Universidade do Minho
Escola de Ciências

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Dissertação de Mestrado
Mestrado em Biologia Molecular, Biotecnologia e Bioempreendedorismo em Plantas

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Resumo

Estratégias de rutura celular e extração de compostos bioativos de fotobiontes de líquenes.

Os líquenes são uma relação simbiótica entre dois organismos, uma microalga (fotobionte) e um fungo (micobionte). As microalgas são organismos simples e com altas capacidades fotossintéticas e capazes de crescer em diferentes ambientes. Elas tornaram-se uma interessante fonte de compostos bioativos, contudo as microalgas isoladas de líquenes ainda não estão muito descritas e, em particular, pouco se sabe sobre o seu potencial de produção de compostos bioativos.

No sentido de tirar o máximo partido das potencialidades das microalgas isoladas a partir de líquenes, é necessário garantir uma adequada rutura celular, de modo a permitir uma libertação eficiente dos compostos intracelulares. O objetivo deste trabalho é determinar quais os métodos de rutura celular com maior eficiência de rutura das células de microalgas isoladas de líquenes e quais garantem a integridade dos compostos orgânicos extraídos.

Aplicaram-se diferentes métodos de rutura celular com diferentes condições a microalgas isoladas de líquenes de modo a determinar as melhores condições de operação para cada método de rutura. Os métodos aplicados foram ciclos de congelamento e descongelamento, sonicação, micro-ondas, homogeneização a altas velocidades e moinho de bolas. A eficiência da rutura celular e extração de compostos bioativos foi analisada por citometria de fluxo, espectroscopia e quantificação de alguns compostos primários (tais como hidratos de carbono e proteínas) produzidos e libertados pelas células após o tratamento. O método que apresentou maior eficiência de rutura e extração foi a sonicação durante 60 min com uma sonda de 40 kHz. Contudo, este método apresenta consumos energéticos e períodos de tratamento bastante elevados.

Assim, surge o moinho de bolas como alternativa, em que foram usadas esferas com 0,5 mm de diâmetro a uma concentração de 32 % durante 6 min. Verificou-se tratar-se de um método simples, rápido e eficaz na extração e rutura celular, capaz de extrair os compostos presentes nas microalgas isoladas de líquenes e de manter a integridade de possíveis compostos de interesse. Este método apresentou ainda a vantagem de apresentar um consumo energético bastante reduzido.

Palavras-chave: Compostos bioativos; Líquenes; Microalgas; Rutura celular

Abstract

Cells disruption strategies for extraction of bioactive compounds from lichens photobionts

Lichens are organisms known by their symbiotic relationship between microalgae (photobiont) and fungi (mycobiont). Microalgae are a simple form of organisms with high photosynthetic efficiency and fast growth. They are a promising source of bioactive compounds, however microalgae isolated from lichens are still poorly understood, in particular their bioactive compounds production potential.

In order to take advantage of this potential of microalgae isolated from lichens, an effective cellular disruption is required. A high disruption efficiency allows an improved release of the intracellular compounds. The main goal of this work is thus the assessment of which method can cause more efficient cellular disruption and maintain the integrity of the intracellular compounds released. Different cell disruption treatments were applied in order to determine the efficiency that they have on the recovery of intracellular compounds from microalgae isolated from lichens. Methods such freezing-thawing, ultrasonication, microwave, high-speed homogenization and bead-milling were applied. All the applied methods were tested with different experimental conditions in order to define a better condition for each treatment.

The efficiency of cellular disruption and extraction of intracellular compounds was analyzed by flow cytometry, spectroscopy at different wavelengths and quantifications of some primary metabolites produced by microalgae cells such as carbohydrates and proteins. The treatment with higher disruption efficiency (in terms of percentage of disrupted cells) and extraction was ultrasonication at 40 kHz for 60 min. Although this treatment was efficient, it presented higher energy consumptions and is time consuming.

As an alternative, bead-milling with 0.5 mm beads at a concentration of 32 % for 6 min showed a similar efficiency in cellular disruption (but with a significantly lower time of operation, which is a significant advantage) and a still satisfactory performance in extracting compounds. It is a simple and efficient treatment capable of maintaining the integrity of the compounds, at a significantly lower level of energy consumption.

Keywords: Bioactive compounds; Cellular disruption; Lichens; Microalgae

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List of Abbreviations and Acronyms

Abs Absorbance

BSA Bovine serum albumin

BM Bead-milling

DNA Deoxyribonucleic acid

dw Dry weight

FS Forward scatter

FT Freezing thawing

HPLC High-performance liquid chromatography

HSH High-speed homogenization

IOMR Intracellular organic matter release

LFR1 Lichen foliose rock 1

LR Lower right

LL Lower left

MW Microwave

OD Optical density

PEF Pulsed electric fields

PI Propidium iodide

PUFAs Polyunsaturated fatty acid

rpm Revolutions per minute

SS Side scatter

TAG Triacylglycerols

UL Upper left

UR Upper right

US Ultrasonication

UV Ultraviolet

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Chapter I - Introduction

1.1 Lichens as a symbiotic organism

Lichens are a symbiotic relationship between a fungus, known as mycobiont and a second organism called photobiont, this one could be microalgae, cyanobacteria or both (Dayan and Romagni, 2001). This relationship is so complex and important for both that they took benefits from this symbiosis (Zambare and Christopher, 2012). The mycobiont protects the microalgae from intense solar radiation and dissection and it can absorb essential nutrients for the photobiont. The photobiont produce organic compounds that are important for the development of the lichen as a whole (Ranković and Kosanić, 2015).

Lichens are capable of growing in very extreme environments and even can resist desiccation for certain periods (Kranner et al., 2008). Around 20% of all fungal species can be lichenized and they have ecological advantages against extreme cold, heat or drought stress (Honegger, 1991; Zambare and Christopher, 2012). Lichens have been found growing in different places, such as rocks, soil, trees and even leaves (Dayan and Romagni, 2001). Some lichens have been found in shells of tortoise in Galapagos Islands, in bugs on New Guinea and other extreme environments around the world. Although this capacity of growing in very extreme environments, lichens have a very slow growth rate. Their growth can be measured in millimeters per year (Ranković and Kosanić, 2015). They show some sensitivity to pollution and it can be used as bioindicators for pollution in the environment, places with low lichen abundance can be associated with higher levels of pollutants. There are three different ways to monitoring pollution with lichens: measure variations on diversity or abundance, assessment of physiological parameters and use lichens as accumulators of pollutants (Pinho et al., 2004). Lichens can be used as biomonitors, bioaccumulators, and bioindicators (Brunialti and Giordani, 2003).

The fungus is responsible to assure the main structure of the lichen, which is known as thallus (Sanders, 2001). This structure has some layers the upper and lower cortex, algal layer and medulla. These layers, depending on the development and species can have different thicknesses. Lichens thallus morphology can have three principal classes of growth. They can be classified as crustose (Figure **1.A**), foliose (Figure **1.B**) or fruticose (Figure **1.C**) each one with they own characteristics (Ranković and Kosanić, 2015). Crustose lichens have the slowest growth rates of all lichens. This type of lichen, as the name indicates, forms a crust which adheres to the substrate and became highly associated with it. This interaction between the lichen and the substrate is so

strong that makes the collection of this type of lichen a complex process. The crustose lichens have a distinct upper cortex and lack a lower cortex (Armstrong and Bradwell, 2010; Ranković and Kosanić, 2015). This type of lichen morphology can be used by archeologist and other researchers to determine the age of a specific substrate, rock or even an old monument (Benedict, 2009). The foliose lichens have a two-dimensional structure, with an upper and lower cortex and an algal layer and medulla, these are attached to the substrate by a loose structure called rhizines (Dayan and Romagni, 2001). The fruticose lichens are common in more humid environments and are characterized by a three-dimensional structure. They are usually hanging and are highly branched (Dayan and Romagni, 2001; Ranković and Kosanić, 2015).

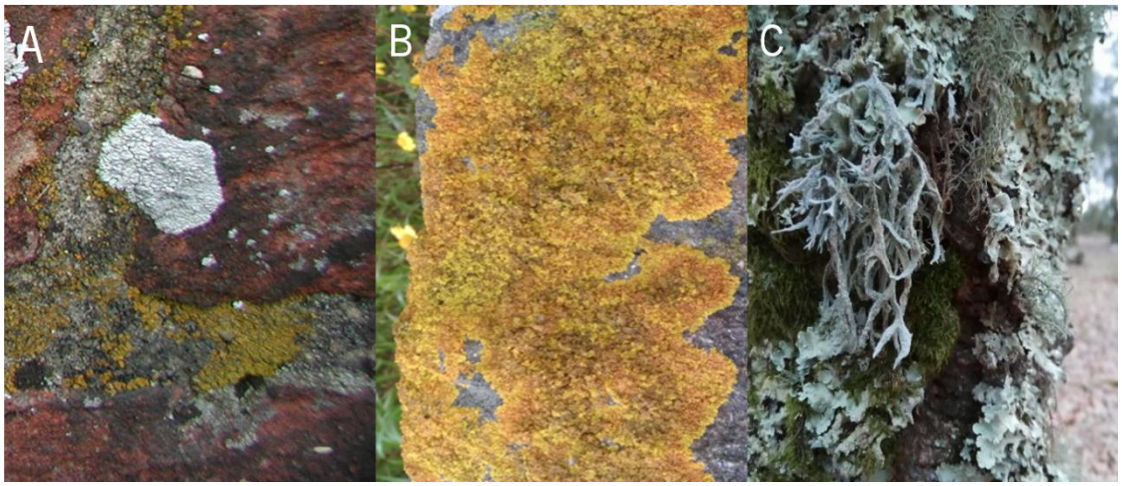

Figure 1. Different types of lichens morphologies, crustose lichen (A), foliose lichen (B) and fruticose lichen (C).

The lichen symbiosis produces different compounds from its primary and secondary metabolism. The primary compounds are essential for the primary metabolism of the organism, those metabolites are also found in some higher plants. In their secondary metabolism, lichens are known to produce more than 800 secondary metabolites. Some of these are only found in this organism (Ranković and Kosanić, 2015). Some of these secondary metabolites were isolated and used in different areas such as pharmaceutical and food industries (Karthikaidevi et al., 2009).

1.2 Main characteristics of microalgae

Microalgae are unicellular and photosynthetic organisms that can grow in a wide range of different environments, such as lakes, ponds, rivers, oceans, and wastewater. They tolerate different temperatures, pH values and even low salinity (Khan et al., 2018). They are capable of rearrange their biochemical composition accordingly with the environment around them (Renaud and Parry, 1994). Microalgae can grow alone or in symbioses with another organism such as a fungus (Khan et al., 2018).

One of the Earth most important energy sources is the sun, the average light intensity from the sun is <1 $kW.m^{-2}$. The best way to take advantage of this energy is through photosynthesis, the theoretical efficiency of photosynthesis for optimal grow on microalgae is 10 %. In natural growth conditions, microalgae have an efficiency of about 3 %. Another advantage of microalgae when compared to other food crops is a short life cycle. Microalgae has a life cycle of 1 to 4 days, while crops like rice and maize have around 90 to 180 days (Barsanti and Gualtieri, 2018).

One of the main advantages of microalgae culture is that they do not need arable land to grow like other plant species (Vaz et al., 2016). At industrial levels, their growth development can be easily optimized in comparison to the terrestrial plants (Günerken et al., 2015). Microalgae do not need any kind of pesticides or any kind of compounds that promotes or control their growth (Vaz et al., 2016). They are still poorly explored in comparison with land plant species. Nowadays land plants are well exploited in terms of production of different compounds, but it is also known that the production of these by algae species is ten times higher than the ones produced by land plants (Fu et al., 2017).

1.2.1 Biotechnological applications of microalgae

In the 1950s with the increase of the world population, there was a need to increase food production and other essential resources. Algae biomass appears like a good way for this problematic (Spolaore et al., 2006). They can be considered a sustainable source for the extraction of several compounds such as fatty acids, phycobiliproteins, chlorophylls, carotenoids, vitamins

and others (Fu et al., 2017). All of these compounds can have commercial value and can be used to meet the energy demands of the population and assist in the health fields (Vaz et al., 2016).

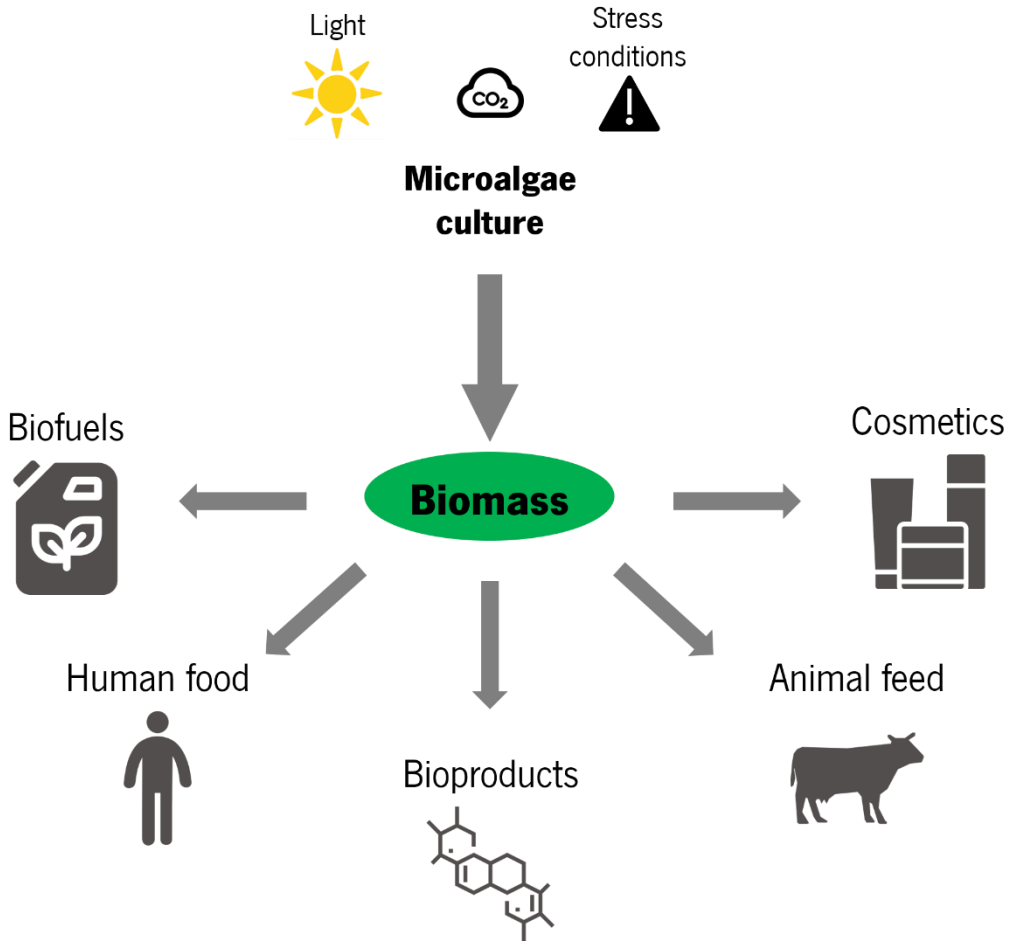

Figure 2. Applications of microalgae. Microalgae can be directly used by humans as food supplements or can be used as a different source of biofuels and even can be used as sources of different bioproducts.

The production of microalgae under control conditions demands some specific reactors, called photobioreactors. This type of reactors differ from the conventional ones due to their need for constant light supply (Loubière et al., 2009). For commercial cultivation of microalgae, it is possible to use different designs depending on what is most needed. The simplest design is the raceways ponds, in this model, the culture is exposed to the air and it simulates the external environment for microalgae growth (Shen et al., 2009). Raceways ponds present low construction and maintenance costs, easy scale-up processes and can be integrated into wastewater treatment. This type of design was described to produce about 27 ton ha^{-1} $year^{-1}$ in south of Spain in the projections of 2016 (Ruiz et al., 2016). The disadvantages of these types of reactors is how easily contamination could happen by other organisms, the low productivity and the high harvesting costs (Shen et al., 2009). This type of photobioreactors are used for commercial-scale production and the more

common cultures are microalgae and cyanobacteria such as *Spirulina* and *Dunaliella*. To produce microalgae with high-value compounds it is more common to use closed systems like tubular photobioreactors. This type of reactor allows a high parameter control and guarantee that the culture is not contaminated and produces higher amounts of biomass. This type of design was described to produce between 34 and 61 ton ha^{-1} $year^{-1}$ in south of Spain also in the projections of 2016 (Ruiz et al., 2016). These photobioreactors have been using for the cultivation of *Porphyridium*, *Phaeodactylum*, *Nannochloropsis*, *Chlorella*, *Haematococcus*, and *Tetraselmis* (Fernández, F.G. Acién; Sevilla, J. M. Fernández; Grima, 2013; Shen et al., 2009). The microalgae production costs on these types of photobioreactors are around US$5 per kg on open raceways and around US$13 per kg on tubular photobioreactors (Llamas et al., 2017).

Microalgae can be used to obtain new clean energy sources (Khan et al., 2018). In the European Union the transportation sector is responsible for more than 20% of gas emissions and the energy sector is responsible for more than 60% of the emissions, for these reasons it was necessary to find alternatives to fossil fuels (Mata et al., 2010; Gouveia and Oliveira, 2009). The biofuels have higher oxygen levels than fossil fuels and lower sulfur levels, this makes the use of them a cleaner source of energy. They can be produced by different types of biomass, such as food crops, crops waste, algae and others (Khan et al., 2018). So, microalgae as becoming an interesting alternative to produce biofuels, because they can produce high amounts of oil when compared to other crops (Raheem et al., 2015).

Microalgae are also important in CO_2 removal from the atmosphere, thus reducing greenhouse gas emissions. There are two different strategies for CO_2 mitigation, chemical reaction-based approaches and biological approach. The focus will be on the biological CO_2 mitigation because it is associated with photosynthetic organisms such as microalgae. Microalgae can fix CO_2 more efficiently than other plants, they have been described as 10 to 50 times more efficient. They are capable of fixing 1.83 tons of CO_2 and produce 1 ton of algae biomass (Mubarak et al., 2014). This is because microalgae have a faster growth rate than terrestrial plants. Using microalgae for CO_2 mitigation has many advantages, they are photosynthetic organisms, so they need CO_2 for their growth, and to produce high-value compounds which can be extracted later. And contrarily to chemical reaction based approaches, it does not produce any type of reaction waste (Wang et al., 2008).

The microalgae production is increasing every year, the microalgae biomass market has already established a production around 5000 t/year of dry matter, and it generated a turnover of US$1.25 x 10^9/year, in the year of 2008 (Raja et al., 2008). Another study done in 2015 showed that between the years 2010 and 2012, the European Union imported algae-based products from Chile around 13 million dollars/year. In the European Union, Ireland, France and the Netherlands were the main exporters, but all with values lower than Asian countries, which were capable of generate more than 125 million dollars per year on algae based products in this period of time (Vigani et al., 2015; Raja et al., 2008).

1.2.2 Compounds extracted from microalgae

Beside the biofuel production and CO_2 mitigation, microalgae are also important in the production of different compounds. These compounds could be from primary or/and secondary microalgae metabolism. The primary metabolism of microalgae are the basic reactions for the organism development, the metabolites produced can be lipids, proteins, vitamins, carbohydrates, and others. The secondary metabolism produces high-value compounds with interesting properties such as antibiotic, antiviral, anti-fungal, anti-inflammatory, anti-oxidative and most important they are safe for human and animal consumption (Barsanti and Gualtieri, 2018).

The primary metabolites produced by microalgae are mainly lipids, proteins, and carbohydrates. Lipids are a class of biomolecules soluble in organic solvents. The amount of lipids in microalgae cells varies with the species, and it can range between 25 % and 75 % of its dry weight (dw) (Mubarak et al., 2014). Algae lipids can be classified as two different types, as neutral lipids, which are mainly triacylglycerols (TAG) and present in cytosolic bodies. The other type of algae lipids is membrane lipids which are present in the cell membranes (Vuppaladadiyam et al., 2018). The microalgae cells can be induced to produce more lipids in their metabolism by altering some properties of the culture conditions. These properties can be: pH, illumination intensity, rate of light/dark cycles, growth temperature, aeration rate, nutrients sources (carbon, nitrogen, phosphorus and silicate), the growth regime (autotrophic, heterotrophic or mixotrophic) and even the species and strain of the microalgae (Perez-Garcia et al., 2011; Mubarak et al., 2014).

