Coupling bead-milling and microfiltration for the recovery of lipids and proteins from Parachlorella kessleri: Impact of the cell disruption conditions on the separation performances

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Abstract

Microalgae biorefinery has become an attracting topic to fully exploit the intracellular compounds such as lipids, proteins, polysaccharides or pigments, etc. Conventionally, microalgae biorefinery involves four major operations; harvesting, cell disruption, fractionation and purification, and final product formulation. This study evaluates the possibility to recover lipids and hydrosoluble compounds from *Parachlorella kessleri* cultivated in starving conditions. The cell disruption by bead-milling was coupled with centrifugation and membrane filtration for the fractionation. The impact of the biomass variation and the bead-milling, centrifugation and

physicochemical conditions on the compounds' recovery into the supernatant and on the filtration performances was investigated. Three different filtration conditions were also compared: dead end, cross-flow and dynamic filtration. Finally, the best scenario allowed to recover 23% of the total lipids from initial biomass in the retentate and 9% of the sugars and 8% of the proteins from the biomass, totally soluble in the permeate.

Keywords

Microalgae biorefinery, bead milling, membrane filtration, lipids, proteins

1. Introduction

Microalgae have shown high potential as an alternative to non-renewable resources, in various sectors such as food, cosmetics, medicine or energy [1]. Intensified studies were carried out over the past decade on microalgae physiological conditions understanding and cultivation optimization [2–7]. Microalgae biorefinery has recently become an increasing research topic to fully exploit their potential. Conventionally microalgae biorefinery processes proposed in literature include the following unit operations: harvesting and biomass dewatering or biomass drying (centrifugation or membrane filtration, oven drying, etc.), cell disruption (by mechanic or enzymatic operations), fractionation by solvent extraction or membrane filtration [1,8].

Many studies have focused on the optimization of the recovery of a single compound by a single operation, such as lipids for biofuels, protein or pigment for cosmetics, etc. [9–12]. The main challenge of the microalgae biorefinery is to maximize the recovery of different purified fractions without damaging them, and to operate efficiently and cost- effectively.

In this study, to avoid energetic biomass-drying before the fractionation, a wet pathway was applied as described by Clavijo Rivera et al., 2018 [13] to recover lipids and proteins from *Parachlorella kessleri* (PK) cultivated in starving conditions. The process involved the bead milling of a diluted biomass for a better compound release, the clarification by centrifugation to remove the cell fragments, the membrane filtration to fractionate the hydrophobic (lipids and a part of pigments) and hydrophilic compounds (salts, soluble proteins and carbohydrates). The combination of bead milling and membrane filtration facilitates the industrial scale up, as they can treat large volumes of suspension.

Membrane filtration is a well-known process for the separation and purification of biomolecules from natural products or agrofood byproducts [14,15]. Considering microalgae culture and biorefining, membranes can be used in both upstream and downstream processes: the culture medium recycling and the cell harvesting, the recovery of extracellular metabolites present in the culture medium, the recovery of a water-soluble fraction from a lysate, etc [16–19].

PK cultured in starving conditions shows high potential for triglycerides (TAG) accumulation (up to 40–50% dry matter) [20]. Recently, a considerable number of studies focused on the optimization of culture conditions to increase lipids accumulation in microalgae cells. However, the transposition of those optimal conditions to large-scale lipids rich biomass production remains difficult, since the system regulation and the risk of contamination are more critical for a larger volume cultivation. The nitrogen deficiency level and the light stress, which determines the harvested cell lipid content, also varies according to the photobioreactor (PBR) geometry and the volume of the culture [3,6,7,20–29].

69 The compromise between biomass productivity and lipid accumulation under
70 depleted conditions is a challenge for biorefinery as this second part requires much
71 biomass for process optimization. As a result, the harvested biomass contains fewer
72 target lipids but more carbohydrates [3,7,26]. For biorefinery, the recovery of those
73 compounds and their fractionation seems necessary.

74 It is already known that the cell disruption conditions significantly impact on the
75 quality of the released intracellular content and the efficiency of the following
76 separation steps [30]. The cell disruption is a decisive step, where the intracellular
77 compounds come in contact with the dilution medium. But the effect of a change in
78 environment and of the reorganization of the whole matter was still poorly studied in
79 literature. It is important to characterize the distribution of each composition (lipids,
80 proteins and carbohydrates) between the cell fragments and the supernatant and their
81 characteristics for the optimization of the downstream separation and purification
82 process.

83 Once a part of the lipids, proteins and carbohydrates are recovered into an
84 aqueous phase (as suspended colloids or solubilized), it is necessary to evaluate whether
85 their separation can be achieved by the membrane process. It was demonstrated that
86 polyacrylonitrile (PAN) 500 kDa or polythersulfone (PES) 300 kDa membranes can
87 retain lipid droplets [31,32]. Lorente et al., 2017 and Safi et al., 2014 [17,33] also
88 showed the possibility to fractionate lipids and carbohydrates with a PES100-500 kDa
89 membrane, but the permeation of proteins is not obvious even with a molar weight cut
90 off (MWCO) of 1000 kDa [18,19]. The impact of hydrodynamic conditions on the
91 separation of microalgae extracts has also been poorly studied.

92 In this context, the objective of this work was to evaluate the possibility to
93 recover lipids and hydrosoluble compounds (proteins and carbohydrates) from *P.*
94 *kessleri* cultivated in starving conditions using bead-milling, centrifugation and
95 membrane filtration. Two biomass under different starving levels were harvested and
96 the impact of the bead-milling and centrifugation conditions on the compounds recovery
97 into the supernatant and on the filtration performances was investigated. Finally, the
98 global performances for the recovery of biomolecules from the biomass and the yield of
99 each step was studied.

100 **2. Material and methods**

101 **2.1. Experimental setup**

102 The overall steps of the wet pathway process and the products recovered in each
103 step are outlined in the Table 1.

104 ***Table 1. Overall steps of wet way process for the fractionation of lipids, proteins and carbohydrates***
105 ***from P. kessleri cultivated in starving conditions. Definition of abbreviations.***

Steps	Recovered fraction	Abbreviation
The harvested culture	raw biomass	0-RB
The storage (freezing, -25°C)	frozen biomass	0-FB
The thawing (4°C, overnight)	thawed biomass	1-TB
The pretreatment (dilution, chemicals) and the bead-milling	Lysate	2-Lys
The clarification by centrifugation (3,000 *g*)	Supernatant and pellet	3-Sup and 3-Pel
The membrane filtration	Retentate and permeate	4-Ret and 4-Per

106

First of all, the *P. kessleri* strain was harvested by centrifugation and the raw biomass (0-RB) was stored under -25°C. Before cell disruption, the frozen biomass (0-FB) was thawed (1-TB), then physiochemical pretreatment steps were performed (biomass concentration adjustment and chemical products introduction). After bead-milling, the lysate (2-Lys) was clarified to recover the supernatant (3-Sup) which was filtrated to separate the lipids into the retentate (4-Ret) from the hydrosoluble compounds (carbohydrates and parts of soluble proteins) into the permeate (4-Per).

