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Application of ultrasound for green extraction of proteins from spirulina. Mechanism, optimization, modeling, and industrial prospects

L. Vernès[a], M. Abert-Vian[a], M. El Maâtaoui[b], Y. Tao[c], I. Bornard[d], F. Chemat[a,*]

[a] Avignon University, UMR 408, Green Extraction Team, 84000 Avignon, France
[b] Avignon University, Qualisud UMR95, 84000 Avignon, France
[c] College of Food Science and Technology, Nanjing Agricultural University, Nanjing 210095, China
[d] INRA PACA, Pathologie végétale, 84143 Montfavet, France

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ABSTRACT

A green and innovative method, manothermosonication (MTS), for proteins extraction from dry *Arthrospira platensis* cyanobacteria assisted by ultrasound was designed in this work. Manothermosonication (probe, 20 kHz) was compared to a conventional process performed in the same conditions without ultrasounds. The extraction was carried out with a continuous flow rate at 15 mL/hour. Extraction parameters were optimized using a central composite design. Moreover, mathematic modelling and microscopic investigations were realized to allow a better understanding of ultrasound physical and structural effects on spirulina filaments and mass transfer phenomena over time. Crude extract and sections stained with toluidine blue were analyzed with optical and scanning electron microscopies. According to experimental results, MTS promoted mass transfer (high effective diffusivity, D_e) and enabled to get 229% more proteins (28.42 ± 1.15 g/100 g DW) than conventional process without ultrasound (8.63 ± 1.15 g/100 g DW). With 28.42 g of proteins per 100 g of dry spirulina biomass in the extract, a protein recovery rate of 50% was achieved in 6 effective minutes with a continuous MTS process. Microscopic observations showed that acoustic cavitation impacted spirulina filaments by different mechanisms such as fragmentation, sonoporation, detexturation. These various phenomena make extraction, release and solubilization of spirulina bioactive compounds easier.

1. Introduction

Microalgae are unicellular photosynthetic organisms, basis of the food chain in the sea. This is a group of heterogeneous microorganisms having a great biodiversity of colors, shapes, and cell characteristics with a size of approximately 3–10 µm [1]. The term microalga includes prokaryotic and eukaryotic organisms, which can grow in fresh or salt water. With their extraordinary biodiversity, more than 50,000 species [2], microalgae represent an important resource with a wide range of active biomolecules [3]. These autotrophic microorganisms use light energy and inorganic nutrients (carbon dioxide, nitrogen, phosphorus, etc.) to develop and synthesize biocompounds with high aggregated nutritional value and therapeutic functions, like lipids, proteins, carbohydrates, pigments, and polymers. Only few species of microalgae are authorized for human consumption and have exceptional nutritional properties such as spirulina or *Arthrospira platensis*, Chlorella or *Chlorella vulgaris*, *Dunaliella*, *Aphanizomenon*, and *Nostoc* [4]. These micro-organisms are announced as major elements in the challenge of global nutrition, thanks to their high protein content and their large-scale and low-cost development.

In recent years, spirulina has attracted the attention of many scientists. It is a non-toxic prokaryotic cyanobacterium with the advantage of containing high value-added compounds such as proteins (50–70% of its dry weight [5]). In 1981, Spirulina was approved by the FDA (Food Drug Administration) by the issuance of a GRAS (generally recognized as safe) certificate. Spirulina is legally marketed as a food or food supplement without risk to human health. Due to its high nutritional value and the presence of active biomolecules, this microorganism is one of the most studied microalgae worldwide [1]. Spirulina represents the second most important commercial microalga in term of US$ after chlorella, however spirulina is more produced than chlorella in terms of total biomass [6]. Spirulina grows in natural environments characterized by brackish, alkaline (8 < pH < 11.5) and natroned waters (highly concentrated in carbonates and bicarbonates) of the intertropical zone. It develops in warm conditions (28–40 °C), with a high light intensity [7]. Commercial production of spirulina has long been

* Corresponding author.
E-mail address: farid.chemat@univ-avignon.fr (F. Chemat).

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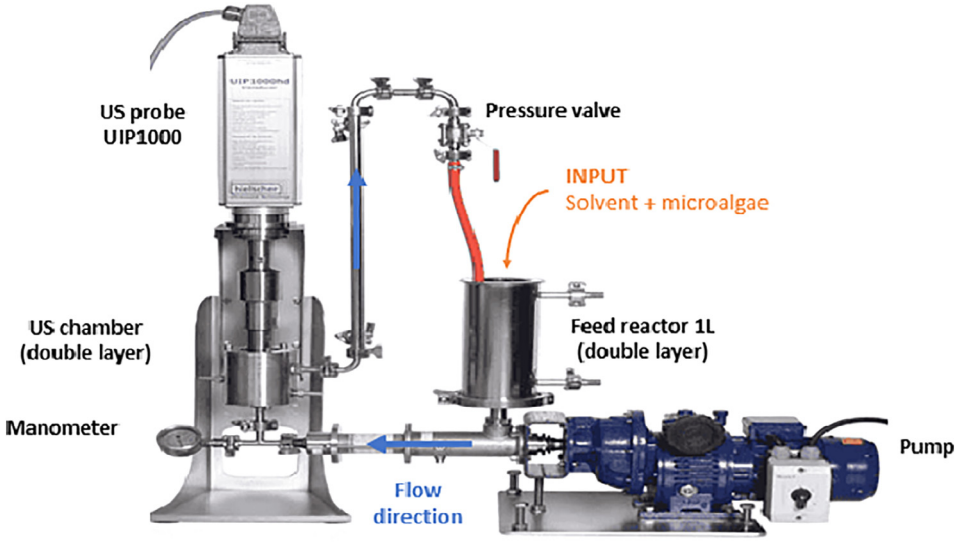

Fig. 1. Manothermosonication (MTS) montage at the laboratory scale.

implemented in open ponds, mostly under greenhouse, in shallow water, continuously brewed but it is also cultivated in closed photobioreactors (PBR). Photobioreactor refers to closed equipment which provides a controlled environment and results in high productivity of algae thanks to culture parameters optimization such as carbon dioxide supply, water supply, optimal temperature, efficient light intensity… [8].

Spirulina biomolecules are particularly desirable for extraction due to their numerous properties and applications. Solid-liquid extraction of water-soluble bioactive compounds is generally carried out by maceration in aqueous solvents. However, this technique requires long extraction time, large amount of solvent and does not lead to an optimal extraction yield [9]. Therefore, it seems important to optimize extraction parameters of spirulina molecules to obtain a fast, efficient, low cost, and environmentally friendly process for a further scale-up [10]. The optimization of protein extraction requires cell wall breakdown, which is very resistant in the case of microalgae. Cell disruption represent a key step essential to improve intracellular compounds extraction efficiency, since it facilitates solvent access to targeted compounds. The different membrane breaking technologies are grouped into three categories: 1) mechanical methods such as beads milling or high pressure, freezing/thawing, pulsed electric field [11], use of ultrasound [12]; 2) chemical methods involving the use of organic acids for example and 3) biochemical methods of lysis such as those based on the use of enzymes [13]. The advantage of ultrasound is that it is possible to damage the cell wall while allowing the release of bioactive compounds in the solvent. Ultrasound can be used as pretreatment to permeabilize the cell wall or as extraction technique. The use of ultrasound has already proved to be effective in plant extraction to target substances of interest such as polyphenols [14] and is considered as an alternative to conventional extraction methods. Ultrasonic waves propagating in a solvent cause a cavitation phenomenon, which is characterized by violent collapse of bubbles in an alternating pressure field. Acoustic cavitation bubbles in microalgae aqueous suspension produces severe and localized short-term pressure increase as well as microstreaming effects (movement of liquid around gas bubbles formed by cavitation) and shock waves that promote the rupture of microalgae cells. Some authors have reported the use of ultrasound in the extraction of numerous bioactive substances such as carotenoids [15,16] and phycobiliproteins from different species of microalgae [17,18].

To intensify water-soluble compounds extraction from spirulina, pressure and temperature were added to ultrasounds intensity. The combination of these three parameters is called the manothermosonication (MTS). In this study, MTS has been selected as a new extraction technique for its effectiveness compared to conventional ultrasonic extraction techniques. The assumption is made that effect of pressure and temperature combined with ultrasound leads to a better cell disruption and increase mass transfer phenomena (increase of effective diffusivity) compared to ultrasound alone. Thanks to MTS compounds of interest could become more bioavailable and the extraction yield could be increased. The use of MTS as extraction technique is considered as a novelty since MTS was mainly known for its application in food preservation. For instance, MTS was employed to inactivate some bacteria such as *Escherichia coli* from food products [19] and only one article mentioned the use of MTS as extraction technique applied to yeast [20]. The present study is the first reporting the use of MTS as extraction tool from microalgae.

