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Integrating wet stirred-bead milling for *Tetraselmis suecica* biorefinery: operating parameters influence and specific energy efficiency

Pauline Delran^{a b c}, **Laurie Barthe**^a, **Jérôme Peydecastaing**^c, **Pierre Yves Pontalier**^c, **Freddy Guihéneuf**^b, **Christine Frances**^{a*1}

^a *Laboratoire de Génie Chimique, Université de Toulouse, CNRS, INPT, UPS, Toulouse, France*

^b *SAS inalve, Nice / Villefranche-sur-Mer, France*

^c *Laboratoire de Chimie Agro-industrielle, Université de Toulouse, INRAE, INPT, Toulouse, France*

Corresponding author : Pr. Christine Frances

Full address :

Université de Toulouse/University of Toulouse

Laboratoire de Génie Chimique INP-ENSIACET

4 allée Emile Monso - CS 84234

F-31432 Toulouse Cedex 4

Tel : +33 (0)5 34 32 36 39

Email : christine.frances@ensiacet.fr or christine.frances@toulouse-inp.fr

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CRedit authorship contribution statement

Pauline Delran: Conceptualization, Investigation, Methodology, Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Laurie Barthe:** Project administration, Supervision, Conceptualization, Methodology, Data curation, 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. **Christine Frances:** Project administration, Supervision, Conceptualization, Methodology, Data curation, Validation, Writing – review & editing.

* Corresponding Author: Christine Frances, christine.frances@toulouse-inp.fr

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Abstract

Stirred bead milling proved to be an efficient cell destruction technique in a biorefinery unit for the extraction of over 95% of proteins and 60% of carbohydrates from the green marine microalga *Tetraselmis suecica*. Optimum conditions, expressed in terms of metabolite yield and energy consumption, were found for average values of bead size and agitator rotation speed. The higher the microalgae concentration, up to 100 g.L⁻¹, which is adequate for biofilm algae growth in an industrial unit, the more efficient the cell destruction process. Cell destruction rates and metabolite extraction yields are similar in pendular and recycling modes, but the pendular configuration reduces the residence time of the suspension in the grinding chamber, which is less costly. With regard to the cell destruction mechanism, it was concluded that bead shocks first damage cells by permeabilizing them, and that after a longer period, all cells are shredded and destroyed, forming elongated debris.

Keywords: Proteins, Cell destruction, Energy consumption, Stress parameters, Microalgae

1. Introduction

Microalgae represent an alternative resource for the production of many molecules due to their high content of proteins, carbohydrates and lipids (Lucakova et al., 2022; Pereira et al., 2019). The exploitation of microalgae for these high added value molecules could be a solution to meet the increasing demand for proteins and health ingredients in the animal feed and human nutrition fields (Guedes et al., 2015). To produce these compounds on an industrial scale, it is necessary to develop a biorefinery process (Tan et al., 2018). A biorefinery is generally divided in two main parts, the upstream processing (USP) and the Downstream processing (DSP). USP refers to the microalgae cultivation and harvest stages (Daneshvar et al., 2021), while DSP includes a series of unit operations to destruct, extract, separate, purify, and recover target products (Chew et al., 2017). One of the first steps of the DSP is the cell destruction (Günerken et al.,

2015), defined as the partial or total loss of cell wall or membrane integrity. This is an essential step to recover the targeted intracellular products. However, the cell destruction step in the microalgae biorefinery is not yet fully optimized, controlled and remains costly (’t Lam et al., 2018). Indeed, due to their great diversity, each type of microalgae has a wall with its own characteristics (de Carvalho et al., 2020). The microalgae physiological state may also affect the cell disruption (Mear et al., 2023). Indeed, the walls of microalgae can be tough and resistant to degradation. They can be more or less thick, formed by several layers and by different polymer networks, resulting in a lack of standardization and optimization of the cell destruction processes (Phong et al., 2018). Chemical, enzymatic or mechanical processes can be applied but, many of these current cell destruction technologies require large amounts of energy, which can increase expenses. Mechanical processes use various forces (e.g. cavitation, shear, frictions) to damage the cells (Günerken et al., 2015). Mechanical methods are maybe less selective for extraction than enzymatic or chemical methods, but offer a greener alternative by allowing to operate in water and thus avoiding the use of polluting or toxic solvents (Tang et al., 2020, Oh et al., 2022). A wide range of methods can be used and are well documented in the literature (Nitsos et al., 2020). Among them, mechanical grinding can be performed using a Stirred Bead Mill (SBM) that subjects the biomass to impact, compression and shear forces (Blecher et al., 1996). This type of mill is typically used for wet grinding of suspensions, primarily for ultrafine grinding of minerals, chemicals and microorganisms (Kwade, 1999a). Stirred bead mills have a grinding chamber partially filled with beads. The grinding chamber is fed with the suspension to be ground by means of a pump, and the suspension and beads are set in motion by an agitator. The speed of the agitator shaft has an influence on the grinding efficiency and the energy consumption (Günerken et al., 2015). The cell destruction is caused by the capture and compression of the cells between the beads. The bead filling volume of the chamber is usually 70 to 85% of the free volume. The size of the beads can be adjusted as well as their composition (steel, glass, ceramic). The suspension flow characteristics and the residence time distribution are also parameters to be taken into account. They depend on the operating mode of the mill. Indeed, the mill can be operated in continuous or batch mode in different configurations. In continuous operation, the suspension passes through the grinding chamber only once. In batch mode, the mill can operate in recycling mode (the same tank is used for the inlet and outlet suspension) or in pendular mode by making several passes through the grinding chamber (using two tanks that are alternatively used for the inlet and outlet suspension). Finally, the initial concentration at which the cell suspension is treated is another parameter that also has a considerable impact on the efficiency and productivity of these processes.

Furthermore, it is necessary to consider the results not only in terms of extraction yields or cell destruction rates, but also in terms of energy efficiency because cell destruction processes can require high energy inputs, which may increase costs. Calculating the specific energy (E_s) consumed by the process based on the results obtained will give a more precise and reliable idea of which operating parameters to select (Günerken et al., 2015). The specific energy consumption can be expressed in units of energy per unit of production, for example in kilowatt-hours per kilogram of product produced. In the context of biorefinery, E_s refers to the amount of energy required to convert biomass

into useful products (Vanthoor-Koopmans et al., 2013). Selecting the optimal operating parameters for the process can decrease energy consumption and increase profitability. In a study on cell destruction of *N. oleoabundans* by SBM, Postma et al., 2017 reported that energy consumption can be reduced from 1.78 kWh.kg⁻¹ to 0.74 kWh.kg⁻¹ only by decreasing the bead size from 1 mm to 0.3 mm, respectively, and without affecting the maximum protein yield obtained. Similarly, Doucha and Lívanský, 2008 showed that increasing the feed rate was sufficient to reduce the energy consumed by the SBM from 8.37 kWh.kg⁻¹ to 3.11 kWh.kg⁻¹. Thus finally, Es is an important metric in industry because it allows for the measurement and comparison of energy efficiency and helps companies to reduce costs, decrease the environmental impact and increase sustainability.

In this paper, the influence of some milling operating parameters was studied on *Tetraselmis suecica*, a green marine microalgae grown as a biofilm, which allows for immediate harvesting as a concentrated paste without the need for further procedures before cell destruction. This study aims to determine the best performing SBM operating parameters on *Tetraselmis suecica* cell destruction, while considering energy consumption. The results will be analyzed and discussed in terms of efficiency on the extraction yields of the molecules of interest and specific energy consumption. Finally, this paper also aims to better understand the mechanisms of cell destruction by this process.

2. Material & Methods

2.1 *Tetraselmis suecica* biomass

Tetraselmis suecica was supplied by the company inalve (Nice, France) as a biofilm (patented rotating algae growing system: WO2021180713A1) with a dry matter concentration of about 15%. All experiments were performed within 4 days of harvesting, and stored at 4°C to preserve biomass quality and avoid bacterial growth. Conditioning and desalting of the biomass was done as described in Delran et al. 2023. Moisture content was determined by drying in an oven at 103°C for 24 hours (Sluiter, 2008a). Ash fraction was measured by calcination at 550 °C for 12 hours (Sluiter, 2008b). Total protein was quantified by the elemental analysis method using the conversion factor N = 6.25 (Schwenzfeier et al., 2011) and the Dubois method (DuBois et al., 1956) was applied to measure total carbohydrates. For each experiment, the initial biomass was diluted with distilled water to the desired final concentration (10 g.L⁻¹, 50 g.L⁻¹ or 100 g.L⁻¹).

