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Content analysis of microalgae *Scenedesmus sp*. UCV strain harvested at different stages of growth.

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Lina Tomás Mochón

Abstract

The demand for renewable energy has driven interest in microalgae as a promising resource. However, commercial production is hampered by two bottlenecks, biomass harvesting and extraction of high-value compounds. This study focuses on the self-flocculating strain *Scenedesmus sp.* UCV, which addresses the first bottleneck. The cell content of this strain from different samples obtained or not, by a two-step culture method was characterized and an extraction protocol was optimized by comparing two cell lysis techniques: acid hydrolysis (AH) and sonication bath (SB), targeting the bottleneck of compound extraction. The results revealed that SB showed superior efficiency in the extraction of carbohydrates, chlorophyll a and b, while no significant differences were observed for proteins, lipids, polyphenols, and carotenoids. Both methods successfully extracted chlorophyll a and b pigments but, were less effective for carotenoids. The strain showed relatively low carbohydrate and protein contents, reaching up to 21.04 ± 5.79 % and 14.59 ± 10.44 %, respectively. However, it showed high lipid storage capacity and antioxidant potential, with lipid content up to 34.06 ± 19.36 % and polyphenol content up to 6.05 ± 4.56 %. Interestingly, samples subjected to two-stage culture did not show the expected increase in lipid and carotenoid content. This was probably because the lysis methods were not as effective due to cell wall thickening with culture under stress. However, further investigations on the cell wall would be necessary to facilitate its breakdown and provide valuable clues for the selection of strains for specific applications.

Keywords: Microalgae; Two-stage cultivation; High-value compounds; *Scenedesmus sp.;* acid Hydrolysis; sonication bath; self-flocculating.

Resumen

La demanda de energías renovables ha impulsado el interés por las microalgas como recurso prometedor. Sin embargo, la producción comercial está obstaculizada por dos cuellos de botella, la recolección de biomasa y la extracción de compuestos de alto valor. Este estudio se centra en la cepa *Scenedesmus sp.* UCV con capacidad de autofloculación, que aborda el primer cuello de botella. Se caracterizó el contenido celular de esta cepa de diferentes muestras obtenidas o no mediante un método de cultivo en dos etapas y se optimizó un protocolo de extracción comparando dos técnicas de lisis celular: hidrólisis ácida (AH) y baño de sonicación (SB), dirigidas al cuello de botella de la extracción de compuestos. Los resultados revelaron que el SB mostró una eficacia superior en la extracción de carbohidratos, clorofila a y b, mientras que no se observaron diferencias significativas en proteínas, lípidos, polifenoles y carotenoides. Ambos métodos extrajeron con éxito los pigmentos de clorofila a y b, pero resultaron menos eficaces para los carotenoides. La cepa mostró un contenido relativamente bajo en carbohidratos y proteínas, alcanzando hasta un 21,04 ± 5,79 % y un 14,59 ± 10,44 %, respectivamente. Sin embargo, mostró una alta capacidad de almacenamiento de lípidos y potencial antioxidante, con un contenido en lípidos de hasta el 34,06 ± 19,36 % y en polifenoles de hasta el 6,05 ± 4,56 %. Curiosamente, las muestras sometidas al cultivo en dos etapas no mostraron el aumento esperado en el contenido de lípidos y carotenoides. Esto se debió probablemente a que los métodos de lisis no fueron tan eficaces debido al engrosamiento de la pared celular en los cultivos sometidos a estrés. Sin embargo, serían necesarias más investigaciones sobre la pared celular para facilitar su ruptura y proporcionar pistas valiosas para la selección de cepas para aplicaciones específicas.

Palabras clave: Microalgas; Cultivo en dos etapas; Compuestos de alto valor; *Scenedesmus sp*.; Hidrólisis ácida; Baño de sonicación; Autofloculación.

1. Introduction

In recent years, demand for fossil fuel resources, as well as carbon emissions, have increased exponentially (Liu et al., 2022). The problem with this feedstock is its finite nature, therefore, it is necessary to find renewable energies that contribute to the development of a circular economy, ensuring a sustainable future, and also capable of addressing environmental and economic issues related to climate change, energy security and oil prices (Filote et al., 2021). Occupying more than 70% of the Earth's surface and with a depth of more than 11,000 meters, the marine environment is a source of potential raw material, harboring more than one billion species of microorganisms and one million macroscopic species representing approximately half of the world's biodiversity (Nguyen et al., 2022). Marine biorefineries use these renewable and living aquatic resources to achieve the necessary sustainability parameters and drive the establishment of this circular bioeconomy (Veríssimo et al., 2021). In fact, more than 1,000 marine compounds are discovered every year and, to date, about 30,000 bioactive compounds of marine origin have been described (Nguyen et al., 2022). In the quest of changing a fossil-based economy for a bioeconomy, algae have gained more and more attention (Filote et al., 2021). In fact, third-generation biofuels derived from microalgae and macroalgae offer several advantages over first- and second-generation biofuels, which are produced from lignocellulosic biomass such as crops or agricultural residues. Third-generation biofuels, therefore, provide a better alternative because the production of biofuels does not compete with the production of food for human consumption (Patle et al., 2020).

Algae can be broadly classified into Rhodophyta (red algae), Phaeophyta (brown algae) and Chlorophyta (green algae). And they can also be classified according to their size, into macroalgae or microalgae. Macroalgae correspond to marine algae, and are large multicellular algae, visible to the naked eye, while on the other hand, microalgae are individual microscopic cells and can be prokaryotic, as cyanobacteria (Chloroxybacteria), or eukaryotic, as green algae (Chlorophyta) (Khan et al., 2018). During the COVID-19 pandemic in 2020, the global microalgae market was estimated at USD (United States dollar) 3.4 billion and is projected to reach USD 4.6 billion by the year 2027 growing at a CAGR (compound annual growth rate) of 4.3% (Markets, 2020). This is due to the increasing awareness of the valuable functional compounds possessed by microalgae and the benefits they can provide in terms of sustainability compared to current methods of food or energy production (Markets, 2021). The

production of algae and microalgae has multiple applications, apart from being an alternative source of fossil fuels, such as wastewater treatment, bioremediation, and CO_2 biofixation to significantly reduce pollution and greenhouse gas emissions (Nguyen et al., 2022). Specifically, microalgae are considered fundamental for the development of sustainable processes that contribute to the global bioeconomy (Özçimen et al., 2018) for three main reasons: first, they have a rapid growth that doubles in less than a day, in fact, the growth rate of microalgae is 5 to 10 times higher than that of conventional food crops, which is one of the most important advantages of their use as a source of biomass (Khoo et al., 2020). Second, they can achieve high biomass productivities of more than 100 t/ha (tonnes per hectare) per year (in dry weight), giving microalgae higher productivity compared to macroalgae and terrestrial plants (Nguyen et al., 2022). And thirdly, they mainly use sunlight as an energy source, with efficiencies of up to 10 % (Özçimen et al., 2018). In addition, this unicellular microorganism is essential in the ecosystem, as they represent the primary product of the aquatic food chain, thus providing food for a great variety of marine creatures (Show, 2022). The contribution of microalgae has supplied approximately half of the earth's atmospheric oxygen (Khoo et al., 2020). Moreover, this photosynthetic organism possesses remarkable adaptability to diverse environments. It can thrive in various aquatic habitats, including lakes, rivers, oceans, and even sewage systems. Microalgae demonstrate tolerance to a wide range of temperatures, salinity levels, and pH values. They can also withstand different light intensities and adapt to conditions found in reservoirs or desert regions. Furthermore, microalgae have the ability to grow independently or form symbiotic relationships with other organisms (Khan et al., 2018). Besides, microalgae have a higher proportional amount of valuable cell compositions such as proteins, lipids, polysaccharides, antioxidants, and pigments as compared to agriculturally grown biomass such as edible food crops and lignocellulosic biomass (Sankaran et al., 2020). Oleaginous microalgae (as *Ankystrodesmus sp., Chlorella sp. and Scenedesmus sp.)* are considered a feedstock for biofuel production as they can accumulate 30–70% lipids per unit dry cell weight (Sun et al., 2018). Microalgae have also been introduced as a source of biofertilizer processing, biochemicals, and biochar for wastewater treatment (Khoo et al., 2021). It has many uses and can suit a wide range of industries, making it a highly valuable resource (Figure 1) (Show, 2022). Nevertheless, the cultivation of microalgae for the commercial production of bioactive compounds and biofuels is hampered by economic unfeasibility due to high production cost and low product yield, in fact, currently, the main products obtained from microalgae that are produced at industrial level, are only carotenoids and algal biomass, which are mainly used for human food, animal feed and aquaculture

(Martínez-Francés & Escudero-Oñate, 2018). This is also the reason why commercial cultivation of microalgae is limited to a few species that are capable of synthesizing lucrative compounds whose market value offsets the cost of production, or to robust strains capable of growing in low-cost cultivation systems with minimal risk of contamination (Liyanaarachchi et al., 2021). Approximately fifteen microalgae species have already been utilized as raw materials and are currently being produced at a commercial scale. This number is expected to grow in the near future due to the extensive research being conducted on these photosynthetic microorganisms (Rocha et al., 2019). Among the microalgae species exploited for commercial purposes is the genus *Scenedesmus* (Chlorophyta). This genus exhibits significant potential for biofuel production owing to its rapid growth rate, high lipid biosynthesis capabilities, resistance to microbial contamination and predators. These characteristics make *Scenedesmus* well-suited for cultivation in open tanks (Rocha et al., 2019). The genus *Scenedesmus* is composed of about 120 species and special attention has been paid to *Scenedesmus obliquus* (Ji et al., 2018).

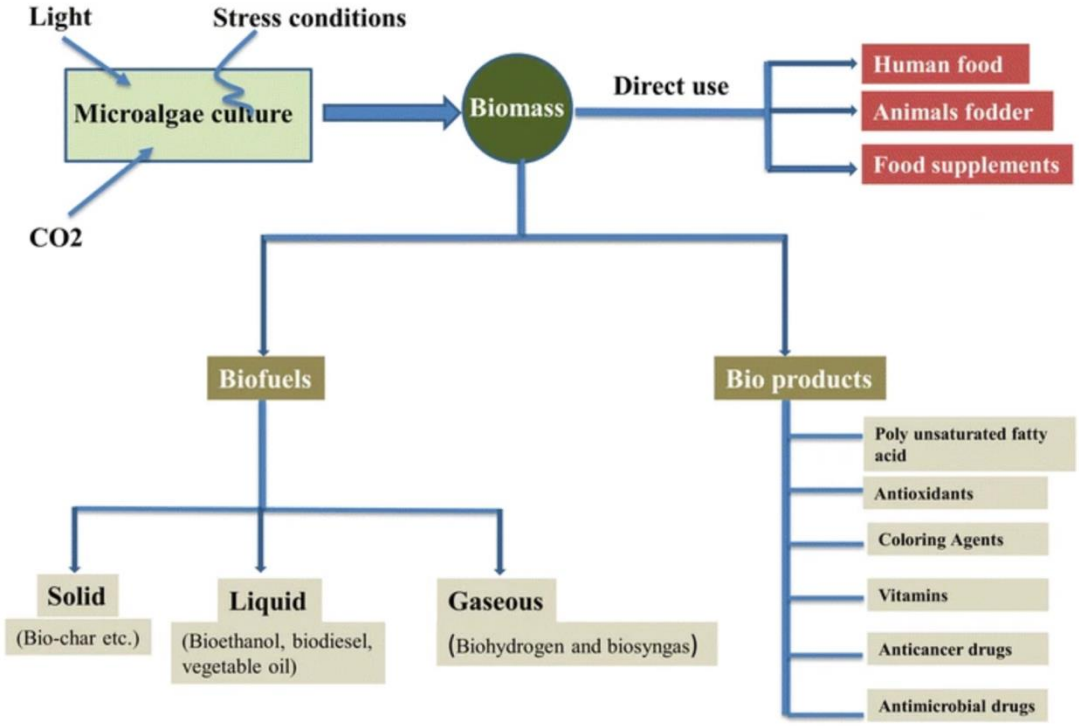

Figure 1: Overview of the development of potential bioactive metabolites from microalgae, and their optimization to generate sustainable and renewable industry for biofuels, animal feed and other products (Khan et al., 2018).