2.2. Microalgae cultivation, harvesting and storage

In this study, two batches of *PK* culture 0-RB-A and 0-RB-B were used; each culture was conducted in a bubble column photobioreactor (100 L, Φ = 30 cm; H = 150 cm) under nitrogen starvation (0.23 g/L of $NaNO_3$) and light intensified conditions (100-150 µmol/m²/s) [20,34]. The pH was maintained at 7.5 through pH-controlled by the addition of carbon dioxide to the airflow, and the culture temperature was maintained between 22 and 25 °C. Then the microalgae were harvested by centrifugation at 5,400 *g* (ROUSSELET ROBATEL DRA320VX, France) into a concentrated suspension containing 1.5 % to 2.0 % dry matter and stored at -25°C before the cell disruption.

2.3. Cell disruption by bead milling

Prior to cell disruption, the frozen microalgae paste was defrosted at 4°C overnight and re-suspended to 5 g/L (total dry weight) within a phosphate buffer (pH 7.4, Conductivity 790 µs/cm). Cell disruption was performed by bead milling (Dyno-mill multi labo, WAB, Switzerland) and operated in a pendulum mode as described by

129 Montalescot et al., 2015 [35]. The milling chamber volume was 600 mL, glass beads
130 0.5-0.75 mm (average: 0.65 mm) were used and the feed flow rate was set at 150
131 mL/min. The filling ratio of the grinding media was 80% and the rotational speed was
132 set at 8 m/s [36]. During cell disruption, the lysate at different residence time was
133 sampled and the composition (lipids, proteins, and carbohydrates) analyzed.

134 The cell disruption rate reached at each pass was characterized by microscopy as
135 described by Zinkone 2018 [37]: the biomass was diluted with ultrapure water and 2%
136 (v/v) Lugol until each cell was separated and clearly presented under the microscope,
137 the same dilution rate was applied for all the lysates. The cell counting was done before
138 and after each pass by combination of Malassez cell microscope (Axio Scope A1,
139 ZEISS, Germany) photography and software Image J data treatment. The intact and
140 broken cells were distinguished by adjusting the circularity greater than 0.85. According
141 to Zinkone 2018, this value makes it possible to distinguish cellular debris and
142 deformed cells during particle detection.

143 **2.4. Clarification by centrifugation**

144 The lysate recovered from the bead milling was then centrifuged (SORVALL
145 LYNX 6000, Thermo Scientific, USA) and fractionated into an aqueous phase – the
146 supernatant (3-Sup) and an insoluble fraction – pellet (3-Pel). The recovered
147 supernatants were then stored at 4 °C in a bucket for subsequent filtration.

148 Three preliminary experiments on centrifugation conditions were carried out
149 with the same lysate: 6,000 *g* at 20 °C; 6,000 *g* at 5 °C and 3,000 *g* at 20 °C, and the
150 supernatants were filtrated. No significant centrifugation impact was observed for the

membrane filtration performances (selectivity and flux). Thus, 3,000 *g* at 20 ° C was selected as the optimal centrifugation conditions for low-cost large-scale applications.

2.5. Fractionation by micro and ultrafiltration

A commercial flat sheet polythersulfone (PES) membrane with a MWCO of 0.1 µm (MFK-618, KOCH Membrane Systems, USA) was used in this study. The PES material was previously selected by Clavijo Rivera et al., 2020 [31] as offering a limited irreversible fouling resistance for the filtration of lipid-rich mixtures. Besides, Clavijo Rivera et al., 2020 [31] demonstrated that the membrane polyacrylonitrile (PAN) 500 kDa (Orelis Environment, France) shows good properties for the filtration of lipid-rich mixtures as well. Thus this membrane was also tested and results are detailed in the Supplementary Material.

The unused membrane was gently rinsed with 96% ethanol and water to remove excessive superficial glycerol and then conditioned in the filtration pilot by the following steps: basic bleach cleaning (10 ppm NaClO at pH 10.5) at 30 °C during 30 min, 5 min water rinsing, then 0.2% (v/v) Ultrasil 110 (Ecolab, USA) cleaning at 45 °C for 30 min and finally water rinsing at 30 °C during at least 30 min. For all experiments of dead-end filtration, the same membrane cut was reused after membrane cleaning (without bleach step) and the pure water permeability was measured before each supernatant filtration. The objective was to limit the membrane coupon variability.

The dead-end filtration (DEF) experiments were performed in a 400 mL Amicon ® Stirred Cell (Milipore, USA). The feed supernatant was filtrated at 30 ± 1 °C and stirred at 300 tr/min, the inlet pressure was maintained at 0.3 bar by compressed air and adjusted by a valve combined with a manometer on the cell inlet, the instant

174 temperature of permeate was measured. All the supernatants were concentrated at a
175 Volume Reduction Ratio ($VRR=V_{supernatant}/V_{retentate}$) equal to 3.

176 The cross-flow filtration (CF) was performed with a Rayflow® pilot as
177 described by Clavijo Rivera et al., 2020 [31], and the rotating disc dynamic filtration
178 (DF) was performed with the pilot as described by Frappart et al., 2011 and Villafaña-
179 López et al., 2019 [32,37] with a disc equipped with eight 6 mm vanes, and a rotation
180 speed of 366 rpm ($\dot{\gamma}_{max}$ = 16,000 s^{-1}). The same membrane cleaning and filtration
181 procedures were followed for all filtration experiments (DEF, CF and DF). The
182 effective membrane area was 42 cm^2, 130 cm^2 and 188 cm^2 for DEF, CF and DF
183 respectively.

184 The transmembrane pressure (TMP) was set at 0.3 bar for all the pilots. This
185 TMP was selected according to preliminary critical pressure measurements carried out
186 on the CF pilot in full recycling mode, with the most complex lysate solutions (4^{th}
187 passes after bead milling). The selected TMP was calculated as 90% critical pressure. It
188 was used with all the pilots to facilitate the results comparison.

189 **2.6. Biochemical sample analysis**

190 To determine the dry weight (DW), the samples are dried to constant mass in an
191 oven where the temperature was controlled by thermostat with forced air ventilation and
192 able to maintain a temperature of 105 ± 5 °C. This DW contains the organic matter that
193 composes the biomass, and the salts from the culture medium. Then, the samples were
194 placed in a muffle furnace at 600 °C for 6 hours for ash determination.

195 The total fatty acids (TFA) were determined by gas chromatography-flame
196 ionization detector (GC-FID) [13]. The triglycerides (TAG) and free fatty acids (FFA)
197 were quantified by a high-performance thin-layer chromatography. The Pierce BCA
198 Protein Assay Kit (Thermo Scientific) was used for the quantification of proteins, and
199 the carbohydrate content was determined by the phenol-sulphuric acid (Dubois) method
200 [38].

201 The total and extractable soluble compounds (proteins, lipids and carbohydrates)
202 were characterized for each fraction. For example, in supernatant 3,000 *g,* the measured
203 total protein concentration was named as total proteins for 3-sup fraction, and the
204 proteins present in the supernatant at 12,000 *g* (centrifugation of 3-sup) were named
205 “soluble” for simplification even if colloids can be present.