2.2 *Stirred-Bead Milling experiments*

Grinding experiments were performed in a laboratory stirred media mill (Labstar from Netzsch) using two operating configurations: recycling and pendular mode. A full description of the experimental setup can be found in Ouattara and Frances, 2014. To ensure proper homogenization prior to starting grinding, the initial suspension was placed in a feed tank equipped with a stirrer at 600 rpm for at least 10 minutes at room temperature.

For the recycling milling configuration, the suspension was continuously pumped from the feed tank through the stirred media mill using a peristaltic pump at a flow rate of 31 kg.h⁻¹. The outlet of the mill is connected to the feed tank, allowing the recycling of the ground suspension. Grinding times up to 90 minutes were performed. The grinding chamber has an effective volume of 0.62 liters. At the outlet of the grinding chamber, the suspension passes through a separation cartridge, allowing the beads to remain in the chamber. The stirrer speed can vary from 1000 to 4000 rpm, and experiments were performed with three different stirring speeds (1000, 2000 and 3000 rpm). Microbeads made of 95% yttrium stabilized zirconia (YSZ, ZrO₂) (Zirmil® Y from WAB-Group) with a density of 6.28 g.cm⁻³ were used. The bead filling volume of the grinding chamber was set to 83%. Different bead sizes (d_{GB}) were considered: 0.8 mm, 1 mm and 1.25 mm. The volume of the treated suspension was 2 liters. Finally, tests were also conducted with three initial suspension concentrations: 10 g.L⁻¹, 50 g.L⁻¹ and 100g.L⁻¹.

Additional experiments were done in a pendular configuration to compare the effect of the milling mode on cell destruction. One, two or three passes were considered; the suspension being pumped at a constant flow rate of 17 kg.h⁻¹ from the feed tank to a separate output tank that allows the outlet suspension to be recovered after each pass. The flow rate at which the suspension is pumped is decreased in order to maintain equivalent residence times between the two operating modes.

The mean residence time (\bar{t}) of the suspension in the grinding chamber for the recycling and pendular configurations is given by Eq.(1) and Eq.(2), respectively.

$$\bar{t} = t \times \frac{V_{GC}-V_B}{V_{susp}} + \frac{V_{GC}-V_B}{q_v} \quad \text{Eq.(1)}$$

$$\bar{t} = t \times \frac{V_{GC}-V_B}{V_{susp}} = \sum_{i=1}^{Np} \frac{V_{GC}-V_B}{q_{v,i}} \quad \text{Eq.(2)}$$

Where, t (s) is the grinding time, V_{GC} (m³) the volume of the grinding chamber, V_B (m³) the bead effective volume, V_{susp} (m³) the volume of the treated suspension, q_v (m³.s⁻¹) the volumetric flow rate of the suspension and Np the number of passes.

Choosing a flow rate of 17 kg.h⁻¹ for the pendular configuration, 1 pass using the pendular mode corresponds to 3 minutes with the recycling configuration, 2 passes to 10 minutes and 3 passes to 17 minutes, respectively. Between each pass, the grinding chamber as well as the pumping hoses were cleaned to obtain accurate characterization and allow comparison of results. Runs in the pendular mode were performed with a cell suspension concentration of 100 g.L⁻¹, a stirrer speed fixed at 2000 rpm, a bead size of 1 mm and a bead filling volume of 83%.

Regardless of the configuration used, samples taken before and after grinding were characterized and then centrifuged for 20 min at 8000 × g. The supernatants were then used for biochemical analyses.

For all configurations, motor power, mill pressure and grinding chamber inlet and outlet temperatures were continuously monitored during the process with the LabDat© software.

To quantify the effect of the operating parameters, the results of each experiment were expressed in terms of specific energy consumed (E_s) per kilogram of Dry Weight (DW) in kWh.kg⁻¹ DW.

For the recycling configuration, the specific energy consumption was calculated as follows:

$$E_s(t) = \frac{E(t)}{m_p} = \int_0^t \frac{(P(\tau) - P_0)d\tau}{m_p} \quad \text{Eq.(3)}$$

Where t is the grinding time (h), $P(\tau)$ the power at time τ (kW), P_0 is the no-load power (kW) and m_p the mass of biomass expressed as dry matter (kg).

For the pendular configuration, the specific energy consumption was calculated as follows:

$$E_s = \frac{(P(t_p) - P_0)}{m_p} \times t_p \times Np \quad \text{Eq.(4)}$$

Where $P(t_p)$ is the power (kW) measured at the corresponding residence time, t_p (h) is the residence time and Np is the number of passes.

Finally, this study used a non-mechanical method with water extraction and alkaline extraction as the references. The 100 g.L⁻¹ suspension was constantly stirred in distilled water and sodium hydroxide solution at pH 12 and at a constant temperature of 40°C with a stirring speed of 900 rpm for 30 minutes.

2.3 Sample analysis and cell destruction characterizations

Most analyses were performed in triplicate. For these experiments, data are provided as mean and standard deviation values. Error bars in the figures represent standard deviations.

2.3.1 Cell size analysis

The evolution of the size distribution of the cells and the debris before and after each milling treatment was characterized by laser diffraction using a Mastersizer 3000 (Malvern Panalytical, UK). A refractive index of 1.45 and an absorption index of 0.10 for the solid phase, and a refractive index of 1.33 for water, were used for the measurements (Aas, 1996).

2.3.2 Cell destruction rate analysis

The cell destruction rate obtained after each treatment was determined by cell counting with a Malassez slide. 10 squares of a Malassez cell (0.25 mm x 0.20 mm x 0.20 mm) were counted and imaged using a Nikon SMZ 1500 at 40 fold magnification. Prior to each count, the analyzed suspension was diluted 100-fold and all analyses were performed in duplicate.

2.3.3 Scanning Electronic Microscopy Analysis

SEM (Scanning Electronic Microscopy) analysis was done on *Tetraselmis suecica* suspensions. Cells were fixed in polylysine buffer and stored at 4°C until analysis. SEM

images before and after each experiment were acquired at 500 x and 5000 x magnification at an accelerating voltage of 5 kV with a QUANTA 250 FEG microscope (FEI company, France).

2.3.4 Biochemical analysis

After sample collection, supernatants were dried in an oven at 40°C for 72 hours.

The rate of extracted protein in the supernatant was assessed by elemental analysis of total nitrogen using a Perkin Elmer 2400 Series II flash combustion analyzer with a conversion factor of N = 6.25 to convert the nitrogen level to protein.

To quantify the polysaccharide release, a Dubois colorimetric assay was performed. Glucose was used as standard solution and absorbance was determined at 490 nm using a SPECTROstar-Nano spectrophotometer (BMG LABTECH SARL, Champs-sur-Marne, France). For 1 h, 10 mg of dry sample was hydrolyzed at 100°C with sulfuric acid. Then, 200 µL of phenol (5% w/w) and 1 mL of sulfuric acid were mixed with 200 µL of the hydrolysate. The mixture was then incubated for 1 h at 100°C.

The extraction yields of proteins and carbohydrates were expressed in relation to their initial concentration in the biomass.

3. Results & discussion

To ensure that the cell destruction of *T. suecica* actually results from the mechanisms linked to the action of the grinder, alternative experiments were carried out by applying various non-mechanical treatments consisting of stirring at 900 rpm a suspension concentrated at 100 g.L⁻¹ in water with a thermal treatment at 40°C or with sodium hydroxide with a pH set at 12 at room temperature for 30 minutes (see supplementary material). The cell size distribution of untreated and treated microalgae was measured by laser diffraction. The initial cell size distribution for untreated biomass is shown in Figure 1.A. The distribution is bimodal with two peaks centered at 60 µm and 13 µm. The untreated biomass is composed of intact round and smooth cells with an average size of around 10 µm and a few cell aggregates represented by the peak at 60 µm. After 30 minutes of treatment, the distribution was still bimodal, with only a slight decrease in the proportion of aggregates, but no difference in the size distribution of the populations was observed compared to the untreated microalgae. Only the sodium hydroxide treatment suggested the beginning of cell lysis with the appearance of a third population at 2 µm. SEM images showed cells with round shapes, with a smooth cell wall and identical in appearance to that of untreated microalgae. None of these treatments seem to damage the cells and cause lysis.

