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1 **Recovery of soluble proteins from *Chlorella vulgaris* by bead-milling and**  
2 **microfiltration: Impact of the concentration and the physicochemical conditions**  
3 **during the cell disruption on the whole process.**

4  
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1 **Abstract**

2 *Chlorella vulgaris* proteins show high potential for food applications. The  
3 challenge of biorefinery is to choose the best processes coupling which can ensure the  
4 integrity of the molecules and limit energy consumption. This study aimed at  
5 understanding the impact of biomass concentration and physicochemical conditions  
6 during cell disruption, on the whole process (bead milling (BM), centrifugation,  
7 microfiltration). The best scenario tested (BM: 60 g/L<sub>DW</sub>, 10 °C, lysate dilution:  
8 30 g/L<sub>DW</sub>, pH 7, 20 °C before centrifugation and filtration of the supernatant at a volume  
9 reduction ratio VRR=3) allowed a soluble protein recovery  $\eta^S=12\%$  with a limited energy  
10 consumption of 10 kWh/g protein. Coupling BM at 90 g/L<sub>DW</sub>, pH 7, a dilution at 20 °C,  
11 with NaCl 0.1 M at 30 g/L<sub>DW</sub> and filtration at VRR=20 would lead to  $\eta^S>25\%$ . The final  
12 soluble proteins contained no green pigment; their purity increased from 5% in the raw  
13 biomass to 22% in the permeate.

14 **Keywords**

15 Soluble proteins, microalgae biorefinery, bead milling, membrane filtration,  
16 physicochemical conditions

17

## 1. Introduction

*Chlorella vulgaris* has been considered as a high potential alternative source of proteins for food and feed because of its high protein content (48–60 %w/w), an excellent amino acid profile and no competition with edible crops and land resources [1]. However, functional proteins blocked in cells with their rigid cell wall, or included into organelles, are not easily accessible. Moreover, crude biomass has a low in vitro protein digestibility (12–25 % and 33–41 % were reported) [1–3]. In contrast Kose and Oncel 2015 [2] have obtained a protein hydrolysate with 67–89% digestibility after enzymatic hydrolysis and centrifugation. It was besides demonstrated that the refined microalgae proteins show good techno-functional properties: Ursu et al. 2014 [4] reported that soluble proteins from *Chlorella vulgaris*, extracted by a combination of high-pressure cell disruption and ultrafiltration, showed better emulsifier properties than a well-known commercial ingredient, sodium caseinate. Waghmare et al. 2016 [5] demonstrated the excellent Foaming Capacity (FC 95%) and foaming stability of the protein concentrate, extracted by three phases partitioning method with *Chlorella pyrenoidosa*, compared to the winged bean protein isolate (FC 36%) and mucuna bean protein concentrate (FC 58%). However, food grade reagents and processes will be required to comply with the regulation of algae as a dietary supplement.

Microalgae biorefinery is a current research topic and aims at extracting intracellular compounds to improve their digestibility and techno-functional properties. A wide variety of simple or combined processes have been proposed like bead milling, high-pressure cell disruption, sonication, enzymatic hydrolysis, solvent extraction, membrane filtration, etc.

1           The implementation of the biorefinery process is still critical, a trade-off has to be  
2 found between microalgae complex biological structure and costly processing  
3 requirements, which limit the industrial scale-up production and product distribution. The  
4 main bottleneck of a biorefinery approach is to adopt innovative strategies for a soft,  
5 efficient and eco-friendly production.

6           In this work, a wet-way process was chosen. It involves bead milling of biomass,  
7 clarification by centrifugation to remove cell fragments and, microfiltration to fractionate  
8 soluble proteins in the permeate from the other hydrophobic fractions (lipids and  
9 pigment), maintained in the retentate [6,7]. This proposition allows avoiding the classical  
10 biomass drying step before extraction, which is either energy consuming or physical-  
11 biological properties affecting. The combination of bead milling and membrane  
12 technologies, which does not involve high pressure and solvent applications, should  
13 assure the extraction of the desired compounds without damaging other fractions, and  
14 allow the valorization of the products for food or therapeutic applications [6,8,9].

15           Bead milling is a multi-parameter dependent process, which offers wide  
16 possibilities for process adjustment and optimization. Depending on the operating  
17 conditions, bead milling can achieve different levels of destruction, ranging from  
18 selective deconstruction to disintegration. It has the advantage of being very efficient in  
19 destroying a wide variety of microalgae without damaging the properties of the  
20 biomolecules [10–13] and allows an easy scale-up. However, the optimization of the  
21 grinding conditions necessitates costly experimental efforts. Different studies dealing  
22 with bead-milling of *Chlorella vulgaris* and some other strains are presented in Table 1.  
23 Most of these works were devoted to the studies of the main parameters of bead milling:  
24 bead material and size, chamber filling rate, DW of suspensions, stirring speed, residence

1 time and/or feed rate. Various methods were used to evaluate grinding efficiency: cell  
2 counting, protein, pigment or sugar analysis, size analysis, fluorescence coupled with  
3 particle size distribution.

4         Although the efficiency of the process is undeniable, the high energy consumption  
5 (up to  $\sim 140 \text{ kWh.kg}_{\text{DW}}^{-1}$ ) has tarnished interest in this method. Nevertheless,  
6 improvements in energy consumption can be made by processing suspensions  
7 concentrated in dry matter ( $145 \text{ g.L}^{-1}$ ) [14] and by choosing the optimal conditions (bead  
8 size, stirring speed, processing times) [10]. The energy consumption estimated in those  
9 works was strongly related to the species of treated microalgae: e.g.  $0.4 \text{ kWh.kg}_{\text{DW}}^{-1}$  for  
10 *T. suecica* versus  $1.42 \text{ kWh.kg}_{\text{DW}}^{-1}$  and  $1.78 \text{ kWh.kg}_{\text{DW}}^{-1}$  respectively for *C. vulgaris* and  
11 *N. oleoabundans* [14].

12         In our case, the recovery of biomolecules (mainly proteins) from *Chlorella*  
13 *vulgaris* was studied using the same scale of bead milling equipment (0.6 L) as described  
14 by Montalescot et al. 2015 and Zinkoné et al. 2018 [10,13].

15         It is difficult to optimize the disruption of microalgae because the process  
16 efficiency is not relied only to the number of broken cells or the rate of compounds  
17 released in the aqueous phase. The physicochemical properties of the recovered  
18 suspension also have a significant impact on the downstream processes. Frequently a  
19 trade-off is necessary between the liberation efficiency and repercussion of downstream  
20 process. For example, the target compounds release efficiency can be maximized by  
21 increasing the intensity of cell disruption, which will simultaneously increase the release  
22 of contaminants and the micronization of cell debris. As consequence, the viscosity of the  
23 product will increase, which will adversely affect downstream process performances such  
24 as centrifugation, filtration or chromatography. Thus, the choice of cell disruption

1 conditions should not be based only on the cell destruction efficiency, the possibility of  
2 extrapolation and the costs of implementation and investment of the method. It should  
3 also be optimized by taking into account the requirements of a coupling with the up and  
4 downstream operations to achieve a high recovery of the different fractions, with an  
5 appropriate purity and physical properties [15,16].

6  
7  
8 In addition to the cell disruption operating parameters, the physicochemical  
9 properties such as pH, ionic strength or temperature can be modified to maximize protein  
10 solubility [17]. Thus, an important challenge is to release a maximum of soluble proteins  
11 from the cells. But it is not sufficient: the compounds also need to be purified. A second  
12 challenge is to evaluate the process efficiency not only through cell disruption, proteins  
13 release and cost effectiveness, but also with the impact of the grinding conditions on the  
14 whole downstream fractionation processes.

15 A centrifugation step is largely used to separate protein-rich aqueous phase from  
16 the solid phase. The main lab parameters are rotational centrifuge force and operating  
17 time. Its scalability depends on these parameters (acceleration  $<10\ 000\ x\ g$ , operating  
18 time  $<30\ min$ ) [18]. In these conditions, a protein-rich supernatant containing fine  
19 particles with chlorophyll, probably dispersed as colloids, is produced [19]. Several  
20 techniques can lead to protein purification and two processes are widely used : isoelectric  
21 precipitation, exploiting a unique property of proteins being less soluble at their  
22 isoelectric point, and membrane filtration based on sieving.

23 The aim of this work was to recover soluble proteins from *Chlorella vulgaris* by  
24 the coupling of bead-milling, centrifugation and microfiltration (see Figure 1). The  
25 impact of the bead milling feed concentration on the soluble proteins recovery of the

1 whole process was studied. The effect of pH, temperature, maceration time and ionic  
2 strength was also investigated. The energy consumption of different scenarios was  
3 evaluated.

4

## 5 **2. Materials and Methods**

### 6 *2.1 Experimental setup*

7 The overall experimental process, the different fractions recovered at each step  
8 and the tested conditions are outlined in Figure 1. The fraction number  $i$  was used to  
9 simplifying equations.

### 10 *2.2 Biomass characterization*

11 The microalgae strain used in this study is *Chlorella vulgaris*. The biomass was  
12 purchased as frozen paste (20–27 %DW), from the company Allmicroalgae (PT). The  
13 purchased lot was cultivated heterotrophically in July 2019 in Pataias (PT) using organic  
14 growth medium.

15 The total protein content  $P_0$  and the extractable soluble proteins  $S_0$  of the initial  
16 biomass were characterized. The biomass paste was diluted at 30 g/L<sub>DW</sub>, disrupted by  
17 high-pressure cell disrupter ( HPH, TS series 2.2 kW from Constant Systems Limited,  
18 Daventry Northants, UK) (3 passes, 2.7 kbar, 10 °C) and centrifuged at 12 000 g, 15 min.  
19 The total lysate (i.e. total protein fraction) and the supernatant (i.e. extractable total  
20 soluble protein fraction) were solubilized in 2 N NaOH at 95 °C for 5 min, mixed with  
21 1.6 N HCl (1v:1v:1v) and subsequently quantified by BCA Protein Assay kit (Thermo  
22 Scientific). The proteins present in the supernatant at 12 000 g were named “soluble” even  
23 if colloids can be present.

