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Biorefinery of microalgal soluble proteins by sequential processing and membrane filtration

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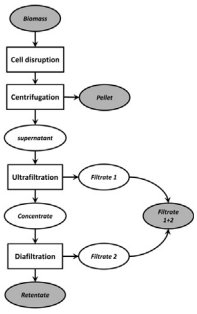
HIGHLIGHTS

- A 100 g.L^{-1} slurry of *N. gaditana* was submitted to protein biorefinery.
- A biorefinery process was tested to disrupt the cell wall and filtrate the samples.
- High-pressure homogenization was more efficient than enzymatic treatment.
- The filtration process was more efficient after enzymatic treatment.
- Increasing the cut off of the membrane does not improve the filtration process.
- The mathematical model corresponded to the experimental data of filtration.

GRAPHICAL ABSTRACT

Schematic representation of the overall process from cell disruption to diafiltration.



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ABSTRACT

A mild biorefinery process was investigated on the microalga *Nannochloropsis gaditana*, to obtain an enriched fraction of water soluble proteins free from chlorophyll. After harvesting, a 100 g.L^{-1} solution of cells was first subjected to cell disruption by either high-pressure homogenization (HPH) or enzymatic treatment (ENZ). HPH resulted in a larger release of proteins (49%) in the aqueous phase compared to the Alcalase incubation (35%). In both cases, an ultrafiltration/diafiltration (UF/DF) was then performed on the supernatant obtained from cell disruption by testing different membrane cut-off (1000 kDa, 500 kDa and 300 kDa). After optimising the process conditions, the combination of ENZ → UF/DF ended in a larger overall yield of water soluble proteins (24.8%) in the permeate compared to the combination of HPH → UF/DF (17.4%). A gel polarization model was implemented to assess the maximum achievable concentration factor during ultrafiltration and the mass transfer coefficient related to the theoretical permeation flux rate.

1. Introduction

The last century witnessed an exponential increase of the world population and an industrial revolution that consumed without limit the resources provided by our planet. This dangerous anthropogenic interference with the climate system resulted in an unprecedented ecological debt that is growing every year, increase in global temperature, increase in oceans level and increase in natural disasters. Hence, with the present rate of consumption alternative measures are strongly required to prevent these problems and build a more sustainable future.

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Microalgae are promising microorganisms that can take part of these measures, due to their large diversity (Barsanti and Gualtieri, 2010) and numerous benefits they can provide (Hu et al., 2008). They can grow in fresh or marine water, do not compete with arable land, multiply rapidly and can accumulate large amounts of valuable components (pigments, polyunsatured fatty acids, proteins, lipids and carbohydrates) within a short period of time compared to conventional crops. Algae mitigate large amounts of carbon dioxide as an amount of 1.8 tons of CO_2 is required to produce one ton of microalgae (Kliphuis et al., 2010).

The last decade, microalgae technology witnessed an intensive focus on the possibility of producing microalgal biofuels. Studies proved that although it is possible to make biofuels from microalgae the major drawback is the high production cost, which currently makes this technology uncompetitive with the fossil fuel market in terms of price (Batan et al., 2016). However, the emphasis on biofuels has indirectly neglected the presence of other valuable components of microalgae such as proteins.

Some studies have tested the separation of microalgae proteins by means of pH shifting (Cavonius et al., 2015; Chronakis, 2001; Ursu et al., 2014), three phase partitioning (Waghmare et al., 2016) or aqueous two-phase extraction (Desai et al., 2014). Nevertheless, despite their efficiency in terms of separation, these methods employ large amount of solvents and other chemicals especially when these processes are scaled up. In addition, separation of components based on chemical-free, low energy (Chronakis, 2001; Waghmare et al., 2016) and mild operating conditions is possible through ultrafiltration; a method that could be scaled-up to an industrial level (Susanto et al., 2008) for example in the dairy industry. The integration of membrane technology in such a context, however, is not highly developed for microalgae (Gerardo et al., 2014). It has been used mainly for harvesting cells (Hwang et al., 2015; Petruševski et al., 1995; Rossignol et al., 1999; Zhang et al., 2010), and its use in separating microalgal biomass components in an integrated process is hardly explored. Only a limited number of studies have investigated this technique to purify components like polysaccharides from *Porphyridium cruentum* (Marcati et al., 2014; Patel et al., 2012), *Spirulina platensis* and *Chlorella pyrenoidosa* (Pugh et al., 2001), or to concentrate proteins from *Chlorella vulgaris* and *Haematococcus pluvialis* in the retentate (Ba et al., 2016; Ursu et al., 2014). The focus of another study conducted on *Tetraselmis suecica* (Safi et al., 2014a) was to obtain proteins in the filtrate instead of the retentate. As the concentration of proteins in the mother solution was very low, this approach led to a smooth process in terms of permeate flow rate and total protein recovery in the filtrate.

