

Understanding the effect of cell disruption methods on the diffusion of Chlorella vulgaris proteins and pigments in the aqueous phase

Carl Safi, Christine Frances, Alina Violeta Ursu, Céline Laroche, Cécile Pouzet, Carlos Vaca-Garcia, Pierre-Yves Pontalier

▶ **To cite this version:**

Carl Safi, Christine Frances, Alina Violeta Ursu, Céline Laroche, Cécile Pouzet, et al.. Understanding the effect of cell disruption methods on the diffusion of Chlorella vulgaris proteins and pigments in the aqueous phase. Algal Research - Biomass, Biofuels and Bioproducts, 2015, 8, pp.61-68. 10.1016/j.algal.2015.01.002 . hal-01658654

HAL Id: hal-01658654
https://uca.hal.science/hal-01658654

Submitted on 9 Nov 2018

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.

OATAO

Open Archive Toulouse Archive Ouverte

OATAO is an open access repository that collects the work of Toulouse researchers and makes it freely available over the web where possible

This is an author's version published in: http://oatao.univ-toulouse.fr/20610

Official URL:

https://doi.org/10.1016/j.algal.2015.01.002

To cite this version:

Safi, Carl and Frances, Christine and Ursu, Alina Violeta and Laroche, Céline and Pouzet, Cécile and Vaca-Garcia, Carlos and Pontalier, Pierre-Yves *Understanding the effect of cell disruption methods on the diffusion of Chlorella vulgaris proteins and pigments in the aqueous phase.* (2015) Algal Research, 8. 61-68. ISSN 2211-9264

Any correspondence concerning this service should be sent to the repository administrator: tech-oatao@listes-diff.inp-toulouse.fr

Understanding the effect of cell disruption methods on the diffusion of *Chlorella vulgaris* proteins and pigments in the aqueous phase

Carl Safi [a,b,*], Christine Frances [c,d], Alina Violeta Ursu [e], Céline Laroche [e], Cécile Pouzet [f], Carlos Vaca-Garcia [a,b], Pierre-Yves Pontalier [a,b]

[a] *Université de Toulouse, INP-ENSIACET, LCA (Laboratoire de Chimie Agro-industrielle), 31030 Toulouse, France*
[b] *INRA, UMR 1010 CAI, F-31030 Toulouse, France*
[c] *Université de Toulouse, INP, UPS, LGC (Laboratoire de Génie Chimique), B.P. 84234, 4, Allée Emile Monso, F-31432 Cedex 4, France*
[d] *CNRS, LGC,F-31432 Cedex 4, France*
[e] *Université Blaise Pascal, Polytech, Institut Pascal UMR CNRS 6022, 24, Avenue des Landais, 63174 Aubière, France*
[f] *Fédération de Recherche 3450, Plateforme Imagerie, Pôle de Biotechnologie Végétale, F-31320Castanet-Tolosan, France*

Keywords:
Cell wall
Ultrastructure
Release kinetics
Aqueous medium
Cell disruption

A B S T R A C T

Cell disruption of microalgae is usually evaluated by microscopic observations and quantification of the target molecules before and after cell disruption. The following study considers a new approach by analysing the diffusion behaviour of proteins and pigments of *Chlorella vulgaris* in an aqueous medium after applying different cell disruption methods. Results were revealed by microscopic observations, quantifying the concentration of the molecules of interest, and calculating their diffusion coefficient. Microscopic observations showed intact cells after applying chemical hydrolysis and ultrasonication. However, the majority of the cells lost their globular shape after bead milling and high-pressure homogenization. The protein concentration increased in the following order: ultrasonication < chemical hydrolysis < high-pressure homogenization < bead milling. Conversely, their diffusion increased in a different order: chemical hydrolysis > bead milling > ultrasonication > high-pressure homogenization. Pigments were not detected in the aqueous phase of the chemical hydrolysis treatment, but their concentration and their diffusion were in the same order as proteins in the mechanical treatments. The study implied that diffusivity of the target molecules was not directly correlated to their increase concentration in the aqueous phase. Therefore, even if the cells were completely broken, diffusivity followed the hindered molecule diffusion phenomenon, which implies that somehow cells are not completely disrupted.

1. Introduction

Intensive research on biofuel production from microalgae over several years is still hindered by high overall production costs, making it uncompetitive in the market, and it'sunsustainable according to several life-cycle assessments (LCA) [1–3]. Finding a solution to reduce production costs has become preponderant. Current thought agrees that microalgae technology would be more beneficial if they were completely valorised within a bio-refinery[4–7]. Species selection is a major criterion and one of the species that has drawn attention during the last century is *Chlorella vulgaris*.

This unicellular microscopic species with a mean diameter ranging from 2 to 10 μm [8] is easy to grow, multiplies rapidly, is resistant to harsh conditions and contamination, and has a variety of highly added-value components. Its high protein content has revealed *C. vulgaris* as an unconventional protein source. Comparison of its protein content and quality (essential and non-essential amino acids) to reference food proteins (eggs, soya, meat) recommended by World Health Organisation [9] and Food and Agriculture Organization [10] has been highly favourable [11,12].

Proteins are located in different parts of the cells: they represent part of the cell wall as well as the cytoplasm, the chloroplast and all the organelles inside the barrier of the cell wall. When *C. vulgaris* is grown under favourable conditions, it is capable of accumulating 1–2% chlorophyll of its dry weight, which gives it the dense green colour that masks the colour of less concentrated pigments, such as astaxanthin and other carotenoids [13]. These pigments are located in the thylakoids (chlorophyll and some carotenoids) of the chloroplast and some (β-carotene) are associated with the lipid droplets synthesised in the chloroplast [14].