Microalgae growing in adverse conditions are more likely to produce secondary metabolites. So far, different factors have been analyzed in order to evaluate whether they improve the carotenoid or target metabolites production performance of algae. Some of these factors are culture medium (nutrient deficit), higher temperature, intense illumination, oxidative stress (H_2O_2) and salt stress (KCl and NaCl) (He at al., 2022).

Playing with these factors, the biochemical components of the microalgae vary (Figure 2), allowing to induce a higher production of the component of industrial interest. For example, Zhang et al. (2022) reported an effective method to increase protein content related to various metabolic pathways, such as photosynthesis, ascorbic acid, alginate metabolism and phenylpropane biosynthesis, is to inhibit the growth and photosynthetic activity of *Scenedesmus obliquus* by applying an 8mg/L dose of H_2O_2. This treatment induces oxidative stress in the microalgae, causing a decrease in cell growth and photosynthetic activity, as well as alterations in chlorophyll fluorescence parameters. In response to oxidative stress, the activity of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) increases, which helps to mitigate the harmful effects. On the other hand, salinity stress has a detrimental effect on the oxygen evolution complex (OEC) and the photosystem II reaction center (PSII), leading to altered electron transport processes on both the donor and acceptor sides of the reaction center. This altered electron transport negatively affects the uptake, transfer, and utilization of light energy by microalgae. As a result, the overall photosynthetic efficiency and performance of microalgae are compromised under salinity stress conditions (Anand et al., 2021). With increasing NaCl concentration, biomass production is compromised, but the content of chlorophyll a, b and carotenoids increases. However above 20 g/L NaCl concentrations the production of these pigments decreases considerably (Elloumi et al., 2020). NaCl treatment also increases the total lipid content of the cells. The highest lipid content (32.26 %) was found in cells grown in the presence of 0.20 M NaCl, approximately 2.52 times higher than that of cells grown in medium without NaCl (12.82 %) (Ji et al., 2018). Adjustments of culture media, therefore, might trigger the lipid biosynthesis and storage in microalgae cells. Indeed, effect of nitrogen (N), phosphorus (P) and sulfur (S) on the biosynthesis of lipids, amino acids, phospholipids, and photosynthetic enzymes have been evaluated over the last years (Ghafari et al., 2018). Microalgae are known to contain three main types of storage products: proteins, starch and lipids. In general, many microalgae use starch as their main carbon storage compound, whereas under nutrient deficient conditions, they shift photosynthetic carbon partitioning towards energy-rich storage compounds such as lipids. Possibly, this is because lipid and starch synthesis

pathways compete for common biosynthetic precursors. Briefly, under starvation conditions (nitrogen and phosphorus deficiency), electron transfer activity is down-regulated and ATP production decreases, resulting in an increase in the NADPH pool that favors the accumulation of the storage compound polyhydroxybutyrate (PHB) or polyhydroxybutyric acid, a product of carbon assimilation from glucose and starch, which serves as an energy store, and as a result, a reduction in the rate of protein synthesis is observed, in spite of, the synthesis of certain proteins necessary for the acclimation process increases with nutrient limitation (García et al., 2020). In addition, tricarboxylic acid (TCA) cycle metabolites decrease, and then acetyl-CoA is diverted to fatty acid biosynthesis under stress, resulting in an increase in stored lipids. Therefore, eliminating polysaccharide biosynthesis (e.g., starch) would be a feasible strategy to improve lipid production (Yang et al., 2018). Indeed, De Jaeger et al. (2014), in an attempt to increase triacylglyceride (TAG) content, productivity and yield, generated starch-free mutants of *S. obliquus* by UV mutagenesis. Other previous studies in *Chlamydomonas reinhardtii* have also shown that blocking starch synthesis results in higher TAG contents, although these TAG contents still do not exceed those of oleaginous microalgae. On the other hand, it has been observed that different forms of nitrogen (NaNO3, NH4Cl and CH4N2O), have different effects on the growth and lipid production of *S. obliquus*. Their growth increases with the addition of NaNO3 and CH4N2O, while it is inhibited with the addition of NH4Cl (An et al., 2020). However, NO3 addition produces chlorophyll pigments and amino acid accumulation, while NO3 limitation has very low amino acid content, but is very effective in increasing neutral lipid content (Figure 2) (Ma et al., 2018). On the other hand, phosphorus limitation in *Scenedesmus sp.* increases the cellular lipid content, but decreases its biomass production, and therefore, also the overall lipid production. Yang et al. (2018) observed that, after 48h of culture of *S. obliquus* under phosphorus-limiting conditions, the production of neutral lipids (approximately 80 % of total lipids) increases. While the proportion of phospholipids is recruited from 6.1 % to 4.3 % of total lipids, saturated fatty acids contents increased from 35.9 % to 38.7 %.

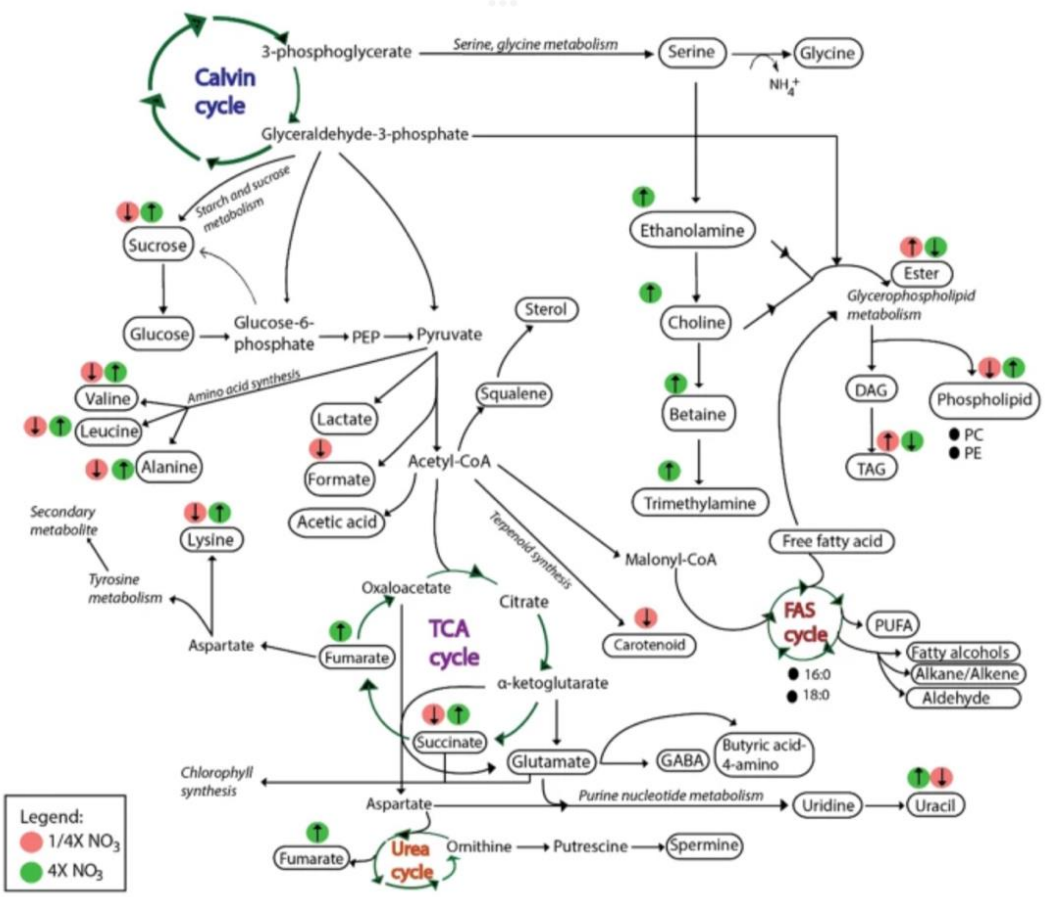

Figure 2: Metabolite regulatory pathways involved in *Scenedesmus regularis* under nitrate treatment. nitrate treatment. FAS: Fatty acid synthesis; PC: Phosphatidylcholine; PE: Phosphatidylethanolamine; PEP: Phosphoenulpyruvate; TCA: tricarboxylic cycle; TAG: triacylglyceride; DAG: diacylglyceride; GABA: γ-aminobutyric acid (Ma et al., 2018).

