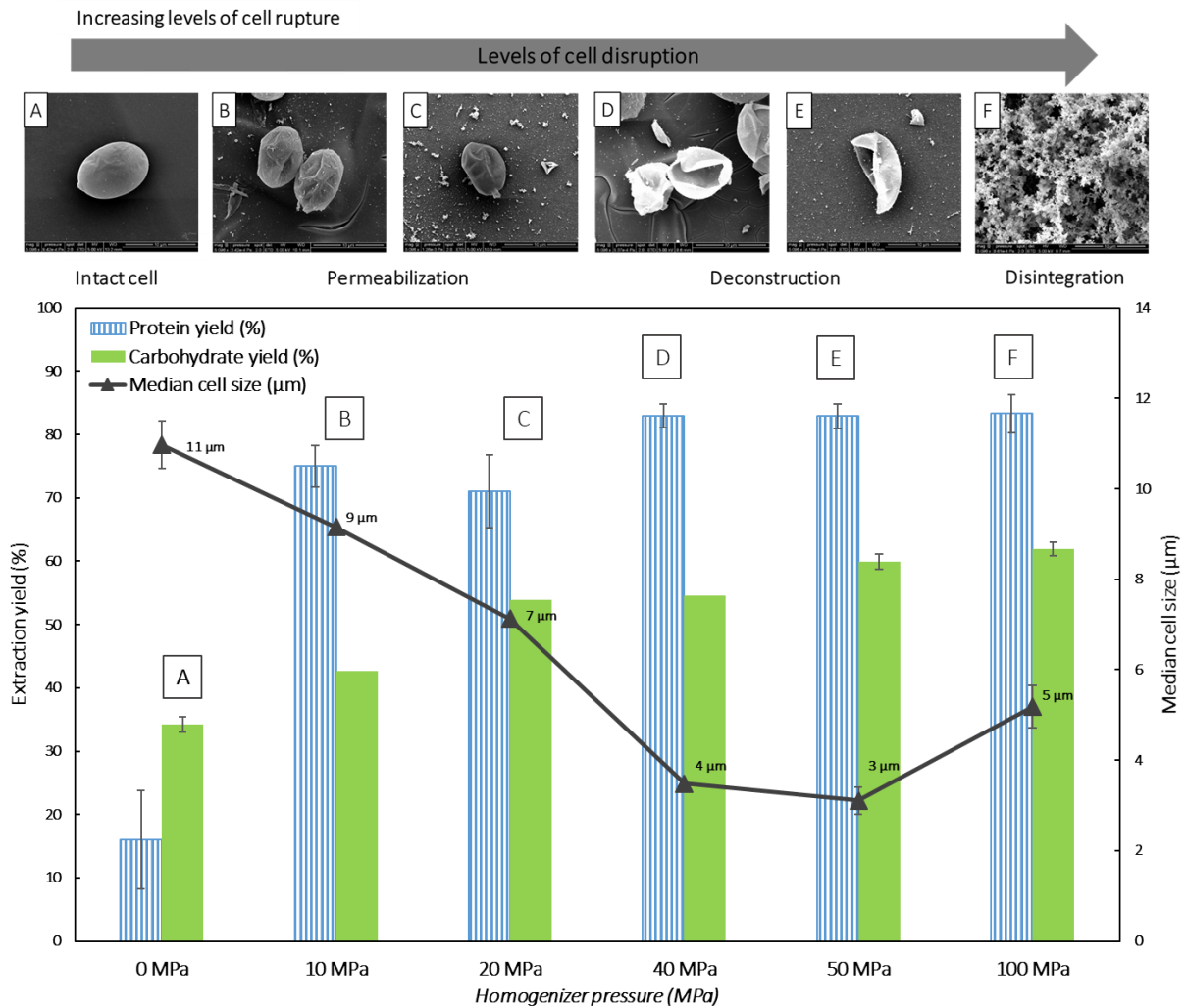


Figure 3: Coupling of analytical methods for monitoring cell destruction in *Tetraselmis suecica*: influence of pressure levels on the carbohydrates and proteins extraction yields and evolution of the mean particule size distribution (μm). (Values are the mean of triplicates ($n = 3$) with error bars representing standard deviation). SEM micrographs illustrate the effect of pressure on cell morphology and allow the different levels of cell destruction to be imaged.