### 24 *2.3 Cell disruption by bead milling*



1 The biomass paste was thawed and diluted with softened water at different  
2 concentrations (30-60-90 g/L<sub>DW</sub>). The 3 biomass concentrations tested were chosen for  
3 their different rheological behaviour [20–22]:

4 30 g/L<sub>DW</sub> (watery solution), with Newtonian behaviour, where the viscosity is  
5 independent of applied stresses (viscosity: 0.002 Pa s);

6 60 g/L<sub>DW</sub>, the transition between Newtonian and non-Newtonian, where a strong  
7 shear thinning behaviour can be observed when the applied stress exceeds a critical value  
8 (viscosity: 0.006 Pa s);

9 90 g/L<sub>DW</sub> (sticky liquid) with non-Newtonian behaviour, where yield stress  
10 behaviour can be observed (viscosity: 0.03 Pa s [11,22])

11 For the cell disruption experiments, Dyno-mill multi lab (WAB, Switzerland) was  
12 operated in pendulum mode as described by Montalescot et al. 2015 [11]. The rotational  
13 speed was kept to 10 m/s, while the feed flow rate was 200 mL/min. The beads used were  
14 made in ceramic (density of 6 g/cm<sup>3</sup>, approved for contact with food products) and  
15 characterized by a diameter of 0.4 mm. The beads diameter was chosen according to the  
16 supplier indications: beads diameter  $\approx 30 \cdot$  cell diameter. The filling ratio of the beads  
17 was 80% to attain a maximum disruption efficiency [10]. The lysate temperature was  
18 maintained at 10 °C.

19 The mean residence time of the microalgae inside the milling chamber was  
20 calculated as a ratio between the free volume inside the chamber and the volumetric flow  
21 rate. At time 0 and after each pass in the bead milling, cell disruption was characterized  
22 as described in Zinkoné et al. 2018 [13]. Moreover, the total and soluble proteins released  
23 were quantified by BCA kit assays. The fraction of the disrupted cells ( $X_d$ ) was calculated  
24 as follows:

$$X_d = 1 - N_t/N_0 \quad \text{Equation 1}$$

Where:  $N_0$  and  $N_t$  are the number of intact cells at the bead-milling inlet and outlet.

The lysate and supernatant particles size distributions were analysed by MASTERSIZER3000 size analyser with a refractive index of 1.44 and an absorption index of 0.003 (Malvern Panalytical, USA).

#### *2.4 Physicochemical conditions modifications*

The physicochemical conditions (temperature, pH and ionic strength) were modified to improve the solubility of proteins. After bead milling cell disruption, the lysate at 60 g/L<sub>DW</sub> was stirred at 4 °C, 20 °C, 40 °C and 60 °C, sampled at different times from 10 min to 3 hours, centrifuged in two conditions (5 000 x g, 5 min or 12 000 x g, 15 min) and subsequently assayed by BCA kit for total and soluble protein content released in the supernatant (5 000 x g).

For pH modification assay, values from 3 to 11 were tested, compatible with the PES membrane operation (purchaser recommendations). The lysate pH was adjusted by 0.1 M NaOH or 0.1 M HCl, and 0.1 M NaCl was added to maintain the same ionic strength. For ionic strength modification assay, NaCl was added from 0 to 0.5 M in the lysate. After physicochemical conditions adjusting, the lysate was kept at 4 °C for at least 1 hour (with manual stirring), centrifuged at 5 000 x g 5 min or 12 000 x g 15 min and assayed by BCA kit for total and soluble protein content released in the supernatant (5 000 x g).

#### *2.5 Centrifugation*

The centrifugation parameters were chosen in order to be in line with large-scale applications. The centrifugation was carried out at 5 000 x g for 4 min (SORVALL

1 LYNX 6000, Thermo Scientific), corresponding to the same settling power of a  
2 commonly used disk stack centrifuge working at 9 700 x g with a flow rate of 30 L/h.  
3 These centrifugation parameters allow the settling of the particles  $\geq 1 \mu\text{m}$  at all the tested  
4 biomass concentration (simulations based on the Stokes law, considering the lysate  
5 characteristics). The centrifugation at 12 000 x g for 15 min was used only for analytic  
6 purposes, in order to differentiate the soluble proteins from the non-soluble proteins  
7 suspended in the supernatant.

## 8 *2.6 Membrane filtration*

9 The filtration experiments were performed in a 500 mL Amicon <sup>®</sup> Stirred Cell  
10 (Milipore, USA). The inlet pressure ( $P_e$ ) was given by compressed air and adjusted by a  
11 valve combined with a manometer on the cell inlet. The supernatant was introduced in  
12 the filtration cell at  $30 \pm 1 \text{ }^\circ\text{C}$  and stirred at 300 tr/min, the pressure was maintained at 0.3  
13 bar, the instant temperature of the permeate was measured. For all experiences, the  
14 previously selected membrane, PES 0.1  $\mu\text{m}$  (Koch Membrane Systems) was reused after  
15 0.2%(v/v) Ultrasil 110 (Ecolab) cleaning ( $45 \text{ }^\circ\text{C}$ , 30 min) and water rinsing ( $30 \text{ }^\circ\text{C}$ ,  
16 30 min). The pure water permeability was measured before each supernatant filtration  
17 experiment and ranged in  $225 \pm 25 \text{ L/h/m}^2/\text{bar}$  at  $30 \text{ }^\circ\text{C}$  for all experiences. All the  
18 supernatant was concentrated at a Volume Reduction Ration ( $\text{VRR} = V_{\text{feed}}/V_{\text{retentate}}$ ) equal  
19 to 3.

## 20 *2.7 Calculation of the recovery yields*

21 The different fractions recovered in the process and related indices are defined in  
22 Figure 1 and the different variables in the following:

23  $T_i$ : the concentration of total proteins quantified into the fraction i

1  $S_i$ : the concentration of soluble proteins quantified into the fraction i

2  $V_i$ : the volume of the fraction i (note that  $V_1 = V_2$ )

3  $\tau_i$ : the dilution rate of the lysate before centrifugation (i=2) or supernatant before  
4 filtration (i=3) with a water volume  $V_w$

5 
$$\tau_i = V_i / (V_i + V_w) \quad \text{Equation 2}$$

6  $\Phi$ : the volume fraction of the supernatant compared to diluted lysate

7 
$$\Phi = \tau_2 \cdot V_3 / V_2 \quad \text{Equation 3}$$

8 EF: the extraction factor of the microfiltration

9 VRR: the volume reduction rate of the microfiltration

10 
$$EF = V_4 \cdot \tau_3 / V_3 = 1 - 1/VRR \quad \text{Equation 4}$$

11 Considering the initial diluted paste,  $T_1$  and  $S_1$  were determined after HPH of the  
12 cells to quantify the maximum of total proteins and soluble proteins which can be  
13 extracted from the biomass. The recovery yield of soluble proteins, respectively  $\eta_{i/j}^S$ , at  
14 step i from step j was calculated using the following equations:

15 The recovery yield from the biomass into the supernatant after bead-milling and  
16 lysate centrifugation at 5 000 x g is:

17 
$$\eta_{3/1}^S = V_3 \cdot S_3 / V_1 \cdot S_1 = \Phi \cdot V_2 \cdot S_3 / (\tau_2 \cdot V_1 \cdot S_1) = \Phi \cdot S_3 / (\tau_2 \cdot S_1) \quad \text{Equation 5}$$

18 The recovery rate of soluble proteins in permeate from the supernatant is:

19 
$$\eta_{4/3}^S = V_4 \cdot S_4 / (V_3 \cdot S_3) = EF \cdot V_3 \cdot S_4 / (V_3 \cdot S_3 \cdot \tau_3) = EF \cdot S_4 / (S_3 \cdot \tau_3) \quad \text{Equation 6}$$

20 The recovery rate of soluble proteins in permeate from the biomass is:

$$\eta_{4/1}^S = V_4 \cdot S_4 / (V_1 \cdot S_1) = EF \cdot V_2 \cdot \phi \cdot S_4 / (\tau_3 \tau_2 V_1 S_1) = \eta_{4/3}^S \cdot \eta_{3/1}^S \quad \text{Equation 7}$$

## 2.8 Statistical analysis

All the values resulting from the samples analysis (dry weight, protein analysis) were calculated by the mean of triplicate measurements. The standard errors (SE) presented in the results were calculated as the standard deviations of the triplicate divided by  $\sqrt{3}$ . The combined standard error of any value y was calculated by equation 8a for additions or 8b for multiplications or divisions of two variables  $X_1$  and  $X_2$ :

$$\text{If } y = X_1 + X_2, SE_y = \sqrt{SE_{X_1}^2 + SE_{X_2}^2} \quad \text{Equation 8a}$$

$$\text{If } y = X_1^{\pm 1} \times X_2^{\pm 1}, SE_y = y \cdot \sqrt{\left[\frac{SE_{X_1}}{X_1}\right]^2 + \left[\frac{SE_{X_2}}{X_2}\right]^2} \quad \text{Equation 8b}$$

## 3. Results and discussion

### 3.1 Bead milling

The bead milling parameters selected allowed the disintegration of more than 80% of the microalgae cells for all the conditions tested after 4 passes, 6 min of residence time (Figure 2.a). The biomass concentrations corresponding to Newtonian, transition and non-Newtonian behaviour didn't affect the cell disruption rate.

The first pass in the milling chamber (residence time: 1.3 min) resulted in a low fraction of disrupted cells (around 20%) if compared to other studies [11,13]. This phenomenon could be associated with the presence of several cells agglomerates we noted at the time 0 (before milling) by microscope observation. Thus, during the first pass most of the milling energy was used to break up the cell agglomerates. Then, the cell disruption rate increased achieving a plateau between 5 and 6.5 min.

### 3.1.1 Cell disruption kinetic and soluble proteins release

The kinetic of cell disruption and protein release were analysed for the three different biomass concentrations. The total and soluble proteins released refer to centrifugation at 5 000 x g and 12 000 x g respectively as defined in the material and methods. The results are reported in the Figure 2.

Cell disruption rate ( $X_d$ ) is presented in the Figure 2.a. It follows a first order kinetic ( $X_d=1-e^{-kt}$ ) for the three-biomass concentrations tested with the same kinetic constant of  $0.005\text{ s}^{-1}$ .

If we analyse the results from the energetic point of view, higher cell concentrations are preferred according to the equipment power consumption per gram of soluble protein produced (Equation 9). For example, the volume to be treated for the biomass at 30 g/L<sub>DW</sub> required three times as long as the equipment running time compared to that at 90 g/L<sub>DW</sub>.

$$E = \Sigma(P_x \cdot t_x) / (S_1 \cdot \eta_{4/1}) \quad \text{Equation 9}$$

where: E is energy consumption per gram of soluble protein produced in permeate in kWh/g S,  $P_x$  is the power of the experimental equipment in kW, x represents each process equipment; t is the equipment running time.