C. vulgaris has a rigid cell wall, mainly composed of cellulose, β1–3 glucan, glucosamine, proteins, lipids and ash [15]. As in terrestrial plants, the most common skeletal polysaccharide is cellulose, but during maturation, the cell wall gradually increases in thickness reaching 17–21 nm [16] where a microfibrillar layer is detected, which represents a chitosan-like layer [17] that brings additional rigidity to its cell

* Corresponding author at: Université de Toulouse, INP-ENSIACET, LCA (Laboratoire de Chimie Agro-industrielle), 31030 Toulouse, France.
E-mail address: carl.safi@wur.nl (C. Safi).

wall and improves resistance. Thickness and composition are not constant because the change is based on different growth and environmental conditions [18].

Many studies have used different methods to break the cell wall of *C. vulgaris*, such as bead milling [19], ultrasonication [20], lysing buffer [21], high-pressure homogenization [22], microwaves [23], enzymatic treatment [24] and surfactants [25] in order to free internal components, such as lipids or proteins [26]. These studies assessed the efficiency of cell disruption by quantifying target molecules before and after cell disruption. However, to our knowledge, understanding the diffusion behaviour of the microalgal target molecules has not been cited in the literature. The role of the ultrastructure of cells over the release of a specific component after cell disruption has been poorly investigated except by Jubeau et al. [27], who considered this approach for the release of proteins and phycoerythrin from *Porphyridium cruentum* after applying high-pressure homogenization.

This study aimed to understand the diffusion behaviour of proteins and pigments (chlorophylls and carotenoids) in the aqueous phase after applying different cell disruption methods. Pigment quantification was used as a marker for chloroplast alteration in order to explore the effect of each cell disruption technique on the integrity of the cell wall as well as the *C. vulgaris* chloroplast. The methods applied were chemical hydrolysis, ultrasonication, bead milling and high-pressure homogenization.

2. Materials and methods

2.1. Microalga and materials

Sueoka culture medium was used for growing *C. vulgaris* (strain SAG 211–19) in batch mode in an indoor tubular Air-Lift PhotoBioReactor (PBR, 10 L) at 25 °C, inoculated from a prior culture in a flat panel Air-LiftPBR (1 L). Culture homogenization was achieved by sterile air injection at the bottom of the PBR. The pH and temperature were recorded by a pH/temperature probe (Mettler Toledo SG 3253 sensor) monitored by the acquisition software LabVIEW. pH was regulated at 7.5 with CO_2 bubbling. Microalgae were harvested by centrifugation during the exponential growth phase and supplied as a frozen paste from Alpha Biotech (Asserac, France). There was no significant effect on the integrity of the cell wall after freezing. The biomass contained 28% dry matter and was composed of 55% proteins, 2% chlorophyll and 1% carotenoids.

Chemicals were purchased from a different distributor: methanol 99.9% (for pigment analysis), HCl 37% and NaOH beads (for chemical hydrolysis) from Sigma Aldrich, Lowry kit (for protein analysis) contained a prepared mixture of Lowry reagent and BSA standards. The 2 N Folin–Ciocalteu reagent was purchased from Thermo Scientific.

2.2. Chemical hydrolysis

Mother solutions were prepared with approximately 500 mL of distilled water. A few drops of 2 N NaOH were added to adjust the solution to pH 12 for maximum protein solubility. A 0.5 g sample of the freeze-dried biomass was added to 25 mL of the mother solution. The mixture was then stirred for 2 h at 40 °C. Separation of the supernatant from the pellet was conducted by centrifugation at 10,000 *g* for 10 min at 20 °C. The supernatant was then adjusted to pH 3 with 0.1 M HCl in order to precipitate the proteins. The protein isolate was collected after centrifugation at 10,000 *g* for 10 min at 20 °C and the pellet was neutralised with 0.01 M NaOH. Samples were taken for protein and pigment analysis.

2.3. Bead milling

Cells were treated in a stirred bead mill (LABSTAR-NETZCH). Disruption was conducted using 1–1.6 mm Zirconium Silicate grinding beads. Milling time for both trials was 1–60 min with a 1/13 solid water ratio (w/v). The process was performed in a batch mode. The initial cell suspension was placed into a pre-dispersion tank, and stirred at 350 rpm in order to avoid cell sedimentation and ensure good homogeneity of the solid concentration. During the runs, the suspension was pumped continuously from the tank to the mill inlet by a peristaltic pump at a flow rate of approximately 30 L/h and sent back into the dispersion tank through a cartridge maintaining the beads inside the chamber. Agitation speed of the cell suspension and the beads within the grinding chamber was 2500 rpm. The bead mill contained an integrated cooling system to prevent overheating and after 1 h of milling, maintained the temperature below 33 °C. At the end of the runs, the broken cells were recovered for further processing.

2.4. Ultrasonication

This treatment was carried out using a VC-750HV (20 kHz, probe 13 mm) ultrasonic processor where a sample of 0.5 g of dry cells was dispersed in 25 mL of distilled water. Ultrasonication time was 30 min with 5 s of ultrasonication and 15 s of resting time to prevent overheating the sample. Separation was conducted by centrifugation at 10,000 *g* for 10 min at 20 °C and the supernatant was analysed for proteins.

2.5. High-pressure cell disintegration

The "TS Haiva series, 2.2-kW" disrupter from Constant Systems Limited, Northants, UK, was used. The operating parameters were pressure (2700 bars), cell concentration (2% dry weight) and number of passages (two passages). Before treatment, cells were well mixed in distilled water to ensure good homogeneity of the sample. All the tests were performed in triplicate. After disruption, samples were centrifuged at 10,000 *g* for 10 min at 4 °C. Supernatant was collected after different contact times of the broken cells with water and then analysed for proteins and pigments.

2.6. Lowry method [28]

The procedure involved the reaction of proteins with cupric sulphate and tartar in an alkaline solution leading to the formation of tetradentate copper protein complexes. Addition of Folin–Ciocalteu reagent led to oxidation of the peptide bonds by forming molybdenum blue with the copper ions. A calibration curve was prepared using a concentration range of bovine standard albumin from 0 to 1500 μg mL^{-1}. In order to measure the protein content, 0.2 mL of each standard or samples containing the crude protein extract was removed and 1 mL of modified Lowry reagent was added to each sample. Each sample was then vortexed and incubated for exactly 10 min. After incubation, 100 μL of Folin–Ciocalteu reagent (1 N) was added and each sample was vortexed again and incubated for exactly 30 min. The blue solution was then measured at 750 nm with a UV-1800 Shimadzu spectrophotometer after being zeroed using a blank sample containing all the chemicals without the extract.