3.1. Effect of bead size

The influence of bead size on cell destruction was studied by laser diffraction (Figure 1). The tests were performed with the same stirring speed in the grinding chamber of 2000 rpm and three bead sizes (0.8, 1 and 1.25 mm).

The treated suspension of *T. suecica* had a concentration of up to 100 g.L⁻¹. After 5 minutes of grinding, the cell size distribution is similar regardless of the bead size used. The proportion of the microalgae population centered at 9 µm increases, due to the

disappearance of the agglomerated cell population at 40 μm . A new population of cells centered at 0.7 μm appears, suggesting the beginning of cell destruction. For longer grinding times, differences in destruction behaviour are observed. The population of intact cells was greatly reduced as well as the subpopulation of cell aggregates after 30 minutes of grinding with the smallest bead size of 0.8 mm. The cells are fragmented into two new size classes: 0.7 μm and 3 μm . These two populations become predominant and their proportion increases continuously until 60 minutes of grinding, suggesting a faster cellular destruction. Indeed, when the size of the beads is higher, the fragmentation of the cells is less significant. With 1 mm beads, although the final rate of fragments at 0.7 μm is approximately the same as with 0.8 mm beads, the fragments generated are not as small in size. Finally, the largest bead size, 1.25 mm, has only a small effect on cell fragmentation. Indeed, throughout the milling process, only two size populations are visible, at 9 μm and 0.8 μm , and the final fragment rate remains very low. These results suggest that reducing the bead size has a positive effect on the cell destruction rate.

Tracking the median fragment size generated (Figure 2.A) and the rate of cell destruction (Figure 2.B) as a function of the specific energy supplied supports these results.

For a specific energy applied lower than $0.5 \text{ kWh.kg}^{-1}_{\text{DW}}$, the size of the fragments remains on average around 10 μm . This suggests that the stress energy applied is not sufficient to allow the cells to fragment. More stress events are needed, requiring more energy input. However, once more energy is supplied, the influence of bead size is visible. Micronization is always higher at a smaller size. This result shows that with beads larger than 1 mm, more energy will be needed to micronize the cells efficiently. The same trend is observed with the cell destruction rates. While almost 100% of the cells are destroyed providing $2 \text{ kWh.kg}^{-1}_{\text{DW}}$ of energy with 0.8 and 1 mm beads, this rate remains slightly lower with 1.25 mm beads. This phenomenon of reduced process efficiency due to larger bead sizes has been observed in previous studies. Grinding work on the microalga *Chlorella vulgaris* by Doucha and Lívanský, 2008 showed that the use of beads larger than 0.5 mm had a negative effect on the efficiency of cell destruction. Indeed, by increasing the beads size from 0.3 mm to 0.7 mm and keeping the same operating parameters such as the feed rate, bead filling and rotor stirring speed, the authors showed that the cell destruction rate decreased significantly from 95% to 70%. Similarly, in the study of Pan et al., 2017, the effect of bead size is also visible on the final rate of destroyed cells on *Nannochloropsis* sp., despite maintaining the operating conditions. The authors report that with 0.4 mm beads, 90% of cells are destroyed after 25 minutes of grinding while with 1.8 mm beads, only 65% of cells are destroyed after 55 minutes of grinding. Finally, Suarez Garcia et al., 2019 showed that 100% of *T. suecica* cells were destroyed after only 400 seconds of grinding using beads of 0.5 mm.

The release of proteins into the medium was also monitored as a function of energy consumed (Figure 2.C). Contrary to previous observations, bead size does not seem to influence protein extraction yields. Dispersion by simple agitation in water at room temperature before grinding leads to a protein extraction rate between 20% and 25%. As soon as energy is supplied, this rate increases to 70% for $0.03 \text{ kWh.kg}^{-1}_{\text{DW}}$ and continues to gradually increase until it reaches 96% for an energy of $2 \text{ kWh.kg}^{-1}_{\text{DW}}$. The

same observation for the microalgae *T. suecica* was made by Postma et al. 2017 using four different bead sizes (0.3, 0.4, 0.65 and 1 mm) in a SBM. The degree of cell fragmentation dependent on bead size did not influence protein release. These results seem to support the hypothesis that once the cell is damaged, regardless of its level of micronization, the proteins are extracted from the cell.

Modelling the forces involved in this process helps to understand and predict the choice of operating parameters, including bead size. Several studies have proposed the stress energy model, which allows optimization of operating parameters (Becker et al., 2001; Breitung-Faes and Kwade, 2014; Kwade and Schwedes, 2002). Cell destruction results from contact between the beads and microalgae. When the bead size is reduced while holding the other operating conditions constant, the impact velocity of these bead-cell collisions is reduced. This effect can be modelled as the Stress Intensity (SI), which depends on the kinetic energy of the beads, and will be lower the smaller the bead size. The stress intensity depends on operating parameters such as the media size d_{GM} , media density ρ_{GM} , and tip speed of discs v_t . It can be defined by the following equation (Becker et al., 2001) (Eq.5) :

$$SI_{GM} = d_{GM}^3 \rho_{GM} v_t^2 \quad (\text{Eq.5})$$

However, for the same bead filling, the number of beads increases, which increases the number of probabilities that a collision will occur. This effect is modelled by the Stress Number (Eq.6), which describes the number of sudden stresses experienced by the cell. SN, is determined by the number of media contacts, N_c , by the probability that a particle is caught and sufficiently stressed at a media contact, P_s , and by the number of product particles inside the mill, N_p (Kwade, 1999b).

$$SN = N_c P_s N_p \quad (\text{Eq.6})$$

Tetraselmis suecica cell wall can be considered a rather fragile material due to its composition (Kermanshahi-pour et al., 2014). Indeed, the cell wall is made up of complex carbohydrates (Becker et al., 1991) and seems to break down within the first minutes of grinding releasing the proteins (Figure 3.C). Therefore, it is not necessary to provide more energy during the collision (SI) but rather to increase the number of shocks (SN). By decreasing the bead size, the cell disintegration process is therefore accelerated, as reported by Pan et al. 2017. In conclusion, in the case of the microalga *T. suecica*, the choice of bead size will only matter for the desired cell fragmentation rate.

However, surprisingly, the effect of bead size studied for a lower speed, $v= 1000$ rpm, showed different results (Figure 3).

Cell size monitoring for $v= 1000$ rpm (see supplementary material) showed less clearly the effect of the size of the beads used on the size distribution of the cell populations. The same size populations were observed regardless of bead size and evolve in the same way during grinding. Figures 3.A and 3.B show the evolution of median fragment size and cell destruction as a function of specific energy for the three bead sizes used 0.8, 1 and 1.25 mm respectively. At low energy consumption, i.e. during the first few minutes of grinding, the 0.8 mm and 1 mm beads seem to produce finer cell fragments than the

1.25 mm beads. In contrast, at $0.5 \text{ kWh}\cdot\text{kg}^{-1}_{\text{DW}}$ and above, the effect of bead size is no longer significant on the fineness of *Tetraselmis suecica* cell fragmentation. Moreover, for the same operating conditions and for the same specific energy consumed, fragments of a slightly larger size than those obtained at $v=2000 \text{ rpm}$ are generated. For a specific energy of $1 \text{ kWh}\cdot\text{kg}^{-1}_{\text{DW}}$, at $v=1000 \text{ rpm}$, the final median size of the generated fragments is about $6.4 \mu\text{m}$, while for $v=2000 \text{ rpm}$, it is $5.5 \mu\text{m}$. Similarly, for $1 \text{ kWh}\cdot\text{kg}^{-1}_{\text{DW}}$, the cell destruction rates (Figure 3.B) are significantly different for the two stirring speeds. With $v=2000 \text{ rpm}$, 99% of the cells are already destroyed while for $v=1000 \text{ rpm}$, this rate is lower (94%). The influence of the bead size in this case, is more pronounced: the smaller the beads size, the higher the rate of destroyed cells for the same energy consumption. The cells show a similar behaviour regarding the size of the beads than for $v=2000 \text{ rpm}$.

