

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

Shuli Liu, Camille Rouquié, Laurence Lavenant, Matthieu Frappart, Estelle

Couallier

▶ To cite this version:

Shuli Liu, Camille Rouquié, Laurence Lavenant, Matthieu Frappart, Estelle Couallier. Coupling beadmilling and microfiltration for the recovery of lipids and proteins from Parachlorella kessleri: Impact of the cell disruption conditions on the separation performances. Separation and Purification Technology, 2022, 287, pp.120570. 10.1016/j.seppur.2022.120570 . hal-03561759

HAL Id: hal-03561759 https://hal.inrae.fr/hal-03561759

Submitted on 28 Sep 2023 $\,$

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers. L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 Coupling bead-milling and microfiltration for the recovery of lipids and proteins

2 from *Parachlorella kessleri*: impact of the cell disruption conditions on the

3 separation performances

- 4 Shuli Liu^{a,b}, Camille Rouquié^{a,c}, Laurence Lavenant^c, Matthieu Frappart^a, Estelle
 5 Couallier^{a,*}
- ^a CNRS, Université de Nantes, ONIRIS, Laboratoire de Génie des Procédés,
 Environnement et Agroalimentaire, GEPEA, F-44600 Saint Nazaire, France

^b Agence de l'environnement et de la Maîtrise de l'Energie 20, avenue du Grésillé-BP
90406, 49004 Angers Cedex 01 France

^c INRAE, BIA, Rue de la Géraudière, BP 71627, 44 316 Nantes Cedex 3, France

11 *Corresponding author at : GEPEA - UMR CNRS 6144 CRTT - 37 boulevard de

12 l'Université - 44602 Saint-Nazaire, France. E-mail address: estelle.couallier@univ-

13 nantes.fr (E. Couallier).

14 Abstract

15 Microalgae biorefinery has become an attracting topic to fully exploit the 16 intracellular compounds such as lipids, proteins, polysaccharides or pigments, etc. Conventionally, microalgae biorefinery involves four major operations; harvesting, cell 17 18 disruption, fractionation and purification, and final product formulation. This study 19 evaluates the possibility to recover lipids and hydrosoluble compounds from 20 Parachlorella kessleri cultivated in starving conditions. The cell disruption by bead-21 milling was coupled with centrifugation and membrane filtration for the fractionation. 22 The impact of the biomass variation and the bead-milling, centrifugation and

¹

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.

28 Keywords

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

30 **1. Introduction**

31 Microalgae have shown high potential as an alternative to non-renewable 32 resources, in various sectors such as food, cosmetics, medicine or energy [1]. Intensified 33 studies were carried out over the past decade on microalgae physiological conditions 34 understanding and cultivation optimization [2–7]. Microalgae biorefinery has recently 35 become an increasing research topic to fully exploit their potential. Conventionally 36 microalgae biorefinery processes proposed in literature include the following unit 37 operations: harvesting and biomass dewatering or biomass drying (centrifugation or 38 membrane filtration, oven drying, etc.), cell disruption (by mechanic or enzymatic 39 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.

45 In this study, to avoid energetic biomass-drying before the fractionation, a wet 46 pathway was applied as described by Clavijo Rivera et al., 2018 [13] to recover lipids 47 and proteins from Parachlorella kessleri (PK) cultivated in starving conditions. The 48 process involved the bead milling of a diluted biomass for a better compound release, 49 the clarification by centrifugation to remove the cell fragments, the membrane filtration 50 to fractionate the hydrophobic (lipids and a part of pigments) and hydrophilic 51 compounds (salts, soluble proteins and carbohydrates). The combination of bead milling 52 and membrane filtration facilitates the industrial scale up, as they can treat large 53 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].

60 *PK* cultured in starving conditions shows high potential for triglycerides (TAG) 61 accumulation (up to 40-50% dry matter) [20]. Recently, a considerable number of 62 studies focused on the optimization of culture conditions to increase lipids accumulation 63 in microalgae cells. However, the transposition of those optimal conditions to large-64 scale lipids rich biomass production remains difficult, since the system regulation and 65 the risk of contamination are more critical for a larger volume cultivation. The nitrogen 66 deficiency level and the light stress, which determines the harvested cell lipid content, 67 also varies according to the photobioreactor (PBR) geometry and the volume of the 68 culture [3,6,7,20-29].

The compromise between biomass productivity and lipid accumulation under depleted conditions is a challenge for biorefinery as this second part requires much biomass for process optimization. As a result, the harvested biomass contains fewer target lipids but more carbohydrates [3,7,26]. For biorefinery, the recovery of those 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 environment and of the reorganization of the whole matter was still poorly studied in 78 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 each103 step are outlined in the Table 1.

104Table 1. Overall steps of wet way process for the fractionation of lipids, proteins and carbohydrates105from 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

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 beadmilling, 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).