Proteins are another class of biomolecules. The use of microalgae for protein extraction began in the 1950s due to their high protein content and ability of synthetized all amino acids. For this reason, they are a good natural source for the production and extraction of proteins (Gouveia et

al., 2010). Mostly proteins in microalgae are synthesized during the culture growth phase (Vuppaladadiyam et al., 2018). Accordingly with some studies the major product from microalgae are the proteins, they can represent between 25 % to 40 % of the microalgae dry weight (Phong et al., 2018).

Another type of primary metabolites produced by microalgae are the carbohydrates, they are synthesized as energy storage molecules (Vuppaladadiyam et al., 2018). These molecules can be found in the cells in the form of starch, sugars, glucose, and polysaccharides (Spolaore et al., 2006). *Chlorella vulgaris* is described to have a total carbohydrate content between 13 % and 17 % (Markou et al., 2012).

Chlorophylls are also considered primary metabolites, they are used by cells as light absorbers and can act as anti-oxidants to repair from the damage caused by photosynthesis (Vuppaladadiyam et al., 2018). Besides this role in the development of the microalgae cells, these pigments also have an aesthetic function, they can give cells different colors (Gouveia et al., 2010). Chlorophylls can be divided into two different classes: chlorophyll *a* and chlorophyll *b* and they are responsible for the green color of the cells. Chlorophylls are the main light absorbers for microalgae and to the other type of plant cells, they absorb solar radiation in the red and blue regions and emits in the green region. Recently chlorophylls have been used in food and pharmaceutical industries, some studies have shown that chlorophyll can accelerate the healing process by 25 % and stimulate tissue growth (Hosikian et al., 2010). In recent years, chlorophyll has been reported to be associated with a decreased risk of colorectal cancer (Balder et al., 2006).

In general, the secondary metabolites produced by plants can be classified as terpenoids, essential oils, alkaloids, phenolics, flavonoids, tannins, glycosides and saponins (Kabera, 2014). In microalgae it is possible to find some of these high-value compounds, they can be pigments (carotenoids and phycobiliproteins), vitamins and minerals, terpenes, polymeric carbohydrates, lectins, polyunsaturated fatty acids (PUFAs), sterols, ketones, waxes and others (Vuppaladadiyam et al., 2018; Fu et al., 2017; Barsanti and Gualtieri, 2018).

Carotenoids are another type of natural pigment from microalgae cells, they belong to the class of terpenoids (Kabera, 2014). In plant cells, they are responsible for the huge variety of colors such as yellow, orange or red (Gong and Bassi, 2016; Gouveia et al., 2010; Vuppaladadiyam et al., 2018). Similar to chlorophylls, carotenoids also have an important protective effect on the photosynthetic apparatus, by prevent lipid peroxidation and promote the stability of the

photosystems (Gong and Bassi, 2016; Gouveia et al., 2010). Carotenoids can be divided into two different classes, primary and secondary carotenoids. The primary carotenoids, such as lutein, can absorb energy and transfer that same energy to chlorophylls, so they can expand the cell light absorption spectrum. The primary carotenoids are also classified as plant primary metabolites (Barsanti and Gualtieri, 2018). The secondary carotenoids (e.g. astaxanthin and canthaxanthin), are involve in protective mechanisms of the plant cells (Gong and Bassi, 2016). These molecules are a good natural additive in food and cosmetic industries, and in the year 2004, its market was estimated at around US$889.9 million (Ye et al., 2008). Carotenoids antioxidant properties can have significant protective effects on immune response, premature aging, arthritis, cardiovascular diseases and even in some cancers. The anti-oxidant properties can also have some effect on reducing the risk of diabetes and neurodegenerative diseases (Gong and Bassi, 2016).

Another two groups of compounds which can be produced by microalgae are vitamins and minerals. Microalgae can produce almost all essential vitamins, such as A, B_1, B_2, B_6, B_{12}, C, E, nicotinate, biotin, folic acid, and pantothenic acid. Similar to other compounds from microalgae, the contents of the vitamin depends on some factors such as state of growth, light intensity and cell nutritional status (Gouveia et al., 2010). Some of these vitamins are also powerful anti-oxidant agents and can prevent strokes, reduce homocysteine levels, reduce thrombosis, cerebrovascular diseases, which can be important in the treatment of pernicious anemia (Raposo and de Morais, 2015; Pyne et al., 2017). Some of the minerals found in microalgae can be sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), zinc (Zn) and other minor minerals (Gouveia et al., 2010).

1.3 Microalgae cell disruption methods

For the extraction of microalgae compounds, it is necessary to assure a successful rupture of the cellular membrane and cell wall. The disruption methods currently available can be divided into two main groups, mechanics and non-mechanics. Different methods can have different efficiencies, both types of methods have their advantages and disadvantages (de Carvalho et al., 2017; Günerken et al., 2015).

The cellular disruption is an essential step for the extraction of several compounds from microalgae and other cells, the amount of compounds extracted from cells varies with the efficiency of the cellular disruption (Lee et al., 2010).

The mechanical methods are more efficient in cell disruption, but they are high energy consumers and more aggressive than the non-mechanicals. These types of methods allow a lower contamination level and the extracted products can maintain their original properties. On the other hand, they all require some sophisticated equipment and high inputs of energy (D'Hondt et al., 2017). Some of these methods are described in Figure **3** (Lorente et al., 2015; Günerken et al., 2015).

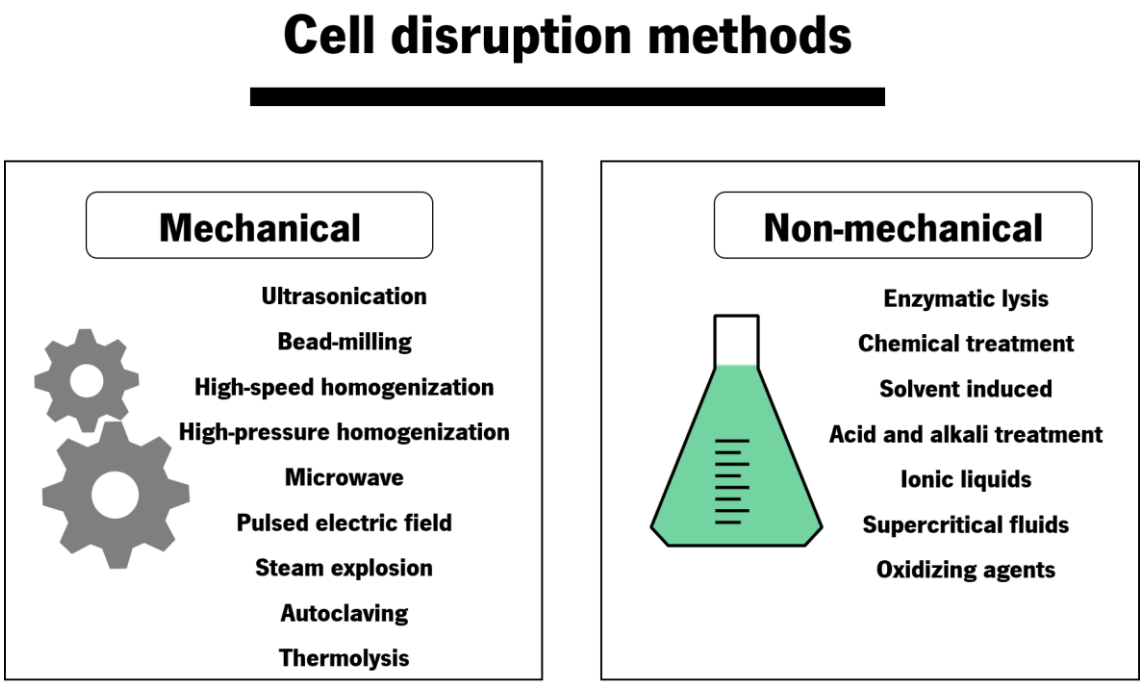

Figure 3. Cell disruption methods, mechanical and non-mechanical. Different cell disruption methods which can be used for cellular disruption on microalgae.

The non-mechanical methods can alter the membrane physiology to enhance the release of a certain product or even to disrupt cell integrity (de Carvalho et al., 2017). These types of methods can be more selective than mechanical treatments because they are capable of interact with the cell walls and membranes. In terms of energy demands, they require lower or no energy to be used. The use of chemicals can make them more expensive and after the extraction, the chemicals used might contaminate the wanted products and there is a need to be removed from the sample, reducing the quality of the extracted products (de Carvalho et al., 2017; D'Hondt et al., 2017). Some of these methods are describe in Figure **3** (D'Hondt et al., 2017; Günerken et al., 2015).

The mechanical methods will be the focus on this work because with this type of treatment there is no need to separate the products released after the treatment from the chemicals used. Another reason for only use the mechanical methods is because they are described as the most efficient on cell disruption, although it can damage some of the products due some heat generated

from the equipment (de Carvalho et al., 2017). All the mechanical cell disruption methods used are described on Figure **4**.

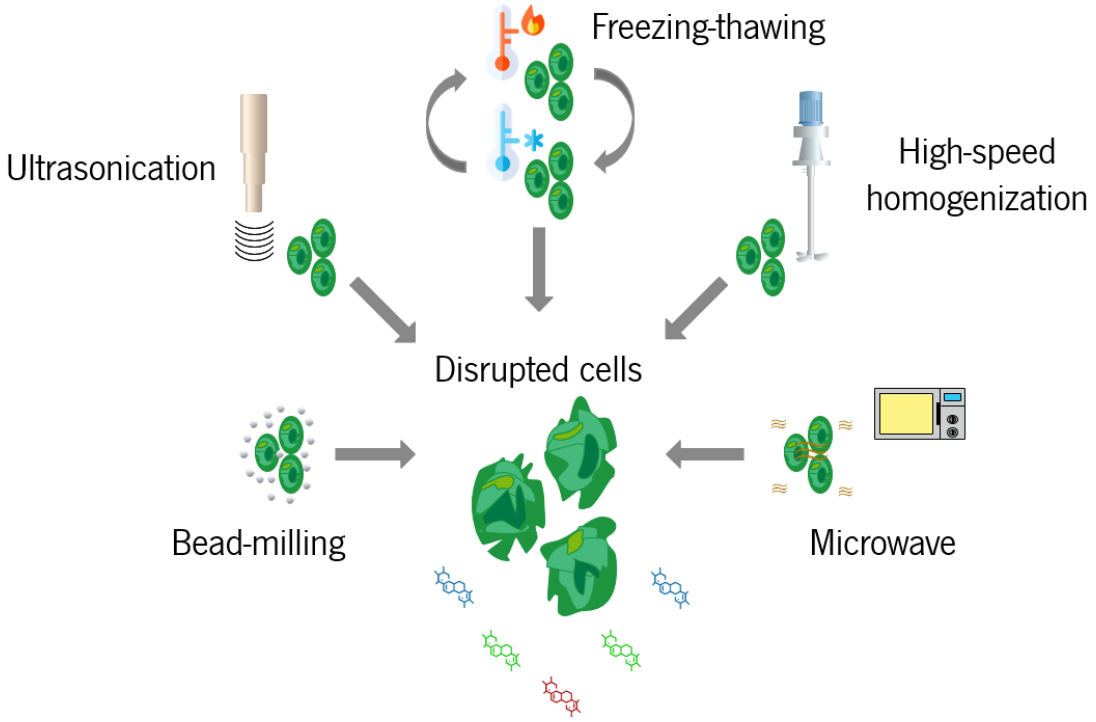

Figure 4. Cell disruption methods applied to microalgae cells. Schematic representation of the disruption methods applied on the microalgae isolated from a foliose lichen.

1.3.1 Freezing thawing

The freezing thawing (FT) is a technique that submits the cells to sudden temperature shocks. During a FT treatment, the cells are submitted to a freezing phase at a certain temperature then they are submitted to a phase of thawing, these temperature differences will cause cellular damage. This method it is not very effective on bacterial cells, it works better on large cells (de Carvalho et al., 2017).

In this method, the cellular rupture is achieved by the formation of ice crystals inside the cells during the freezing phase, and during the thawing process the cell expands and it damages the cellular structure (D'Hondt et al., 2017). In most cases this technique is done by different cycles of freezing and thawing, the number of cycles and temperatures can vary between studies (Keane et al., 2015).

Although it is a quite simple rupture method, it is not a very effective method to cause cellular disruption (Chotipan et al., 2016).

1.3.2 Ultrasonication

In the ultrasonication (US), ultrasound waves with high frequency initiate a cavitation process and the propagation of the waves in the medium causes cellular disruption (Günerken et al., 2015). The ultrasound waves used in this method are at a frequency higher than the maximum range of human earing, which is between 15 kHz and 20 kHz (Montalbo-lomboy et al., 2010). Sonication has the advantage to cause cellular disruption at lower temperatures in comparison with other methods such as microwave and autoclaving. These two methods could lead to higher protein denaturation due to the high temperatures. Another characteristic of US is that it do not necessary require any kind of chemical or bead to assist the disruption process, this represent an advantage by reducing the operational costs (Gerde et al., 2012). The effectiveness of the US is associated with the cavitation process and not to the effect of the ultrasounds (Greenly and Tester, 2015).

US is easy to scale-up and to operate *in continuo*, when the sonication starts two different mechanisms are happening in the solution: cavitation and acoustic streaming. The cavitation is caused by the production of microbubbles in the medium due to the application of energetic sound waves, these microbubbles will expand and contract and will become unstable and implode. After this violent implosion, the resulting shock waves will disrupt the cells around. And the acoustic streaming allows a better mixture of the solution and it can facilitate uniform dispersion of the ultrasonic waves in the solution (Montalbo-lomboy et al., 2010; Gerde et al., 2012).

For the US treatment, two types of devices can be used, the sonication baths and probes. In the sonication probes, it is possible to achieve the highest amplitudes of the ultrasonic waves but uses small sample volumes (between 10 mL to 100 mL). In the sonication baths, it is possible to use volumes up to 3 L. Both sonication methods are usually used in batch operations but also can be used in continuous operations after some adaptations (Lee et al., 2012).

The effectiveness of US depending on the microalgae species cell and membrane characteristics. This method can also produce some heat in the medium which could alter some of the compounds of interest, however, the heat production problem can be minimized by keeping the sample on an ice bath (Miranda et al., 2012). This method can also be associated with others to improve its effectiveness, for example adding glass beads can increase the crushing (Lee et al., 2012; Günerken et al., 2015).

1.3.3 Microwaves

The microwaves (MW) treatment for microalgae disruption it is a non-contact form of heating the sample. This method can damage the cell walls and allows the extraction of certain intracellular metabolites (Günerken et al., 2015). The main difference between microwave heating to conventional heating is the form of heat dispersion. In conventional heating, the heat goes from the outside to the inside of the sample, on the MW the heat direction it is inverted, it starts from the inside of the sample (Teo and Idris, 2014). Another important factor is the uniform heat dispersion through all the sample (Günerken et al., 2015).

The MW have been described as an efficient method to disrupt cell walls and some describe it as a good method for the extraction of vegetable oil (Rakesh et al., 2015). This method is capable of interact selectively with the dielectric or polar molecules of a solution and the resulting friction causes the local heating, this heating will result in an increase of pressure which will cause the rupture of the cell membranes (Balasubramanian et al., 2011; Günerken et al., 2015) Depending on the concentration of the solvent in the sample, the heat generated also differs, for example, higher water concentration means higher free molecules to react and higher temperature achieved (Günerken et al., 2015).

The use of MW for cell disruption reduces some operational costs, it allows lowest energy inputs, lowest extractions time and there is no need to use large quantities of solvents. In comparison to conventional heat, MW heating can reach the same temperatures ten times faster (Balasubramanian et al., 2011). It also allows using more simple equipment on a smaller scale, although it is an easy process to scale up (Virot et al., 2008; Günerken et al., 2015).

The MW are widely used to assist some extraction techniques of different compounds of interest, these techniques of extraction were described by: Hara and Radin (Hara and Radin, 1978), Chen et al. (Chen et al., 1981), Folch et al. (Folch et al., 1956), Bligh and Dyer (Bligh and Dyer, 1959) and the Soxhlet extraction (Balasubramanian et al., 2011).

1.3.4 High-speed homogenization

The high-speed homogenization (HSH) can be used for cellular disruption, the devices used are made by two different components. The first component it is a static steel tube, and the second component it is a high-speed rotating blade inside the static tube, these components are the stator

and rotor respectively. The high-speed homogenization can be operated in continuous, semi-batch and batch (Lee et al., 2012).

During the operation with a high-speed homogenizer, the cells in the solution go throw the gaps between the rotor and the stator and due to the high shearing forces the cells are disrupted. Like the other mechanical methods, several variables can affect the efficiency of this homogenization. Those variables can be the design of the rotor-stator and their size, the speed used, the sample initial volume and concentration, the processing time, the shape of the vessel and others (Park et al., 2015).

HSH is a simple but aggressive method for cellular disruption. This method can be used for short periods and its very effective in disrupting the sample cells, but to have this effect it requires large amounts of energy inputs to operate all of the system (Günerken et al., 2015). Another drawback of this technique it is that we cannot combine with another technique such as glass beads because the homogenizer can destroy the beads and those fragments become hard to remove (Lee et al., 2012).

1.3.5 Bead-milling

The bead-milling (BM) treatment is another mechanical method for cellular disruption, this method uses small beads that can be made of different materials such as glass, steel or ceramic (Günerken et al., 2015). The basic principle of this method is to take advantage of the beads capability to collide to the cells and consequently causing cellular disruption. BM can be used for cells more rigid, and harder to damage (Lee et al., 2012). It can be used in batch experiments and in continuous (Kim et al., 2013; Postma et al., 2015).

For this technique two different approaches can be made, the first approach is the shaking vessels and the second is the agitated beads. The shaking vessels are usually to rupture at a laboratory scale, and it consists on a vibrating platform where the culture is placed inside the vessels with beads. These vessels can cause similar cellular damage to different samples at the same time. The second approach consists in a vessel which is fixed, and it is filled with the cell culture and beads. This type of method requires forms of cooling in order to avoid the denaturation of some compounds and it can be used at a laboratory scale and in a few cubic meters (Lee et al., 2012).

The efficiency of rupture of bead-milling is affected by diverse factors, like the size of the container, the beads diameter, the type of beads used, the density of those beads, the shaking rate and even the amount of beads used (Montalescot et al., 2015; Kim et al., 2013). Due to all of these variable factors, this method can be easily altered and optimized, some authors have tested these different parameters such as the bead diameter, the material of the beads, the rotational speed and others (Montalescot et al., 2015).

In some literature, it is been defined that the optimal bead diameter to disrupt microalgae is around 0.5 mm. Beside of using beads of glass, steel or ceramic, they can be more specific and improved if they are made of zirconia-silica, zirconium oxide or titanium carbide, these specific materials are the densest and presumably the ones capable of higher cell damage. After the bead-milling, the beads are easily removed from the cellular extract (Lee et al., 2012).

1.4 Flow cytometry

The flow cytometry is a sophisticated technique capable of measuring several cell parameters by taking advantage of light scattering features. The main principle of the flow cytometer is the light scattering and fluorescence emission by the cells. This method is capable of several analyses, such as phenotypic characterization of blood cells, measurement of apoptosis markers, DNA fragmentation and others (Adan et al., 2017).

A flow cytometer is composed of four main elements. The fluidics, responsible for directing the cells from de sample to the optical system. While directing the cells flow, this component also assure that the cells reach the laser in a single file. The optics, where the lasers (excitation optics) and lenses (collection optics) are fixed in specific positions, these components are the main responsible for the light scattering, this scattering is done in two different ways: the forward scatter (FS) and the side scatter (SS). The FS sensor is placed in the same axe as the laser and after the laser beam goes through the cells and reaches the sensor, the user receives information about the size of the cells. The SS sensor is placed in an angle of 90 degrees within the laser, this sensor takes advantage of the cell capacity the reflect and refract the light. It gives the user information about the structural complexity of the cells in the sample. And finally the electronic network and the computer which are responsible to convert the light information into digital data and to analyze that data, respectively (Wilkerson, 2012; Adan et al., 2017)

Flow cytometry can give information about the microalgae cells, such like concentration and size (Günerken et al., 2017). Flow cytometry also gives information about the number of cells, with this it simplifies the time used on counting cells in a certain sample. For example, the microscopy cellular counting it is a time consuming technique and it presents low accuracy, while the flow cytometry it is more precise (Wang et al., 2010).