For $v=1000 \text{ rpm}$, the 1.25 mm beads seems to slightly improve the proteins extraction (Figure 3.C). The final yield reached about 85% with the 1.25 mm beads, 5 points higher than with the 0.8 and 1 mm beads. However, the error bars for the experiments performed with the 1 mm beads seem to indicate the effect of the beads remains not very significant. Thus, finally, the 1 mm beads are selected for further experiments. The stirring speed of the rotor seems to have an effect on the protein extraction rates, independently of the degree of micronization and cell destruction. Indeed, the final rate of 85% protein extracted at $v=1000 \text{ rpm}$ is lower than the rate obtained for $v=2000 \text{ rpm}$ equal to 91%. These experimental works concerning the effect of bead size at two different stirring speeds suggest a combined effect of the two operating parameters. The effect of the bead size only becomes visible and effective after a certain speed, which suggests that the energy input must be sufficiently large to influence the results. Indeed, studies have shown that increasing the stirrer speed tends to increase the total number of particles stressed (Strobel et al., 2018). This would make the SN no longer the only parameter to consider. One must also take into account the probability of particle capture, which can be influenced by the stirring speed and not just the size of the grinding beads (Kwade, 2003).

3.2 Effect of the agitation speed

Rotor stirring speed is another important operating parameter in decision making. The influence of three speeds (1000, 2000 and 3000 rpm) was studied on extractive yields, cell destruction, cell fragmentation and energy consumption. The results of cell fragmentation as a function of speed for different specific energies consumed are shown in Figure 4.

The mechanisms of mill-induced fragmentation for the three rotational speeds were also studied by SEM. Untreated cell size is approximately $10 \mu\text{m}$ and cells are round in shape and with a smooth cell wall. With a low energy supplied, $0.2 \text{ kWh}\cdot\text{kg}^{-1}_{\text{DW}}$, the grinding mainly allows deagglomeration of the population at $45 \mu\text{m}$ and initiates the beginning of cell lysis, but the energy input is still too low. The majority of cells ground at $v=1000 \text{ rpm}$ appear still intact or with a damaged and deflated cell wall, having lost their initial round and smooth shape. Some cell fragments are also visible for $v=2000 \text{ rpm}$ (and $v=3000 \text{ rpm}$) but the majority of cells are still round in shape, but with a

deflated and tearing appearance. For an energy equivalent to $0.5 \text{ kWh.kg}^{-1}_{\text{DW}}$, cell destruction is more evident regardless of speed, with always a higher proportion of destructed cells or debris at $v= 3000 \text{ rpm}$. For $0.5 \text{ kWh.kg}^{-1}_{\text{DW}}$, the round-shaped cells are rarer especially for highest rotation speeds and appear torn and severely damaged. In contrast, for $v= 1000 \text{ rpm}$, the degree of fragmentation is much less severe and the cells appear deflated. Finally, at $3 \text{ kWh.kg}^{-1}_{\text{DW}}$ and a speed of 3000 rpm , all initial intact cells were fragmented and resulted in two sub-populations of $6 \mu\text{m}$ and $0.7 \mu\text{m}$. The final rate of fragments generated is higher than for the two other speeds. Indeed, SEM observations for $v= 3000 \text{ rpm}$ showed a very severe level of cell fragmentation with a large proportion of cell wall fragments and no whole cells left. The effect of repeated bead-cell shocks seems to initially damage the cells by permeabilizing them. After repeated shocks for a longer period of time, the cells are totally shredded and destroyed, as seen in SEM images at $3 \text{ kWh.kg}^{-1}_{\text{DW}}$. Cells for $v= 1000 \text{ rpm}$ appear less damaged, with deflated cells and few fragments. In conclusion, the higher the rotation speed of the agitator and the higher the specific energy supplied, the higher the initial cell fragmentation rate.

Regarding cell destruction (Figure 5.A), destruction is slower at 1000 rpm than at 2000 and 3000 rpm . At $1.5 \text{ kWh.kg}^{-1}_{\text{DW}}$, only 87% of the cells are destroyed at 1000 rpm compared to 96% for 2000 and 3000 rpm . At $3 \text{ kWh.kg}^{-1}_{\text{DW}}$, 100% of the cells are destroyed for 2000 and 3000 rpm , while this maximum rate is not yet reached at 1000 rpm . In a study concerning the influence of operating parameters on *Saccharomyces cerevisiae* microorganisms, Melendres et al. 1991 have observed a strong dependence of the destruction rate on the agitation speed and explained this result in terms of the collision frequency of the beads. In addition, Zinkoné et al. 2018 reported that different cell populations may have an influence. Larger and older cells are more fragile and break down easily. On the contrary, small, newly divided cells are perturbed at different rates depending on the collision intensity (stress intensity in the stress model).

Furthermore, here, tracking the median fragment size (Figure 5.B), shows that there is no clear dependence between fragment size and rotation speed. The final median size of the generated fragments is approximately the same, around $4 \mu\text{m}$. Therefore, to achieve a high destruction rate as well as a significant reduction in cell size, an average specific energy of $3 \text{ kWh.kg}^{-1}_{\text{DW}}$ will be required.

While stirring speed induces severe cell damage in a similar manner, protein and carbohydrate release seems to be influenced by this parameter. Protein (Figure 5.C) and carbohydrate (Figure 5.D) yields are reported as a function of energy supplied.

Metabolite extraction started to increase during the first few minutes of grinding. The initial protein content is about 15% , and then varies with the stirring speed. The dotted lines highlight the yield rates for the same specific energy input. The yield of extracted protein for the 1000 rpm experiment is always lower than for the 2000 and 3000 rpm experiments. From approximately $1.5 \text{ kWh.kg}^{-1}_{\text{DW}}$, the yields reach a plateau. These maximum values reach 90% for 3000 rpm , 96% for 2000 rpm and 84% for 1000 rpm . Consuming more energy does not increase the efficiency. The release behaviour of

carbohydrates is similar; the extraction kinetics is progressive but does not reach a plateau in the energy range between 1.5 kWh.kg⁻¹_{DW} and 3 kWh.kg⁻¹_{DW} as for proteins. The speeds 2000 and 3000 rpm, give higher extraction yields than at 1000 rpm, whatever the energy consumed. For a low energy consumption, 0.5 kWh.kg⁻¹_{DW}, the three rates are similar, then, for an input of 1.5 kWh.kg⁻¹_{DW}, the rates increase for 2000 and 3000 rpm. Similarly, at 3 kWh.kg⁻¹_{DW}, these rates increase again and the gap widens between 1000 rpm and high speeds of 2000 and 3000 rpm. It is interesting to note that, unlike proteins, total carbohydrate extraction rates are significantly lower, with only 62% of carbohydrates extracted versus 96% of the total protein extracted for the same energy input of 3 kWh.kg⁻¹_{DW}. This difference in yield could be a consequence of the different distribution of these molecules within *Tetraselmis suecica* cells. Suarez Garcia et al. 2018 reported that proteins are distributed into three pools in the cell: aqueous, function and structure proteins that are located in the cytosol. In contrast, studies have shown that carbohydrates are stored and aggregated as starch granules in the cytosol of cells, making their solubilization more difficult (Kermanshahi-pour et al., 2014; Postma et al., 2017). The stirred bead mill is therefore an efficient technology for protein extraction, but less so for carbohydrate extraction, where a maximum rate of 70% of extracted carbohydrates seems to be reached even with more energy input by increasing the stirring speed. The accessibility and location of the molecules can be an obstacle to their extraction and require combining this mechanical process with a more selective extraction process such as enzymatic or chemical extraction (Alavijeh et al., 2020; Nitsos et al., 2020). However, for both proteins and carbohydrates, the optimal extraction rate seems to be obtained at 2000 rpm. Surprisingly, final rates are higher at 2000 rpm than at 3000 rpm, suggesting excessive cell destruction. The choice of working at 2000 rpm seems to be a good compromise to achieve maximum extraction yields while decreasing total energy consumption. However, if the extraction of maximum protein is the main criterion for selecting mill parameters, a halved energy consumption of 1.5 kWh.kg⁻¹_{DW} is sufficient.

3.3 Effect of initial cell concentration

Finally, the last operating parameter studied is the initial concentration of the treated suspension. The post-harvest biomass is usually concentrated in a range of 100 g.L⁻¹ to 150 g.L⁻¹ due to seasonal variability. Thus, various biomass concentrations were tested, 10 g.L⁻¹, 50 g.L⁻¹ and 100 g.L⁻¹ with a stirring speed set at 2000 rpm, a bead size of 1 mm and a filling volume maintained at 83%. Figure 6 shows how the initial concentration affects both the extraction yields and the cell destruction rates.

