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

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Abstract

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

Keywords

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

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

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

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

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

It is difficult to optimize the disruption of microalgae because the process efficiency is not relied only to the number of broken cells or the rate of compounds released in the aqueous phase. The physicochemical properties of the recovered suspension also have a significant impact on the downstream processes. Frequently a trade-off is necessary between the liberation efficiency and repercussion of downstream process. For example, the target compounds release efficiency can be maximized by increasing the intensity of cell disruption, which will simultaneously increase the release of contaminants and the micronization of cell debris. As consequence, the viscosity of the product will increase, which will adversely affect downstream process performances such 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 $<10\,000 \times g$, 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

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

2. Materials and Methods

2.1 Experimental setup

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

2.2 Biomass characterization

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

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

2.3 Cell disruption by bead milling

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

30 g/L_{DW} (watery solution), with Newtonian behaviour, where the viscosity is independent of applied stresses (viscosity: 0.002 Pa s);

60 g/L_{DW}, the transition between Newtonian and non-Newtonian, where a strong shear thinning behaviour can be observed when the applied stress exceeds a critical value (viscosity: 0.006 Pa s);

90 g/L_{DW} (sticky liquid) with non-Newtonian behaviour, where yield stress behaviour can be observed (viscosity: 0.03 Pa s [11,22])

For the cell disruption experiments, Dyno-mill multi lab (WAB, Switzerland) was operated in pendulum mode as described by Montalescot et al. 2015 [11]. The rotational speed was kept to 10 m/s, while the feed flow rate was 200 mL/min. The beads used were made in ceramic (density of 6 g/cm³, approved for contact with food products) and characterized by a diameter of 0.4 mm. The beads diameter was chosen according to the supplier indications: beads diameter $\approx 30 \cdot$ cell diameter. The filling ratio of the beads was 80% to attain a maximum disruption efficiency [10]. The lysate temperature was maintained at 10 °C.

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

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

2.6 Membrane filtration

The filtration experiments were performed in a 500 mL Amicon ® Stirred Cell (Milipore, USA). The inlet pressure (P_e) was given by compressed air and adjusted by a valve combined with a manometer on the cell inlet. The supernatant was introduced in the filtration cell at $30 \pm 1 \text{ }^\circ\text{C}$ and stirred at 300 tr/min, the pressure was maintained at 0.3 bar, the instant temperature of the permeate was measured. For all experiences, the previously selected membrane, PES 0.1 μm (Koch Membrane Systems) was reused after 0.2%(v/v) Ultrasil 110 (Ecolab) cleaning ($45 \text{ }^\circ\text{C}$, 30 min) and water rinsing ($30 \text{ }^\circ\text{C}$, 30 min). The pure water permeability was measured before each supernatant filtration 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 supernatant was concentrated at a Volume Reduction Ration ($\text{VRR} = V_{\text{feed}}/V_{\text{retentate}}$) equal to 3.

2.7 Calculation of the recovery yields

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

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

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

6 Φ : the volume fraction of the supernatant compared to diluted lysate

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

8 EF: the extraction factor of the microfiltration

9 VRR: the volume reduction rate of the microfiltration

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

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:

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

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

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

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 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_{DW} required three times as long as the equipment running time compared to that at 90 g/L_{DW}.

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

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⁻¹ for 30, 60, 90 g/L_{DW} respectively.

7 The higher release of the soluble proteins (70%) was observed after 5 min at
8 90 g/L_{DW} and 60 g/L_{DW} while, at 30 g/L_{DW}, a maximum of 60% was reached. At 90 g/L_{DW}
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⁻¹ 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 (ZrO₂ beads 0.4 mm, biomass
13 concentration 90 g/L_{DW}, 25 °C). Zinkoné 2018 [16] reported a disruption constant
14 between 0.003 and 0.017 s⁻¹ for *Chlorella sorokiniana* (fresh biomass, 1 g/L_{DW}, 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⁻¹
21 at cell concentration near 90 g/L_{DW}.

