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ABSTRACT

Low (20 kHz) and intermediate (100 kHz) frequency ultrasound (US) were studied for their efficiency on cell destruction and metabolites extraction of the microalga *T. suecica*. This study revealed different levels of cell destruction. Firstly, the prolonged irradiation of US at low frequency allowed the extraction of 90% of total proteins and 70% of carbohydrates by rapidly inducing at high power (100 W or 200 W) a coiling up phenomenon of the cell walls on themselves. A low power (50 W) over short times allows extracting proteins by the perforation of the cells without destroying them, opening the perspective of milking. Furthermore, the use of 100 kHz frequency, showed lower yields of metabolites as well as a low level of cell destruction, resulting in a simple deflation of the cells.

1. Introduction

New projections from the Food and Agriculture Organization (FAO) predict that nearly 670 million people will face hunger by 2030 [19]. Simultaneously, the demand for protein is increasing in some agro-sectors. For example, an additional 37.4 million tons of aquafeeds will be needed by 2025, competing with existing protein resources [29]. To face these predictions, it is necessary to develop new protein alternative sources to ensure food safety. Many efforts have been made to respond to these global challenges, and among the new possibilities explored, microalgae seem to be a very promising and suitable option to contribute to the increasing protein needs. *Tetraselmis suecica*, is a green marine microalga, presenting many benefits to be included in animal feed, such as a high protein content [45], as well as benefits in terms of health [26]and zootechnical performance improvements [57].

However, there are still many scientific obstacles for an efficient and sustainable use of these resources [56]. The production of microalgal proteins on an industrial scale must be integrated in a biorefinery approach to become competitive on the world market and allow the simultaneous valorization of multiple molecules of interest. Several unit operations compose a biorefinery system, ranging from the cultivation and harvesting of the microalgae to the final purification of the desired products. One of these steps, cell destruction, is particularly considered critical because it induces the highest costs in the process [53]. Indeed, most industrially exploited microalgae have more or less rigid cell walls leading to a different destruction efficiency and thus having negative consequences on the final extraction rates [66]. Günerken et al. [25] described several techniques of mechanical cell destruction using solid–liquid interfacial shear forces which have a high cell disruption efficiency. Ultrasound-assisted extraction (UAE) is one of them. Ultrasound (US) is a widely used technology in various fields for the destruction of biological cells to release intracellular compounds [14]. The action of ultrasound in liquid media is based on the phenomenon of cavitation [39]. The increase of pressure, temperature and shear force in a localized manner in the medium causes cell destruction and improves the extraction of molecules of interest by maximizing the surface area exchange between the solid/liquid phases [59].

Ultrasound-assisted extraction (UAE) is a green and easily scalable technique with many advantages such as high extraction yield, low amount of solvent needed, possibility to operate in water, relatively short extraction times [32,55]. UAE can also be easily combined with other enzymatic, mechanical or chemical extraction methods. It is a technology widely used on all types of vegetal biomass for the extraction more or less selective of high added value molecules of interest [46]. A

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non-exhaustive reading of the literature shows that ultrasound are particularly suitable for the cellular destruction and the protein extraction of microalgae. However, this also shows the importance of knowing the characteristics of the biomass as well as controlling the operating parameters of the US that can influence the results. For example, *Arthrospira platensis* protein extraction and cell morphology changes were studied by Vernès et al. [58], using a US system at a frequency of 20 kHz. Microscopic observations showed various levels of cells destruction named as fragmentation, sonoporation and detexturation phenomena during the treatment. Moreover, Safi et al. [48] highlighted the variable final extraction yields between different microalgae for the same extraction conditions: *Chlorella vulgaris*, *Haematococcus pluvialis*, *Porphyridium cruentum* and *Nannochloropsis oculata*. This variability can be explained by the difference in cell wall rigidity of each microalgae and would therefore have required longer processing times. The cell walls can have various compositions, with the presence of several layers of polymers more or less resistant to external attacks justifying the necessity of a pre-treatment for the extraction of metabolites [10]. Other studies [37,65] report that the initial biomass concentration has an effect on the final UAE yields. Only few studies have focused on the extraction of *T. suecica* metabolites by UAE and the effect of US waves on the cells. The majority of these studies focused on lipid extraction at very low frequencies, commonly 20 kHz [43,42] or 40 kHz [3]. A recent study focused on determining if US could be used as a pre-treatment to break down *T. suecica* cells more easily, again at 20 kHz and keeping the US power fixed at 500 W [44].

This benchmark shows that most of the studies were conducted on very diluted algal suspensions, which is not representative of the industrial constraints, sometimes requiring to work at high cell concentrations. There is also a lack of information about the influence of ultrasound physical parameters on the mechanism of cell destruction. Similarly, most of the studies were conducted with low-frequency US pilots at 20 kHz. It would be interesting to work at intermediate frequencies to study the behavior towards the cells. Indeed, US can induce cell lysis in various ways, by fragmentation, erosion, sonocapillary effects, by local shear stress or by detexturation [8,38]. It is important to understand and control these cell lysis mechanisms in order to optimize the process and adapt it to the desired results.

The objective of this study was to evaluate the efficiency of ultrasound-assisted extraction on protein yields and destruction levels of *Tetraselmis suecica* cells. Various ultrasonic operating parameters were studied. The irradiated power (W), the sonication time as well as the ultrasonic power density (W mL^{-1}) in the medium were monitored and compared for two frequencies: a low frequency (20 kHz) and an intermediate frequency (100 kHz). Two modes of ultrasonic irradiation, continuous or pulsed, were also studied. Finally, the effect of the initial cell concentration was also considered in this study in order to determine the most adequate concentration range to operate.

2. Material & method

2.1. Tetraselmis suecica biomass characterization and conditioning

Tetraselmis suecica, produced as a biofilm (patented rotating algae growth system WO2021180713A1) with a dry matter content of about 15%, was supplied by the company inalve (Nice, France). After each harvest, the biomass was carefully stored at 4 °C and all experiments were performed within 4 days to ensure its freshness and avoid bacterial development.

The biomass was characterized to determine its initial protein, carbohydrate and lipid composition as described by Delran et al. [13]. Briefly, moisture content was determined by drying in an oven at 103 °C for 24 h. The ash fraction was measured by calcination at 550 °C for 12 h. Total protein was determined by the elemental analysis method using the conversion factor N = 6.25 [49]. Total carbohydrates were measured by the Dubois method [15]. Before each experiment, a protocol for desalting the biomass has been performed to remove the salts and the extracellular matrix composing the biofilm. For each experiment, the desalted biofilm was then rediluted with distilled water to the desired final concentration (10 g L^{-1}, 50 g L^{-1} or 100 g L^{-1}).

All chemicals, sulfuric acid, phenol and standards sugars (glucose), were purchased from Sigma Aldrich Chimie (Saint-Quentin, France) and used as received.

2.2. Ultrasonic assisted extraction pilot

Ultrasound-assisted extraction was performed with two ultrasonic devices each operating at low (20 kHz) and intermediate (100 kHz) frequencies.

The low frequency experiments were done using a 20 kHz transducer (SinapTec, Lezennes, France) and a 35 mm immersed probe. A 51D36 booster was added allowing a maximum US power of 400 W. The transducer was driven by a computer-controlled ultrasonic generator (SinapTec NexTgen Inside 500). Quantification of the real acoustic power was determined by calorimetry by following the temperature rise for 5 min in a 2 L volume of water for various vibration amplitudes (30, 40, 50, 60, 70, 80, 90 and 100%). Fig. 1 – Supplementary data, shows the real acoustic power as a function of the ultrasound irradiation time. Three amplitudes 33%, 51% and 84% corresponding respectively to 50 Watts (W), 100 W and 200 W were chosen to perform the experiments in "continuous" mode. The "pulsed" mode was performed with the 200 W power using the same conditions as previously described but with cycles of 30 s irradiation followed by 30 s in silent conditions.

All the experiments were performed in a double jacketed glass reactor with a total capacity of 2.5 L. The probe was immersed by 5 cm into the suspension in exactly 1 or 2 L (depending on the experimental design) of *Tetraselmis* suspension, constantly homogenized by a magnetic bar stirring system (900 rpm). The reactor was thermostated by a recirculating cooling bath (Microcool MC250, Lauda) to control the temperature evolution throughout the experiment to avoid overheating of the medium above 40 °C. The suspension was irradiated for up to 60 min, and several samples were collected over time.

High frequency experiments were performed using a 100 kHz Lab500 transducer in a Cup Horn reactor (0.37 L) from SinapTec (Lezennes, France) with a maximum US power of 120 W. The real acoustic power was determined by calorimetry as described before (Fig. 2 – Supplementary data). 0.3 L of *Tetraselmis* suspension was irradiated continuously by three different US powers (30 W, 60 W and 120 W) up to 60 min and samples were collected over time. A "pulsed" mode was also performed with the 120 W power, applying irradiation cycles of US of 30 s followed by 30 s under silent conditions.

The Cup Horn was thermostated by a recirculating cooling bath (FC Chiller, Julabo). The transducer was driven by a computer-controlled ultrasonic generator (SinapTec NexTgen Inside 500) to select the US amplitude/power and to directly monitor the temperature rise with a temperature probe immersed in the suspension.