Working with the lowest concentration, 10 g.L⁻¹, only 80% of total protein and 50% of total carbohydrates are extracted. This concentration never allows achieving the maximum extraction yields of 96% protein (Figure 6.A) and 60% carbohydrate (Figure 6.B) obtained with the higher concentrations, regardless of the specific energy supplied. In their study on *Chlorella vulgaris* grinding, Liu et al. 2021 reported similar observations on the influence of concentration. The kinetics of soluble proteins were considerably better for concentrations of 90 g.L⁻¹ and 60 g.L⁻¹ while for a lower concentration of 30 g.L⁻¹, these kinetics decreased significantly. Thus, this suggests that there is an optimal cell density for this milling process. Similarly, the concentration has consequences on

cell destruction (Figure 6.C). The kinetics of cell destruction are significantly slower with dilute cell concentrations than with denser cell concentrations. Furthermore, working at such a low concentration critically increases the energy consumption from 3 kWh.kg⁻¹_{DW} to 20 kWh.kg⁻¹_{DW}. To achieve 100% of cell destruction, 10 times more energy will be required when working at 10 g.L⁻¹ instead of 100 g.L⁻¹. The stress model could explain this (Montalescot et al., 2015). For the same operating conditions, collision events are more frequent and more efficient at higher concentrations (Becker et al., 2001; Kwade and Schwedes, 2002). A higher number of cells increases the probability of cell-bead shocks. Of these three concentrations, only the two highest, 50 g.L⁻¹ and 100 g.L⁻¹, make the process efficient in terms of yield and energy consumption. For efficient grinding, it is therefore recommended to operate with high concentration ranges. The advantage of the stirred bead mill is that it can be used in a high concentration range directly after harvesting, thus avoiding an expensive biomass dilution step. In addition, the efficiency of the stirred bead mill on concentrated biomass results in a significant saving in energy consumption, which can be divided by up to 10.

3.4 Influence of residence time and operating milling configuration

In order to analyze the effect of the operating mode, experiments were also performed in a pendular configuration (Quesada-Salas et al., 2021). It is possible to relate the number of passes in pendular mode to the residence time when the stirred mill operates in a recycling mode. 1 to 3 passes of the suspension were performed, representing different residence times of 3, 10 and 17 minutes, respectively, in a recycling mode. Table 1 provides a comparison of the protein and carbohydrates extraction yields and cell destruction rates obtained using the two types of mill configurations. The operating conditions were kept identical between the two modes with a stirring speed of 2000 rpm, an initial concentration of 100 g.L⁻¹, beads of size 1 mm and a filling volume of 83%. Only the feed rate was reduced from 31 kg.h⁻¹ in recycling mode to 17 kg.h⁻¹ in pendular mode, in order to have similar residence times between the two modes of operation. Ouattara and Frances, 2014 showed that the feed rate usually has no effect on the grinding results. First, under identical operating conditions, no significant difference is visible in protein and carbohydrate yields and cell destruction rate (Table 1).

The rates evolve in the same way whatever the recycling or pendular configuration used. The residence times calculated in recycling mode are well equivalent to the number of passes in pendular mode. Only the results related to cell destruction rates seem to indicate a slightly greater destruction efficiency by the pendular mode. These differences remain sufficiently small enough to be attributed to the accuracy of the analysis (cell counting). However, the energy consumption per pass in pendular mode is lower than in recycling mode because the grinding and processing times are reduced. In pendular mode, the residence time equivalent to one pass is 1.14 min, compared to 3 minutes in recycling mode. As a result, the total specific energy consumed for each pass is always lower than that consumed for equivalent grinding times in recycling mode. 17 minutes of milling in recycling mode consumes 0.572 kWh.kg⁻¹_{DW} while 3 passes in pendular mode consume only 0.114 kWh.kg⁻¹_{DW} for equivalent results in terms of cell destruction and metabolite extraction yield. In the case of *Tetraselmis suecica* in suspension, stirred bead milling is a process that requires a short residence time to

effectively break up the cells and is able of consuming limited energy. Therefore, the configuration of the grinder and the effective residence time are key parameters (Monteiro et al., 2013). According to Kwade, 1999a the residence time distribution inside the mill and the operation configuration are the main factors that determine the stress number. Some parameters such as bead size and speed offer the possibility of making a compromise, for example, achieving a high protein yield while minimizing energy cost. Regarding the results of the different parameters considered in the present study, the selection of operating parameters such as bead size, rotor stirring speed, cell concentration or mill configuration will depend on the desired objectives.

However, even if a specific energy consumption saving can be achieved, the use of the pendular mode is questionable and depends on the material to be ground. *Tetraselmis suecica* is a relatively easy to destroy microalga that allows 90% of the total protein to be extracted in only 3 passes. However, this mode of milling configuration would lose its interest for harder materials or for nano-grinding operations. It would require a much higher number of passes to achieve very fine cell size, and therefore a too frequent interruption of the process, which would make it more complicated to use compared to the recycling mode. Thus, chemical industries often prefer to use continuous processing or recycling mode for large-scale production. For use in pendular mode, automation, for example using two tanks for product inlet and outlet interconnected by a pump, would be required (Monteiro et al., 2013).

4. Conclusion

In the context of microalgal biorefineries, it was shown that stirred bead milling is an efficient process for the cell destruction of *Tetraselmis suecica*, enabling the release of 96% of proteins and 60% of carbohydrates. Optimum conditions were obtained with 1 mm grinding beads, a stirring speed of 2000 rpm and an initial suspension concentration of 100 g.L⁻¹. The operating parameters with the greatest influence on process energy consumption were rotation speed, initial cell concentration and suspension residence time. Cell destruction rates and metabolite yields are identical in pendular and recycling modes, but pendular mode consumes less energy.

E-supplementary data for this work can be found in e-version of this paper online.

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Figures, Table and Captions

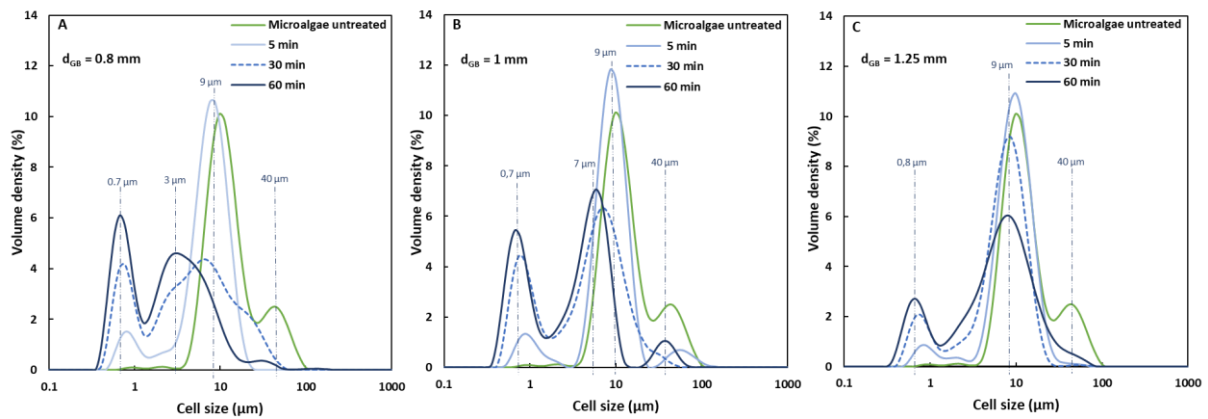


Figure 1 : Evolution of cell size as a function of grinding time for three bead sizes (d_{GB}), for a stirring speed of $v = 2000$ rpm and for a cell concentration of 100 g.L^{-1} .