206 Three types of electrophoresis, Native-PAGE (Native PolyAcrylamide Gel
207 Electrophoresis), SDS-PAGE (Sodium DodecylSulphate PolyAcrylamide Gel
208 Electrophoresis) and IEF (Isoelectric Focusing Electrophoresis), were carried out as
209 described in the Supplementary Material .

210 The sample particle size distributions were analyzed by a MASTERSIZER 3000
211 size analyzer (Malvern Panalytical, UK), the particle refractive index (IR) was set at
212 1.43, and the particle absorption index (IA) was set at 0.01 for lysates and 0.001 for
213 supernatants.

2.7. Variation of the composition (lipids, proteins and carbohydrates content) in the biomass (0-RB):

*Table 2. Composition of the two raw biomasses compared to literature**

Biomass	0-RB-A	0-RB-B	Deng et al., 2019	Sharma et al., 2019	Kandilian et al., 2019		Huo et al., 2012
Conditions	Starvation	Starvation	Autotrophic	Autotrophic	No starvation	Starvation	Starvation
Strain	*PK*	*PK*	*CK*	*PK*	*PK*	*PK*	*CZ*
Dry matter g/L	*5.27	*5.33	0.5	1-1.5	2.1	0.98	0.7
Total lipid %DW	20.9	14.6	25.1	20.14	11	27	31
Protein %DW	21.2	19.1	51.6	41.29	58	9	-
Carbohydrate %DW	39.1	43.3	16.3	34.15	25	46	10.3

**(0-RB-A: raw biomass lot A, 0-RB-B: raw biomass lot B, PK: Parachlorella kessleri, CK. Chlorella kessleri, CZ: Chlorella zofingiensis. (*dry matter: *medium + biomass)*

In our study, two 100 L culture batches A and B were produced. The culture of *PK* in starving conditions at large scale to produce lipids is still difficult to master and the two batches, cultivated in similar conditions, however presented different results. The starving was more efficient for 0-RB-A than for 0-RB-B. More lipids were produced even if in both cases, a large quantity of sugars was present (mostly starch recovered in the pellet after bead-milling and centrifugation). The biomasses compositions are compared to literature in Table 2. It must be mentioned that the %DW of the carbohydrates, lipids and proteins could vary depending on the concentration of salts, which depends itself on the culture medium and the rinsing of the biomass before characterization. Anyway, the comparison with the composition of *PK* cultured in autotrophic conditions in the literature shows that the starving conditions in this study

231 led to the accumulation of starch but did not reach the optimized conditions to have a
232 high content of lipids.

233 The physiological state of the harvested biomass had an impact on the cell
234 disruption and the liberation of the target compounds. This point will be detailed in
235 section 3.1 “Impact of the bead-milling residence time”.

236 Besides, the impact of the storage and the thawing step on the thawed biomass
237 (1-TB) composition was also studied and the results are shown in the Supplementary
238 Material.

239 **2.8. Statistical analysis**

240 Due to the limitation of the quantity of microalgae raw material, the experiments
241 in this study were carried out in duplicate with different batches of harvested biomass.
242 All the values resulting from the samples analysis (dry weight, ash, protein, lipids and
243 carbohydrates analysis) were calculated by the means of triplicate measurements. The
244 standard errors (SE) presented in the results were calculated as the standard deviations
245 of the triplicate divided by $\sqrt{3}$. The combined standard error of any value y was
246 calculated by Equation 1a for additions or 1b for multiplications or divisions of two
247 variables X_1 and X_2:

248 $$\text{If } y = X_1 + X_2,\ SE_y = \sqrt{SE_{X1}^2 + SE_{X2}^2}$$

249 *Equation 1.a*

250 $$\text{If } y = X_1^{\pm 1} \times X_2^{\pm 1},\ SE_y = y \cdot \sqrt{\left[\frac{SE_{X1}}{X1}\right]^2 + \left[\frac{SE_{X2}}{X2}\right]^2}$$ *Equation 1.b*

3. Results and discussion

3.1 Impact of the bead-milling residence time on the compounds recovery into the supernatant and on the filtration performances

In this paragraph, the impact of the residence time (lined to the number of passes) into the bead milling on the cell disruption and the compounds recovery into the centrifugation supernatant 3-Sup is presented. Considering the same ground biomass concentration, the centrifugation conditions had low impact on the following results (see 2.4).

The rate of cell disruption depended on the raw biomass. The number of passages of biomass from 1 to 4 passes represents different residence times of 1.9; 3.7; 5.6; 7.4 minutes. After 3 passes (5.6 min), 65% of the cells were disrupted in the ground biomass A (2-Lys-A) whereas only 2 passes (3.7 min) were needed to disrupt 69% of the biomass B cells in 2-Lys-B. Comparing to the first batch of biomass (A), the second batch (B) had an insufficient deficiency level, the cells accumulated a lot of carbohydrates or starch as energy storage. The cells disruption was easier with a culture containing less lipids, which indicates that the cells under a better nitrogen deficiency were more difficult to break during bead milling process.

3.1.1 Impact on the compounds recovery in the supernatant 3-Sup

The percentage of biomass 1-TB dry weight recovered in the supernatant 3-Sup increased with the number of passes, from $35\%_{DW}$ to $44\%_{DW}$ for biomass A, and from $35\%_{DW}$ to $39\%_{DW}$ for biomass B. It is already known that increasing the bead milling process intensity has a significant impact on the downstream filtration process [40].

Thus, in order to evaluate the effect of the number of passes on the quality of the supernatant, the particles volume size distribution was characterized and the different compounds released during the bead milling process were quantified. The results are shown in Figure 1 and 2.

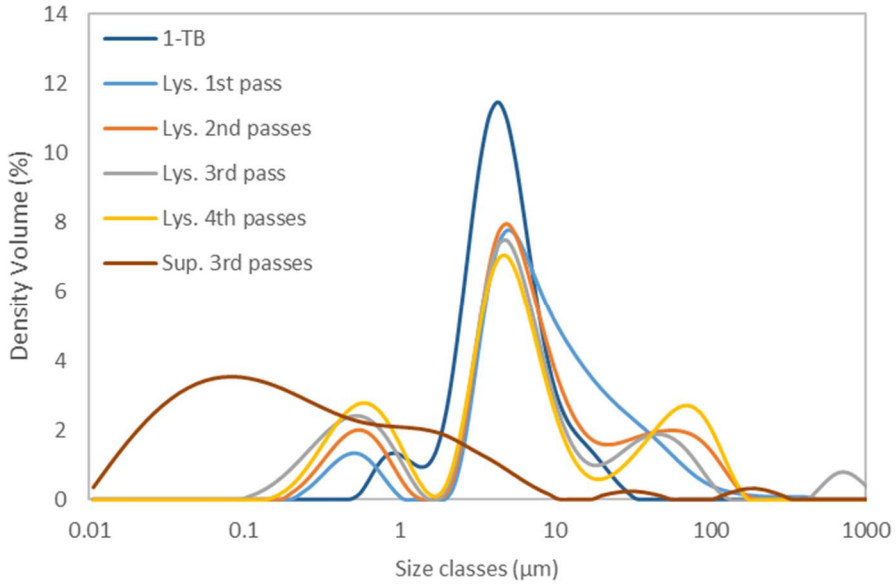
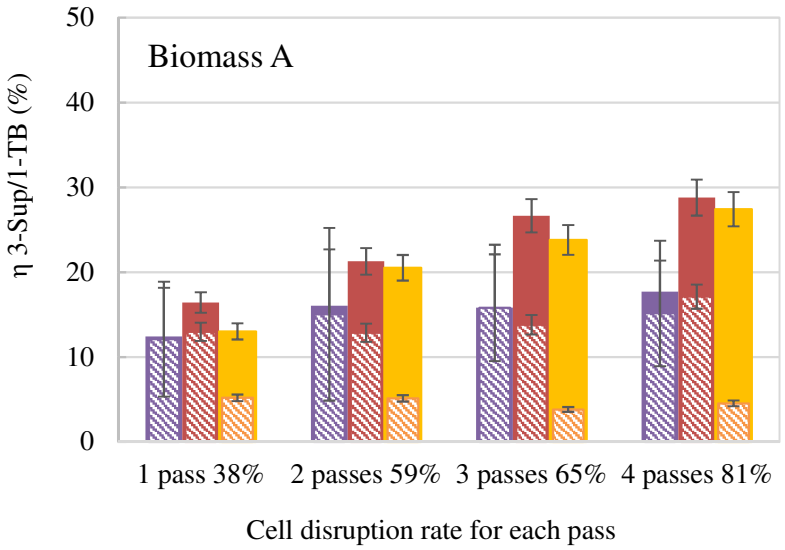