The objective of this study is to conduct a process that releases relatively high amount of proteins in the supernatant, and then apply a two-step filtration in order to obtain a protein rich fraction in the filtrate, free from polysaccharides and chlorophyll. The process has been tested on different membranes pore size, and supernatants obtained after applying two different cell disruption methods on *Nannochloropsis gaditana*.

2. Materials and methods

Nannochloropsis gaditana CCFM-01 (Microalgae Collection of Fitoplancton Marino S.L., CCFM) was grown outdoors by Fitoplancton Marino in horizontal tubular 2000 L reactors and harvested in the exponential phase. The reactors used pure CO_2 injection to control pH in the culture by a pH controller and flowmeters. pH was set at 7.5, while natural light-dark cycles and ambient temperature were used (10–11 h of light, temperatures ranging from 10 to 22 °C). The reactors were inoculated with cultures grown in a growth chamber with the standard conditions of Fitoplancton Marino S.L. Cells were harvested by centrifugation during the exponential growth phase and supplied as a frozen paste (20% (w/w) dry weight).

Alcalase® was purchased from Sigma Aldrich, gels and reagents for SDS- and Native PAGE were purchased from BioRad.

2.1. Cell disruption

A 100 $g.L^{-1}$ microalgae suspension was prepared and subjected to high-pressure homogenization (HPH) using a GEA Niro Soavi PandaPLUS 2000. The pressure applied was 1500 bar with a permeate flow rate of 9 $L.h^{-1}$. Only one passage was sufficient to break the cell walls of *N. gaditana* To prevent overheating of the suspension, a cooling system was integrated into the homogeniser to keep the temperature below 30 °C during the process.

Enzymatic treatment was performed by incubating the cells for 4 h with 5% (v/w) Alcalase (per dry matter) at pH 8 and 50 °C.

In both cases, the mixture was centrifuged at 10,000*g* (10 min, 20 °C) and the collected supernatant was analysed in terms of proteins, carbohydrates and chlorophyll content. The amount of algal proteins in the supernatant were calculated as the amount of total protein measured minus the amount of enzymes added.

2.2. Ultrafiltration/diafiltration (UF/DF)

The filtration process was conducted using a Labscale™ TFF system (Millipore, Billerica, MA) fitted with a membrane with a cut-off of 300 kDa, 500 kDa or 1000 kDa at a fixed transmembrane pressure (TMP) of 2.07 bar and with a filtration area of 50 cm^2 (A) for all the tested membranes (Pellicon XL Ultrafiltration biomax). The transmembrane pressure (TMP) was chosen on the basis of preliminary tests carried out at different TMP.

During concentration, a feeding volume of the supernatant obtained after cell disruption of 0.4 L (V^0) was concentrated to 80% by ultrafiltration. The remaining retentate was used for diafiltration up to two diavolumes to recover more proteins in the filtrate (Fig 1). During both filtration steps, the feeding solution was constantly stirred in the feeding chamber to constantly insure the complete solubilisation of the components in the extract. Permeate flow rate (J) and TMP were monitored every 5 min, and the permeate flow rate and permeability of the membranes were calculated with the following equations:

$$Permeate\ Flow\ rate = \frac{Permeate\ mass\ recovered\ (kg)}{Time\ (h) \times\ membrane\ surface\ (m^2)} \quad (1)$$

$$Permeability\ (Lp) = \frac{Permeate\ volume\ recovered\ (L)}{Time\ (min) \times membrane\ surface\ (m^2) \times pressure\ (bar)} \quad (2)$$

After each trial, the membranes were flushed with distilled water. Subsequently circulated during 60 min with 0.1 M NaOH and finally flushed with distilled water.

2.3. Protein quantification

Protein nitrogen was quantified by Kjeldahl method (Gerhardt Analytical Systems – Germany). Dried samples of 200 mg were digested by means of sulfuric acid and high temperature (420 °C) in a KJELDATHERM® block heating system. Once the digestion step was completed, the samples were transferred to a VAPODEST® 50 s fully automated system in terms of dilution, filling and titration. A conversion factor of 5.20 was used to calculate the total protein from total nitrogen. The N-to-protein conversion factor was obtained by determination of the amino acid composition of *N.* gaditana according to Gilbert-Lopez et al. (2015).