A non-mechanical water extraction was performed and used as a reference for this study. Extraction was performed by placing the 100 g L^{-1} suspension in water with a constant temperature set at 40 °C and a mechanical stirring at 900 rpm for 30 min.

Before and after each treatment, samples were characterized and then centrifuged at 8000*g* for 20 min to collect the supernatant for biochemical analysis and cell destruction characterizations.

2.3. Sample biochemical analysis and cell destruction characterizations

For each experiments, data are expressed as mean ± standard deviation. The error bars, shown on the figures, correspond to the standard deviations.

2.3.1. Cell size analysis

The changes in cell size of the suspension before and after each

treatment were monitored by wet laser diffraction with the Mastersizer 3000 (Malvern Panalytical, UK). A refractive index of 1.45 and an absorption index of 0.100 were used for the measurements.

2.3.2. Cell destruction rate analysis

Cell counts were performed by counting 10 squares of a Malassez cell (0.25 mm × 0.20 mm × 0.20 mm) and imaged using a Nikon SMZ 1500 at 40 fold magnification. The suspensions were diluted 100 times before observations and all the analysis were performed in duplicate.

Trypan blue exclusion test of cell viability was also performed before and after each treatment at 20 kHz. The suspension was diluted at 1:10 and 1 mL was mixed with 1 mL of 0.4% Trypan blue solution (ThermoFisher Scientific). 20 μL of sample was placed on a glass face with a coverslip. Observation was done using a Nikon SMZ 1500 at ×200 magnification. Images were captured by a Nikon Eclipse E600 camera. Trypan blue is a stain that binds to membrane proteins when the membranes are no longer intact [1].

2.3.3. Cell morphology analysis

The cell morphology of *T. suecica* cells in suspension before and after treatment was characterized using the Morphologi G3 (Malvern Panalytical, UK) which is an optical microscope associated with a software allowing to scan and save the image of all measured objects. The suspensions were diluted 1:10 and then placed on a wet cell plate. 5000 particles were scanned and the observations were done at ×200 magnification. Different shape parameters can be calculated from the measured area and perimeter of the individual objects and their convex hull. Two morphological criteria, circularity and elongation, were selected for particle shape characterization. Circularity quantifies how close the shape is to a perfect disk. A perfect circle has a circularity of 1.0, while an elongated or irregular object has a circularity closer to 0. Elongation has values in the range 0–1. A symmetrical shape, such as a circle or square, has an elongation value of 0; shapes with large aspect ratios have an elongation closer to 1.

2.3.4. Scanning Electronic Microscopy analysis

SEM (Scanning Electronic Microscopy) images of the *Tetraselmis* suspension were acquired at ×5096 magnification at an accelerating voltage of 5 kV with a QUANTA 250 FEG microscope (FEI company, France). Cells were previously fixed in polylisin buffer and stored at 4 °C until analysis.

2.3.5. Biochemical analysis

After water evaporation, the rate of protein release in the supernatant was evaluated by elemental analysis of total nitrogen using a Perkin Elmer 2400 Series II flash combustion analyzer. The conversion factor N = 6.25 was selected to convert the nitrogen level to protein.

The Dubois colorimetric assay was used to determine the polysaccharide content of each supernatant dried sample, using glucose as the standard solution. The absorbance was determined at 490 nm using a BMG-LabtechSpectrostar-Nano spectrophotometer (BMG LABTECH SARL, Champigny s/Marne, France). Briefly, 10 mg of dry sample was hydrolyzed for 60 min at 100 °C with sulfuric acid. 200 μL of the hydrolysate was then mixed with 200 μL of phenol (5% w/w) and 1 mL of sulfuric acid and incubated for 60 min at 100 °C.

The extraction yields of each compound (proteins and carbohydrates) were then calculated relative to their initial composition in the dried biomass.

3. Results & discussion

3.1. Selection of operating conditions and preliminary results

In this study, all experiments were performed in water in order not to modify the nature of the initial solvent in which the biomass is grown and thus avoid the use of harmful solvents. As mentioned earlier, the cell destruction and the increase of extraction yields by ultrasound-assisted extraction is caused by the propagation of ultrasonic waves through the liquid medium and the implosion of cavitation bubbles, resulting in mechanical and thermal effects. The energy dissipated due to the propagation of ultrasonic waves can cause a rapid increase in the temperature and can have in some cases a positive effect on the extraction [8]. Shirsath et al. [50] showed a beneficial effect on ultrasonic extraction with temperatures ranging from 30 to 40 °C. In a study of *T. suecica* cell destruction for proteins recovery, Safi et al. [47] showed that a temperature increase of the cell suspension to 46 °C was not sufficient to cause the denaturation of *T. suecica* proteins. However, it has already been observed that a too large increase in temperature can lead to a decrease in the sonochemical effects, as cavitation is much less efficient due to high vapor content in collapsing bubbles [6]. An excessive increase in temperature can also cause in some cases the denaturation of the extracted molecules. Thus, temperature control of the extraction medium is an important parameter to consider, both to maximize the efficiency of the ultrasound and to preserve the molecules from denaturation. Preliminary work was performed to ensure that the temperature remains acceptable during the experiment. Monitoring of the temperature evolution was performed during 60 min as a function of the irradiated ultrasonic power (50 W, 100 W or 200 W) and mode (continuous or pulsed) in a 100 g L^{-1} cell suspension of *Tetraselmis suecica*. Whatever the US power, the temperature of the medium never exceeded 43 °C and an average temperature of 35 °C was thus retained for the further experiments.

Moreover, as the acoustic streaming was not sufficient to ensure a constant homogenization of the suspension over time, the tests were performed under stirring. To verify that the cell destruction observed after US treatment was the result of US and not stirring, a maceration was performed to estimate the resistance of the *T. suecica* cells. This maceration was performed by placing the 100 g L^{-1} suspension in water at 40 °C and with a mechanical stirring at 900 rpm. The cell size distribution of the *T. suecica* suspension before and after this treatment is described in Fig. 3 – Supplementary data. A bimodal distribution is visible for both untreated and treated cells. Overall, the same cell size distribution is visible, with an intact cell population between 9 and 11 μm, and a second size population, surely cell agglomerates, of about 60 μm, indicating that this heat treatment did not cause cell destruction. SEM observations seem to confirm these results, for both untreated and treated suspensions, the cells still appear intact, with a circular and relatively smooth shape. Maceration in heated water (40 °C at 900 rpm) is insufficient to destroy or at least alter *T. suecica* cell wall.

3.2. Effect of low frequency 20 kHz on cell destruction and metabolites extraction efficiency

3.2.1. High power 200 W

As previously described, *T. suecica* cells are enclosed by a wall described in the literature as relatively resistant to attack by its surrounding, osmotic shock, temperature elevation or alkaline lysis [13]. The cell wall of *T. suecica* is of glycoprotein type [33]. Depending on the culture conditions, it has been observed that it can be formed by 5 layers [4]. These different layers of the cell wall are composed by carbohydrates and proteins, forming a complex network and providing a more resistant cell structure [35]. *T. suecica* required more extreme conditions to be destruct and a high energy input to induce cell wall damages. The effect of three ultrasonic powers (200 W, 100 W and 50 W) at a frequency of 20 kHz on the cell destruction of *T. suecica* was studied.

Fig. 1 represents the evolution of cell size of a *T. suecica* suspension measured by laser granulometry. The cell suspension concentrated to 100 g L^{-1}, was irradiated by a US power of 200 W equivalent to a power density of 0.1 W mL^{-1}, during 60 min. The initial suspension before US treatment is composed of two main types of cell populations. A population of 10 μm cells corresponding to the intact cells and a population of 52 μm size corresponding to the agglomerates that may form in the

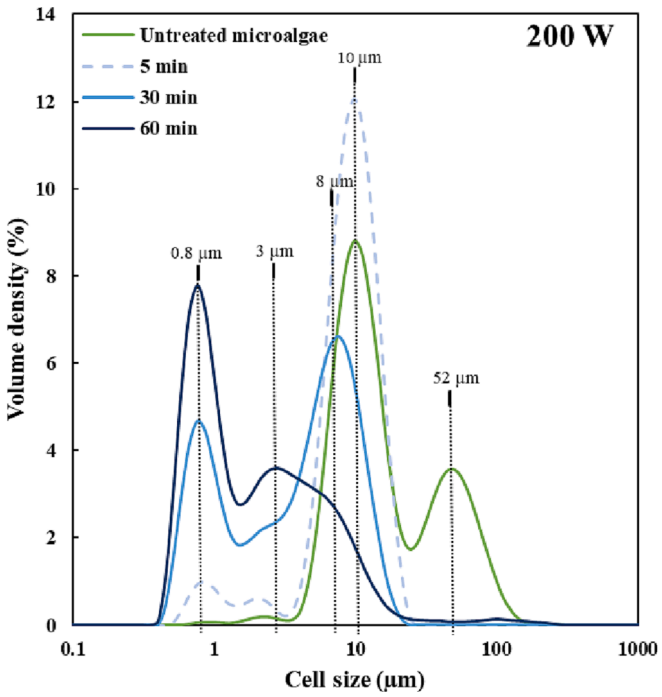

Fig. 1. Evolution of the cell size distribution of a suspension of *T. suecica* at 100 g L^{-1} irradiated by 200 W.

biofilm. A third population of size is visible, at 3 µm, which can indicate the presence of some cell fragments. This trimodal size distribution is still visible after 5 min of US treatment at 200 W but the population shift to smaller fragment sizes. The population of cell agglomerates initially present at 52 µm disappears in favor of a larger population of cells at 10 µm. From the first minutes, US seems to break the cell agglomerates. This phenomenon was also reported by Sivaramakrishnan and Incharoensakdi, [52]. The ultrasound irradiation, by causing a cavitation and vibration effect on the cell walls, separates the agglutinated cells. The US also generates two new fragment sizes, one at 3 µm and one at 0.8 µm, indicating a beginning of cell destruction. After 30 min of US treatment, the peak of intact cells at 10 µm shifts slightly to the left to reach a cell size of 8 µm; this slight decrease may indicate that the cells have deflated. Finally, after 60 min of US treatment, no more intact cells are visible; these having been reduced to 3 µm. Cell fragments of 0.8 µm become the majority population, indicating a strong cell destruction caused by the US power of 200 W.

