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A classroom experimental game to improve the understanding of asymmetric common-pool resource dilemmas in irrigation water management

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Fractionation of A*rthrospira platensis (Spirulina)* water soluble proteins by membrane diafiltration

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Abstract:

Membrane diafiltration process was developed in order to fractionate the water soluble proteins of the supernatant obtained after *Spirulina* bead milling disruption and centrifugation. The obtained crude extract was filtered with 50 kDa, 150 kDa, 300 kDa or 0.2 µm membranes. Phycocyanin purified extracts were produced after filtration on the 300 kDa or 0.2 µm membranes. The filtration on 0.2 µm membrane allowed high recovery (more than 89 %) of Phycocyanin A and C into the retentate and permeate as well as separation of both molecules (depletion of C-PC into the retentate relatively to the A-PC; enrichment into the permeate). Blue colour permeates, poor in chlorophyll, were successfully produced. Concentrated solutions with more than 80% (dry weight basis) of proteins were obtained. The most severe fouling occurred with the 300 kDa membrane leading to low permeate fluxes (65 $L.h^{-1}.m^{-2}$). A fractionation of proteins by the size was possible for the 50, 150 and 300 kDa diafiltrations.

Keywords: *Spirulina*, biorefinery, ultrafiltration, protein, phycocyanin

1. Introduction

Microalgae are one of the most promising renewable feedstocks of healthy food ingredients and functional food products. Due to their richness of highly valuable bioactive compounds, they can be used to enhance the nutritional value of food and, thus, to favorably affect human health by improving the well-being and quality of life, but also curtailing disease and illness risks [1]. Among them, *Arthrospira platensis* (*Spirulina*), a blue-green alga, is a photosynthetic filamentous cyanobacterium often considered for biomass production due to its high cell growth rate, facility of harvesting and potential market as a human food supplement and animal feed. The microalga *A. platensis* possesses high protein content, unique composition of fatty acids, pigments (phycocyanin and β-carotene), and high carbohydrate content [2]. Besides, this microalga contains 46-71% (w/w) (dry weight basis) of protein and has interesting nutritional properties due to its content in essential amino acids, vitamins (particularly vitamin B12) as well as minerals [3, 4, 5].

Given its interesting characteristics, the cultivation of *Spirulina* could be a solution to improve human health, especially nutrition in Third World countries and also to develop industrial crops. This cyanobacterium currently seems one of the best solutions for the simple production of a dietary supplement of high quality and, as a result, large companies have embarked on the culture of this organism on an industrial scale. In addition to proven nutritional properties, *Spirulina* is now enjoying a revival of interest from the international scientific community because of its possible use as a source of pharmaceuticals, and nutraceuticals compounds. Indeed, the potential of this microalga seems to be important and this mainly thanks to its two phycobiliproteins, C-phycocyanin (C-PC) and allophycocyanin (A-PC), acting as a component

of light-collecting complexes in the cyanobacteria [6, 7, 8]. C-PC is the major photosynthetic pigment compared to A-PC [9]. C-PC has demonstrated a great interest for both nutraceutical and health food industries (natural blue colorant), as well as in biomedical research and clinical diagnostics (fluorescent probes) [10, 11, 12]. Furthermore, it has also been highlighted that this pigment has positive effects on impaired physiological conditions such as antioxidation, anti-inflammation, antitumor, and immunity enhancements [13].