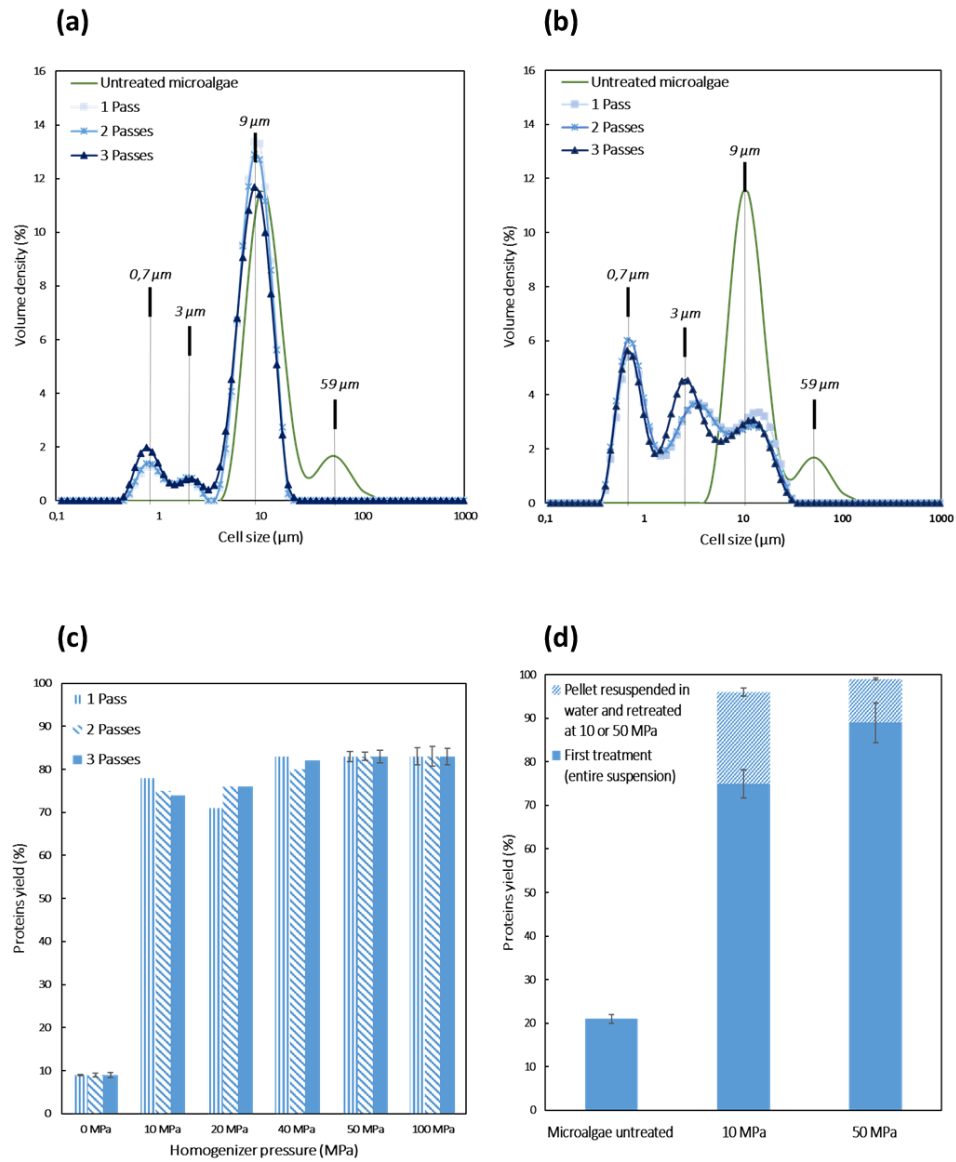


Figure 4: Cell size distribution of *Tetraselmis suecica* for 1 to 3 treatment passes for a pressure of 10 MPa (a) and 50 MPa (b). Protein extraction yields for 1 to 3 homogenization cycle for a pressure of 50 MPa (Values are the mean of triplicates ($n = 3$) with error bars representing standard deviation) (c). Effect of resuspending the pellet in water and a second pass through the homogenizer at 10 or 50 MPa on protein extraction yields (d).