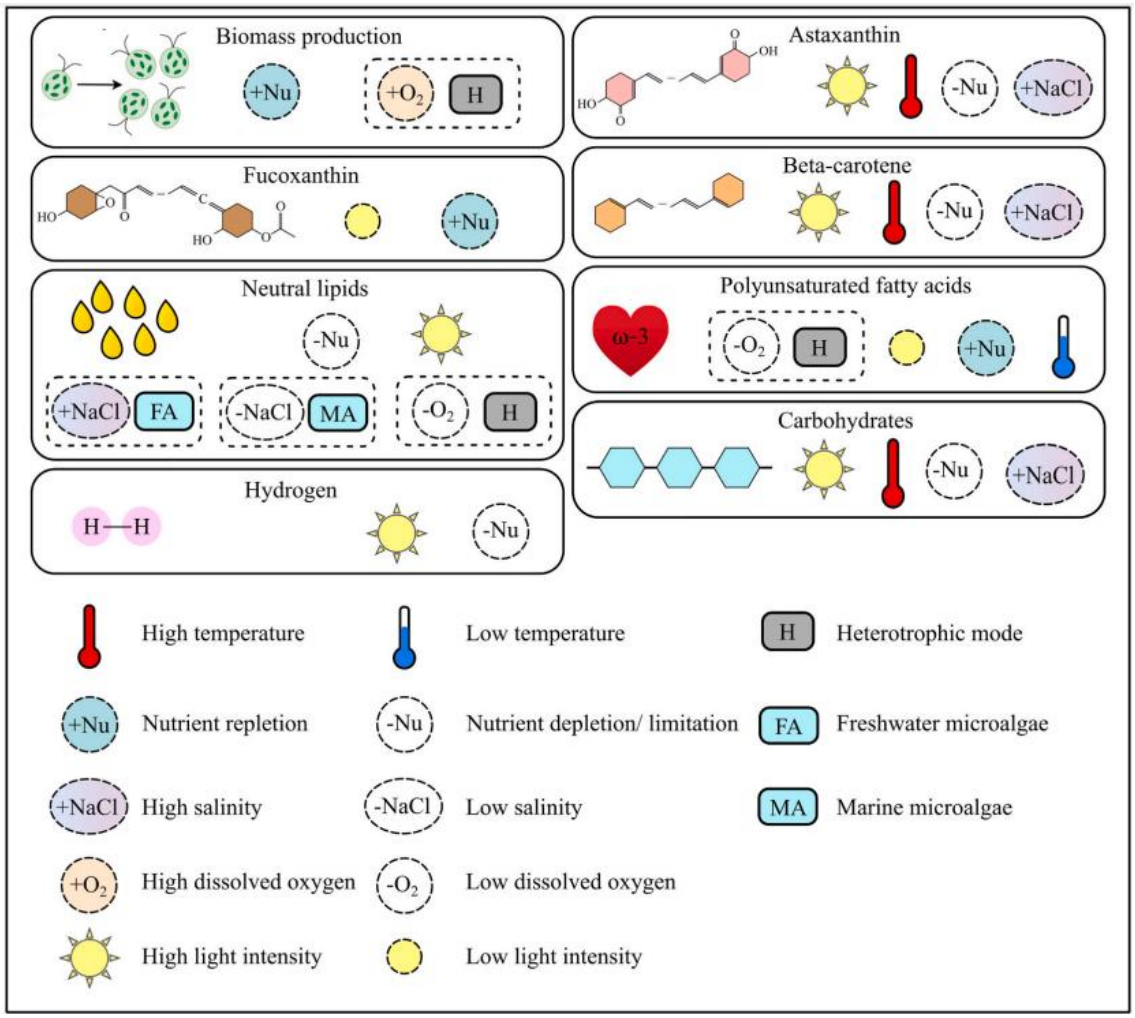

Figure 3: Physicochemical culture conditions used for microalgal biomass production and target metabolite biosynthesis. Depending on the conditions that are altered during cultivation, the production of the metabolites of interest shown at the top of the image will be induced (Liyanaarachchi et al., 2021).

The process of extracting microalgae biomolecules from biomass begins with the selection of the appropriate microalgae strain as well as the culture conditions. The main problem is that the growth conditions necessary to increase biomass productivity are detrimental to the accumulation of desired metabolites, and vice versa, the synthesis of desired metabolites, such as lipids, carotenoids, carbohydrates, etc., is often increased under stress conditions such as nutrient starvation, nutrient deprivation, extreme irradiation, high temperatures, etc., leading to inhibition or cessation of microalgal growth. Due to the opposing culture conditions necessary for rapid cell proliferation and accumulation of desired compounds, extensive studies have been conducted on two-stage culture strategies (Liyanaarachchi et al., 2021). These two-stage cultures consist of combining a series of factors that increase the production of microalgal biomass in the first stage and induce an increase in the production of the

metabolite of interest in the second stage, using the tools shown in Figure 3. For this, the change of culture from the first to the second stage is achieved by altering one or more of the physicochemicals factors used with the culture (nutrients, light intensity, salinity, temperature and pH), the growth factors (photoautotrophy, heterotrophy, mixotrophy), the mode of operation of the culture (batch, semi-continuous, fed-batch and continuous), and the different culture system (closed or open systems) (Figure 4). In addition, different combinations of these factors can be incorporated to develop new two-stage cultivation strategies for the production of various target compounds from microalgae.

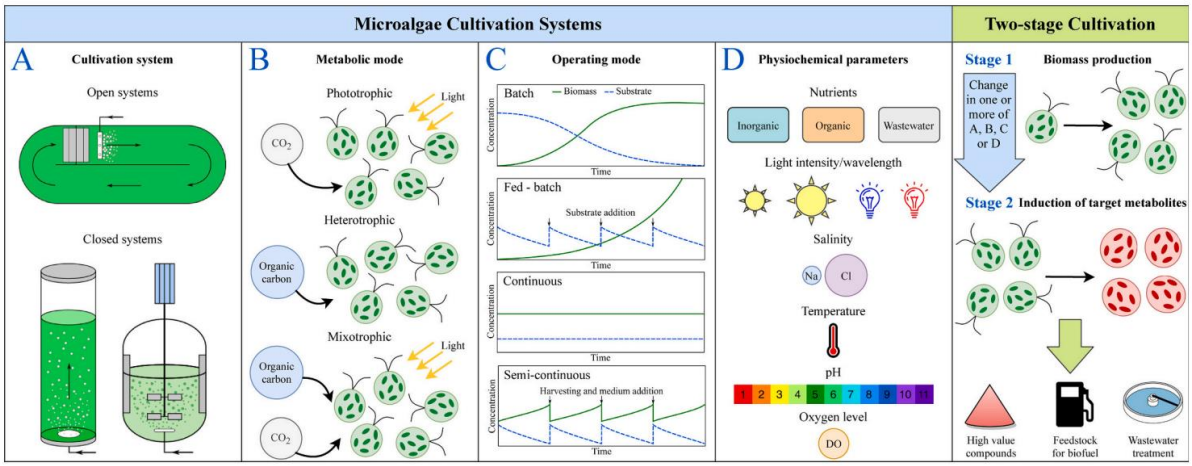

Figure 4: Two-stage cultivation of microalgae. In the first stage, biomass production is induced, and involves the alteration of culture systems, growth metabolism, mode of operation and physicochemical conditions. To achieve in the second stage, the induction of target metabolites (Liyanaarachchi et al., 2021).

In current algae treatment processes, there are two bottlenecks: harvesting the algae and extracting high-value compounds from the cells (Canelli et al., 2021). Once the microalgae reach their mature stage, harvesting, is an important step to separate the microalgae biomass from the culture medium, and continues with biomass processing, and extraction of high-value compounds, which usually involves pretreatment of the biomass (e.g., to break the algal cell wall and access the components inside the cell) (Tang et al., 2020). Downstream processing of the microalgal biomass (harvesting and product extraction) accounts for 70-80% of the total process cost, and carries the largest weight in terms of energy consumption (Kapoore et al., 2018). The harvesting step involves the separation of the microalgae biomass from the large volume of culture medium in which it is contained. This coupled with their small cell size, negative surface charge and low biomass concentration achieved during photosynthetic autotrophic culture, all pose a great challenge for their biorefinery (Matter et al., 2019).

Different methodologies have been developed that allow the recovery of microalgal biomass, which usually involves exogenous flocculants, such as mechanical, chemical, biological by predatory organisms, or electrical methods (Chen et al., 2020a; Calhoun et al., 2021). In this sense, gravity sedimentation facilitated by flocculation of some microalgal cells is more competitive from an economic point of view, and more environmentally friendly. Some microalgal strains that can spontaneously flocculate or aggregate have been previously described (Rashid et al., 2019; Yuan et al., 2019; Chen et al 2020b; Leite & Daniel, 2020; Dias et al., 2021; Kumar et al., 2021). In addition, previous studies have shown that many of these self-flocculating microalgae have considerable potential to flocculate non-flocculent microalgae and thus could be employed as bioflocculants (Lv et al 2019), which makes these strains very interesting on an industrial scale. This growing interest in these microalgal strains has led several studies to focus on studying their behavioral changes. Kumar et al. (2021) reported that, the self-flocculation behavior of these microalgae increases parallel to the microalgal growth phases and increases significantly when the pH increases above 10. The pH-dependent variation of flocculation could be due to a change in the surface charge (Zeta potential) of the microalgal cell under modified pH conditions, which would have affected the flocculation ability of the microalgal cells. Kumar et al. (2021) also observed that the temperature also influenced self-flocculation efficiency, since it decreases at higher temperatures. On the other hand, various metal ions such as aluminum (Al^{3+}), iron (Fe^{3+}), magnesium (Mg^{2+}), can also play an important role in the development of this self-flocculation capacity, given that, it has seen that they can improve microalgae harvest when added to microalgae culture suspension (Sheng et al., 2010; Kumar et al., 2020; Kumar et al., 2021). That is because, the stability of the microalgae cell is affected by cationic metal ions, and, therefore, its flocculation capacity. This interaction is made generally by two mechanisms, firstly, cationic metals neutralize the microalgae interacting directly with negatively charged cells (Kumar et al., 2020). And secondly, metals hydrolyze to metal hydroxides or interact with phosphate to produce positively charged ligand, which flocculate the microalgae through bridging or electrical patch mechanisms (Rashid et al., 2019). Other compounds that may influence this self-flocculation are found inside the cell and may be bound to membranes and/or the cell wall. So far, microalgal cell wall polysaccharides and other extracellular polymeric substances containing sugars and proteins have been identified as flocculating agents (Aljuboori et al., 2016).

The step after harvesting the microalgae in downstream processing of the microalgal biomass, is the target compounds extraction, which represents the second bottleneck of the process (Canelli et al., 2021). Extraction methods are classified as mechanical,

chemical, thermal/thermochemical, electromagnetic, biological and current (Figure 5). The most effective methods are mechanical shearing of solids, such as grinding and cryogenic grinding with liquid nitrogen, which are extremely efficient, but are expensive and impractical for industrial applications (Ryckebosch et al., 2011). To date, Chemical methods are widely used for the isolation of intracellular microalgal products (Singh et al., 2021), as they are very effective at breaking down the cell wall, they can be used on an industrial scale and require low energy consumption. However, the traditional solvent extraction techniques commonly employed are labor-intensive, time-consuming and, unless carefully controlled, are susceptible to cause degradation or undesirable chemical changes in the products, especially in carotenoids, since they use large amounts of toxic organic solvents, may expose the bioactive components to excess light, heat and oxygen, and may cause changes in stereochemistry (Kapoore et al., 2018).

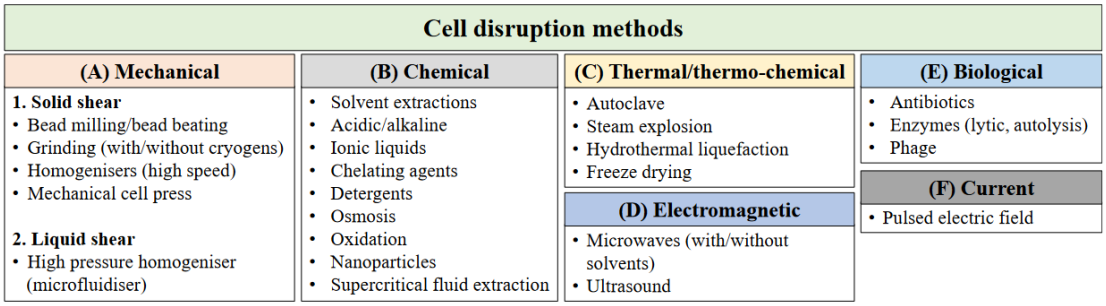

Figure 5: Summary of cell disruption methods that have been used in the microalgae industry. (Kapoore et al., 2018).

Among thermochemical methods, such as freeze-drying, although it does not interfere with the chemistry of cellular components, cell disruption is variable and often the integrity of the cell wall is weakened but not disrupted, therefore in algae with a thick cell wall, this technique is not effective (Al Hattab et al., 2015). Electromagnetic methods are effective and, also, preserve bioactive components excellently, however, these methods are not yet suitable for use on an industrial scale. The sonication bath, for example, allows efficient cell wall disruption and excellent recovery of bioactive components relatively fast and does not require hazardous substances. However, operating costs and energy consumption are very high, making it difficult to use on an industrial scale (Al Hattab et al., 2015). On the other hand, microwaves are also suitable for commercial applications, as they allow efficient cell wall disruption, require relatively low energy input, provide rapid heating with short reaction time, and have the advantage of reducing the use of solvents. Nevertheless, microwaves generate heat

and have a high maintenance cost, which also hinders their use on an industrial scale (Kapoore et al., 2018). Due to the different structural properties of the various biochemical components, identifying an optimal solvent and extraction technology to extract the desired products remains one of the major challenges in microalgal biotechnology.