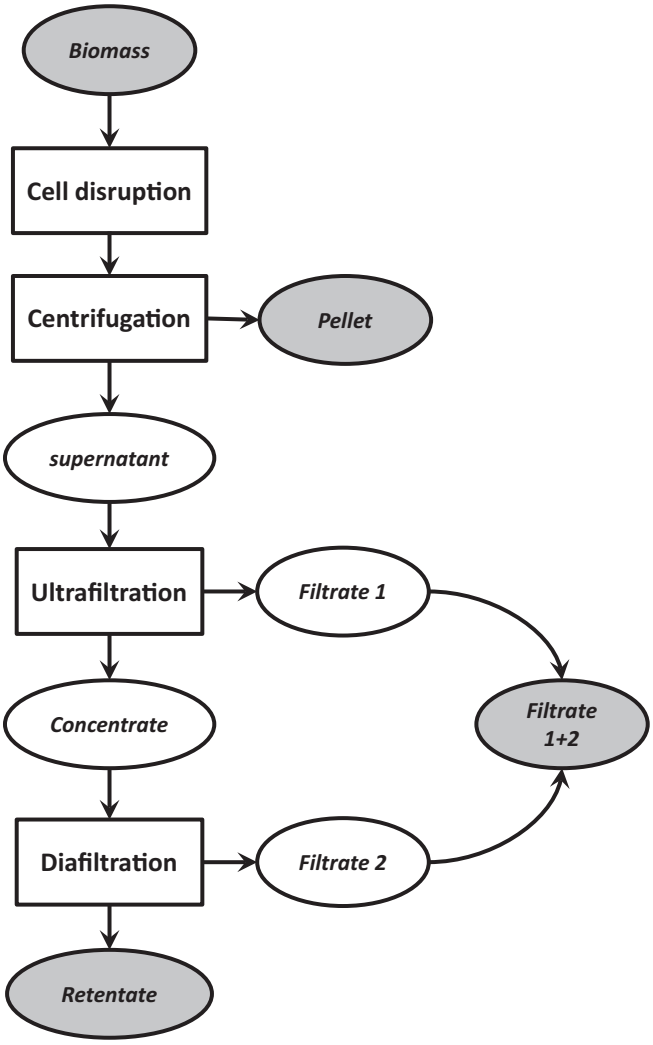

Fig. 1. Schematic representation of the overall process from cell disruption to diafiltration.

2.4. Native PAGE

Native PAGE analysis was performed on a 4–20% Criterion TGX gel by using the method of Postma et al. (2016). Fifty μL Native sample buffer (Biorad) and 125 μg proteins were mixed and adjusted to 100 μL using Milli-Q water. Twenty-five μg protein was loaded per lane, and Tris/Glycine (Biorad) was used as running buffer at 200 V for 35 min. Invitrogen™ Novex™ NativeMARK™ unstained protein standard was used to identify the molecular weight of proteins.

Bio-Safe Coomassie stainBiorad was used to stain the Native PAGE gel for 120 min followed by overnight rinsing with Milli-Q water.

2.5. SDS-PAGE

The samples were dissolved in 1 mL Milli-Q water and centrifuged (10,000*g*, 10 min, RT). The sample mixture, which contained 6.25 μL NuPAGE LDS Sample Buffer (4×), 2.5 μL NuPAGE Sample Reducing Agent (10×) and 16.25 μL sample, was heated at 70 °C for 10 min and centrifuged. The running buffer was prepared by diluting MOPS SDS Running Buffer (20×) in Milli-Q water. Electrophoresis was conducted at 200 V for 50 min. After electrophoresis the gel was washed three times with Milli-Q water for 5 min at 100 rpm using an orbital shaker. Afterwards, the gel was stained for 1 h at 100 rpm using SimplyBlue SafeStain, and the process of destaining of the gel was performed overnight using Milli-Q water.

2.6. Starch analysis

Starch analysis consists of mixing 0.25 mL sample with 5 mL iodine reagent (mixture of iodine and potassium iodide). The mixture was mixed for 5 s and placed for 2–5 min at RT so that the colour stabilized. A blank containing distilled water instead of the sample with the iodine reagent was used and the absorbance was measured at 620 nm. The concentration ($mg.L^{-1}$) is calculated by plotting the calibration curve using standard solution of potato starch at different concentrations.

2.7. Chlorophyll analysis

Two hundred μL supernatant was mixed with 1300 μL methanol and incubated in the dark for 1 h at 45 °C. Subsequently, the samples were centrifuged at 10,000*g* for 10 min at 20 °C. The organic phase (methanol) containing the pigments was recovered, and the chlorophyll content was determined according to the equation proposed by Ritchie (2006).

$$Total\ chlorophyll\ mg.L^{-1} = (9.3443 \cdot A652) + (4.3481 \cdot A665) \quad (3)$$

2.8. Sugar analysis

Sugars were quantified by using a BRIX digital refractometer HI96803 (Hanna instruments) that reports the sugar content of an aqueous solution as % glucose by weight (% w/w), with ±0.2% measurement accuracy. The method consists of addition of 100 μL of the aqueous solution in a sealed stainless steel well with a high-grade optical prism made of flint glass. The readings are displayed within 1.5 s response time.