Thus, the whole *Spirulina* can be used but its refining, that is to say the extraction and purification of molecules from this bio-resource bring real added value. Currently, microalgal soluble proteins are recovered using chemical methods such as pH shifting [5, 14], three phases partitioning [15] or aqueous two-phase extraction [16]. Unfortunately, these environmentally unfriendly techniques use a large amount of solvents and chemicals and can lead to the extraction of low-purity proteins with high salt content, denaturation and poor functional properties, or to be incompatible with industrial-scale applications. In this context, membrane filtration technologies, especially ultrafiltration, are presented as advantageous methods to purify and concentrate components of microalgal biomass with mild operating and chemical-free conditions [17, 18, 19]. The membrane filtration is notably useful for the concentration of large volumes and can be easily automatized and scaled up to an industrial level [20]. Also, this reduces the possible denaturation, deactivation and/or degradation of interest biological molecules. To date, several works have employed this technique on the separation and concentration of microalgal proteins, notably from *Chlorella vulgaris* [21], *Haematococcus pluvialis* [14], *Tetraselmis suecica* [22] and *Nannochloropsis gaditana* [23, 24]. Concerning *Spirulina*, a water-soluble protein-pigment complex such as phycocyanin can be recovered by membrane filtration after cell disruption. Herrera *et al*. (1989) [25] used ultrafiltration membrane with a molecular weight cut-off of 50 kDa to concentrate up to a volume reduction factor of 1.9 a *Spirulina maxima*‘s extract. This methodology made it possible to obtain food

grade phycocyanin. Jaouen *et al.* (1999) [26] obtained 96 % of phycocyanin retention during the filtration on a 100 kDa tubular membrane of an extract resulting from ultrasonic breakage of *Spirulina platensis*. More recently, Chaiklahan *et al.* (2011) [27] obtained 99 % of phycocyanin retention during the filtration of a *Spirulina sp.* extract, on a membrane with a molecular weight cut-off equal to 50 kDa. These works show that membrane filtration can be used for an almost complete retention of phycocyanin. An original work would be to show that it is possible to use membranes for fractionation/separation of proteins of *Spirulina*. In this last case, a part of proteins should pass through the membranes while the other part should be retained. The fractionation could in particular make it possible to produce different protein extracts of different size classes: each class could have different bio-activities. Nevertheless, severe membrane fouling can induce a non-selective separation and low permeate fluxes. Proteins would be difficult to recover in case of membrane adsorption, protein-protein or protein-polysaccharide interactions in the bulk solution and/or membrane [28]. Moreover, filtration performance will depend on the membrane chosen (material, pore diameters), feed protein concentration, physico-chemical characteristics of solutions to be filtered (pH, ionic strength) as well as operating conditions of filtration (pressure, cross-flow velocity, temperature, etc). Thus, only an experimental study can account for extending of membrane fouling and the feasibility to fractionate proteins coming from crushed *Spirulina*.

Thus, the aim of this study was to evaluate the feasibility to use diafiltration for protein fractionation of *Spirulina* (*A. platensis*). Ultrafiltration in diafiltration mode was used to selectively let pass proteins through the membranes and produce different protein extracts more or less purified, notably in salt and chlorophyll. Various molecular weight cut-offs of membranes were used. Membrane fouling extend was evaluated in relationship with the selectivity of operations as well as hydraulic performances.

2. Material and methods

Food grade dried *Spirulina* (*A. platensis*) powder was purchased from Bio Gatrana Society (Sidi Bouzid, Tunisia). Solutions of sodium hydroxide and nitric acid were prepared from analytical grade pellets and 70% of nitric acid respectively. These solutions were used for the cleaning of membranes. All other chemicals used are of analytical grade and were purchased from Sigma Aldrich Chimie (Lyon, France).

2.1. *Spirulina* protein extraction by bead milling

Spirulina suspensions were prepared in Milli-Q® water and used as feed for the disruption experiment. The suspensions contained 20 grams of dried *Spirulina* per liter of water. Cell disruption was conducted in a Dyno®-mill multi-lab from Willy A Bachofen AG (Muttenz, Switzerland) containing 0.5-0.75 mm glass beads at 80% filling percentage. Experiments were run in continuous mode in 3 passes. Samples were pumped from an agitated feed tank to a 600 mL grinding chamber containing the grinding medium at a flow rate of 150 mL/min and a rotation speed of 10 $m.s^{-1}$. The system was operated in batch recirculation mode, with a constant agitation speed of 2986 rpm. At the end of the first passage the suspension was recovered in a second tank and transferred to the first agitated tank to be pumped again. The temperature of the suspension was maintained at ~10 °C with a cold glycerol solution circulating in the cooling jacket of the grinding chamber. After cell disruption the suspensions were centrifuged using a Sorvall RC6 Plus centrifuge (Thermo Fisher Scientific Inc, Illkirch-Graffenstaden, France) at 12,000 x g for 30 min at 5 °C. The supernatant was stored at -20 °C before thawing at ambient temperature for at least 12 h, just before the membrane filtration experiments.