Figure1. Volume size distribution of thawed biomass (1-TB), lysate (Lys.) and supernatant (Sup.) before and after bead milling and clarification (biomass B)

We observed that when the number of grinding passes increases, the proportion of large particles (between 50 to 200 µm) with a diameter larger than the diameter of the intact cells also rises. Our hypothesis is that parts of cell fragments gathered together and formed large aggregates; At the same time, more and more fine particles were generated, which were difficult to eliminate by the centrifugation and were recovered in the supernatant (Figure 1).

The quantification of the released compounds during bead milling process showed that, in the case of biomass A, either total sugars, total proteins or total lipids

289 kept on increasing in 3-Sup when grinding number increased; and for biomass B, the
290 maximum of total lipids and soluble proteins into the 3-Sup was reached after 3 passes
291 in the bead milling i.e. a residence time of 5.6 min (Figure 2. A and B).



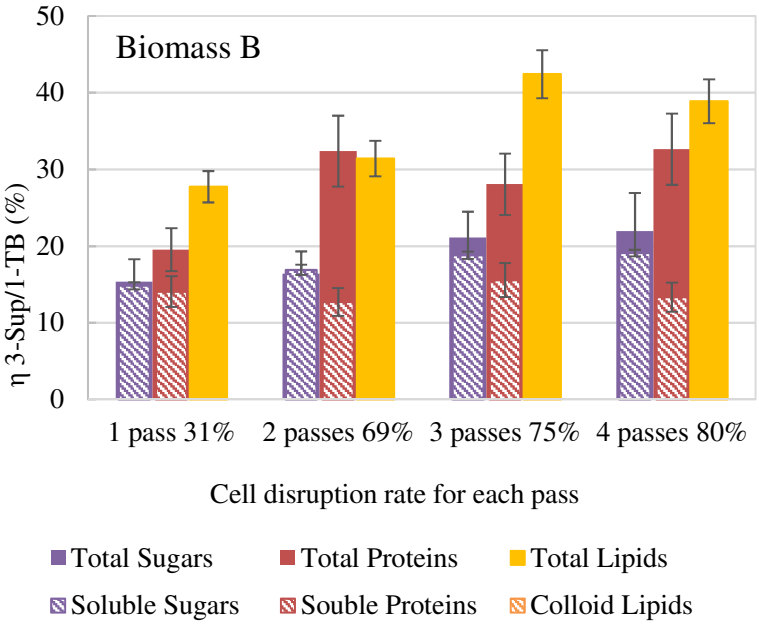


Figure 2. Recovery rate of lipids, proteins and sugars into the supernatant versus the number of passes in the bead milling for the two biomasses A and B. *3-sup: supernatant after bead milling and centrifugation. Triplicate measurements, +/- SE.*

In both cases, the dry matter recovered in the 3-Sup reached between 2.1 and 2.2 g/L with $41\%_{DW} - 43\%_{DW}$ of ashes and $57\%_{DW} - 59\%_{DW}$ of biomolecules. Most of the compounds were released after 3 passes: 15.8-21.1% of carbohydrates (0.34-0.51 g/L), 26.7-28.1% of proteins (0.30-0.31 g/L) were recovered in the 3-Sup. The

301 percentage of proteins recovered into supernatant is comparable to those recovered by
302 Suarez Garcia et al in 2018 with *Tetraselmis suecica (22.5%)* [41] and Liu et al in 2021
303 with *Chlorella vulgaris* (24-36%) [42]. Only 23.8% of lipids were recovered in the
304 supernatant from 0-RB-A whereas 42.4% of lipids from 0-RB-B were in 3-Sup.

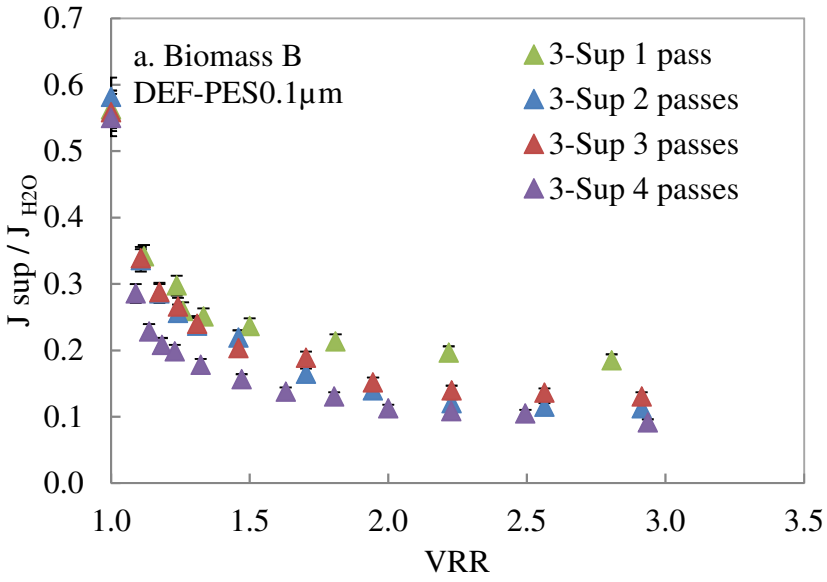
305 As described previously, the mass distribution in the supernatant increased with
306 the grinding pass number. The total carbohydrates, total proteins or total lipids all
307 increased in the supernatant with the grinding number. However, the water-soluble
308 compounds (still in the supernatant after centrifugation at 12,000 *g*) have slightly
309 increased in case of biomass A and remained constant after three passes for biomass B.
310 But for all the cases, the total quantity of biomolecules recovered in the supernatant
311 increased more markedly than the quantity of soluble molecules, which means that most
312 of the increased part was composed of finer aggregates. As a consequence, the purity of
313 the water-soluble fraction into the supernatant decreased.