2.9. Flow cytometry

The disruption efficiency was determined by using the method of Postma et al. (2015). Microalgae samples were analysed using a flow cytometer (BD Accuri C6). The suspensions obtained after cell disruption were diluted to a total volume of 1 mL using ultrapure water. A fixed volume of 15 μL was measured at a fluidics rate of 35 μL min^{-1} and a core size of 16 μm. All samples were analysed before and after cell disruption of *N. gaditana*.

2.10. Microscopic observation

A Leica DM 2500 light microscope equipped with a Leica DFC 450 camera was used for microscopic observations to determine the efficiency of cell disruption. Magnification used was 1000 times.

2.11. Gel polarization model

The UF process was described according to the assumption of gel-polarization (Bhattacharjee et al., 1996; Gosh, 2009): the permeate flow rate (J) has been expressed as a function of the concentration (c) of all the retained polysaccharides according to the following equation

$$J = \rho \cdot k \cdot \ln\frac{c_G}{c} \quad (4)$$

ρ is the liquid density, k ($L.m^{-2}.h^{-1}$) is the mass transfer coefficient under gel polarization condition and c_G is the concentration of the retained polysaccharides in the gel layer.

According to the mass balances on the liquid and on the polysaccharides, the permeate flow rate along the time can be described as a function of the retained volume (V) as follows:

$$J = k \cdot A \cdot \ln\left(\frac{c_G}{c^0} \cdot \frac{V}{V^0}\right) \quad (5)$$

k gives an estimation of the expected range of values of J: the higher the k, the more the membrane polarization can be prevented. According to Lewis (1996) c_G/c^0 represents the upper limit of the concentration factor ($CF = c/c^0$) achieved by the volume reduction degree ($V/V^0 = CF^{-1}$).

2.12. Statistical analysis

All experiments were conducted in duplicates. Statistical analyses were carried out using Minitab 17 software. ANOVA and Tukey test were carried out and measurements of duplicates for each sample were reproducible for ±5% of the respective mean values.

3. Results and discussion

3.1. Cell disruption methods and supernatant characterisation

Frozen cells of *N. gaditana* were used for cell disruption to release protein. The total amount of protein present was 50% (w/w) dry matter as determined by Kjeldahl. The freezing process did hardly have an effect on the integrity of the cell wall of *N. gaditana* as only a negligible amount of proteins (<2% (w/w) total proteins) was released in the supernatant before applying any cell disruption method.

HPH was performed and flow cytometry indicated that the majority of *N. gaditana* cells were disrupted (>90% disintegration) after applying one passage at 1500 bar. Increasing the pressure to 2000 bar or applying two or three passages did not improve the disintegration efficiency, which implies that one passage was enough to disintegrate the majority of the cells. Microscopic observations showed also the degree of disintegration of the cells after high-pressure homogenization (Fig 2). Indeed most of the cells appeared completely disintegrated, which is in concordance with the results of flow cytometry (data not shown). *N. gaditana* was incubated with the commercial protease Alcalase that can weaken the cell wall integrity as protein is part of its cell wall composition. Microscopic observations showed that the cells kept their spherical shape after the treatment (Fig 2). This is not very surprising since the enzymatic treatment is a soft method that peels off the cell wall proteins, hydrolyse the intracellular proteins, and can form protoplasts (Honjoh et al., 2003; Liu et al., 2006) without altering the morphological shape of the cells. The amount of protein released after HPH treatment was 49 ± 1% (w/w total proteins) in the aqueous phase. The release of all proteins was not achieved due to the rigidity of the cell wall (Scholz et al., 2014) and the insoluble nature of some proteins that remained in the pellet. However, the performance of HPH in this study was more efficient than the HPH used to disrupt the cells of *Nannochloropsis oculata* (Safi et al., 2014b). The yield of proteins after two passages was 52 ± 1% with a specific energy input of 7.5 kWh.kg^{-1} (Safi et al., 2014b), whereas we found 49 ± 1% protein yield and a specific energy input of 0.44 kWh.kg^{-1} after one passage. Indeed, between both studies, the protein yield is statistically similar (95% confidence level), but the lower energy input is due to the number of passages, the configuration difference of the homogenizer, and the higher cell concentration used in this study more efficient. The use of Alcalase solely significantly increased the concentration of proteins in the supernatant by releasing 35 ± 1% (w/w) of total proteins. The protein yield was lower compared to HPH treatment due to the complex cell wall composition of *N. gaditana* that is composed of proteins, cellulose and algaenans (Scholz et al., 2014). Hence, hydrolysing the proteins of the cell wall most likely was not sufficient enough to completely weaken the integrity of the cell wall and to release the majority of proteins. In Table 1, an overview is shown of the amounts of sugars, starch and chlorophyll released for both methods applied.