In this context, manothermosonication (MTS) process was used to obtain proteins from *Arthrospira platensis*. The aim of this study was to optimize protein extraction from spirulina thanks to MTS process and to better understand physical and structural effects of ultrasound on cell and mass transfer phenomenon thanks to microscopic analysis and mathematic modeling. Once the process optimized at laboratory scale, industrialization tracks will be proposed to support a further process scale up.

2. Material and methods

2.1. Microalgae

Arthrospira platensis (spirulina) was obtained from Cyane (France) in dry powder form and was stored in sealed, air tight packages at room temperature in the dark prior to use. The average granulometry of the powder was a diameter of 70 μm and the Activity water (Aw) was 0.321 at 25.1 °C.

2.2. Ultrasonic apparatus

Sonication and manothermosonication (MTS) were performed in a continuous ultrasonic apparatus (Fig. 1). The ultrasonic device was used at low frequencies (20 kHz) with a 1000 W ultrasonic processor (UIP1000hd, Hielscher Ultrasonics, GmbH, Germany) equipped with a versatile power meter. The apparatus consisted of a stainless-steel cylindrical feed reactor of 1 L linked to a SEEPEX pump to insure the flow of microalgae in the system and connected to a stainless-steel cylindrical ultrasound chamber connected to a sonication horn (BS2d34

standard titanium sonotrode, Hielscher Ultrasonics). The treatment chamber (250 mL) and the feed reactor (1 L) were surrounded by a double layer with a water circulation to control the temperature thanks to a chiller/heater module system (Huber, GmbH, Germany). A manual pressure valve was connected between the output of the ultrasonic reactor and the feed reactor to maintain the desired pressure due to the perilstatic pump. A manometer was connected to the output of the pump to control the level of the pressure upstream of the US chamber.

Microalgae flow rate was regulated by adjusting speed of the peristaltic SEEPEX pump. Considering the actual input, power from the device is converted to heat which dissipated in the medium. Calorimetric measurements were performed to assess actual ultrasound power P ($J{\cdot}min^{-1}$), calculated by the following (Eq. (1)).

$$P = m \times Cp \times \frac{dT}{dt} \quad (1)$$

where Cp is the heat capacity of the solvent at constant pressure (for the water: $C_p = 4180\ J.K^{-1}{\cdot}kg^{-1}$), m is the mass of solvent (kg) and dT/dt is temperature increase per minutes ($K{\cdot}min^{-1}$).

Then, the applied ultrasonic intensity (UI) was determined using the calculated power as shown in the (Eq. (2))

$$UI = \frac{4P}{\pi D^2} \quad (2)$$

where UI is the ultrasonic intensity (W/cm^2), P is the ultrasound power (W) as calculated by Eq. (1) and D is the internal diameter (cm) of ultrasonic probe.

2.3. MTS protein extraction

The extraction was conducted by mixing spirulina and solvent at the ratio biomass/solvent 1:20 (g/g): 40 g of spirulina powder were mixed with 760 g of sodium phosphate buffer (0.1 M; pH 7.0). A mechanical stirring (IKA, Eurostar) was used during the extraction process to avoid microalgae sedimentation in the system. The pump flow rate was set at 15 mL/h (speed 5). With this flow rate, transit time in the US chamber is calculated as in the (Eq. (3)) below:

$$t = \frac{V}{Q} \quad (3)$$

where t, is the transit time in the US chamber (s), V the US chamber's volume (mL) and Q the pump flow rate (mL/s).

For one cycle with a 15 mL/s flow rate, the transit time in the US chamber is approximately 15 s. After extraction, samples were centrifuged at 8000 rpm for 10 min and the supernatant was collected for further analysis.

The reference (conventional treatment) was carried out in the same continuous system, with the same extraction parameters except ultrasound (UI = 0 W/cm^2).

2.4. Protein quantification and aminogram

Protein were quantified according to the method of Lowry [21] as modified by Price [22]: 40 µL of each standard and sample replicates were introduced into microplate wells, then 200 µL of Modified Lowry Reagent (Thermo Fischer Scientific) were added to each well at nearly the same moment using a multi-channel pipettor. Immediately after, microplate was mixed on plate mixer for 30 s. The microplate was then covered and incubated at room temperature (RT) for exactly 10 min. Finally, 20 µL of prepared 1X Folin-Ciocalteu Reagent (1:1 ratio of 2 N Folin-Ciocalteu phenol regent: ultra-pure water) was added to each well. Microplate was mixed on plate mixer for 30 s and was incubated at RT for 30 min. The absorbance was measured at 750 nm on the plate reader (FLUOstar Omega, BMG LABTECH). Average 750 nm absorbance value of the blank standard replicates was subtracted from the 750 nm value of all other individual standard and sample replicates using Mars software for data analysis. Calibration curves were prepared for each assay with bovine serum albumin (BSA) stock solution (2000 µg/mL) and using a polynomial line of best fit. The standard curve's equation was used to determine the protein concentration of each sample. Then protein content (%) was calculated following (Eq. (4)).

$$\text{Protein}(\%) = \frac{Protein\ concentration * V}{m} \times 100 \quad (4)$$

where V is the extract volume (in mL) and m, the masse of dry spirulina used for the extraction (in g).

Protein recovery rate is calculated according to (Eq. (5)).

$$\text{Protein recovery rate}(\%) = \frac{Protein\ extracted}{Total\ protein\ in\ the\ initial\ biomass} \times 100 \quad (5)$$

Aminogram was performed by Eurofins Analytics France according to ISO 13903:2005 method.

2.5. C-phycocyanin analysis by spectrophotometry

Raw extracts were centrifuged, and the optical density of the supernatant was determined spectrophotometrically at 620 and 652 nm using Biochrom Libra S22 UV/Vis Spectrophotometer. C-phycocyanin concentration was calculated according to (Eq. (6)) [23]:

$$CPC = \frac{OD620 - 0.474 * OD652}{5.34} \quad (6)$$

where PC is the C-phycocyanin concentration (mg/mL), OD 620 is the optical density of the sample at 620 nm, and OD 652 is the optical density of the sample at 652 nm.

Extraction yield was calculated according to the following equation:

$$\text{Yield} = \frac{CPC * V}{DB} \quad (7)$$

where Yield is the extraction yield of phycocyanin in mg of C-phycocyanin per gram of dry biomass (DB, g), V is the solvent volume (mL).

2.6. Experimental design

The further investigation of the performance of UAE was obtained by the response surface methodology (RSM), using Statgraphics software (Rockville, USA, 2000). To evaluate the relevance and influence of operating parameters on extractions, a central composite design (CDD) was used to identify the optimal parameters for protein extraction from spirulina. The influence of three controlled factors, namely ultrasonic intensity (UI), temperature (T) and pressure (P), was evaluated. A prestudy was done to select other optimal parameters for protein extraction such as solvent nature, pH and biomass/solvent ratio (g/g). These parameters were selected experimentally in accordance with scientific literature sources [24]. The CDD application consists of a two-level full factorial design (coded ± 1), superimposed by center points (coded 0) and star points (coded ± α) located on variable axes at a distance α (α = 1 in this case) from the center (Table 1). Limit values of each variable range were chosen as function of ultrasonic apparatus limitations (to high ultrasound amplitude might damage the probe and reduce efficiency), protein extraction temperature (which might degrade

Table 1
Experimental design parameters.

	Parameter	Low (−1)	High (+1)	−α	+α	Center	Step	Unit
M	Pressure	1	3	0.318	3.682	2	1	bar
T	Temperature	10	30	3.18	36.82	20	10	°C
S	Ultrasonic intensity	20	60	6.36	73.64	40	20	W/cm^2

above 40 °C) and pressure depending on the valve position and pipe resistance of the set-up as presented in Table 1. This experimental design involved a total of 20 experiments; including six replications at the center point to evaluate experimental error measurements and randomize according to a CCD configuration to avoid extraneous variables effects. For each experiment, the response was the total protein content.

The surface response obtained were analyzed by ANOVA and presented as standardized Pareto chart. Experimental and predicted values of total protein were compared in order to determine the validity of the model.

2.7. Mathematical mass transfer modeling

In a well-agitated liquid system, the main resistance for extraction comes from the internal diffusion of target component within raw materials [25]. Thus, the diffusion model based on Fick's second law was used to model the extraction of protein from microalgae. The following assumptions were made before modeling [26]:

a. Microalgae samples had a symmetrically cylindrical shape and proteins were initially homogeneously distributed inside microalgae particles. This assumption can also be inferred from microscopy image.
b. Diffusion coefficient of protein stayed constant during extraction. Meanwhile, protein content in microalgae particles varied with position and time.
c. No external mass resistance was considered due to the flowing of solvent, agitation and sonication.
d. No ultrasonic degradation of protein was considered.