To corroborate these observations, the morphology of the cells before and after this treatment was monitored by SEM (Fig. 2.A). SEM observations were used to understand the mechanisms of cell destruction that can be caused by US waves at 200 W. Fig. 2.A shows untreated microalgae cells appearance. *Tetraselmis suecica* have intact cells with an ovoid shape of about ten micrometers. From 5 min of US, two main types of population are visible, intact cells and coiled-up cells. After 30 min of exposure at 200 W, a radical change in the cells shape is observed: the cells appear as coiled-up on themselves (white arrow) such as a "burst balloon", with a size inferior to 10 µm. At 60 min of US, intact cells are no more visible, the coiled-up cells became the majority and seem to aggregate to each other. The classical pattern of mechanical cell destruction could be expected to destroy the cell in thousands of small fragments as observed by the granulometry particle size analysis. However, these observations show that study of the size of the fragments generated is not sufficient to understand cell destruction. Moreover, in this case, the cells do not tend to fragment but rather adopt another shape by tearing. To better understand this phenomenon, an analysis was performed with the automated microscope Morphologi G3 (Malvern, UK) to obtain a detailed description of morphological properties of the cells. Mean circularity and elongation values are reported in Fig. 2.B. For untreated microalgae, the value of the circularity factor is 0.96, which is very close to 1, allowing to describe a perfect circle, consistent with the observed cell shape. A lower mean elongation value of 0.14 was also calculated, probably corresponding to the rare fragments generated. After 30 min of treatment, this trend is reversed. The mean circularity value almost halves, indicating the loss of the circular structure of the cells. In addition, a clear increase in elongation occurs, from 0.1 to 0.7. These results are consistent with SEM observations, where the cells take the form of rods. In literature, Natarajan et al. [42] have previously observed this phenomenon of cell coiling into rod-shaped structures caused by exposure to US with *T. suecica* cells. This phenomenon can be explained by the fact that *T. suecica* cells have a flexible plasma membrane, and therefore are able to coil up after being degraded.

3.2.2. *Intermediate power 100 W*

A power of 100 W giving a power density of 0.05 W mL^{-1} was also

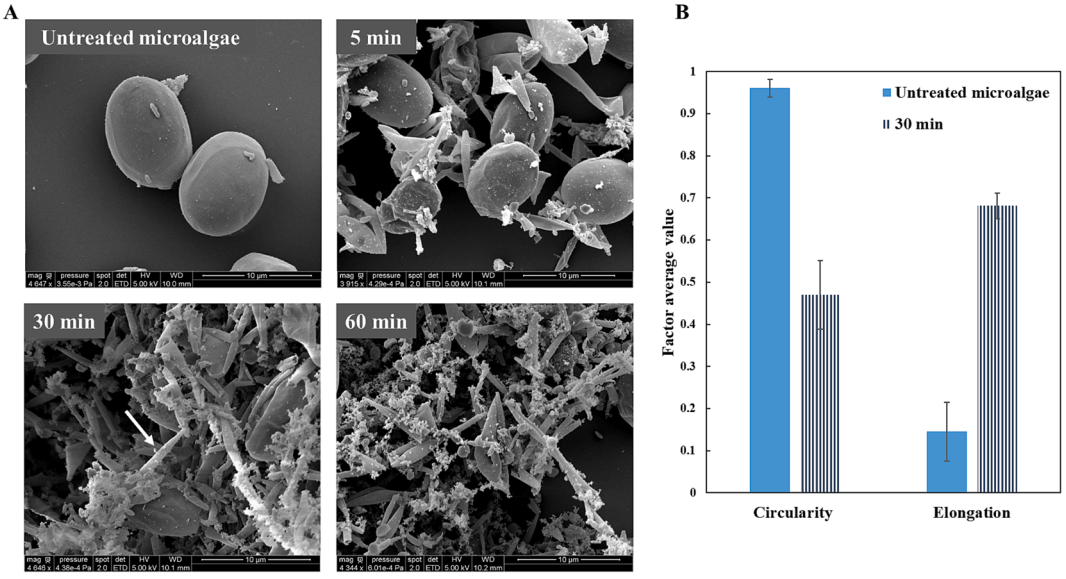

Fig. 2. (A) SEM images of the morphology evolution of cells irradiated with 200 W; (B) Evolution of mean circularity and elongation factors measured by morphological study of untreated cells and after 30 min of irradiation with 200 W.

studied. Fig. 3.A shows the evolution of cells size during 60 min of exposure to US at 100 W. The micronization behavior of the cells is relatively the same with 100 W and with 200 W. The cell destruction induced by 100 W seems to be only slightly slower than for 200 W. During the first 5 min, cell agglomerates (peak at 52 μm) are disaggregated into single cells (peak at 10 μm), and cell debris (peak at 0.8 μm) starts to appear. Considering the kinetics, cell destruction increases with time, with an increasing volume of destroyed cells at 0.8 μm and a decreasing volume of intact cells at 10 μm, between 30 and 60 min. Fig. 3.B illustrates this phenomenon, with the SEM image at 5 min showing both still intact cells as well as destroyed and coiled up cells. After 30 min of treatment, a large proportion of the cells appear destroyed and at 60 min the majority of the cells are fragmented, and no more round cells are visible.

3.2.3. Low power 50 W

Finally, a lower power, 50 W (power density of 0.025 $W.mL^{-1}$), was also compared to 100 W and 200 W. As observed in Fig. 4.A, the cell destruction kinetics is significantly slower than for higher powers. After 60 min of treatment, only a small proportion of cellular debris smaller than 1 μm is generated. The SEM image (Fig. 4.B) at 5 min of treatment confirms a slower destruction mechanism. The appearance of the cells is similar to the untreated ones. Furthermore, SEM observations shows that a majority of the cells still maintain a circular shape without major changes in their morphology even after 30 min of sonication. Cellular debris seems to become the majority only after 60 min of treatment.

A more detailed study of the cell morphology after 5 min of sonication was performed (Fig. 5). Low US power emitted over short times seems to result in a novel mechanism of cell destruction by not causing cell coiling but rather a membrane perforation for a minority of the cells (Fig. 5.A). Most of the cells appear intact (Fig. 5.B) some are perforated (Fig. 5.C) or shrunk (Fig. 5.D).

Staining with Trypan blue was used to check the integrity of the membranes. Blue Trypan can only penetrate when the membrane is no longer intact, indicating the loss of integrity of the cell membranes. After 5 min of sonication at 50 W, the cells retain their green color as well as their circular shape characteristic of untreated microalgae, indicating that application of the ultrasound treatment did not produce cell wall disruption (Fig. 6). After 30 min, although the majority of cells retain their original shape and staining, the presence of blue in the membranes indicates the loss of cell integrity. In contrast, with 200 W, significant morphological changes are visible from the first few minutes. The few remaining cells are blue and no longer impermeable, with fragmented and bleached cells visible, indicating losses of intracellular components and so strong cell destruction.

Regardless of these observations showing that at 50 W a large proportion of the cells have conserved their membrane integrity (Fig. 6), protein liberation monitoring (Fig. 7.A) shows an increase in protein released into the medium as a function of sonication time. Between 0 and 5 min, the protein extraction yield increases from about 15% to 40%. A low power ultrasound and a short treatment can extract part of the proteins without affecting cell membrane integrity. These observations raise the question whether *T. suecica* could be exploited in a milking mode. Milking is a concept defined by the fact that the extraction of the compounds should not kill the cells. This mode of extraction makes it possible to extract the target molecules and to rebloom the cells to start another cycle. Milking allows optimizing the culture and harvesting steps of microalgae whose costs are still too high compared to the whole biorefinery chain [60]. As pulsed electric field technology, ultrasound could be used as a biocompatible and non-destructive technology for protein extraction from living microalgae cells. Indeed, studies have demonstrated the ability of some microalgae to release the target compounds into the extraction medium without being destroyed or too severely micronized [2,9]. These studies have also shown a reversible permeabilization of their membrane, with rapid recovery and reintegration into cultivation systems [22]. These first results are very promising but complementary tests, to verify the cell viability, are necessary before being able to consider this method of valorization. The Trypan blue test only allows controlling the integrity of the membrane, but does not guarantee cell viability. The growth potential of *T. suecica* after such treatment have to be investigated [54] and further tests such as flow cytometry, the use of more specific markers or a measurement of enzymatic activity are necessary before a conclusion can be made [16].