In the Figure 2.b, the ratio between the release of total proteins ( $T_3$ ) in the supernatant 5 000 g and the total proteins available in the biomass ( $T_1$ ) is reported as function of the residence time in the milling chamber, for the different biomass concentration. The protein concentration in each fraction is shown in Table 1. A first order kinetic could also be assumed for the release of total proteins in the supernatant. A plateau for the total proteins release almost 10% of the biomass protein content is reached

1 between 4 and 5 min. It suggests a limited access to the proteins from *Chlorella vulgaris*  
2 and possibly a limited solubility of proteins.

3 In the Figure 2.c, the release of soluble proteins ( $S_3/S_1$ ) in the supernatant, after  
4 centrifugation at 12 000 x g for 15 min is reported. The release of the soluble proteins  
5 also follows the first order kinetic, but different kinetic constants were calculated for the  
6 different cell concentrations: 0.004, 0.007, 0.005 s<sup>-1</sup> for 30, 60, 90 g/L<sub>DW</sub> respectively.

7 The higher release of the soluble proteins (70%) was observed after 5 min at  
8 90 g/L<sub>DW</sub> and 60 g/L<sub>DW</sub> while, at 30 g/L<sub>DW</sub>, a maximum of 60% was reached. At 90 g/L<sub>DW</sub>  
9 the kinetic constant of soluble proteins release is similar to the disruption kinetic constant.

10 Postma et al. 2017 [14] reported a disruption constant of 0.041 s<sup>-1</sup> for the same  
11 strain, but produced in a laboratory culture system and a different trophic mode (change  
12 in the cell wall size), milled in similar conditions (ZrO<sub>2</sub> beads 0.4 mm, biomass  
13 concentration 90 g/L<sub>DW</sub>, 25 °C). Zinkoné 2018 [16] reported a disruption constant  
14 between 0.003 and 0.017 s<sup>-1</sup> for *Chlorella sorokiniana* (fresh biomass, 1 g/L<sub>DW</sub>, 20 °C).  
15 Postma et al. 2017 [14] got a disruption rate higher than the one here reported. Significant  
16 differences in the disruption efficiency are related to the quality of the biomass processed  
17 (culture grown at a lab scale or biomass grown at industrial scale in different culture  
18 media), different methodology of biomass storage (fresh or frozen) and the temperature  
19 during bead-milling. This is a key issue when technological transfer from the lab to the  
20 industry is considered. Postma et al. 2017 [14] also reported a kinetic constant of 0.02 s<sup>-1</sup>  
21 at cell concentration near 90 g/L<sub>DW</sub>.

22 It is interesting to point out that the first two passes in the milling chamber were  
23 sufficient to release 60% of the soluble proteins in all the tested conditions, while, from  
24 the 3<sup>rd</sup> pass (3.9 min) major differences are observed among the cell concentrations. These

1 data could support the hypothesis reported by Zinkoné et al. 2018 [13]: in a heterogeneous  
2 cell population, bigger cells are more fragile and easily disrupted in the first passes,  
3 whatever the milling parameters applied. It should be noted that at the same operating  
4 conditions, higher cell concentration leads to a higher number of efficient collision events  
5 (stress number of the known stress model by Kwade and Schwedes 2002 [23]). On the  
6 contrary, little cells are disrupted at different rates depending on the intensity of the  
7 collisions (stress number of the stress model).

### 8 3.1.2 Granulometric analysis

9 In order to better characterize the cell disruption process and the quality of the  
10 supernatant, which will feed the membrane filtration tests, a granulometric analysis was  
11 performed.

12 The granulometry of the biomass at 30 g/L<sub>DW</sub> fed to the bead milling and after  
13 each milling pass is reported in Figure 3.a. The microalgae paste diluted at 30 g/L<sub>DW</sub> (F1)  
14 is characterized by an important quantity of cell aggregates up to 600 µm (highest peak  
15 F1). The granulometries of the lysate after 1 to 5 passes in the bead milling (F2-1<sup>st</sup> to 5<sup>th</sup>)  
16 show that the first pass in the milling chamber disassembled all the aggregates so that the  
17 two peaks at 600 µm and 20 µm disappeared. At F2-1<sup>st</sup>, the highest peak corresponds to  
18 3 µm, the standard size of *Chlorella vulgaris* cells and the second peak is around 0.1 µm  
19 reasonably corresponding to the debris generated by disrupted cells.

20 As the number of passes in the milling chamber increases, the volumetric density  
21 of the entire cells (peak at 3 µm) was reduced and the volumetric density of the cell debris  
22 increased (peak at 0.1 µm).

23 Figure 3.b compares the cell lysate of the 5<sup>th</sup> pass for the two conditions 30 g/L<sub>DW</sub>  
24 and 60 g/L<sub>DW</sub>. The data show a slight increase of the disruption efficiency for higher



1 biomass concentration (reduced peak at 3  $\mu\text{m}$ ), which also supports the improved  
2 efficiency in soluble proteins release at 60  $\text{g/L}_{\text{DW}}$  compared to 30  $\text{g/L}_{\text{DW}}$  (Figure 2.c).

3 The Figure 3.c shows the granulometric distribution of the supernatant of the 5<sup>th</sup>  
4 pass, at 5 000 g for 4 min, for the different biomass concentrations (30-60-90  $\text{g/L}_{\text{DW}}$ ). A  
5 similar mean particle size distribution is observed for the three conditions tested (peak  
6 from 0.01 to 1  $\mu\text{m}$ , with a maximum around 0.1  $\mu\text{m}$ ). Additional particles of bigger size  
7 are recovered, especially in the supernatant of 90  $\text{g/L}_{\text{DW}}$  (peak between 4 and 100  $\mu\text{m}$ ).  
8 This could be explained by reduced centrifugation efficiency at higher biomass  
9 concentration (90  $\text{g/L}_{\text{DW}}$ ). Indeed, high biomass concentration induced an increased  
10 viscosity of the lysate, which negatively affected the centrifugation step (Stokes's law).

### 11 3.2 *Bead milling - centrifugation*

#### 12 3.2.1 *Biomass concentration effect*

13 The biomass concentration during bead milling has an impact on the soluble  
14 protein release rate into the supernatant. The possible reasons are the difference in the  
15 quantity of soluble proteins released from cells during the grinding process; the variations  
16 of the viscosity of the suspension that limit the solubilization and diffusion of proteins  
17 according to the Stokes-Einstein law; or the interactions between proteins and suspended  
18 material leading to their migration to the pellet during centrifugation. Golovanov and al.,  
19 [24] elucidated that 25–57% of soluble proteins aggregate or precipitate during  
20 concentration because of low solubility of proteins. Both Grossmann and Schwenzfeier  
21 [25,26] demonstrated with extracted microalgae proteins that part of polysaccharides  
22 attach to the proteins, which will affect the proteins' solubility.

23 Table 2 shows the mass balance results of the grinding and centrifugation process.  
24 After centrifugation, the concentration of total and soluble proteins in the supernatant T<sub>3</sub>

1 and  $S_3$  increased with the concentration of the initial biomass  $DW_1$ , but the absolute  
2 volume of supernatant decreased. With higher biomass concentration, the quantities of  
3 cell fragments and released compounds increased, thereby the liquid viscosity increased,  
4 thus during centrifugation, the particles in suspensions did not settle well, which  
5 decreased centrifugation efficiency and led to a larger volume of the pellet. Thus the  
6 supernatant viscosity strongly impacted the soluble protein recovery rate in the  
7 supernatant.

8 In order to verify our hypothesis, the lysate from the biomass ground at 60 g/L<sub>DW</sub>  
9 was diluted at 5 g/L<sub>DW</sub> and 30 g/L<sub>DW</sub> before centrifugation.

10 Comparing assays A30 a/b and A60\_di.ly\_30, it appears that for the same lysate dilution  
11 (same  $DW_2$ ), a higher concentration of soluble proteins  $S_3$  and a higher soluble protein  
12 recovery rate  $\eta_{3/1}^S$  were obtained with a bead milling at 60 g/L<sub>DW</sub>. So the first hypothesis  
13 is validated: the release of soluble proteins is more efficient with a higher concentration  
14 of the biomass. An optimum may exist but it was not deepened in this study. The  
15 comparison between the assay A60\_a/b with A60\_di.ly\_30 also shows that the dilution  
16 before centrifugation and after the grinding process improved the soluble protein recovery  
17 rate  $\eta_{3/1}^S$ . The second hypothesis is also validated and the interaction between proteins  
18 and suspended material (hypothesis 3) seemed limited with dilution.

19 The protein recovery rate  $\eta_{3/1}^S$  obtain in this study is comparable with literature,  
20 Schwenzfeier et al. 2011 [26] grounded the biomass *Tetraselmis* sp. at 120 g/L and  
21 recovered around 21% protein in the supernatant, slightly lower than our conditions at  
22 90 g/L which yielded 24–36% protein in the supernatant. Besides, Zinkoné et al. 2018  
23 [13] grounded the biomass *Chlorella sorokiniana* at 1 g/L and obtained a yield of more

1 than 90% in the supernatant, which once again confirms our previous hypothesis that the  
2 increased grounded biomass concentration will decrease centrifugation efficiency.

3 The soluble protein purity in supernatant (purity<sub>3</sub><sup>S</sup>) for the assay A30, A60 and  
4 A90 increased gradually with the biomass concentration during bead milling. Two factors  
5 can influence the purity of the soluble proteins, which means the proteins recovered by  
6 centrifugation at 12 000 x g: the elimination of the other compounds in the pellet with  
7 diluted biomass, this could explain the differences between assays A60\_a/b and  
8 A60\_di.ly\_30 (same bead-milling and different dilutions); but also the recovery of non-  
9 soluble proteins that were not eliminated in the pellet because of the increased viscosity.  
10 This can explain the apparent increased purity of the 'soluble fraction' from the assay  
11 A90 (confirmed by the granulometry of the supernatant in 3.1). The purity<sub>3</sub><sup>S</sup> values must  
12 be taken with caution.

13 So coupling a bead milling at a concentration near 60 g/L<sub>DW</sub> and dilution to  
14 30 g/L<sub>DW</sub> for centrifugation was efficient to simultaneously enhance the soluble protein  
15 recovery, purity and decrease energy consumption.