2.2. Filtration experiments

A diafiltration process was conducted using an experimental set-up previously described by Guilbaud *et al.* (2012) [29], equipped with a 0.032 m^2 tubular ceramic membranes (TAMI

Industries, Inside CeRAM, Cell 60, 7 channels) with different molecular weight cut offs (50 kDa, 150 kDa, 300 kDa) or pore size diameters (0.2 µm) at a fixed trans-membrane pressure (TMP) of 4 bar and with a cross-flow velocity of 5 $m.s^{-1}$. All filtrations were carried out at 20 °C, in batch mode. The TMP and cross-flow velocity were kept constant during experiments. The supernatant obtained after cell disruption then centrifugation (2 L) was diafiltrated with MilliQ water having a pH and a conductivity respectively equal to 6.5 and 0.12 $mS.cm^{-1}$. A constant volume of the solution (2 L) was maintained within the feed tank during the diafiltrations. Five diavolumes were added before stopping filtration (addition of 10 L of MilliQ water). During this step, retentate and permeate samples were taken for future analysis, at the beginning and at the end as well as in the course of the filtration. The permeate is weighted during the filtration using an electronic balance with the accuracy of 0.01 g. Thus, the permeate flux expressed in $L.h^{-1}.m^{-2}$ was evaluated from the permeate mass according to equation 1:

$$J = M_{permeate} / (\rho \, . \, t \, . \, A) \tag{1}$$

where $M_{permeate}$ (kg) is the permeate mass, ρ ($kg.L^{-1}$) is the volume mass of the permeate, t (h) is the permeation time and A (m^2) is the membrane area.

After each diafiltration experiment, the membrane was chemically cleaned according to the manufacturer recommendations. Firstly, the membrane was cleaned with sodium hydroxide solution (pH = 12 – Sigma-Aldrich S8045) at 60 °C for 30 minutes in closed loop (recirculation of permeate and retentate into the feed tank). Then, the pilot plant was rinced with MilliQ water until neutral pH at 20 °C in the open loop (discharge of the permeate). Afterward, the membrane was cleaned using a nitric acid solution (pH = 1.2 – Sigma-Aldrich 695041) at 50 °C for 15 minutes in closed loop. Finally, the membrane was rinced by MilliQ water until neutral pH. This procedure was also used for the membrane deconditioning. More than 90 % of the water fluxes were recovered after the cleaning.

The mean retention rate (R) of components was calculated as described in equation 2:

$$R = (C_R - C_P) / C_R \times 100 \quad (2)$$

C_R and C_P refer to the concentration within the retentate and permeate respectively.

The recovery rates within the retentate (Y_R) and the permeate (Y_P) were calculated following equations 3 and 4, respectively.

$$Y_R = (C_{Rend} . V_{Rend}) / (C_{R0} . V_{R0}) \times 100 \quad (3)$$

$$Y_P = (C_{Pend} . V_{Pend}) / (C_{R0} . V_{R0}) \times 100 \quad (4)$$

C_{R0} and V_{R0} refer to the concentration and volume of the crude extract to be filtered respectively. C_{Rend} and C_{Pend} refer to the concentrations within the retentate and the whole permeate obtained at the end of diafiltrations. V_{Rend} and V_{Pend} refer to all the volumes of retentate (2 L) and permeate recovered at the end of diafiltrations (10 L). Total recovery rate (Y_T) corresponds to the retentate plus the permeate recovery rates.