314 Also, the small aggregates below 8 µm, generated in a rising quantity during the
315 bead milling and probably containing cell fragments, were more difficult to separate
316 with the 3,000 *g* centrifugation.

317 The differences of the lipid release percentage into the aqueous phase for the
318 two biomasses A and B may be due to the physiological state of the microalgae, their
319 resistance to the milling, and the lipid nature (TAG, FFA or polar lipids). Anyway, in
320 both cases, 0.27 to 0.34 g/L of lipids were recovered in the supernatant, a bit higher than
321 the quantity (0.16-0.17g/L) presented by Villafaña-López et al., 2019 and Clavijo
322 Rivera et al., 2020 [31,32], after a similar bead milling with an initial thawed biomass
323 near 5 g/L.

3.1.2 Impact on the membrane performances

The supernatants obtained after the bead-milling and centrifugation were filtered using the dead-end filtration system and the impact of the number of passes on the membrane performances was evaluated. The Figure 3 presents the permeate flux during concentration and the retention rates of the different compounds.



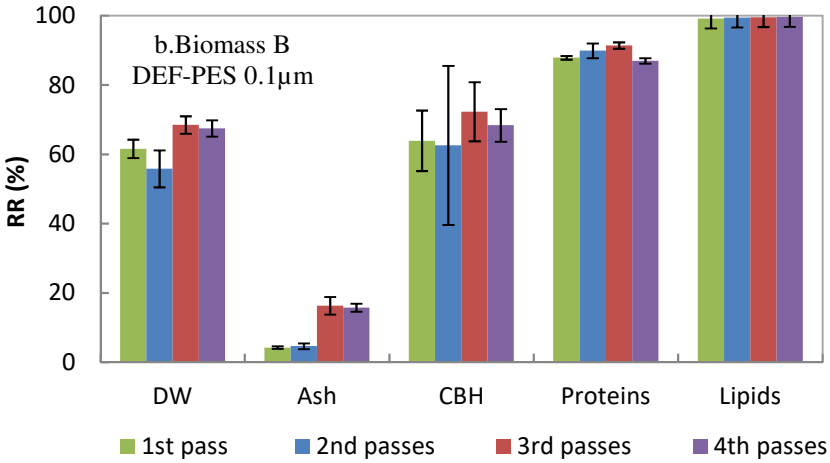


Figure 3. Impact of the biomass B bead-milling intensity on the supernatant dead-end filtration performances. *a: normalized supernatant flux with initial membrane pure water flux J_{SUP}/J_{H2O} versus volume retention ratio (VRR) during the filtration of 3-Sup-B; b: retention rates (RR) of dry weight (DW), ash, carbohydrates (CBH), proteins and lipids. (PES0.1µm, MFK-618, 0,3bar, 30°C, VRR=3), triplicate measurements, +/- SE.*

Regarding the permeation flux, the differences between the flux after 2, 3 or 4 passes into the bead-milling and centrifugation were small, because the filtration was carried out in a dead-end filtration system. This pilot was chosen because it requires less operation volume but this filtration mode attenuate the flux variations. The same behavior was observed with the filtration of 3-sup-A with a more hydrophilic PAN 500 kDa membrane (see Supplementary Material).

However, in both cases, the higher the number of passes in the bead milling before centrifugation, the lower the flux. More and more colloidal cell fragments may disperse into the aqueous phase and generate membrane fouling. The flux decline and thus the membrane fouling also depends on the membrane and the biomass: at VRR=3, the normalized supernatant flux J_{sup}/J_{H2O} reached 0.3 with 3-Sup-A and 0.1 with 3-Sup-B despite a similar cell disruption rate (80%).

Regarding the membrane selectivity, the ash, which mainly represents the salts contained in the aqueous phase, had a low retention rate with both membranes. But it reached 19% after 4 passes (7.4 min residence time) of the biomass in the bead-milling,

showing a strong membrane fouling limiting the salt permeation. The retention rates of dry matter and carbohydrates (CBH) also tended to rise with the number of passes.

The lipids were fully retained by the membrane whatever the number of passes. A fraction of CBH (around 28–37%) could permeate through the membrane whereas maximum 13% of proteins was quantified in the permeate at VRR3 after 4 passes (7.4 min residence time) of the biomass in the bead-milling.

In conclusion, the choice of the number of passes in the bead-milling should result from a compromise between the compound's release and the membrane filtration performances (flux and retention rate). Here, the choice made in this step was the biomass bead milling for 3 passes (residence time equal to 5.6 min) then the centrifugation and filtration of the supernatant.

3.1.3. Nature of the released compounds

1) Proteins

The proteins from different fractions were characterized using different electrophoresis methods. The results of native electrophoresis showed that the major proteins identified in the supernatant were around 480 kDa (see Supplementary Material) and remained totally in the retentate (PES0.1µm) after filtration at VRR3. The same sample was also analyzed with SDS-PAGE electrophoresis and two main bands with molar weights near 25kDa and 60kDa were found.

The IEF showed two groups of proteins: one group with an isoelectric point (IEP) between 6.9 and 7.4 which was totally retained by the PES membrane 0.1µm

during filtration of the supernatant; the other one with an IEP between 5.2 and 6 with a partial permeation through the PES membrane.

According to Teuling et al., 2017 [43], in microalgae or other photosynthesis plants, Rubisco is a well-known photosynthesis proteins containing 8 large and 8 small subunits. In microalgae, the molar weights of large subunits seem constant around 50 kDa and the small subunits are more variable in size and structure between 10 and 20 kDa. It was also demonstrated that some protein complexes, active in photosynthetic organisms, have a major molar weight of 22-27 kDa [43].

Ursu et al., 2014 [44] also detected two groups of proteins with different IEP in *Chlorella vulgaris*, with IEPs in the range of 4.0–5.5 for the main group and of 6.0–8.0 for the minor group.

Thus, the detected major protein released in the supernatant is probably Rubisco. The size and IEP of proteins can slightly change from one strain to another. Fragments of Rubisco but also other proteins and enzymes may also be released [41,45].

According to the size of the larger protein measured by electrophoresis (480 kDa) and the PES membrane cut-off (0.1µm), a permeation of the large protein could be expected. But it was not the case. This was probably due to the membrane fouling by lipids, colloids but also by proteins or modified proteins due to oxidation. Indeed, some large proteins did not migrate during the electrophoresis, showing that large compounds could not be characterized. The retained proteins have an IEP near from pH 7, thus at this pH, adsorption may occur on PES membrane. Proteins can also have interactions with lipids or carbohydrates [46] that lead to structures with larger molar weights.

The hydrodynamics conditions in the Amicon cell could also induce a high fouling resistance that limits membrane selectivity. Thus, the cross-flow and dynamic filtrations were tested (see section 3.3).