### 16 *3.3 Modification of the lysate physicochemical conditions*

17 The recovery rate of microalgae soluble proteins depends on the milling  
18 conditions but also strongly on the physicochemical conditions. Three conditions were  
19 studied: pH, temperature and maceration time and ionic strength.

#### 20 *3.3.1 pH*

21 Figure 4.a shows the total proteins concentration T<sub>3</sub> and soluble protein  
22 concentration S<sub>3</sub> in supernatant after 1 hour at 4 °C at the corresponding pH and  
23 centrifugation. In alkaline conditions, the total and soluble protein concentrations in the

1 supernatant were the highest ( $T_3 = 9.2$  g/L,  $S_3 = 1.3$  g/L at pH 11) whereas in acidic  
2 conditions they were low ( $T_3 = 0.7$  g/L,  $S_3 = 0.6$  g/L at pH 3). This is probably due to the  
3 protein aggregation and then migration in the pellet in acid conditions. This behaviour  
4 was already observed by Ursu et al. 2014; Cavonius, Albers, and Undeland 2015;  
5 Benelhadj et al. 2016 [4,27,28] with the same or other strains like *N. oculata* and *A.*  
6 *platensis*. According to Becker 2007 [1], microalgae proteins contain more acidic amino  
7 acids, thus their negatively charged protein surface determines their surface  
8 hydrophilicity. Under acid conditions, the acid functions are protonated, decreasing the  
9 electrostatic repulsion interactions and increasing the protein-protein hydrophobic  
10 interaction, which leads to the aggregation. Under alkaline conditions, negatively charged  
11  $\text{OH}^-$  increases the electrostatic repulsion, which enhances the solubility of  
12 hydrophobic/insoluble proteins [29]. These phenomena were confirmed here.

13         However, in this work, the increase of soluble proteins  $S_3$  recovered in the  
14 supernatant was rather small between pH 7 and pH 11. And in basic condition pH 11,  
15 much more non-soluble proteins were recovered in the supernatant. Thus, to facilitate the  
16 membrane filtration downstream process and to avoid the membrane degradation in basic  
17 conditions, the optimal pH chosen here was neutral (pH 7).

### 18 3.3.2 Maceration time and temperature

19         After cell disruption, the released molecules may reorganize in the aqueous phase.  
20 The effect of temperature and time during the lysate storage, between milling and  
21 centrifugation, on soluble proteins recovery rate was evaluated. Four temperatures (4 °C,  
22 20 °C, 40 °C and 60 °C) were tested, and the protein solubilization kinetics were  
23 monitored. Bead milling was carried out at 60 g/L<sub>DW</sub>, 10 °C, and then the lysate was  
24 transferred to heating plates (60 °C), in a water bath (40 °C), at room temperature (20 °C)

1 or in the fridge (4 °C). The lysate was sampled at different times, immersed in ice and  
2 centrifuged at 12 000 g for 15 min before the BCA proteins test. The results are shown in  
3 Figure 4.b.

4 Comparing the storage of lysate at 4, 20 and 40 °C, it appears that the higher the  
5 temperature, the faster the soluble proteins release. For those temperatures, a similar  
6 maximum release was reached after 1 hour ( $1.4 \text{ g/L} < S_3 < 1.6 \text{ g/L}$ ). No significant  
7 difference was observed between 4 °C and 20 °C kinetics. At 60 °C, soluble protein  
8 degradation was observed. Thus, 20 °C was used as reference storage conditions between  
9 milling and centrifugation process for the following experiments.

### 10 3.3.3 Ionic strength modification by NaCl

11 Proteins solubility was also affected by ionic strength as shown in Figure 4.c,  
12 where the concentrations of total and soluble proteins  $T_3$  and  $S_3$  in supernatant 5 000 x g  
13 are presented versus increasing sodium chloride concentration from 0 to 0.5 M, added in  
14 the lysate after bead-milling at 30 g/L<sub>DW</sub>. When the ionic strength of lysate increased,  $S_3$   
15 remained stable, whereas  $T_3$  decreased. This means that the hydrophobic proteins formed  
16 larger aggregates, which transferred to the pellet by centrifugation 12 000 g (probably  
17 due to salting out effect).

18 Teuling et al. 2017 [30] found similar results: the protein concentration (with  
19 centrifugation 4 700 x g 30 min) decreased with an increasing ionic concentration (from  
20 0.01 M to 0.5 M), and this was more evident in acidic conditions. While Schwenzfeier,  
21 Wierenga, and Gruppen 2011 [26] found that the soluble protein concentration in  
22 supernatant 10 000 x g 4 °C remained stable between ionic strengths 0.03 M, 0.2 M and  
23 0.5 M in *Tetraselmis sp.*

24 Thus two possible effects of high ionic strength conditions were supposed:

- 1           1. less hydrophobic or colloidal protein recovery may increase soluble protein  
2           purity in supernatant (considering a diafiltration to remove salts);
- 3           2. membrane microfiltration flux may be improved in high ionic strength  
4           conditions with fewer colloid proteins in the feed, which means less fouling.

5           From these hypotheses, filtration experiments were carried out at NaCl 0.1 M  
6           above which the total protein concentration  $T_3$  was constant and the results are compared  
7           with conditions without ionic strength modification in Table 3. The purity of soluble  
8           proteins in the supernatant for A60a+NaCl and A90a+NaCl reached 12.5% and 13.2%, but  
9           after salt elimination by a diafiltration, it could reach respectively 19% and 17%  
10          (calculated by subtracting 0.1 M NaCl added in lysate, considering NaCl concentration  
11          did not change after centrifugation). The slight increase of soluble protein purity  
12          compared to the purity from experiments without salt (17% for A60a and 15% for A90a)  
13          tends to confirm the first hypothesis. The filtration performances of those supernatants  
14          are also discussed in 3.4.1.

### 15    3.4 *Bead milling-centrifugation-filtration*

#### 16    3.4.1 *Biomass dilution effect on filtration*

17          Considering the three tested biomass concentrations (30, 60 and 90 g/L<sub>DW</sub>), after  
18          bead milling and centrifugation, the reorganization of the molecules in the supernatant  
19          may be different and impact the availability of the released hydrophobic, amphiphilic and  
20          water-soluble molecules (proteins, lipids and sugars).

21          To study the consequence of those reorganizations (solubilization, aggregation,  
22          micelle formation, etc.) on the membrane filtration process (flux and membrane  
23          selectivity), the supernatants produced with or without ionic strength modification were  
24          filtrated and the results are detailed in tables 3, 4 and figure 5.a.

1 Comparing A30a, A60a and A90a, taking into account only filtration process, the  
2 soluble protein recovery yield in permeate related to supernatant  $\eta_{4/3}^S$  (Equation 6) was  
3 not significantly different and equal to  $17.7\pm 1\%$ . But for the yield of the whole processes  
4  $\eta_{4/1}^S$  (Equation 7):  $A30 > A60 > A90$  which means that the soluble protein recovery was  
5 improved by lysate dilution or initial biomass concentration adjusting.

6 In all cases (A30a, A60a and A90a), the soluble protein purity in the permeate  
7 compared to supernatant was slightly improved, but the nature of predominant proteins  
8 and impurities was strongly modified. After filtration separation, 100% proteins were  
9 soluble whereas they were only 30–55 % in the supernatant. The composition of  
10 impurities was different, which should improve the techno-functional properties of the  
11 fractions [4,31,32]. In the supernatant, the impurities were mainly composed of lipids,  
12 green pigment, sugar and salts, but in the permeate, it was only salts and sugar (RR=100  
13 % for lipids and pigments) (internal results, not detailed here). The green pigment  
14 elimination from the supernatant can be an advantage for food marketing and consumer  
15 perception [2]. To improve the soluble protein purity, a second stage of ultrafiltration  
16 could be applied to retain the proteins in the retentate and discard the soluble sugar and  
17 salts in permeate. Besides, the concentrate with chlorophyll pigments could also be  
18 valorized as healthy food because it was reported as a cancer preventative agent [33].

19 Concerning the effect of ionic strength modification on the whole process, the  
20 comparison of  $A60a_{+NaCl}$  to A60a and  $A90a_{+NaCl}$  to A90a shows  $\eta_{4/3}^S$  and  $\eta_{4/1}^S$  increased.  
21 But a further downstream diafiltration step would be required to ensure product purity.

22 Figure 5.a shows the results of normalized permeate flux during supernatant  
23 concentration versus the volume reduction ratio VRR. A decrease of the flux was  
24 observed for the filtration of all supernatants from A30a, A60a and A90a, which means

1 that during dead-end filtration, a part of the particles, colloids and macromolecules  
2 present in the supernatant quickly formed a significant fouling layer. The resistance of  
3 the fouling to water permeation was more important when the supernatant was more  
4 concentrated. At this stage it was not possible to know if the nature of the particles and  
5 colloids produced during bead-milling played a major role in the fouling resistance. The  
6 fluxes were not significantly different between supernatants with and without NaCl,  
7 which means that the elimination of the hydrophobic compounds using NaCl was not  
8 sufficient to avoid fouling. Despite a different fouling resistance, the protein retention  
9 rates were similar in all the experiments (86%-89%, see Table 4).

#### 10 *3.4. 2 Supernatant dilution effect on filtration*

11 In order to verify if a higher dilution allowed lower fouling, higher fluxes and  
12 lower protein retention rates, and if the bead-milling in different conditions generated  
13 particles with different fouling properties at the same DW concentration, three  
14 supernatants from 30, 60 and 90 g/L<sub>DW</sub> biomass cell disruption were diluted to the same  
15 dry weight about 5 g/L and filtered.

16 Comparing assay A30/60/90 to A30/60/90\_di sup, the filtration performances (i.e.  
17  $J/J_w=0.15$  to  $0.18$  at  $0.3$  bar,  $RR=88-90\%$  and  $R_{fouling}=0.8-1.1 \times 10^{13} \text{ m}^{-1}$ ) at  $DW_3=5$  g/L  
18 were similar to the performances at  $DW_3=30$  g/L (see Table 4). The soluble protein  
19 recovery for filtration ( $\eta_{4/3}^S$ ) with the diluted supernatant at 5 g/L was similar to the initial  
20 supernatant without dilution (see Table 3). Thus it is not necessary to dilute the  
21 supernatant at a DW concentration below 30 g/L. When the bead-milling was done at  
22 90 g/L<sub>DW</sub>, the fouling was slightly more important than when the bead-milling was done  
23 at 30 or 60 g/L<sub>DW</sub> (see the resistance in Table 4 and the flux Figure 5.b). However, all  
24 those experiments were done with a stirred dead-end batch processing, it would be



1 interesting to see whether this slightly increased fouling at 90 g/L<sub>DW</sub> will be resisted or  
2 not under improving hydrodynamic conditions, for example by cross flow filtration. Thus  
3 the choice of cell disruption at 90 g/L<sub>DW</sub> will result from a compromise between energy  
4 consumption limitation for bead-milling and filtration performances.