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 3rd pass (3.9 min) major differences are observed among the cell concentrations. These

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

3.1.2 Granulometric analysis

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

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

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

Figure 3.b compares the cell lysate of the 5th pass for the two conditions 30 g/L_{DW} and 60 g/L_{DW}. The data show a slight increase of the disruption efficiency for higher

biomass concentration (reduced peak at 3 μm), which also supports the improved efficiency in soluble proteins release at 60 g/L_{DW} compared to 30 g/L_{DW} (Figure 2.c).

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

3.2 Bead milling - centrifugation

3.2.1 Biomass concentration effect

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

Table 2 shows the mass balance results of the grinding and centrifugation process. After centrifugation, the concentration of total and soluble proteins in the supernatant T₃

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_{DW}
9 was diluted at 5 g/L_{DW} and 30 g/L_{DW} 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_{DW}. 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_3^S) 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_3^S values must
12 be taken with caution.

13 So coupling a bead milling at a concentration near 60 g/L_{DW} and dilution to
14 30 g/L_{DW} 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_3 and soluble protein
22 concentration S_3 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

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

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

3.3.2 Maceration time and temperature

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

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

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

3.3.3 Ionic strength modification by NaCl

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

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

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_{DW}), 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.

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

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

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

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

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

3.4. 2 Supernatant dilution effect on filtration

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

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

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

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

coupling a bead-milling at 60 g/L_{DW}, a dilution at 30 g/L_{DW} and centrifugation 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 during at least 30 min (or shorter at 40 °C), the filtration recovery rate $\eta_{4/3}^S$ could reach 21%; then the global soluble protein recovery rate $\eta_{4/1}^S$ could reach 12.2%.

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

Nerveless, a better analyse of the organization of the soluble proteins in the lysate and supernatant becomes essential to understand why 80% are retained by a membrane with a cut-off of 0.1 µm. This is the key to solve the problem of a high soluble protein recovery.

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

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

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

3.5 Overall process balance and energy consumption

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

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

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

The bead-milling coupled with centrifugation and filtration, all at a concentration of 90, 60 and 30 g/L_{DW} are presented with colourful bars.

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

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

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

Considering the average energy cost of 0.1 €/kWh, the impact of the energy on the price of proteins will slow down from 1.4 to 0.7 €/g_{proteins} by using the optimum sequence of disruption-dilution-filtration. The improvement of the VRR will reduce this cost to 0.35€/g_{proteins},

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

The analysis presented in this study, shows that the energetic cost related to microalgal proteins extraction and purification could be reduced to 0.35 €/g_{proteins} by using the correct sequence of disruption-dilution-centrifugation-filtration and pushing the filtration step to a VRR of 20. The proteins recovered with this process are defined as soluble proteins with potential techno-functional properties [12] which can justify high values. At this stage, the cost of microalgal proteins is still high and it cannot compete with the current vegetal sources (soya, pea, whey) as presented by Préta et al. 2020 [39]. Nevertheless, the authors wanted to propose the present analysis of the energy 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_{DW} 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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3