2.7. Pigment analysis

200 μL of aqueous extract was mixed with 1300 μL of pure methanol and incubated in the dark for 1 h at 45 °C. Samples were then centrifuged at 10,000 *g* for 10 min at 20 °C. The organic phase containing the pigments was recovered and analysed using the following equations [29]:

$$\text{Total chlorophyll } \mu g/mL = (19,3443 \times A652) + (4,3481 \times A665) \quad (1)$$

$$\text{Total carotenoids } \mu g/mL = 4 \times A480 \quad (2)$$

where A is the absorbance at a given wavelength.

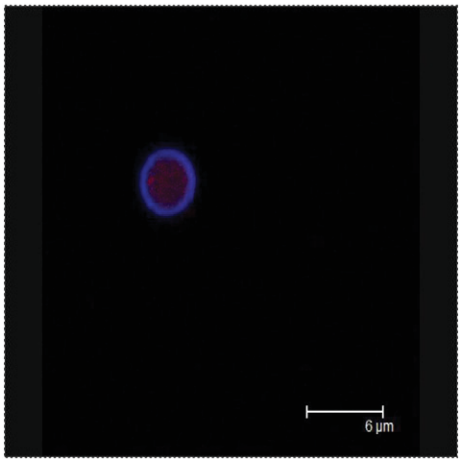


Fig. 1. Isolated cell of *Chlorella vulgaris* before applying a cell disruption treatment.

2.8. Diffusivity

Understanding the behaviour of the molecules after cell disruption is possible by evaluating experimental diffusivities. Diffusion of neutral macromolecules in a dilute solution is well described by an expression that employs the frictional coefficient of the molecule, such as the Stokes–Einstein equation. However, for biological macromolecules, such a simple equation cannot be used because these molecules show strong, non-ideal behaviour in diluted solutions [30]. However, the experimental results were similar to the models; for lysozyme, the value is approximately 1.1 10^{-10} m^2/s [31]. In the present work, it was difficult to define a standard value because our analysis was carried out on total proteins.

The global diffusion coefficient was calculated using the following equation:

$$\frac{\Delta C}{\Delta t} = D\frac{\Delta C^*}{\Delta x} \tag{3}$$

where

Δt	is the lapse time (s)
ΔC	is the concentration difference obtained over time (Δt) for a considered solute (kg/m^3)
ΔC^*	is the gradient concentration between the concentration at equilibrium in the liquid phase and the concentration at the instant t (kg/m^3)
Δx	is the length of the boundary layer (m)
D	is the diffusivity of the macromolecule (m^2/s).

Definition of the boundary layer thickness was rather difficult due to the change in cell size and hydrodynamic conditions. It was calculated using the classic equation in the case of laminar flow:

$$\Delta x = \frac{5 \cdot L}{\sqrt{Re}} \tag{4}$$

[32] where

Δx	is the length of the boundary layer (m)
L	is the diameter of the particle (m)
Re	is the Reynolds number.

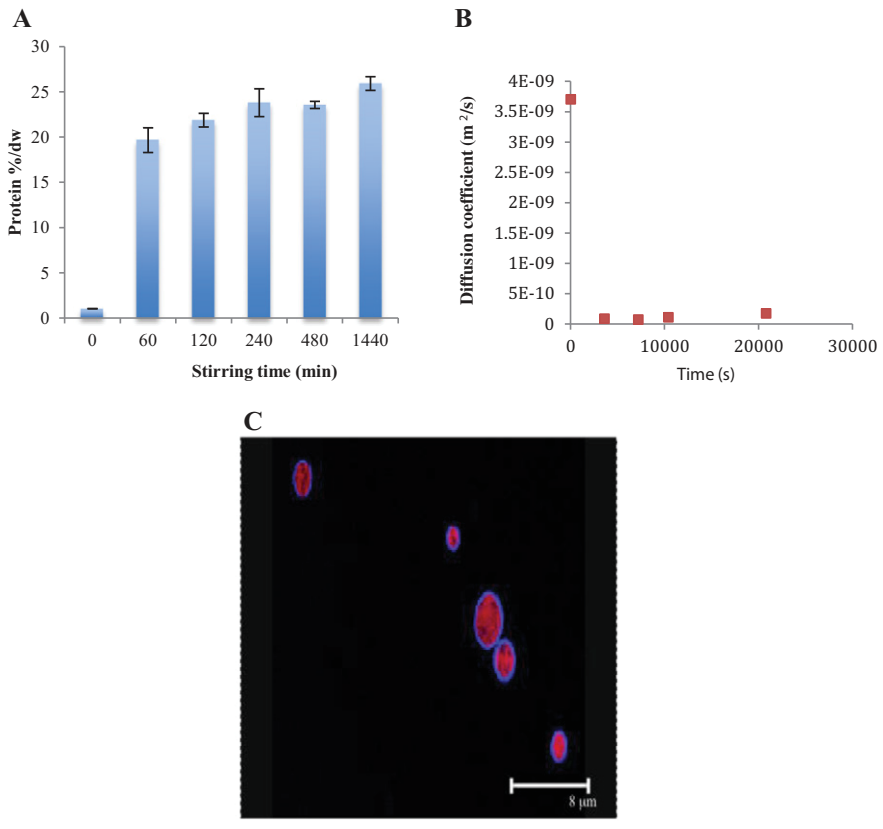


Fig. 2. Assessment of chemical hydrolysis. A — Percentage of hydro-soluble protein concentration per dry weight of biomass. B — Evolution of protein diffusion coefficients in terms of extraction time. C — Microscopic observation of the cells after treatment.