5 To conclude on the selection of the operating conditions to maximize the soluble  
6 proteins from *Chlorella vulgaris* without taking into account the energy consumption:

7 coupling a bead-milling at 60 g/L<sub>DW</sub>, a dilution at 30 g/L<sub>DW</sub> and centrifugation  
8 should lead to  $\eta_{3/1}^S = 58 \pm 6\%$ . If the dilution is done with NaCl 0.1 M, at pH 7 and 20 °C  
9 during at least 30 min (or shorter at 40 °C), the filtration recovery rate  $\eta_{4/3}^S$  could reach  
10 21%; then the global soluble protein recovery rate  $\eta_{4/1}^S$  could reach 12.2%.

11 It is important to point out that all those protein yields were calculated considering  
12 the filtration stopped at VRR3. In large-scale production, a better protein recovery yield  
13 could be expected considering a higher VRR. In our case,  $\eta_{4/3}^S$  was calculated as 43% at  
14 VRR equal to 20 and led to  $\eta_{4/1}^S = 25\%$  for the whole process.

15 Nevertheless, a better analyse of the organization of the soluble proteins in the lysate  
16 and supernatant becomes essential to understand why 80% are retained by a membrane  
17 with a cut-off of 0.1  $\mu\text{m}$ . This is the key to solve the problem of a high soluble protein  
18 recovery.

19 Suarez Garcia et al. 2018 [12] reported  $\eta_{3/1}^S = 22.5\%$  after bead milling and  
20 centrifugation (14 000 rpm, 30 min) with *Tetraselmis suecica* (100 g/L<sub>DW</sub>), near from our  
21 results for A90a/b with 24–36 %. Schwenzfeier, Wierenga, and Gruppen 2011 [26]  
22 recovered 13% soluble proteins in retentate after bead milling, centrifugation ( 40 000 x g,  
23 30 min) and dialyses (14 kDa). For filtration processes, Kulkarni and Nikolov 2018 [34]

1 obtained similar  $\eta_{4/3}^S = 20\text{--}22\%$  (PES 300 kDa, after HPH cell disruption and  
2 centrifugation with *Chlorella vulgaris*).

3 Postma et al. 2015; 2016; Safi et al. 2014 [11,35,36] also worked on water-soluble  
4 protein extraction from *Chlorella vulgaris*, and tested bead milling- centrifugation (25–  
5 145 g/L<sub>DW</sub>) ; pulsed electric field extraction (25 g/L<sub>DW</sub>) and high-pressure homogenizer -  
6 centrifugation (20 g/L<sub>DW</sub>) with simple or multistage processes. They estimated the global  
7 protein yield using  $y = S_3/S_1$  and obtained soluble proteins yield respectively of 24–32 %;  
8 3–5% and 52%. Considering the volume of the pellet was not negligible after  
9 centrifugation at high biomass concentration, the final soluble protein recovery yields  
10 may be lower. But results are comparable to ours.

### 11 *3.5 Overall process balance and energy consumption*

12 The overall process for soluble proteins recovery can be summed up by the  
13 process scheme in the Figure 1.

14 The effect of the dilution of the biomass paste, at 30-60-90 g/L<sub>DW</sub>, on each process  
15 step (milling, centrifugation, membrane filtration) has been deeply analysed in the  
16 previous paragraphs. The results suggest that interesting improvements could come from  
17 the introduction of a dilution step between the bead milling and the centrifugation steps.  
18 In fact, the bead milling is the sole unit operation attaining the maximum efficiency at  
19 high biomass concentration, while centrifugation and filtration performances are  
20 negatively affected. In order to support this hypothesis, the quantification of the energetic  
21 consumption of the single operation unit and of the overall process, at different biomass  
22 concentration, was carried out. The power consumption (kW) of each unit was obtained  
23 from the technical data sheets of the equipment and then multiplied by the operating time.  
24 The operating time was calculated on the basis of the bead milling kinetics and filtration

1 flux at different biomass concentrations (data from sections 3.1 and 3.4). For the  
2 calculation of the energy consumption per gram of soluble proteins, the global protein  
3 recovery at the end of the filtration step was considered. The results are shown in the  
4 Figure 6.

5 The bead-milling coupled with centrifugation and filtration, all at a concentration  
6 of 90, 60 and 30 g/L<sub>DW</sub> are presented with colourful bars.

7 Then, three different scenarios were simulated (presented with shaded bars): one  
8 with the biomass disrupted at 90 g/L<sub>DW</sub>, the lysate diluted at 60 g/L<sub>DW</sub>, centrifuged and  
9 filtrated; a second with the biomass disrupted at 90 g/L<sub>DW</sub>, the lysate diluted at 30 g/L<sub>DW</sub>,  
10 centrifuged and filtered; the other with the biomass disrupted at 60 g/L<sub>DW</sub>, diluted at  
11 30 g/L<sub>DW</sub>, centrifuged and filtered. Those scenarios assumed the hypothesis that the  
12 filtration performances were the same if the biomass dilution was applied before or after  
13 the cell disruption. Moreover, the protein recoveries for those scenarios were supposed  
14 equal to the proteins recoveries when all the process was carried out at 60 or 30 g/L<sub>DW</sub>.

15 When the dilution before the milling step was considered, the biomass  
16 concentration of 60 g/L<sub>DW</sub> resulted in the best operating conditions for attaining the  
17 production objective of 1 g of proteins with the lowest energy consumption for the overall  
18 process (14 kWh/g<sub>proteins</sub>). When a dilution step was introduced between the milling and  
19 the centrifugation, allowing each operation unit to work at its favourable operating  
20 conditions, the best performance (7 kWh/g<sub>proteins</sub>) was achieved disrupting the biomass at  
21 90 g/L<sub>DW</sub> while performing the following steps at 30 g/L<sub>DW</sub>.

22 This last scenario could be further improved achieving a VRR=20. It would lead  
23 to an energy consumption of 3.5 kWh/g<sub>proteins</sub>. It means a reduction of 75% of the  
24 energetic costs associated to the process.

1           Considering the average energy cost of 0.1 €/kWh, the impact of the energy on  
2 the price of proteins will slow down from 1.4 to 0.7 €/g<sub>proteins</sub> by using the optimum  
3 sequence of disruption-dilution-filtration. The improvement of the VRR will reduce this  
4 cost to 0.35€/g<sub>proteins</sub>,

5           Safi et al. 2017 [37] tried to quantify the energy consumption for proteins release  
6 during *Nannochloropsis gaditana* cell disruption. They achieved an energy cost of 0.4-1  
7 €/g<sub>proteins</sub> but, their energetic analysis only considered the disruption step and did not  
8 consider the loss of proteins yield during the purification steps. The same kind of  
9 energetic analysis was performed by Postma et al. 2017 [14] on *Chlorella vulgaris*, but  
10 they always focused on the milling step without analysing the overall process to obtain  
11 industrially exploitable proteins. On the contrary, a recent paper by Gifuni et al. 2020 [38]  
12 analysed the complete operating costs for the purification of microalgal proteins by a  
13 three-step membrane filtration with the impact of the scale up. They reached a total  
14 operating cost of 2.6 €/g<sub>proteins</sub>, but it did not consider the bead billing pretreatment step  
15 for cell disruption.

16           The analysis presented in this study, shows that the energetic cost related to  
17 microalgal proteins extraction and purification could be reduced to 0.35 €/g<sub>proteins</sub> by using  
18 the correct sequence of disruption-dilution-centrifugation-filtration and pushing the  
19 filtration step to a VRR of 20. The proteins recovered with this process are defined as  
20 soluble proteins with potential techno-functional properties [12] which can justify high  
21 values. At this stage, the cost of microalgal proteins is still high and it cannot compete  
22 with the current vegetal sources (soya, pea, whey) as presented by Préta et al. 2020 [39].  
23 Nevertheless, the authors wanted to propose the present analysis of the energy  
24 consumption as key tool for orienting the process choices.

### 3.6 Conclusion

The biorefinery to recover soluble proteins from microalgae is still challenging: we need to conciliate product characteristics and a large-scale energy cost limitation. The process with the best soluble protein recovery rate and the lowest energy consumption was here the coupling of biomass disruption at 90 g/L, lysate dilution at 30 g/L<sub>DW</sub> for centrifugation and filtration. This would allow a soluble protein recovery rate above 25% at VRR=20. Nevertheless, the key point to improve the recovery efficiency will be the decrease of the soluble proteins retention rate during filtration, which is strongly related to their interaction with other compounds in complex aggregates. The evolution of the organization of all compounds during the processes will have to be deepened.