Tables and Figures

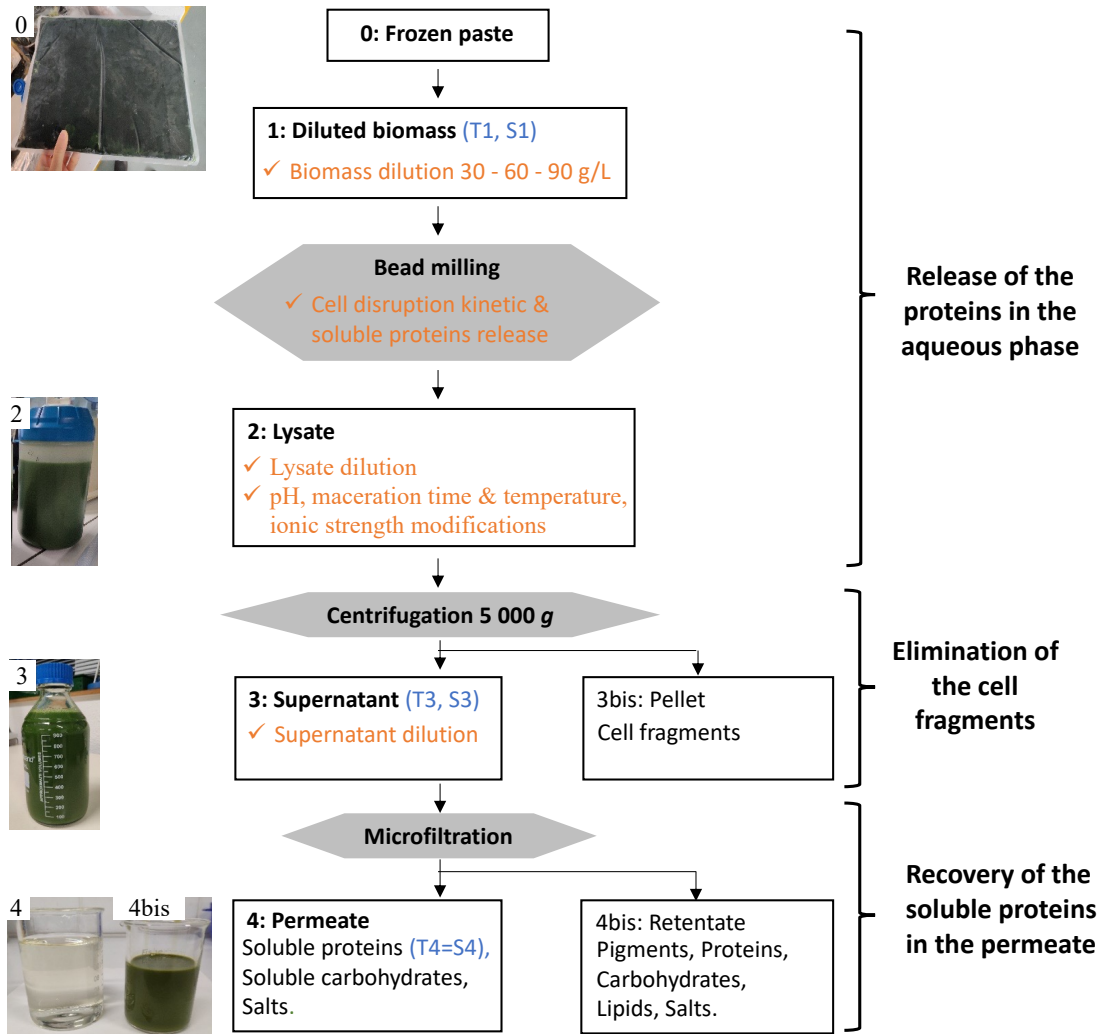


Figure 1: Process flow diagram applied for the recovery of soluble proteins (Pictures correspond to milling concentration at 90 g/L)