Accordingly, the diffusion equation for cylindrical geometry is written as [26]:

$$\frac{\partial C_s(x,\,t)}{\partial t} = De\left[\frac{1}{x}\frac{\partial C_s(x,\,t}{\partial x} + \frac{\partial^2 C_s(x,\,t)}{\partial x^2}\right] \tag{8}$$

where C_s is protein concentration within microalgae particle (g/m^3), De is effective diffusion coefficient for protein (m^2/s), x is radial distance in the diffusion direction (m), referring to the half thickness value of cylindrical microalgae particle, and t is time (s).

The initial conditions are $C_S = C_{S,0}$ for the dispersed phase and $C_L = 0$ for the continuous phase. $C_{S,0}$ is the initial concentration of protein in microalgae particles (mg/m^3) and C_L is the concentration of protein in solvent (mg/m^3).

The boundary condition at the central axis of microalgae ($x = 0$) is:

$$\left(\frac{\partial C_S}{\partial x}\right)_{x=0} = 0 \;\forall\; t \tag{9}$$

At the interface ($x = r$), the outcoming flux of protein from microalgae particles was equal to the incoming flux in the liquid. The equality relationship about flux is expressed as:

$$-\,D_e A\left[\frac{\partial C_S(x,\,t)}{\partial x}\right]_{x=r} = V\frac{dC_L(t)}{dt} \tag{10}$$

where A is the surface area of microalgae particles (m^2), V is the solvent volume (m^3), r is the radius of the cylindrical microalgae particles (m).

The "*pdepe*" function in Matlab, R2010a (The MathWorks, Inc., MA, USA) was used to solve the aforementioned parabolic partial differential equation [27]. To be exact, the original partial differential Eq. (11) was first discretized spatially. After that, the resulting ordinary differential equations were integrated in time and solved by the *pdepe* solver. The De value was adjusted iteratively to fit the experimental data, thus minimizing the root mean square error (*RMSE*) between experimental and predicted content of protein in microalgae particles:

$$RMSE\,(\mathrm{g/m^3}) = \sqrt{\frac{1}{n}\sum_{i=1}^{n}\left[C_{S,p}(t) - C_{S,e}(t)\right]^2} \tag{11}$$

where $C_{S,e}$ and $C_{S,p}$ is the experimental and predicted values of protein content in microalgae particles (g/^{m3}), respectively. n is the number of experimental points.

Once the optimized De value was obtained, three statistical indicators, including R^2 (coefficient of determination), RMSE and Absolute average deviation (AAD) were calculated using the data about protein extraction yield to test the predictive accuracy of the diffusion model:

$$R^2 = 1 - \frac{\sum_{i=1}^{n}(Y_e - Y_p)^2}{\sum_{i=1}^{n}(Y_e - Y_m)^2} \tag{12}$$

$$RMSE\,(\mathrm{g/g}) = \sqrt{\frac{1}{n}\sum_{i=1}^{n}\left[Y_p(t) - Y_e(t)\right]^2} \tag{13}$$

$$AAD(\%) = \left[\frac{\sum_{i=1}^{n}(|Y_e - Y_p|)/Y_{S,e}}{n}\right] \times 100 \tag{14}$$

where Y_e and Y_p are the experimental, predicted values of protein extraction yield, respectively (g/g). Y_m is the average protein extraction yield (g/g).

2.8. Microscopic tools

2.8.1. Optical microscopy (OM)

To follow the gross US-induced alterations on spirulina cells, samples from treated and untreated (control) material were mounted on glass slides in 50% aqueous glycerin and microscopically analyzed as described below. Structural effects of ultrasound have been studied on thin sections of untreated and treated spirulina filaments. Microalgal suspensions were centrifuged, the pellets were collected and included into agar (1% solution) which was then excised in small fragments (width of 5–7 mm) and immediately immersed in a fixative mixture composed of: 20% paraformaldehyde, 4% glutaraldehyde and 1% caffeine in a phosphate buffer (0.2 M, pH 7) for 48 h. After fixation, fragments were rinsed with distilled water (3 × 1 h) and dehydrated in graded alcohol series (70–100%). Agar fragments were then infiltrated and embedded in historesine (Technovit 7100, Kultzer). After historesine polymerization, 3 µm thick sections were cut using a rotating microtome (Supercut 2065, Reichert-Jung, Leica Microsystems, Germany) and mounted on glass slides. Two different microscopic procedures were used to analyze these sections: toluidine blue [28] staining and fluorescence. Toluidine blue is a metachromatic stain, which reveals different cell components such as proteins, and fluorescence (excitation filter: 450–490 nm, emission: 515 nm) specifically highlights the presence of secondary metabolites.

All OM analyses were performed using Leica DMR photomicroscope equipped for brightfield darkfield, phase contrast and UV illuminations. Images were captured using Leica DFC 300 FX digital camera and processed using LAS software (Leica, Wetzlar, Germany). For each treatment, 5 samples were analyzed.

2.8.2. Scanning electron microscopy (SEM)

Changes in the cellular structure of spirulina were assessed by scanning electron microscopy (SEM, XL30, FEI Philips, France). Samples, fixed to the metal support, were covered using a carbon adhesive by means of a sputtering apparatus (BD SCS 004, BALZERS). Filaments were then scanned with SEM under partial vacuum (7 Pa) at 10 kV. All experiments were done in triplicate.

3. Results and discussion

3.1. Optimal controlled protein extraction parameters

Different measures were carried out on the continuous system with a US chamber transit time of 15 s. According to experimental design

results, higher protein content was achieved with a pressure of 2 bars, temperature of 24 °C and ultrasound intensity of 55 W/cm^2. The second-order polynomial equation (Eq. (15)) that allowed calculation of the total protein content according to the model is written as follows:

$$\text{Total proteins} = 2.59 + 0.45 \times \text{Temperature} + 0.17 \times \text{Ultrasound intensity} - 0.61 \times \text{Pressure}^2 - 0.0015 \times \text{Ultrasound intensity}^2 \quad (15)$$

The highest protein content, reached in 15 s of transit time in the US chamber, was 16.10 g of total proteins per 100 g of dry spirulina.

According to Pareto diagram on Supplementary material (Fig. S1A), ultrasound intensity and temperature are two factors that had a positive influence on the total protein yield, whereas pressure had an insignificant effect on the total protein yield if one referred to the cumulative curve. The pressure had no important impact on protein yield since it was very low, close to atmospheric pressure. Indeed, lab scale equipment is not able to endure high pressure because of junction's fragility between different modules. The product of the variables (Intensity US × temperature) which was not significant, suggests the absence of interaction between these variables. On the other hand, the positive quadratic effects revealed the presence of an optimum protein yield for each of the variables studied as shown in the protein response surface (Fig. S1B). Temperature, ultrasonic intensity and pressure increase has led to total protein yield rise. Nevertheless, once protein yield optimum achieved, temperature, ultrasonic intensity and pressure increase has caused protein content reduction in the extract. This decrease in the protein content can be explained by the phenomenon of thermal degradation: the tertiary structure of protein was irreversibly impacted. Indeed, the protein has a three-dimensional conformation conferring a biological activity such as antioxidant capacity. This specific conformation involves covalent bonds like disulfide and hydrogen bond, but also weak bonds such as Van der Waals interactions which can be broken when the temperature exceeds a certain threshold [29].

3.2. Protein extraction kinetic

Extraction kinetics were performed using optimal extraction parameters. MTS process was compared to a reference carried out in the same experimental conditions without ultrasound. Initially, spirulina biomass contained 57.1% of total proteins. Protein kinetics are presented in Fig. 2A for MTS and conventional extraction. Protein yield increased following a logarithmic function in the case of MTS treatment whereas protein content stayed constant for the reference indicating that few proteins were extracted. According to these results, after 20 min of MTS treatment, protein yield was 229% higher with MTS extraction (28.42 ± 1.15 g/100 g DW) than the reference without ultrasound (8.63 ± 1.15 g/100 g DW).

The effect of pressure was investigated on the graph below (Fig. 2B). In accordance with these results, there was no significant difference between MTS and thermosonication (TS) treatments since there is an overlap of standard deviations for both treatments. However, there is a trend where protein yield tended to be higher with pressure than without pressure, in accordance with starting assumption. Under MTS treatment, protein yield was 6% higher (28.42 ± 1.15%) than under TS extraction (26.72 ± 1.69%).

Due to the movement of solvent in the extractor, the extraction time did not correspond to the ultrasonic exposure time in the reactor. Instead, it referred to the transit time, which meant cumulative effective time for a spirulina cell was exposed to ultrasound. For 20 min MTS extraction at a flow rate of 15 mL/s with a reactor volume of 220 mL and a volume of 5% MS microalgal solution of 760 mL, the cumulative flow time was approximately 6 min (5 min and 49 s more precisely). Thus, in 20 min of extraction, the same microalga cell was subjected to ultrasounds 23.81 times (number of cycles) in the reactor and that corresponded to a sonication time of 5 min and 49 s.