114 **2.2. Microalgae cultivation, harvesting and storage**

115 In this study, two batches of PK culture 0-RB-A and 0-RB-B were used; each 116 culture was conducted in a bubble column photobioreactor (100 L, $\Phi = 30$ cm; H = 150 117 cm) under nitrogen starvation (0.23 g/L of NaNO₃) and light intensified conditions 118 (100-150 µmol/m²/s) [20,34]. The pH was maintained at 7.5 through pH-controlled by 119 the addition of carbon dioxide to the airflow, and the culture temperature was 120 maintained between 22 and 25 °C. Then the microalgae were harvested by 121 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 122 123 before the cell disruption.

124 **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 μ s/cm). Cell disruption was performed by bead milling (Dynomill multi labo, WAB, Switzerland) and operated in a pendulum mode as described by Montalescot et al., 2015 [35]. The milling chamber volume was 600 mL, glass beads 0.5-0.75 mm (average: 0.65 mm) were used and the feed flow rate was set at 150 mL/min. The filling ratio of the grinding media was 80% and the rotational speed was set at 8 m/s [36]. During cell disruption, the lysate at different residence time was 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.

Three preliminary experiments on centrifugation conditions were carried out 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 supernatants were filtrated. No significant centrifugation impact was observed for the 151 membrane filtration performances (selectivity and flux). Thus, 3,000 g at 20 ° C was 152 selected as the optimal centrifugation conditions for low-cost large-scale applications.

153

2.5. Fractionation by micro and ultrafiltration

154 A commercial flat sheet polythersulfone (PES) membrane with a MWCO of 155 0.1 µm (MFK-618, KOCH Membrane Systems, USA) was used in this study. The PES 156 material was previously selected by Clavijo Rivera et al., 2020 [31] as offering a limited 157 irreversible fouling resistance for the filtration of lipid-rich mixtures. Besides, Clavijo 158 Rivera et al., 2020 [31] demonstrated that the membrane polyacrylonitrile (PAN) 159 500 kDa (Orelis Environment, France) shows good properties for the filtration of lipid-160 rich mixtures as well. Thus this membrane was also tested and results are detailed in the 161 Supplementary Material.

162 The unused membrane was gently rinsed with 96% ethanol and water to remove 163 excessive superficial glycerol and then conditioned in the filtration pilot by the 164 following steps: basic bleach cleaning (10 ppm NaClO at pH 10.5) at 30 °C during 165 30 min, 5 min water rinsing, then 0.2% (v/v) Ultrasil 110 (Ecolab, USA) cleaning at 166 45 °C for 30 min and finally water rinsing at 30 °C during at least 30 min. For all 167 experiments of dead-end filtration, the same membrane cut was reused after membrane 168 cleaning (without bleach step) and the pure water permeability was measured before 169 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 171 ® Stirred Cell (Milipore, USA). The feed supernatant was filtrated at 30 ± 1 °C and 172 stirred at 300 tr/min, the inlet pressure was maintained at 0.3 bar by compressed air and 173 adjusted by a valve combined with a manometer on the cell inlet, the instant temperature of permeate was measured. All the supernatants were concentrated at a
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 ($\mathring{y}_{max} = 16,000 \text{ s}^{-1}$). The same membrane cleaning and filtration 181 procedures were followed for all filtration experiments (DEF, CF and DF). The effective membrane area was 42 cm², 130 cm² and 188 cm² for DEF, CF and DF 182 183 respectively.

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

189 **2.6. Biochemical sample analysis**

To determine the dry weight (DW), the samples are dried to constant mass in an oven where the temperature was controlled by thermostat with forced air ventilation and able to maintain a temperature of 105 ± 5 °C. This DW contains the organic matter that composes the biomass, and the salts from the culture medium. Then, the samples were placed in a muffle furnace at 600 °C for 6 hours for ash determination. The total fatty acids (TFA) were determined by gas chromatography-flame ionization detector (GC-FID) [13]. The triglycerides (TAG) and free fatty acids (FFA) were quantified by a high-performance thin-layer chromatography. The Pierce BCA Protein Assay Kit (Thermo Scientific) was used for the quantification of proteins, and the carbohydrate content was determined by the phenol-sulphuric acid (Dubois) method [38].

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

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

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

214 **2.7.** Variation of the composition (lipids, proteins and carbohydrates content) in

215 the biomass (0-RB):

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	СК	PK	РК	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

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

217 *(0-RB-A: raw biomass lot A, 0-RB-B: raw biomass lot B, PK: Parachlorella kessleri, CK. Chlorella

218 *kessleri, CZ: Chlorella zofingiensis. (*dry matter: *medium + biomass)*

219

216

220 In our study, two 100 L culture batches A and B were produced. The culture of 221 *PK* in starving conditions at large scale to produce lipids is still difficult to master and 222 the two batches, cultivated in similar conditions, however presented different results. 223 The starving was more efficient for 0-RB-A than for 0-RB-B. More lipids were 224 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 225 226 compositions are compared to literature in Table 2. It must be mentioned that the %DW 227 of the carbohydrates, lipids and proteins could vary depending on the concentration of 228 salts, which depends itself on the culture medium and the rinsing of the biomass before 229 characterization. Anyway, the comparison with the composition of PK cultured in 230 autotrophic conditions in the literature shows that the starving conditions in this study

led to the accumulation of starch but did not reach the optimized conditions to have ahigh content of lipids.