On the contrary, prolonged exposure to US causes serious damages to cell morphology and leads to a release of more metabolites. Ultrasonic power had a positive impact on carbohydrates extraction efficiency (Fig. 7.A). The highest power, 200 W, is the most efficient to extract the carbohydrates, from the first minute of sonication. Contrary to proteins, there is no progressive increase of the carbohydrate concentration, but a maximum value is reached after 5 min of treatment, with a yield of about 60%. Only the lower powers, 50 W and 100 W, allow the gradual extraction of carbohydrates from the medium, from 20% for 1 min to 40 and 55% respectively after 60 min. Extraction yields of carbohydrates remain lower than those of proteins even after 60 min of US. This demonstrates that the sonication time has an essential role in compound extraction and this different extraction behavior could be explained by the location of carbohydrates in the cells. Indeed, microalgal carbohydrates can be divided into three major classes (structural or reserve

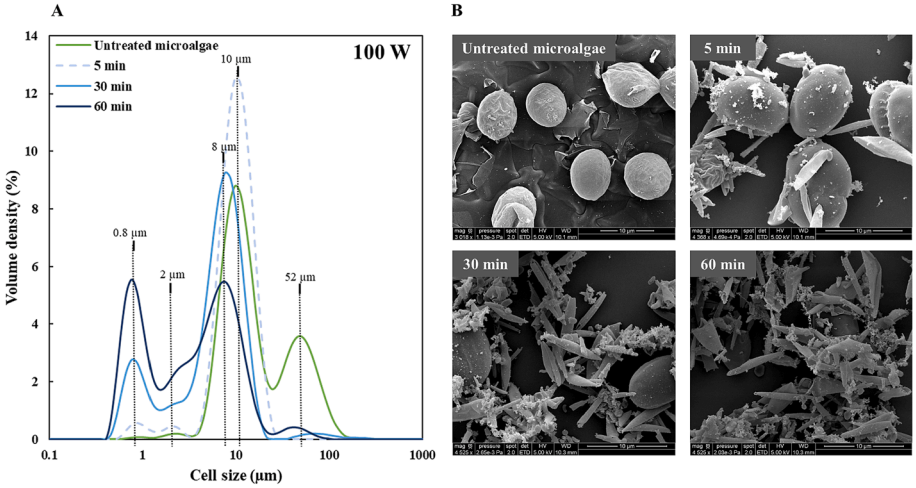

Fig. 3. (A) Evolution of the cell size distribution of a suspension of *T. suecica* at 100 g L^{-1} irradiated by 100 W; (B) SEM images of the morphology evolution of cells irradiated with 100 W.

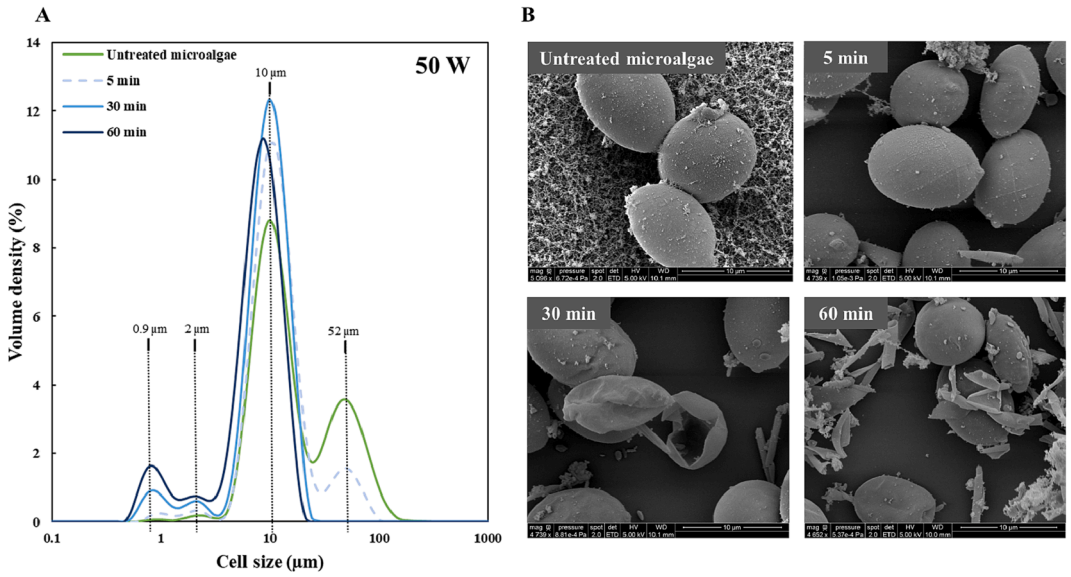

Fig. 4. (A) Evolution of the cell size distribution of a suspension of *T. suecica* at 100 g L^{-1} irradiated by 50 W; (B) SEM images of cells irradiated with 50 W.

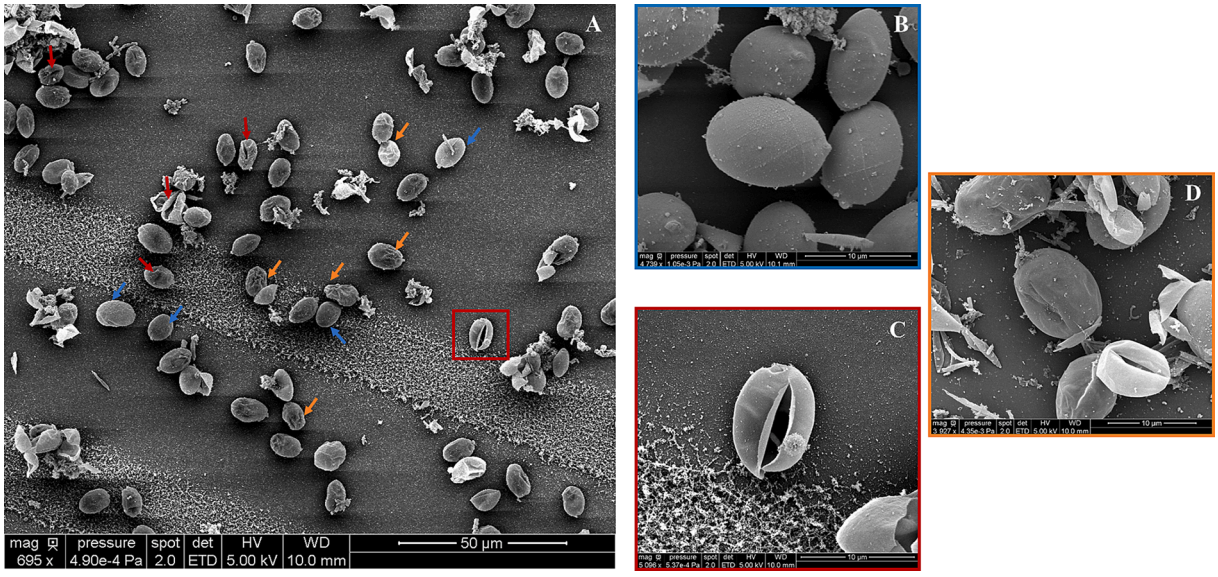

Fig. 5. SEM of cells after 5 min of irradiation of the suspension by 50 W (A), and representative zooms of the different possible cell states, intact (B), perforated (C), and shrunken (D).

polysaccharides and exopolysaccharides) based on their physiological roles in the cell [21]. Concerning structural polysaccharides, in the case of *Tetraselmis suecica*, the theca is mostly composed of monosaccharides: the Kdo (3-deoxy-D-*manno*-oct-2-ulosonic acid) and DHA (3-deoxy-lyxo-2-heptulosaric acid), galacturonic acid and galactose [35]. In the microalga *T. suecica*, reserve polysaccharides are intracellular consisting of a main chain of α 1–4 linked glucose with α 1–4-6 branched glucose residues [45]. Finally, in some cases exopolysaccharides are found [62]. Since the majority of carbohydrates are intracellular, the following hypothesis can be considered. Carbohydrates release would be faster and more important at 200 W because this power damages the cells more rapidly and severely than with lower powers, leading more easily to the release of this various types of carbohydrates into the medium.

Protein yields are also impacted by ultrasound power (Fig. 7.B). Extraction yields increase with time, reaching about 88% with high powers 100 W and 200 W, and 78% with 50 W, after 60 min of sonication. In the first 10 min, the high power 200 W is significantly more efficient compared to 50 W and 100 W by extracting almost twice as much protein. However, it can be noted that after 15 min of sonication, the difference in protein yields decreases between the three powers. Several studies have shown that the efficiency of US tends to decrease when the number of destroyed cells increases [23,24]. Indeed, the 200 W power, by destroying and releasing the intracellular contents from the first 5 min, causes a more rapid modification of the irradiated medium. The viscosity increases causing a bad propagation of the waves in the medium and a decrease in the intensity of cavitation. Another study conducted by De Souza-Barboza [11] showed that beyond a certain US power (and US power density) an acoustic shielding phenomenon could occur. It decreases the efficiency by the formation of a large amount of cavitation bubbles next to the probe that will disperse the acoustic energy and therefore decrease the power transmitted to the medium. This information shows the importance of optimizing the irradiation intensity of the medium to have a satisfactory extraction efficiency-energy consumption ratio.