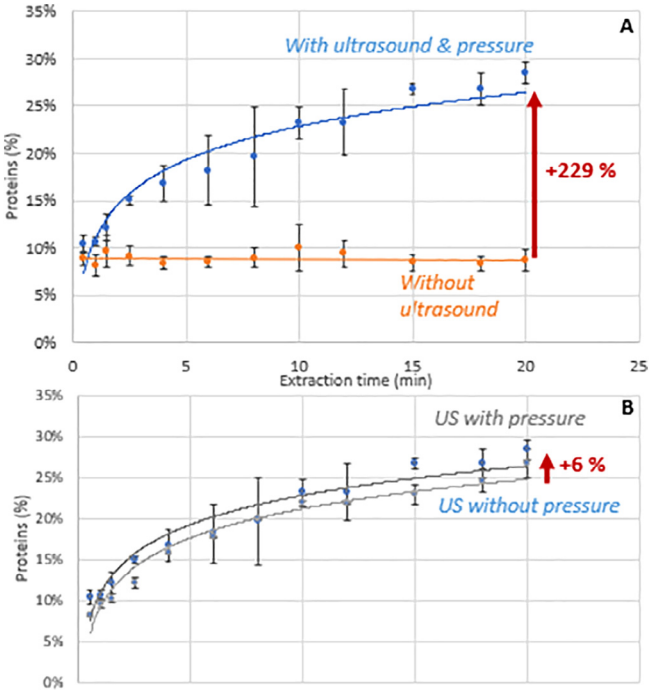

Fig. 2. Protein kinetics obtained after MTS treatment compared to reference (A), and comparison between protein kinetics with and without pressure (B).

With a continuous MTS extraction system, 20 min are required to achieve a protein yield of 28.42 ± 1.15%, namely a protein recovery rate of 49.77%.

3.3. Protein quality and amino acid profile

Proteins are an indispensable component of the diet since they represent a major source of nitrogen for the body. The primary structure of a protein is made of amino acids assembly connected to each other *via* peptide bonds. Among the 20 amino acids existing, nine of these are said to be "indispensable" for human since he is incapable of synthesizing them naturally: histidine, isoleucine, leucine, lysine, sulfur amino acids (methionine and cysteine), aromatic amino acids (phenylalanine and tyrosine), threonine, tryptophan and valine [30]. Nutritional value of a protein ingredient depends on its ability to provide amino acids of its proteins, in proportions adapted to the needs of the individual. Nutritional value of a protein is therefore function of its essential amino acid composition, but also of its bioavailability [31]. Bioavailability reflects the fraction of ingested compounds available at sites of action for use in various physiological functions. To evaluate the nutritional quality of a protein the chemical index is used; it indicates protein ability to contain all the essential amino acids. As part of this study, aminograms are performed for each extract to quantify the different amino acids and compare them to nutritional recommendations.

Spirulina proteins are in a size range between 25 and 100 kDa and 75% of spirulina proteins have a size of 65–60 kDa and 20–25 kDa [32]. Spirulina contains about 500 different types of proteins. According to this study, 37 out of 570 proteins could be identified by LC-MS/MS, the others having too low a concentration to reach the limit of detection. They have been classified into 13 categories according to their function: oxidoreductase, transport, ligases, regulatory proteins, proteins involved in carbohydrate metabolism, transfer proteins, growth proteins, lyases, carboxylases, proteins involved in chlorophyll biosynthesis, proteins ribonucleic [33]. According to Ismaiel et al., phycocyanin is the major protein, which represents up to 50% of total spirulina soluble proteins [32]. These different proteins come from various cellular regions such as protein complexes, cytoplasm, membrane or cytosol

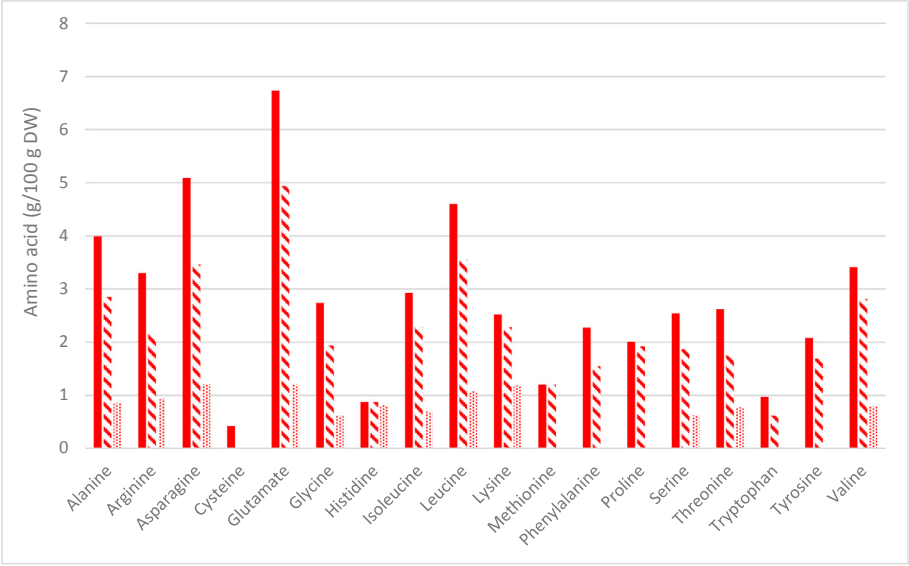

Fig. 3. Aminogram of MTS and CV extract compared to reference (initial spirulina biomass). Continuous: reference; stripped: MTS extract; dotted: CV extract.

where they act specifically [34]. The aminogram (Fig. 3) highlights MTS effectiveness compared to conventional extraction. Not only, amino acids were not degraded by ultrasound treatment, but they are present in a larger quantity in the case of MTS contrary to conventional extract. Thus, the amino acids methionine, phenylalanine, tryptophan, tyrosine (essential amino acids) and proline are only present in MTS extract and were not extracted during conventional maceration. Therefore, MTS extraction present the advantage of being an efficient and non-destructive extraction technique since it does not damage essential amino acids and thus preserves the nutritional quality of extracts produced.

3.4. Mathematical mass transfer model to explain protein diffusion during US extraction

Experimental and simulated results about protein extraction kinetics are illustrated in Supplementary Fig. S2. As can be seen, the diffusion model generally provided a satisfactory description of the evolution of protein yield during CV, TS and MTS extractions, although there were divergences in some areas. Meanwhile, R^2 values for both cases exceeded 0.93, and AAD values were also acceptable (Table 2). Accordingly, the diffusion model was qualified to model the extraction process and investigate the mass mechanism under MTS and TS treatments. D_e values for CV, TS and MTS extractions were 0.67 × 10–10, 0.83 × 10–10 m2/s and 1.67 × 10–10 m^2/s, respectively. All the three values stayed in the same order of D_e of phenolics under ultrasound-assisted solvent extraction from chokeberry [27]. D_e value for conventional extraction was the lowest, implying the low extraction efficiency. Meanwhile, D_e value for MTS extraction was one-fold higher than that of D_e for TS extraction, indicating that pressure treatment could facilitate the diffusion of protein within microalgae particles. Generally, pressure treatment can produce structural damages in raw materials, increase the permeability of solutes, change the diffusivity and concentration gradient and intensify the permeation of extraction solvent into raw materials [35].

Table 2
Effective diffusion coefficients of protein at different extraction conditions and accuracy of diffusion model.

Treatment	D_e (m^2/s)	R^2	RMSE (g/g)	AAD (%)
CV	0.67 × 10–10	0.951	0.008	6.4
MTS	1.67 × 10–10	0.931	0.023	11.5
TS	0.83 × 10–10	0.960	0.016	11.2

3.5. Microscopic investigation for US physical effects illustration

To better understand mechanisms involved in MTS extraction and to complete data from mathematic mass transfer model, microscopic observations were performed over time; the objective was to identify physical phenomena involved and to obtain additional information.