Since hydrodynamic conditions are different for all the apparatus and the cell size can change with time, an available standard condition has been defined for all the conditions with a Reynolds number of 0.2 and a particle size of 10^{-6} m. Comparison of this value between all the extraction procedures was difficult especially when the mixing of the solution was intense and when the cell size was highly reduced, as in bead milling. Diffusion was calculated for protein and, when possible, for chlorophyll and carotenoids.

2.9. Confocal microscopy

Cells were observed with an SP2-AOBS confocal laser-scanningmicroscope from Leica microsystems (Nanterre-France). Fluorochrome calcofluor white that binds to the cell wall was added to the samples. Observed at 488 nm, cell walls were light blue in colour and at 633 nm, the internal parts of the cells were red.

2.10. Statistical analysis

Triplicates of each experiment were conducted separately on *C. vulgaris* and the proteins and pigments were analysed. Statistical analyses were carried out using Statgraphics Sigma Express. An ANOVA test was carried out and measurements of three replicates for each sample were reproducible for ±5% of the respective mean values.

3. Results and discussion

The cell wall is a complex entity with unique characteristics related to the growth phase of a given microalga species: it differs in thickness, rigidity and its constituents. A microalga cannot exist long unless its body is firmly covered and its organelles possess the collective mechanical strength of the cell walls in order to ensure a defence mechanism as well as control of the intracellular and extracellular transport of molecules. The multiple variations observed in microalgal cell walls, ultrastructures and compositions distinguish them from each other. *C. vulgaris* is distinguished basically by its rigid cell wall, and its high chlorophyll and protein concentration. Conducting treatment on its cell wall is necessary to increase assimilation and bioavailability of the intracellular molecules into the extraction solvent. The unit operation of cell disruption cannot be applied without considering the integrity of the molecules of interest in the downstream process. All the techniques applied to *C. vulgaris* in this study took into consideration temperature in order to preserve molecule integrity.

Microscopic observations represented a qualitative approach to the success of the different cell disruption techniques. Before treatment, the cell wall appeared as a ring-like shape at 488 nm, coloured blue, which surrounded the internal part, which was excited at 633 nm and coloured red at this wavelength (Fig. 1). Their diameter ranged from 3 to 7 μm, which corresponded to the findings reported in the literature [16].

3.1. Chemical hydrolysis

It is estimated that 20% of *C. vulgaris* proteins are bound to the cell wall [33], and overall proteins of this species have molecular weights ranging from 12 to 120 kDa [34,35]. During chemical hydrolysis, the concentration of hydro-soluble proteins in the aqueous phase increased with increasing stirring time and reached 26 ± 0.8% of the dry weight after 24 h. This suggested that the alkaline solution slowly weakened the cell wall of *C. vulgaris* by partially penetrating its structure and by recovering the proteins bound to the cell wall. Then, it recovered some

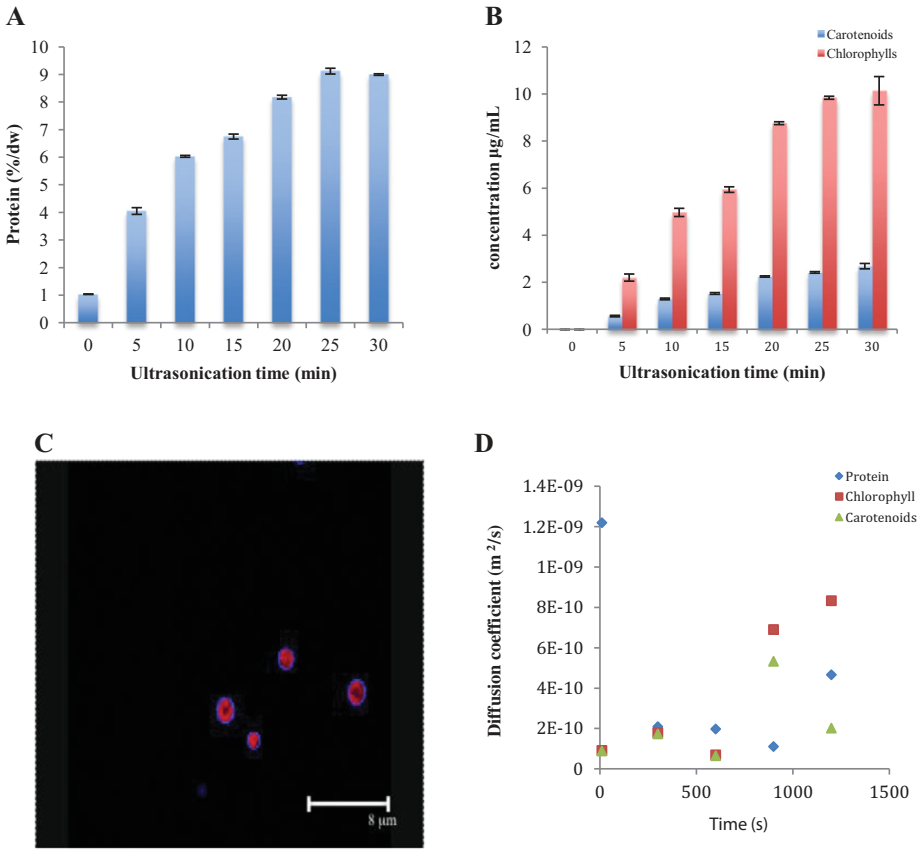


Fig. 3. Assessment of ultrasonication. A — Percentage of hydro-soluble protein concentration per dry weight of biomass. B — Quantification of pigments released in the aqueous phase. C — Microscopic observation of the cells. D — Evolution of proteins and pigment diffusion coefficients in terms of extraction time.