3.2. Ultrafiltration/diafiltration performance

The next step investigated was the effect of UF and DF using different membranes (Fig 1) of the protein fractions obtained after the two disruption methods. Fig. 3A and B show the observed values of the permeate flow rate (J), while maintaining a constant TMP pressure at 2.07 bar for the soluble protein fractions obtained after HPH (Fig. 3A) or protease treatment (Fig. 3B). The sharp permeate flow rate decrease in the beginning (t < 0.3 h) of the process may be related to the onset of a polarization concentration layer due to the action of large molecules (Eteshola et al., 1996) that got retained by the membranes and contribute to the fouling phenomenon (Morineau-Thomas et al., 2002). However, the fouling was not very severe for all membranes tested since J did not strongly decline in time (Fig 3).

Furthermore, the permeate flow rates differed significantly ($p < 0.05$) between the three membranes tested. Tukeys' honest significance test showed that all trials tested differed from each other in terms of permeate flow rate. Moreover, two additional conclu-

Table 1
Release of components after high-pressure homogenization (HPH) and protease (Alcalase) treatment of *Nannochloropsis gaditana*.

Yields (mg.L^{-1})	Alcalase	HPH
Sugars	45.5 ± 3.4	58.3 ± 5.5
Starch	9.0 ± 0.1	12.0 ± 0.1
Chlorophyll	18.0 ± 1.0	56.0 ± 5.8

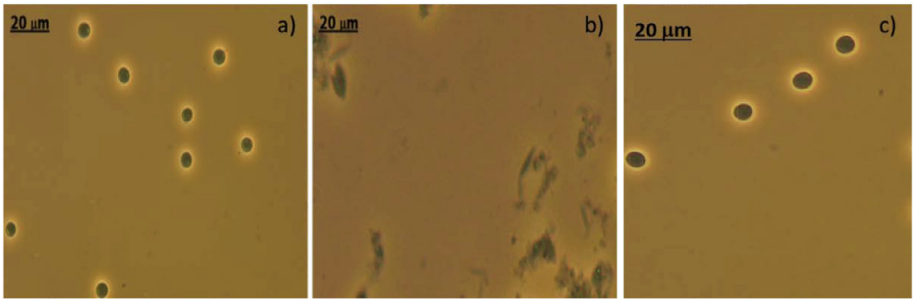

Fig. 2. Microscopic observations of *Nannochloropsis gaditana* (a) before cell disruption, (b) after high-pressure homogenization and (c) after protease treatment. Bar represents 20 µm.

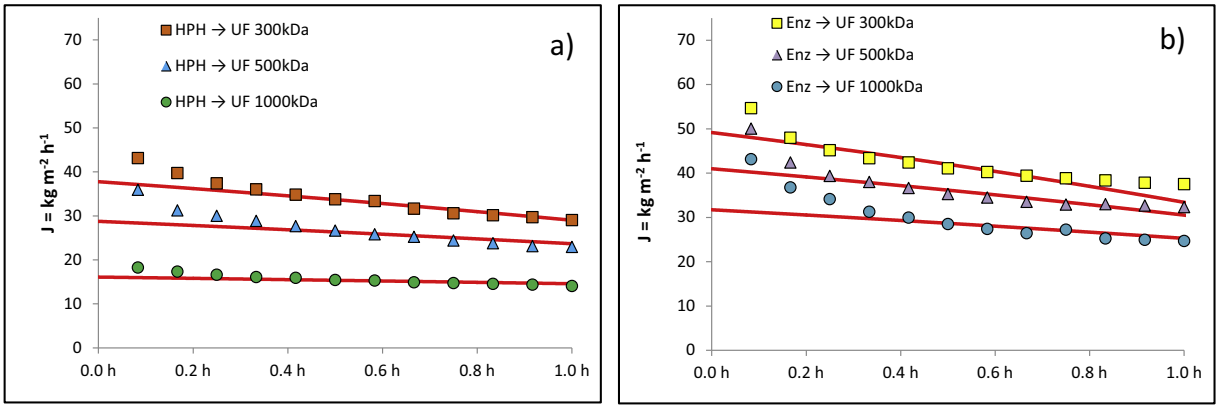

Fig. 3. Permeate flow rate screening at a fixed TMP for all membranes tested. Protein fractions from *Nannochloropsis gaditana* obtained after high-pressure homogenization (HPH; A) and protease (Enz, B) treatment. Red curves report the trend of the predicted value of J according to the mathematical model. Results are based on 2 replicates for 2 experiments ± SD ($n = 2$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