After each diafiltration, 9.5 L of its permeate were introduced into a tank in order to be concentrated on a 5 kDa tubular ceramic membrane (TAMI Industries, Inside CeRAM, Cell 60, 7 channels, 0.032 m²). All concentrations were carried out at 20 °C, in batch mode, at a TMP and cross-flow velocity equal to respectively 6 bar and 5 $m.s^{-1}$.

Thus, for the rest of the article, the “crude extract” will refer to the supernatant obtained after the centrifugation of the microalga grinding solution. The crude extracts will be diafiltered. The “raw permeate of diafiltration” will refer to the permeate of diafiltration without further treatment. The “mean raw permeate” refer to the whole permeate recovered at the end of a diafiltration process. The “concentrated permeate of diafiltration” will refer to the permeate recovered after diafiltration and concentrated on a 5 kDa membrane. It corresponds to the final retentate of a concentration experiment.

2.3. Soluble protein quantification

Protein content was quantified by Lowry's method, using a Sigma- Aldrich kit (BCA1-1KT) [30]. Basically, in alkaline conditions, proteins react with copper to form a cupric complex which is then reduced in cuprous ions, giving a purple color to the solution. First, a solution was prepared by adding 50 mL of BCA solution (Sigma-Aldrich, B9643, bicinchoninic acid, sodium tartrate and sodium bicarbonate in NaOH at 0.1 N, pH = 11.25) and 1 mL of copper sulfate (Sigma-Aldrich, C2284, $CuSO_4.5H_2O$, 40 $g.L^{-1}$). Then, 100 µL of the sample was added to 2 mL of test solutions in a 15 mL polypropylene falcon tube. The blank was made with Milli-Q® water. The tubes were homogenized for 5 seconds (vortex at 3000 rpm) before being incubated at 37 °C for 30 minutes. The calibration curve was carried out using Bovine Serum Albumin (BSA) (Sigma-Aldrich, P0914). Finally, the absorbance at 562 nm was measured using V-630 spectrophotometer (Jasco, Bouguenais, France). Each sample was analysed in triplicate.

2.4. Phycocyanin analysis

The concentrations of A-PC and C-PC were measured using a UV–VIS spectrophotometer (Jasco, Bouguenais, France) at the wavelengths of 620 and 652 nm. The total amount of C-PC and A-PC were calculated using equations (5) and (6) [27, 31]:

$$C_{C\text{-}PC} = [A_{620} - 0.474\ (A_{652})] / 5.34 \quad (5)$$

$$C_{APC} = [A_{652} - 0.208\ (A_{620})] / 5.09 \quad (6)$$

Where $C_{A\text{-}PC}$ is the Allophycocyanin concentration ($g.L^{-1}$), $C_{C\text{-}PC}$ is the C-phycocyanin concentration ($g.L^{-1}$), A_{620} is the optical density of the sample at 620 nm and A_{652} is the optical density of the sample at 652 nm.

2.5. Chlorophyll analysis

A total of 200 µL of supernatant was mixed with 1,3 mL of pure methanol and then 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 concentration ($mg.L^{-1}$) was determined by using the equation (7) according to Ritchie (2006) [32]:

$$C_{chlorophyll} = (9.3443\ A_{652}) + (4.3481\ A_{665}) \quad (7)$$

2.6. Dry matter and ash content determination

All recovered fractions (permeates and retentates) were freeze-dried (Christ Alpha 1-2 LD Plus, France) then placed in pre-weighed crucible and dried at 105 °C then 550 °C until constant mass. Thus, the dry matter and ash were determined respectively. The values presented are the average of three measurements.

2.7. Total carbon and total nitrogen measurements

Total Carbon and Total Nitrogen were determined with an automated dry combustion method (Dumas method) using an elemental analyser (vario MICRO cube, Elementar Analysensysteme, Hanau, Germany).