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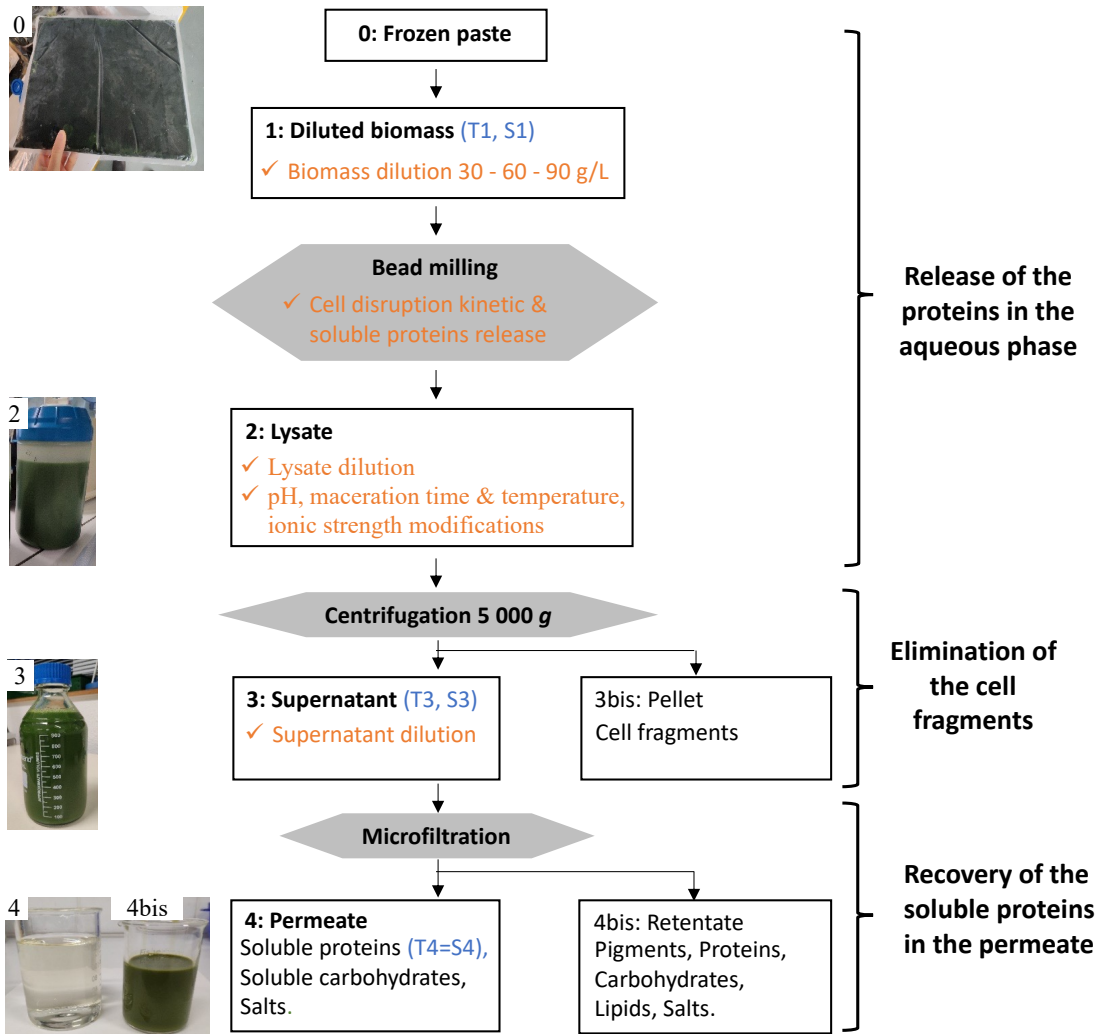


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1 **Tables and Figures**

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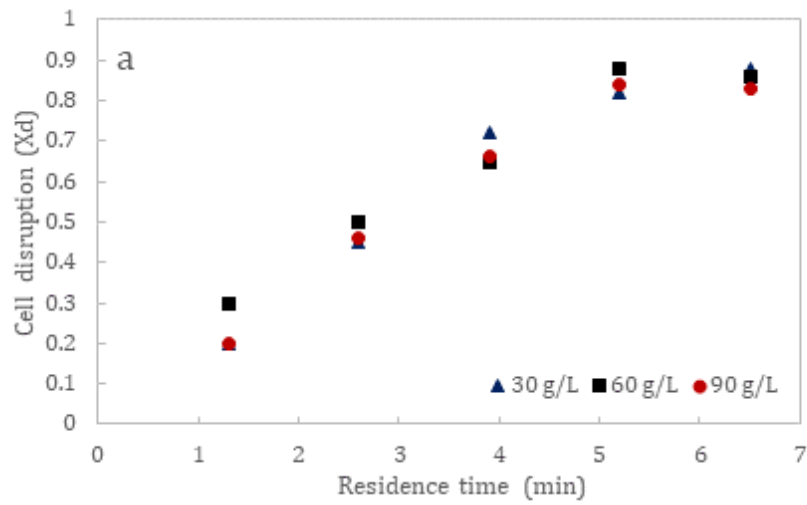
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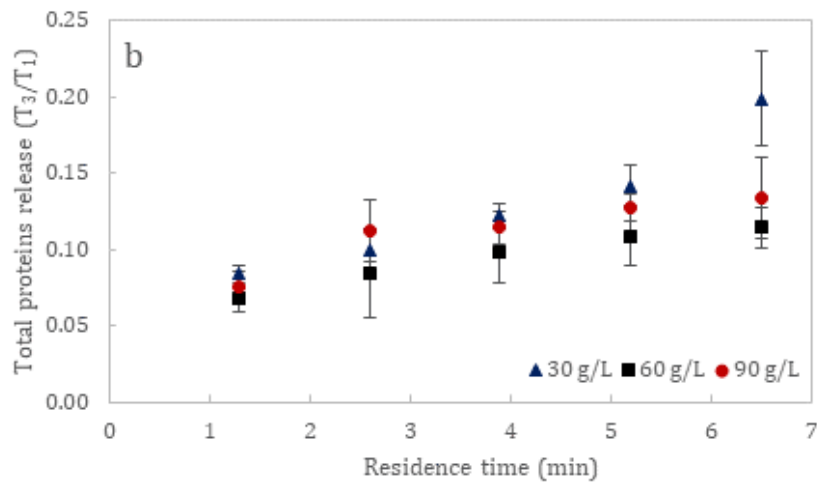
Figure 1: Process flow diagram applied for the recovery of soluble proteins (Pictures correspond to milling concentration at 90 g/L)

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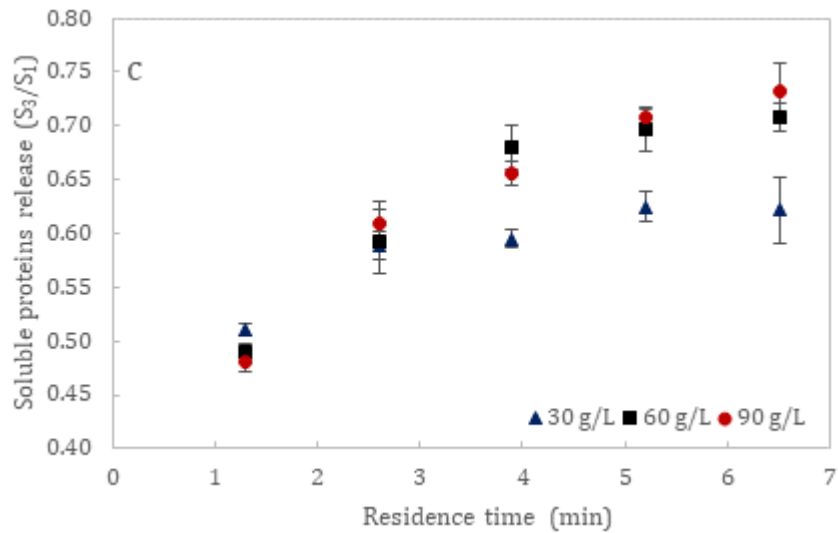
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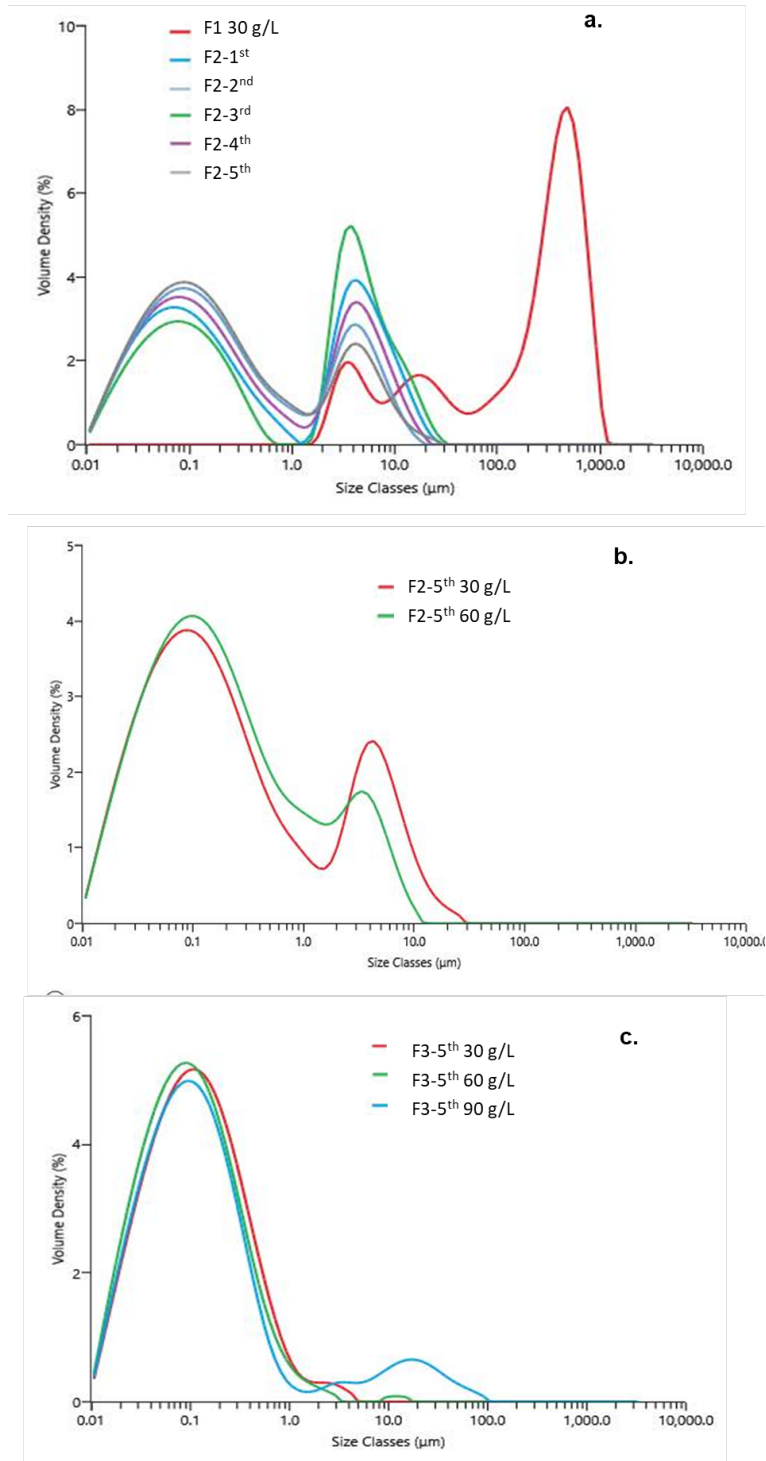