4. Spirulina initial state

In its natural state, fresh and wet spirulina is in the form of multicellular filaments, regular spirals wrapped (Fig. 4A). Fig. 4B shows thin longitudinal and transverse sections of spirulina filaments at the fresh initial states: filaments are well compartmented with a lot of proteins partitioned into the cells revealed thanks to toluidine blue. Dry spirulina was also observed on picture C and D (Fig. 4). Filaments are shorter than in the fresh state due to fragmentation. Ripping of the filaments was due to post-harvest treatments. During harvest, spirulina is filtered, pressed to remove excess water, extruded and dried on racks at low temperatures [36]. These successive treatments were responsible for a first alteration of biomass: filaments unfold and begin to fragment (Fig. 4C). Thus, dry spirulina powder is already altered and contains many fragmented filaments, nevertheless the cells remain intact. These observations allow to understand why the initial protein yield is not zero in the solvent at the beginning of the kinetic measurement. Prior to extraction, biomass-solvent mixture was homogenized for 1 min at 24° C with mechanical stirring and hydraulic convection: imbibition of dry biomass. This preliminary step initiated the solubilization and extraction of some water-soluble compounds, including some membrane proteins and non-compartmented low molecular weight molecules. Indeed, the rehydration of spirulina powder as well as the osmotic shock undergone by cells facilitated the exit and the solubilization of some compounds. Mass transfer was also increased thanks to hydraulic convection generated by the peristaltic pump and temperature increase.

After a conventional treatment of 20 min (Fig. 5D), without ultrasound nor pressure, filaments fragmentation was accentuated compared to the reference, which is the dry initial state (Fig. 5B); there were no more regular and complete spirals, only fragments of different sizes remained, and the cells stayed intact. TS treatment led to isolated cells and some cell debris (Fig. 5E). This can be due to acoustic cavitation

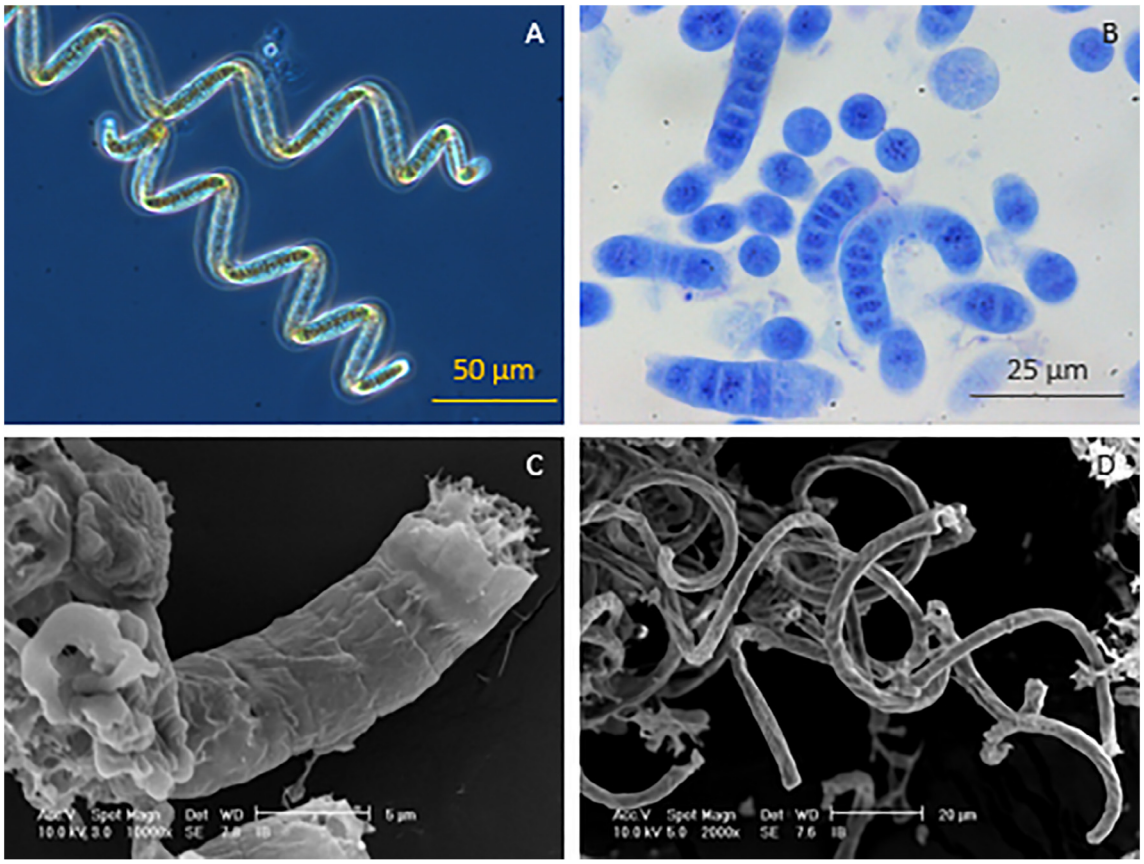

Fig. 4. Microscopic observations of spirulina initial state: fresh (A and B) and dry (C and D). A and B were analyzed with optical microscope: A, Phase Contrast Microscope (PCM); B, spirulina section stained with toluidine blue. C and D were observed with scanning electron microscope.

which affect cell structure when occurring near a cell wall. After MTS treatment, spirulina filaments were totally destructured: only cell debris remained (Fig. 5C). According to these observations, MTS engendered more cell damage than TS. In accordance with modeling results, pressure had a positive impact on protein extraction. This information can be connected to microscopic observations since samples treated with pressure were more destructed than others (conventional and TS), meaning that bioactive compounds can be easily released and solubilized in the extraction solvent with the use of pressure.

5. Focus on MTS effect on filament structure

In the initial state, spirulina was in the form of pluricellular filaments wound in regular spirals. After 3 min of MTS treatment, fragments of different sizes appeared, and remaining filaments were unfolding. From 3 to 7 min of MTS extraction, fragments size decreased until it remained only individualized and isolated cells in the solvent. From 17 min, cells exploded gradually under the effect of acoustic cavitation and released their content directly into the extraction solvent. MTS was responsible for an intense fragmentation of spirulina filaments over time according to Fig. 6. The more filaments are fragmented, the more important is mass transfer and protein diffusion. When a new spirulina filament break, it allows solvent entrance in the solid matrix, the solute is then released into the solvent, the enriched solution diffuse outwardly of the particle and is distributed throughout the environment. According to microscopic observations (Fig. 6), it was found that 20 min were enough to completely degrade spirulina biomass.

Cytology was performed to study ultrasound effect on internal spirulina cell structures. Spirulina filaments were analyzed after toluidine blue's reagent revelation (Fig. 7). Deep blue fluorescence indicates the presence of proteins in the cells. According to Fig. 7 above, proteins were well compartmented inside spirulina filament with a high concentration at the initial state: intact filaments without any protein diffusion in the medium. After 20 min of conventional extraction (reference without US in Fig. 7B), spirulina filaments were still intact but deflated compared to the initial state and protein diffusion in the medium was observed. On Fig. 7C, filament structures were indistinguishable: there was a total destructuring due to MTS treatment and protein were solubilized into the extraction solvent. These observations are in accordance with previous results with the high effective diffusivity and mass transfer in the case of MTS extraction.

Excitation wavelenghts 450–490 nm highlights specifically the presence of secondary metabolites. Natural fluorescence observations underscore 2 effects of MTS treatment: destructuring and compounds extraction. These observations were carried out on thin sections of spirulina filaments. Yellow fluorescence can be correlated to secondary metabolites accumulation in spirulina cell. Initial state, presented Fig. 7D, showed well compartmented whole filaments with an intense yellow fluorescence emission suggesting a high bioactive content. After a conventional treatment (Fig. 7E), yellow color decreased, meaning that few metabolites have been extracted. However, filament cells remained intact and still contained noticeable quantity of bioactive compounds. The Fig. 7F clearly highlights the destructuring effect of MTS since none of initially spirulina structures were distinguishable: all the filaments and cells had been destructed by the treatment. Moreover, an important decrease of yellow fluorescence was noticed suggesting that secondary metabolites of cells had been released in the solvent.