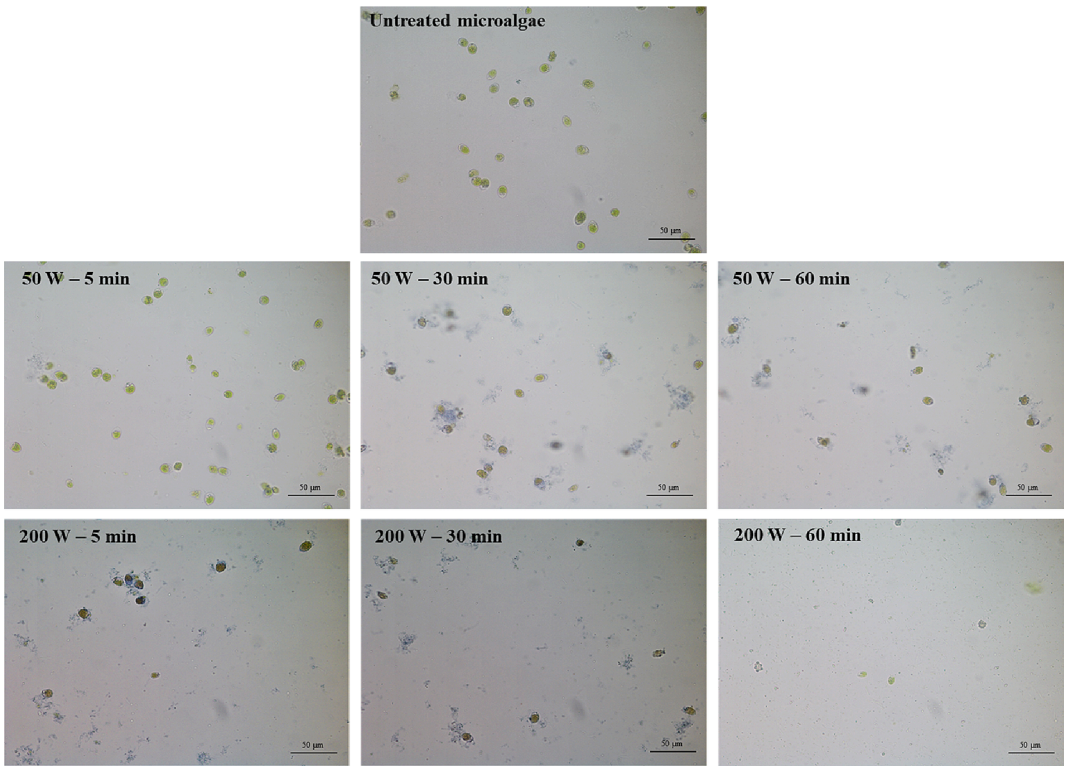

Fig. 6. Membrane integrity study of *T. suecica* cells by blue Trypan staining, before and after US treatment at 50 W and 200 W, as a function of time. Microscopic observation was performed at x200 magnification. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

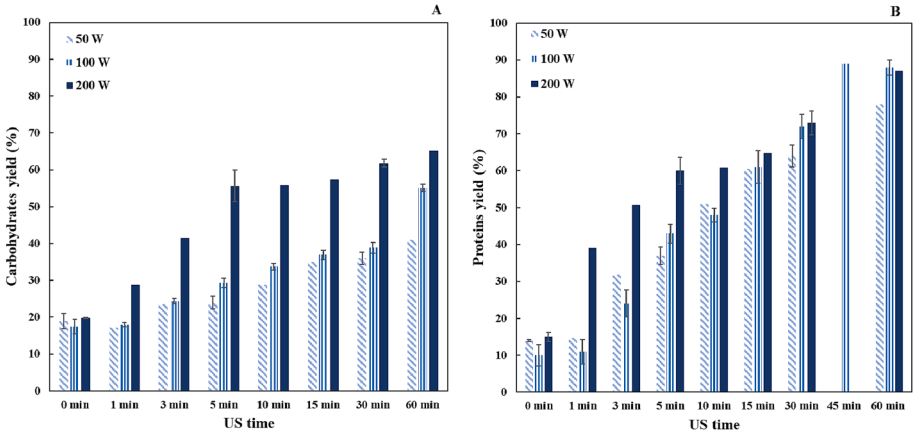

Fig. 7. Maximum carbohydrates (A) and proteins (B) extraction yields obtained as a function of applied ultrasound power and over treatment time.

3.2.4. *Effects of sonication in a continuous or a pulsed mode*

As discussed in the previous part *3.2.3*, optimization of the irradiation intensity of ultrasound seems to be a crucial parameter and applying ultrasound by a pulsed mode can influence the efficiency of the process and limit energy consumption. Several studies show that this mode of operation is sometimes more efficient in terms of extraction yield but also in terms of reduction of specific energy consumption than applying US in a continuous mode [18]. In Caprio [7] study, sonication was tested in pulsed mode (T_{on} = 0.1 s, T_{off} = 0.5 s) and the extraction yield of carbohydrates from the microalgae *Tetradesmus obliquus* and *Chlorella* sp. was monitored. They showed that the pulsed mode was more efficient than the continuous mode, achieving about 3 times higher carbohydrate extraction while consuming 6 times less kWh per kg of extracted carbohydrates. Therefore, the effect of pulsed mode at the highest power which was the more efficient was investigated on *T. suecica* (US power = 200 W and T_{on} = 30 s, T_{off} = 30 s) and compared to the continuous mode, in order to determine if cell destruction, extraction yields and energy consumption could be improved. Indeed, the 200 W pulsed mode consumes as much energy as the 100 W continuous mode and half as much as the 200 W continuous mode.

Fig. 8.A shows the evolution of the cell destruction rate according to the US power and the application mode. The pulsed mode at 200 W clearly gives better cell destruction rates compared to 100 W in a continuous mode and a cell destruction at least equivalent to that obtained at 200 W continuously. These results validate that a high US power is needed to achieve the destruction of *T. suecica* cells. It can be noted that the results become very close with long US treatment times but the evolution of proteins release follows the same trend (Fig. 8.B). The maximum efficiency reached after 30 min is about 73% for the three conditions tested. However, by using the pulsed mode, the power

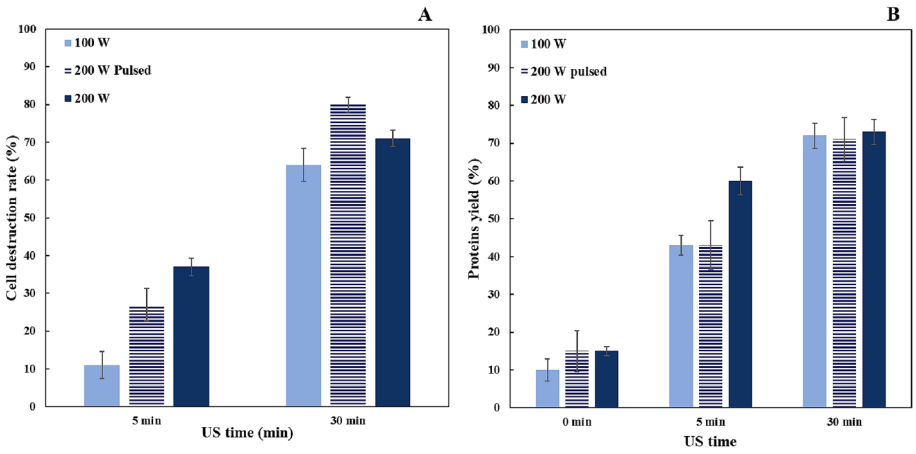

Fig. 8. Effect of continuous and pulsed mode on cell destruction rates (A) and proteins extraction yield (B) over sonication time.

consumption decreases by 50% compared to the continuous mode for 200 W for an equivalent protein yield. This value decreases from 0.5 kWh kg^{-1} to 0.25 kWh kg^{-1} when using the pulsed mode.