In our laboratory, a microalgal strain *Scenedesmus sp.* UCV was identified that appeared to have changed its behavior and even showed a different shape than the original species (*Scenedesmus obliquus*). The strain showed characteristics that made it very interesting at an industrial level, among these, a high resistance to contamination, a tendency to self-flocculation, and a high metabolic flexibility to external stressors. Therefore, in this project, six samples of this microalgae genus cultured under different conditions were studied, and a metabolite extraction protocol was optimized by comparing two methods, ultrasonication bath (SB) extraction method and extraction with acid hydrolysis (AH).

2. Objectives

2.1. General objective

Evaluation and comparison of the composition of the new strain *Scenedesmus sp.* UCV under different growth conditions with different extraction methods in order to test its interest to work with it on an industrial scale.

2.2. Specific objectives

- To grow the new *Scenedesmus sp.* UCV strain using two-stage cultivation method to induce a biochemical and phenotypic change.
- Analysis and selection of different cell lysis techniques as pretreatment prior to the quantification of components.
- Quantification of proteins, carbohydrates, lipids, pigments and phenolic compounds of *Scenedesmus sp.* UCV and comparison between sonication and acid hydrolysis optimized extraction methods.

3. Material and Methods

3.1. *Scenedesmus sp.* UCV culture conditions

3.1.1. Obtaining the strain and culture conditions

For this research we used a variant of the chlorophycean *Scenedesmus sp.* The strain used is part of the microalgae collection of the laboratory of the Catholic University of Valencia (UCV), Spain.

The green microalgae *Scenedesmus sp*. UCV was isolated using the triple-striated Petri dish technique as described by Andersen & Kawachi, (2005). Using the inoculating loop, free colonies were selected and transferred to new Petri dish. This task was repeated three times to ensure successful isolation of a single type of microalgae. Then, the inoculation loop was used to pick the colony with the completely isolated strain, and it was initially inoculated in basal Blue-Green 11 culture medium (BG-11). Briefly, the culture was performed in photobioreactors with a capacity of 2 L continuously illuminated with fluorescence light at 80 mmol m^2 s^2. It was incubated at a temperature of 19 °C, with air bubbled at 0.6 L min^{-1} of CO_2 as a carbon source and to maintain pH stable, and a photoperiod of 12 h light: 12 h dark. Samples were taken once a week for observation or determination. The volume of evaporated culture was reconstituted with distilled water.

3.1.2. Monitoring biomass concentration

To determine the population growth of *Scenedesmus sp*. UCV cultures, two population growth curves were made. The first one (a.) related the number of cells to the absorbance of the culture, and the second one (b.) related the dry biomass weight to the absorbance of the culture.

For both curves, 15 mL falcon tubes were correctly labeled including their stoppers, and weighed on a gravimetric balance. Dilutions were made in these tubes from an exponential phase culture, and the absorbance of each tube was measured with the spectrophotometer (ZUZI 4111RS, Quercus labo, Ghent, Belgium) at OD680, being shaken vigorously with a vortex before each measurement. Then, to determine the first standard curve (a.), 10 µL of each of homogeneous sample tube was deposited on different slides, and the number of cells of each dilution was counted using a Neubauer chamber (NEUBAUER-IMPROVED, MARIENFELD, Germany) and a binocular microscope, observations were made at 40X magnification, and the cells present in the

four corner areas were quantified. The formula 1 to determine the number of cells/mL was as follows:

$$C = N \times 10^4 \times dil \quad (1)$$

Being C: N° of cells/mL; N: Average number of cells in 1 mm^2 (0.1 µL); 10^4: Conversion factor from 0.1 µL to 1 mL; dil: Dilution factor. (International Council for Standardization in Hematology, 1998).

Afterwards, to determine the second standard curve (b.) the tubes with the dilutions were centrifuged (Centrifuga Medifuge 8, Thermo Scientific, USA) at 3,000 xg for 10 minutes, the supernatant was discarded and frozen at - 80 °C, for subsequent lyophilization. For quantification of the total dry biomass, the tubes with the freeze-dried dilutions were reweighed on the same precision balance and the weight of each corresponding empty tube was subtracted according to the following equation 2:

Total dry Biomass (mg/mL) = W2 – W1 (2)

Being W1 the weight of the empty falcon tube, and W2, the weight of the filled falcon tube.

3.1.3. Effect of chemical nitrogen supply and irradiance.

Standard BG-11 medium (Allen, 1968) was used as control (Table 1).

Table 1: Components and quantities of the culture media used (own elaboration).

Culture medium	Components
BG-11 medium	- $NaNO_3$ 150 mg L^{-1} - K_2HPO_4 40 mg L^{-1} - MgSO4 75 mg L^{-1} - $CaCl_2$ 36 mg L^{-1} - Na_2CO_3 20 mg L^{-1} - Ferric citrate 6 mg L^{-1}

Table 2: Different samples used, name, growth conditions and characteristics of each one. The numbers shown after the name of the sample correspond to the month and year, respectively, in which they were harvested. The “Ss” samples correspond to *Scenedesmus sp*. strains that showed changes in their behavior and shape, and therefore, had differentiated from the original *Scenedesmus obliquus* species. The “So” samples correspond allegedly to the original *Scenedesmus obliquus* strains (own elaboration).

Sample	Culture conditions	Characteristics
SsP 10,21. Primitive	First phase in BG-11 medium, 25 ◦C.	Orange 
SsD 12,22. Golden	First phase in BG-11 medium, 19 ◦C. Second phase in water, during one week of growth	Golden 
SsFT 12,22. Terminal Phase	First phase in BG-11 medium, 19 ◦C. Second phase in water, during two weeks of growth	Brown 
SsC 10,22. Control	Growth only in BG-11, 19 ◦C. One week.	Green 
SoD 1,23. Golden	First phase in BG-11 medium, 19 ◦C. Second phase in water, during one week of growth.	Golden 
SoC 7,18. Control	Growth only in BG-11, 19 ◦C. One week.	Green 

The SsP sample changed its color spontaneously in our laboratory. This culture grew during the summer months without being supplemented with new growing medium, and with increasing summer temperatures. Subsequently, the same conditions were reproduced with SsD, SsFT and SoD samples, to be able to repeat this event.

The “Ss” samples (SsD and SsFT) as shown in Table 2, were produced with the two-stage cultivation method (Liyanaarachchi et al., 2021). Shortly, the first stage consisted of culturing the algae in BG-11 medium until they reached the logarithmic phase. The second stage consisted of harvesting and transferring the algae to a new flask where the necessary conditions for this change of metabolism were provided. Once a density of 10^5 cells mL^{-1} (exponential phase) is reached, the first stage ends and the second stage begins, in which we transferred the algae to 250 mL Erlenmeyer flasks containing 150 mL of ultrapure water (10% inoculum by volume, average cell concentration of 0.45 g/L dry weight). At this stage, the lack of nutrients favors

induction to accelerate the accumulation of lipids and other secondary products. It is advisable to increase the culture temperature to achieve a better effect.
In order to compare the culture of the new variant *Scenedesmus sp.* UCV, with the original algae grown in the laboratory, the “So” samples (SoD and SoC) were used in this experiment. The SoC sample (Table 2), was from a culture harvested in 2018, freeze-dried and cold-stored, presumably belonging to the original Scenedesmus obliquus. The SoD sample (Table 2) was obtained from cold-stored cultures in slant agar tubes, along with the rest of the collection, and was subjected to the same metabolism shift induction process explained above for *Scenedesmus sp.* UCV.

3.2. Quantification of the microalgae *Scenedesmus sp.* UCV content

3.2.1. Methods for cell disruption

Cell rupture is necessary for the release of biological compounds from inside the cells of many microalgae (Silva et al., 2021). Specifically, *Scenedesmus sp.* has a very resistant cell wall (Dunker & Wilhelm, 2018). Therefore, the biomass was processed with physical methods (ultrasonication and microwave), and with physicochemical processes (acid hydrolysis with trichloroacetic and hydrochloric acid, deep eutectic solvents, and lysis buffer).

For all cell disruption treatments, the lyophilized biomass of each of the six samples collected under the different culture conditions was used in a concentration of 10 mg/ml, and the optical microscope (Leitz Biomed, Leica Biosystems, Wetzlar, Germany) was used as an analytical method to evaluate the capacity of each of the proposed methods to break the cell wall of this specific microalgae.

3.2.1.1. Sonication

Sonication generates a series of waves and energy, which produce movement in the molecules, along with pressure changes in the liquid solution. This molecular movement consists of a series of compression and rarefaction cycles. During the rarefaction cycle, a negative pressure is produced, which drops until it falls below the vapor pressure of the liquid. At this moment, small bubbles filled with gas or vapor are created in the liquid, which will grow in successive cycles of compression and rarefaction to a size in which ultrasonic energy is insufficient to retain the vapor inside,

which means that, during a compression cycle, a collapse occurs that will release a lot of energy which finally, through mechanical/physical and chemical effects, ends with cell rupture (Wang et al., 2020).

In order to use sonication to achieve cell disruption, needle ultrasonication and sonication bath were tested. For the first one, 10 mg of each of the six samples of the dry biomass of the microalgae were weighed, and resuspended in 0.5 ml of ultrapure water, the extraction was carried out following the protocol of Silva et al. (2021), with a tip ultrasound (Hielscher Ultrasonics GmbH, UP100H) under the following conditions: frequency 20 kHz, amplitude 90%; always in an ice bath to avoid overheating of samples. Tests were carried out at 10, 20, 30 and 40 minutes. Every 10 minutes, the samples were allowed to settle for 5 minutes, in order to avoid overheating of the samples and the consequent degradation of the compounds of interest. Subsequently, the extracts were centrifuged at 3,800 ×g for 10 min and the supernatant was collected. The pellet was resuspended in 0.5 ml of the same solvent and extracted once more following the same procedure, obtaining a final concentration of 10 mg/ml.

A similar methodology was followed using sonication bath (SONOREX SUPER RK, BANDELIN electronic GmbH & Co. KG Heinrichstraße, Berlin, Germany). In this case, 5 mg of biomass was resuspended in 2 mL of ultrapure water, and the samples were left sonicating in parallel in the bath. Every 10 min, 10 µL of each tube were placed on a slide for microscopic analysis.

3.2.1.2. Microwave

Microwaves cause vibration of polar molecules such as water, within the wet biomass, which causes an increase in temperature in the intracellular fluids, causing water to evaporate and exert pressure on the cell walls, ultimately leading to cell rupture. In addition, it allows greater penetration of the solvent into the sample because the microwaves break hydrogen bonds causing migration of dissolved ions (Kapoore et al., 2018). To achieve cell lysis with this technique, freeze-dried algal biomass was resuspended in ultrapure water in 15 mL glass tubes, obtaining a concentration of 10 mg/ml in each of the six samples. Then, following the protocol of Kapoore et al. (2018), with the Microwave Synthesizer (Discover® 2.0, CEM, USA) different temperatures were tested: 90°C, 110°C, 130°C and 150°C; under the following conditions: 40 W, 1 bar, 20 minutes. At each of the different temperatures, a sample was taken and observed under the microscope to find the threshold temperature at which the microwaves could break the cell wall of the microalgae.