The cytometer capability to measure the cellular integrity it is important in order to distinguish intact cells from the non-intact. A very common way to measure the cellular integrity in flow cytometry is by using a fluorescent dye like propidium iodide (PI). This compound is capable of interacting with genomic DNA, and when excited with blue light it will produce red fluorescence. Although PI is capable of interact with the cell DNA, it cannot go through the cell membrane, so in order this interaction could happen the cell membrane must be vulnerable. This means that the PI only interacts with genomic DNA from cells with damage in their structure, which allow the interaction of PI with the DNA (Suman et al., 2015). For microalgae cells, due to the presence of photosynthetic pigments, it is possible to take advantage of those to distinguish between the intact from non-intact cells. This way avoids the use of PI and other fluorescent dyes (Dashkova et al., 2016).

1.5 Objectives

The main objective of this work is to understand how mechanical methods are capable of inducing damage to a species of microalgae isolated from a lichen symbiosis. The cells will be submitted to different disruption methods at different conditions in each one. The objective is to know which is the best condition in each one of these methods to assure the maximum cellular disruption and higher extraction of bioactive compounds from the microalgae cells. The method applied must be efficient in disrupting the cells, not too aggressive to assure the integrity of the extracted bioactive compounds and it should be simple and not time-consuming.

All the samples will be analyzed by scanning spectroscopy, flow cytometry and quantification of metabolites from microalgae primary metabolism, such as carbohydrates and proteins.

Chapter II – Materials and methods

2.1 Microalgae culture and maintenance

The photobiont culture used was isolated from a foliose lichen found in a rock and it has a two-dimensional structure. The nomenclature attribute to this photobiont was LFR1. The lichen came from a mountain region in the north of Portugal (Castro de São Lourenço, Esposende) and it was collected on April 19, 2018. The isolated microalgae are *Eukaryotic* cells from the filo of *Chlorophyta* and by microscopy observation have around 20-25 µm of diameter with the magnification used in Figure **5.C** is possible to see the cell size, which is bigger than other well know microalgae. The *Chlorella vulgaris* cells, which are one of the most studied microalgae cells, have a diameter of around 5 to 10 µm (Scragg et al., 2003).

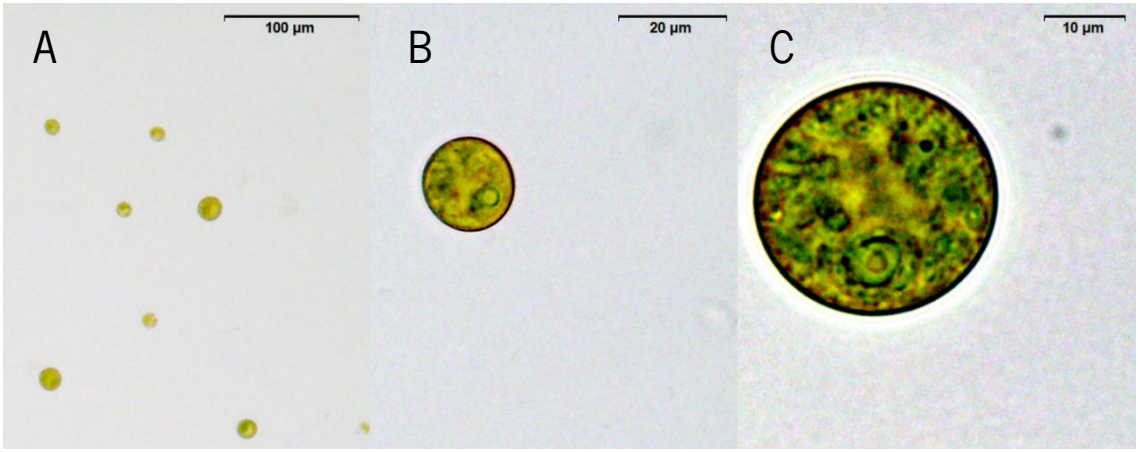

Figure 5. Photobiont LFR1 isolated from a foliose lichen. Microalgae cells used in this work with three different magnification: 100x (A); 400x (B); 600x (C)

The microalgae growth is performed in autotrophic conditions with constant light supply at 100 µm photons.m^{-2}.s^{-1} and air flow. The growth medium is composed by macro and micronutrients important for microalgae growth and maintenance. The macronutrients present in the medium are 18 mM $(NH_2)_2CO$, 1.70 mM KH_2PO_4, 0.83 mM $MgSO_4 \cdot 7H_2O$, 0.12 mM EDTA NaFe, 0.79 mM $CaCl_2 \cdot 2H_2O$. The micronutrientes presente in the médium are: 6.73 mM H_3BO_3, 3.0 mM $CuSO_4 \cdot 5H_2O$, 8.32 mM $MnCl_2 \cdot 4H_2O$, 1.99 mM $CoSO_4 \cdot 7H_2O$, 1.17 mM $ZnSO_4 \cdot 7H_20$, 6.96e-8 mM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ and 5.18e-8 mM $(NH_4)VO_3$. The medium is autoclaved for 20 min at 121 °C before utilization.

2.2 Characterization of microalgae cultures

A calibration curve was done in order to establish a relationship between the optical density (OD) and cell concentration in number of cells per mL. The wavelengths used to measure the OD was 670 nm and 750 nm. At 670 nm is in the chlorophylls emission spectra, allowing their detection (Gregor and Maršálek, 2004). At 750 nm it is possible to evaluate the culture age and development phase (Griffiths et al., 2011). The OD was measured at both wavelengths and at different concentrations. The calibration curve between optical density and cell concentration obtained for both wavelengths is shown in Figure **6**.

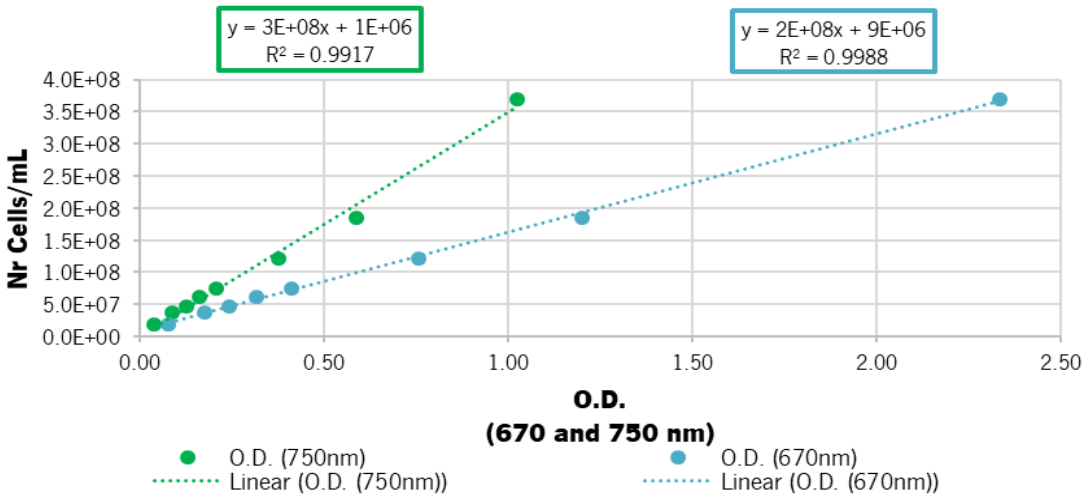

Figure 6. Calibration curve between cellular concentration and optical density. The wavelengths used were 670 nm and 750 nm. The blue curve is at 670 nm and the green curve is at 750 nm.

Besides the OD at 670 nm and 750 nm, it was also measured dw of microalgae. A total volume of 5 mL of culture was vacuum filtered with the aid of a pump, for this filtration was used nylon filters with a diameter of 47 mm and pore size of 0.22 µm. The filters were weighed before the filtration and after the filtration, they went to the stove at 105 °C overnight, then they were weight again and the weight difference was measure. The dw calibration curve is shown on Figure **7**.

The cells were also counted with a microscope *Nikon Eclipse Ci-L* and a Neubauer hemocytometer from *Marienfeld.*, the cells were counted in quadruplicate. The total volume used in the chamber was 20 µL, and the number of squares counted was 89 for each 20 µL.

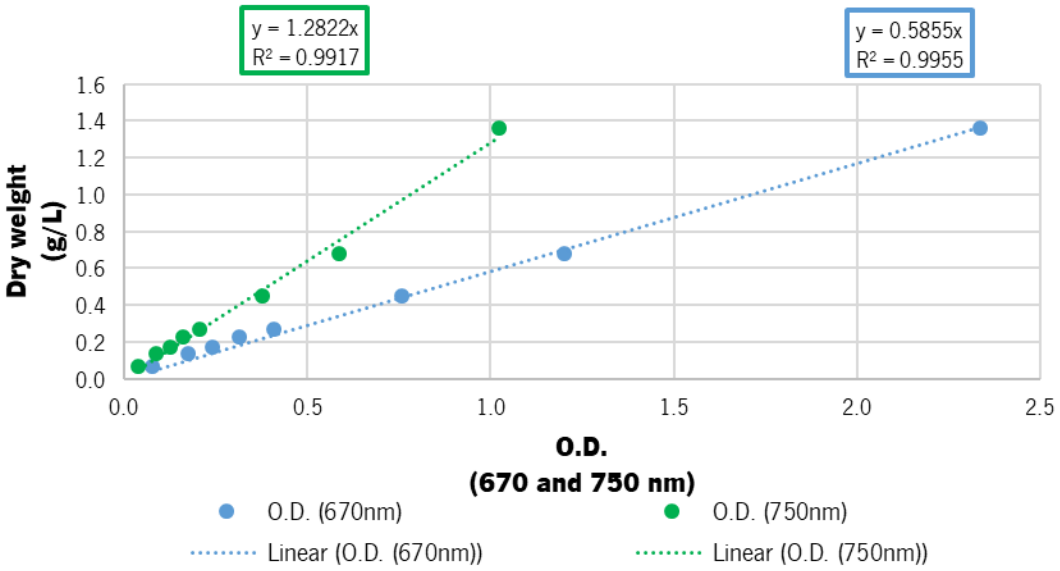

Figure 7. Calibration curve between dry weight and optical density. The wavelengths used were 670 nm and 750 nm. The blue curve is at 670 nm and the green curve is at 750 nm.

2.3 Microalgae disruption methods

2.3.1 Freezing thawing

In FT, four different experimental conditions were tested. Different number of cycles and different temperatures of freezing and thawing were used. The two different freezing temperatures were, -20 °C and -80 °C and the two different thawing temperatures were 20 °C and 40 °C in a water bath for exactly two hours. In all the experiments the cell concentration was established at 10^8 cells/mL. All the experimental conditions are represented on Table **1**.

Table 1. Experimental conditions used in the FT disruption method

Experimental condition	Temperature of freezing (°C)	Temperature of thawing (°C)	Cycles
1	-20	20	6
2	-80	20	6
3	-20	40 (2 h)	10
4	-80	40 (2 h)	10

All the conditions were done in triplicate, and all the samples were analyzed by spectroscopy and flow cytometry.

2.3.2 Ultrasonication

The ultrasonic device used in these experiments was a *Sonics Vibra-cell Processor,* model VCX 500, and two different probes were used, a 20 kHz and a 40 kHz ultrasonic probe, model CV33. With the 20 kHz probe, the cells were exposed to 60 min of treatment. The 40 kHz probe was used for three different experimental conditions: 60 min, similar to the one done with at 20 kHz. The other two conditions were 20 min without pulses and 20 min with 5 s pulses *on/off*. In all the experiments the cell concentration used was constant at 10^8 cells/mL. The different experimental conditions are described in Table **2**.

Table 2. Experimental conditions used in the US with the 20 kHz and 40 kHz ultrasonic probe

Experimental condition	Time (min)	Pulses (*on/off*)	Ultrasonic probe
1	60	Continuous (no pulses)	20 kHz
2	60	Continuous (no pulses)	40 kHz
3	20	Continuous (no pulses)	
4	20	5 s	

All the experimental conditions were done in triplicate and the samples were analyzed by spectroscopy and flow cytometry.

2.3.3 Microwave treatment

In the MW cell disruption treatment was used a conventional microwave oven, *Samsung MS23K3513AW*, with an output power of 800 W. In all the experimental designs the cell concentration was constant at 10^8 cells/mL. Two types of experimental designs were made, a non-continuous operation where the sample had a cooling time on ice before returning to the microwave and a continuous operation where the cells went to the microwave oven one single time during a certain period of exposure to the microwaves, the times are shown in Table **3**, as experimental condition 2.

Table 3. Experimental conditions used in MW treatment

Experimental condition	Time (min)	Power
1	12	800 W
2	2	
	4	
	6	
	8	
	10	
	12	

All the experimental conditions were done in triplicate and the samples were analyzed by spectroscopy and flow cytometry.

2.3.4 High-speed homogenization

In the HSH treatment was used a high-speed homogenization device, *IKA T18 digital ULTRA TURRAX*, capable of reach 25000 rpm. The different experiments were also done with a concentration of 10^8 cells/mL. For this rupture method, two different conditions were tested. The first condition was done with different rpms during one minute of treatment. The other condition was at the best rpm of the first condition for a maximum of 80 min. These two conditions are described in Table **4**.

Table 4. Experimental conditions used in HSH treatment

Experimental condition	Time (min)	rpm
1	1	16000
		18000
		20000
		25000
2	80	25000

All the experimental conditions were done in triplicate and all the samples were analyzed by spectroscopy and flow cytometry.

2.3.5 Bead-milling

For the BM treatment, the cells at a concentration of 10^8 cells/mL were submitted to direct contact with glass beads with different diameter. There was used two different beads used, 0.5 mm of diameter and between 0.149 and 0.250 mm. The total volume used was constant at 5 mL and was used different beads concentration. The time of treatment was established at 7 min, which were described as an optimal time for treatment with bead-milling (Geada et al., 2019). After testing the different beads concentration and diameter, the minimum time for cells disruption were tested. For this, the cells were submitted to different times from 1 to 10 min in direct contact with the beads. In all the experiments was used a vortex *Reamix 2789* capable of reaching 2800 rpm. All the conditions tested are described in Table **5**.

Table 5. Experimental conditions used in the BM treatment

Experimental condition	Diameter of beads used (mm)	% of beads	Time (min)
1	0.5	15 %; 24 %; 32 %; 42 %; 52 %	7
2	0.149 – 0.250	15 %; 24 %; 32 %; 42 %; 52 %	7
3	0.5	32 %	Máx. 10

All the experimental conditions were done in triplicate and all the samples were analyzed by spectroscopy and flow cytometry.

2.4 Evaluation of cellular disruption

During the different treatments some samples were collected in order to evaluate the progression of the cellular disruption, those samples were analyzed by different methods: spectroscopy, cellular integrity by flow cytometry and quantification of some primary metabolites.

2.4.1 Spectroscopy analysis

After the treatment, the samples were centrifuged at 6000 rpm for 10 min in a microtube centrifuge. The pellet with the cellular debris and the remaining cells was discarded, and the supernatant was used for the spectral analysis.

The spectroscopy analysis was done in two different ways. The first one was with a single read at a wavelength of 254 nm, which is at ultraviolet (UV) range. At this wavelength, it is possible to evaluate the intracellular organic matter release (IOMR) from the cells, the release of organic compounds from the cells will increase the absorbance (Abs) values at 254 nm (Huang et al., 2016). For this analysis, the supernatant is collected and measure the absorbance at 254 nm for the control sample before the cellular disruption, and for the different times after treatment. With the absorbance values, it is possible to calculate the IOMR factor according to the following equation:

$$\text{IOMR factor} = \frac{\text{Abs}_t - \text{Abs}_0}{\text{Abs}_0}$$

In this equation, Abs_0 corresponds to the absorbance before the cellular disruption and the Abs_t corresponds to the absorbance in a certain period after the treatment (Geada et al., 2019). A 96 well quartz plate was used to measure the absorbance in the UV range, each well had 200 µL of supernatant for analysis. This analysis was done in triplicate.

The second spectroscopy analysis was a pigments release spectrum between 400 nm and 800 nm, which is in the visible range. This spectrum shows the difference between the pigments at different times during the cellular disruption and in the control before rupture. A 96 well plate was used to measure the absorbance of the samples, each well had 200 µL of supernatant for analysis.

2.4.2 Cellular integrity by flow cytometry

The cellular integrity was measured by flow cytometry, through a *Cell analyzer Sony EC800.* The cytometry analysis was done for every experimental condition in triplicate. In each run, a volume of 100 µL was used by the flow cytometer for analysis of cells in the sample.

A specific protocol was designed in the cytometer software to evaluate the cell rupture of the culture used. This protocol can identify the different cell populations present in the culture and the disrupted cells of those populations. Several parameters were analyzed with this protocol: the cell complexity (SS), the cell size (FS) and the cell disruption.

Each cell that enters the cytometer is read as a single event, and the light that passes through the cell reaches the filter that corresponds to the emission zone of chlorophylls, the filter is FL3. The FL3 signal is used to plot a graph capable of show the cells disruption, the graph was plot with the axes FL3-Lin and FL3-Peak-Lin, given by the software, an example of this type of graphs are shown in Supplementary Information **1** and **2**. The cells disruption efficiency was measured by drawing gates manually in these graphs, given four different quadrants. These quadrants are upper-right (UR), upper-left (UL), lower-right (LR) and lower-left (LL). In the UR were plot the healthy microalgae cell population, with their normal metabolism working. In the LL quadrant were the cells that lost their integrity after the treatment those are the disrupted cells. And in the other two quadrants is essentially cellular debris (Günerken et al., 2017).

The cytometer software can also give the cell percentage on each run. With the use of a control sample before the treatment it is possible to establish the total cell percentage of cells in the culture. By analyzing of the different conditions of treatment, it is possible obtain the cellular disruption percentage.

2.5 Determination of metabolites

2.5.1 Determination of carbohydrates

For the determination of carbohydrates in the treated samples, were used the Dubois method (Dubois et al., 1956). A sample from the microalgae culture at 10^8 cells/mL was used as a control for the total amount of carbohydrates present in the sample. These cells were hydrolyzed by a chemical method to release intracellular compounds. An aliquot of 1 mL was centrifuged at 10000 rpm for 5 min, washed and then centrifuged again at same conditions. The supernatant was removed and then added 1 mL of HCl at 37 %. This control sample were then hydrolyzed for 2 h at 100 °C water bath, in triplicate (Fernández-Linares et al., 2017).

A calibration curve was done from a stock solution of glucose at 250 mg/L. The concentrations used in the standard curve were between 5 - 250 mg/L.

An anthrone solution at a concentration of 2 g/L was prepared in 75% sulphuric acid (H_2SO_4). Samples from control after hydrolysis and from all cell disruption treatment were collected, 200 µL each. To these samples and to the standard curve were added 200 µL of H_2SO_4 and mixed in the vortex. After mixing, 400 µL of the anthrone solution was added to the samples and to the standard curve and went for incubation in a hot water bath for 15 min at 100 °C. The OD was read at 578 nm and the carbohydrate concentration was calculate using the calibration curve (Yemm and Willis, 1954; Chen and Vaidyanathan, 2013).

2.5.2 Determination of proteins

The protein determination was done by the Lowry method, which is widely used on quantification of proteins of a sample. This method is simple and take advantage of the Biuret reaction (Lowry et al., 1951). This method requires two different solutions, the Lowry solution and the Folin-phenol reagent. The Lowry solution is made by three different stock solutions, 0.48 M Na_2CO_3, 0.07 M NaK Tartrate and 0.06 M $CuSO_4H_2O$ in the ratio 100:1:1.

The Folin-phenol reagent must be prepared right before utilization, because its light sensitive. The solution is 5 mL of 2 N Folin and Ciocalteu's Phenol reagent and 6 mL of distillated water.

For the calibration curve was done a BSA standard curve with a stock solution of 100 mg/L. The different concentrations used were between 10 - 100 mg/L.