Thanks to these microscopic analysis, hypothetical US mechanism was proposed (Fig. 8). When a cavitation bubble implodes near a cell wall it damages and erodes its surface which becomes gradually thinner and thinner. When the cavitation occurs at the same location, a pore is created by the microjets effects: this is the sonoporation. Thus,

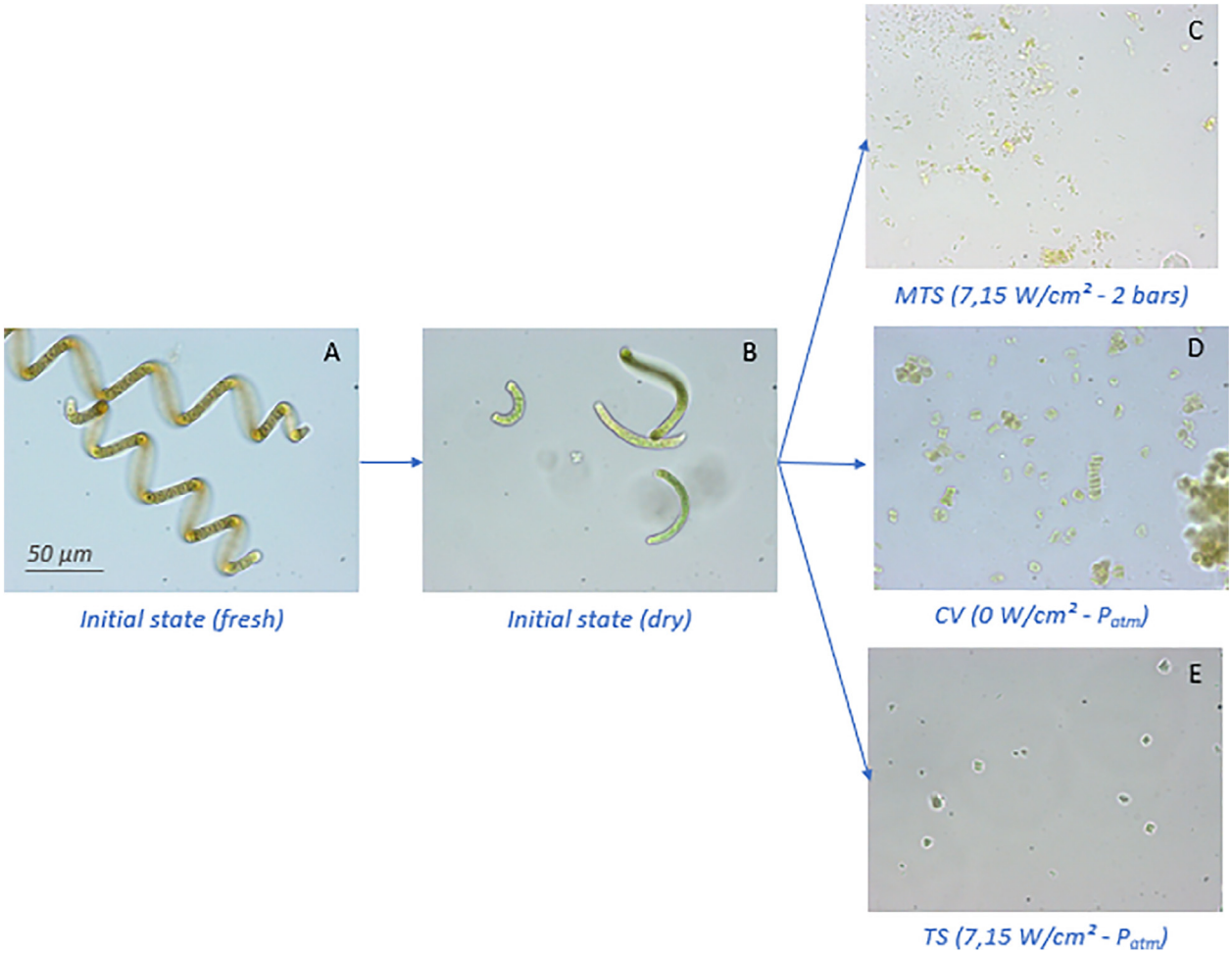

Fig. 5. OM analysis of spirulina subjected to different treatments. Scale bar (picture A) = 50 µm for all pictures.

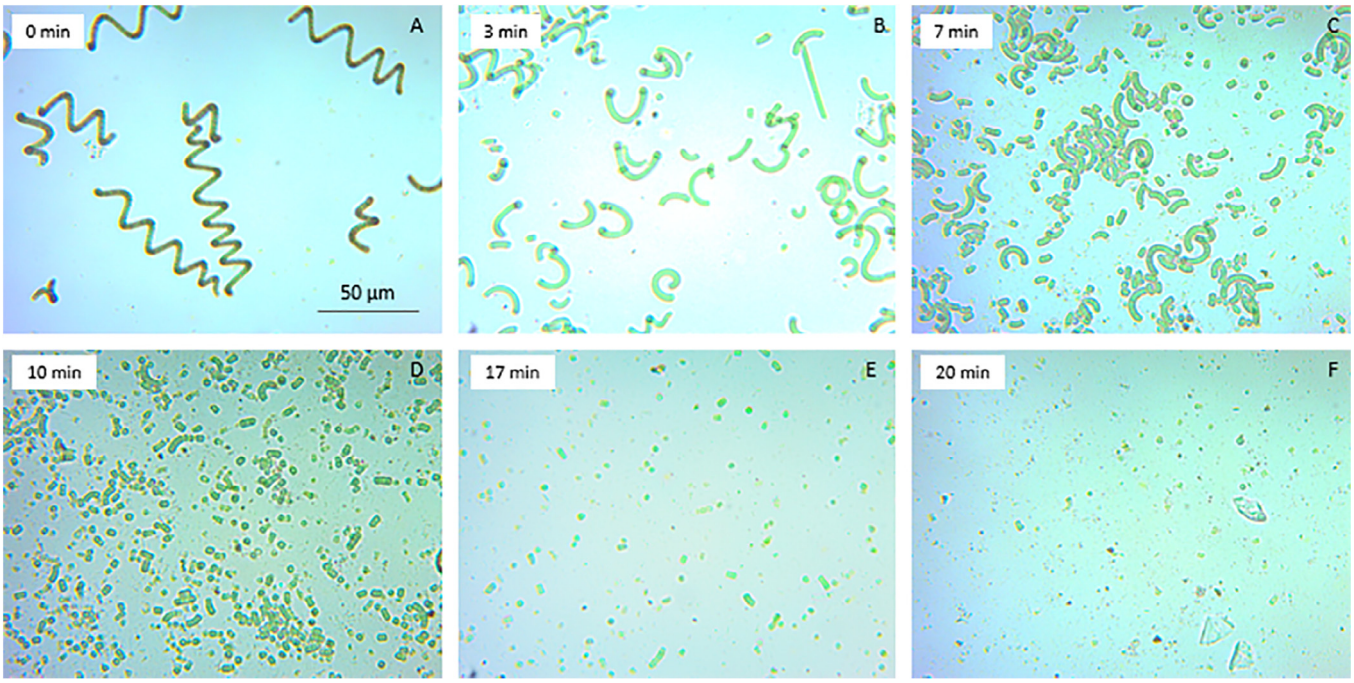

Fig. 6. OM observations of whole mounted spiurulina filaments subjected to MTS treatment over time. Scale bar (picture A) = 50 µm for all pictures.

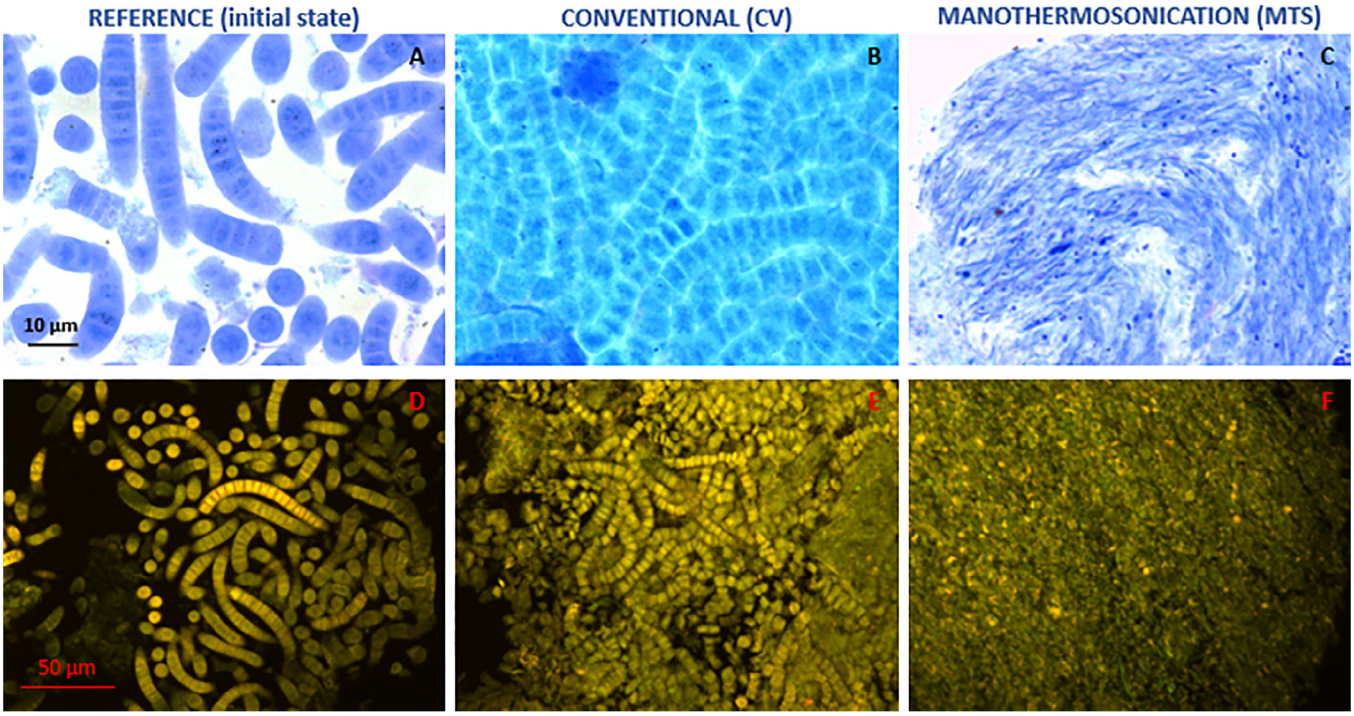

Fig. 7. OM observations of spirulina filaments sections after toluidine blue staining (A, B, C), and with natural fluorescence (D, E, F). Scale bar (picture A) = 10 µm for pictures A, B, C. Scale bar (picture D) = 50 µm for pictures C, D, E.