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

Besides, the impact of the storage and the thawing step on the thawed biomass (1-TB) composition was also studied and the results are shown in the Supplementary 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. All the values resulting from the samples analysis (dry weight, ash, protein, lipids and 242 243 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 of the triplicate divided by $\sqrt{3}$. The combined standard error of any value y was 245 246 calculated by Equation 1a for additions or 1b for multiplications or divisions of two 247 variables X₁ and X₂:

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

249

Equation 1.a

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

259 The rate of cell disruption depended on the raw biomass. The number of 260 passages of biomass from 1 to 4 passes represents different residence times of 1.9; 3.7; 261 5.6; 7.4 minutes. After 3 passes (5.6 min), 65% of the cells were disrupted in the ground 262 biomass A (2-Lys-A) whereas only 2 passes (3.7 min) were needed to disrupt 69% of 263 the biomass B cells in 2-Lys-B. Comparing to the first batch of biomass (A), the second 264 batch (B) had an insufficient deficiency level, the cells accumulated a lot of 265 carbohydrates or starch as energy storage. The cells disruption was easier with a culture 266 containing less lipids, which indicates that the cells under a better nitrogen deficiency 267 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.



277

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

280

We observed that when the number of grinding passes increases, the proportion of large particles (between 50 to 200 μ m) 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
- in the bead milling i.e. a residence time of 5.6 min (Figure 2. A and B).





Cell disruption rate for each pass





percentage of proteins recovered into supernatant is comparable to those recovered by
Suarez Garcia et al in 2018 with *Tetraselmis suecica (22.5%)* [41] and Liu et al in 2021
with *Chlorella vulgaris* (24-36%) [42]. Only 23.8% of lipids were recovered in the
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.

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

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

324 **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.







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 ofdry 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.

362 **3.1.3. Nature of the released compounds**

363 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 apartial 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].

386 According to the size of the larger protein measured by electrophoresis 387 (480 kDa) and the PES membrane cut-off (0.1µm), a permeation of the large protein 388 could be expected. But it was not the case. This was probably due to the membrane 389 fouling by lipids, colloids but also by proteins or modified proteins due to oxidation. 390 Indeed, some large proteins did not migrate during the electrophoresis, showing that 391 large compounds could not be characterized. The retained proteins have an IEP near 392 from pH 7, thus at this pH, adsorption may occur on PES membrane. Proteins can also 393 have interactions with lipids or carbohydrates [46] that lead to structures with larger 394 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).

398

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).

405 After grinding and centrifugation, the total lipids in the supernatant dry matter 406 decreased compared to initial biomass 1-TB. But the TAG proportions in 3-Sup were 407 higher than in 1-TB: after first pass, TAG/(TAG+FFA) = 5.2% in the 3-Sup-A and 45% 408 in the 3-Sup-B. TAG liberation in the supernatant seemed easier than that of other 409 lipids. The proportion of TAG diminished when the number of passes in the bead-410 milling increased, TAG/(TAG+FFA) = 4.6% in the 3-Sup-A and 38.4% in the 3-Sup-B 411 after 3 passes (see Supplementary Material). Two possibilities can be considered: the 412 enzymes may be released by the grinding process and lead to the degradation of TAG; 413 or the increasing degree of grinding released more other lipids into the supernatant such 414 as the polar lipids from the cell membrane. Thus, in the purpose of TAG recovery for 415 energy conversion applications, it is important to adjust the grinding intensity to keep a 416 high proportion of TAG released and limit the polar lipid recovery.

417 **3.2. Impact of the chemical conditions**

418 During the bead milling, the proteins are released from cells and have a contact 419 with oxygen. According to Stadtman et Levine 2003 [47,48], the polypeptide chain may 420 dissociate and generate cross-linked protein aggregates. B-mercaptoethanol, sodium 421 diethyl dithioncarbamate, DTT and sodium metabisulfite (SMBS) can maintain a strong 422 reducing environment to counteract oxidase activities [49,50]. To facilitate the 423 separation of lipids and proteins, physicochemical modifications were tested: the 424 experiments performed at pH 7, presented before, were compared to experiments with 425 the addition of SMBS at pH7 before bead-milling, in order to limit the protein 426 oxidation, thus a strong fouling of the membrane. According to previous study [44,51], 427 basic pH could also enhance the protein solubilization. pH 9 was also tested to recover 428 more proteins in the supernatant 3-Sup and hopefully in the permeate 4-Per.