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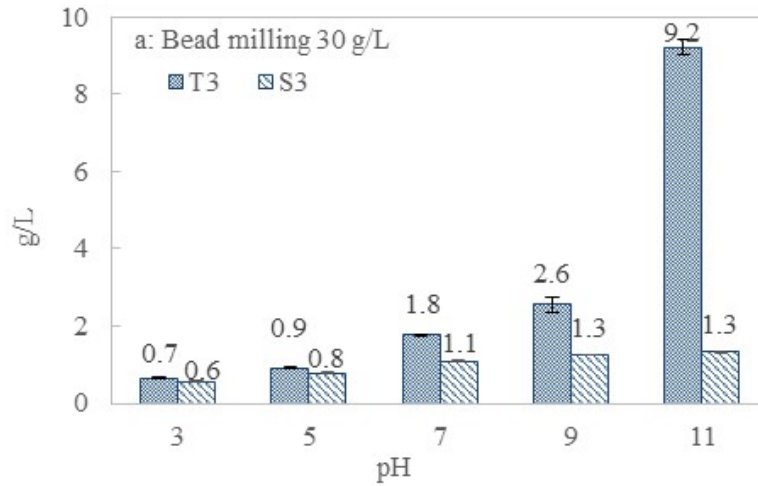
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5 Figure 2: The kinetic of cell disruption (a), total proteins (b) and soluble proteins (c) as  
6 function of residence time for different biomass concentrations during bead milling

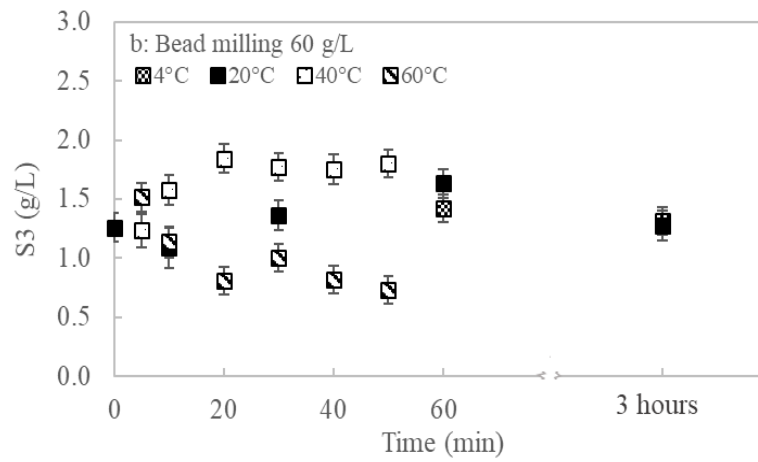


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 2 Figure 3: Lysate and supernatant particles size distribution. a: Granulometric analysis of  
 3 the diluted biomass fraction (F1) and the lysate fraction after each pass (F2-x pass) in the  
 4 bead milling for the 30 g/L test; b: Granulometric analysis of the lysate in 5<sup>th</sup> pass at  
 5 different cell concentrations (30–60 g/L); c: Granulometric analysis of the supernatant (5  
 6 000 g 4 min) from lysate 5<sup>th</sup> pass at different cell concentrations (30-60-90 g/L)  
 7 (color for print)

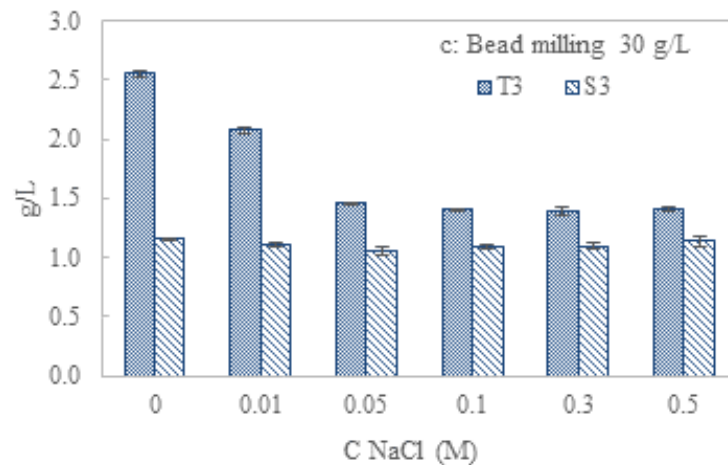
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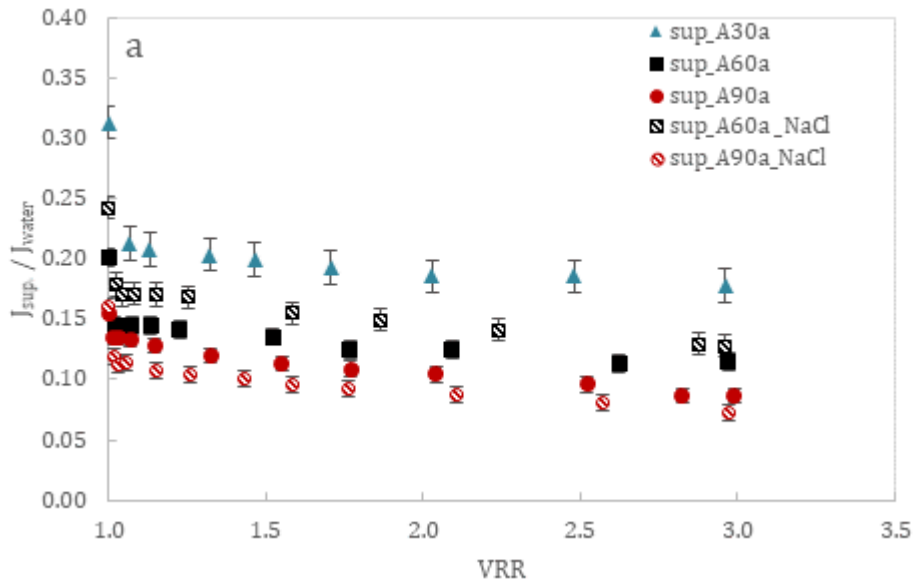


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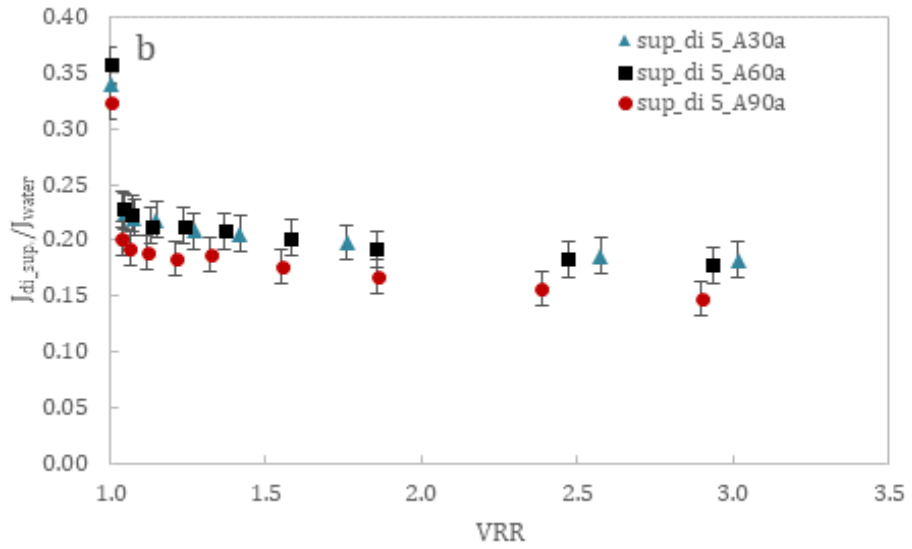


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5 Figure 4: Modification of the physicochemical conditions in the lysate, results. a:  
6 quantification of total and soluble proteins in the supernatant (T<sub>3</sub> and S<sub>3</sub>) after bead  
7 milling, pH modification and centrifugation 5 000 x g. b: soluble proteins concentration  
8 in supernatant 5 000 x g under different maceration times and temperatures. c: total and  
9 soluble proteins concentrations in supernatant 5 000 x g after modification of ionic  
10 strength by NaCl.

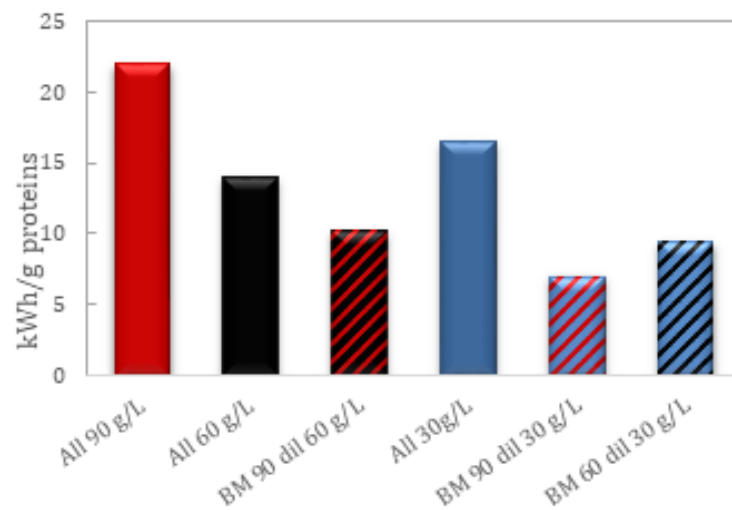


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3 Figure 5. Normalized permeate flux  $J_{sup.}/J_{water}$  during concentration. a: filtration of not  
 4 diluted supernatant, b: filtration of diluted supernatant, after centrifugation.