The control microalgae samples, at a concentration of 10^8 cells/mL were hydrolyzed with an alkaline method, NaOH 1 M was added to cells in a 1:1 ratio and incubated for 10 min in a 100 °C hot water bath. After hydrolysis, the samples from control and from the different disruption treatment were centrifuged at 6000 rpm for 10 min, and a sample of supernatant was collected. To each sample collected were added 1.25 mL of Lowry solution and it incubate for 10 min in the dark at room temperature, then were added 250 µL of Folin-phenol reagent and it incubated for another 30 min in the dark at room temperature. The OD was read at 750 nm in a spectrophotometer.

2.6 Statistical analysis

All the statistical analysis was done with the informatic software *GraphPad Prism*, version 6.01. The results will be presented in triplicate with mean of the triplicate and the respective standard deviation.

Chapter III – Results

3.1 Freezing thawing

In the FT treatment, the influence that successive cycles of freezing and thawing have on cellular disruption was tested. In the first experimental condition, cells were submitted to 6 cycles of FT, and the results are shown in Figure **8**. In this condition, thawing was done at 20 °C. Freezing was at -20 °C in a common freezer and at -80 °C in a deep freezer.

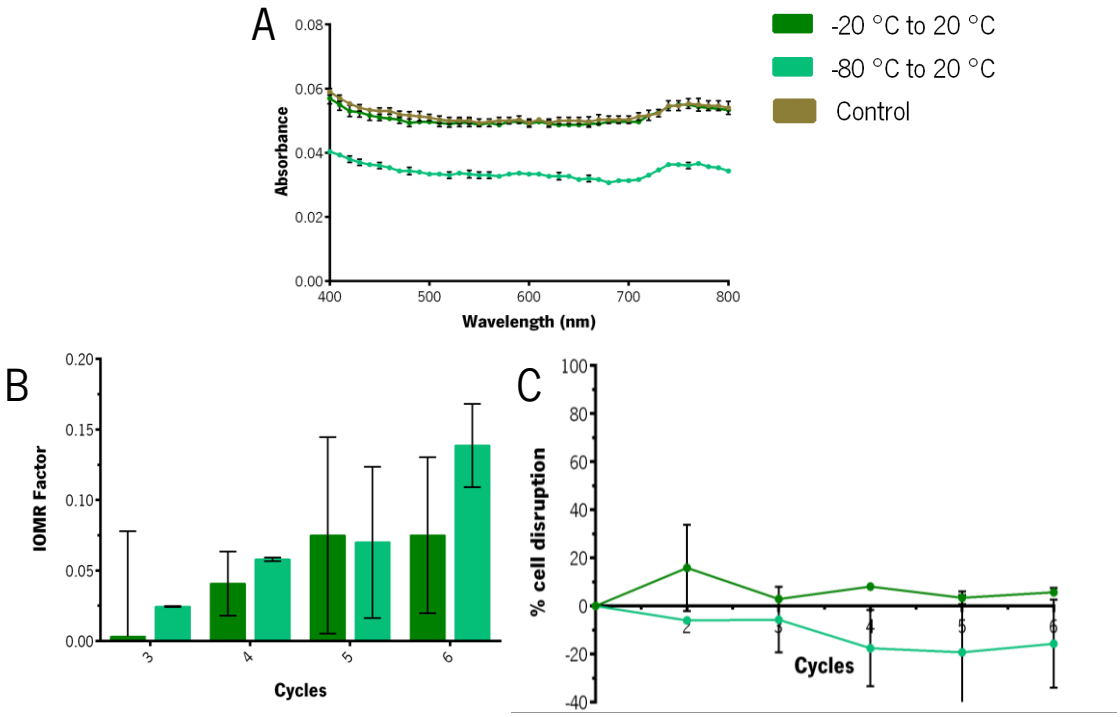

Figure 8. Results of pigments release (A), IOMR factor (B) and percentage of cell disruption (C) from FT treatment after 6 cycles with two freezing temperatures. The IOMR factor (B) is only showed after the third cycle.

The results from the pigments release spectra after 6 cycles are shown in Figure **8.A**. It reveals that no release of any photosynthetic pigments from the microalgae cells occurred after the treatment.

From the analysis of the IOMR of both conditions, on Figure **8.B**, it is possible to observe a low release of organic matter from the cells after the treatment (is only showed the values starting after the third cycle, because it was not possible to observe any difference in the first two cycles). On both experiments the IOMR factor was lower than 0.14 ± 0.03. This value was achieved after 6 cycles of the experimental condition where the cells were submitted to a freezing temperature of -80 °C and a thawing temperature of 20 °C.

From the analysis of the flow cytometry data, on Figure **8.C**, in both experimental conditions the cells did not suffer any kind of damage and the percentage of cell disruption was low or none after the treatment.

Figure **9** shows the spectroscopy and flow cytometry results from the other two FT conditions. In these conditions, the freezing temperatures were the same as previously, but the number of cycles was increased to 10 and the thawing temperature was 40 °C in a water bath for exactly 2 h.

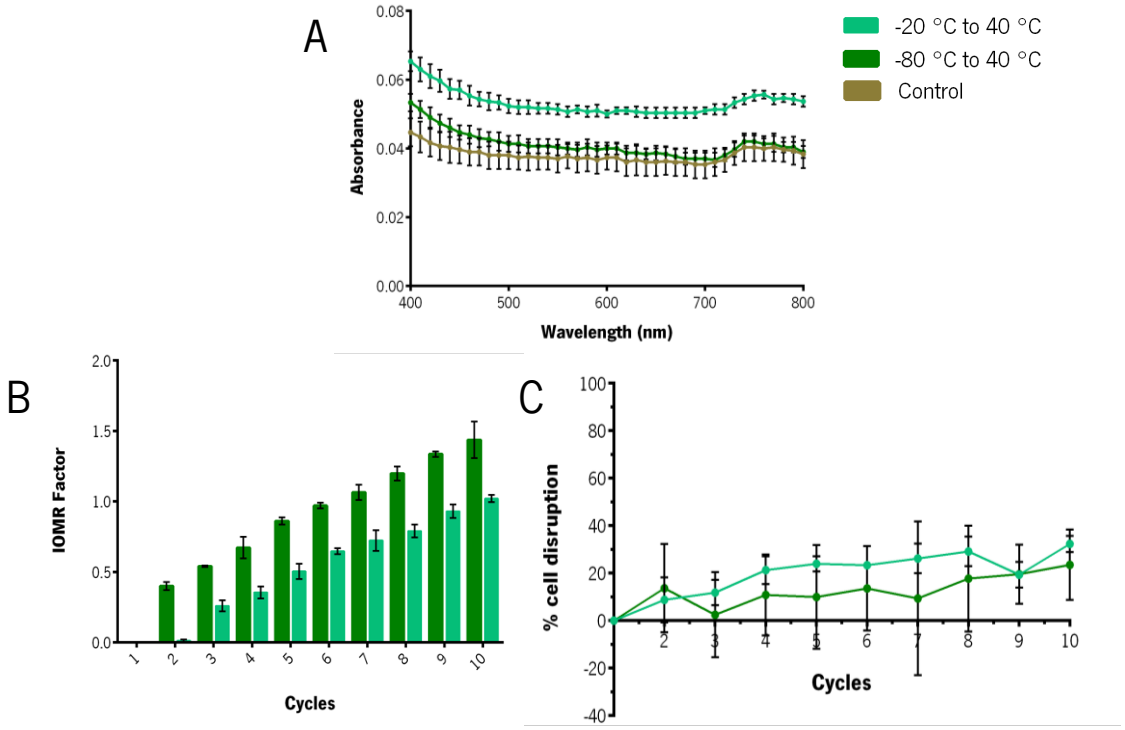

Figure 9. Results of pigments release (A), IOMR factor (B) and percentage of cell disruption (C) from FT treatment after 10 cycles with two freezing temperatures.

The pigments release spectra on Figure **9.A**, after 10 cycles was low, just like the one observed with the 6 cycles. These results indicate that there was no release of photosynthetic pigments in both treatments.

On the other hand, the IOMR (Figure **9.B**) showed some release of intracellular organic compounds after 10 cycles. In the condition where the cells were frozen at -20 °C, the IOMR factor after the 10 cycles was around 1.02 ± 0.03. After the same number of cycles, but with a freezing temperature of -80 °C, the intracellular organic matter release was higher. The IOMR factor after 10 cycles in this condition was 1.44 ± 0.13.

The flow cytometry data on Figure **9.C**, show some rupture after the 10 cycles of FT. The cycles between -20 °C to 40 °C presents a percentage of cell disruption of around 32.26 ± 3.44 % after 10 cycles. In the treatment between -80 °C and 40 °C, after the same number of cycles, was only achieve a percentage of cell disruption of 23.50 ± 14.85 %.

3.2 Ultrasonication

In the US treatment, two different ultrasonic probes were tested at different frequencies, 20 kHz and 40 kHz, in order to understand which probe and condition shows higher cellular disruption and release of compounds. Both probes were used for 60 min of exposure to the ultrasonic waves. The results are shown in Figure **10**.

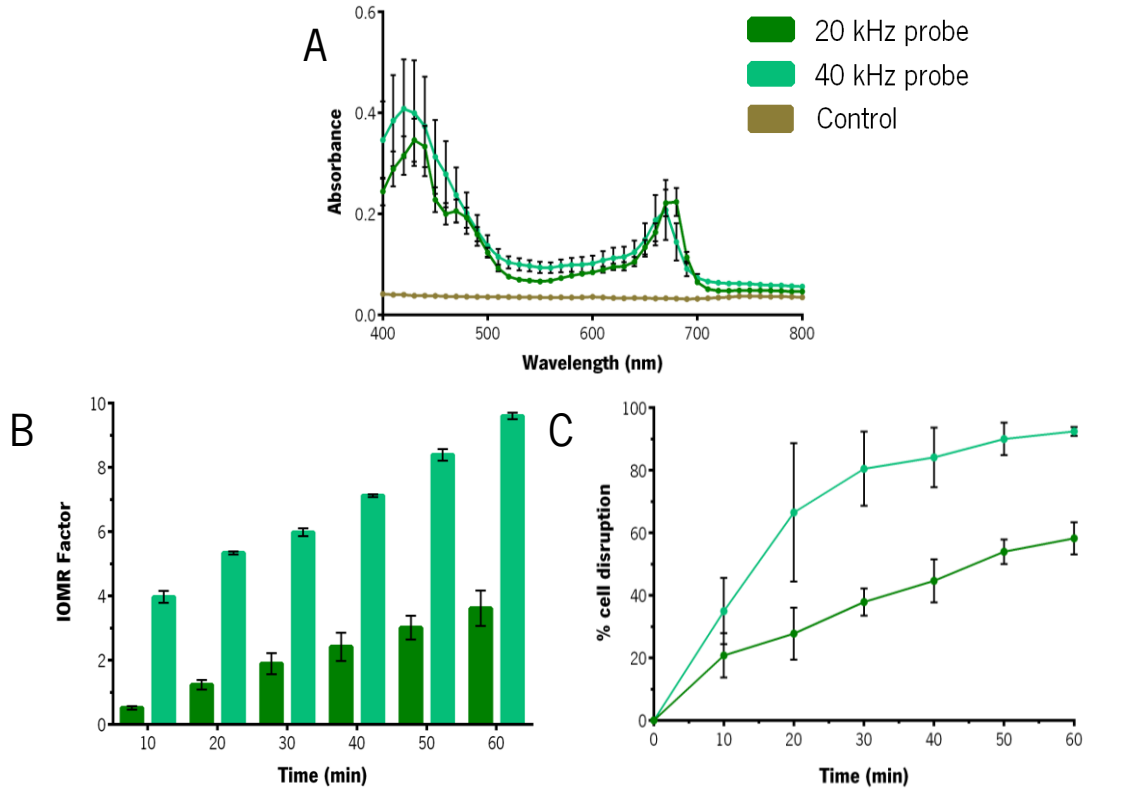

Figure 10. Results of pigments release (A), IOMR (B) and percentage of cell disruption (C) from US treatment after 60 min using a 20 kHz and 40 kHz ultrasonic probe.

Pigments release spectra after 60 min (Figure **10.A**) show some differences in the chlorophyll's emission peaks. With the 20 kHz ultrasonic probe the spectra show two peaks, with different height, between the 400 and 500 nm, while with the 40 kHz ultrasonic waves there is only one between the same wavelength. Although this difference, the 40 kHz ultrasonic probe shows a higher release of photosynthetic pigments than the 20 kHz probe. The pigments release

spectra of the other times of US with both probes are available on the Supplementary Information **3** and **4**.

The results from the IOMR factor (Figure **10.B**) shows a significant difference between both probes. The 40 kHz ultrasonic probe after 10 min of ultrasounds already have an IOMR factor of 3.97 ± 0.18, while the 20 kHz ultrasonic probe after the same time has only a value of 0.52 ± 0.05. And after the 60 min, the 40 kHz ultrasonic probe could reach an IOMR factor value of 9.60 ± 0.10. This value is significant different from the one obtained, after the same time, with the 20 kHz ultrasonic probe which was only of 3.62 ± 0.55.

In flow cytometry results (Figure **10.C**) after the 60 min of treatment, both probes show a difference on the percentage of cellular disruption. The 40 kHz probe was capable of cause a cellular disruption above 90 % after 50 min and after the 60 min of treatment, the maximum achieve was 92.46 ± 1.45 %. The percentage of cell disruption with the 20 kHz ultrasonic probe after 60 min of treatment was only 58.27 ± 5.14 %.

After concluding which probe has the best result on cellular disruption and higher release of intracellular compounds, were tested how pulses *on/off* could influence the cell disruption, IOMR and pigments release. These experiments were done for 20 min with and without pulses of 5 s with the 40 kHz ultrasonic probe. The results are shown on Figure **11**.

The pigments release spectra (Figure **11.A**) show similar release of pigments with and without the pulses. The only difference observed between using the pulses and not using is between 400 and 500 nm, where without pulses show a little more release of pigments. In these results it is also observed two peaks between 400 and 500 nm, which is not observed after the 60 min with the 40 kHz probe (Figure **10.A**). The pigments release spectra from the other times of the treatment are available on Supplementary Information **5** and **6**.

The IOMR factor (Figure **11.B**) shows similar values on both conditions. These results show smaller differences between the two tested conditions. With the pulses applied the release of organic matter, after 20 min, is 4.28 ± 0.04. After the same time, without pulses, the IOMR factor is 5.16 ± 0.51.

The results from flow cytometry (Figure **11.C**) shows almost the same percentage of cell disruption for both conditions. The condition where the pulses were applied showed a cell disruption of 60.62 ± 9.78 %, while without the pulses a cell disruption was 61.05 ± 10.82 %.

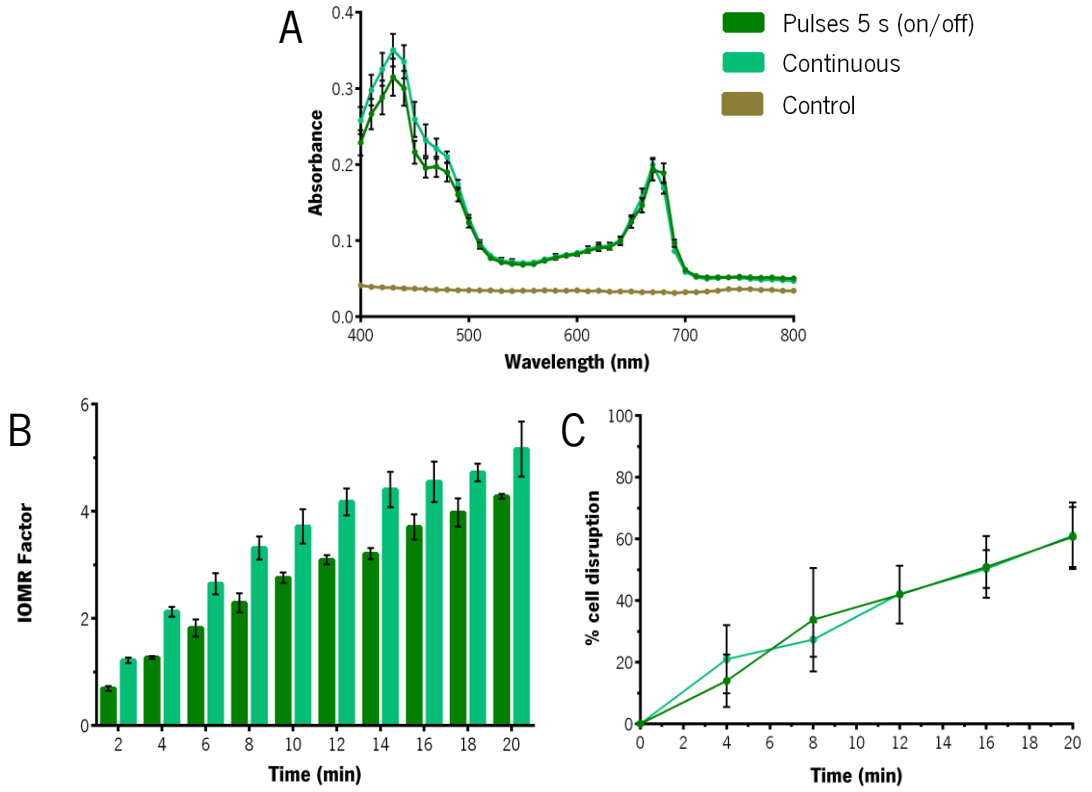

Figure 11. Results of pigments release (A), IOMR factor (B) and percentage of cell disruption (C) from US treatment after 20 min using a 40 kHz ultrasonic probe with and without 5 s pulses *on/off*.

3.3 Microwave

In the MW treatment was used a conventional microwave oven with a maximum output of 800 W. The cells went to the microwave in two experimental conditions, a continuous with different times and a non-continuous with interruptions for cooling down before return to the microwave oven. The results are shown on Figure **12**.

The pigments release spectra (Figure **12.A**) after the treatment on both conditions there was no release of photosynthetic pigments.

The results from IOMR factor (Figure **12.B**) shows similar values on the release of organic matter. In the experimental design were the cells were submitted to a continuous treatment the

maximum value achieve was 1.88 ± 0.02. In the non-continuous experimental condition, the IOMR factor was establish at 1.75 ± 0.19.

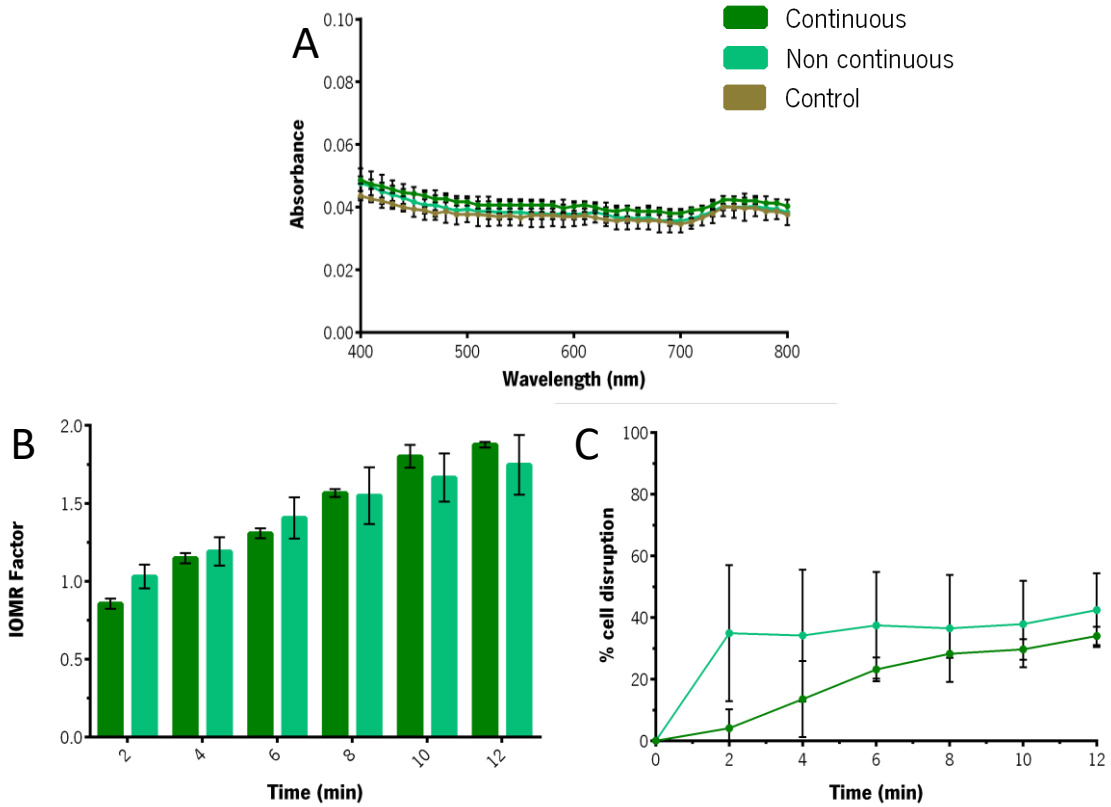

Figure 12. Results of pigments release (A), IOMR factor (B) and percentage of cell disruption (C) from MW treatment after 12 min in continuous and non-continuous operation.