3.2.1.3. Acid Hydrolysis

The chemical lysis is a different approach to hydrolyze biomass in its constituent molecules. In acid hydrolysis, the acid reagent attacks the polysaccharide fibers making them soluble and hydrolyzing the sugar polymers that produce sugar monosaccharides (Stirk et al., 2020).

To determine the efficiency of the acid pretreatment, acid hydrolysis was performed with two different reagents (trichloroacetic and hydrochloric acid), and the results obtained were evaluated and compared in subsequent protein quantification assays.

For treatment with trichloroacetic acid (TCA), the protocol proposed by Slocombe et al. (2013) was followed, optimized for subsequent protein quantification. Therefore, 5 mg of each lyophilized microalga sample were weighed and resuspended by shaking in 200 µl of 24% (p/v) TCA. The homogenized solutions were incubated in a water bath at 95 °C for 15 min and after allowed to cool down to room temperature. Then, 600 µL of ultrapure water were added to dilute the samples to 6% (w/v), following by centrifugation at 15,000 xg for 20 min at 4 °C (Microcentrifuge CT15E/CT15RE, VWR Hitachi®, USA) and their supernatants were discarded. The sediment was stored at -20°C for later analysis.

For treatment with hydrochloric acid (HCl), the biomass was processed mixing 50 mg of each of the 6 samples, with 5 ml of 1N HCl in glass tubes, following the protocol of Stirk et al. (2020). Samples were incubated in parallel for 2 hours at 90 °C with 200 rpm shaking using a carousel (carousel 12 Plus Reaction Station™, Radleys, United Kingdom). Subsequently, they were neutralized with a 1N NaOH solution, measuring with a pHmeter (Orion 3 STAR, Thermo Scientific, USA) until the pH was 7, and dialyzed overnight in a dialysis bag pre-treated RC Tubing MWCO: 5kD (Spectra/Por® Dialysis Membrane, Spectrum Laboratories, USA). Salinity was controlled with a refractometer (HI 96801, HANNA Instruments®, France) measuring before and after dialysis.

3.2.1.4. Lysis Buffer

For the analysis of microalgae cell wall rupture using a lysis buffer, freeze-dried biomass samples were vigorously mixed with extraction buffer in 2 mL eppendorf. The buffer consisted of PBS (sodium phosphate buffer) with 1% Triton X-100 and 0.1%

SDS (sodium dodecylsulfate). Subsequently, the samples were subjected to a sonication bath and samples were taken from each sample every 10 min during 1h, to analyze the cell wall integrity of the microalgae under a microscope.

3.2.1.5. Deep eutectic solvents (DES)

Four different protocols were tested using cholinium and urea deep eutectic solvent (DES). For this purpose, dry biomass of the microalgae was distributed in four test tubes and resuspended according to the following methodologies: a) Heating to boiling with thermal pistol in pure DES; b) Dry biomass suspended in water + HCl, heated again; c) HCl + DES, heating to boiling with thermal pistol; d) DES dissolved in water, heating to boiling with thermal pistol.

Subsequently, by thin layer chromatography, the release of biochemical components in the media was analyzed by eluting with ethyl acetate and methylene chloride. The pH of each of the tubes was checked with the same pHmeter (Orion 3 STAR, Thermo Scientific, USA). Finally, the samples were observed under the microscope.

3.2.2. Biochemical analysis

Both methods, (1) sonication bath (SB) 45 minutes; and (2) acid hydrolysis (AH) 2h, 90°C, were selected as the most capable of achieving cellular lysis. Therefore, were used as pretreatment for all subsequent biochemical assays. Due to the limiting factor of dry biomass quantity, a protocol for biochemical quantification assay was optimized to use as little biomass as possible. In this way, in (1) SB and (2) AH, the same process was followed for the quantification assays in parallel (Figure 6). A stock tube of each sample and extraction method (12 in total) was used for all subsequent quantification assays. This tube contained 2 mL of each of the microalgae extracts dissolved in water, at a concentration of 10 mg/mL. From this tube the protein (which required a 1:3 dilution to stay within the standard curve), carbohydrate, and lipid (which required a 1:10 dilution to stay within the standard curve) assays were performed. For the quantification of phenolic compounds, it was diluted 1:2 in acetone to achieve a 50% acetone extraction solvent, and for pigments, it was diluted 2.5 times to achieve an 80% acetone extraction solvent (Figure 6).

All treatments described were performed in triplicate and repeated twice for greater accuracy of the results. In all cases, the absorbance was measured with the

spectrophotometer (Fluostar Omega, BMG Labtech, Ortenberg, Germany), in a 96-well microplate (NUNC 96 A/S, Thermo Scientific, Denmark).

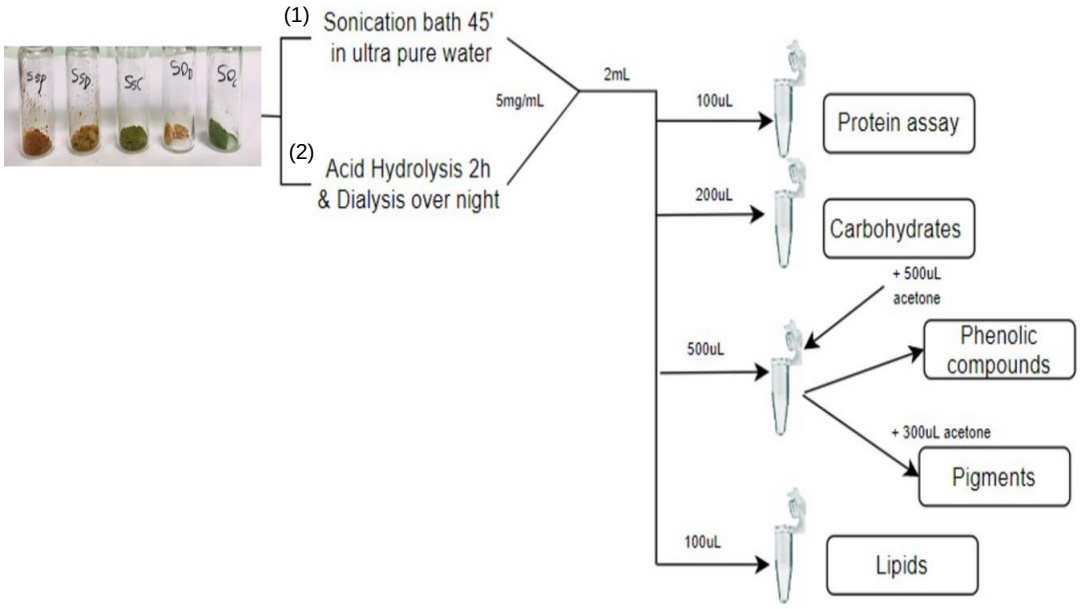

Figure 6: Flowchart of the extraction and quantification process of the different components. 2 mL of each of the microalgae extracts dissolved in water, at a concentration of 10 mg/mL, was placed in an 1.5 mL eppendorf. This tube (used as a reserve for all subsequent quantification assays), was labeled and stored at - 20°C. For protein quantification, 100 µL was taken and placed in a new tube, which was labeled with the sample name and corresponding assay. This volume was diluted 1:3 in ultrapure water to keep it within the standard curve. For carbohydrate quantification, 200 µL were taken and added to a new, properly labeled eppendorf, and this volume was used directly in the assay. For the quantification of phenolic compounds, 500 µL were taken. Another 500 µL of acetone was added to obtain samples diluted 1:2 in 50% acetone solvent. 300 µL were taken and used for polyphenol quantification. The remaining 200 µL were diluted 2.5 times, adding 300 µL of pure acetone to obtain the sample in 80% acetone solvent. Pigment quantification was performed from this solution. For lipid quantification, 100 µL of each extract stock solution was taken, and diluted 1:10 to perform the lipid assay and stay within the standard curve (own elaboration).

3.2.2.1. Analysis of total protein content

To determine the efficiency of the acid pre-treatment, a quantitative acid hydrolysis for the total extraction of proteins was performed, and the results obtained compared to those of the disruption essays.

In all cases, the proteins were analyzed using a standard curve of BSA dilutions of 50 to 600 µg ml^{-1}. Pellets obtained with the TCA extraction protocol of Slocombe et al. (2013), were resuspended in 0.5 mL of Lowry's Reagent D, consisting of the proportions: 100:1:1 of Lowry Reagents A (2% (w/v) Na_2CO_3 (anhydrous) in 0.1 N NaOH); B (1% (w/v) NaK Tartrate tetrahydrate) and C (0.5% (w/v) $CuSO_4$ $5H_2O$ in H_2O), respectively. The homogenized samples were then incubated for 3 hours at 55°C. Subsequently, they were centrifuged at 15 000 xg for 20 minutes at room temperature and the pellet was discarded and the supernatant retained. The second one was prepared in a 96-well microplate for Lowry analysis in triplicate. In each well, 0.1 mL of 1 M Folin-Ciocalteu 1 M phenol reagent diluted 1/20 in ultrapure water (mixture of phosphotungstic acid $H_3PW_{12}O_{40}$ and phosphomolybdic acid $H_3PMo_{12}O_{40}$) prepared daily, were added and well mixed using the pipette tip. After 30 min at room temperature, the absorbance of each sample was read at 600 nm with the spectrophotometer.

For the rest of the assays, protein concentration was measured by the Lowry method using Bio-Rad solution (DC Protein Assay Reagents Package, Bio-Rad Laboratories, USA). In a 96-well microplate, 25 µl of sample and standard curve were added in triplicate to 20 µl of Lowry's reagent and 200 µl of Folin's reagent. It was incubated for 20 min at room temperature and in the dark, and absorbance was measured at 750 nm.

3.2.2.2. Analysis of total carbohydrates content

The determination of total sugars is carried out by the spectrophotometric method of the phenol/sulfuric acid mixture (Dubois et al., 1951), which is based on the formation of yellow-orange compounds, due to the interaction of phenolic compounds. With furfuric compounds (furfural and 5-hydroxymethylfurfural), formed due to the dehydration of the osidic units (hexose and pentose) in the presence of concentrated sulfuric acid. And its appearance is checked by measuring the increase in absorbance at 485 nm.

Prior to carbohydrate quantification, a calibration curve was performed with a glucose solution, used as a standard, Glucosa Anhidra 98%. In triplicate, 80 µL of sample and standard solution were prepared in opaque glass vials with 80 µL of 5 % phenol and 400 µL of 96 % sulfuric acid (Sigma-Aldrich, USA). The reaction mixture was incubated for 30 min at 90°C in the dark using a heating plate (Carousel™ Stirring Hotplates,

Radleys Standard, United Kingdom). Subsequently, the samples were allowed to cool on ice, and in a 96-well plate the absorbance was measured at 485 nm.

3.2.2.3. Analysis of total lipid content

In hot sulphosphoric medium, lipids develop with vanillin a pink color that can be measured with a spectrophotometer (Chabrol & Charonnat, 1937). This assay is based on the interaction between lipids and vanillin in a sulfosphoric medium. Fatty acids react with sulfuric acid to produce 2 types of carbocations: alkyl and alkenyl. The vanillyl reacts with phosphoric acid to give an aromatic phosphoric ester and the carbocation reacts with the activated carbonyl group of phosphovanillin to form an aromatic phosphoric ester which absorbs at 540 nm, and the absorbance is proportional to the lipid concentration.