small sized cytoplasmic proteins that managed to pass through the pores of the weakened membrane, and hindered simultaneously the diffusion of larger size proteins. Nonetheless, taking into account the standard deviation of the three samples considered for chemical hydrolysis, the concentrations of hydro-soluble proteins obtained from 4 h to 24 h (Fig. 2A) were equivalent statistically ($p > 0.05$). The lack of pigments (Eqs. (1) and (2)) in the aqueous phase also implied that the alkaline solution was not capable of penetrating the phospholipid bilayer of the chloroplast in which pigments, such as chlorophyll and carotenoids, are embedded inside the thylakoids. Analysis of their diffusivity in the aqueous phase reinforced the previous approach to proteins. By following the evolution of protein diffusivity over time (Eqs. (3) and (4)), a first set of proteins diffused rapidly (after 10 s) in the aqueous phase (Fig. 2B), but a few minutes afterwards, protein diffusion became very slow with a very low diffusion coefficient value (4.10^{-9} m^2/s), which is lower than the diffusion coefficient of proteins in water [30, 31]. Decrease of the diffusion coefficient was also discussed when the protein concentration increased in the extract, which was not the case. A plausible explanation must consider the decrease of the gradient concentration. It appeared that the extraction lead to the solubilisation of surface proteins, which diffused rapidly in the solution. When the pool of accessible proteins decreased, the recovery rate decreased simultaneously as presented in Fig. 2. Through this assessment, sodium hydroxide supposedly did not completely hydrolyse the cell wall of *C. vulgaris*, which explained the low recovery yield as well as the lack of pigments in the solution. Such values usually describe hindered protein diffusions inside a pore, and confirmed that no cell disruption occurred due to the resistance of the rigid cell wall. Microscopic observations at different wavelengths showed intact cells that maintained their globular shape after chemical hydrolysis (Fig. 2C). This observation supported the evaluation mentioned previously for this method.

3.2. Ultrasonication

The functionality of ultrasonication dwells on creating cavitation in the cell wall. In another term, it occurs when the vapour bubbles of a liquid form in an area where pressure of the liquid is lower than its vapour pressure. These bubbles grow when the pressure is negative and compress under positive pressure, which causes a violent collapse of the bubbles. If it occurs close to cell walls, possible damage could be inflicted on the cell wall allowing the release of intracellular components [23]. Some points, such as the cell wall characteristics of the species (thickness, composition and rigidity) prohibit or contribute to the effectiveness of this technology. In this study, after 30 min of ultrasonication (Fig. 3A), the concentration of hydro-soluble proteins in the aqueous phase followed the same trend as chemical hydrolysis, but with a lower quantity of liberated proteins (9% per dry weight after 25 min). The presence of pigments (Fig. 3B) indicated that the treatment produced small cavities in the cell wall as well as in the chloroplast, allowing some pigments to penetrate through the cell membrane. In addition, by following the trend of chlorophyll release over time, the highest concentration obtained after 25 min of ultrasonication was statistically similar to the concentration obtained after 30 min($p > 0.05$), but significantly different from the concentration obtained after 20 min($p < 0.01$). Taking into account the diffusivity of all the molecules, proteins were diffused rapidly with a coefficient value of 2.10^{-9} m^2/s (Fig. 3C). This suggested that such a behaviour is linked to the diffusion of soluble proteins after cell wall disruption. After 500 s of treatment, the pigment diffusion coefficients increased (Eqs. (3) and (4)), indicating an alteration of the chloroplast membrane. Diffusion of both molecules was rapid for hydrophobic molecules, which was similar to the value obtained for proteins. Usually, these molecules are linked to the proteins in complexes that are much more hydrophilic and increase the diffusion

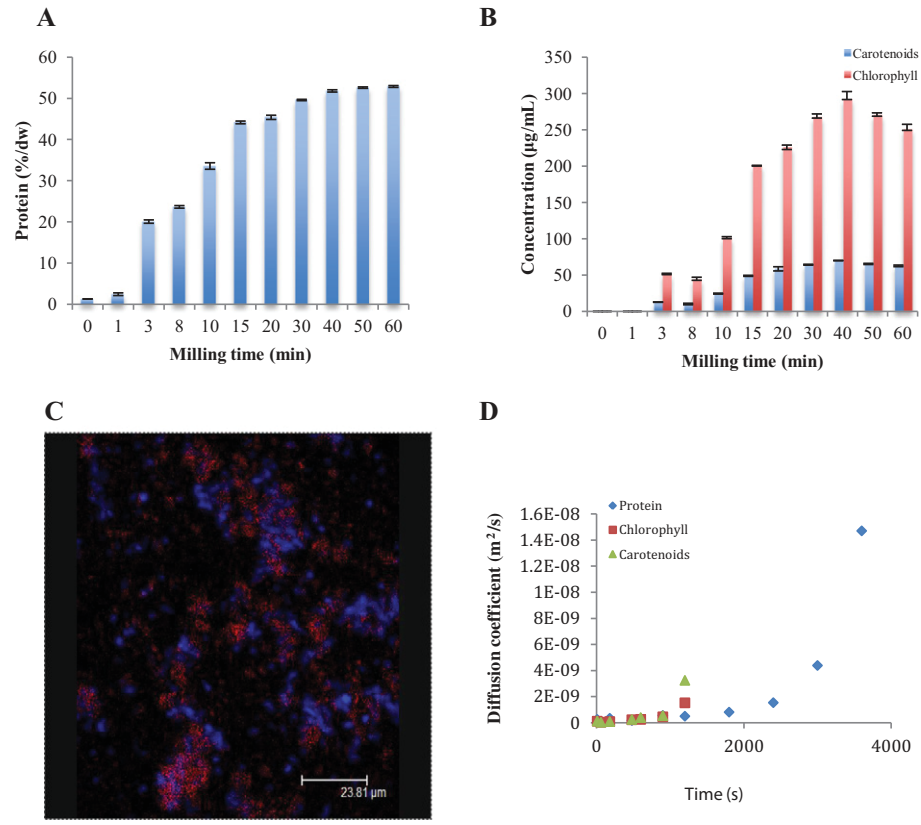


Fig. 4. Assessment of bead-milling. A — Percentage of hydro-soluble protein concentration per dry weight of biomass. B — Quantification of pigments released in the aqueous phase. C — Microscopic observation of the cells after 30 min of bead milling. D — Evolution of proteins and pigment diffusion coefficients in terms of extraction time.

rate. The diffusion coefficient of these complexes was evaluated in the cell membrane at approximately 3.10^{-14} m^2/s, but with a much faster transfer from grana to stroma in a few seconds [36].