The percentage of cell disruption (Figure **12.C**) it shows better results in the non-continuous treatment in the first minutes. After 2 min, the cellular disruption is 34.93 ± 22.06 %, when in the continuous treatment, the 2 min in the microwave oven has a percentage of cellular disruption of 4.10 ± 6.09 %. In both conditions, after 6 min of treatment both conditions started to present a constant linear increase in cellular disruption. In the end, the final disruption with this treatment was 34.03 ± 2.98 % for the continuous operation and 42.40 ± 11.93 % for the non-continuous.

3.4 High-speed homogenization

In the HSH treatment, two experiments were done, the initial one was with different rpm for 1 min, these results are shown on Table **6**. The best rpm is at 25000 rpm and higher IOMR factor and percentage of cell disruption, with 0.16 ± 0.02 and 30.11 ± 6.05 %, respectively. This condition did not show release of any photosynthetic pigments, as shown in Supplementary Information **7**.

Table 6. Results from HSH at different rpm for 1 min. Were tested four different rpm, as shown in the table and the respective results from IOMR factor and % of cell disruption

rpm	IOMR factor	% cell disruption
16000	0.059 ± 0.008	16.410 ± 2.900
18000	0.051 ± 0.027	15.394 ± 4.649
20000	0.080 ± 0.017	17.060 ± 4.668
25000	0.155 ± 0.024	30.113 ± 6.047

A second experiment, at 25000 rpm, was conduct in order to realize the time needed to assure a total cellular disruption. The treatment was conduct for 80 min and these results are shown on Figure **13**.

The graph **13.A** shows the pigments release spectra before and after 80 min of treatment. The highest release of photosynthetic pigments was after 80 min of treatment. The other tested times show less release of photosynthetic pigments, as shown in Supplementary Information **8**.

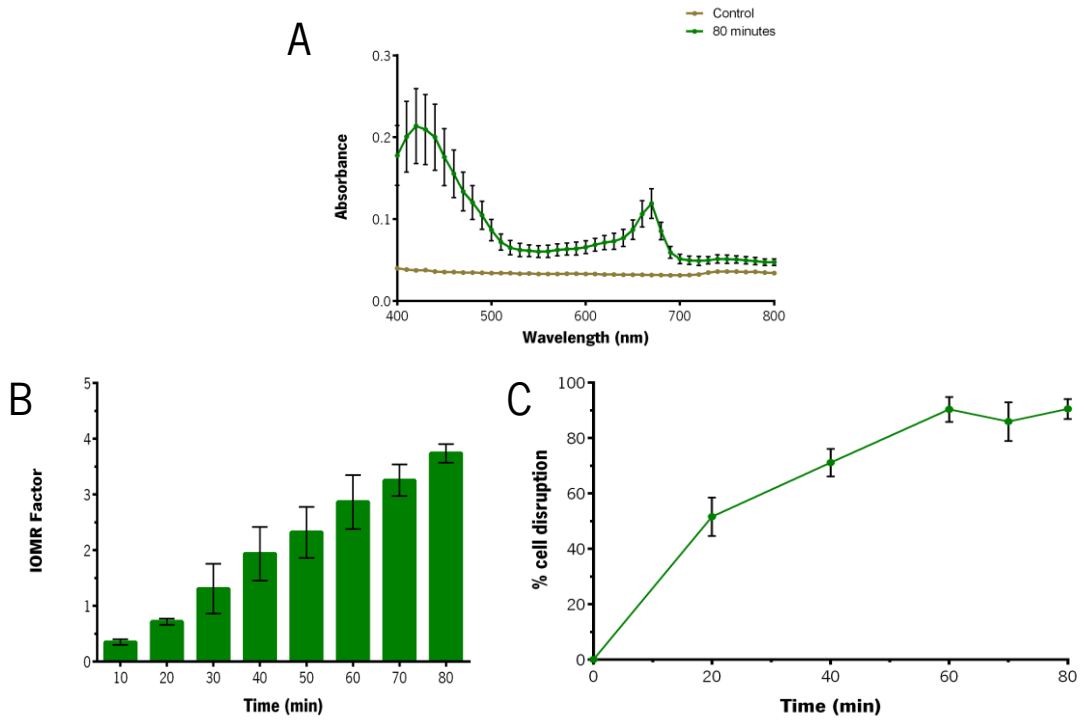

Figure 13. Results of pigments release spectra (A), IOMR factor (B) and percentage of cell disruption (C) from HSH treatment after 80 min at 25000 rpm.

In the graph **13.B** is shown the IOMR factor after the treatment, this shows a gradually release of organic compounds after each 10 min of treatment. After the 80 min of treatment was achieve the highest value of IOMR factor with this experimental condition, which was 3.74 ± 0.17.

The cell disruption efficiency was evaluated by flow cytometry, shown in graph **13.C**. After 60 min of treatment it is achieved a percentage of cellular disruption of 90.34 ± 4.51 %. At 70 and 80 min the cell disruption is 85.93 ± 6.95 % and 90.50 ± 3.58 %, respectively. These results indicate that after 60 min of treatment, this method can cause enough cellular damage to achieve the 90 % of cell disruption.

3.5 Bead-milling

In the BM treatment two different experiments were done, the first with two types of beads and different concentrations of those beads all the experiments were done for 7 min. The second experiment was done with the best bead concentration and size for different times until 10 min. The results from the concentration and diameter of the beads are shown on Figure **14**.

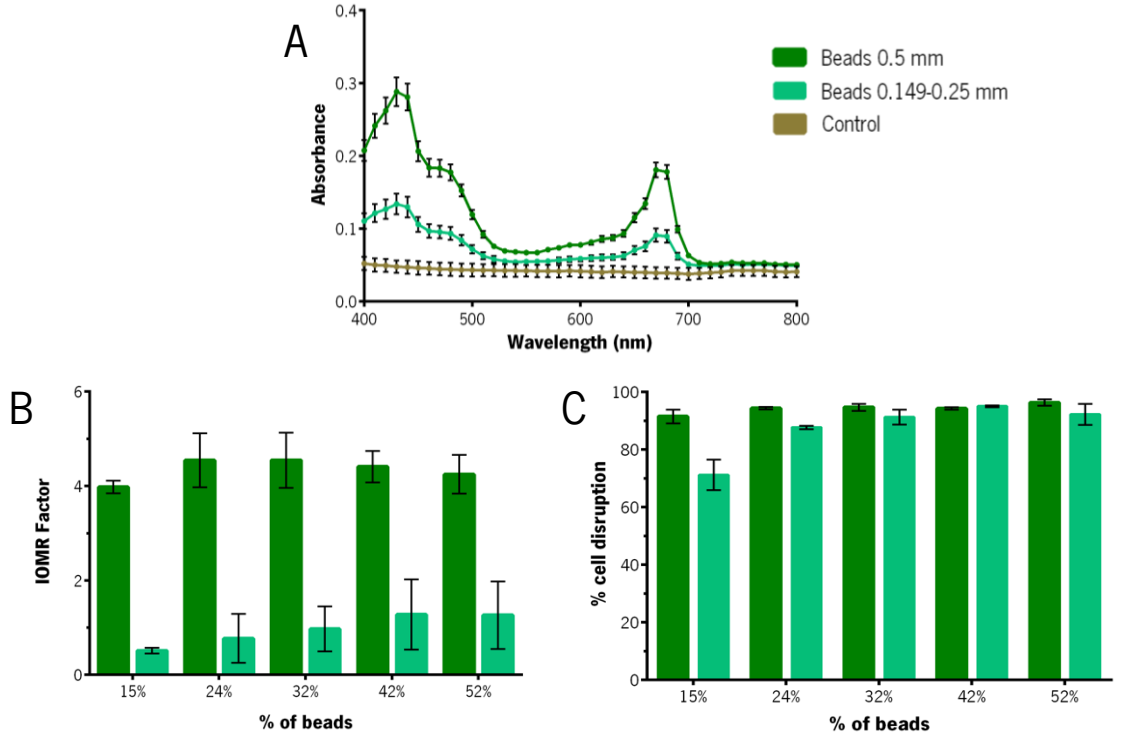

Figure 14. Results of pigments release spectra (A), IOMR factor (B) and percentage of cell disruption (C) from BM treatment with different beads at different concentrations.

The graph **14.A** shows the pigments release spectra after the cellular disruption, it is possible to see the difference in photosynthetic pigments release between the two types of beads used and from the control which did not suffer from the treatment. The higher pigments release was done with 0.5 mm diameter beads, at a concentration of 32 % of beads. With the 0.149-0.250 mm beads, there is a difference in pigments release when compared to the 0.5 mm beads. In the smaller beads, the concentration which released more pigments was 42 % of beads. The pigments

release spectra from the other tested concentrations are shown in Supplementary Information **9** and **10**.

In Figure **14.B**, it is possible to compare the IOMR between the two types of beads and at different concentrations used. The difference between beads is significant, with the 0.5 mm beads, all the beads concentration used presented similar IOMR factor. The release of organic compounds did not show significant differences with the increase of beads concentration. These results show that with a minimum of 24 % of beads in the chamber is already achieved a maximum in IOMR factor. The other beads concentration presented similar values when compared to the 24 %, this beads concentration shows an IOMR factor of 4.54 ± 0.57. In the case of the 0.149-0.250 mm beads different results were obtained, this bead diameter shows lower values of IOMR. With these beads, the IOMR factor was low with all the concentrations used, the values were all below 1.28 ± 0.75 which were obtain with 42 % of beads.

In Figure **14.C**, we have the data from the flow cytometry analysis, here it is possible to observe that both types of beads have high percentages of cell disruption independent of the concentration used. With the 0.5 mm diameter beads, we reach a percentage of cell disruption above 91 % in all the concentrations tested. With the smaller beads, the cellular disruption was not so efficient, to achieve a percentage of cell disruption above 90 %, is needed at least 32 % of beads to achieve a 91.31 ± 2.60 % of cellular disruption.

After tested the beads diameter and concentrations, were tested with the 0.5 mm at 32 % of beads in the chamber for different times until 10 min. The 32 % were chosen because it was the concentration that showed higher peaks in the pigment release spectra, as shown in Supplementary Information **9**.

In the pigments release spectra (Figure **15.A**), it is possible to see that all the times used show a similar spectrum to the previous one in Figure **14.A** for the same beads concentration at 32 %. From the analysis of the pigments release spectra, it is possible to see that the cells after 6 min of BM released the higher amount of photosynthetic pigments.

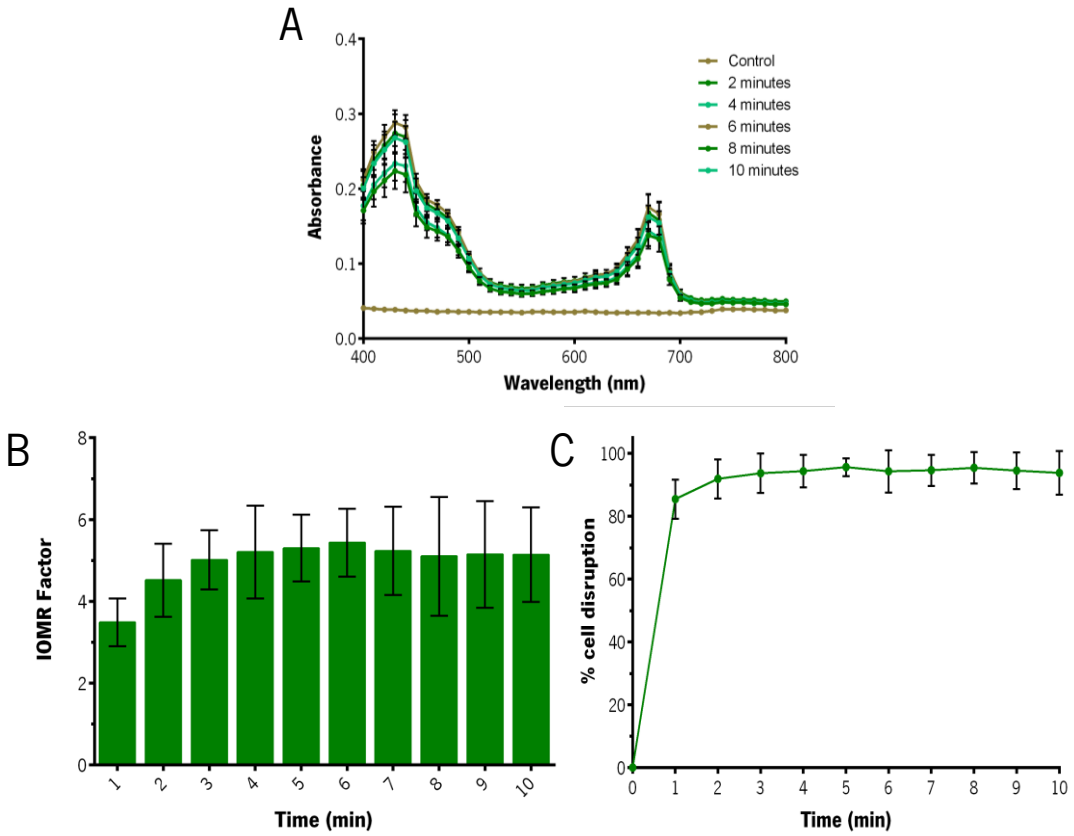

Figure 15. Results of pigments release spectra (A), IOMR factor (B) and percentage of cell disruption (C) from BM treatment with 0.5 mm beads at a concentration of 32%.

In Figure **15.B** it is shown the IOMR at different times. The highest pigments release was done at the 6 min of bead-milling, with an IOMR factor of 5.44 ± 0.83. All the other times showed values above 5.01 ± 0.73, except for 1 and 2 min. At these times, the release of intracellular organic compounds was 3.49 ± 0.58 and 4.52 ± 0.90, respectively.

The results from flow cytometry in Figure **15.C** show high values of cell disruption just at the initial times, which means that this method is very effective in causing cellular disruption on the microalgae cells. These results show that after 2 min of treatment the cellular disruption is already above 91.93 % ± 6.22, and there is no significantly difference in the percentage of cell disruption and in the IOMR factor after 2 min.

3.6 Quantification of metabolites

3.6.1 Quantification of carbohydrates

For the quantification of carbohydrates, a calibration curve with different glucose concentrations were done. The glucose stock solution was at 250 mg/L, and the calibration curve is shown on Supplementary Information **11**. The equation from the standard curve is:

$$\text{Abs} = 1.5 \times 10^{-3}[\text{glucose}] + 5.1 \times 10^{-3}$$

With this equation it is possible to calculate de total concentration of carbohydrates present in the different samples from the cell disruption treatment. The results are shown in Figure **16**.

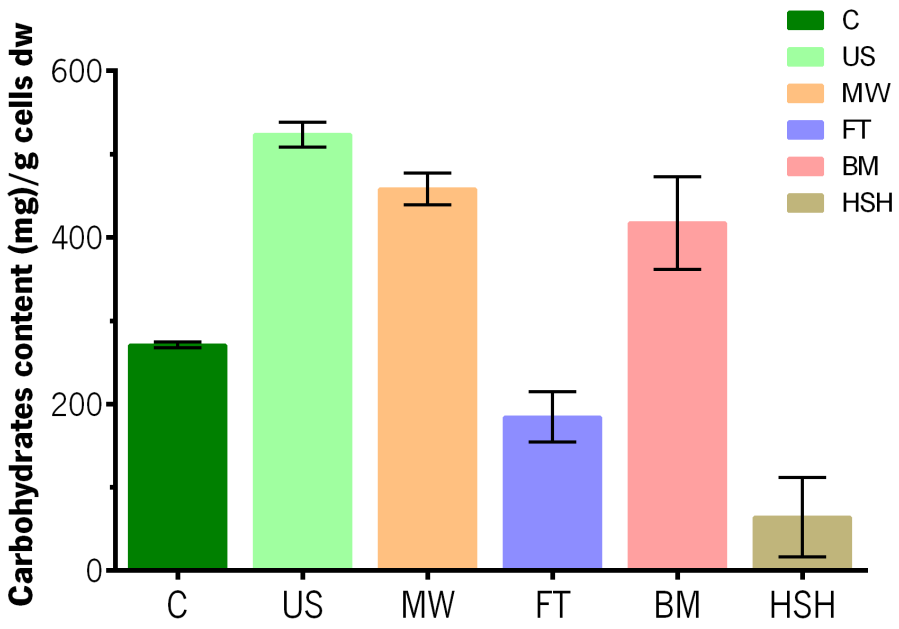

Figure 16. Total concentrations of carbohydrates extracted after each cell disruption treatment. The different conditions used were, control samples with chemical hydrolysis (C), US at 40 kHz for 60 min, MW for 12 min in continuous, 10 cycles of FT between -80 °C and 40 °C, BM with 0.5 mm beads at 32 % concentration for 6 min and HSH for 80 min at 25 000 rpm.

The results from carbohydrates quantification are presented in milligrams of carbohydrates per gram of cells dw. The US treatment released 523.23 ± 15.11 mg/g of cells dw, the MW treatment released 458.06 ± 19.14 mg/g of cells dw, the BM treatment released 417.13 ± 55.50 mg/g of cells dw. All these treatments presented a higher release of carbohydrates than the chemical hydrolysis with HCl used as control treatment, the control had a release of 270.98 ± 3.54 mg/g of cells dw. The treatment with FT and HSH had the lower release of the disrupter methods. The FT had a release of 184.46 ± 30.13 mg/g of cells dw and the HSH had a release of 64.16 ± 47.85 mg/g of cells dw.

3.6.2 Quantification of proteins

The protein quantification was done with the Lowry method, for this method was also done a calibration curve with a BSA stock solution at 100 mg/L. The calibration curve is available in Supplementary Information **12**. The equation from the standard curve is:

$$\text{Abs} = 2.50 \times 10^{-3}[\text{BSA}] + 2.14 \times 10^{-2}$$

With this equation, was possible to calculate the protein concentration of the different samples after each disruption treatment. The results are shown in Figure **17**.

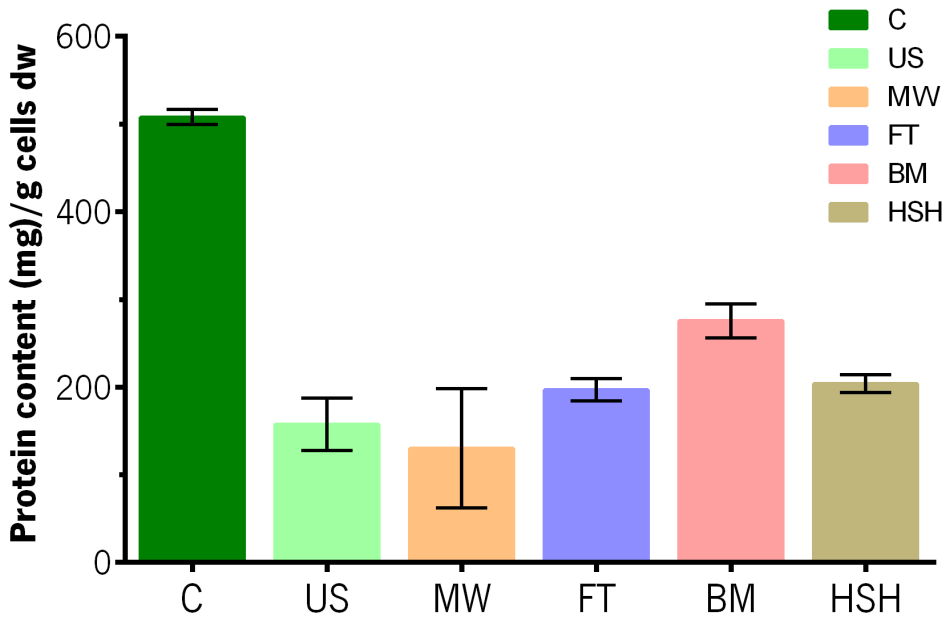

Figure 17. Total concentrations of proteins extracted after each cell disruption treatment. The different conditions used were, control samples with chemical hydrolysis (C), US at 40 kHz for 60 min, MW for 12 min in continuous, 10 cycles of FT between -80 °C and 40 °C, BM with 0.5 mm beads at 32 % concentration for 6 min and HSH for 80 min at 25 000 rpm.