429 **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.

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

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

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

437

433

434

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 all447 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.

	3-Sup	3-Sup pH7 SMBS	3-Sup
$J_{Sup}/J_{H2O} (VRR=1 \rightarrow 3)$	$0.60 \rightarrow 0.14$	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

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

453 *3-Sup using PES 0.1 μ m membrane (0,3bar, 30°C, VRR=3). 3-Sup is the supernatant coming from 454 thawed biomass B, after physicochemical modifications, 2 passes in the bead-milling and centrifugation. 455 SMBS: sodium metabisulphite, J_{Sup}/J_{H2O} : normalized supernatant flux with initial membrane pure water 456 flux, VRR: volume reduction ratio, RRx: retention rate of the compounds x, DW: dry weight, CBH: 457 carbohydrates. Triplicate measurements, +/- SE.

458 Regarding the retention rates: despite a higher apparent solubility of proteins and lipids in the 3-Sup at pH9 centrifuged at 12,000 g, the retention rates of both 459 460 compounds are like their RR at pH 7. In the case of pH7 with SMBS, the concentration 461 of proteins in the supernatant was lower and those proteins were less retained by the 462 membrane. It could be interesting, but the concentration of proteins in the permeate was 463 0.03 g/L whereas it was 0.06 g/L in 4-Per at pH 7. As described by Tamayo Tenorio et 464 al., 2018 [45], under alkaline conditions, in our case at pH 9, protein solubility in 465 supernatant after clarification was maximum. The lipids may be partly saponified and a 466 part of sugars like cellulose can be hydrolyzed. But these compounds may reorganize 467 and generate a new membrane fouling, which may be the reason for the important flux 468 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.

476 **3.3. Impact of the membrane filtration conditions on the fractionation**

477 In order to study the impact of the hydrodynamic conditions on the filtration 478 performances, the same supernatant 3-Sup-B2 was produced by bead-milling with 3 479 passes (5.6 min) at pH 7 and centrifugation. It was filtrated using three types of 480 filtration: DEF, CF and DF. The comparison of normalized permeate fluxes (J_{sup}/J_{H2O}) 481 and retention rates is presented in Figure 4.





493 due to the formation of a cake-like fouling. J_{DEF} varied from 39 Lh⁻¹m⁻² to 17 Lh⁻¹m⁻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.

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

Despite the variation of filtration hydrodynamic conditions, the proteins and carbohydrates retention rates were not significantly different in the three conditions: $RR_P = 85.4, 84.5$ and 87.4% and $RR_{CBH} = 65.1, 60.7$ and 71.0% respectively for a DEF, CF and DF (Figure 4.b). The retention of the salts reduced with an increase of the shear rate, showing a lower fouling. Thus the high shear rate induced with dynamic filtration favored an increase in flux and therefore in productivity, but did not enhance the 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 516 87%-95% protein retention rates with *C. vulgaris* with a PES 300 kDa membrane, Safi 517 et al., 2017 [18] yielded 17% proteins in permeate with a MWCO 1000-300 kDa 518 membranes, whereas Kulkarni and Nikolov, 2018 [19] also obtained 78–80% protein 519 retention rates with a PES 300 kDa membrane. Liu et al, 2021 [42] btained 85-90% 520 protein retention rates from *C. vulgaris* coupling bead milling, centrifugation and 521 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.

528 The characterization of those structures will be one of the key issues in the 529 objective of an efficient fractionation of proteins, carbohydrates and lipids from 530 *P.kessleri* grown in starving conditions.

531 **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 μ m at 0.3 bar with DF at 30 °C. 537 The process efficiency of this scenario was evaluated by calculating the mass 538 balance obtained at each step. The overall evaluation of the coupling of bead milling 539 and membrane filtration is provided in Table 5.

540

541 542

543

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*.

Drocess	V DW		W	Ash		Protein		carboh	carbohydrates		Lipids	
1100055	(L)	η	М	η	М	η	М	η	Μ	η	Μ	
		(%)	(g)	(%)	(g)	(%)	(g)	(%)	(g)	(%)	(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.

548

549 For filtration until VRR 3, 25%_{DW} was recovered in the retentate and 18%_{DW} in 550 the permeate. 48%_{DW} was initially recovered in the supernatant, thus the part of the lost 551 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 processcombined 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].