sions can be drawn from Fig. 3: First, the permeate flow rate is always faster for the samples pre-treated with Alcalase compared to high-pressure homogenization. Proteases reduce the molecular weight of proteins and this leads to a facile filtration and a higher permeate flow rate. Second, among all membranes tested, the highest permeate flow rate obtained was for the 300 kDa membrane both for the fractions obtained after protease and high-pressure homogenization treatment. The lowest permeate flow rate was observed for the 1000 kDa membrane. Therefore, the results are in contrast with the hypothesis that increasing the molecular weight of the membrane would lead to a higher permeate flow rate.

Fig. 3 also shows the results of the predicted permeate flux rate with respect to the gel polarization model. The agreement has been satisfactory for almost all the tests with a relative error of <5% between the model and experiments. Consequently, a good estimation of the mass transfer coefficient and the gel concentration for both cell disruption methods and the different membranes cut-off has been derived. When enzymatic treatment was applied, k decreased from 21 to 15 $L.m^{-2}.h^{-1}$ while increasing the cut-off from 300 to 1000 kDa. Moreover, while performing HPH, lower values of $\boldsymbol{k}$ were observed: from 16 to 7.1 $L.m^{-2}.h^{-1}$ when increasing the membrane cut-off from 300 to 1000 kDa. Therefore the data confirm that: i) the enzymatic cell disruption can better prevent membrane fouling/polarization compared to HPH; ii) the performance of the ultrafiltration operation for both cell disruption methods is negatively affected when larger membrane cut-off are adopted.

In addition, the value of the upper limit of the concentration factor (c_G/c^0) was equal to 9.81 ± 0.57 for all the performed UF tests, regardless of the pre-treatment technique and the membrane cut-off. According to that value, not more than 90% of the initial volume can be ultra-filtered with the concentration mode.

The behaviour of the membranes was also tested by evaluating their permeability (Lp) during the process. Hence, similar to the permeate flow rate, the permeability followed the same trends (Fig 4) due to the formation of the polarization layer that reduces the pores surface and simultaneously reduces the permeability of the membranes. The lowest permeability was for the 1000 kDa membrane after protease and HPH treatment with 0.34 $L.min^{-1}.bar^{-1}.m^{-2}$ and 0.14 $L.min^{-1}.bar^{-1}.m^{-2}$, respectively. Whereas the highest permeability after either protease or HPH treatment for the 300 kDa membrane was 0.34 $L.min^{-1}.bar^{-1}.m^{-2}$ and 0.43 $L.min^{-1}.bar^{-1}.m^{-2}$, respectively (Fig 4).

The characterisation of the permeate after the first step of filtration showed that for all the membranes tested starch and chlorophyll were retained in the retentate (data not shown), which corresponds to the results obtained in other studies on different microalgal species (Ba et al., 2016; Safi et al., 2014a; Ursu et al., 2014). The retention of starch was expected given their large molecular weight. The retention of chlorophyll might be due to the hydrophilic characteristics of the membrane (polyethersulfone; PES) leading to the complete retention of chlorophyll that is mainly hydrophobic. Another explanation is that chlorophyll was present in small lipid droplets or in very small cell debris particles that remained in the supernatant as both are larger than the cut-off of the membranes (Safi et al., 2014a).

Proteins were quantified at each step of the process in order to calculate the mass balance and evaluate the process efficiency. Fig. 5a shows that after applying ultrafiltration using 300, 500 and 1000 kDa membranes the amount of proteins quantified in the filtrate was 23 ± 0.21 and 12 ± 0.01%, respectively, of the soluble proteins initially obtained in the supernatant after HPH. The results obtained with the 300 kDa membrane are 10% higher than the results obtained in the study of Ursu et al. (Ursu et al., 2014) that used similar cut off and pH. The higher amount of proteins recovered is due to the higher TMP (2.07 bar) employed in our study. Moreover, the amount of protein obtained was slightly lower with the 500 kDa membrane and the lowest amount was obtained with the 1000 kDa membrane. Subsequently, the collected retentates were submitted to diafiltration and two diavolumes were necessary to recover more proteins in the filtrates up to 37 ± 0.01% for the 300 kDa membrane and 15 ± 0.01% for the 1000 kDa membrane (Fig 5). Indeed, the amount of proteins found in the filtrates is rather low regardless the type of membrane that was tested. Several reasons can explain these results; some glycoproteins are simultaneously retained with, as a consequence of their covalent linkages, with large polysaccharides (Heaney-Kieras et al., 1977; Liu et al., 2005). Another explanation is that the supernatant consist of a complex mixture of unequally charged proteins, which leads to a strong interaction between the positively and negatively charged proteins that form aggregates (van den Berg and Smolders, 1990) with a large molecular weight (Ursu et al., 2014). This renders them large enough to be retained by the membranes and contributes to the increase in resistance due to the concentrated layer formed near the membrane interface (van den Berg and Smolders, 1990).