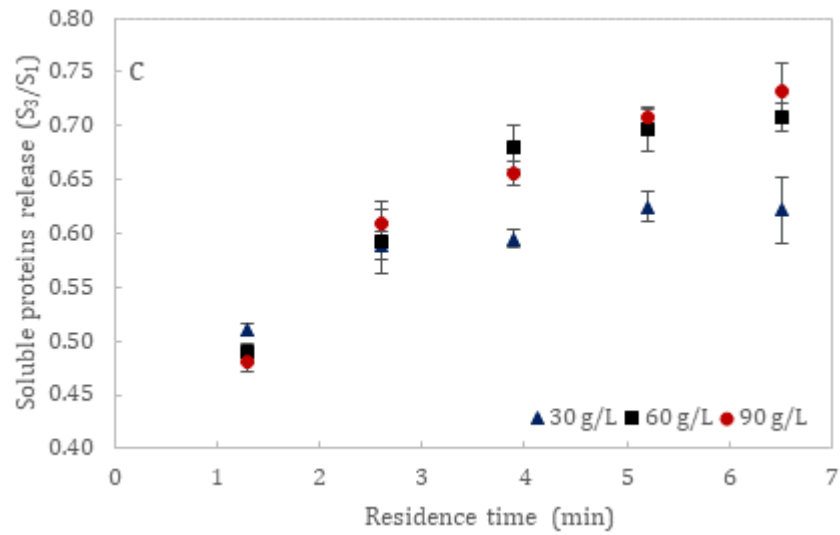
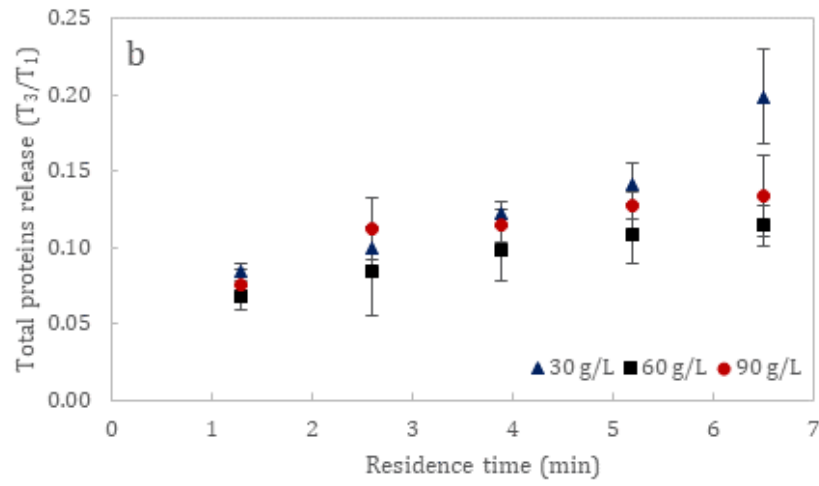
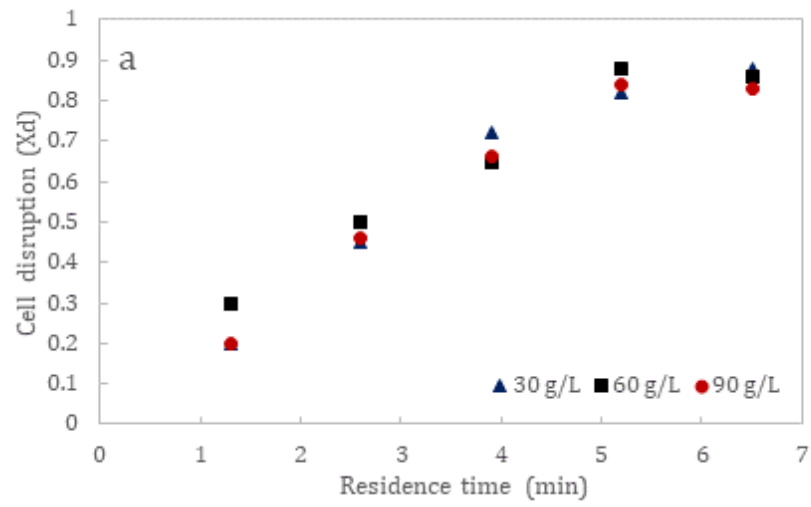


Figure 2: The kinetic of cell disruption (a), total proteins (b) and soluble proteins (c) as function of residence time for different biomass concentrations during bead milling