567 Finally, the fractionation of lipids and salts was achieved, and comparing the 568 retention rate of lipids, soluble proteins and sugars, the fractionation of lipids and 569 hydrosoluble compounds was also partially achieved. A higher recovery of 570 hydrosoluble compounds could be enhanced by increasing the volume reduction rate, or 571 maybe by diafiltration. However, the composition of the complex aqueous extracts and 572 the interactions between the compounds and the membrane strongly impact the filtration 573 performances. The main bottleneck to recover separated fractions of lipids in the 574 retentate and proteins and polysaccharides in the permeate is their aggregation after 575 bead-milling. The classical modification of the physicochemical conditions was not 576 sufficient to destabilize them. A fine characterization of these aggregates would be 577 helpful to develop new strategies to overcome this limitation to the microalgae 578 valorization. This question should be the priority of the future works in this field. 579 Innovative solvent extraction has been considered (aqueous two phase extraction-ATPS, 580 ionic liquids for example), and their coupling to membrane filtration could be 581 interesting.

582 **4. Conclusion**

583 This study shows the feasibility to fractionate the hydrophobic lipids and water-584 soluble proteins, carbohydrates and salts from a starving biomass Parachlorella kessleri 585 by microfiltration. The best scenario, bead milling at pH 7 5.6 min residence time, 586 centrifugation at 3,000 g 20 min, dynamic filtration with PES 0.1 µm at 0.3 bar at 587 VRR3, allowed the recovery of 23% of total lipids in the retentate and 18% of dry 588 weight of initial biomass in the permeate, corresponding to a permeate containing 589 63%DW of ash, 27%DW of soluble sugars and 10%DW of soluble proteins (with total 590 retention of lipids, 61-71% RR of sugars and 85-88% RR of proteins). The purity of 591 water-soluble compounds could easily be increased by diafiltration. However, as 592 noticed in this study, it is necessary to pay attention to the storage and thawing of 593 biomass to ensure the integrity of lipids for biofuel applications. The complexity and 594 variability of the mixture are the major bottleneck for the global process optimization, a 595 new strategy should be developed to overcome this. In the future, the understanding of 596 the interactions and reorganization of molecules after their release in the complex 597 mixture would be necessary to optimize the solubilization of the water-soluble 598 compounds and improve the compounds' fractionation.

599

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

- 610 No conflicts, informed consent, or human or animal rights are applicable to this study.
- 611
- 612 Authorship contributions : E. Couallier, M. Frappart and S. Liu were responsible for the
- 613 conception and design of the study. The data were acquired by S. Liu, C. Rouquié and L.
- 614 Lavenant. The data were analyzed and interpreted and the manuscript written by S. Liu,
- 615 E. Couallier and M. Frappart. All the authors approved the final manuscript.
- 616

617 References

618 [1] C. Dixon, L.R. Wilken, Green microalgae biomolecule separations and
619 recovery, Bioresources and Bioprocessing. 5 (2018) 14. https://doi.org/10.1186/s40643620 018-0199-3.

[2] X. Deng, B. Chen, C. Xue, D. Li, X. Hu, K. Gao, Biomass production
and biochemical profiles of a freshwater microalga Chlorella kessleri in mixotrophic
culture: Effects of light intensity and photoperiodicity, Bioresource Technology. 273
(2019) 358–367. https://doi.org/10.1016/j.biortech.2018.11.032.

625 B. Fernandes, J. Teixeira, G. Dragone, A.A. Vicente, S. Kawano, K. [3] 626 Bišová, P. Přibyl, V. Zachleder, M. Vítová, Relationship between starch and lipid 627 accumulation induced by nutrient depletion and replenishment in the microalga 628 Parachlorella kessleri. Bioresource Technology. 144 (2013)268 - 274.629 https://doi.org/10.1016/j.biortech.2013.06.096.

[4] R. Kandilian, A. Taleb, V. Heredia, G. Cogne, J. Pruvost, Effect of light
absorption rate and nitrate concentration on TAG accumulation and productivity of
Parachlorella kessleri cultures grown in chemostat mode, Algal Research. 39 (2019)
101442. https://doi.org/10.1016/j.algal.2019.101442.

634 [5] A. Melis, L. Zhang, M. Forestier, M.L. Ghirardi, M. Seibert, Sustained
635 Photobiological Hydrogen Gas Production upon Reversible Inactivation of Oxygen
636 Evolution in the Green Alga *Chlamydomonas reinhardtii*, Plant Physiology. 122 (2000)
637 127–136. https://doi.org/10.1104/pp.122.1.127.

638 [6] K.N. Sorokina, Y.V. Samoylova, V.N. Parmon, Comparative analysis of
639 microalgae metabolism on BBM and municipal wastewater during salt induced lipid

640 accumulation, Bioresource Technology Reports. 11 (2020) 100548.
641 https://doi.org/10.1016/j.biteb.2020.100548.

642 [7] T. Takeshita, S. Ota, T. Yamazaki, A. Hirata, V. Zachleder, S. Kawano,
643 Starch and lipid accumulation in eight strains of six Chlorella species under
644 comparatively high light intensity and aeration culture conditions, Bioresource
645 Technology, 158 (2014) 127–134. https://doi.org/10.1016/j.biortech.2014.01.135.