3.2.5. Effect of initial biomass concentration on the efficiency of ultrasound for the extraction of metabolites

Biomass conditioning is important and can have an impact on the efficiency of extraction and cell destruction. Indeed, the initial concentration of the biomass is known to mitigate the efficiency of the US treatment. An increase in the cell concentration can induce a decrease in the yields of molecule extractions as well as in the level of cell destruction rates [64]. In this study, the effect of three initial cell concentrations, for a 100 W US power, was studied on protein yields (Fig. 9. A). During the first 10 min of sonication, the protein extraction kinetics is faster for the lowest concentration, 10 g L^{-1}, than for 50 g L^{-1} and 100 g L^{-1}. Then, this difference is reduced for 15 min and 60 min of US for the two lower concentrations, respectively 10 g L^{-1} and 50 g L^{-1}. The maximum extraction rate reached, after 60 min of US, is 90% and is, surprisingly, the same for each concentration tested. Monitoring carbohydrates yield rate (Fig. 9.B) also seems to lead to the same conclusions as for proteins. Yields are slightly better with the lowest concentrations 10 g L^{-1} although the difference between the two lowest

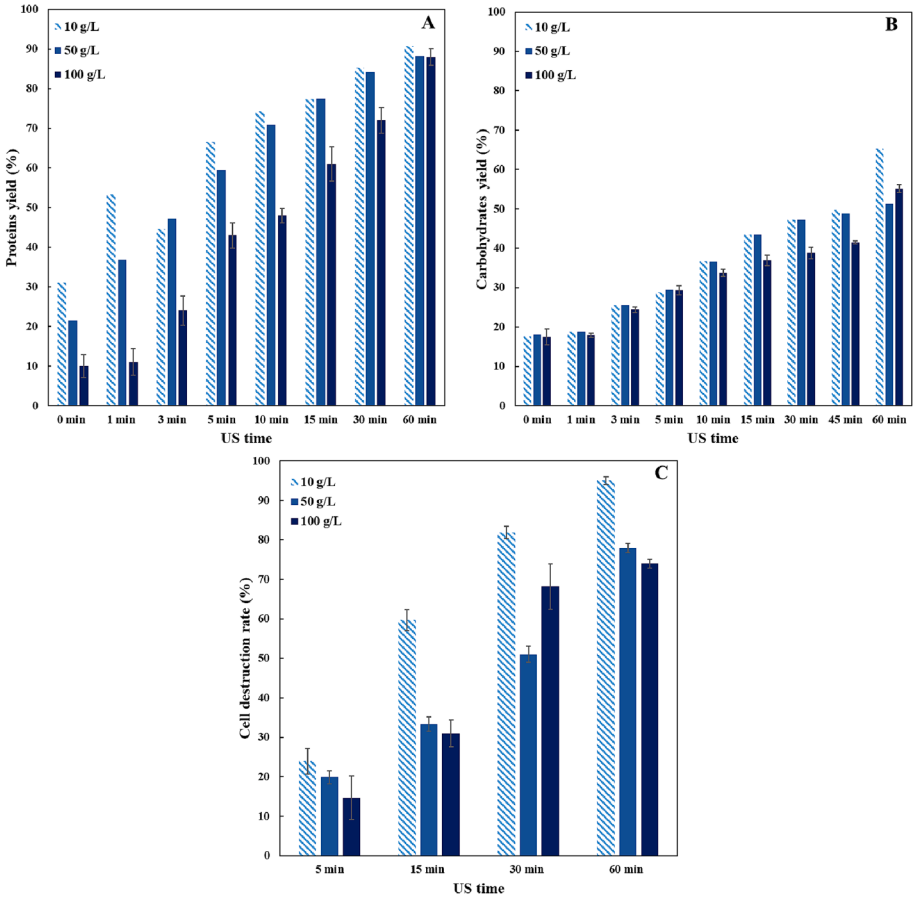

Fig. 9. Effect of initial biomass concentration for a US treatment operated at 100 W for 60 min on. Proteins (A) and carbohydrates (B) extraction yields and cell destruction rate (C).

concentrations is much smaller. The final rate of the three concentrations tested do not stabilize even after 60 min of US contrary to the protein one. These results seem to be consistent with the cell destruction rates (Fig. 9.C). Indeed, almost 100% of cells are destroyed after 60 min of US with the lowest cell concentration, compared to only about 80% for the two concentrations above 10 g L^{-1}. These results indicate that the suspension concentration does affect the efficiency of US and to a lesser extent the release of the molecules, depending on their location.

Several studies have shown that the use of US was not necessarily more effective at high concentrations. In a study dealing with the extraction of carbohydrates from *Chlorella vulgaris* [65], a maximum glucose yield of 36.85 g/100 g DW (Dry Weight) was obtained. However, this value decreased when the cell concentration increased from 0.3 to 3.0 g L^{-1}, leading to a final yield of 31.35 g/100 g DW for a suspension at a concentration of 1 g L^{-1}, for example. Natarajan et al. [42] showed that doubling the concentration of a suspension of *Chlorella* sp. irradiated by US, from 6.84 g L^{-1} to 12.2 g L^{-1}, did not significantly increase the efficiency of cell destruction. Similarly, another study conducted on *Chlorella vulgaris* sonicating the medium at 400 W (24 kHz) [37] brings similar conclusions. Another study from Greenly and Tester, [24] showed that on *Isochrysis* sp. suspensions, a slight decrease in cell destruction is visible when the concentration of the suspension is increased from 5 g L^{-1} to 70.5 g L^{-1} indicating that more energy will have to be supplied to destruct the cells at higher cell concentration. This observed phenomenon can be attributed to the fact that above a certain level of concentration, the cell population becomes too dense and attenuates the efficiency of the propagation of the shock waves resulting from the implosion of cavitation bubbles. Thus, the vibration of the shock wave is spread over a larger number of cells, which results in a decrease in the amount of energy distributed to each cell [27]. These results show the crucial importance of considering both the physical parameters (power, sonication time, power density) and the intrinsic parameters of the biomass (initial concentration), in order to operate under optimal conditions.

3.3. Intermediate frequency: 100 kHz

3.3.1. Effects of the power of ultrasound

The effectiveness of ultrasound-assisted extractions also depends on the ultrasound frequency. Generally, the ultrasound frequencies used for industrial applications are between 20 kHz and 100 kHz and are commonly referred to as « conventional power ultrasound » [40]. Some studies have shown that microalgae cell disruption is frequency dependent and that its effectiveness can vary according to the characteristics of the microalgae [36,61]. Even if low and high frequency can have both a significant impact on microalgae cell disruption and metabolite extraction [20,63], other studies show that sometimes high frequencies are less effective for cell destruction [38]. Indeed, it has been shown that at high frequencies, the acoustic cycle is shorter than at low frequencies, making the bubbles formed during cavitation smaller that lead to less violent cavitation collapse [41]. The mechanical effects induced are therefore less important than at low frequency.

In this study, the effect of an intermediate frequency of 100 kHz on cell destruction, protein and carbohydrate extraction was compared to the lower frequency of 20 kHz. Three power levels, 30 W, 60 W and 120 W were tested. In order to compare the two frequencies from an energetic point of view, the minimum power at 100 kHz was chosen to be 30 W, as this corresponds to the same power density of 0.1 W mL^{-1} irradiated in the previous experiment at 200 W and 20 kHz. Fig. 10.A shows the evolution of cell size after 60 min of irradiation by 30 W power. The median size of untreated microalgae is 7 μm. There is no visible change in cell size even after 60 min of treatment. The cells do not seem to fragment. Fig. 10.B appears to confirm these observations. The SEM images selected at the four times studied (3, 5, 30 and 60 min) show that the cells hardly fragment, but rather acquire a deflated cell appearance with heavily impacted surface and no visible perforation.

When applying a power of 60 W (Fig. 11.A), no cell destruction is observable up to 30 min of US. No fragments are generated and the median cell size remains 7 μm. SEM observations (Fig. 11.B) show similar cell morphological damage at 30 W and 60 W, with a cell shrinking effect up to 30 min. In contrast, at 60 min, the cells appear fragmented and perforated on SEM observations with a large proportion of fragments generated with a median size of 1 μm. Contrary to what has been observed for a lower frequency (20 kHz), the cells are not affected in the same way even for low powers depending on the frequency used. The absence of membrane fragmentation and coiling observed at 20 kHz in favor of cell deflation and shrinkage has already been described in the literature [20,30] for *Chlorella vulgaris*. This milder way of damaging cells by US could possibly be a way for the extraction and valorization of other high added value molecules like exopolysaccharides [12], using the capacity of cavitation bubbles implosion to generate a surface peeling effect [8]. Indeed, some studies have shown that these molecules secreted outside the cells, most often in an extracellular matrix, can be extracted by US. Bagher Hashemi et al. [5] showed that the use of US on

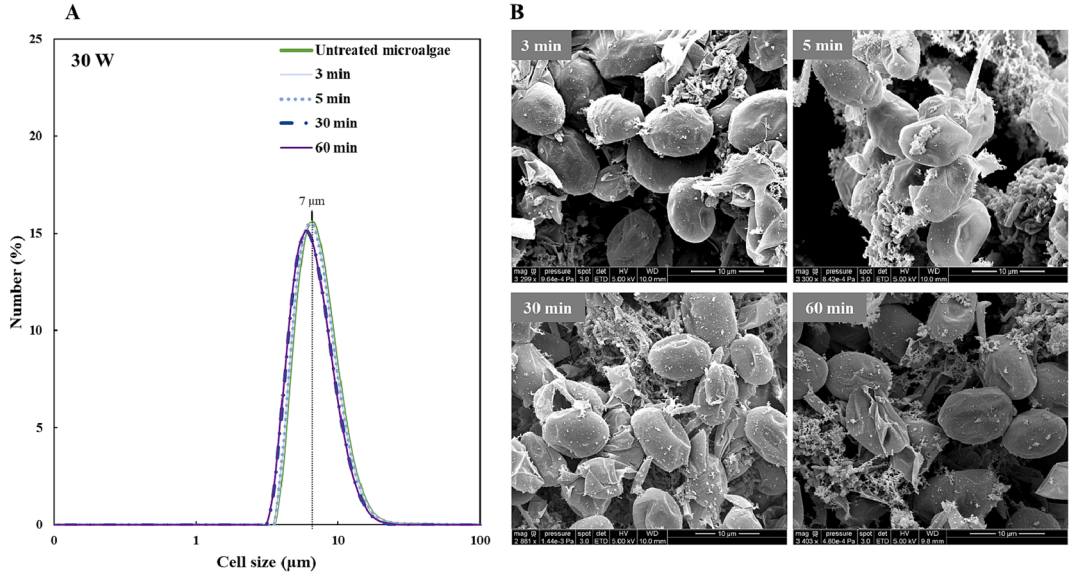

Fig. 10. (A) Evolution of the cell size distribution of a suspension of *T. suecica* at 100 g L^{-1} irradiated by 30 W; (B) SEM images of cells irradiated with 30 W.