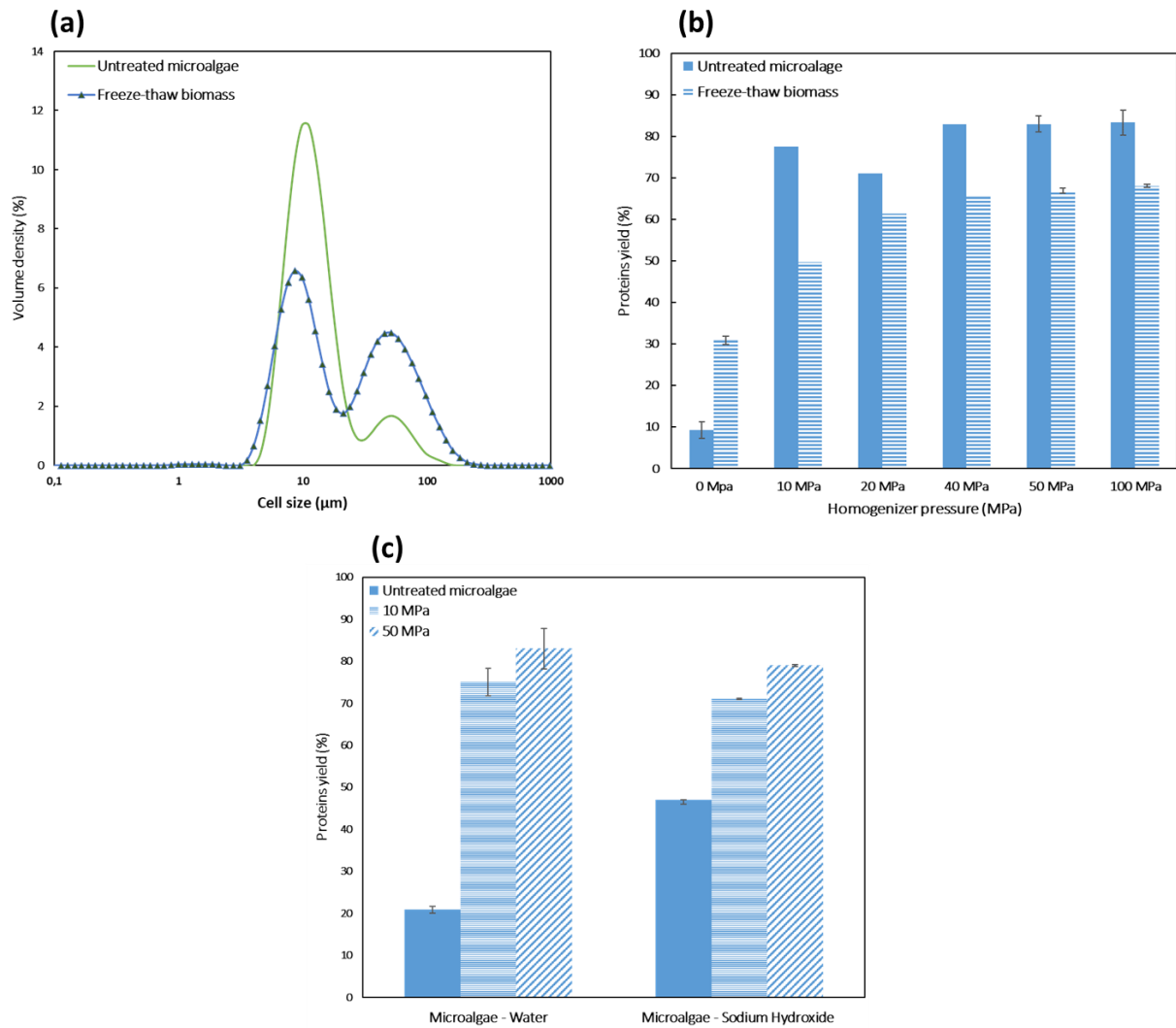


Figure 5: Cell size distribution of untreated biomass and after one of freeze-thaw cycle (a). Protein extraction yield (b) for the same biomass, with or without a cycle of freeze-thaw. (Values are the mean of triplicates ($n = 3$) with error bars representing standard deviations). (c) Protein extraction yield for 2 different solvents, water and sodium hydroxide, on a biomass