2.8. Molecular weight distribution determination

Molecular weight distribution of the *Spirulina* extracts was performed by gel permeation chromatography on a Superdex™ Peptide 10/300 GL column (GE Healthcare, Baie-D'Urfé, Qc, Canada) using a FPLC system (Akta Avant, GE Healthcare, Baie-D'Urfé, Qc, Canada). The mobile phase consisted of 50 mM sodium phosphate buffer containing 150 mM of NaCl at pH 7.0. The column was calibrated according to the manufacturer's guidelines with proteins of known molecular weight as reference samples. Ribonuclease A (1 $g.L^{-1}$), aprotinin (1 $g.L^{-1}$),

vitamin B12 (0.1 $g.L^{-1}$) and bovine serum albumin (1 $g.L^{-1}$) (Sigma-Aldrich, Oakville, ON, Canada) were mixed together and injected (0.5 mL) within the column for the calibration. Briefly, the *Spirulina* fraction was solubilized at 1 $mg.mL^{-1}$ with the mobile phase and then injected (0.5 mL) within the column. Samples were eluted (isocratic) at a flow rate of 0.8 $mL.min^{-1}$. Proteins were detected by monitoring the absorbance at 214 nm. UNICORNTM 7.0 software was used for data collection and results analysis. The samples were analysed in duplicate. The samples freeze dried (Christ Alpha 1-2 LD Plus, France) then analysed using a FPLC method were:

- The final retentate of diafiltration experiments
- The final retentate of concentration experiments. This sample corresponds to the "concentrated permeate of diafiltration".

2.9. Description of separation of compounds

Several parameters were calculated in order to evaluate the degree of separation between a first compound (1) and a second one (2). The extent of separation (ξ) links the permeate (Y_P) and retentate (Y_R) recovery rates following the equation (8) [33] :

$$\xi_{1/2} = \text{abs}\,(Y_{R.1} \,.\, Y_{P.2} - Y_{P.1} \,.\, Y_{R.2}) \qquad (8)$$

Since the highest value of the recovery rate is 1 and the smallest value is 0, ξ varies between 1 and 0. The perfect separation will induce an extent separation equal to 1.

The separation factor (α) was defined as the ratio of recovery rates. The separation factor related to the retentate is defined as the ratio of the retentate recovery rates (Y_R) as shown in the equation (9) [34].

$$\alpha_{R.1/2} = Y_{R.1} / Y_{R.2} \qquad (9)$$

The separation factor related to the permeate is defined as the ratio of the permeate recovery rates (Y_P) using the equation (10).

$$\alpha_{P.1/2} = Y_{P.1} / Y_{P.2} \quad (10)$$

Finally, sugar or nitrogen contents of dry matter were calculated within the concentrated permeate of diafiltration.

2.10. Statistical analysis

All experiments were conducted in triplicate. Statistical analyses were carried out using the professional edition of the Statistical Package for the Social Sciences (SPSS) version 16.0 software package (SPSS Inc. Chicago, IL, USA) and expressed as means ± standard deviation (SD). ANOVA test was carried out and a p-value lower than 0.05 ($p<0.05$) was considered significant.

3. Results and discussion

3.1. Biochemical characterization and mass balance at the in/outlet of diafiltrations

The pH and conductivity of the crude extract were respectively equal to 7.27 and 3.95 $mS.cm^{-1}$. The mass volume and dynamic viscosity at 20°C were equal to 1001 $kg.m^{-3}$ and $1.6.10^{-3}$ Pa.s, respectively. The nitrogen and carbon represented 10.9 and 46.0 % of the dry matter weight. The total protein, C-PC, A-PC and chlorophyll-a concentrations into the crude extract were respectively equal to 11.78, 2.99, 3.06 and 0.030 $g.L^{-1}$ (Table 1). The presence of chlorophyll induced the green colour of samples. Ash contents of dry matter was initially equal to 9.3 % and decreased up to 2-3 % into all final retentates after diafiltrations (Data not shown).