K.W. Chew, J.Y. Yap, P.L. Show, N.H. Suan, J.C. Juan, T.C. Ling, D.-J.
Lee, J.-S. Chang, Microalgae biorefinery: High value products perspectives,
Bioresource Technology. 229 (2017) 53–62.
https://doi.org/10.1016/j.biortech.2017.01.006.

[9] L.M.L. Laurens, M. Quinn, S.V. Wychen, D.W. Templeton, E.J.
Wolfrum, Accurate and reliable quantification of total microalgal fuel potential as fatty
acid methyl esters by in situ transesterification, Analytical and Bioanalytical Chemistry.
403 (2012) 167–178. https://doi.org/10.1007/s00216-012-5814-0.

[10] W.N. Phong, P.L. Show, T.C. Ling, J.C. Juan, E.-P. Ng, J.-S. Chang,
Mild cell disruption methods for bio-functional proteins recovery from microalgae—
Recent developments and future perspectives, Algal Research. 31 (2018) 506–516.
https://doi.org/10.1016/j.algal.2017.04.005.

[11] P.R. Postma, T.L. Miron, G. Olivieri, M.J. Barbosa, R.H. Wijffels,
M.H.M. Eppink, Mild disintegration of the green microalgae Chlorella vulgaris using
bead milling, Bioresource Technology. 184 (2015) 297–304.
https://doi.org/10.1016/j.biortech.2014.09.033.

662 [12] C. Safi, B. Zebib, O. Merah, P.-Y. Pontalier, C. Vaca-Garcia,

Morphology, composition, production, processing and applications of Chlorella
vulgaris: A review, Renewable and Sustainable Energy Reviews. 35 (2014) 265–278.
https://doi.org/10.1016/j.rser.2014.04.007.

E. Clavijo Rivera, V. Montalescot, M. Viau, D. Drouin, P. Bourseau, M.
Frappart, C. Monteux, E. Couallier, Mechanical cell disruption of Parachlorella kessleri
microalgae: Impact on lipid fraction composition, Bioresource Technology. 256 (2018)
77–85. https://doi.org/10.1016/j.biortech.2018.01.148.

[14] R. Castro-Muñoz, G. Boczkaj, E. Gontarek, A. Cassano, V. Fíla,
Membrane technologies assisting plant-based and agro-food by-products processing: A
comprehensive review, Trends in Food Science & Technology. 95 (2020) 219–232.
https://doi.org/10.1016/j.tifs.2019.12.003.

674 [15] O. Akoum, M.Y. Jaffrin, L.-H. Ding, Concentration of total milk proteins
675 by high shear ultrafiltration in a vibrating membrane module, Journal of Membrane
676 Science. 247 (2005) 211–220. https://doi.org/10.1016/j.memsci.2004.09.021.

677 [16] G. Singh, S.K. Patidar, Microalgae harvesting techniques: A review,
678 Journal of Environmental Management. 217 (2018) 499–508.
679 https://doi.org/10.1016/j.jenvman.2018.04.010.

[17] E. Lorente, M. Hapońska, E. Clavero, C. Torras, J. Salvadó, Microalgae
fractionation using steam explosion, dynamic and tangential cross-flow membrane
filtration, Bioresource Technology. 237 (2017) 3–10.
https://doi.org/10.1016/j.biortech.2017.03.129.

684 [18] C. Safi, G. Olivieri, R.P. Campos, N. Engelen-Smit, W.J. Mulder, L.A.M.
685 van den Broek, L. Sijtsma, Biorefinery of microalgal soluble proteins by sequential

processing and membrane filtration, Bioresource Technology. 225 (2017) 151–158.
https://doi.org/10.1016/j.biortech.2016.11.068.

[19] S. Kulkarni, Z. Nikolov, Process for selective extraction of pigments and
functional proteins from Chlorella vulgaris, Algal Research. 35 (2018) 185–193.
https://doi.org/10.1016/j.algal.2018.08.024.

[20] A. Taleb, R. Kandilian, R. Touchard, V. Montalescot, T. Rinaldi, S. Taha,
H. Takache, L. Marchal, J. Legrand, J. Pruvost, Screening of freshwater and seawater
microalgae strains in fully controlled photobioreactors for biodiesel production,
Bioresource Technology. 218 (2016) 480–490.
https://doi.org/10.1016/j.biortech.2016.06.086.

G. Benvenuti, R. Bosma, M. Cuaresma, M. Janssen, M.J. Barbosa, R.H.
Wijffels, Selecting microalgae with high lipid productivity and photosynthetic activity
under nitrogen starvation, Journal of Applied Phycology. 27 (2015) 1425–1431.
https://doi.org/10.1007/s10811-014-0470-8.

[22] X. Li, P. Přibyl, K. Bišová, S. Kawano, V. Cepák, V. Zachleder, M.
Čížková, I. Brányiková, M. Vítová, The microalga Parachlorella kessleri—A novel
highly efficient lipid producer, Biotechnology and Bioengineering. 110 (2013) 97–107.
https://doi.org/10.1002/bit.24595.