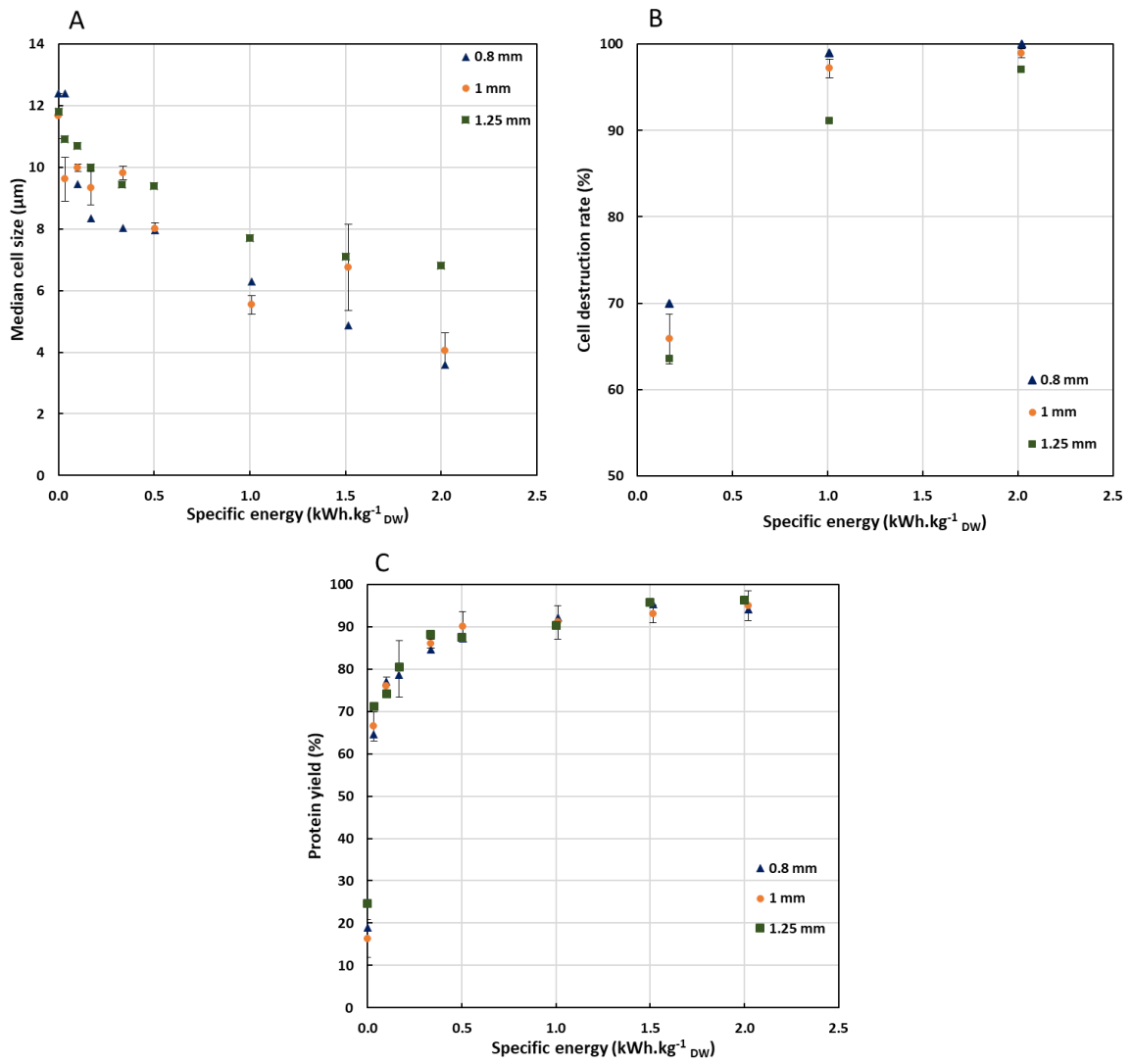


Figure 2: Effect of bead size on A: median cell size; B: cell destruction rate; C: protein yield, as a function of specific energy consumed for a stirring speed of $v = 2000$ rpm and for a cell concentration of 100 g.L^{-1} .

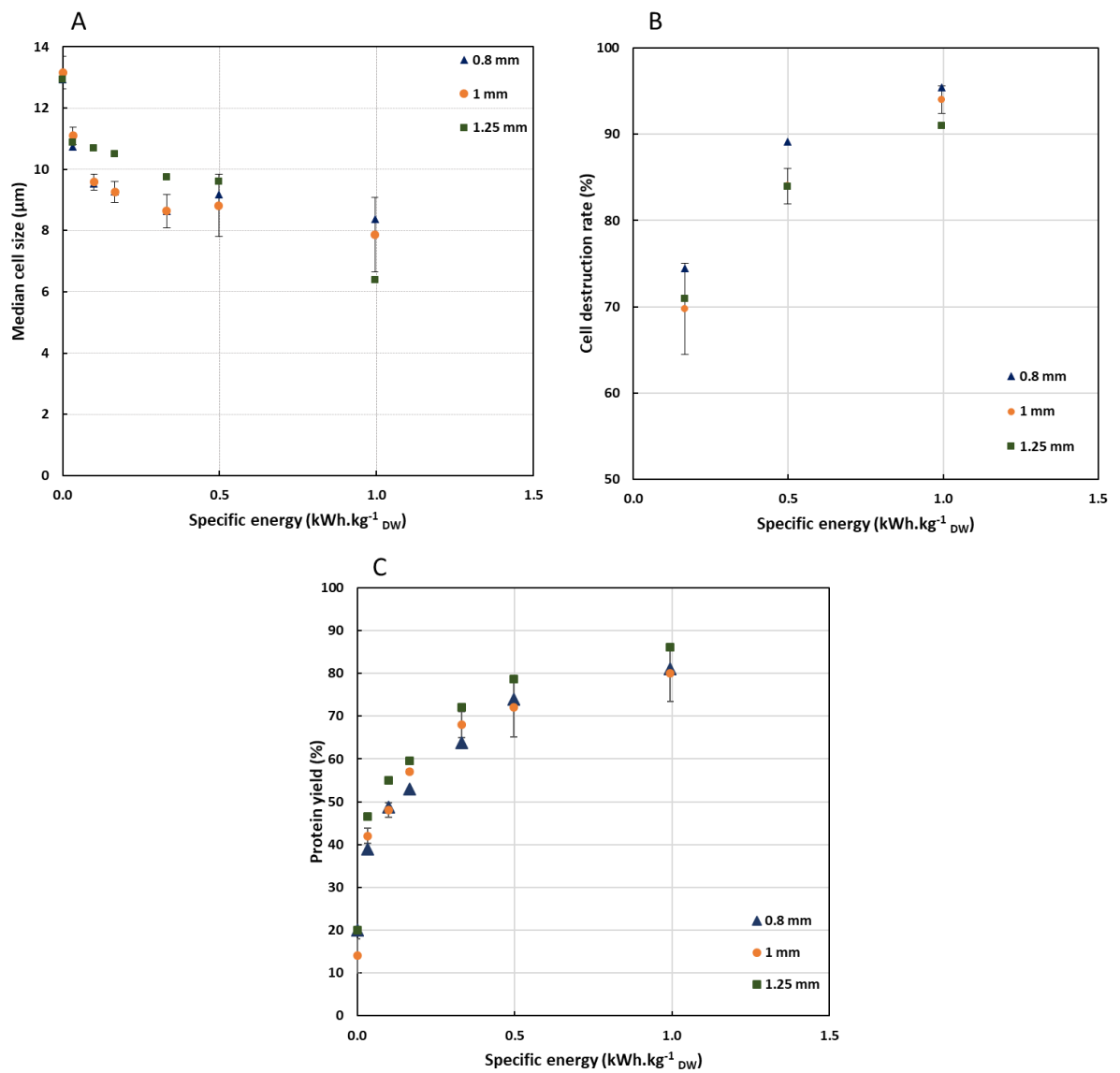


Figure 3: Effect of bead size on A: median cell size; B: cell destruction rate; C: protein yield, as a function of specific energy consumed for a stirring speed of $v=1000$ rpm and for a cell concentration of 100 g.L^{-1} .