Desalination of retentates by diafiltration may probably participate in this drop. In the course of diafiltrations, a transmission of proteins through the membranes occurred and particularly through the 0.2 µm membrane since the protein concentration of the permeate was the highest

(0.68 $g.L^{-1}$ – Table 1). Thus, 29 % of proteins were recovered within this mean raw permeate. On the contrary, few proteins passed through the 50 kDa membrane. Thus, the final retentate of 50 kDa diafiltration remained the most protein concentrated (9.99 $g.L^{-1}$) while its permeate was the lowest protein concentrated (0.19 $g.L^{-1}$). Almost the same protein concentrations (7.50 and 7.27 $g.L^{-1}$) were found, respectively, into the final retentate of the diafiltrations on 150 or 300 kDa membranes. Unfortunately, 8 to 28 % of proteins were lost (not recovered within retentate or permeate) during diafiltrations (Table 2). A part of proteins would stay into the pilot plant, that is to say traped on the membrane or piping surface (participation to the fouling), inside the pump, sensors, etc. The highest recovery rate was obtained during the diafiltration with the 50 kDa membrane (92 %) whereas the lowest (72 %) was obtained in the case of the diafiltration with the 300 kDa membrane. As mentioned later, the 300 kDa membrane seemed the most prone to the fouling since the drop of permeate fluxes was the highest. Consequently, the unrecovered proteins could participate to the fouling of this membrane and increase the resistance formed by the layer near the membrane interface.

Each diafiltration induced good rejection of chlorophyll-a (more than 99%). Thus, the blue colour has been strengthened in all the permeates and especially those from 0.2 µm as shown in the photos of table 3. Thus, permeates could be used as blue dye in food industry. Whatever the diafiltration, 87 to 88 % of chlorophyll-a were recovered, mainly within the retentates (Table 2). Global recovery rates showed no significant difference ($p \geq 0.05$) between all membranes used.

The 0.2 µm membrane showed a better performance in terms of recovery of C-PC and A-PC than the three other membranes (Table 2). This diafiltration process seems interesting for the recovery and pre-purification of *Spirulina* phycobiliproteins because it induced little loss of these biomolecules. This diafiltration step appears also more interesting because it allowed the fractionation of C-PC from A-PC. Indeed, the separation factor ($\alpha_{C\text{-}PC/A\text{-}PC}$) was the lowest for

the mean retentate of 0.2 µm (0.65) while it was the highest for its permeate (2.43) (Table 4). In other terms there was a depletion of C-PC into the 0.2 µm retentate relatively to the A-PC and an enrichment in the 0.2 µm permeate. The same conclusion was made by the extent separation parameter ($\xi_{C\text{-}PC/A\text{-}PC}$) which was equal to 0.001, 0.012, 0.041 and 0.220 for respectively diafiltrations on the 50, 150, 300 kDa and 0.2 µm membranes.

The dry matter of the concentrated permeate of 0.2 µm diafiltration was protein rich (approximately 80 % of dry matters); the content of sugars into this dry matter (1.5 %) was the highest but it remained low (Table 4). By taking a ratio of 6.27 between protein and nitrogen, it would seem that more than 80 % of this dry matter (from the concentrated permeate of 0.2 µm diafiltration) would be composed of proteins [35]. It should be noted that a difference of composition exists between mean raw permeate of diafiltration and the concentrated permeate. Indeed, during the concentration step, a part of compounds can be loose because they can foul the membrane but also because a transmission through the membrane occurs notably salts; desalination takes place during the concentration step.

Finally, the diafiltrations allowed to produce extracts where the proportion of phycocyanin among proteins was increased. Thus, antioxidant activity of the dry matter would be increased comparatively to the crude extract. Indeed, the 0.2 µm diafiltration permeate was enriched in C-PC since the separation factor ($\alpha_{C\text{-}PC/Protein}$) was equal to 1.38 against maximum 1.03 for other ones (Table 4). Retentate of 300 kDa was the most enriched in C-PC with a separation factor ($\alpha_{C\text{-}PC/Protein}$) equal to 1.25 versus maximum 1.02 for others. On the other hand, the 300 kDa permeate and 0.2 µm retentate were A-PC purified since the separation factors ($\alpha_{A\text{-}PC/Protein}$) were the highest that means respectively equal to 1.29 and 1.56.