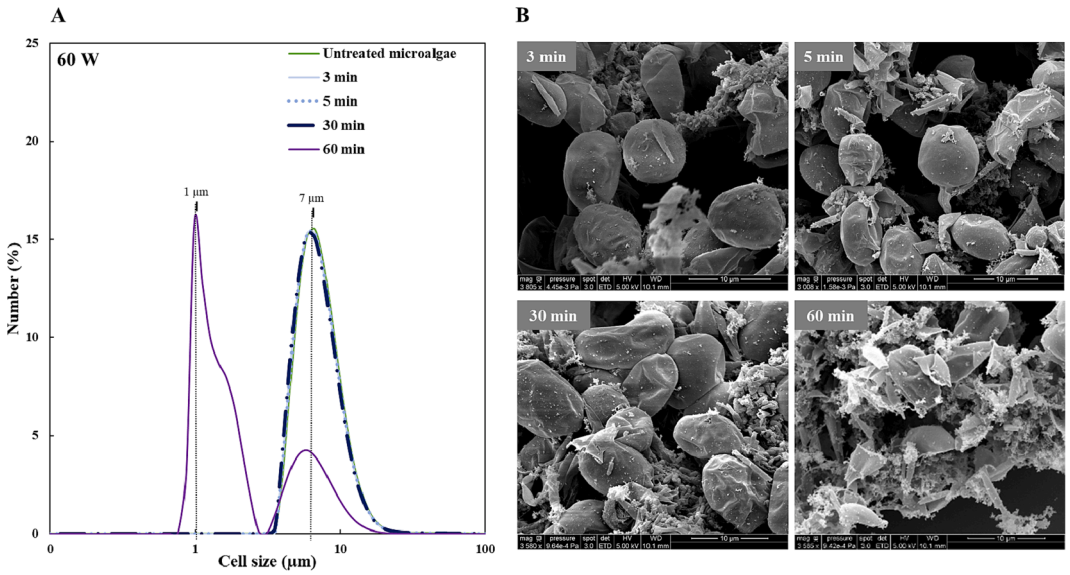

Fig. 11. (A) Evolution of the cell size distribution of a suspension of *T. suecica* at 100 g L^{-1} irradiated by 60 W; (B) SEM images of cells irradiated with 60 W.

Lactiplantibacillus plantarum cultures led to an increase in exopolysaccharide production. Similarly, Hasheminya and Dehghannya, [28] showed that combining US irradiation and a moderate temperature (68 °C) increased the yields of extractions of kefiran exopolysaccharides.

Finally, cells irradiated by a higher power, 120 W, show a rapid fragmentation (Fig. 12.A). From 3 min to 5 min of US, a high proportion of fragments of about 1 µm are generated. Between 5 and 60 min, this fragment rate increases and the proportion of intact cells (peak size of 7 µm) decreased drastically. As observed in Fig. 12.B, from 3 min and up to 60 min of US, SEM micrographs show the presence of perforated cells, fragmented cells and cellular debris.

Protein and carbohydrate yields and cell destruction rates correlate with these observations. For 30 W and 60 W, the protein yield rates remain low, about 18% during the first 30 min and increase only slightly to 20% at 60 min (Fig. 13.A).

Release kinetics are also slow for carbohydrates (Fig. 13.B). A slight increase in yield is observed for 30 W and 60 W from 5 min of US. The cell destruction rates (Fig. 13.C) also remain very low during the 60 min of treatment. At 30 W, only 13% of the cells are destroyed from 10 min of US, and this rate remains constant until the end of the treatment. At 60 W, cell destruction is higher than at 30 W, with 20% of cells already destroyed after 10 min and with a final rate of 30% of cells destroyed in total. The trend is different with the 120 W power. From the first minutes, the rate of extracted proteins increases from 23% at 1 min to 90% at 60 min. The extraction kinetics are similar for the extraction of carbohydrates, with a maximum yield reached at 60 min of US of 80%. However, the rates of cell destruction at 120 W remain quite low (40% at 60 min) compared to the amount of metabolites extracted. This can be explained by the fact that the morphology of the cells destroyed at 120 W and 100 kHz is very different from those destroyed at 200 W and 20 kHz (perforated cells instead of coiled cells). It is therefore more difficult to determine by a counting technique which cells are destroyed or not.

3.3.2. *Effect of pulsed or continuous mode at intermediate frequency*

As previously, the effect of the pulsed mode was studied in order to reduce the specific energy consumption and to compare the process performances. For this, two ultrasonic powers were studied in pulsed mode and compared to the continuous mode corresponding to the same energy consumption: 60 W pulsed compared to 30 W and 120 W pulsed compare to 60 W, for 60 min of US treatment (T_{on} = 30 s; T_{off} = 30 s). Fig. 14 shows the effect of the pulsed mode on the cell destruction rate and on protein extraction yields. The cell destruction is always faster

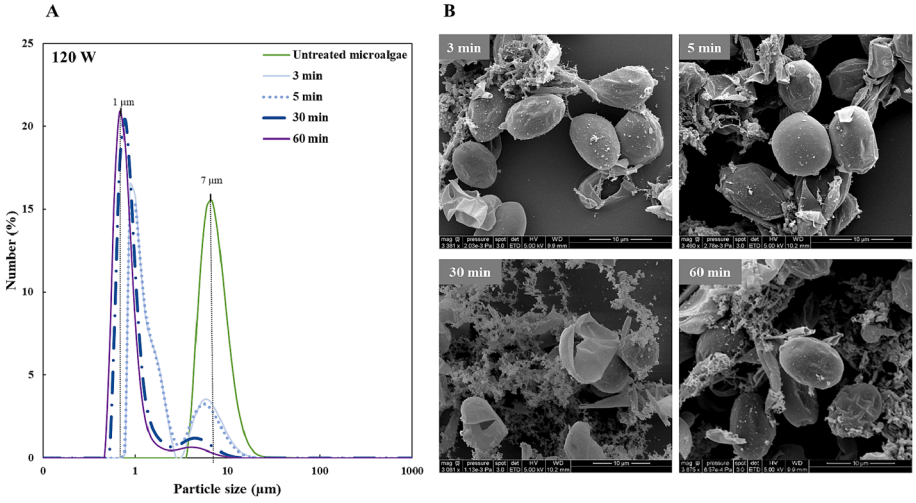

Fig. 12. (A) Evolution of the cell size distribution of a suspension of *T. suecica* at 100 g L^{-1} irradiated by 120 W; (B) SEM images of the morphology evolution of cells irradiated with 120 W.

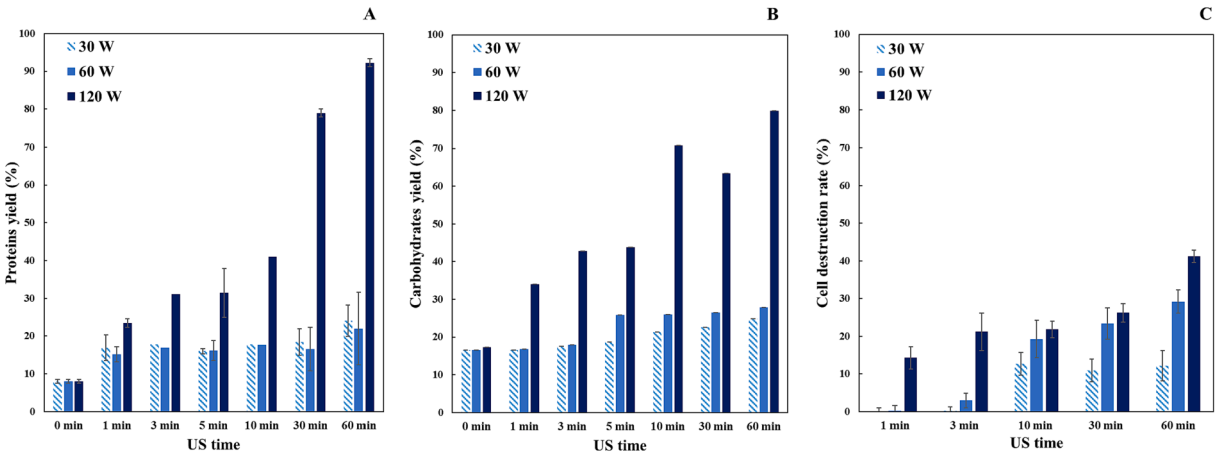

Fig. 13. Effect of irradiated power on proteins (A) and carbohydrates (B) extraction yields and (C) cell destruction rate.