Quantitative assessment of the results supported by microscopic observations (Fig. 3D) indicated that ultrasonication was not a reliable method to increase the recovery of the molecules of interest because this method degraded only a few cells and not the absolute majority of cells. This is consistent with the results of other studies that used this technology for cell disruption in *C. vulgaris*[37] to recover lipids for bioenergy purposes. These studies concluded that the technology was poorly effective in increasing the lipid recovery yield and breaking the cell wall. Conversely, ultrasonication was effective on other species with different cell wall characteristics, such as *Spirulina platensis*[38].

3.3. Bead milling

As expected, bead milling proved to be effective in inflicting serious damage to the cell wall of *C. vulgaris*. Protein (Fig. 4A) and pigment (Fig. 4B) concentrations started to increase after 5 min and continued to increase with bead milling time to reach a maximum recovery at 40 min (96% of proteins from total proteins). The latter was statistically equivalent to the concentrations obtained after 50 and 60 min ($p > 0.05$), which implied that water had access to the different intracellular organelles and recovered the majority of proteins after 40 min. In addition, the strong concentration of pigments (Eqs. (1) and (2)), especially chlorophyll in the aqueous phase, signalled a strong alteration of the chloroplast allowing the release of intra-thylakoid pigments. Moreover, microscopic observations revealed some broken cells after 5 min of bead milling and total disruption was observed after 30 min where cell wall debris were coloured blue and the interior fragments were coloured red having lost their blue cover. The results also indicated that the target molecules diffused rapidly out of the cells (Eqs. (3) and (4)), which signalled that the cell wall, together with the intracellular membranes, was disrupted for some cells. The diffusion coefficient remained low since it may have been hindered by the media organisation (Fig. 4C). This suggested that the concentration gradient was low and increased with respect to the increasing number of disrupted cells. Indeed, chlorophyll and carotenoids are hydrophobic pigments; their presence in the aqueous phase indicated the formation of micellar structures and an alteration of the chloroplast. The other indication is that some cell debris containing green pigment were extremely reduced in size and did not precipitate in the pellet after centrifugation at 10,000 *g*, leading to a greenish supernatant as shown in previous work [39].

This method remains highly efficient especially for microalgae with a rigid cell wall, such as *C. vulgaris*, due to the high contact surface between the microalgal cells and the beads. The setup parameters, such as bead diameter, composition and agitation speed, also play a key role for the efficiency of cell disruption.

Despite its high efficiency, and being considered as a highly energetic method, a recent study successfully broke the cells of *C. vulgaris* by bead milling, and significantly reduced the energy consumption (0.81 $kWh \cdot kg^{-1}$) by employing the optimum parameters required for an efficient cell disruption [40].

3.4. High-pressure homogenization

High-pressure cell disruption is also a reliable method for cell disruption [41,42]. It acts by applying high pressure on a piston that violently and rapidly breaks the cells on the top of the feeding chamber.

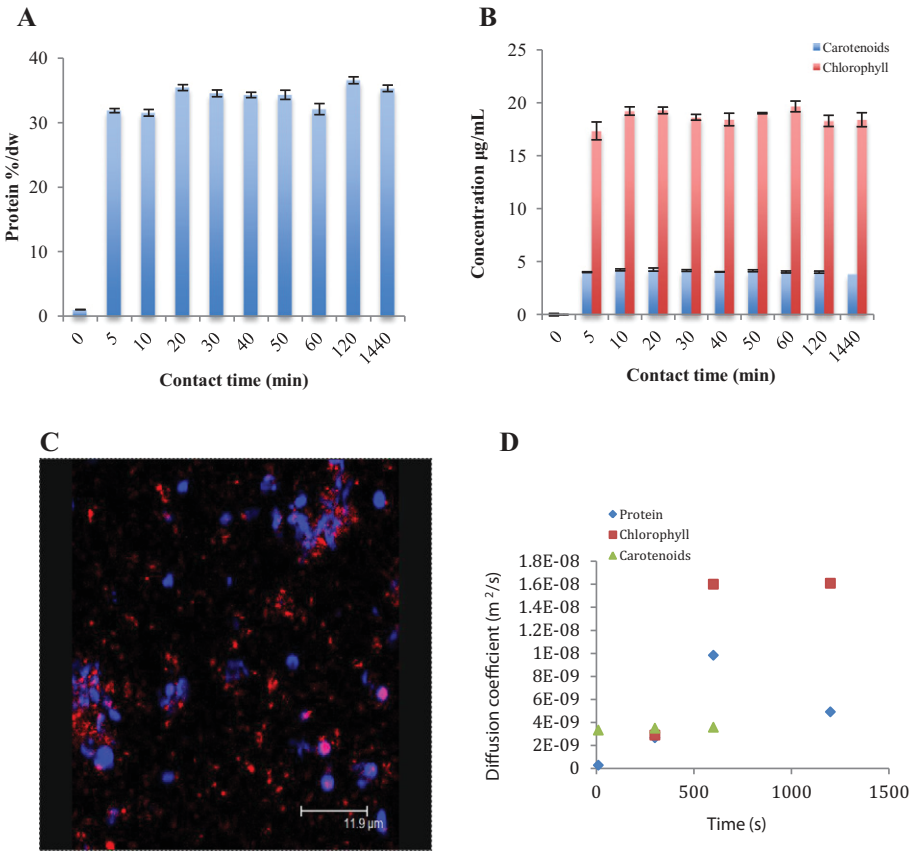


Fig. 5. Assessment of high-pressure homogenization. A — Percentage of hydro-soluble protein concentration per dry weight of biomass. B — Quantification of pigments released in the aqueous phase. C — Microscopic observation of the cells after cell disruption treatment. D — Evolution of proteins and pigment diffusion coefficients in terms of extraction time.