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 2 Figure 6: Energy consumption (kWh) per gram of soluble proteins recovered in permeate  
 3 considering all the process operating at a unique biomass concentration of 90-60-30 g/L  
 4 (“All”, with colourful bar); considering the bead milling (BM) at 90 or 60 g/L and then  
 5 the lysate diluted at 60 or 30 g/L for the following operation units (with shaded bar).  
 6



1 Table 1. Summary and comparison of case studies on bead milling for microalgae with our studies

Microalgae strain	Conditions	Scale	Indicator for optimization	Results	Energy consumption	Ref.
<i>Chlorella vulgaris</i>	DW: 30–90 g.L <sup>-1</sup> d <sub>B</sub> : 0.4 mm ceramic Q: 200 mL/min φ: 80% v/v v: 10 m.s <sup>-1</sup>	0.6 L	Soluble protein recovery in the supernatant and permeate			This work
<i>Chlorella</i> sp.	DW: 69–158 g.L <sup>-1</sup> d <sub>B</sub> : 0.3-0.75mm glass/ 0.3-0.8 mm et ceramic Q: 3–1220 kg.h <sup>-1</sup> φ: 60–85% v/v v: 10–16 m.s <sup>-1</sup>	0.6L- 18.3L	Cell disruption rate	More than 90% cell disruption	NM	[40]
<i>Tetraselmis</i> sp.	DW: 120 g.L <sup>-1</sup> d <sub>B</sub> : 0.4-0.6 mm ceramic Q: 1.5 L.min <sup>-1</sup> φ: 65% v/v 30 min recirculation Tris/HCl buffer, EDTA, DTT.	0.3L	Soluble protein recovery	Complete disruption of cell, 21% proteins recovered in supernatant after centrifugation	NM	[41]
<i>Chlorella vulgaris</i>	DW :25–145 g.L <sup>-1</sup> d <sub>B</sub> : 1 mm ceramic φ: 65% v/v v: 6–12 m.s <sup>-1</sup> phosphate buffer saline	0.08 L	Soluble protein recovery	Over 90% cell disruption 32–42% proteins in supernatant 20 000 g (Ci/Cj)	Lowest Specific energy consumption for 145 g/L 6m.s <sup>-1</sup> : 0.8 kWh kgDW <sup>-1</sup> (only BM)	[11]
<i>Porphyridium cruentum</i> <i>Nannochloropsis oculata</i>	DW: >5g.L <sup>-1</sup> d <sub>B</sub> : 0.375-2.15mm glass /0.2-1.25mm ceramic Q:102-200 mL.min <sup>-1</sup> φ :35–85% v/v v: 8–14 m.s <sup>-1</sup>	0.6 L	Cell disruption rate	Over 90% cell disruption for two strains	2.10 <sup>10</sup> J/kg for P.C 8.10 <sup>11</sup> J/kg for NO Only BM	[10]
<i>Chlorella vulgaris</i> <i>Tetraselmis suecica</i> <i>Nannochloropsis oleoabundans</i>	DW: 25–145 g.L <sup>-1</sup> d <sub>B</sub> : 0.3-1 mm ceramic φ: 65% v/v v: 6–12 m.s <sup>-1</sup> phosphate buffer saline	0.08 L	Proteins and carbohydrates recovery	d <sub>B-optimal</sub> : 0.3 mm for minimum specific energy consumption of 60 kWh.kgDW <sup>-1</sup> resulting in a Y <sub>Prot</sub> of 28% and a Y <sub>Carb</sub> of 52% in supernatant 20 000 g (Ci/Cj)	The lowest specific energy: 0.45 kWh kgDW <sup>-1</sup> for C. vulgaris	[14]

<i>Chlorella sorokiniana</i>	DW: 1 g/L d <sub>B</sub> : 0.2-1.3 mm glass/ ceramic Q: 200 mL.min <sup>-1</sup> φ: 80% v/v v: 8-14 m.s <sup>-1</sup>	0.6 L	Cell disruption, Starch, proteins chlorophyll recovery	d <sub>B</sub> -optimal: 0.4 mm glass beads v: 14 m.s <sup>-1</sup> for better liberation. up to 90% proteins recovered in supernatant 3 000 g	Stress model: combined effect of stress intensity, stress number and specific energy	[13]
<i>Tetraselmis suecica</i> <i>Chlorella vulgaris</i>	DW:30- 155 g.L <sup>-1</sup> d <sub>B</sub> : 0.3-1 mm φ: 2.5-75% v/v phosphate-buffer saline	0.08 L 0.5L	Protein Carbohydrates Cell	The kinetics of biomolecules release is proportional to the kinetics of cell disintegration . 28% proteins in supernatant 20 000 g for lowest energy consumption (C <sub>i</sub> /C <sub>j</sub> )	high bead filling ratio (>65%) is crucial to ensure an energy efficient process	[42]

- 1 \*d<sub>B</sub>: diameter of the beads; DW: dry matter of biomass suspensions; Q: feed rate; φ: chamber filling ratio; v: stirring speed. Protein yield calculation in several literature:  
2 C<sub>i</sub>/C<sub>j</sub> : protein concentration ratio in fraction i (biomass) relative to fraction j (supernatant); NM: no mentioned

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Table 2: Bead milling-centrifugation mass balance: DW<sub>1</sub>: biomass dry weight before bead milling; DW<sub>2</sub>: lysate dry weight before centrifugation (\* diluted lysate); DW<sub>3</sub>: supernatant dry weight; T<sub>1</sub> and T<sub>3</sub>: total protein concentrations in the biomass and in the supernatant; S<sub>1</sub> and S<sub>3</sub>: soluble protein concentrations in the biomass and in the supernatant; S<sub>3</sub>/S<sub>1</sub>: ratio between the two concentrations; Φ: supernatant volume recovery rate after bead milling and centrifugation;  $\eta_{3/1}^S$ : soluble protein recovery rate in supernatant related to biomass; purity<sub>3</sub><sup>S</sup>: soluble proteins purity related to supernatant dry weight.

N°Assay	DW <sub>1</sub>	DW <sub>2</sub>	DW <sub>3</sub>	T <sub>1</sub>	S <sub>1</sub>	T <sub>3</sub>	S <sub>3</sub>	S <sub>3</sub> /S <sub>1</sub>	Φ	$\eta_{3/1}^S$	purity <sub>3</sub> <sup>S</sup>
	g/L	g/L	g/L	g/L	g/L	g/L	g/L	%	%	%	%
<b>Biomass concentration effect</b>											
A30_a	30	30	8.3	14.8	1.5	2.9	0.9	60	74±7	44±4	11±0.3
A30_b	32	32	8.5	16.1	1.6	3.2	1.0	63	74±7	46±5	12±0.4
A60_a	66	66	12.8	33.0	3.3	4.2	2.2	67	57±6	37±4	17±0.1
A60_b	64	64	20.5	32.2	3.3	6.0	2.9	-	48±5	43±5	14±0.4
A90_a	89	89	20.3	44.4	4.5	5.9	3.3	73	32±5	24±4	16±0.6
A90_b	78	78	19.3	39.1	4.0	5.4	3.8	96	37±6	36±5	20±0.6
<b>Lysate dilution effect on centrifugation</b>											
A60_di.ly_5	66	*6	1.1	33.0	3.3	0.3	0.2	36	96±10	68±8	19±1.2
A60_di.ly_30	66	*33	5.1	33.0	3.3	1.6	1.2	6	81±8	58±6	23±1.6

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1 Table 3: Filtration mass balance from three different biomass concentrations (30, 60 and  
 2 90 g/L): DW<sub>3</sub>: supernatant dry weight for filtration; T<sub>i</sub>: total protein concentration in  
 3 fraction i, S<sub>i</sub>: soluble protein concentration in fraction i;  $\eta_{4/3}^S$  - soluble protein recovery  
 4 rate in permeate related to supernatant;  $\eta_{4/1}^S$ : soluble protein recovery rate in permeate  
 5 related to initial biomass; purity<sub>3</sub><sup>S</sup>: purity of soluble protein in the supernatant; purity<sub>4</sub><sup>S</sup>:  
 6 purity of soluble protein in permeate; (\* diluted supernatant)

N°Assay	DW <sub>3</sub> g/L	T3 g/L	S3 g/L	T4=S4 g/L	$\eta_{4/3}^S$ %	$\eta_{4/1}^S$ %	purity <sub>3</sub> <sup>S</sup> %	purity <sub>4</sub> <sup>S</sup> %
<b>A30a</b>	8.3	2.9	0.9	0.25	18.5±0.7	8.1±0.8	10.7±0.2	14.1±0.4
<b>A60a</b>	12.8	4.2	2.2	0.56	17.2±0.5	6.4±0.6	17.0±0.1	17.7±0.1
<b>A90a</b>	20.3	5.9	3.3	0.86	17.5±0.8	4.2±0.4	15.0±0.4	17.4±0.5
<b>A60a+NaCl</b>	17.5	3.3	2.2	0.66	20.2±0.5	7.5±0.8	12.5±0.1	6.6±0.1
<b>A90a+NaCl</b>	24.1	4.2	3.2	1.03	21.6±0.3	5.4±0.5	13.2±0.3	8.3±0.1
<b>A30 di. sup.</b>	*5.3	2.9	0.9	0.16	18.3±0.3	8.0±0.8	*12.4±1.8	14.0±0.4
<b>A60 di. sup.</b>	*4.9	6.0	2.9	0.17	16.2±1.1	7.0±0.8	*14.5±0.2	22.3±1.3
<b>A90 di. sup.</b>	*4.5	5.9	3.3	0.17	16.0±0.6	3.8±0.4	*17.2±0.4	16.0±0.3

7

1 Table 4: Membrane filtration performances during the filtration of supernatants after the  
 2 bead-milling and the centrifugation of three different biomass concentrations (30, 60 and  
 3 90 g/L):  $J_{w\ 30\ ^\circ C}$  : pure water flux at 0.3 bar at 30 °C before concentration;  $J_{VRR3\ 30\ ^\circ C}$ :  
 4 supernatant filtration permeate flux at VRR3 at 30°C;  $J_{VRR3}/J_w$ : normalized permeate  
 5 flux with initial pure water flux; RR: soluble protein retention rate calculated by 1-  
 6  $C_{permeate}/C_{retentate}$  at VRR3;  $R_{fouling}$  : fouling resistance at VRR3

N°Assay	$J_w\ 30\ ^\circ C$	$J_{VRR3\ 30\ ^\circ C}$	$J_{VRR3}/J_w$	RR	$R_{fouling}$
	L/h/m <sup>2</sup> 0.3bar	L/h/m <sup>2</sup> 0.3bar	-	%	m <sup>-1</sup>
<b>A30a</b>	70.9	12.6	0.18	87.1	$0.9 \times 10^{13}$
<b>A60a</b>	66.2	7.6	0.11	-	$1.6 \times 10^{13}$
<b>A90a</b>	68.7	5.9	0.09	86.9	$2.1 \times 10^{13}$
<b>A60a+NaCl</b>	60.8	7.8	0.13	85.8	$1.5 \times 10^{13}$
<b>A90a+NaCl</b>	66.8	4.8	0.07	85.1	$2.6 \times 10^{13}$
<b>A30 di. sup.</b>	75.2	13.7	0.18	88.9	$0.8 \times 10^{13}$
<b>A60 di. sup.</b>	68.9	12.2	0.18	89.9	$0.9 \times 10^{13}$
<b>A90 di. sup.</b>	71.3	10.6	0.15	90.4	$1.1 \times 10^{13}$

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