Molecular weight distributions of concentrated permeates were obtained by size exclusion chromatography in FPLC mode (Fig. 1). Chromatogram profiles were different depending on the membrane used for the diafiltration. Indeed, the shape of curves related to the diafiltration

steps with the 50, 150 and 300 kDa membranes were almost similar but relatively different from that obtained with the 0.2 µm membrane. Except for the diafiltration with the 0.2 µm membrane, molecular weight distributions of retentates and permeates were more tight compared to the clarified crude extract. Steric exclusion chromatograms have shown that diafiltrations on 50, 150 and 300 kDa make it possible to almost completely exhaust the retentates in proteins smaller than 0.9 kDa while permeates were almost exclusively composed of proteins of less than 19 kDa (Fig. 1). Thus, molecular-weight fractionation seems to occur during the diafiltration on the 50, 150 and 300 kDa membranes. On the other hand, the diafiltration with the 0.2 µm membrane induced different results. Indeed, proteins larger than 19 kDa would pass through the membrane and the mass distribution remains widespread. UV-visible spectra directly performed on the final retentates of diafiltrations confirmed that the 0.2 µm membrane behaves differently from other membranes (Table 3). Indeed, part of the compounds which absorb between 500 and 670 nm disappears from the final 0.2 µm retentate while no loss seems to occur for the other retentates.

3.2. Performance monitoring in the course of filtrations

3.2.1. Evaluation of the hydraulic performances

All diafiltration experiments were achieved after 287 minutes (Table 1). The diafiltration process using the four membranes (50, 150, 300 kDa and 0.2 µm) was stopped with the same manner after 5 diavolumes. The recovered volume of permeate was monitored in the course of diafiltrations (Fig. 2.a). It was a quasi-linear function of time for all filtrations. So, no drastic change of production capacity occurred during operations. The 0.2 µm membrane appears as the most permeable since its mean permeate flux was equal to 119 $L.h^{-1}.m^{-2}$; 70, 105 and 65 $L.h^{-1}.m^{-2}$ for respectively the 50, 150 and 300 kDa membranes. Interestingly, the mean permeate flux of the 300 kDa membrane was the lowest and close to the 50 kDa one whereas the

molecular weight cut-off of membranes is relatively different. In fact, the polarisation concentration and/or the fouling of the membrane could control the filtration behaviour and induce almost similar permeate fluxes.

The permeate volumes obtained during the concentration step on a 5 kDa membrane were also monitored (Fig. 2.b). All concentration operations were stopped after the same volume reduction factor. The permeate volumes were not proportional to the time since the slopes of the curves decreased showing the presence of a fouling and/or a change in the polarisation concentration from the beginning to the end of the concentration. Interestingly, the mean permeate fluxes were the highest (61 – 63 $L.h^{-1}.m^{-2}$) for the concentration step of the permeates coming from the 50 kDa and 300 kDa diafiltrations. These diafiltration membranes would allow producing the permeate with the lowest fouling potential of the 5 kDa membrane. On the other hand, the mean permeate fluxes ranged between 44 and 48 $L.h^{-1}.m^{-2}$ during the concentration step of the permeates obtained after diafiltration on 150 kDa and 0.2 µm membranes.

3.2.2. Evaluation of the selectivity performances

The conductivities of retentates were also monitored in the course of each diafiltration (Fig. 3). Initially, it was equal to 3.9 $mS.cm^{-1}$ for the crude extract. The retentate conductivities decreased as diafiltration progressed and finally reached around 157 to 244 $\mu S.cm^{-1}$. During the diafiltrations, pH remained relatively constant between 7.1 and 7.4 (Fig. 3). Almost similar retentate conductivity was reached at the end of the 50 and 300 kDa diafiltrations as well as 150 kDa and 0.2 µm ones. Diafiltrations on 150 kDa and 0.2 µm as well as 50 and 300 kDa membranes seem again to behave very closely. Charged compounds could preferentially be retained by the latter two membranes.