R. Coat, V. Montalescot, E.S. León, D. Kucma, C. Perrier, S. Jubeau, G.
Thouand, J. Legrand, J. Pruvost, O. Gonçalves, Unravelling the matrix effect of fresh
sampled cells for in vivo unbiased FTIR determination of the absolute concentration of
total lipid content of microalgae, Bioprocess and Biosystems Engineering. 37 (2014)
2175–2187. https://doi.org/10.1007/s00449-014-1194-5.

[24] S. Huo, Z. Wang, S. Zhu, W. Zhou, R. Dong, Z. Yuan, Cultivation of
Chlorella zofingiensis in bench-scale outdoor ponds by regulation of pH using dairy
wastewater in winter, South China, Bioresource Technology. 121 (2012) 76–82.
https://doi.org/10.1016/j.biortech.2012.07.012.

[25] G.J.O. Martin, D.R.A. Hill, I.L.D. Olmstead, A. Bergamin, M.J. Shears,
D.A. Dias, S.E. Kentish, P.J. Scales, C.Y. Botté, D.L. Callahan, Lipid Profile
Remodeling in Response to Nitrogen Deprivation in the Microalgae Chlorella sp.
(Trebouxiophyceae) and Nannochloropsis sp. (Eustigmatophyceae), PLOS ONE. 9
(2014) e103389. https://doi.org/10.1371/journal.pone.0103389.

[26] S. Zhu, W. Huang, J. Xu, Z. Wang, J. Xu, Z. Yuan, Metabolic changes of
starch and lipid triggered by nitrogen starvation in the microalga Chlorella zofingiensis,
Bioresource Technology. 152 (2014) 292–298.
https://doi.org/10.1016/j.biortech.2013.10.092.

[27] G. De Bhowmick, G. Subramanian, S. Mishra, R. Sen, Raceway pond
cultivation of a marine microalga of Indian origin for biomass and lipid production: A
case study, Algal Research. 6 (2014) 201–209.
https://doi.org/10.1016/j.algal.2014.07.005.

[28] R. Halim, B. Gladman, M.K. Danquah, P.A. Webley, Oil extraction from
microalgae for biodiesel production, Bioresource Technology. 102 (2011) 178–185.
https://doi.org/10.1016/j.biortech.2010.06.136.

[29] P. Přibyl, V. Cepák, V. Zachleder, Production of lipids in 10 strains of
Chlorella and Parachlorella, and enhanced lipid productivity in Chlorella vulgaris,
Applied Microbiology and Biotechnology. 94 (2012) 549–561.

732

https://doi.org/10.1007/s00253-012-3915-5.

[30] C. Safi, C. Frances, A.V. Ursu, C. Laroche, C. Pouzet, C. Vaca-Garcia,
P.-Y. Pontalier, Understanding the effect of cell disruption methods on the diffusion of
Chlorella vulgaris proteins and pigments in the aqueous phase, Algal Research. 8 (2015)
61–68. https://doi.org/10.1016/j.algal.2015.01.002.

[31] E. Clavijo Rivera, L. Villafaña-López, S. Liu, R. Vinoth Kumar, M. Viau,
P. Bourseau, C. Monteux, M. Frappart, E. Couallier, Cross-flow filtration for the
recovery of lipids from microalgae aqueous extracts: Membrane selection and
performances, Process Biochemistry. 89 (2020) 199–207.
https://doi.org/10.1016/j.procbio.2019.10.016.

[32] L. Villafaña-López, E. Clavijo Rivera, S. Liu, E. Couallier, M. Frappart,
Shear-enhanced membrane filtration of model and real microalgae extracts for lipids
recovery in biorefinery context, Bioresource Technology. 288 (2019) 121539.
https://doi.org/10.1016/j.biortech.2019.121539.

[33] C. Safi, A.V. Ursu, C. Laroche, B. Zebib, O. Merah, P.-Y. Pontalier, C.
Vaca-Garcia, Aqueous extraction of proteins from microalgae: Effect of different cell
disruption methods, Algal Research. 3 (2014) 61–65.
https://doi.org/10.1016/j.algal.2013.12.004.

750 [34] A. Taleb, Production de biodiesel à partir des microalgues : recherche des
751 souches accumulatrices des lipides et optimisation des conditions de culture en
752 photobioréacteurs, thesis, Nantes, 2015. http://www.theses.fr/2015NANT2007
753 (accessed April 1, 2019).

754

[35] V. Montalescot, T. Rinaldi, R. Touchard, S. Jubeau, M. Frappart, P.

Jaouen, P. Bourseau, L. Marchal, Optimization of bead milling parameters for the cell
disruption of microalgae: Process modeling and application to Porphyridium cruentum
and Nannochloropsis oculata, Bioresource Technology. 196 (2015) 339–346.
https://doi.org/10.1016/j.biortech.2015.07.075.