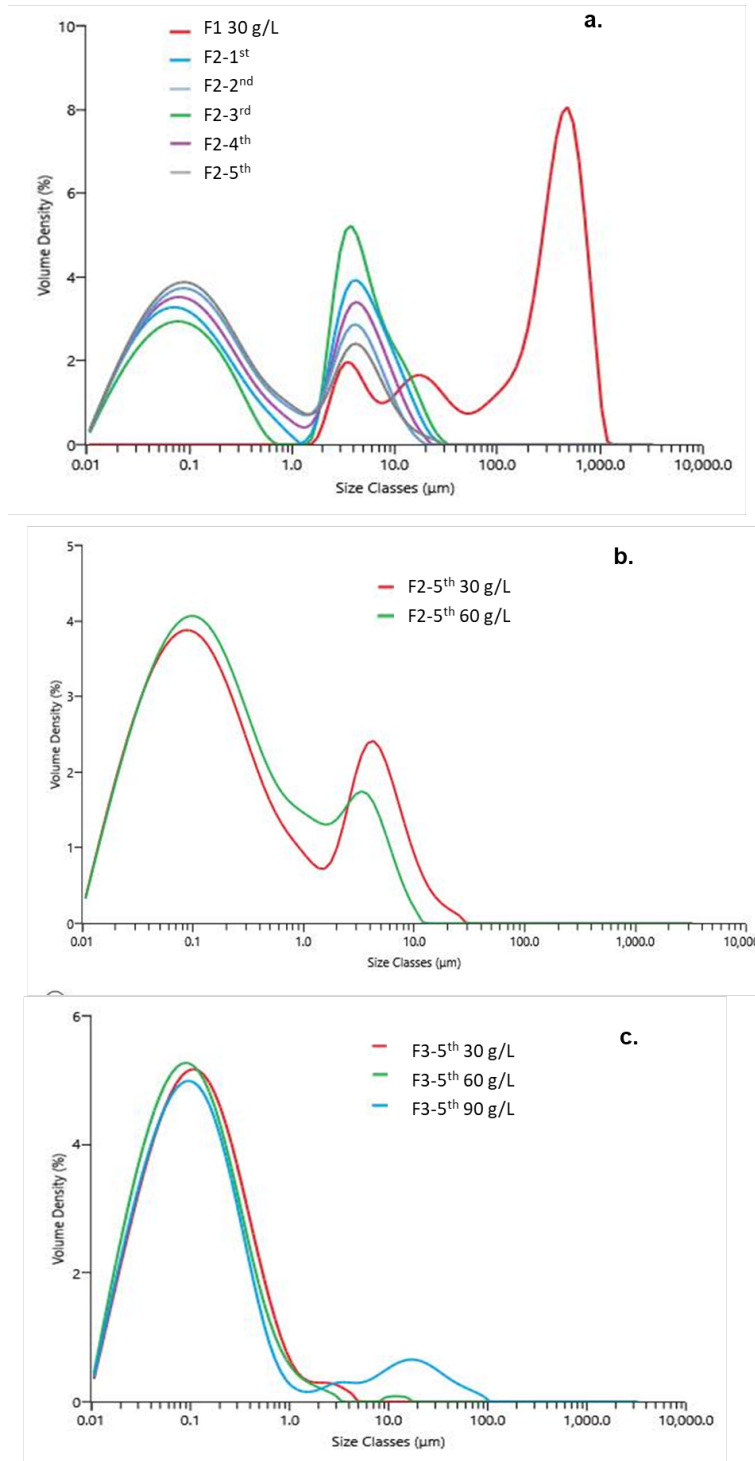


Figure 3: Lysate and supernatant particles size distribution. a: Granulometric analysis of the diluted biomass fraction (F1) and the lysate fraction after each pass (F2-x pass) in the bead milling for the 30 g/L test; b: Granulometric analysis of the lysate in 5th pass at different cell concentrations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