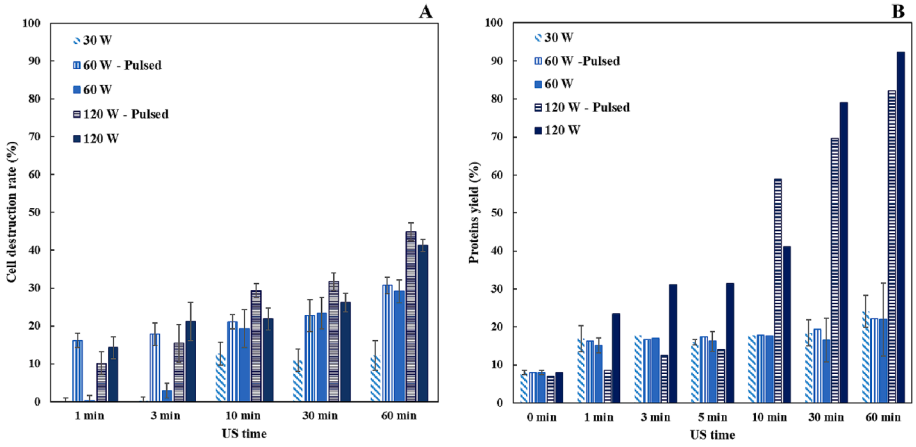

Fig. 14. Effect of continuous and pulsed mode on cell destruction rate (A) and proteins extraction yield (B).

using the pulsed mode compared to the continuous irradiation but the final rate of destroyed cells remains relatively low, approximately 50%. The protein yields are similar up to 10 min of treatment whatever the mode and the power applied.

This rate remains constant at about 20%. However, from 10 min of treatment, a clear difference in efficiency according to the mode used is visible between the powers of 60 W in continuous mode and its equivalent of 120 W in pulsed mode. The cell destruction rates with 120 W pulsed are up to 4 times higher than with 60 W. The extracted protein levels are significantly higher with the pulsed mode from 10 min onwards, resulting in a final protein yield of 82%. These results show the interest of using the pulsed mode. This mode gives equivalent protein yields for a specific energy consumption of 2.6 kWh kg^{-1} compared to 5.2 kWh kg^{-1} obtained with the highest power 120 W in continuous mode.

3.3.3. *Influence of the power density*

In order to achieve similar extraction efficiencies (protein yield of 90%) to those obtained with previous work conducted at 20 kHz with a power of 200 W, it was necessary to increase the power density from 0.1 W mL^{-1} to 0.4 W mL^{-1}. Indeed, when working at high frequency, it is necessary to increase the irradiation amplitude to be able to maintain an equivalent cavitation rate in the system [41]. Compared to a lower frequency, more power must be delivered into the treatment medium for a higher frequency. Mason and Peters [41], concluded that to maintain the same cavitation effects from a low frequency to a frequency above 40 kHz, it may be necessary to put 10 times more power into the medium.

To better quantify the effect of acoustic power density, two additional experiments were performed at the 20 kHz frequency. The US power of 100 W and 200 W were applied in the same conditions as before but in a volume of suspensions of 1 L in order to achieve equivalent power density between the different experimental conditions. Protein yields for equivalent US power density irradiated into the medium for the frequencies of 20 kHz and 100 kHz and for power densities of 0.1 W mL^{-1} and 0.2 W mL^{-1} were compared (Fig. 15).

First, the comparison of the power density of 0.1 W mL^{-1} and 0.2 W mL^{-1} for the same irradiated power level of 200 W at 20 kHz shows a faster kinetic extraction at the highest US power density during the first 30 min. It is assumed that for an equal suspension concentration, the density of cells to be destroyed is less important in a reduced volume of 1 L, thus the release of the proteins requires less time. However, the extraction yield is not clearly improved after 60 min of sonication at high US power density (even if using the same power of 200 W but doubling the power density to 0.2 W mL^{-1}). Moreover, the yields are even slightly lower at 0.2 W mL^{-1} (86%) with 200 W than at 0.1 W mL^{-1} with 100 W (92%). These results suggest that an optimum power density exists and that an excessive power level can decrease cavitation effect. This conclusion was also drawn by Keris-Sen et al. [34] and Sivakumar and Pandit [51].

For the same power density of 0.1 W mL^{-1} and for a frequency of 20 kHz, 100 W allows to obtain higher protein yields than at 200 W. After 5 min of sonication, the rates are equivalent for 100 W and 200 W with 60% of total proteins extracted. Then, at 30 and 60 min, the 100 W – 1 L

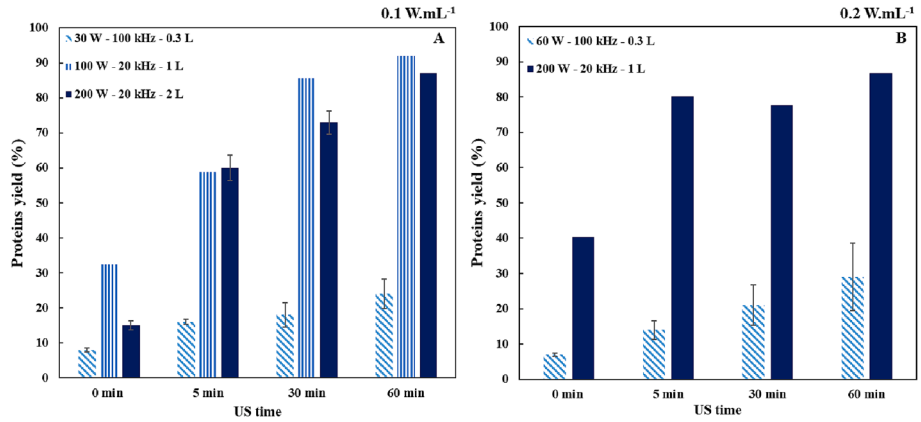

Fig. 15. Comparison of US powers according to the frequency used for the same US power density of 0.1 $W.mL^{-1}$ (A) and 0.2 $W.mL^{-1}$ (B).

condition achieves higher yields with more than 90% of total protein extracted. The kinetics is faster when emitting 100 W in a 1 L volume than when emitting 200 W in 2 L of suspension with similar operating conditions. Indeed, the acoustic cavitation and so the extraction behaviour is different according to the reactor filling and the US power even if the experiments were performed using the same reactor, the same probe immersion and the same US power density. These results indicate that the distribution and the intensity of the acoustic field depend on many other factors such as the liquid level and the position of the probe in the reactor, confirming that the scale-up cannot be exclusively based on the US power density.

Finally, whatever the irradiated power density, the results obtained at the 100 kHz frequency are always much lower than those obtained at 20 kHz. The protein levels extracted at 100 kHz are 2 to 4 times lower than those extracted at 20 kHz. Extraction yields remain low (about 25%), and the same phenomenon is visible at 0.2 $W\ mL^{-1}$ with only about 30% of the total proteins extracted. The extraction efficiency does not only depend on the power, but also on the selected frequency. These observations are in agreement with previous results [31], indicating that the frequency influences the performance of the UAE. Low frequency ultrasound seems to be more adapted and efficient for the cell destruction stage, allowing to break the cells more quickly.

Other process parameters than ultrasound frequency and power can also influence the process efficiency, such as the wave power absorbed by the irradiated medium or the ultrasonic intensity (power per square meter of emitting surface) as well as the shape of the reactor and the type of US probe [8,17,55]. However, ultrasonic power density remains an important parameter to limit the energy consumption and to control cell destruction depending on the US pilot (reactor and US equipment).

4. Conclusion

Low-frequency ultrasound, 20 kHz, was effective for cell destruction of the microalgae *Tetraselmis suecica*. This process allowed the extraction of 90% of total proteins and 70% of total intracellular carbohydrates. The use of three US powers (50 W, 100 W and 200 W) allowed to highlight different levels of cell lysis. Phenomena of cell perforation were observed at low US power for short treatment times, opening the perspective of milking, and membranes coiling at high US power. A too high initial concentration of the suspension slows down the metabolite extraction kinetics. The use of the pulsed mode showed a significant decrease of the energy consumption while keeping a similar efficiency to the continuous mode. Intermediate frequency (100 kHz) resulted in lower extraction yields. Only 25% of the total proteins are extracted at this intermediate frequency for the same irradiated power density as at low frequency. However, this original frequency revealed an interesting cell lysis mechanism, with cells deflated without visible perforation. This could be interesting to extract other types of metabolites than proteins and carbohydrates like exopolysaccharides. Finally, the article shows that many process parameters have an impact on cell destruction and metabolite extraction during US-assisted extraction such as US frequency, power, power density, reactor size and US probe position as well as treatment time. In addition, specific parameters such as biomass concentration also have significant effects on the yields and energy costs incurred. All these factors must be taken into consideration to ensure an optimal use of ultrasound in the extraction process.

CRediT authorship contribution statement

Pauline Delran: Conceptualization, Investigation, Methodology, Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Christine Frances:** Supervision, Conceptualization, Methodology, Validation, Writing – review & editing. **Jérôme Peydecastaing:** Investigation, Validation, Writing – review & editing. **Pierre Yves Pontalier:** Writing – review & editing. **Freddy Guihéneuf:** Supervision, Resources, Data curation, Writing – review & editing. **Laurie Barthe:** Project administration, Supervision, Conceptualization, Methodology, Data curation, Validation, Writing – review & editing.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ultsonch.2023.106492.

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