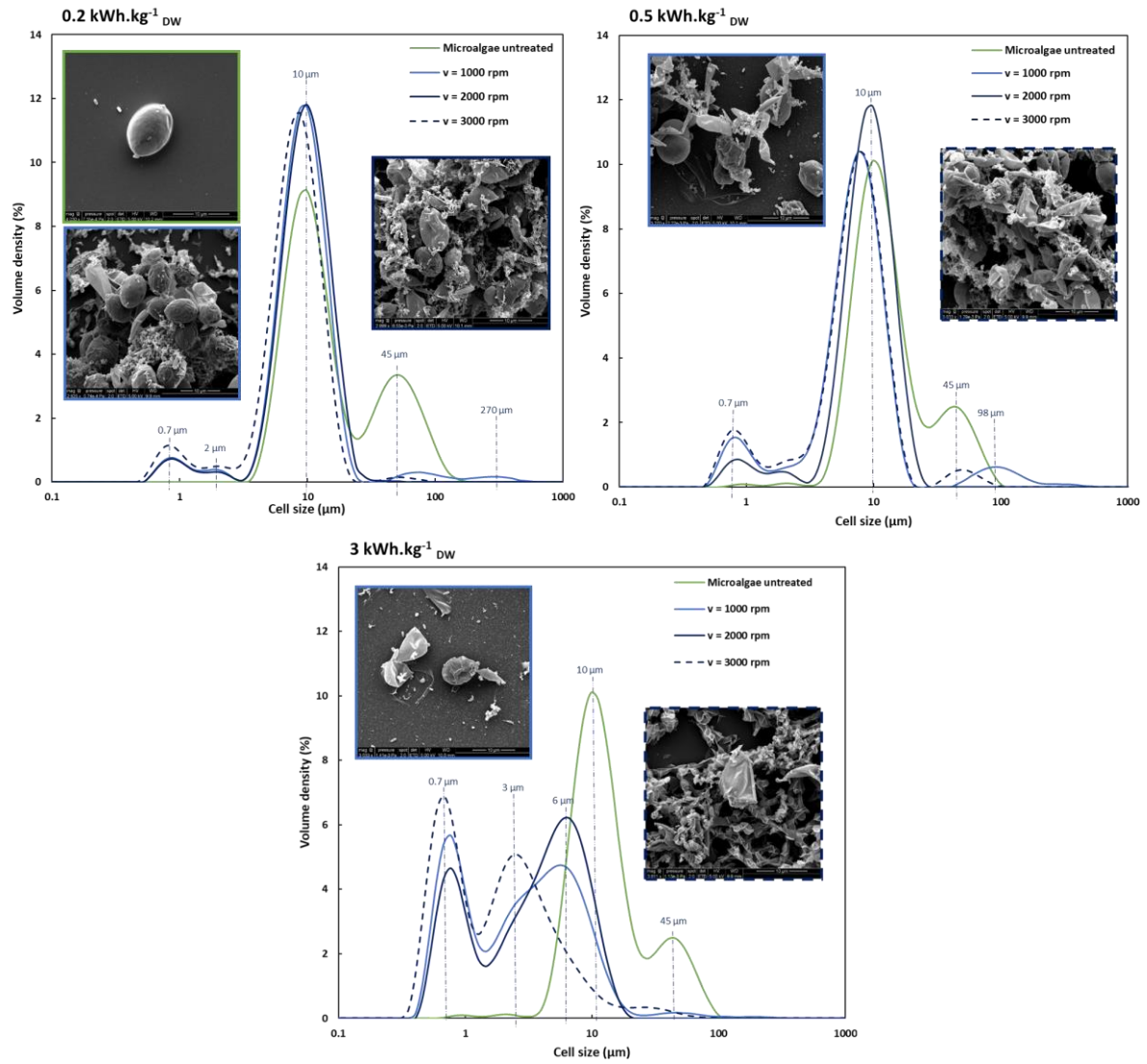


Figure 4: Evolution of cell size as a function of specific energy consumption ($\text{kWh.kg}^{-1} \text{DW}$) for three different stirring speeds 1000, 2000 and 3000 rpm, for $d_{GB} = 1 \text{ mm}$ and for a cell concentration of 100 g.L^{-1} . The evolution of cell morphology is shown by SEM images.

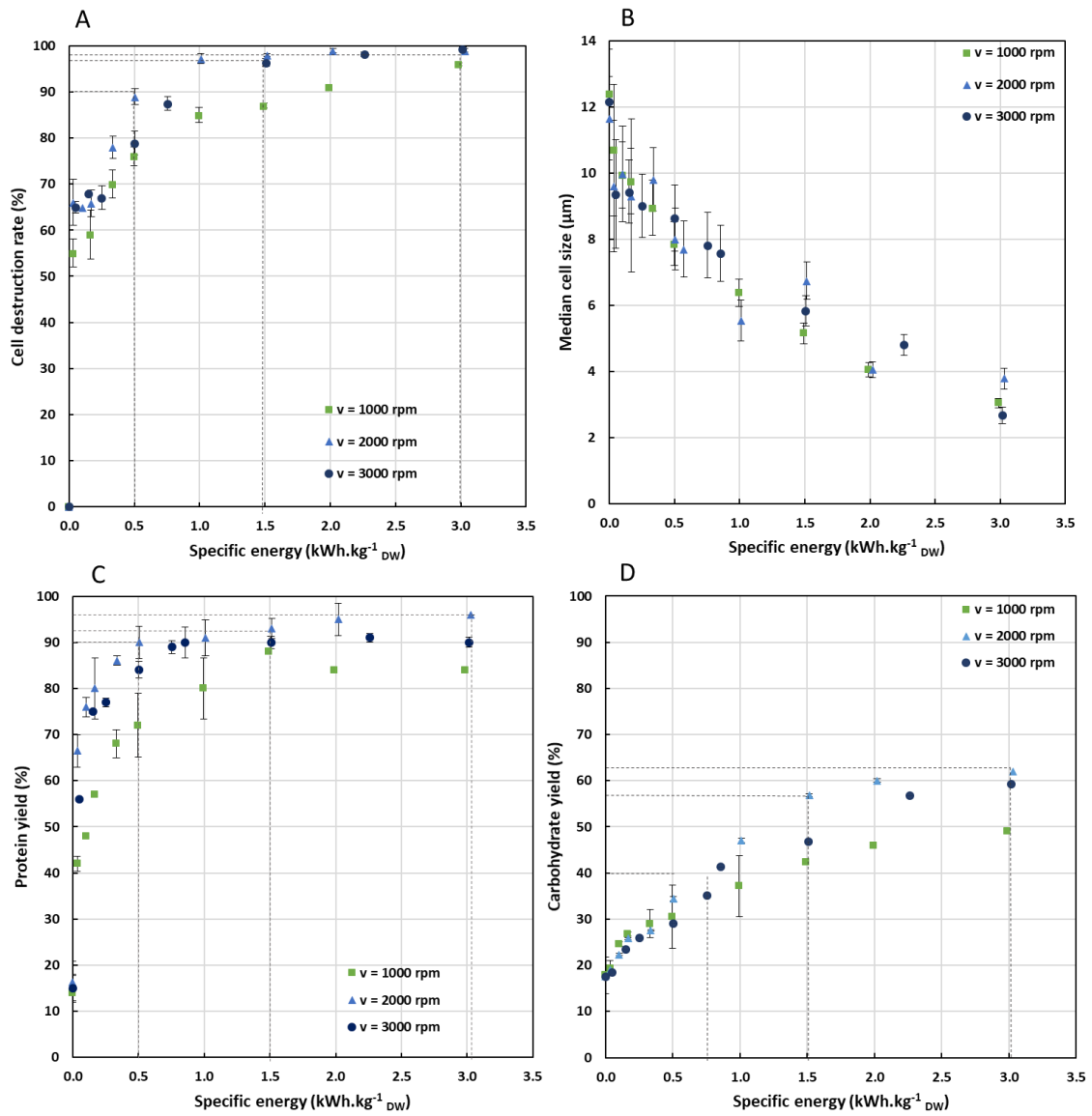


Figure 5: A = Cell destruction rate; B = Evolution of median cell size; C = Protein yield and D = Carbohydrate yield for three stirring speeds, $d_{GB} = 1$ mm and for a cell concentration of 100 g.L^{-1} as a function of specific energy consumed.

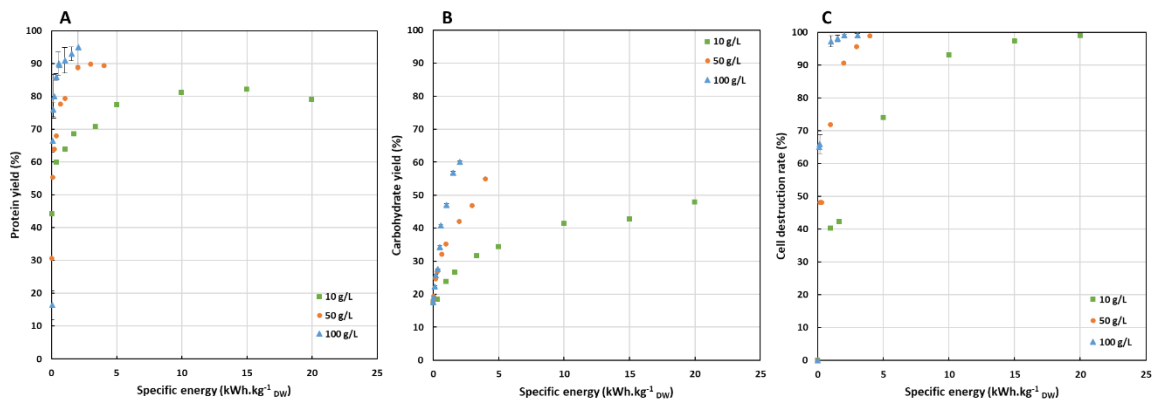


Figure 6: Effect of initial cell concentration on A = protein and B = carbohydrate extraction yields and C = cell destruction rates as a function of specific energy consumed.