T.R. Zinkone, Broyage à billes de microalgues : étude et modélisation par
classe de taille, application au bioraffinage, These de doctorat, Nantes, 2018.
http://www.theses.fr/2018NANT4084 (accessed November 11, 2020).

[37] M. Frappart, A. Massé, M.Y. Jaffrin, J. Pruvost, P. Jaouen, Influence of
hydrodynamics in tangential and dynamic ultrafiltration systems for microalgae
separation, Desalination. 265 (2011) 279–283.
https://doi.org/10.1016/j.desal.2010.07.061.

[38] Michel. DuBois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, Fred. Smith,
Colorimetric Method for Determination of Sugars and Related Substances, Analytical
Chemistry. 28 (1956) 350–356. https://doi.org/10.1021/ac60111a017.

[39] S. Sharma, S.-F. Cheng, B. Bhattacharya, S. Chakkaravarthi, Efficacy of
free and encapsulated natural antioxidants in oxidative stability of edible oil: Special
emphasis on nanoemulsion-based encapsulation, Trends in Food Science & Technology.
91 (2019) 305–318. https://doi.org/10.1016/j.tifs.2019.07.030.

[40] B. Balasundaram, S. Harrison, D.G. Bracewell, Advances in product
release strategies and impact on bioprocess design, Trends in Biotechnology. 27 (2009)
477–485. https://doi.org/10.1016/j.tibtech.2009.04.004.

[41] E. Suarez Garcia, J. van Leeuwen, C. Safi, L. Sijtsma, M.H.M. Eppink,
R.H. Wijffels, C. van den Berg, Selective and energy efficient extraction of functional

proteins from microalgae for food applications, Bioresource Technology. 268 (2018)
197–203. https://doi.org/10.1016/j.biortech.2018.07.131.

[42] S. Liu, I. Gifuni, H. Mear, M. Frappart, E. Couallier, 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, Process Biochemistry. (2021).
https://doi.org/10.1016/j.procbio.2021.05.021.

[43] E. Teuling, P.A. Wierenga, J.W. Schrama, H. Gruppen, Comparison of
Protein Extracts from Various Unicellular Green Sources, Journal of Agricultural and
Food Chemistry. 65 (2017) 7989–8002. https://doi.org/10.1021/acs.jafc.7b01788.

[44] A.-V. Ursu, A. Marcati, T. Sayd, V. Sante-Lhoutellier, G. Djelveh, P.
Michaud, Extraction, fractionation and functional properties of proteins from the
microalgae Chlorella vulgaris, Bioresource Technology. 157 (2014) 134–139.
https://doi.org/10.1016/j.biortech.2014.01.071.

792 A. Tamayo Tenorio, K.E. Kyriakopoulou, E. Suarez-Garcia, C. van den [45] 793 Berg, A.J. van der Goot, Understanding differences in protein fractionation from 794 conventional crops, and herbaceous and aquatic biomass - Consequences for industrial 795 use, Trends in Food Science & Technology. 71 (2018)235-245. 796 https://doi.org/10.1016/j.tifs.2017.11.010.

797 [46] P.L. Yeagle, Chapter 12 - Lipid–Protein Interactions in Membranes, in:
798 The Membranes of Cells (Third Edition), Academic Press, Boston, 2016: pp. 291–334.
799 https://doi.org/10.1016/B978-0-12-800047-2.00012-7.

800

[47] E.R. Stadtman, R.L. Levine, Free radical-mediated oxidation of free

amino acids and amino acid residues in proteins, Amino Acids. 25 (2003) 207–218.
https://doi.org/10.1007/s00726-003-0011-2.

803 [48] H.R. Griffiths, Antioxidants and protein oxidation, Free Radical
804 Research. 33 Suppl (2000) S47-58.

805 [49] M.P. Deutscher, Guide to Protein Purification, Gulf Professional806 Publishing, 1990.

807 [50] H.G. Pontis, Chapter 2 - Preparation of Protein Extracts, in: H.G. Pontis
808 (Ed.), Methods for Analysis of Carbohydrate Metabolism in Photosynthetic Organisms,
809 Academic Press, Boston, 2017: pp. 31–44. https://doi.org/10.1016/B978-0-12-803396810 8.00002-8.

[51] S. Benelhadj, A. Gharsallaoui, P. Degraeve, H. Attia, D. Ghorbel, Effect
of pH on the functional properties of Arthrospira (Spirulina) platensis protein isolate,
Food Chemistry. 194 (2016) 1056–1063.
https://doi.org/10.1016/j.foodchem.2015.08.133.

815

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

Shuli Liu^{a,b}, Camille Rouquié^{a,c}, Laurence Lavenant^c, Matthieu Frappart^a, Estelle Couallier^{a,*}

Graphical abstract