In glass vials, 100 µL of sample and standard curve were added with 200 µL of chloroform-hexane solvent (2:1, v/v) and 200 µL of 96% sulfuric acid before incubating for 10 min at 90°C using the heating plate (Carousel™ Stirring Hotplates, Radleys Standard, United Kingdom). Subsequently, 200 µL of acid vanillin solution (5 mg mL^{-1} in 68% phosphoric acid) was added to the glass vials and incubated for 5 min at room temperature. Finally, absorbance was measured at 540 nm in a 96-well microplate.

3.2.2.4. Analysis of total pigments content

The addition of small amounts of a polar solvent (acetone) to the extraction solvent prevents the loss of β-carotene and some chlorophyll a, and allows complete extraction of chlorophylls and carotenoids (Lichtenthaler & Hartmut, 1987). Natural chlorophylls generally show two notable absorption maxima in the visible region, a major peak at about 400 nm in the blue region, and a minor peak in the red region, around 650 nm. In both cases, the maximum wavelength position of chlorophyll b lies between those of chlorophyll a. Although the major peak has a higher sensitivity, the wavelength of the minor peak has been commonly adopted for the determination of chlorophylls, in order to avoid significant interference from carotenoids, which are mainly absorbed between 400-500 nm (Chen & Vaidyanathan, 2013).

For the extraction and quantification of pigments, including carotenoids, the methodology proposed by Wellburn, (1994) was used. The extraction was initiated by adding 300 µL of pure acetone to the eppendorf that already contained the extract in

50 % acetone, to form a solution in 80 % acetone, and as a consequence the sample was diluted 2.5 times, a factor that was considered for subsequent calculations. After, extracts were centrifuged for 10 min at 7,100 xg.

For the quantification of chlorophyll a, chlorophyll b and total carotenoids, equations 3, 4 and 5 were used, respectively:

$$Ca = 12{,}25 \times A663{,}2 - 2{,}79 \times A646{,}8 \quad (3)$$

$$Cb = 21{,}5 \times A646{,}8 - 5{,}1 \times A663{,}2 \quad (4)$$

$$Cc = (1000A470 - 1{,}82Ca - 85{,}02Cb)/198 \quad (5)$$

Where Ca is the concentration of chlorophyll a (µg mL^{-1}), Cb is the concentration of chlorophyll b (µg mL^{-1}), Cc is the concentration of total carotenoids (µg mL^{-1}).

After extracting and quantifying the extract, the concentration was converted to % using equation 6, as follows:

$$\% = [(\text{mg pigment} \times \text{dil}) \times (100 \text{ mg microalgae})^{-1}] \quad (6)$$

Using the total volume of the extract and the mass of biomass extracted and, being dil = Dilution factor = 2.5.

3.2.2.5. Analysis of total phenolic compounds content

The extraction and quantification of phenolic compounds was carried out following the protocol of Singleton & Rossi, (1965), using the Folin-Ciocalteu 2 N reagent (Folin & Ciocalteu's phenol reagent, Sigma-Aldrich, USA), which is a mixture of phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PMo_{13}O_{40}$), which, during the oxidation of phenols, is reduced to a mixture of blue oxides of tungsten and molybdenum (Ribereau-Gayon, 1968), and the color produced, whose maximum absorption is between 725 and 750 nm, is proportional to the amount of polyphenols present in the different fractions.

To form a 50% solution in acetone, 500 µL of acetone was added to 500 µL of the microalgae extracts and mixed vigorously. The same procedure was followed with the standard curve, made with different dilutions of gallic acid (GAE) (Sigma-Aldrich, USA), at concentrations between 0 and 1 mg mL^{-1}. Subsequently, the samples were centrifuged for 10 min at 7,100 xg, and 200 µL of the supernatant was introduced into centrifuge tubes, together with 100 µL of Folin-Ciocalteu 2 N reagent. The tubes were incubated at room temperature for 3 min and 400 µL of 20 % Na_2CO_3 (Sigma-Aldrich,

USA) was added. They were left 45 min in darkness, and finally, the absorbance was measured at 730 nm.

The analyses were performed in triplicate and the results was expressed as gallic acid equivalents in milligrams per 100 milligrams of sample dry weight (mg GAE 100 mg^{-1} DW).

3.3. Statistical analysis

Normality and variance homogeneity of data distribution were checked by Shapiro-Wilk and Levene tests, respectively. Differences between the different samples taken from *Scenedesmus sp.* UCV cultures were determined by two-way analysis of variance (ANOVA) and Tukey's multiple comparisons test, with a significance level of $p < 0.05$ using GraphPad Software, Inc® Prism version 8.3.1.

4. Results

4.1. Results of cell disruption methods

The results of the cell lysis tests showed that only three of the five methods tested were able to break the microalgae cell wall of *Scenedesmus sp.* UCV, according to microscopic observations (Table 3), being the most effective method, acid hydrolysis in HCl for two hours at 90 ºC, which lysed all the cells. It was followed by ultrasonication bath for 45 minutes, which lysed approximately 50% of the cells, and finally, microwave synthesizer for 20 minutes at 130 ºC, which was the least effective of the three methods.

Table 3: Results of the different cell lysis assays, and most effective conditions for each one. Cell breakage capacity: (*) Some broken cells; (**) Many/almost all broken cells, (***) All broken cells.

METHOD	CELL BREAK	CONDITIONS
ULTRASONICATION & SONICATION BATH	Yes **	45 min in H2O ultra pure
MICROWAVE	Yes *	130 ºC, 20 min in H2O ultra pure
ACID HYDROLYSIS	Yes ***	90 ºC, 2h in HCl 1N
LYSIS BUFFER	No	1h, in PBS (Sodium Phosphate Buffer) with: - 1% Triton X-100 - 0.1% SDS (Sodium Dodecyl Sulfate)
DEEP EUTECTIC SOLVENTS (DES)	No	DES made of cholinium and urea +: - Heating until boiling with thermic pistole in pure DES - Dry biomass Suspended in water + HCl, then heated again - HCl + DES, Heating until boiling with thermic pistolet DES dissolved in water, Heating until boiling with thermic pistolet

4.2. Results of biochemical analysis

The results of the biochemical quantification analysis of each component generally showed a higher extraction efficiency using sonication bath (SB) pretreatment for 45 minutes, in comparison with acid hydrolysis (AH) pretreatment for 2h at 90 ºC (Table 4). Our results showed no significant differences in any of the groups, in relation to the type of sample (conditions of growth). However, in relation to the extraction method used, there were significant differences for carbohydrates, chlorophyll a and b, with the sonication method showing the best results compared to acid hydrolysis.

Table 4: Results of proteins, carbohydrates, lipids and phenolic compounds, of the 6 different samples with both pre-treatments methods 1) after 2h of AH, and 2) after SB 45 minutes, are expressed as percentage of the algal sample in dry weight (mean ± SD). Prot: Proteins; Carb: Carbohydrates; Lip: Lipids; Pc: Phenolic compounds.

Samples	1) Prot.	2) Prot.	1) Carb.	2) Carb.	1) Lip.	2) Lip.	1) Pc	2) Pc
SsP	4.21±2.85	8.45±4.58	4.08±3.62	16.02±2.61	21.73±19.57	28.26±11.91	2.08±2.80	4.14±2.36
SsD	5.29±2.57	8.56 ± 5.83	4.94±4.49	20.82±9.35	13.09± 1.11	21.37±16.27	3.24±2.41	4.67±2.96
SsFT	4.02±0.86	4.90± 3.03	3.63±2.32	20.03±8.63	20.48±10.20	28.48±3.03	1.18±1.07	2.50±1.64
SsC	6.15±0.51	5.40± 3.60	7.45±4.51	17.83±2.12	34.06±19.36	17.80± 7.25	2.78±0.34	3.32±2.50
SoD	4.22±2.37	9.65± 5.51	3.24±2.31	21.04±5.79	18.13±16.83	32.45±15.48	1.05±0.87	5.20±3.87
SoC	12.24±6.07	14.59±10.44	6.24±6.01	14.07±8.52	6.32 ± 3.59	6.73±3.98	5.47±3.07	6.05±4.56

The results of protein quantification by the Lowry method showed no significant differences between groups in relation to growth conditions, nor in relation to the extraction method, however, the results of protein quantification showed in general, a lower extraction efficiency with the AH method (Figure 7). The samples that showed the highest amount of protein were SoD and its control, SoC. Especially, SoC showed the highest amount, with 14.59 ± 10.44 mg sample per 100 mg dry weight (DW), and 12.24 ± 6.07 mg sample per 100 mg DW by means of AH method. Then, SoD, SsD and SsP samples showed a protein amount of 9.65 ± 5.51; 8.56 ± 5.83 and 8.45 ± 4.58 mg 100 mg^{-1} DW, respectively, by SB method. In these same samples, the results by the AH method were approximately half, showing a protein extraction of 4.22 ± 2.37, 5.29 ± 2.57 and 4.21 ± 2.85 mg 100 mg^{-1} DW in the SoD, SsD and SsP samples, respectively. On the other hand, the SsC and SsFT samples showed the lowest protein quantification results, with an amount of 5.40 ± 3.60 and 6.15 ± 0.51 mg 100 mg^{-1} DW, obtained from the SsC sample by the AH and SB, respectively. Sample SsFT showed the lowest amount of protein in both extraction methods, with a protein percentage of 4.90 ± 3.03 % with SB, and 4.02 ± 0.86 % with AH (Table 4).

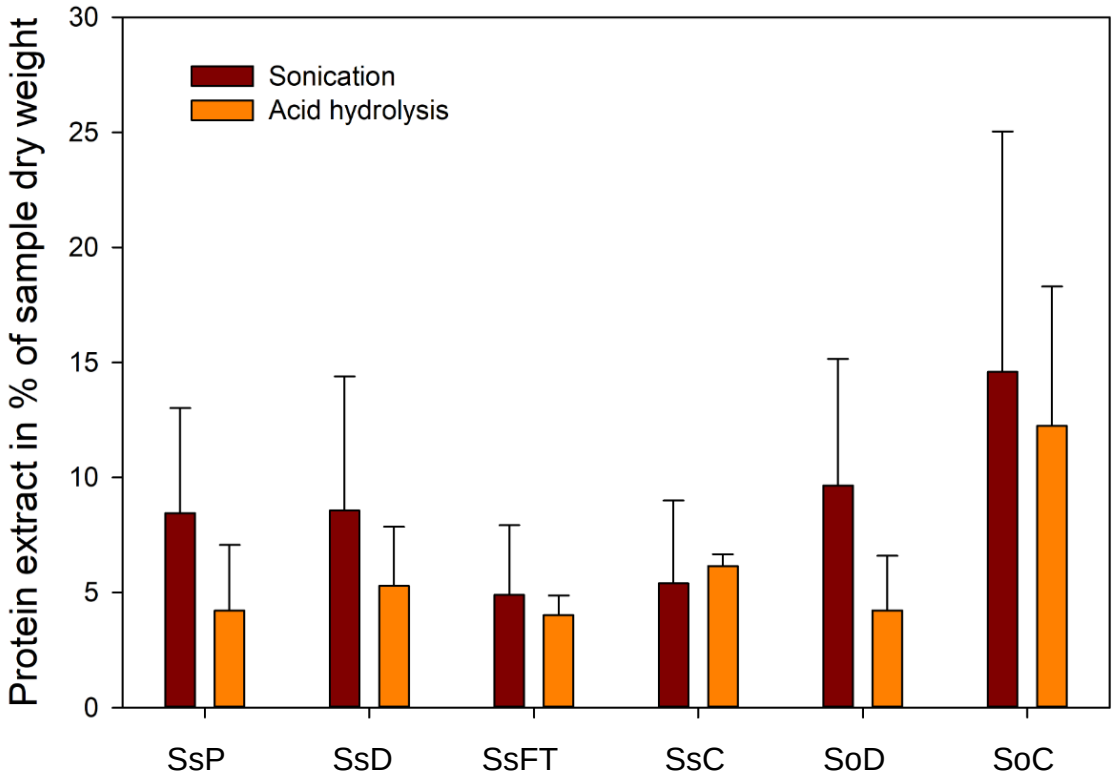

Figure 7: Results of proteins quantification by the Lowry method (A) after 2h of acid hydrolysis, and (B) after sonication 45 minutes, of the 6 different samples, Bovine Serum Albumin (BSA) equivalent. Quantities are expressed as percentage of the algae sample in dry weight (mean ± SD).