at $100 \text{ g}\cdot\text{L}^{-1}$ and for a pressure up to 50 MPa (Values are the mean of triplicates ($n = 3$) with error bars representing standard deviations).

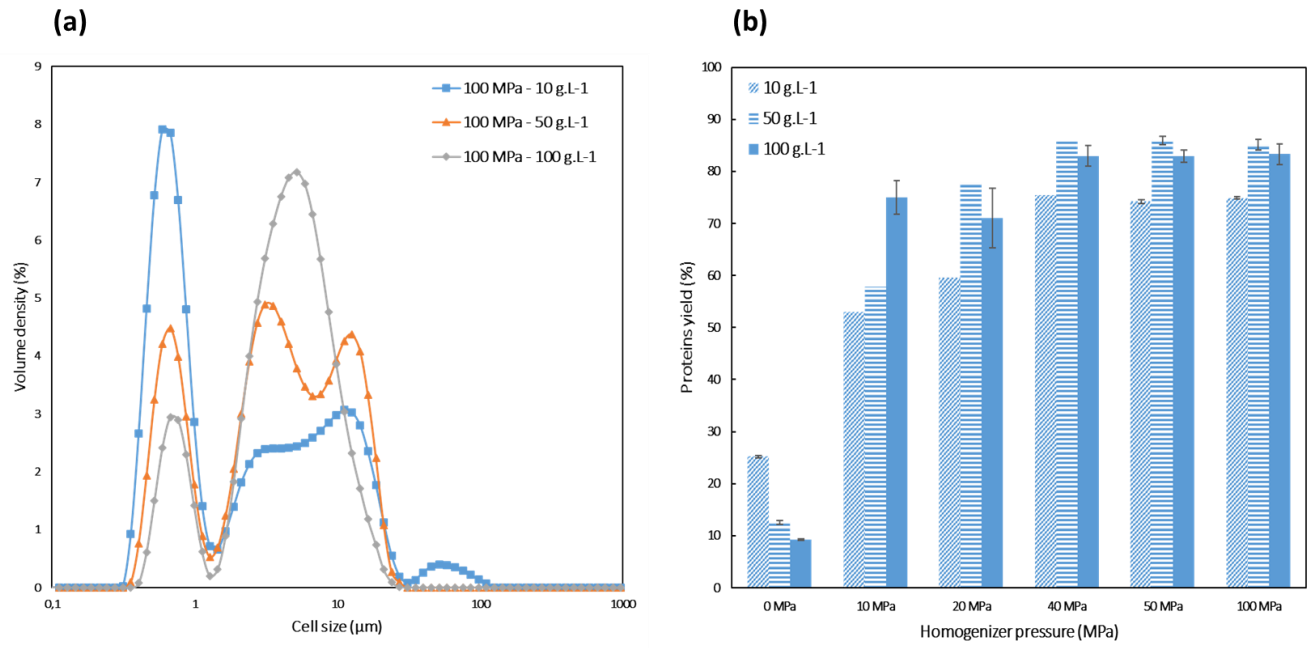


Figure 6: (a) Influence of the biomass concentration on the cell size distribution after one pass at 100 MPa. (b) Influence of the biomass concentration, 10 g.L⁻¹, 50 g.L⁻¹ and 100 g.L⁻¹, on the protein yield.