ultrasounds are responsible for sonoporation and erosion phenomena which leads to localized cell wall permeabilization [37]. At the location of the pores, weakened filaments can break under the impact of acoustic cavitation and hydraulic convection (pump flow rate) combined together creating fragments of different sizes. The subsequent entry of solvent causes an increase in pressure in the cell which, combined with the implosion of cavitation bubbles, leads to filaments rupture at le level of pores and the intercellular junctions. These various effects act synergistic during ultrasound treatment and they are amplified adding pressure (MTS) according to the previous results. Acoustic cavitation is also responsible for the destructuring of spirulina filament cells. All these effects lead to filaments destruction and bioactive compounds extraction [37].

6. Industrial prospects: Process upscaling

6.1. Feasibility study and cost estimate

To ensure safety, sustainability, economic and greener methods, the design of an efficient ultrasound-assisted large-scale extraction requires process intensification and energy consumption reduction. Ultrasounds are already widely used industrially in different sectors such as food. Hielscher (Germany) develops large-scale probe ultrasound extraction devices for continuous processes and commercializes devices of a wide power range: from 50 to 400 W for analytical scales, and from 500 to 16,000 W for industrial scales.

According to lab scale results, the use of pressure enabled to get 229% more protein than the reference, and sonication without pressure led to 210% more protein than the reference. Hence, the use of pressure allowed to extract only 6% more of total spirulina proteins compared to a process without pressure. However, to ensure and maintain such a constant pressure during extraction process, a worm pump (or peristaltic pump) is needed contrary to sonication alone where a classical pump (lobe pump) is sufficient. Is this efficiency gain significant compared to the costs generated using a specific pump to extract under pressure? A worm pump costs about 2500 € (Seepex prices), 10 times more than a classical pump. As worm pump is much more expensive than a lobe pump of the same capacity and considering the protein gain with pressure, it would be more profitable to use a process without pressure enabling to achieve a protein recovery rate of 47% and consuming less energy. Elsewhere, prices of industrial ultrasonic reactors vary from € 18,000 (5 L batch or 5 L/h continuously) to € 200,000 (1000 L batch or 1000 L/h continuously). Ultrasonic reactor induces 25% investment more than conventional reactor regarding extraction unit operation; however, US extraction time is shorter than conventional techniques from 10 to 100 times, and US enables energy saving and reduces pollution by a factor of 10 [38]. Moreover, if one considers the whole production chain, in both cases, conventional and ultrasound assisted extraction, production line consists of five unit operations as presented in Supplementary material (Fig. S3). Thus, the use of ultrasound generates an investment increase of only 5% (25%/5 = 5%). Considering these results, the use of ultrasound is favorable to an energy saving leading to a cost reduction for the industrial production of spirulina extracts with a high protein content. Moreover, focusing on

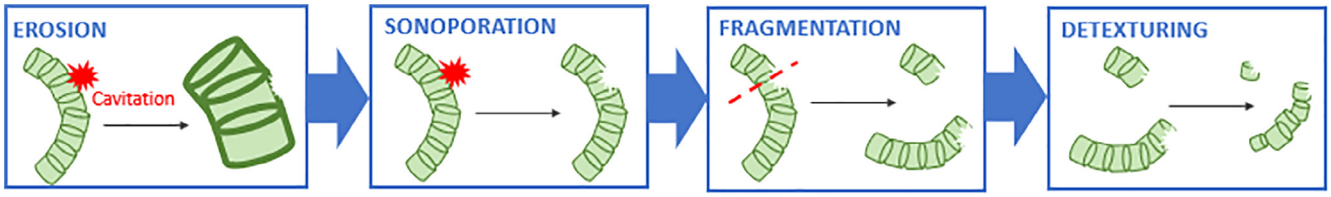

Fig. 8. Mechanism of ultrasound physical effects.

Fig. 9. Scaling up of manothermosonication: from laboratory equipment (A) to pilot scale equipment (B, C & D). On picture D is schematized a transversal section of the US chamber.

the global process, the unit operation "extraction" is not the one that generates the biggest expenses. In terms of cost, solid liquid separation and drying are the most expensive steps. Indeed, for a plate separator operating at a rate of 15–70 hL/h it is necessary to count 122,000 € (Flottweg); and for a spray drying generating about 6 kg of powder per hour the price is about 90,000 € (REUS, Techni-process).

6.2. Pilot scale tests

For a further industrialization, pilot experiments were performed using a continuous process equipped with a US probe (UIP4000hd, Hielscher). According to previous section, experiments were conducted without pressure. Fig. 9 depicts the equipment used for pilot trials. This extraction was realized with 30 L of solvent.

The most important parameter to consider for scaling up a continuous process is the space-time in the US chamber. Furthermore, parameters such as flow rate, US chamber and US amplitude must be optimized to increase protein yield. According to the results, protein extraction yield obtained with pilot equipment was lower than the one generated at laboratory scale. Indeed, 40 min were needed at the pilot scale to achieve 19.63 ± 1.23 g of total protein per 100 g of dry spirulina, whereas only 20 min were required to reach 27.72 ± 1.15% of total protein at lab scale. Focusing on transit time, there is an important difference between pilot and lab scale: lab scale transit time is 6 times longer (≈6 min) than pilot scale transit time (≈1 min). To perform a good scaling up, according to the rules, dimensionless number such as Sherwood number or ratio of characteristic variables need to be considered. In addition, it is important to pay attention to certain parameters such as reactor geometry, flow nature (Reynolds number, *Re*) or stirring type.

7. HACCP and HAZOP study toward ultrasound process industrialization

Ultrasonic techniques are finding increasing use in the food industry. As presented in this study, ultrasound can serve to extract and produce high value-added ingredient from different biomass such as microalgae. Ultrasound operations in food engineering require the establishment of a hazard analysis and critical control point (HACCP) program in which the critical control points of food processing are identified. This section will be focused on the concepts of hazard analysis and critical control points (HACCP), and hazard and operability (HAZOP) [39]. These tools will be described in relation to the use of ultrasound in the food industry and a list of issues and recommendations for the prevention of each problem will be provided.

The design of any ultrasound-assisted method for food processing must be supported by HACCP and HAZOP analysis. Toward standardization and certification, a food safety program should be based on principles of risk analysis and critical points study, as stated in the current edition of the *Codex Alimentarius*. Indeed, the design of a US facility based on HACCP concept is a preliminary step towards approval by regulatory agencies such as the European Community. Hazard analysis of critical control points (HACCP) is the most secure and cost-effective method for controlling possible product contamination or cross-contamination, due to physical or chemical hazard during production. In the case of US food processing facility, it defines critical control points (CCPs). HACCP is applied from the raw material reception stage to final storage before final product distribution. Hazard analysis concerns any biological, chemical, or physical agent reasonably likely to cause illness or injury unless controlled. Critical control points (CCPs) are essential to prevent or eliminate a food safety hazard or to reduce it till an acceptable level. HACCP is based on seven principles (P):

P1. List the steps in the process where significant hazards occur and describe preventive measures.
P2. Identify the CCPs among raw material, ingredients and process step.
P3. Determine the critical limits or tolerances for preventive measures associated with each CCP.
P4. Establish monitoring procedures for each CCP.
P5. Establish corrective actions to be taken when monitoring indicates a deviation from a critical limit.
P6. Establish effective procedures to verify that HACCP system is working correctly.
P7. Establish record-keeping and documentation procedures [40].

In the case of ultrasound, the equipment must ensure product processing according to safety and quality requirements, without contaminating final product. US parameters such as temperature, physical properties of the product, treatment time, US frequency and power, nature of probe are points which need to be monitored as part of

HACCP measures. Indeed, ultrasonic bath or probe employed during the operation are likely to contaminate final products. Unacceptable accumulation of any material is a potential source of contamination. For instance, ultrasound equipment can release toxic elements and cause chemical migration (e.g., titanium) in the food product. This phenomenon may occur by corrosion of US equipment, which can be damaged during extraction process, or by contact with raw materials or cleaning equipment.