The protein normalized concentrations and retentions were determined throughout diafiltrations (Fig. 4). Protein normalized concentrations of retentates ranged from 48 to 90 %. Whatever the diafiltration, the protein concentrations of retentate were the highest at the beginning of the filtration and then decreased; at the beginning, a part of proteins could clog the membrane and/or pass through the membrane. Interestingly, it should be noted that the rejection rates increased while diafiltrations progressed may be because small compounds were already passed through the membrane or because the fouling of the membrane induced a more impermeable barrier than the membrane itself. It could be interesting to carry out backwashing during diafiltration in order to know if the selectivity performances could be maintained in the course of diafiltrations and consequently if a larger portion of proteins could pass through the membrane. The diafiltration with the membrane having the lowest molecular weight cut-off (50 kDa) induced the highest protein concentrations into the retentate while the permeate was the least concentrated. Consequently, the mean rejection rate was the highest (97.3 %). The highest drop of protein concentration into the retentate concerned the diafiltration on the 0.2 µm. This operating condition would allow let pass the highest quantity of proteins through the membrane. As shown into the figure 4, the protein concentrations into the retentates of all diafiltrations seemed to more or less stabilize after 3 diavolumes. To pursue the diafiltration beyond this level could not be judicious since few proteins would pass through the membranes. In the same way, A-PC was also monitored during diafiltrations (Fig. 5). Rejection rates increased in the course of diafiltrations. A-PC concentrations were globally the lowest for the 300 kDa retentate. As reminder the permeate fluxes were also the lowest for this membrane (Fig. 2). Consequently, these compounds could participate to the fouling of the membrane that's why they are found in small quantities in the retentate; they could be in significant quantity on the surface of the membrane. After a sharp decrease of A-PC concentration in the 300 kDa

retentate, a slight increase would occur. This increase could be due to a release of A-PC foulants into the retentate.

Normalized concentrations (C_R/C_{R0}) of C-PC in the retentate were ranged from 50-66, 55-77, 55-87, 47-60 %, for respectively the 50, 150, 300 kDa and 0.2 µm membranes (Fig. 6). The 0.2 µm permeate was always the most C-PC rich which induces the lowest retention rate. This membrane seems very interesting in order to fractionate proteins and more specifically to isolate the A-PC into the retentate. Unfortunately, the passage of C-PC through the membrane decreased during the diafiltration. It may be interesting to carry out backwashing to maintain the membrane in the initial state of filtration in order to keep the C-PC rejection rates as low as possible. Finally, in view of the results of the transmission rates of C-PC, A-PC, proteins through the membranes and notably 0.2 µm membrane, one can ask whether it is economically sustainable to continue the diafiltrations beyond 3 diavolumes because from this, protein, A-PC and C-PC transmission is low.

4. Conclusion

This study demonstrated the potentiality of membrane diafiltration process for the fractionation of water-soluble proteins from *A. platensis* after cell disruption by bead milling. Diafiltration with the 0.2 µm membrane proved to be extremely useful and satisfactory alternative in obtaining phycobiliproteins (C-PC and A-PC) enriched fractions free from chlorophyll. Protein recovery and production of various water soluble protein fractions with different sizes, purity and concentration levels was obtained. Several concentrated filtrates could be valorised in food formulation, notably as natural colorant. Nevertheless, future studies will be led in order to demonstrate that several fractions have specific bioactivities and technofunctional properties.

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Credit authorship contribution statement

R. Balti : Conceptualization, Methodology, Validation, Investigation, Formal analysis, Writing - original Draft, N. Zayoud : Investigation, F. Hubert : Investigation, L. Beaulieu : Investigation, Writing, Anthony Massé : Conceptualization, Methodology, Resources, Investigation, Writing, Supervision

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Statement of informed consent

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

Declaration of author's agreement to authorship and submission

All authors have agreed to authorship and the submission of this manuscript for peer review.

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