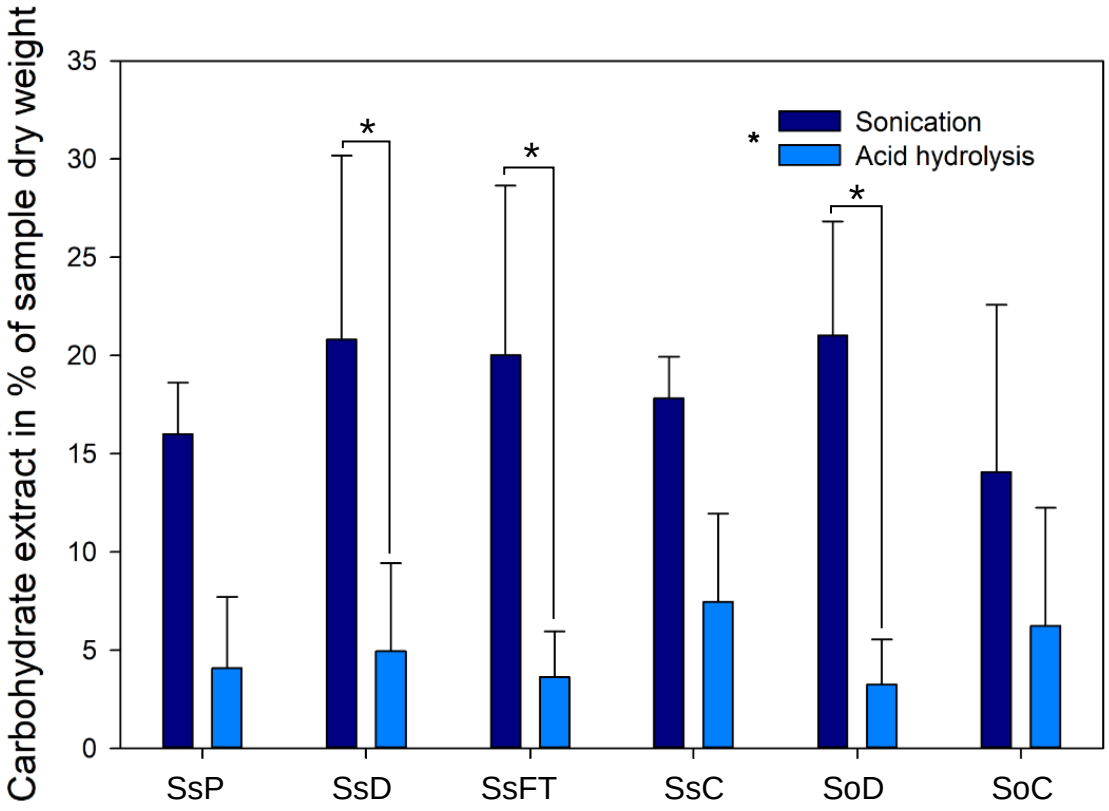

Figure 8: Results of carbohydrates by Dubois method, (A) after 2h of acid hydrolysis, and (B) after Sonication 45 minutes, of the 6 different samples, Glucose equivalent. Quantities are expressed as percentage of the algae sample in dry weight (mean ± SD). The symbol (*) above the bars and among the extraction techniques, indicates significant differences $p < 0.05$.

The results of carbohydrate quantification by the Dubois method showed no significant differences between groups in relation to growth conditions, however, two-way analysis of variance (ANOVA) showed significant differences in relation to extraction method (Figure 8). The carbohydrate content of the microalgal biomass, was again generally higher in the samples that were subjected to SB, with the golden algae samples (SoD and SsD) showing the highest quantities of polysaccharides. The highest amount was shown by the SoD sample, which obtained a carbohydrate amount of 21.04 ± 5.79 mg

per 100 mg DW, followed closely by SsD, with a percentage of 20.82 ± 9.35 %, and in decreasing order, SsFT with 20.03 ± 8.63 %, and SsC which showed 17.83 ± 2.12 mg of glucose per 100 mg DW. Finally, SsP and SoC showed the lowest amounts of glucose, with an amount of 16.02 ± 2.61 % and 14.07 ± 8.52 %, respectively (Figure 8). The efficiency of carbohydrate extraction after AH decreases in comparison with sonication by 64-86%. In the samples pretreated with AH, the highest amounts were obtained by the green samples, SsC and SoC, showing a carbohydrate amount of 7.45 ± 4.51 and 6.24 ± 6.01 mg per $100\,mg^{-1}$ DW respectively. The orange samples, in decreasing order, showed a polysaccharide percentage of 4.94 ± 4.49 %, 4.08 ± 3.62 %, 3.63 ± 2.32 and 3.24 ± 2.31 % for samples SsD, SsP, SsFT and SoD, respectively (Table 4). After performing Tukey's multiple comparisons test post hoc analysis by rejecting the null hypothesis of equality of means, to analyze differences at the individual sample type level, carbohydrates showed significant ($p < 0.05$) effect on the extraction method in SsD, SsFT and SoD (Figure 8).

The results of lipid quantification by spectrophotometer method showed no significant differences between groups in relation to growth conditions, nor in relation to the extraction method (Figure 9). Generally, higher results were showed in the samples treated with SB, with SoD showing the highest concentration of 32.45 ± 15.48 mg per $100mg^{-1}$DW, followed by SsFT, SsP, SsD and SsC samples, that showed a lipid amount of 28.48 ± 3.03; 28.26 ± 11.91; 21.37 ± 16.27 and 17.80 ± 7.25 mg per $100\,mg^{-1}$DW, respectively. In contrast, the highest amount of total lipids was obtained with AH, in the control sample SsC, with a percentage of 34.06 ± 19.36 %. Quite higher than the other HA samples, which showed, in decreasing order, amounts of 21.73 ± 19.57; 20.48 ± 10.20; 18.13 ± 16.83 and 13.09 ± 1.11 mg of lipids per $100\,mg^{-1}$DW, in the samples SsP, SsFT, SoD, SsD, respectively. The lowest amounts of lipids were observed in the SoC sample, both with SB and AH, with a percentage of 6.73 ± 3.98 and 6.32 ± 3.59 %, respectively (Table 4).

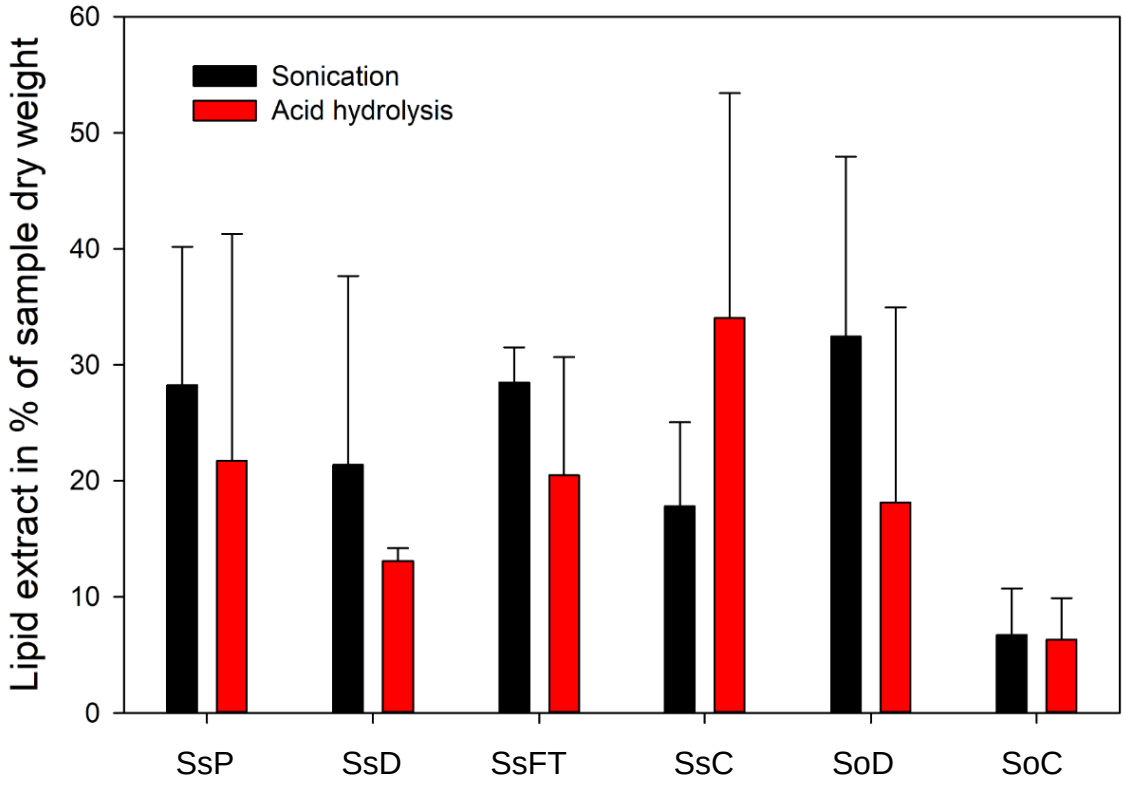

Figure 9*:* Results of lipids quantification by the spectrophotometer method of (Chabrol & Charonnat, 1937), (A) after 2h of acid hydrolysis, and (B) after Sonication 45 minutes, of the 6 different samples, olive oil equivalent. Quantities are expressed as percentage of the algae sample in dry weight (mean ± SD).

The results of the quantification of phenolic compounds also showed no significant differences in the statistical analysis of variance in relation to growth conditions, nor in relation to the extraction method (Figure 10). The highest concentration of polyphenols corresponded to the SoC sample in both methods, with 5.47 ± 3.07 mg GAE per $100\,mg^{-1}$ DW quantified with AH and 6.05 ± 4.56 mg GAE per $100\,mg^{-1}$ DW quantified with SB. With SB the amounts of polyphenols extracted were generally higher, showing percentages of phenolic compounds of 5.20 ± 3.87; 4.67 ± 2.96; 4.14 ± 2.36 and 3.32 ± 2.50 %, from the SoD, SsD, SsP and SsC samples, respectively. With AH on the other hand, the amounts extracted were much lower, showing percentages of 3.24 ± 2.41; 2.78 ± 0.34; 2.08 ± 2.80 and 1.18 ± 1.07 %, from samples SsD, SsC, SsP, SsFT, respectively. The lowest amounts were found in the SsFT sample, in which 1.18 ± 1.07 mg per $100\,mg^{-1}$ DW was extracted by AH and 2.50 ± 1.64 mg per $100\,mg^{-1}$ DW by SB (Table 4).