The results from protein quantification are expressed in milligrams of protein per grams of cells dw. After the different treatments, the BM was the method which had the highest release of proteins, 275.49 ± 19.46 mg/g of cells dw, the HSH had a released of proteins of 203.91 ± 10.21 mg/g of cells dw, the FT had a release of 196.94 ± 12.55 mg/g of cells dw, the US treatment achieve a release of 157.44 ± 29.99 mg/g of cells dw and the MW was the treatment with the lower release of proteins, with 130.23 ± 68.16 mg/g of cells dw. The control sample, which was treated with NaOH for 10 min was a more effective way to extract proteins from the cells than the five disruption treatments used, with 507.86 ± 8.36 mg/g of cells dw.

Chapter IV – Discussion

After applying the different disruption treatments on microalgae cells, the results showed different disruption efficiencies and release of compounds. Some of the applied methods worked better in disrupting microalgae cells, while others seem not to have caused much damage to the cells.

In FT treatment, the cells were submitted to 6 cycles of freezing at two different temperatures, -20 °C and -80 °C, and thawing at 20 °C. The pigments release spectra did not show a release of any photosynthetic pigments from the cells (Figure **8.A**). After the treatment, a lower or none release of organic matter was observed, achieving a maximum value of IOMR factor of 0.14 ± 0.03 (Figure **8.B**). This value was obtained with a freezing temperature of -80 °C after 6 cycles. The percentage of cellular disruption after the treatment was low (Figure **8.C**). These results could indicate that these microalgae cells are capable of survive in environments where the temperatures are several degrees below zero. These cells are isolated from lichens, which are organisms capable of growing in extremophile environments like the Arctic and Antarctic (Kranner et al., 2014). So, both treatments with 6 cycles with two different freezing temperatures were ineffective with these microalgae cells. This could indicate that the formation of ice crystals inside the microalgae cells was not enough to damage the cell wall. Accordingly with Pestana and co-authors, freezing at -20 °C for 2 h is enough to form ice crystals on cell wall from cyanobacteria, leading to disruption, our results may indicate a presence of a more rigid cell wall in this microalgae (Pestana et al., 2014). The other experimental condition using the FT method was done in 10 cycles, instead of the 6 tested previously (Figure **9**). Besides the increase in number of cycles, the temperature of thawing was also increased, which now was 40 °C for exactly 2 h in a water bath. The best results in FT treatment were achieved with these conditions. The photosynthetic pigments released was not observed in this experimental condition (Figure **9.A**), with results that were similar to those obtained after 6 cycles. In these experimental conditions was observed a release of organic matter from the cells in both conditions (Figure **9.B**). In the condition were the cells were submitted to a freezing temperature of -80 °C, the IOMR factor reached a value of 1.44 ± 0.13, which is the highest IOMR factor value achieved with this treatment. The experimental condition with a freezing temperature of -20 °C, the IOMR only reach 1.02 ± 0.03. After 10 cycles, the percentage of cell disruption increased (Figure **9.C**). The condition were the cells were submitted to freezing temperatures of -20 °C the percentage of cell disruption was 32.26 ± 3.44 % and at -80 °C was 23.50 ± 14.85 %. This treatment presented a low efficiency on extracting carbohydrates and proteins from microalgae cells it was not effective for extraction carbohydrates, the other methods

were more efficient (Figure **16**). In the proteins extraction FT presented a better result when compared to the other methods applied, BM is the only method with a significantly better extraction of proteins (Figure **17**).

In the US treatments two probes were used: a 20 kHz and a 40 kHz ultrasonic probe. As mentioned before, the mechanism which leads to cell disruption by US is cavitation. The high acoustic waves will generate vacuum bubbles, which will propagate through the sample; when these bubbles find an obstacle they will burst. The obstacles are the microalgae cells, which will be damaged (Skorupskaite et al., 2019). The comparison of probes was done during 60 min of treatment (Figure **10**). The results show a more efficient cellular disruption with the 40 kHz ultrasonic probe, reaching 92.46 ± 1.45 %. The other probe used, the 20 kHz, only reached a cellular disruption of 58.27 ± 5.14 % (Figure **10.C**). The results with the 20 kHz ultrasonic probe are identical to results obtained by Skorupskaite and co-authors with *Chlorella vulgaris* (Skorupskaite et al., 2019). Skorupskaite et al obtain values of 52.26 % of cell disruption after 60 min of treatment at 20 kHz (Skorupskaite et al., 2019). These authors also report that in the first minutes of ultrasonication they were capable of breaking cells of *C.vulgaris*, but in order to achieve higher disruption efficiency it is necessary higher frequencies (Skorupskaite et al., 2019). The 40 kHz ultrasonic probe caused the best disruption on the microalgae cells isolated from lichens used in this work. Geada and co-authors used this frequency in cyanobacteria, *Microcystis aeruginosa*, and it was their best results on cellular disruption with US treatment (Geada et al., 2019). The 20 kHz ultrasonic probe was also tested for 180 min, and after this treatment the cellular disruption was only at 87.78 ± 1.68 %, which is lower than the results obtain for 60 min with the 40 kHz ultrasonic probe (Supplementary Information **13**). These two probes also show significant differences on the IOMR factor (Figure **10.B**), the 40 kHz ultrasonic probe does a stronger cavitation process to cause more cellular damage to release more intracellular organic matter. As described in literature, high frequencies and higher times of treatment will release higher concentrations of organic matter from the cells (Huang et al., 2016). The 20 kHz ultrasonic probe does a weaker cavitation process, which led to less release of organic matter after the 60 min of treatment. This treatment also shows release of photosynthetic pigments (Figure **10.A**), the pigments spectra shows small difference between the use of both probes. Although the 40 kHz ultrasonic probe present higher peaks between 400 and 500 nm, their photosynthetic pigments seem to be more degraded than the pigments detected in the same wavelength with the 20 kHz probe. The photosynthetic pigments are sensible to high temperatures, so with the heat generated

from the 40 kHz ultrasonic probe, it is possible that some of the pigments were damaged. This could explain why the 40 kHz probe shows only one peak between 400 and 500 nm while the 20 kHz probe shows two peaks in the same wavelength (Rahimi et al., 2017). The use of pulses during the treatment was also tested to understand if there is a less aggressive way to cause cellular disruption using the 40 kHz ultrasonic probe in less time of treatment (Figure **11**). The percentage of cellular disruption (Figure **11.C**) with and without the pulses were around the same value, 60.62 ± 9.78 % and 61.05 ± 10.82 % respectively. The use of the 5 s pulses *on/off* did not cause any significance difference when compared to the continuous US. The other results with this condition also present similar results between the use or non-use of the 5 s pulses *on/off* (Figure **11.A** and **11.B**). Using the 40 kHz probe with shorter exposure times seem to be less aggressive for the photosynthetic pigments, the spectra are more defined and the two peaks between 400 and 500 nm are easily observed (Supplementary Information **5** and **6**). The use of pulses caused a percentage of disruption of 60.62 ± 9.78 % after 20 min of treatment. This value was similar to the one obtained previously during the 60 min treatment, at 20 min the percentage of cell disruption was 66.54 ± 22.12 %. This treatment was very effective on the extraction of carbohydrates: with US it was possible to extract more carbohydrates then with the chemical hydrolysis used as positive control (Figure **16**). In the extraction of proteins, this treatment was not very efficient, presenting a lower value when compared to chemical hydrolysis and to other disruption treatments applied (Figure **17**).

The MW treatment was done only at 800 W, it was the higher capacity of the microwave oven used, other intensities were tested but did not present a significant cellular disruption efficiency, so they were immediately discarded. At a power of 800 W was possible to disrupt 32.03 ± 2.98 % of cells in the continuous operation for 12 min and 42.40 ± 11.93 % in the non-continuous for 12 min with cooling time after each minute (Figure **12.C**). McMillan et al. worked with *Nannochloropsis oculata* cells, they treated the cells with microwave. In their work they were capable to achieve a cellular disruption of 94.92 % ± 1.38 after 20 min of treatment with a conventional microwave oven capable of reaching 1025 W of power. The total mechanism of microwaves for cell disruption is not yet fully understood (McMillan et al., 2013). After the microwave treatment the release of photosynthetic pigments was not detected (Figure **12.A**), possibly implying that this method may be too aggressive for those pigments, because they are easily damaged by heat (the temperature of the sample after leaving the microwave oven achieved temperatures close to 90 °C) (Rahimi et al., 2017). The IOMR factor did not show a significantly

difference between both tested conditions (Figure **12.B**). The MW treatment is considered one of the best ways to extract compounds from microalgae, such as lipids for biofuel production, these results are achieved if it is used a non-polar solvent like hexane or others (Rakesh et al., 2015). In our experiments we only used distillated water as solvent, so the IOMR factor was below 2 in both conditions of MW treatment. Balasubramanian et al. in their work with microwaves on *Scenedesmus obliquus*, used hexane after treating the cells in a microwave processing system for a few seconds and a residence time in a water bath to maintain the temperature. They extracted 76 % to 77 % of total of recoverable oil from the cells, indicating that MW treatment is better if is used other types of solvent (Balasubramanian et al., 2011). Since both conditions with MW treatment present similar results, the simplest condition is to use *in continuo* because it does not require successive cooling times on ice. The MW treatment could extract high quantities of carbohydrates, it was one of the best treatments for this purpose (Figure **16**). While in protein extraction, MW did not seem to be an efficient way on these microalgae cells (Figure **17**).

The HSH treatment was used at different values of rpm for 1 min, in order to understand which value of rpm can cause more damage to these microalgae cells. These results are shown on Table **6** and the best results were obtained at the highest rpm, the cellular disruption efficiency being 30.11 ± 6.05 %. The IOMR factor was also the highest and it was 0.16 ± 0.03. Skorupskaite et al. described an increase of cellular disruption on *Ankistrodesmus fusiformis* cells with the increase of the rpm used, in these microalgae were observed an increase between the lower values of rpm and 25000 rpm (Skorupskaite et al., 2017). The cellular disruption treatment was then conducted for 80 min with the same device at 25000 rpm. The percentage of cellular disruption (Figure **13.C**) increased rapidly between the different times of treatment, and after the first 20 min it is already observed more than 50 % of cell rupture. After 1 h of treatment the percentage of cellular disruption was 90.34 ± 4.51 % (Figure **13.C**), and the cell disruption after 80 min was at 90.50 ± 3.58 %. Skorupskaite et al. also had a significant cell disruption on *Chlorella* sp. in the first 20 min of treatment at 24000 rpm, their cells reached a rupture of 43.5 % (Skorupskaite et al., 2019). Skorupskaite et al. also worked with *Ankistrodesmus fusiformis* cells at 24000 rpm. After 60 min of treatment, it was possible observe a cellular disruption around 90 % (Skorupskaite et al., 2017). The IOMR in this condition was 3.74 ± 0.17 after 80 min (Figure **13.B**). The pigments release show an increase in pigments peaks height during the several times of treatment applied (Supplementary Information **8**). These spectra also present one single peak between 400 and 500 nm, similar to those obtained with the 40 kHz probe. This may again result in some degradation

of pigments due to the heat generated by the device (Rahimi et al., 2017). The HSH was the least efficient treatment for extracting carbohydrates (Figure **16**), the total amount of carbohydrates was significantly lower when compared to the other methods. HSH is not a recommended method for extracting carbohydrates from the cells. The extraction of proteins with HSH was more efficient when compared to other methods; the only treatment with better results was BM (Figure **17**).

The fifth treatment used was BM with two types of beads, and with different beads concentration. With the 0.5 mm beads, it was possible to achieve near total cellular disruption, above 91 %, with all the beads concentrations tested (Figure **14.C**). Lee et al. described the 0.5 mm beads as the optimal size for microalgae disruption and Byreddy et al. with their work with *C. vulgaris*, define an optimal bead diameter between 0.3 and 0.7 mm for *C. vulgaris* cells (Byreddy et al., 2016; Lee et al., 2012). The 0.149-0.250 mm beads were less effective in disrupting these microalgae cells, only the three highest concentrations of beads used were capable of achieving similar results to the ones obtained with 0.5 mm beads. Doucha and Lívanský used 0.149-0.250 mm beads on *Chlorella* cells in a *Dyno-Mill KDL-Pilot A*, they worked on a large-scale operation and the disintegration of microalgae cells was less than 70 % with 82 % of beads in the total volume of the chamber (Doucha and Lívanský, 2008). The results obtained permits conclude that 0.5 mm beads were better to disrupt these cells, all the concentrations used with these beads had a high percentage of cellular disruption. By analyzing the photosynthetic pigments spectra (Figure **14.C**) it is observed that the bead concentration of 32 % seems to be the best in releasing pigments from the cells. This assumption is done because in the spectra, the highest pigments absorption peaks occur at this concentration (Supplementary Information **9**). The IOMR (Figure **14.B**) for the different beads concentration and size shows significant differences between the two beads diameter, but not significant between different concentrations with the same type of beads used. These results also show that although the 0.149-0.250 mm beads show similar cellular disruption with the 0.5 mm beads above the 32 % of beads, the IOMR factor was not identical. These could indicate that the 0.5 mm beads do more intracellular damage than only cell wall and membrane disruption. Some previous studies have proved that the total bead volume in the chamber has a crucial role in a better efficiency during cellular disruption, while the type of beads used it is also important in improving the efficiency of the treatment (Byreddy et al., 2016; Günerken et al., 2015). The last tested condition was different times of treatment with the 0.5 mm beads at 32 % of the total chamber volume (Figure **15**). In this condition, 2 min were enough to cause more than 91.93 ± 6.22 % of cellular disruption (Figure **15.C**), all the other times of treatment present higher cellular

disruption. With 1 min of treatment was achieve less than 90 % of disruption. In the IOMR results (Figure **15.B**), the 1 min treatment was also the lower value achieve with this condition, it was at 3.49 ± 0.90. The other tested times present IOMR factor above 4.52 ± 0.90, which is the correspond value to the 2 min of treatment. The 6 min treatment showed the highest peak from the photosynthetic pigments spectra (Figure **15.A**). The BM treatment was efficient on extracting carbohydrates (Figure **16**) and the extraction of proteins with this treatment is significantly higher when compared to the other four methods (Figure **17**).

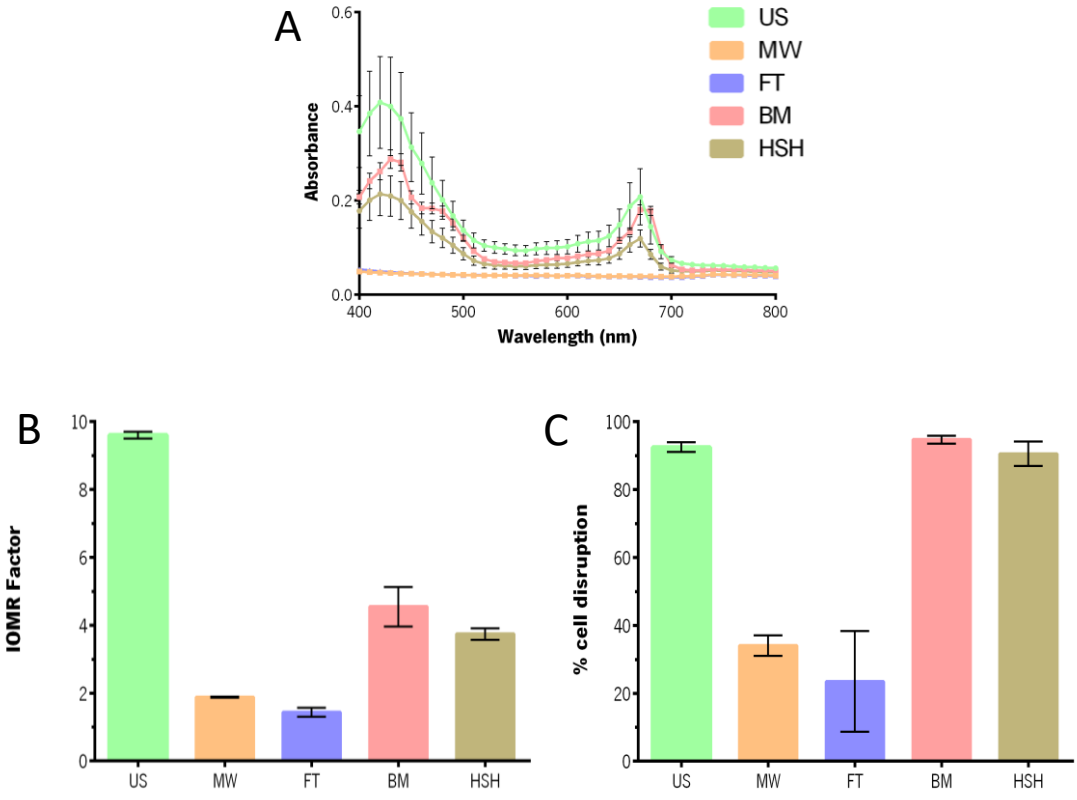

Figure 18. Comparison of photosynthetic pigments spectra (A), IOMR factor (B) and percentage of cellular disruption (C) between the best conditions of each disruption method. US at 40 kHz for 60 min, MW for 12 min in continuous, 10 cycles of FT between -80 °C and 40 °C, BM with 0.5 mm beads at 32 % concentration for 6 min and HSH for 80 min at 25000 rpm.

After all the five methods with the different conditions tested and analyzed, it is possible to conclude what is the best condition of each method based on pigments release, IOMR factor and percentage of cell rupture. The methods and conditions are present on Figure **18**, where the results are shown side by side. The condition which shows the best results in the three parameters analyzed was US. During this treatment the device produced some excessive heat, which could damage some bioactive compounds, this effect could be observed in the photosynthetic pigments release spectra (Figure **18.A**). This heat damage on photosynthetic pigments maybe observed with

the HSH; the percentage of cell disruption is also high and the spectra of pigments present a similar profile as the one in the US treatment (Rahimi et al., 2017). BM was also a method which showed to be efficient on the analyzed parameters. It reached a high percentage of cellular disruption (Figure **18.C**), and for the analysis of the pigments spectra, the photosynthetic pigments peaks are more defined, all of this with a significantly lower heat production when compared to US and HSH.

The quantification of primary metabolites was done in order to understand in a more precise way the real amount of IOMR quantified at 254 nm by spectroscopy. The absorbance at 254 nm will increase in the same proportion as the IOMR. This has a drawback in that does not quantify the type of intracellular compound, so more specific treatments are needed (Huang et al., 2016). Carbohydrates (Figure **16**) were quantified with the Dubois method; a difference can be observed in the amount of carbohydrates extracted after each disruption method. Generally, the carbohydrates content in microalgae are between 5 % to 30 % of its dw, these percentages vary between species (Blackburn and Volkman, 2012). Microalgae cells are known to have high photosynthetic efficiency, for this reason their carbohydrates content in some cases could be higher than 50 % of its dry matter, if their growth conditions are optimized (Yen et al., 2013; Ho et al., 2012). The different treatments used on microalgae cells, presented different efficiency in the release of carbohydrates. The higher amount of carbohydrates detected was after the US treatment: 53.2 % of the cells dry weight corresponded to the amount of carbohydrates released. The other two treatments with high content of carbohydrates were MW (where each gram of cell dw released 458 mg of carbohydrates) and BM (where each gram of cell dw released 417 mg of carbohydrates). These three disruption treatments were more effective than the chemical hydrolysis with HCl for 2 h which was used as control treatment. These results allowed concluding about the better efficiency of these treatments when compared to chemical treatments. The other two treatments (FT and HSH) showed lower efficiencies when considering carbohydrate extraction: 184 mg and 64 mg of carbohydrate extracted per gram of cell dw, respectively. In terms of proteins content (Figure **17**), microalgae are described to have a percentage of protein between 25 % to 40 % of their dry weight. Microalgae are rich in almost all of the essential amino acids and they have their advantageous in presence of different amino acids profile when compared to other conventional crops (Blackburn and Volkman, 2012; Becker, 2007). With the Lowry method, was possible to quantify the amount of proteins release after each disruption treatment, the most efficiency treatment on extract proteins was BM (where each gram of cells dw released 275 mg of proteins). The other treatment

presents a lower extraction of proteins, HSH, FT, US and MW for each gram of cells dw were extracted 203 mg, 196 mg, 157 mg and 130 mg of proteins. Contrarily to the quantification of carbohydrates, the chemical treatment with NaOH 1 M, used as control was more efficient on extracting proteins than the five mechanical methods studied.