In this study, after two passages, water had access to cytoplasmic proteins and infiltrated the chloroplast to recover 66% of proteins from the total proteins (Fig. 5A). The presence of chlorophyll (Eq. (1)) in the aqueous phase was 12-fold lower than for bead milling (Fig. 5B), which suggested that the intensity of the chloroplast alteration was lower compared to bead milling. In addition, microscopic observations revealed that the majority of the cells were broken while some of them remained intact, which helped explain the lower concentration of proteins and pigments in the aqueous phase (Fig. 5).

Contrarily to all the methods assessed previously, the results obtained after high-pressure homogenization showed that the mechanical action of this method rapidly (300 s) increased the diffusion (Eqs. (3) and (4)) of proteins and chlorophyll out of the cells while the diffusion of carotenoids did not change significantly with contact time (Fig. 5C). Within few minutes, molecules were found in the aqueous phase, with almost unchanged concentrations with regard to contact time, and the diffusion led to zero afterwards. Cells appeared to be destroyed rapidly, allowing faster recovery of the components and metabolites; therefore, molecule transfer was not hindered by any structures. The high diffusivity coefficient values indicated that the initial hypothesis for their calculation was no longer valid. In particular, the hydrodynamic conditions for the calculation of the boundary layer were not the same during high-pressure homogenization or chemical extraction, and the particle sizes were different compared to the initial cell. In the case of extraction, mass transfer limited the recovery rate while this was not the case during high-pressure homogenization. Therefore, using this method, diffusion of the molecules seemed related to the speed of the cell disruption method, which allowed faster diffusion of the molecules in the aqueous phase compared to all the other methods tested in this study.

4. Conclusions

Bead milling was the most efficient method to break the cell wall of *C. vulgaris* and to release the target molecules into the aqueous phase. The study revealed that despite the high efficiency of a cell disruption method on breaking the cell wall and maximizing the recovery of intracellular molecules, diffusion of these molecules does not follow the same trend. The results showed that chemical hydrolysis led to a sharp decrease in the diffusion coefficient and the fastest diffusion of the target molecules occurred with high-pressure homogenization and not with bead milling. This suggested that the transfer of the molecules was no longer limited by the cell membrane regardless of the high efficiency of the cell disruption method. Mass transfer seemed to occur according to hindered internal diffusion, as if the cell disruption was not completed. This new approach towards microalgal cell disruption is still in its infancy and requires further, in-depth studies.

Acknowledgements

This work was funded by the French National Research Agency (ANR- 10 - BLAN - 0936) in the framework of the “Algoraffinerie project”. The authors are grateful to Alpha Biotech for providing the biomass.