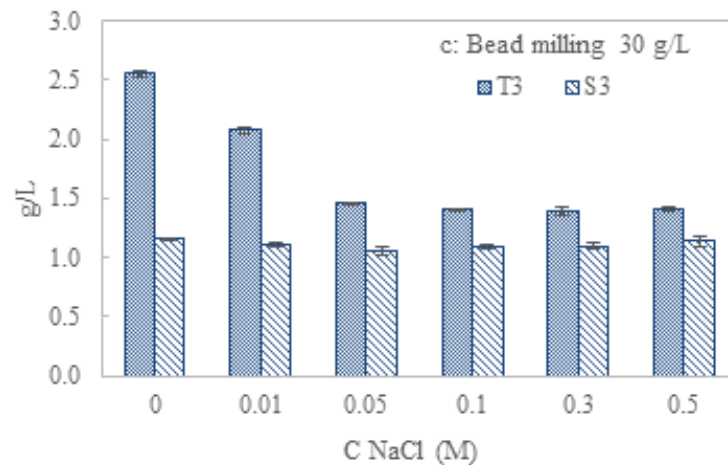
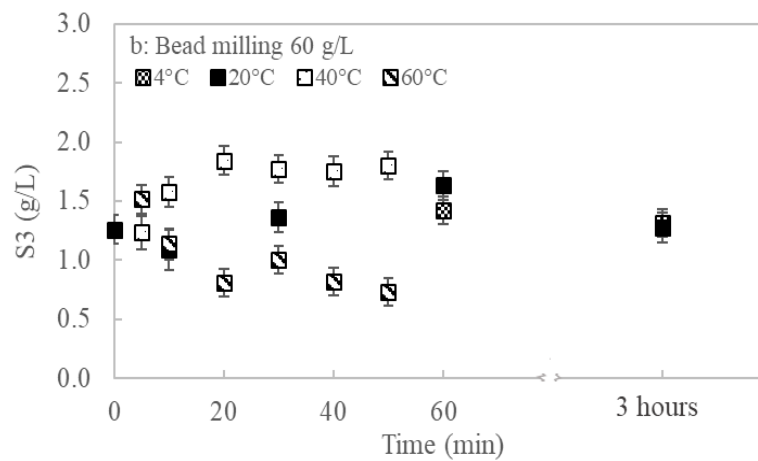
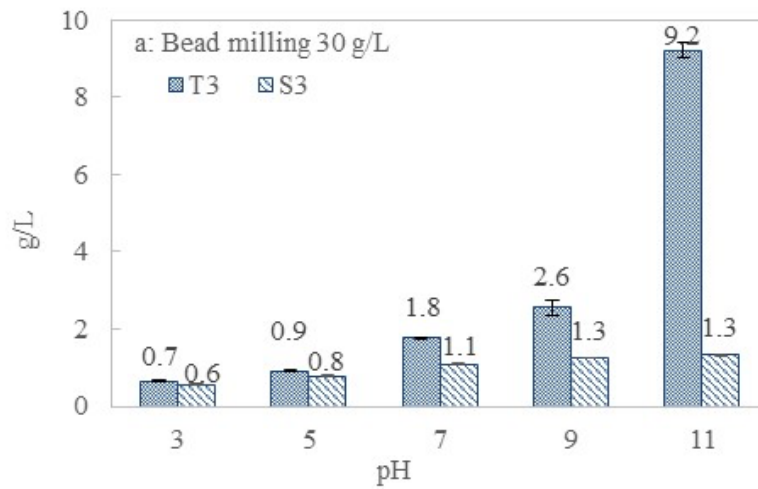


Figure 4: Modification of the physicochemical conditions in the lysate, results. a: quantification of total and soluble proteins in the supernatant (T₃ and S₃) after bead milling, pH modification and centrifugation 5 000 x g. b: soluble proteins concentration in supernatant 5 000 x g under different maceration times and temperatures. c: total and soluble proteins concentrations in supernatant 5 000 x g after modification of ionic strength by NaCl.