Table 7 . Energy consumptions of the different treatments used on microalgae cell disruption. Values of power, time of usage and energy consumption of the different methods applied. The processing time is the usage of the device for each disruption, US requires 1 h, MW requires 12 min, FT requires 1 week (2 cycles per day), HSH requires 80 min and BM requires 6 min

Method	**US**	**MW**	**FT**		**HSH**	**BM**
Device used	*Sonics Vibra-cell Processor,* model VCX 500, 40 kHz probe	*Samsung MS23K3513 AW*	Deep freezer	Water bath	*IKA T18 digital ULTRA TURRAX*	*Reamix 2789*
Power of the device (W)	500	800	750	825	500	30
Operation time	60 min	12 min	1 week	4 h per day	80 min	6 min
Energy consumed per utilization (kWh)	0.5	0.16	90	16.5	0.67	0.003
Energy (kJ)	1800	576	324000	59400	2400	10.8

One of the main disadvantages of using these types of treatments is the high energy consumption of the different devices used. Table **7** shows the energy consumption, in kWh and in kJ, of the best experimental conditions of each method applied. The US treatment was the method which presented the best results on cellular disruption and extraction of compounds from the cells, but this treatment is time consuming. The highest percentage of cellular disruption was only achieved after 60 min of treatment. We also observed heat production during the treatment, and this heat may be responsible for damages in some of the extracted compounds. Almost all the methods applied were time consuming, which led to more energy supply or heat production during the process (Table **7**). BM is the treatment with less energy consumed and it was the simplest treatment, only requiring 6 min to achieve the highest percentage of cellular disruption. FT is the longest treatment and presents more energy consumption, because it demands a constant use of equipment to maintain the sample frozen. These energy consumption values correspond to one utilization to obtain a given extract. Despite of these differences in energy consumption of the

different cellular disruption methods applied, another aspect which can be considered is the sample volume of each method can operate. These methods were applied in a laboratory scale process, and the energy consumption was significantly variable between different treatments. For large scale processes this consumption of energy must be considered and the relation between energy consumption and production of the extracts must be viable.

Chapter V – Final remarks and future perspectives

As final remarks of this work, it is possible to conclude from the five treatments used that FT was the least efficient for rupturing microalgae isolated from lichens. This was the longest treatment of all, it required a full week and constant energy supply on the deep freezer and four hours a day in the water bath. Lack of efficiency of this treatment may be associated with the fact that these microalgae cells are isolated from a lichen, which is an organism capable of resisting to high variations of temperature. This treatment was not the ideal way to extract carbohydrates and proteins from these microalgae cells because it presents low efficiencies. The US is the treatment with best results in all of the analyzed parameters; however, the heat generated during the process of disruption makes this a less preferred treatment when compared to others also tested during this work, as high temperatures may damage some of photosynthetic pigments extracted from the cells. This was also the best treatment for extraction of carbohydrates from microalgae cells with the highest quantity of extracted carbohydrates. The MW treatment was another of the less effective treatments when considering the disruption of microalgae cells, but it was efficient in extracting carbohydrates from them. MW became one of the less preferable treatments because the excessive heat production during the process could cause several damages on compounds present in cells during the treatment. The HSH is also a time consuming treatment and capable of generating heat that could damage some metabolites. Despite of this drawback, this treatment achieved 50 % of cellular disruption in the first 20 min. In terms of extraction of primary metabolites, when compared to other methods HSH only presents good efficiency in extracting proteins. The last treatment applied was BM, this treatment could overcome all the drawbacks from the other four methods applied. BM could achieve high percentage of cellular disruption above 91 % in short periods of time and low energy consumption. This treatment did not present relevant heat production like the other treatments, so the extracted metabolites were not affected by heat even after the disruption. The BM conditions which allowed achieving the best results were: 0.5 mm beads at a concentration of 32 % of the total volume of the treatment chamber, treatment time between 2 to 10 min (best treatment time: 6 min). This method was also efficient in the extraction of carbohydrates and proteins from microalgae cells.

As future work for the extracted metabolites a more precise quantification (e.g. using high-performance liquid chromatography) would be interesting. The spectroscopy used for quantification of extracted metabolites is not as precise as HPLC, so these results could be improved, allowing a precise identification of the extracted bioactive compounds. The extraction of bioactive compounds could be optimized in the different treatments by using different types of solvents. A wide variety of

solvents can be used to assist the extraction of intracellular organic matter from microalgae cells. The use of non-polar and ionic solvents could improve the extraction efficiencies of some of the interest bioactive compounds.

In short, more work should be done in order to optimize some of the treatments used. The treatment with ultrasonic waves could be improved in order to maintain its good efficiency and extraction of compounds, but with less generated heat and lesser times of treatment. The use of pulses *on/off,* seem to cause cellular damage and they seem to maintain a better integrity of intracellular compounds and reduce the heat generated. US can also be combine with some beads, which can increase the disruption (Lee et al., 2012).

Also, the application of pulsed electric fields (PEF) should be considered; this method uses external electric fields to induce variation in the cell wall electric potential, and these variations will in turn induce the formation of pores across the cell wall and membrane (Günerken et al., 2015). It will be interesting to work with this type of treatment because the pulses will induce the formation of pores in the cell wall and membrane, which will be capable of efficiently releasing at least some intracellular compounds (Carullo et al., 2018). Another method that could show some interesting results is ohmic heating; this method consists in a faster and uniform way of heating a sample through the application of an electric field. Because of that, ohmic heating will also induce an electroporation process on cells, similar to that of PEF treatment. Besides this electroporation process, the temperature and variation of solvents could increase the extraction efficiency (Yodsuwan et al., 2018).

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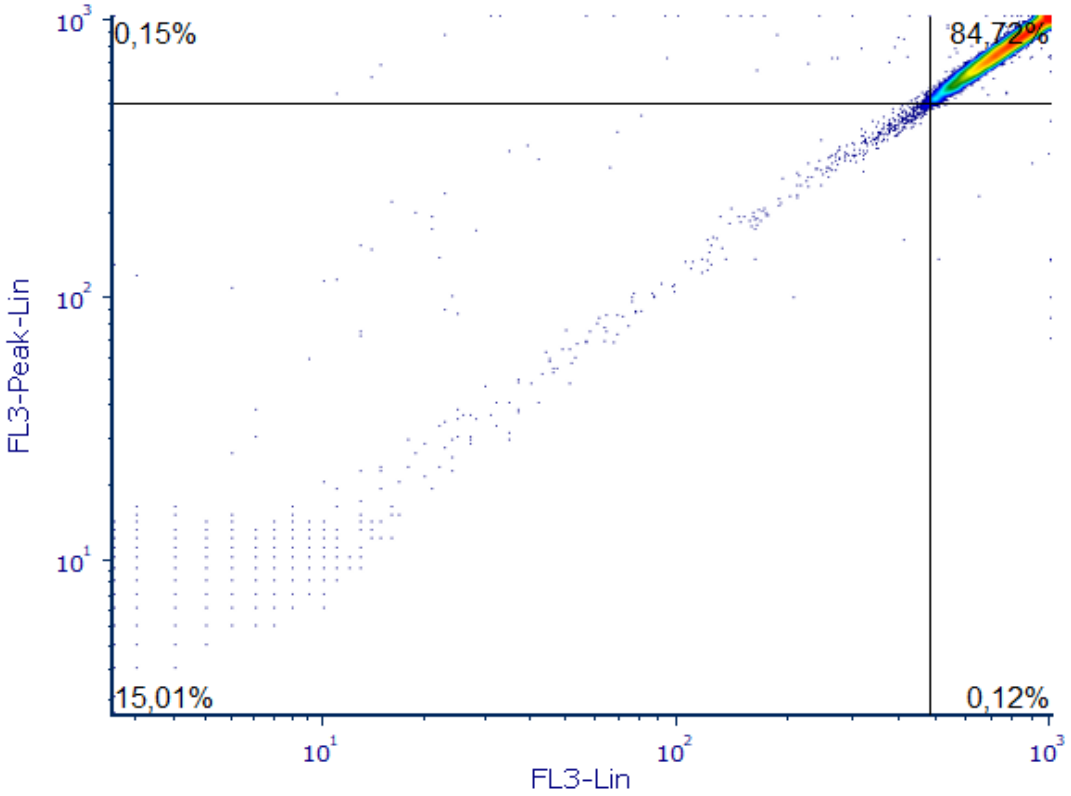
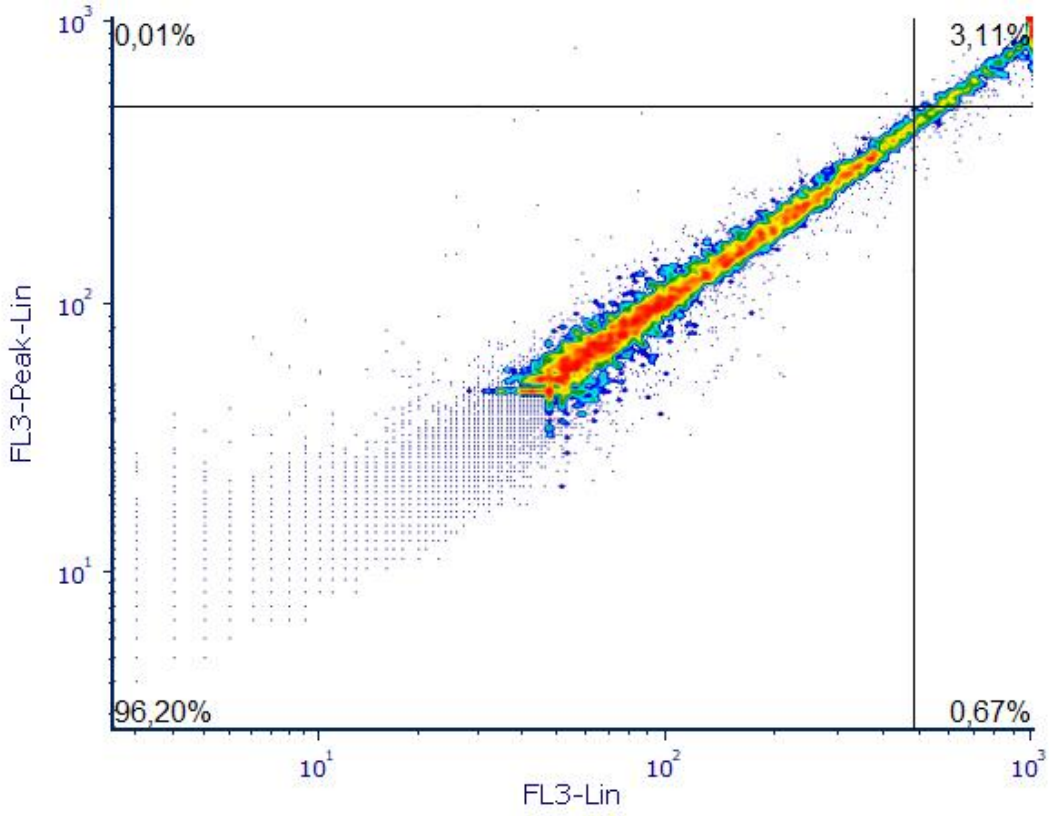
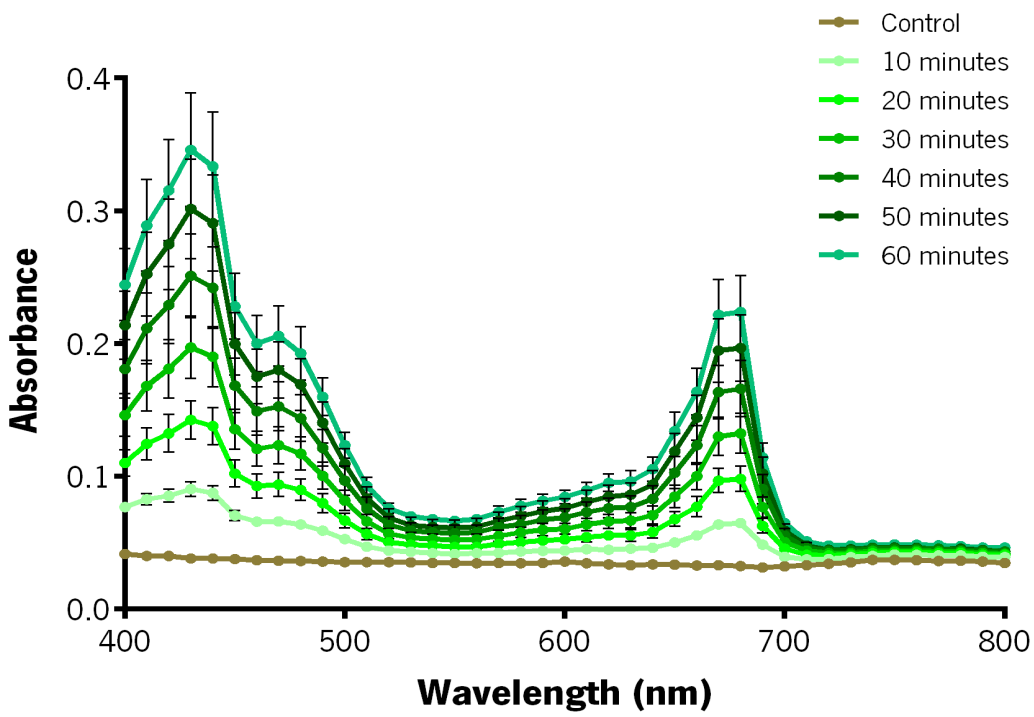
Supplementary Information



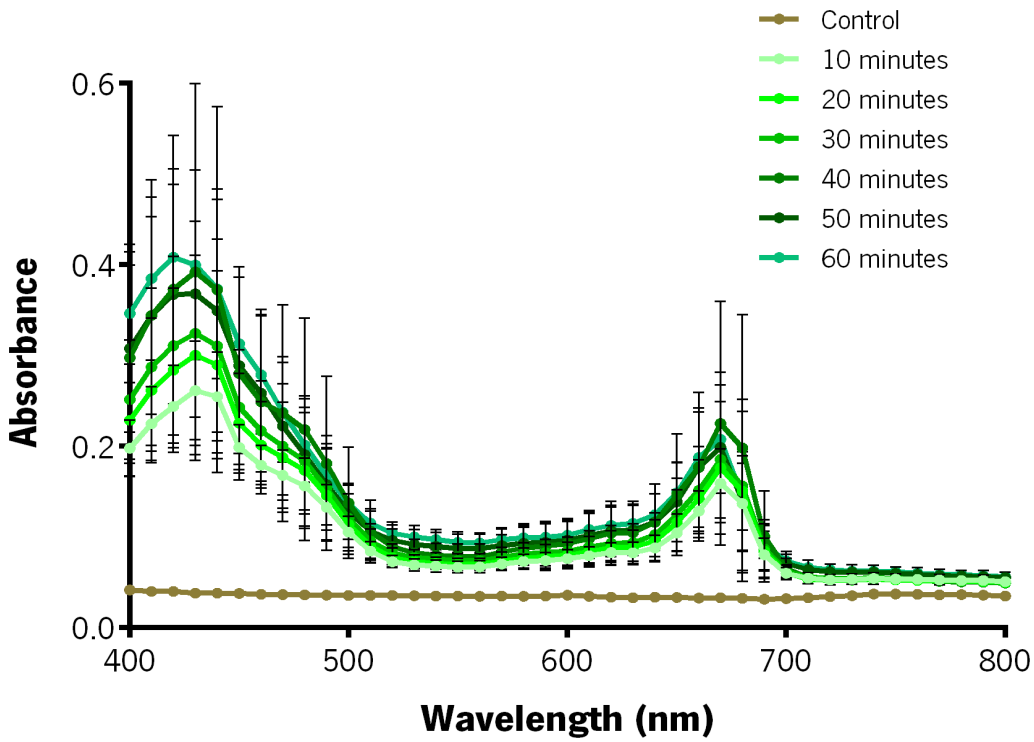
Supplementary Information 1. Graph generated by flow cytometer of a control sample of microalgae with the defined protocol.



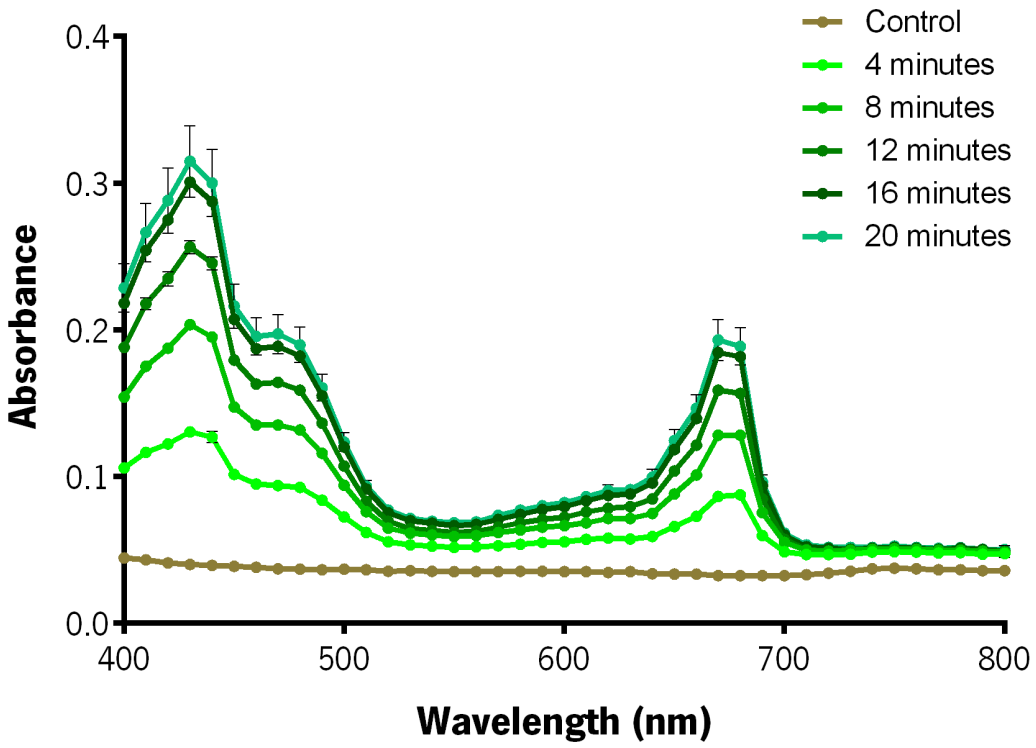
Supplementary Information 2. Graph generated by flow cytometer of a treated sample of microalgae with the defined protocol.