Nevertheless, against all expectations the 1000 kDa membranes showed the lowest permeate flow rate and protein yield in the filtrate especially for the fraction obtained after HPH treatment. This phenomena has been reported as adsorptive fouling: Susanto et al. (2008) performed UF tests with a PES membrane on protein-polysaccharide mixtures and they found that membranes with large cut-off were more susceptible to a decrease of permeate flux rate due to adsorptive fouling of polysaccharides. So additionally to the polarization layer, it is possible that for the 300 kDa membrane

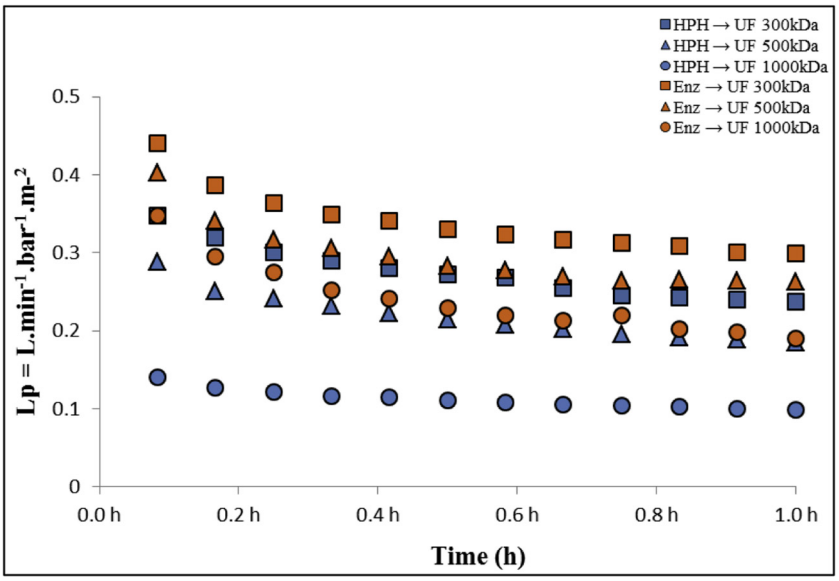

Fig. 4. Permeability screening at a fixed TMP for all the membranes tested after HPH and enzymatic treatment. Results are based on 2 replicates for 2 experiments ± SD ($n = 2$).

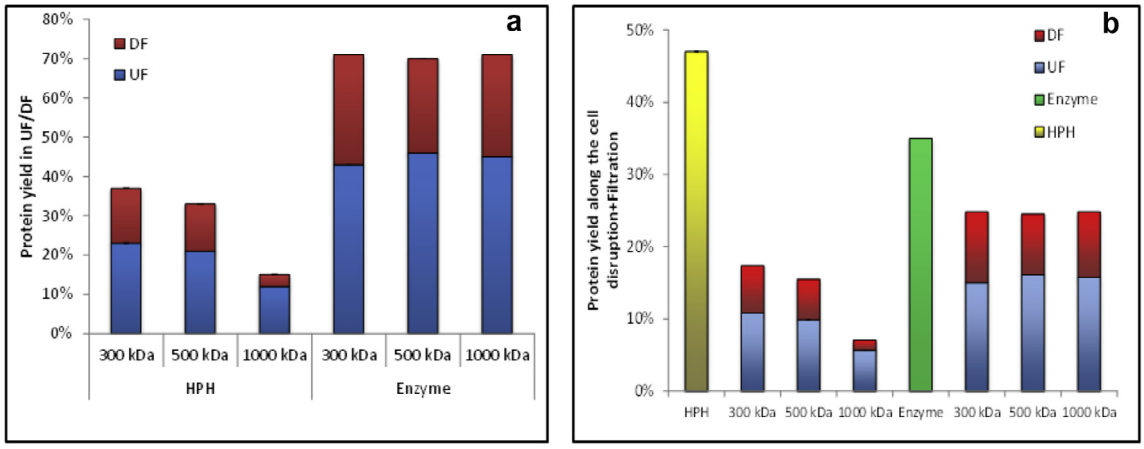

Fig. 5. Recovery of *Nannochloropsis gaditana* proteins in the filtrate after a two-step filtration with different membranes and two cell disruption methods. (a) Protein yield is expressed as% (w/w) of proteins in the filtrate out of total proteins present in the supernatant after cell disruption. (b) Protein yield is expressed as % (w/w) of proteins out of total proteins in the biomass. Results are based on 2 replicates for 2 experiments ± SD ($n = 2$). DF: diafiltration; UF: ultrafiltration; HPH: high-pressure homogenization; Enzyme: protease treatment.