References

[1] P. Collet, A. Hélias, L. Lardon, M. Ras, R.-A. Goy, J.-P. Steyer, Life-cycle assessment of microalgae culture coupled to biogas production, Bioresour. Technol. 102 (2011) 207–214.
[2] M.K. Lam, K.T. Lee, Microalgae biofuels: a critical review of issues, problems and the way forward, Biotechnol. Adv. 30 (2012) 673–690.
[3] A.L. Stephenson, E. Kazamia, J.S. Dennis, C.J. Howe, S.A. Scott, A.G. Smith, Life-cycle assessment of potential algal biodiesel production in the United Kingdom: a comparison of raceways and air-lift tubular bioreactors, Energy Fuel 24 (2010) 4062–4077.
[4] I. Hariskos, C. Posten, Biorefinery of microalgae — opportunities and constraints for different production scenarios, Biotechnol. J. 9 (2014) 739–752.
[5] L. Soh, M. Montazeri, B.Z. Haznedaroglu, C. Kelly, J. Peccia, M.J. Eckelman, et al., Evaluating microalgal integrated biorefinery schemes: empirical controlled growth studies and life cycle assessment, Bioresour. Technol. 151 (2014) 19–27.
[6] M. Vanthoor-Koopmans, R.H. Wijffels, M.J. Barbosa, M.H. Eppink, Biorefinery of microalgae for food and fuel, Bioresour. Technol. 135 (2013) 142–149.
[7] H.W. Yen, I.C. Hu, C.Y. Chen, S.H. Ho, D.J. Lee, J.S. Chang, Microalgae-based biorefinery—from biofuels to natural products, Bioresour. Technol. 135 (2013) 166–174.
[8] M. Yamamoto, M. Fujishita, A. Hirata, S. Kawano, Regeneration and maturation of daughter cell walls in the autospore-forming green alga *Chlorella vulgaris* (Chlorophyta, Trebouxiophyceae), J. Plant Res. 117 (2004) 257–264.
[9] WHO, Protein and Amino Acid Requirements in Human Nutrition, World Health Organisation, Geneva, 2007. 284.
[10] FAO, A Review on Culture, Production and Use of *Spirulina* as Food for Humans and Feeds for Domestic Animals and Fish, FAO Fish Aquacult, Rome, 2008. 20.
[11] E.W. Becker, Microalgae: Biotechnology and Microbiology, Cambridge University Press, Cambridge; New York, 1994.
[12] C. Safi, M. Charton, O. Pignolet, F. Silvestre, C. Vaca-Garcia, P.-Y. Pontalier, Influence of microalgae cell wall characteristics on protein extractability and determination of nitrogen-to-protein conversion factors, J. Appl. Phycol. (2012) 1–7.
[13] C. Safi, B. Zebib, O. Merah, P.-Y. Pontalier, C. Vaca-Garcia, Morphology, composition, production, processing and applications of *Chlorella vulgaris*: a review, Renew. Sust. Energ. Rev. 35 (2014) 265–278.
[14] E.P. Solomon, L.R. Berg, D.W. Martin, Biology, 5th ed. Fort Worth, Saunders College Pub., 1999
[15] M. Blumreisinger, D. Meindl, E. Loos, Cell wall composition of chlorococcal algae, Phytochemistry 22 (1983) 1603–1604.
[16] M. Yamamoto, I. Kurihara, S. Kawano, Late type of daughter cell wall synthesis in one of the Chlorellaceae, *Parachlorella kessleri* (Chlorophyta, Trebouxiophyceae), Planta 221 (2005) 766–775.
[17] N. Yvonne, K. Tomas, Cell wall development, microfibril and pyrenoid structure in type strains of *Chlorella vulgaris*, *C. kessleri*, *C. sorokiniana* compared with *C. luteoviridis* (Trebouxiophyceae, Chlorophyta), Arch. Hydrobiol. 100 (2000) 95–105.
[18] S. Liu, C. Liu, X. Huang, Y. Chai, B. Cong, Optimization of parameters for isolation of protoplasts from the Antarctic sea ice alga *Chlamydomonas* sp. ICE-L, J. Appl. Phycol. 18 (2006) 783–786.
[19] J.Y. Lee, C. Yoo, S.Y. Jun, C.Y. Ahn, H.M. Oh, Comparison of several methods for effective lipid extraction from microalgae, Bioresour. Technol. 101 (Suppl 1) (2010) S75–S77.
[20] G.S. Araujo, L.J. Matos, J.O. Fernandes, S.J. Cartaxo, L.R. Goncalves, F.A. Fernandes, et al., Extraction of lipids from microalgae by ultrasound application: prospection of the optimal extraction method, Ultrason. Sonochem. 20 (2013) 95–98.
[21] H. Packeiser, C. Lim, B. Balagurunathan, J. Wu, H. Zhao, An extremely simple and effective colony PCR procedure for bacteria, yeasts, and microalgae, Appl. Biochem. Biotechnol. 169 (2013) 695–700.
[22] S. Yamada, T. Nakamura, Y. Tanaka, Y. Isogai, T. Nishio, T. Oku, Characterization and amino acid sequences of cytochromes c(6) from two strains of the green alga *Chlorella vulgaris*, Biosci. Biotechnol. Biochem. 64 (2000) 628–632.
[23] P. Mercer, R.E. Armenta, Developments in oil extraction from microalgae, Eur. J. Lipid Sci. Technol. 113 (2011) 539–547.
[24] H.G. Gerken, B. Donohoe, E.P. Knoshaug, Enzymatic cell wall degradation of *Chlorella vulgaris* and other microalgae for biofuels production, Planta 237 (2013) 239–253.
[25] T. Coward, J.G.M. Lee, G.S. Caldwell, Harvesting microalgae by CTAB-aided foam flotation increases lipid recovery and improves fatty acid methyl ester characteristics, Biomass Bioenergy 67 (2014) 354–362.
[26] 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 Res. 3 (2014) 61–65.
[27] S. Jubeau, L. Marchal, J. Pruvost, P. Jaouen, J. Legrand, J. Fleurence, High pressure disruption: a two-step treatment for selective extraction of intracellular components from the microalga *Porphyridium cruentum*, J. Appl. Phycol. 25 (2013) 983–989.
[28] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[29] R.J. Ritchie, Consistent sets of spectrophotometric chlorophyll equations for acetone, methanol and ethanol solvents, Photosynth. Res. 89 (2006) 27–41.
[30] Y.-C.b. Kim, A. Myerson, Diffusivity of protein in aqueous solutions, Korean J. Chem. Eng. 13 (1996) 288–293.
[31] D. Brune, S. Kim, Predicting protein diffusion coefficients, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 3835–3839.
[32] H. Schlichting, K. Gersten, Boundary-layer Theory, 8th rev. and enl. ed. Springer, Berlin; New York, 2000.
[33] M.D. Berliner, Proteins in *Chlorella vulgaris*, Microbios 46 (1986) 199–203.
[34] M.K. Hanan, Comparative effects of autotrophic and heterotrophic growth on some vitamins, 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity, amino acids and protein profile of *Chlorella vulgaris* Beijerinck, Afr. J. Biotechnol. 10 (2011) 13514–13519.
[35] H.J. Morris, O.V. Carrillo, Á. Almarales, R.C. Bermúdez, M.E. Alonso, L. Borges, et al., Protein hydrolysates from the alga *Chlorella vulgaris* 87/1 with potentialities in immunonutrition, Biotecnol. Apl. 26 (2009) 162–165.
[36] H. Kirchhoff, S. Haferkamp, J.F. Allen, D.B. Epstein, C.W. Mullineaux, Protein diffusion and macromolecular crowding in thylakoid membranes, Plant Physiol. 146 (2008) 1571–1578.
[37] R. Halim, R. Harun, M.K. Danquah, P.A. Webley, Microalgal cell disruption for biofuel development, Appl. Energy 91 (2012) 116–121.

[38] T. Furuki, S. Maeda, S. Imajo, T. Hiroi, T. Amaya, T. Hirokawa, et al., Rapid and selective extraction of phycocyanin from *Spirulina platensis* with ultrasonic cell disruption, J. Appl. Phycol. 15 (2003) 319–324.
[39] J.A. Gerde, M. Montalbo-Lomboy, L. Yao, D. Grewell, T. Wang, Evaluation of microalgae cell disruption by ultrasonic treatment, Bioresour. Technol. 125 (2012) 175–181.
[40] 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, Bioresour, Technol, 2014.
[41] H. Komaki, M. Yamashita, Y. Niwa, Y. Tanaka, N. Kamiya, Y. Ando, et al., The effect of processing of *Chlorella vulgaris*: K-5 on in vitro and in vivo digestibility in rats, Anim. Feed Sci. Technol. 70 (1998) 363–366.
[42] N. Samarasinghe, S. Fernando, R. Lacey, W.B. Faulkner, Algal cell rupture using high pressure homogenization as a prelude to oil extraction, Renew. Energy 48 (2012) 300–308.