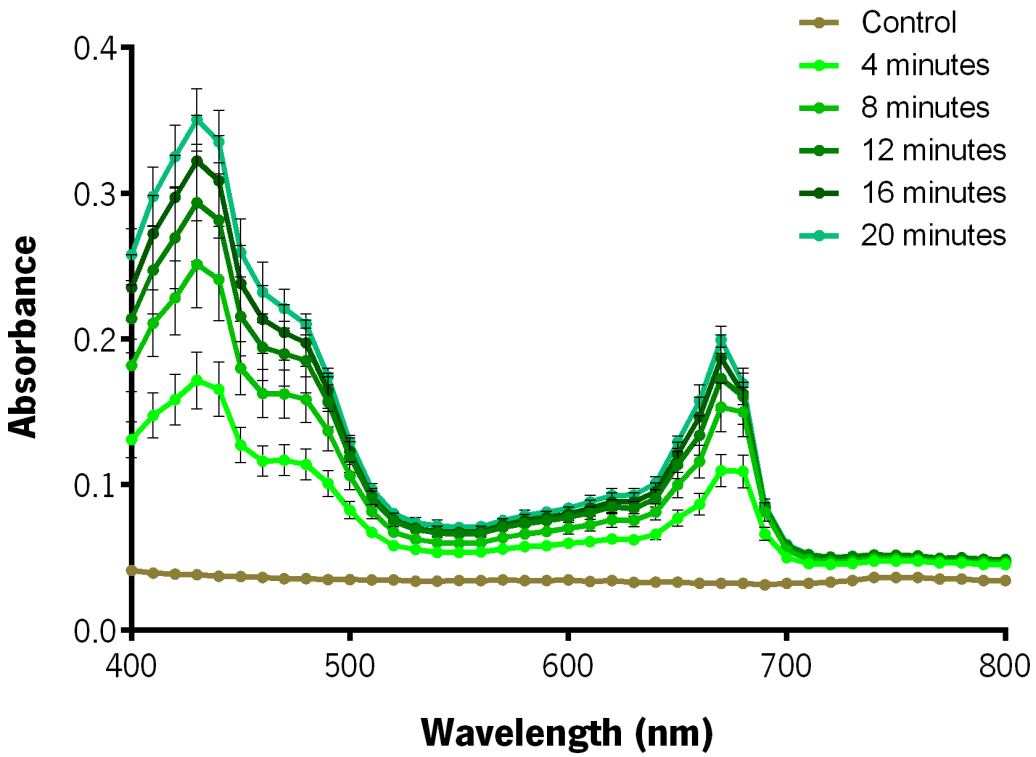
Supplementary Information 3. Pigments release spectra from ultrasonication with the 20kHz ultrasonic probe for 60 min.



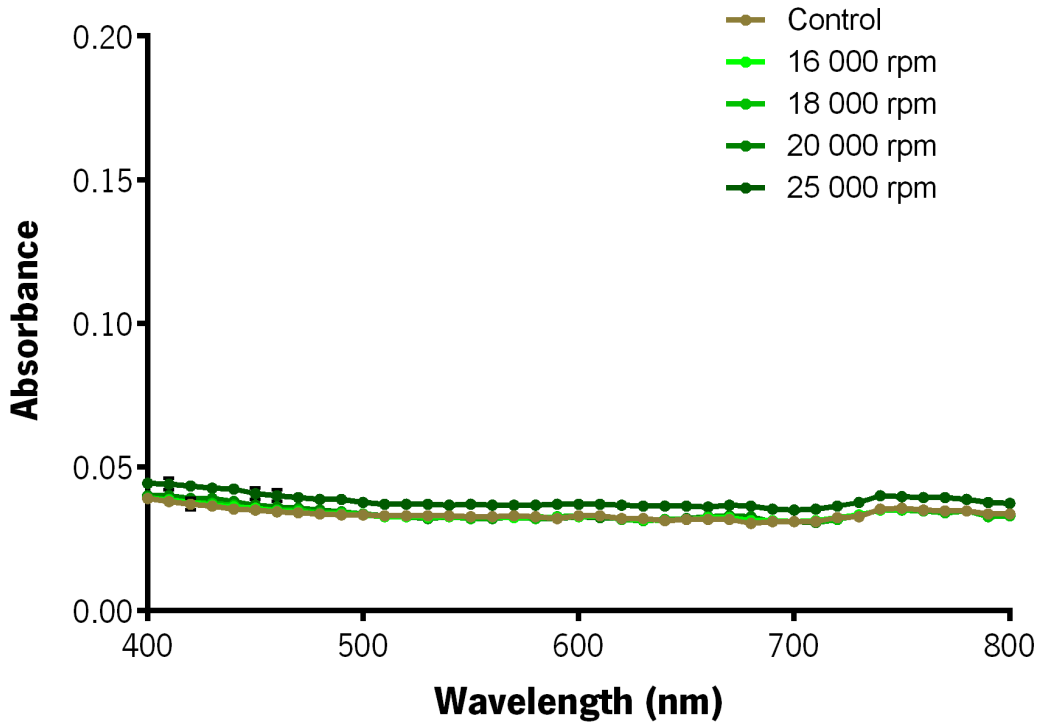
Supplementary Information 4. Pigments release spectra from ultrasonication with the 40kHz ultrasonic probe for 60 min.



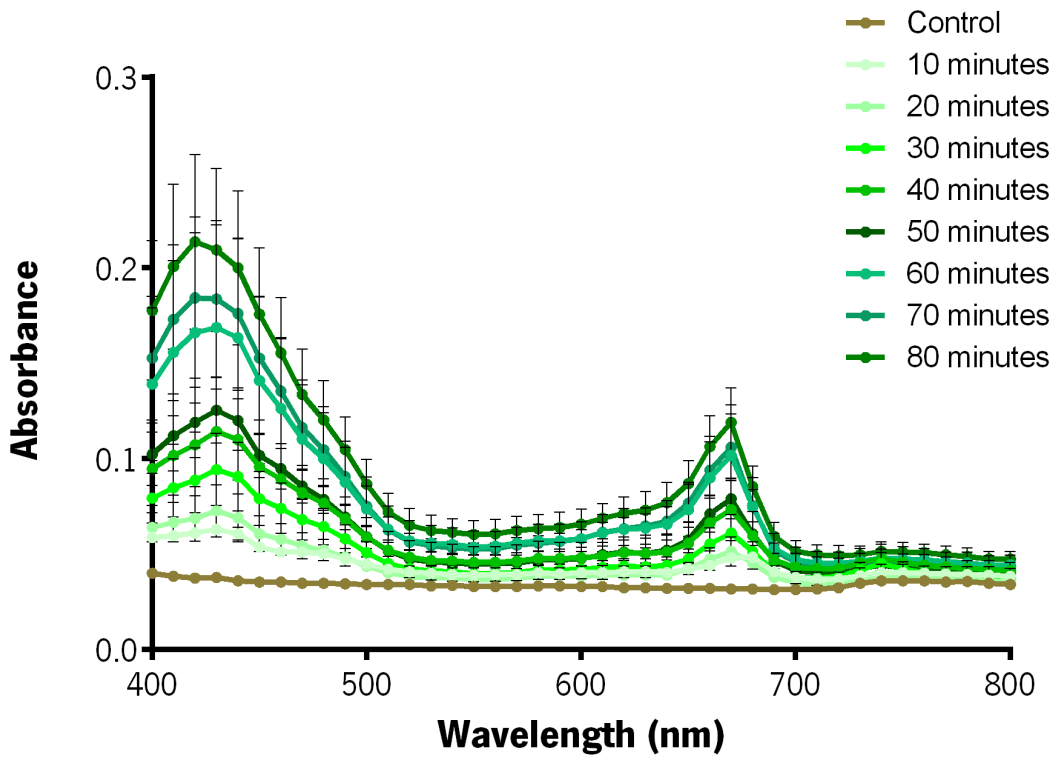
Supplementary Information 5. Pigments release spectra from ultrasonication with the 40kHz ultrasonic probe for 20 minutes with 5 seconds pulses on/off.



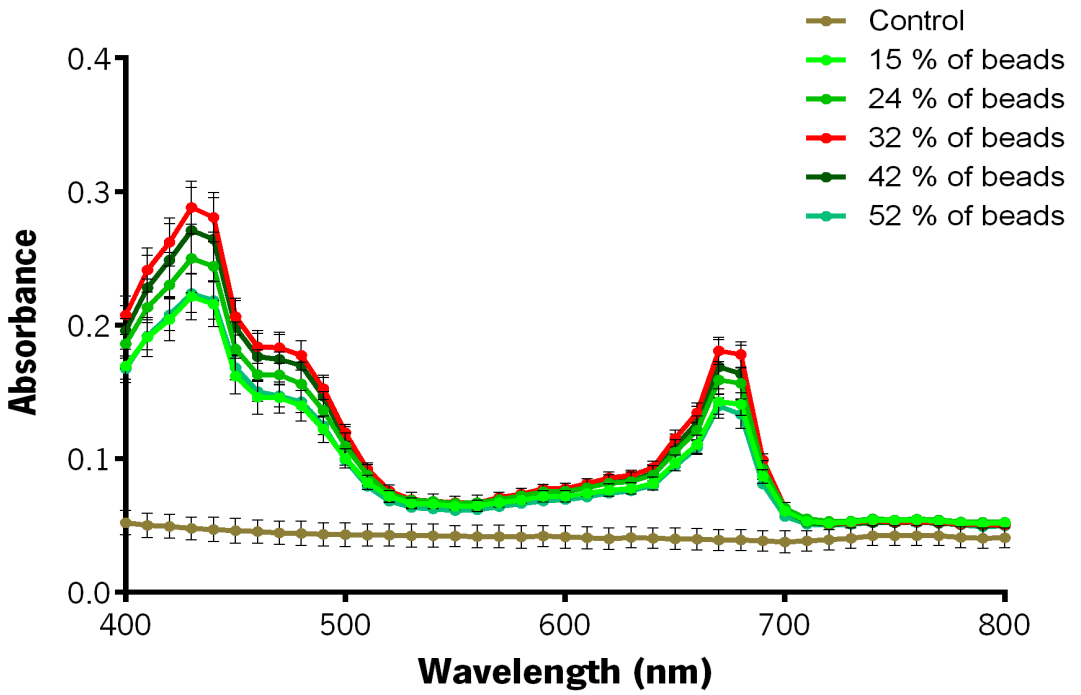
Supplementary Information 6. Pigments release spectra from ultrasonication with the 40kHz ultrasonic probe for 20 min.



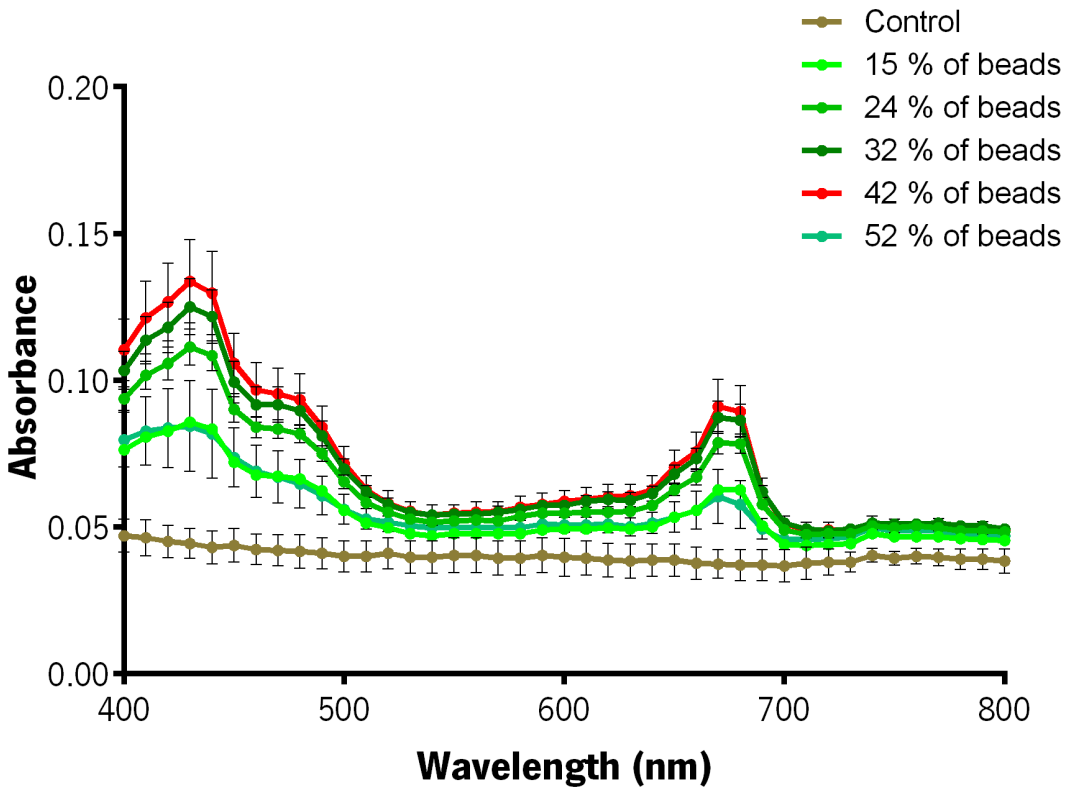
Supplementary Information 7. Pigments release spectra from high-speed homogenization at 16000, 18000, 20000 and 25000 rpm for 1 min.



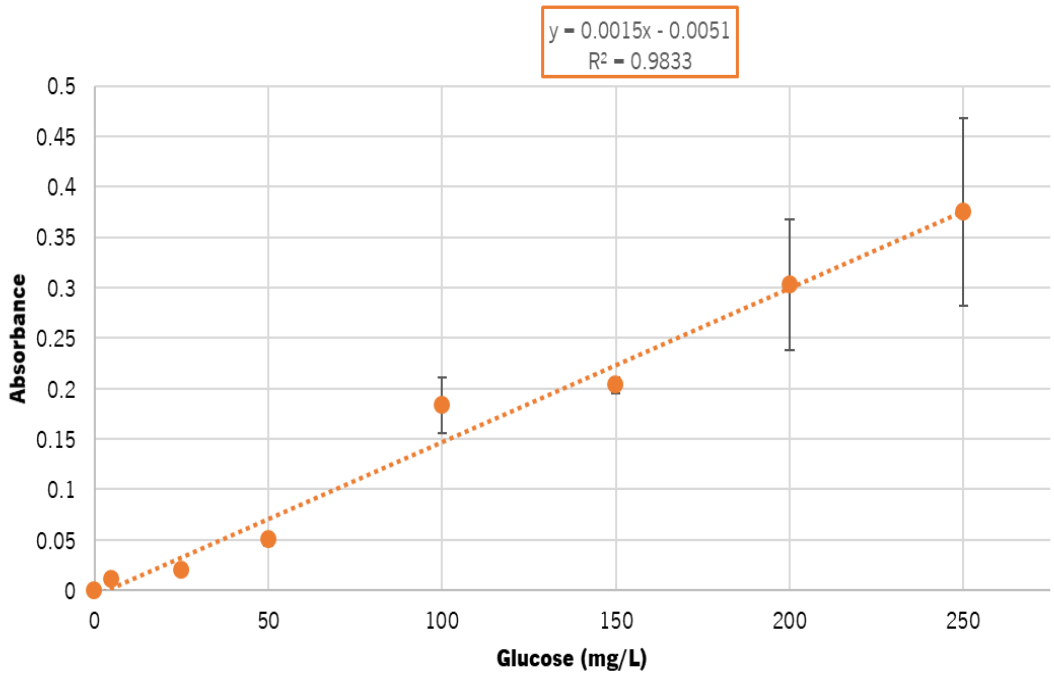
Supplementary Information 8. Pigments release spectra from high-speed homogenization at 25000 rpm for 80 min.



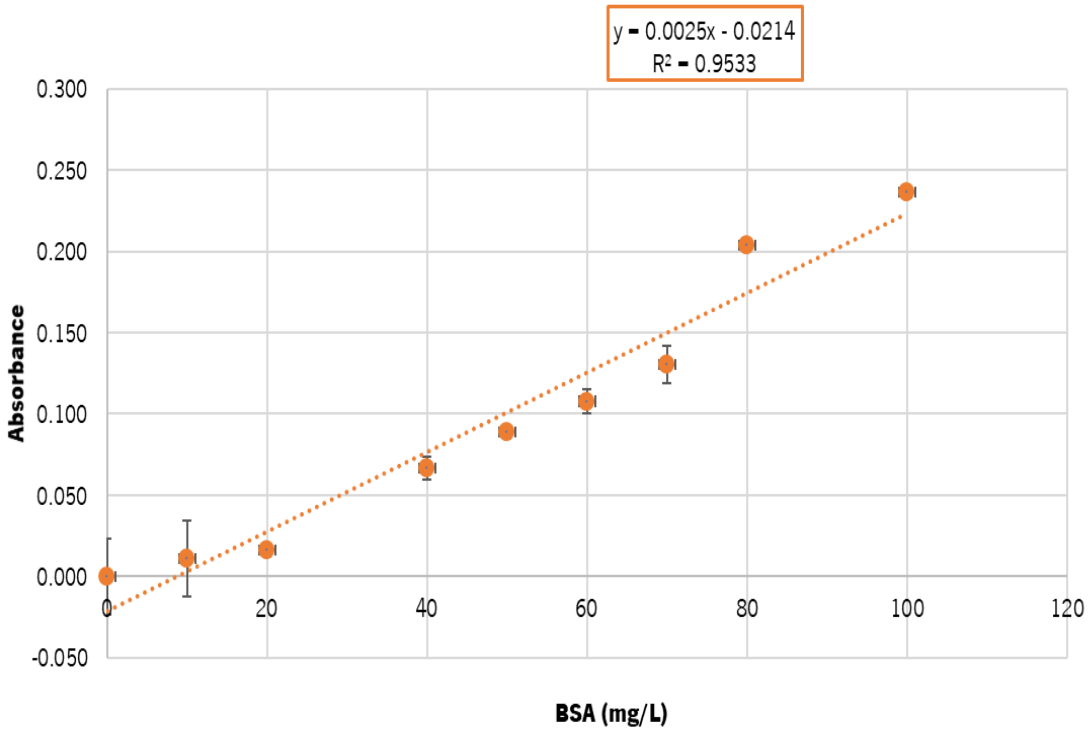
Supplementary Information 9. Pigments release spectra from bead-milling with 0.5 mm beads and with different beads concentration.



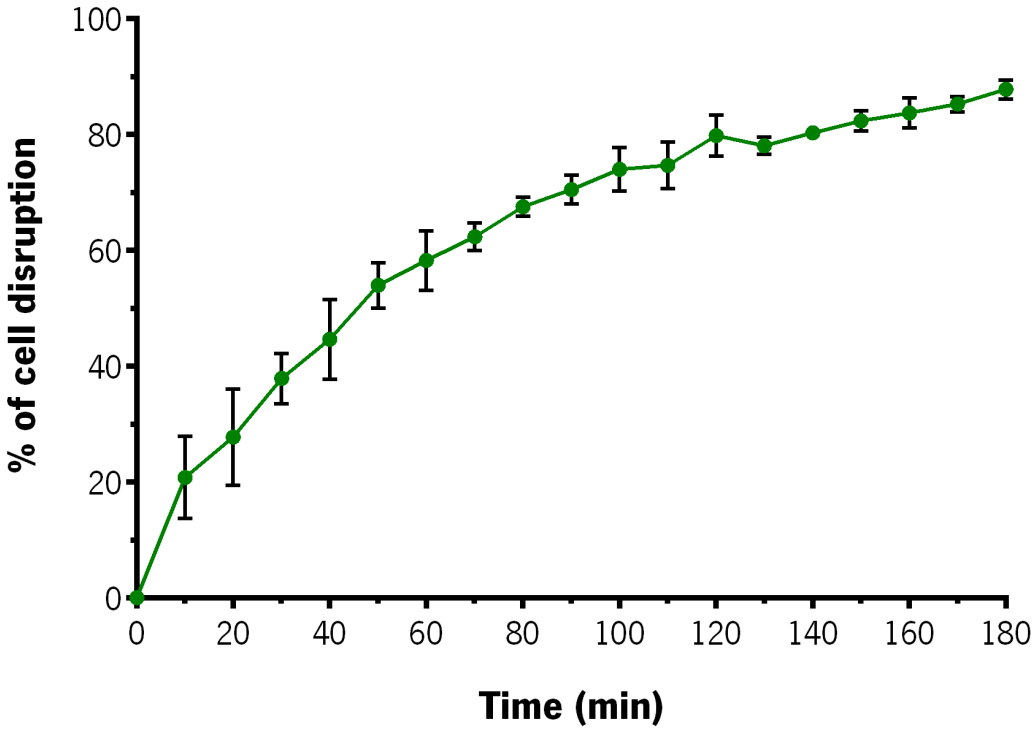
Supplementary Information 10. Pigments release spectra from bead-milling with 0.149-0.250 mm beads and with different beads concentration.



Supplementary Information 11. Carbohydrates calibration curve using different concentrations of a glucose stock solution of 250 mg/L..



Supplementary Information 12. Proteins calibration curve using different concentrations of a BSA stock solution of 100 mg/L..



Supplementary Information 13. Percentage of cellular disruption with the 20 kHz ultrasonic probe for 180 min of treatment.