the adsorption can occur only in front of the membrane surface. However, for the 1000 kDa membrane a fraction of retained molecules can penetrate into the membrane pores and contribute to the membrane fouling as well as to the low yield of proteins in the filtrate (De la Torre et al., 2009; Susanto et al., 2007).

3.3. Overall process performance

The proposed biorefinery alternatives were compared in terms of overall protein yield along the different steps of the process. The yield of proteins in the filtrates (UF and DF) after protease treatment was similar ($p > 0.05$) for all membranes with up to 71 ± 0% protein yield in the filtrate (w/w total proteins in the supernatant). This is, however, two to three fold higher compared to HPH (Fig 5a). Indeed, the higher protein yield found could be due to the smaller size of proteins as determined by NATIVE (proteins undetected) and SDS PAGE (6–98 kDa), which simplifies their passage through the membrane. At the end of the filtration process, the protein mass balance (on average) for all the membranes tested was up 90 ± 3% for HPH and protease, respectively.

The overall yield of the proposed biorefinery for extracting and fractionating proteins depending on the total protein present in *N. gaditana* is reported in Fig. 5b. The yields always refer to the initial amount of protein content within the algae biomass. As expected the combination of enzymes → UF → DF assures the highest final yield of 24.8% protein (w/w). Since after protease treatment the membrane cut-off did not affect the overall protein yield, it would be suitable to operate at 300 kDa where larger permeate flow rate can be achieved, which assures a faster operation (low energy consumption) and a lower amount of membrane area (less capital investment).

The proposed biorefinery scheme represents a good candidate for the extraction and fractionation of soluble protein and protein hydrolysates from microalgae. The key points of this process are:

1) Mildness in terms of energy consumption in case of enzymatic cell disruption.
2) Use of membranes instead of solvents can strongly reduce the cost of the process, while keeping it green and mild. Alternative techniques that can keep the proteins in their

native state include aqueous two-phase extraction and fractionation of water-soluble proteins based on mixture of polymer/polymer, polymer/salt or ionic liquid/polymer (Benavides J, 2011; Desai et al., 2014; Vanthoor-Koopmans et al., 2013). However, a large quantity of chemicals is required, especially for the upscaling process.

3) Realistic operating conditions were tested, especially with respect to the initial biomass concentration (100 $g.L^{-1}$). Coons et al. (2014) reported that a feasible microalgal biorefinery should treat a biomass stream significantly concentrated to reduce the cost of the process. To our knowledge, this is the first case of a complete microalgal biorefinery aiming at soluble proteins that has been demonstrated for an initial biomass concentration of 100 $g.L^{-1}$. In other cases, only cell disruption has been tested at comparable level of biomass concentration.

Nonetheless, it is worthwhile mentioning that the proteins of HPH kept their native structure all along the process without being affected by the shear stress induced by the homogenizer. NATIVE PAGE detected a protein profile in the supernatant that ranged between 40 and 720 kDa, the most concentrated protein was observed at 480 kDa. Whereas the proteins obtained after enzymatic treatment lost their native structure and were in the form of peptides or even amino acids, but all these valuable fractions could be valorised in the market of food, feed, nutraceuticals and pharmaceuticals depending on the properties required.

Further improvement of the process will be required to augment the quantity of proteins in the supernatant after cell disruption, and in the filtrate afterwards. A plausible line of unit operations is to combine both disruption methods and find a synergistic effect that would increase the amount of proteins released in the supernatant. Hence, by following the proposed sequence of unit operations, a higher amount of hydrolysates will be simultaneously obtained in the filtrate.

4. Conclusions

The main objective of this study was to carry out a mild biorefinery process with concentrated microalgae to obtain a protein fraction in the filtrate after testing two cell disruption methods. The results demonstrated that homogenization was more efficient than the protease treatment in terms of cell disintegration and release of soluble proteins. However, the filtration process revealed higher efficiency for the protease treated samples with a 25% protein yield obtained in the filtrate of the 300 kDa membrane. The study also concluded that increasing the cut off of the membrane does not necessarily improve the performance of the process, especially for native proteins.

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