2) Lipids

The lipids contained in the raw biomass 0-RB and the supernatants 3-Sup were characterized by HPTLC. The polar lipids could not be quantified because of their low proportions. Most lipids were TAG and FFA. The biomass A was richer in lipids (1.1 g/L) than the biomass B (0.78 g/L, see Table 2), but because of the lipid's degradation during thawing, most of them were FFA. The ratio TAG/(TAG+FFA) was equal to 3.7% in the 1-TB-A and 17.5% in the 1-TB-B (see Supplementary Material).

After grinding and centrifugation, the total lipids in the supernatant dry matter decreased compared to initial biomass 1-TB. But the TAG proportions in 3-Sup were higher than in 1-TB: after first pass, TAG/(TAG+FFA) = 5.2% in the 3-Sup-A and 45% in the 3-Sup-B. TAG liberation in the supernatant seemed easier than that of other lipids. The proportion of TAG diminished when the number of passes in the bead-milling increased, TAG/(TAG+FFA) = 4.6% in the 3-Sup-A and 38.4% in the 3-Sup-B after 3 passes (see Supplementary Material). Two possibilities can be considered: the enzymes may be released by the grinding process and lead to the degradation of TAG; or the increasing degree of grinding released more other lipids into the supernatant such as the polar lipids from the cell membrane. Thus, in the purpose of TAG recovery for energy conversion applications, it is important to adjust the grinding intensity to keep a high proportion of TAG released and limit the polar lipid recovery.

3.2. Impact of the chemical conditions

During the bead milling, the proteins are released from cells and have a contact with oxygen. According to Stadtman et Levine 2003 [47,48], the polypeptide chain may dissociate and generate cross-linked protein aggregates. B-mercaptoethanol, sodium diethyl dithioncarbamate, DTT and sodium metabisulfite (SMBS) can maintain a strong reducing environment to counteract oxidase activities [49,50]. To facilitate the separation of lipids and proteins, physicochemical modifications were tested: the experiments performed at pH 7, presented before, were compared to experiments with the addition of SMBS at pH 7 before bead-milling, in order to limit the protein oxidation, thus a strong fouling of the membrane. According to previous study [44,51], basic pH could also enhance the protein solubilization. pH 9 was also tested to recover more proteins in the supernatant 3-Sup and hopefully in the permeate 4-Per.

3.2.1 Impact of the chemical conditions on the composition of 3-Sup

The concentration of lipids, proteins and carbohydrates in 3-Sup, after thawing of biomass B, modification of the physicochemical conditions, bead-milling with two passes and centrifugation are presented in Table 3.

433 ***Table 3. Composition of the supernatant after thawing of biomass B, modification of the***
434 ***physicochemical conditions, bead-milling (two passes) and centrifugation*.***

g/L	1-TB	3-Sup, pH 7	3-Sup, pH7, SMBS	3-Sup, pH9, SMBS
*Dry matter	5.33±0.23	2.31±0.10	5.94±0.07	6.95±0.14
Ash	1.02±0.07	0.95±0.05	5.00±0.15	5.77±0.14
Total lipids	0.78±0.02	0.26±0.01	0.18±0.01	0.28±0.02
Total proteins	1.02±0.14	0.34±0.05	0.15±0.01	0.31±0.01
Proteins in 12,000 *g*	-	0.13±0.02	0.08±0.01	0.31±0.06
Total carbohydrates	2.31±0.15	0.39±0.04	0.30±0.01	0.30±0.01
Carbohydrates in 12,000 *g*	-	0.39±0.04	0.30±0.01	0.30±0.01

435 ** 1-TB: thawed biomass, 3-Sup: supernatant, SMBS: sodium metabisulphite (dry matter: medium +*
436 *biomass). Triplicate measurements, +/- SE*

437

438 Regarding experiments at pH 7 and pH 7 with SMBS, it can be seen that only
439 half proteins were extracted from biomass into the supernatant (3,000 *g*) with
440 antioxidants. It means that metabisulfite had an opposite effect to the initial hypothesis.

441 At pH9, even in presence of SMBS, the same amount of total proteins as at pH 7
442 was extracted, but most of the proteins extracted at 3,000 *g* at pH 9 were still in
443 suspension at 12,000 *g*. If the proteins are more solubilized, it could be easier to recover
444 them into the filtration permeate. More lipids were also recovered into the supernatant
445 at pH9, the saponification of some FFA may happen.

446 The carbohydrates were less extracted in presence of SMBS but they were all
447 soluble whatever the pH.

448 **3.2.2 Impact of the chemical conditions on the filtration performances**

449 Regarding the permeation flux (Table 4), it appears that the best fluxes were
450 reached at pH7 (J_{Sup}/J_{H2O} = 0.6), whatever the presence of SMBS.

Table 4. J_{Sup}/J_{H2O} ratio and retention rates of dry weight, lipids, proteins and carbohydrates during dead-end filtration of the supernatants*.

	3-Sup pH 7	3-Sup pH7 SMBS	3-Sup pH9 SMBS
J_{Sup}/J_{H2O} (VRR= 1→3)	0.60 → 0.14	0.64 → 0.18	0.41 → 0.09
RR_{DW}	56±2	21±1	33±1
RR_{Ash}	5±1	6±1	4±1
RR_{Lipids}	99±3	94±3	98±4
$RR_{Proteins}$	90±2	79±4	91± 3
RR_{CBH}	63±3	63±2	70±2

**3-Sup using PES 0.1µm membrane (0,3bar, 30°C, VRR=3). 3-Sup is the supernatant coming from thawed biomass B, after physicochemical modifications, 2 passes in the bead-milling and centrifugation. SMBS: sodium metabisulphite, J_{Sup}/J_{H2O}: normalized supernatant flux with initial membrane pure water flux, VRR: volume reduction ratio, RRx: retention rate of the compounds x, DW: dry weight, CBH: carbohydrates. Triplicate measurements, +/- SE.*

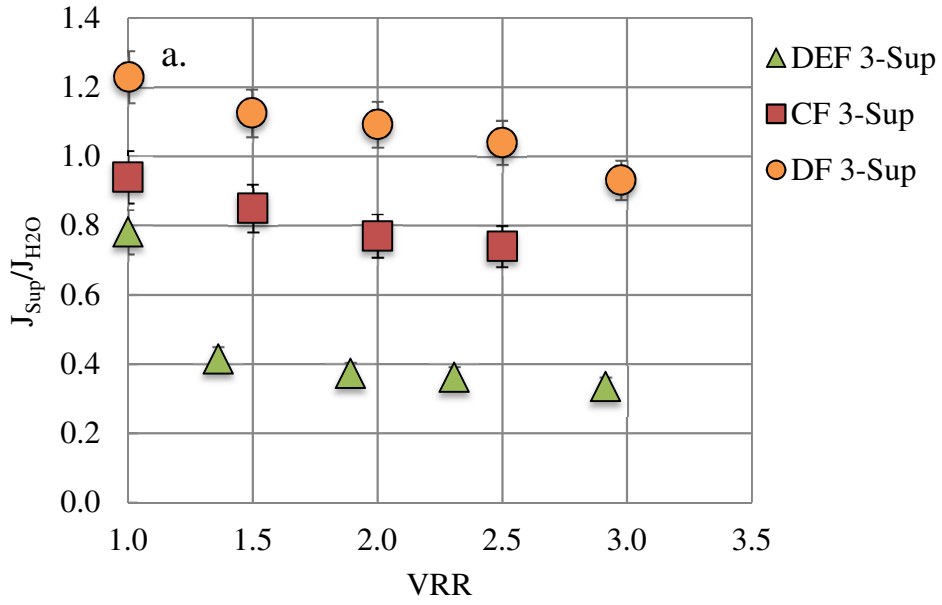
Regarding the retention rates: despite a higher apparent solubility of proteins and lipids in the 3-Sup at pH 9 centrifuged at 12,000 *g*, the retention rates of both compounds are like their RR at pH 7. In the case of pH7 with SMBS, the concentration of proteins in the supernatant was lower and those proteins were less retained by the membrane. It could be interesting, but the concentration of proteins in the permeate was 0.03 g/L whereas it was 0.06 g/L in 4-Per at pH 7. As described by Tamayo Tenorio et al., 2018 [45], under alkaline conditions, in our case at pH 9, protein solubility in supernatant after clarification was maximum. The lipids may be partly saponified and a part of sugars like cellulose can be hydrolyzed. But these compounds may reorganize and generate a new membrane fouling, which may be the reason for the important flux reduction for 3-Sup pH9 SMBS.