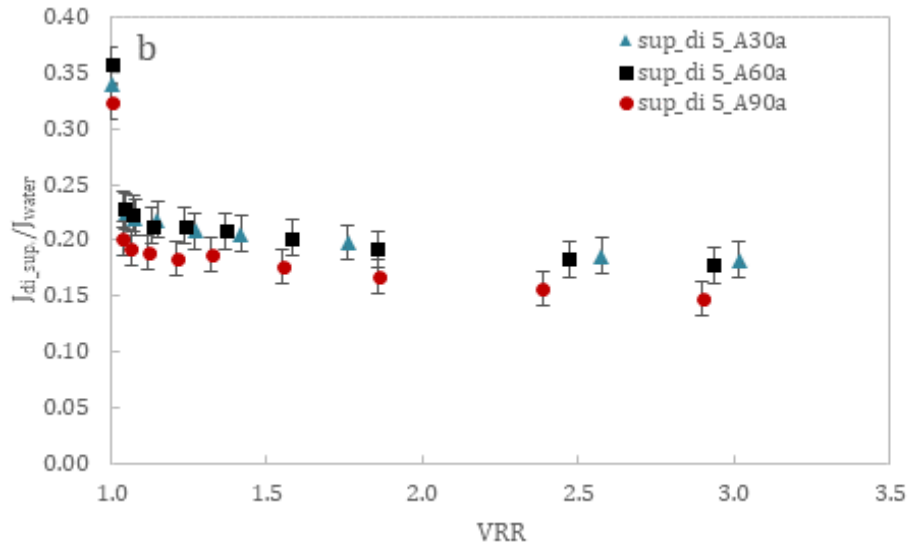
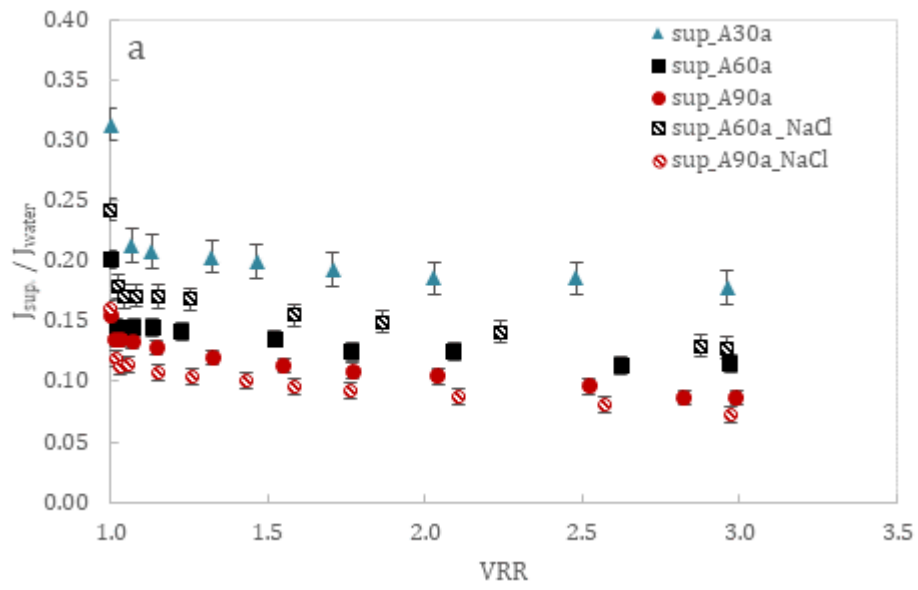


Figure 5. Normalized permeate flux $J_{sup.}/J_{water}$ during concentration. a: filtration of not diluted supernatant, b: filtration of diluted supernatant, after centrifugation.

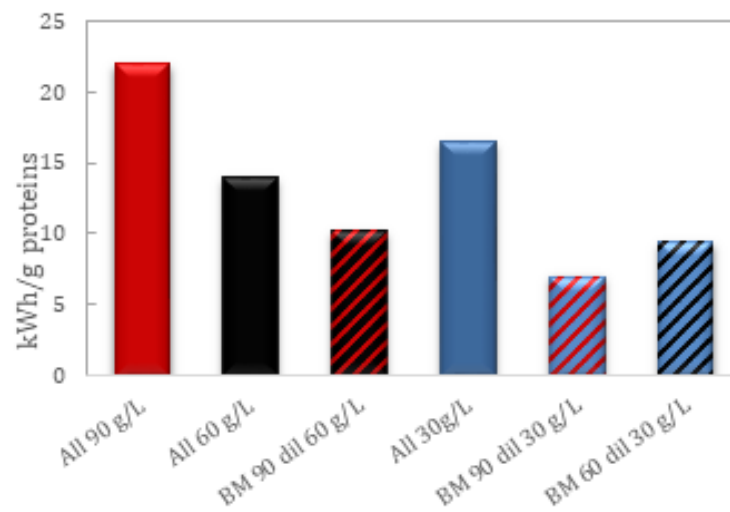


Figure 6: Energy consumption (kWh) per gram of soluble proteins recovered in permeate considering all the process operating at a unique biomass concentration of 90-60-30 g/L (“All”, with colourful bar); considering the bead milling (BM) at 90 or 60 g/L and then the lysate diluted at 60 or 30 g/L for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