Hazard and operability (HAZOP) system investigates potential deviations of operations from design conditions that could lead to process operation problems and hazards. HAZOP is a structured investigative study which serve as key critical tool used throughout processing industries worldwide. HAZOP study analysis is based on four main objectives:

1. For each deviation from the intended design function, determine the reasons.
2. Identify all major hazards and operability problems associated with any identified deviations.
3. Decide whether action is required to control the hazard or operability problems.
4. Ensure that actions decided on are implemented and documented [40].

In the case of ultrasound manipulation, users are exposed to various hazards, where the most important is contact exposure to ultrasonic waves. Accidental immersion of a body part in a liquid treated by ultrasound can cause tissue damage and is responsible for sharp pain of the operator within seconds. It is thus important to maintain an air gap between the transducer and operator tissue. Moreover, ultrasounds are generated by an electrical device which can be harmful as it can cause electrical shock, burns and represent a source of fire. Thus, only qualified operators should be allowed to access these apparatuses. Ultrasound is also responsible for adverse effects such as ear damages and body temperature increase due to cavitation. To avoid noise pollution and hearing side effects, workers are recommended to wear soundproof helmet.

All these measures allow to produce a healthy and qualitative food product, free of contaminant, in a safe approach to avoid any risk for the worker as well as for the final consumer.

8. Eco-footprint of ultrasound assisted extraction process

The term “green extraction” refers to a safe, environment-friendly and economic way to produce extract.

A green approach aims to reduce or to eliminate the use and generation of hazardous substances. Green extraction is based on the design of innovative extraction processes which reduce energy consumption, allow use of alternative solvents and renewable natural products, and ensure a safe and high-quality extract. Green extraction of natural product is governed by the 6 following principles:

1. Principle 1: Innovation by selection of varieties and use of renewable plant resources.
2. Principle 2: Use of alternative solvents and principally water or agro-solvents.
3. Principle 3: Reduce energy consumption by energy recovery and using innovative technologies.
4. Principle 4: Production of co-products instead of waste to include the bio- and agro-refining industry.
5. Principle 5: Reduce unit operations and favour safe, robust and controlled processes.
6. Principle 6: Aim for a non-denatured and biodegradable extract without contaminants [41].

Keeping in mind the six principles of eco-extraction, global environmental impact of MTS treatment for protein extraction from spirulina was assessed in this study. To measure MTS eco-footprint, raw material, value of by-products, extract quality, extraction time, solvent quantity and energy required were considered. To evaluate environmental impact, MTS was compared to conventional extraction in the same conditions without ultrasounds (reference). According to this study, there is no change of raw material, by-products and solvent quantity and nature for both techniques (Fig. S4). Compared to conventional process, ultrasound enabled to get higher protein extraction yield in only 20 min instead of 5 h. Indeed, ultrasound permitted to get 210% more proteins (26.72 ± 1.69 g/100 g DW) than conventional process without ultrasound (8.63 ± 1.15 g/100 g DW). These results are in accordance with other authors where it has been found that extraction yields generated by ultrasound are 25–50% higher than in the case of a conventional extraction. Regarding energy consumption, ultrasound treatment also lead to a reduction of more than 50% compared to conventional extraction according to Sicaire et al. concerning oil extraction from rapeseeds and Fabiano-Tixier et al. [38,42]. In accordance with Fabiano-Tixier works, the calculated quantity of carbon dioxide rejected in the atmosphere is higher in the case of conventional extraction than for UAE. Ultrasonic extraction produces 200 g of CO_2 in the atmosphere, while conventional process rejects more than 4000 g. These calculations have been carried out based on the following assumptions: to obtain 1 kW·h from coal or fuel, 800 g of CO_2 will be rejected in the atmosphere during combustion of fossil fuel [38]. Following all these aspects, this study suggests that the use of ultrasound allow a reduction of the eco-footprint in comparison with the reference as shown in Supplementary material Fig. S4. The positive impact of US on extraction has been studied at lab scale on a dry microalgal matrix suspended inside the solvent. Moreover, the solid-liquid ratio optimized at lab scale could be different in the industry. Hence, environmental impact in industrial conditions needs to be further investigated to assess real and concrete eco-footprint of the full process. For instance, a life cycle assessment could be realized considering all unit operations.

9. Conclusions

In conclusion, ultrasound technologies enabled to extract more efficiently protein from *Arthrospira platensis* compared to conventional techniques: 229% more protein with ultrasound. Furthermore, with pressure (MTS) protein yield was higher than without pressure (TS) in accordance with the initial assumption. This can be explained by mathematic mass transfer modeling. Indeed, effective diffusivity (D_e) of MTS is higher than the one of TS according to the model: pressure has a positive effect on protein extraction. Moreover, microscopic analysis support kinetic results: 20 min of ultrasound treatment has led to a maximal protein extraction yield since the kinetic curve plateau had been reached. Thus, ultrasound appears as a non-destructive and efficient technique since protein extracted still contained essential amino acids of the initial biomass. Microscopic investigations also offered a better understanding of ultrasound physical and structural effects. Thanks to various observations with different microscopic techniques (SEM and OM fluorescence), a mechanism was proposed including the following physical and structural effects: erosion, sonoporation, shear effects and fragmentation.

Toward a future industrialization, a cost study, safety measures related to the use of ultrasound and an estimate of process environmental footprint have been proposed. It emerges from this study that the use of a pressurized process is not justified regarding the costs generated. Ultrasound alone is enough to produce a high-quality extract rich in spirulina proteins. Ultrasound assisted extraction is thus proposed as an “environmentally friendly” extraction method suitable for protein extraction from spirulina at laboratory scale but could also be transposed to pilot and industrial scale.

As a perspective, we could imagine a greener process by reducing unit operations number. Indeed, if one implanted the extract

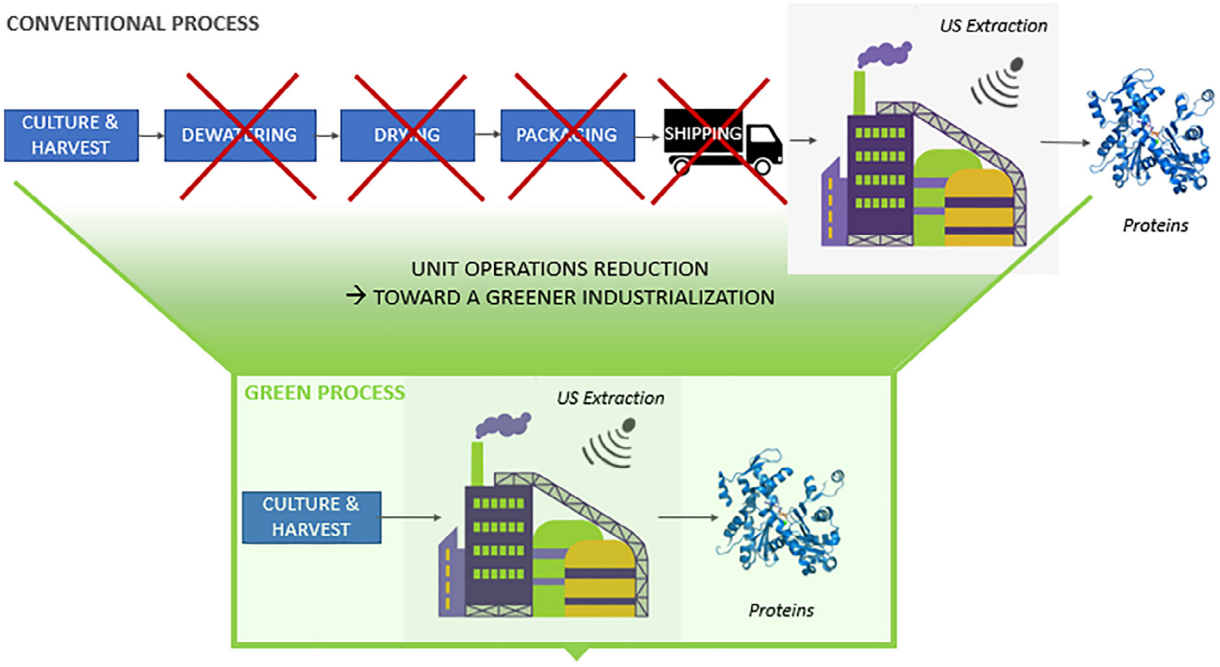

Fig. 10. Toward a greener process production of spirulina protein extract.

industrialization near spirulina production site, it would be possible to extract fresh spirulina directly after harvest, without any drying step as presented on Fig. 10.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ultsonch.2019.02.016.

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