The modification of the chemical conditions was not efficient enough to enhance the separation of lipids and hydrosoluble compounds. Thus the modification of the hydrodynamic conditions during the membrane filtration was tested: the performances of the dead-end filtration (DEF), cross-flow filtration (CF) and dynamic filtration (DF)

were compared using a single supernatant produced from biomass B: 3-Sup-B2. The conditions to produce the supernatant were chosen based on the former results as described in 3.3.2.

3.3. Impact of the membrane filtration conditions on the fractionation

In order to study the impact of the hydrodynamic conditions on the filtration performances, the same supernatant 3-Sup-B2 was produced by bead-milling with 3 passes (5.6 min) at pH 7 and centrifugation. It was filtrated using three types of filtration: DEF, CF and DF. The comparison of normalized permeate fluxes (J_{sup}/J_{H2O}) and retention rates is presented in Figure 4.



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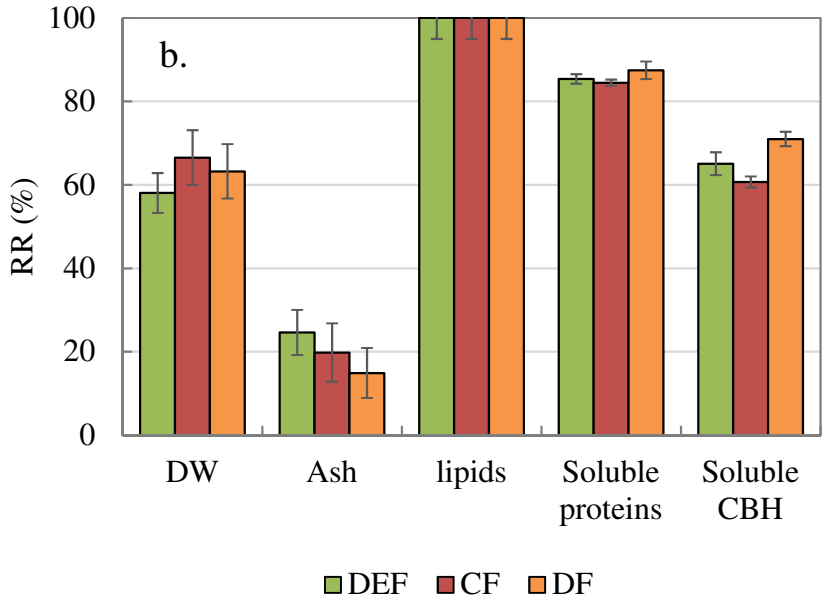

483

484 ***Figure 4. Comparison of three hydrodynamic filtration conditions on membrane performance.***

485 *a: normalized permeate flux for dead-end (DEF), cross-flow (CF, γ max=16,000 s-1) and dynamic*
486 *filtration (DF, γ max=16,000 s-1) b: retention rates of dry weight (DW), ash, lipids, soluble proteins and*
487 *soluble carbohydrates (CBH) measured at volume redaction ratio VRR=3 during filtration of a*
488 *supernatant after biomass B thawing, bead-milling (3 passes) and centrifugation, triplicate*
489 *measurements, +/- SE.*

490 The modification of the hydrodynamics during filtration had a significant impact

491 on the flux compared to water flux [37]. During agitated dead-end filtration, the shear

492 rate near to the membrane was low and a strong fouling occurred, which was probably

493 due to the formation of a cake-like fouling. J_{DEF} varied from 39 $Lh^{-1}m^{-2}$ to 17 $Lh^{-1}m^{-2}$.
494 The normalized permeate flux J_{Sup}/J_{H2O} started at 0.8 to decrease to 0.3 at VRR3. The
495 cross-flow filtration was performed with an apparent cross-flow velocity of 1 ms^{-1} with
496 a laminar flow and a maximum shear rate of 16,000 s^{-1}. The dynamic filtration was
497 performed with a similar maximum shear rate (16,000 s^{-1}) but in turbulent flow, with a
498 maximum velocity of 2.5 ms^{-1}.

499 The Figure 4.a shows that the higher the shear rate and turbulent flow, the higher
500 the ratio J_{Sup}/J_{H2O}. J_{DF} varied from 44 $Lh^{-1}m^{-2}$ to 43 $Lh^{-1}m^{-2}$ and J_{CF} from 38 $Lh^{-1}m^{-2}$ to
501 24 $Lh^{-1}m^{-2}$. These results were expected because the shear rate limits the fouling. They
502 are also consistent with the ratio J_{Sup}/J_{H2O} between 2 and 3 given by Villafaña-López et
503 al., 2019 [32], for supernatants using a dynamic filtration with a PAN membrane and a
504 maximum shear rate of 66,000 s^{-1}.

505 Despite the variation of filtration hydrodynamic conditions, the proteins and
506 carbohydrates retention rates were not significantly different in the three conditions:
507 RR_P = 85.4, 84.5 and 87.4% and RR_{CBH}= 65.1, 60.7 and 71.0% respectively for a DEF,
508 CF and DF (Figure 4.b). The retention of the salts reduced with an increase of the shear
509 rate, showing a lower fouling. Thus the high shear rate induced with dynamic filtration
510 favored an increase in flux and therefore in productivity, but did not enhance the
511 membrane selectivity.

512 Similar retention rates were obtained in literature for microalgae intracellular
513 compounds fractionation in three similar publications setting up with the same biomass
514 treatment: high-pressure homogenizer (HPH) for cell disruption, clarification to remove
515 cell fragments then filtration for protein fractionation. Ursu et al., 2014 [44] obtained

87%-95% protein retention rates with *C. vulgaris* with a PES 300 kDa membrane, Safi et al., 2017 [18] yielded 17% proteins in permeate with a MWCO 1000-300 kDa membranes, whereas Kulkarni and Nikolov, 2018 [19] also obtained 78–80% protein retention rates with a PES 300 kDa membrane. Liu et al, 2021 [42] btained 85-90% protein retention rates from *C. vulgaris* coupling bead milling, centrifugation and membrane filtration with the same PES 0.1 µm membrane in DEF mode.