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 ⁻¹ d _B : 0.4 mm ceramic Q: 200 mL/min φ: 80% v/v v: 10 m.s ⁻¹	0.6 L	Soluble protein recovery in the supernatant and permeate			This work
<i>Chlorella</i> sp.	DW: 69–158 g.L ⁻¹ d _B : 0.3-0.75mm glass/ 0.3-0.8 mm et ceramic Q: 3–1220 kg.h ⁻¹ φ: 60–85% v/v v: 10–16 m.s ⁻¹	0.6L- 18.3L	Cell disruption rate	More than 90% cell disruption	NM	[40]
<i>Tetraselmis</i> sp.	DW: 120 g.L ⁻¹ d _B : 0.4-0.6 mm ceramic Q: 1.5 L.min ⁻¹ 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 ⁻¹ d _B : 1 mm ceramic φ: 65% v/v v: 6–12 m.s ⁻¹ 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 ⁻¹ : 0.8 kWh kgDW ⁻¹ (only BM)	[11]
<i>Porphyridium cruentum</i> <i>Nannochloropsis oculata</i>	DW: >5g.L ⁻¹ d _B : 0.375-2.15mm glass /0.2-1.25mm ceramic Q:102-200 mL.min ⁻¹ φ :35–85% v/v v: 8–14 m.s ⁻¹	0.6 L	Cell disruption rate	Over 90% cell disruption for two strains	2.10 ¹⁰ J/kg for P.C 8.10 ¹¹ J/kg for NO Only BM	[10]
<i>Chlorella vulgaris</i> <i>Tetraselmis suecica</i> <i>Nannochloropsis oleoabundans</i>	DW: 25–145 g.L ⁻¹ d _B : 0.3-1 mm ceramic φ: 65% v/v v: 6–12 m.s ⁻¹ phosphate buffer saline	0.08 L	Proteins and carbohydrates recovery	d _{B-optimal} : 0.3 mm for minimum specific energy consumption of 60 kWh.kgDW ⁻¹ resulting in a Y _{Prot} of 28% and a Y _{Carb} of 52% in supernatant 20 000 g (Ci/Cj)	The lowest specific energy: 0.45 kWh kgDW ⁻¹ for C. vulgaris	[14]

<i>Chlorella sorokiniana</i>	DW: 1 g/L d _B : 0.2-1.3 mm glass/ ceramic Q: 200 mL.min ⁻¹ φ: 80% v/v v: 8-14 m.s ⁻¹	0.6 L	Cell disruption, Starch, proteins chlorophyll recovery	d _B -optimal: 0.4 mm glass beads v: 14 m.s ⁻¹ 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 ⁻¹ d _B : 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 _i /C _j)	high bead filling ratio (>65%) is crucial to ensure an energy efficient process	[42]

- 1 *d_B: diameter of the beads; DW: dry matter of biomass suspensions; Q: feed rate; φ: chamber filling ratio; v: stirring speed. Protein yield calculation in serval literature:
2 C_i/C_j : 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: DW₁: biomass dry weight before bead milling; DW₂: lysate dry weight before centrifugation (* diluted lysate); DW₃: supernatant dry weight; T₁ and T₃: total protein concentrations in the biomass and in the supernatant; S₁ and S₃: soluble protein concentrations in the biomass and in the supernatant; S₃/S₁: 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; purity₃^S: soluble proteins purity related to supernatant dry weight.

N°Assay	DW ₁ g/L	DW ₂ g/L	DW ₃ g/L	T ₁ g/L	S ₁ g/L	T ₃ g/L	S ₃ g/L	S ₃ /S ₁ %	Φ %	$\eta_{3/1}^S$ %	purity ₃ ^S %
Biomass concentration effect											
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
Lysate dilution effect on centrifugation											
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

1 Table 3: Filtration mass balance from three different biomass concentrations (30, 60 and
2 90 g/L): DW₃: supernatant dry weight for filtration; T_i: total protein concentration in
3 fraction i, S_i: 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; purity₃^S: purity of soluble protein in the supernatant; purity₄^S:
6 purity of soluble protein in permeate; (* diluted supernatant)

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

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

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