Table 1: Influence of the mill configuration on protein, carbohydrate yields, cell destruction rate and specific energy consumption. The results are expressed for 1, 2 and 3 passes and compared to the same residence times corresponding to milling in the recycling configuration. Values are reported as mean \pm SD ($n = 3$).

SBM Mode	SBM time - passes	Protein yield (%)	Carbohydrate yield (%)	Cell destruction rate (%)	Specific energy (kWh.kg ⁻¹ _{DW})
Recycling Mode	0 min	17 \pm 4.41	19 \pm 0.13	-	-
	3 min	73 \pm 2.12	23 \pm 0.21	61 \pm 1.62	0.101
	10 min	86 \pm 1.01	30 \pm 0.11	74 \pm 2.37	0.336
	17 min	88 \pm 2.36	43 \pm 0.48	85 \pm 1.13	0.572
Pendular Mode	0 pass	16 \pm 4.94	18 \pm 0.07	-	-
	1 pass eq. 3 min	76 \pm 0.92	22 \pm 0.14	65 \pm 3.71	0.038
	2 passes eq. 10 min	86 \pm 2.31	28 \pm 0.15	78 \pm 3.33	0.076
	3 passes eq. 17 min	90 \pm 1.21	41 \pm 0.51	90 \pm 2.26	0.114

e-Supplementary data

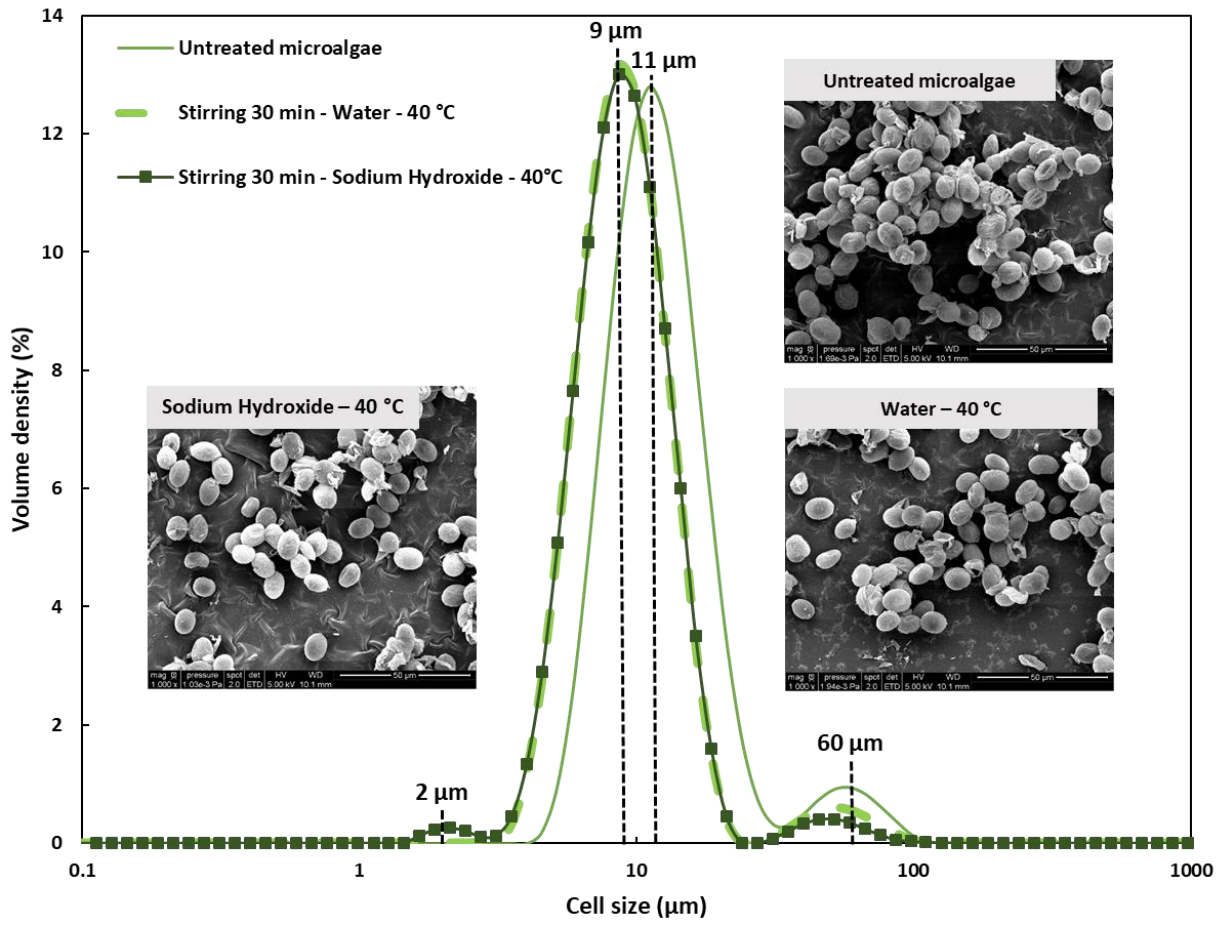


Figure A.1: Effect of non-mechanical treatments on the cell size distribution of a cell suspension of *Tetraselmis suecica* at 100 g.L^{-1} .

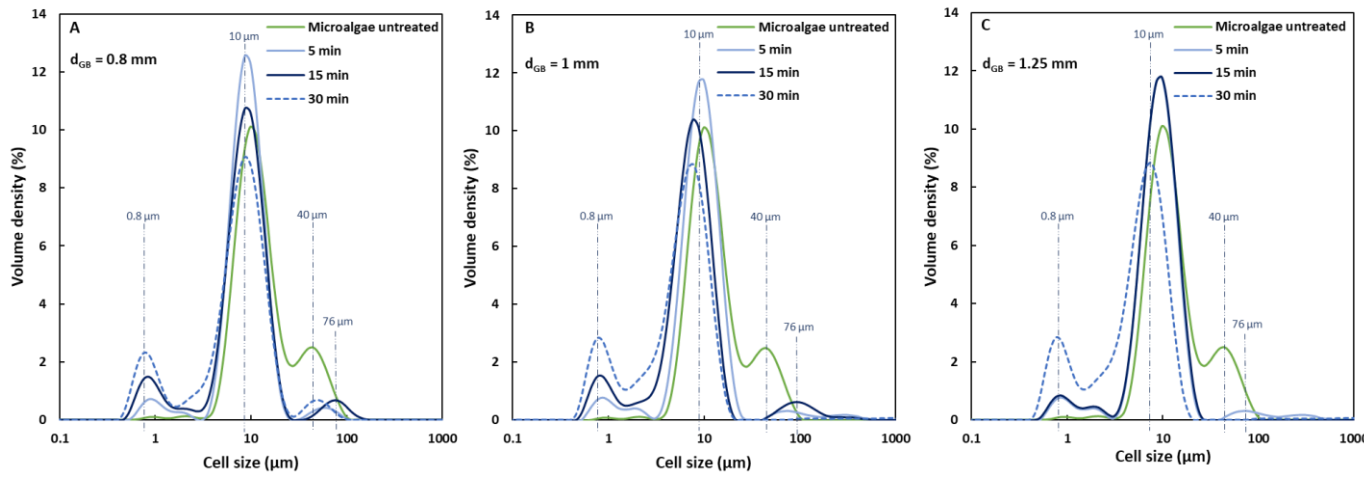


Figure A.2: Evolution of cell size distribution as a function of grinding time for three bead sizes (d_{GB}), for $v = 1000$ rpm and for a cell concentration of 100 g.L⁻¹.