With a limited membrane fouling as demonstrated above, the compounds smaller than the pores should have enhanced their permeation through the membrane. This work demonstrated that a large fraction of proteins and carbohydrates that were released in the aqueous phase during the cell disruption, belonged to structures more complex and larger than expected, existing inside the cells or coming from a strong reorganization of the biomolecules.

The characterization of those structures will be one of the key issues in the objective of an efficient fractionation of proteins, carbohydrates and lipids from *P.kessleri* grown in starving conditions.

3.4. Recovery rates of the target compounds in the selected conditions

After the analysis of the above results, the best scenario recommended in our study to recover a maximum of apolar lipids in the retentate and proteins and carbohydrates in the permeate was the biomass bead milling at 3 passes (5.6 min residence time), the centrifugation at 3,000 *g* and 20 min duration, and the filtration with PES 0.1 µm at 0.3 bar with DF at 30 °C.

The process efficiency of this scenario was evaluated by calculating the mass balance obtained at each step. The overall evaluation of the coupling of bead milling and membrane filtration is provided in Table 5.

Table 5. Mass balance of the whole process: bead milling of thawed biomass B at 3 passes, centrifugation and filtration with polyethersulfone 0.1µm membrane until volume reduction ratio of 3 using dynamic filtration*.

Process	V (L)	DW		Ash		Protein		carbohydrates		Lipids	
		η (%)	M (g)	η (%)	M (g)	η (%)	M (g)	η (%)	M (g)	η (%)	M (g)
1-TB	1.0	100	5.03	100	0.94	100	1.13	100	2.83	100	0.14
SE 1-TB		7		22		7		2		3	
3-Sup	0.97	48	2.42	94	0.88	47	0.54	25	0.70	23	0.01
SE 3-Sup		6		35		1		1		0.5	
4-Ret	0.32	**25**	1.24	25	0.23	33	0.37	14	0.40	**23**	0.01
SE 4-Ret		2		8		2		2		0.4	
4-Per	0.65	**18**	0.91	**43**	0.40	**8**	0.09	**9**	0.24	0	0.00
SE 4-Per		2		11		0.4		0.4		0	

**V: volume, DW: dry weight, η: recovery rate related to initial mass of each compound in biomass; M: mass of each compound, it was calculated from the product of measured total compounds (ash, proteins, carbohydrates, lipids) concentrations and volume of each fraction, 1-TB: thawed biomass B, 3-Sup: supernatant, 4-Ret: retentate, 4-Pe: permeate, Triplicate measurements, +/- SE.*

For filtration until VRR 3, 25%$_{DW}$ was recovered in the retentate and 18%$_{DW}$ in the permeate. 48%$_{DW}$ was initially recovered in the supernatant, thus the part of the lost mass (5%) should be accumulated in the filtration device or blocked on the membrane.

Moreover, 23% of the total lipids were recovered in the retentate, and about 43% of the ash, 8% of the proteins and 9% of the sugars were recovered in the permeate. It is important to point out that the protein and sugar yields were related to the total proteins and sugars present in the biomass. Most of them were not soluble, whereas all of them in the permeate were dissolved in water. It will be possible to increase the soluble proteins and sugars recovery by increasing VRR. But for lipids yield in retentate, the

key point is to increase the lipids release by a more efficient cell disruption process combined with a suitable clarification process.

Lorente et al., 2017 [17] filtrated the lysate from a steam exploded *Nannochloropsis gaditana* through a dynamic filtration with a membrane in Polyvinylidene fluoride (PVDF) with a MWCO 100 kDa. They obtained 7% lipids compared to 23% in retentate in this work. They also confirmed that dynamic filtration significantly improved the filtration performances in terms of fouling reduction, compared to cross-flow filtration. The same protein recovery rate (8%) was obtained by Liu et al 2021 in their best conditions [42].

Finally, the fractionation of lipids and salts was achieved, and comparing the retention rate of lipids, soluble proteins and sugars, the fractionation of lipids and hydrosoluble compounds was also partially achieved. A higher recovery of hydrosoluble compounds could be enhanced by increasing the volume reduction rate, or maybe by diafiltration. However, the composition of the complex aqueous extracts and the interactions between the compounds and the membrane strongly impact the filtration performances. The main bottleneck to recover separated fractions of lipids in the retentate and proteins and polysaccharides in the permeate is their aggregation after bead-milling. The classical modification of the physicochemical conditions was not sufficient to destabilize them. A fine characterization of these aggregates would be helpful to develop new strategies to overcome this limitation to the microalgae valorization. This question should be the priority of the future works in this field. Innovative solvent extraction has been considered (aqueous two phase extraction-ATPS, ionic liquids for example), and their coupling to membrane filtration could be interesting.

4. Conclusion

This study shows the feasibility to fractionate the hydrophobic lipids and water-soluble proteins, carbohydrates and salts from a starving biomass *Parachlorella kessleri* by microfiltration. The best scenario, bead milling at pH 7 5.6 min residence time, centrifugation at 3,000 *g* 20 min, dynamic filtration with PES 0.1 µm at 0.3 bar at VRR3, allowed the recovery of 23% of total lipids in the retentate and 18% of dry weight of initial biomass in the permeate, corresponding to a permeate containing 63%DW of ash, 27%DW of soluble sugars and 10%DW of soluble proteins (with total retention of lipids, 61-71% RR of sugars and 85-88% RR of proteins). The purity of water-soluble compounds could easily be increased by diafiltration. However, as noticed in this study, it is necessary to pay attention to the storage and thawing of biomass to ensure the integrity of lipids for biofuel applications. The complexity and variability of the mixture are the major bottleneck for the global process optimization, a new strategy should be developed to overcome this. In the future, the understanding of the interactions and reorganization of molecules after their release in the complex mixture would be necessary to optimize the solubilization of the water-soluble compounds and improve the compounds' fractionation.

Acknowledgements

The authors would like to thank Marie Cueff and Laura Herve (Algosolis platform) for their assistance for the biomass cultivation, Delphine Drouin (CNRS) and Guillaume Roelens (GEPEA) for their assistance for lipids analysis and bead milling experience. This work was supported by the French Environment and Energy

605 Management Agency (ADEME), the French region of Pay de la Loire, the Challenge
606 Food For Tomorrow/Cap Aliment, Pays de la Loire, France (project 3MFOODGY) and
607 the Process Engineering for Environment and Food Laboratory (GEPEA), University of
608 Nantes (France).

609

No conflicts, informed consent, or human or animal rights are applicable to this study.

Authorship contributions : E. Couallier, M. Frappart and S. Liu were responsible for the conception and design of the study. The data were acquired by S. Liu, C. Rouquié and L. Lavenant. The data were analyzed and interpreted and the manuscript written by S. Liu, E. Couallier and M. Frappart. All the authors approved the final manuscript.

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Coupling bead-milling and microfiltration for the recovery of lipids and proteins from *Parachlorella kessleri*: impact of the cell disruption conditions on the separation performances

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Graphical abstract