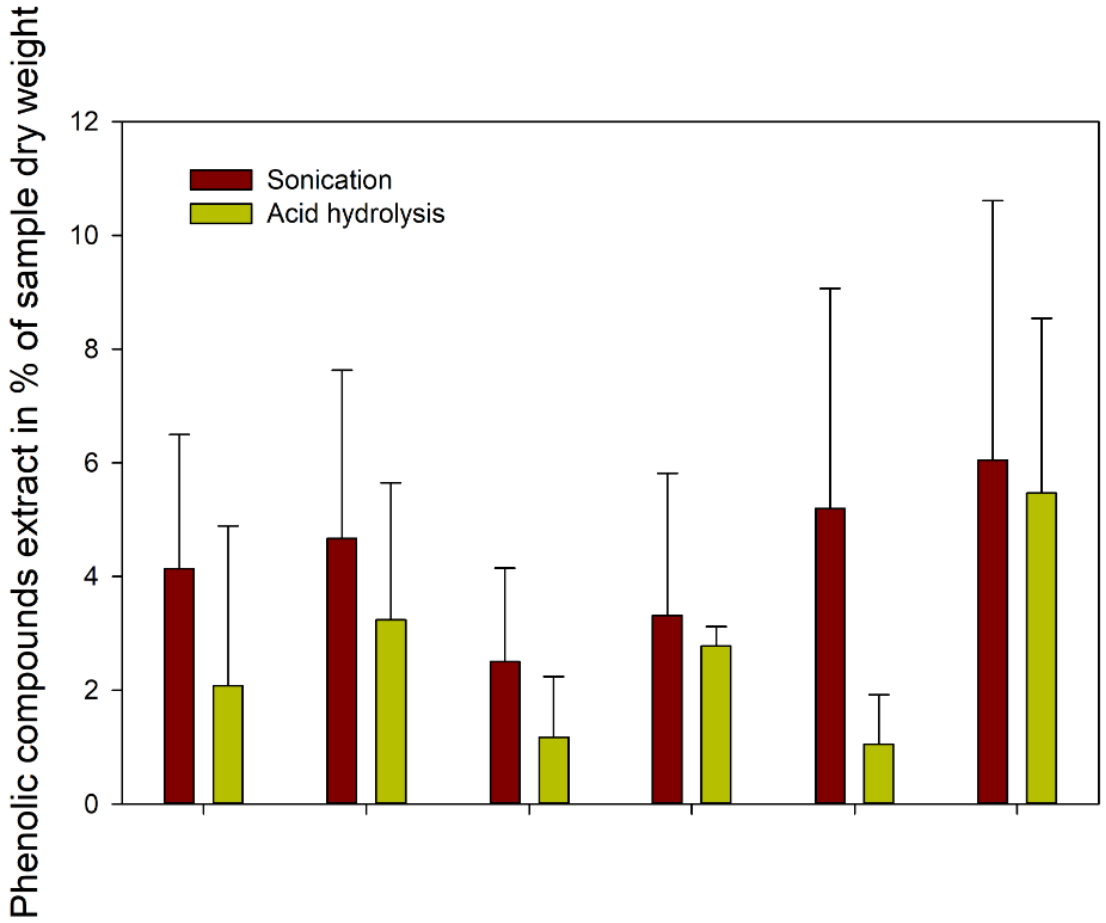

Figure 10: Results of phenolic compounds quantification with the Folin-Ciocalteu reagent (Singleton & Rossi, 1965), (A) after 2h of acid hydrolysis, and (B) after Sonication 45 minutes, of the 6 different samples Gallic acid (GAE) equivalent. Quantities are expressed as percentage of the algae sample in dry weight (mean ± SD).

Regarding the quantification of pigments, again, the statistical analyses in relation to the type of sample, showed no significant differences in any of the groups. However, in relation to the extraction method used, the quantification tests for chlorophyll a (Figure 11) and chlorophyll b (Figure 12), showed significant differences at the general level in the ANOVA test, although carotenoids did not show any significant difference (Figure 13).

Table 5*:* Results of chlorophyll a, chlorophyll b and total carotenoids, of the 6 different samples with both pre-treatments methods 1) after 2h of AH, and 2) after SB 45 minutes, are expressed as percentage of the algal sample in dry weight (mean ± SD). Ca: chlorophyll a; Cb: chlorophyll b; Cc: total carotenoids.

Samples	1) Ca	2) Ca	1) Cb	2) Cb	1) Cc	2) Cc
SsP	0.30±0.10	0.62±0.35	0.50±0.16	1.01±0.58	0.02±0.00	0.02±0.01
SsD	0.40±0.06	0.67±0.31	0.65±0.08	1.06±0.51	0.002± 0.00	0.01±0.00
SsFT	0.18±0.21	0.32±0.00	0.28±0.31	0.51±0.59	0.02±0.00	0.003±0.01
SsC	0.41±0.05	0.45±0.00	0.57±0.10	0.65±0.01	0.02±0.00	0.01±0.02
SoD	0.34±0.23	0.75±0.36	0.54±0.37	1.22±0.59	0.003±0.00	0.01±0.01
SoC	0.73±0.07	1.31±0.89	0.93±0.03	1.73±5.79	0.05± 0.02	0.07±0.06

Pigment quantification results, as shown in (Table 5), generally exhibit higher extraction efficiency in SB compared to AH.

Although chlorophyll a showed significant differences ($p < 0.05$) on the extraction method in the analysis of variance, in the post hoc analysis, no significant differences were shown when comparing multiple and individual samples (Figure 11). The results by the spectrophotometer method of Wellburn, (1994), showed the % chlorophyll a was 0.30 ± 0.10 % in SsP; 0.40 ± 0.06 % in SsD; 0.41 ± 0.05 % in SsC; 0.34 ± 0.23 % in SoD, extracted with AH. While it was 0.62 ± 0.35% in SsP; 0.67 ± 0.31 % in SsD; 0.45 ± 0.00 % in SsC; 0.75 ± 0.36 % in SoD, extracted with SB. The highest amount of chlorophyll a was in the SoC sample, obtaining a percentage of 1.31 ± 0.89 mg per $100\,mg^{-1}$ DW by SB, while with AH, it was obtained 0.73 ± 0.07 mg per $100\,mg^{-1}$ DW. The lowest amount was shown by SsFT, in which, only 0.18 ± 0.21 mg per $100\,mg^{-1}$ DW was quantified using AH, and 0.32 ± 0.00 mg per $100\,mg^{-1}$ using SB.

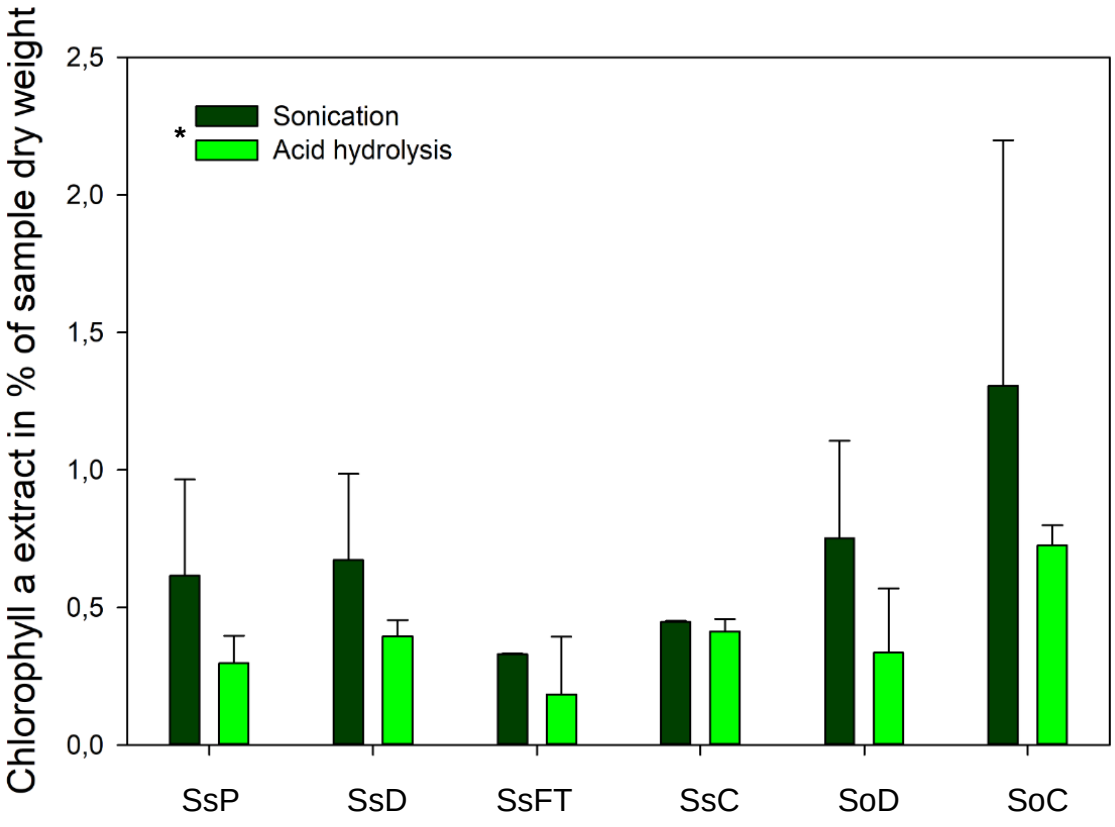

Figure 11*:* Results of Chlorophyll a quantification by the spectrophotometer method of Wellburn, (1994), (A) after 2h of acid hydrolysis, and (B) after sonication 45 minutes, of the 6 different samples, olive oil equivalent. Quantities are expressed as percentage of the algae sample in dry weight (mean ± SD). The symbol (*) among the extraction techniques, indicates significant differences $p < 0.05$.

As shown in (Figure 12), as with chlorophyll a, chlorophyll b also showed no significant differences at the individual level between groups in the post hoc analysis, although it had shown significant differences ($p < 0.05$) on the extraction method in the analysis of variance test. The results are assimilated to those of chlorophyll a, showing a % of chlorophyll b of 0.50 ± 0.16 % in SsP; 0.65 ± 0.08 % in SsD; 0.57 ± 0.10 % in SsC; 0.54 ± 0.37 % in SoD, extracted with AH. While it was 1.01 ± 0.58 % in SsP; 1.06 ± 0.51 % in SsD; 0.65 ± 0.01 % in SsC; 1.22 ± 0.59 % in SoD, extracted with SB. The highest amount of chlorophyll b was another time in the SoC sample, obtaining a percentage of 1.73 ± 5.79 mg per $100\,mg^{-1}$ DW by SB, while with AH, it was obtained 0.93 ± 0.03 mg per $100\,mg^{-1}$ DW. The lowest amount, as with chlorophyll a, was

shown by SsFT, in which, only 0.28 ± 0.31 mg per $100\ mg^{-1}$ DW was quantified using AH, and 0.51 ± 0.59 mg per $100\ mg^{-1}$ using SB (Figure 12).

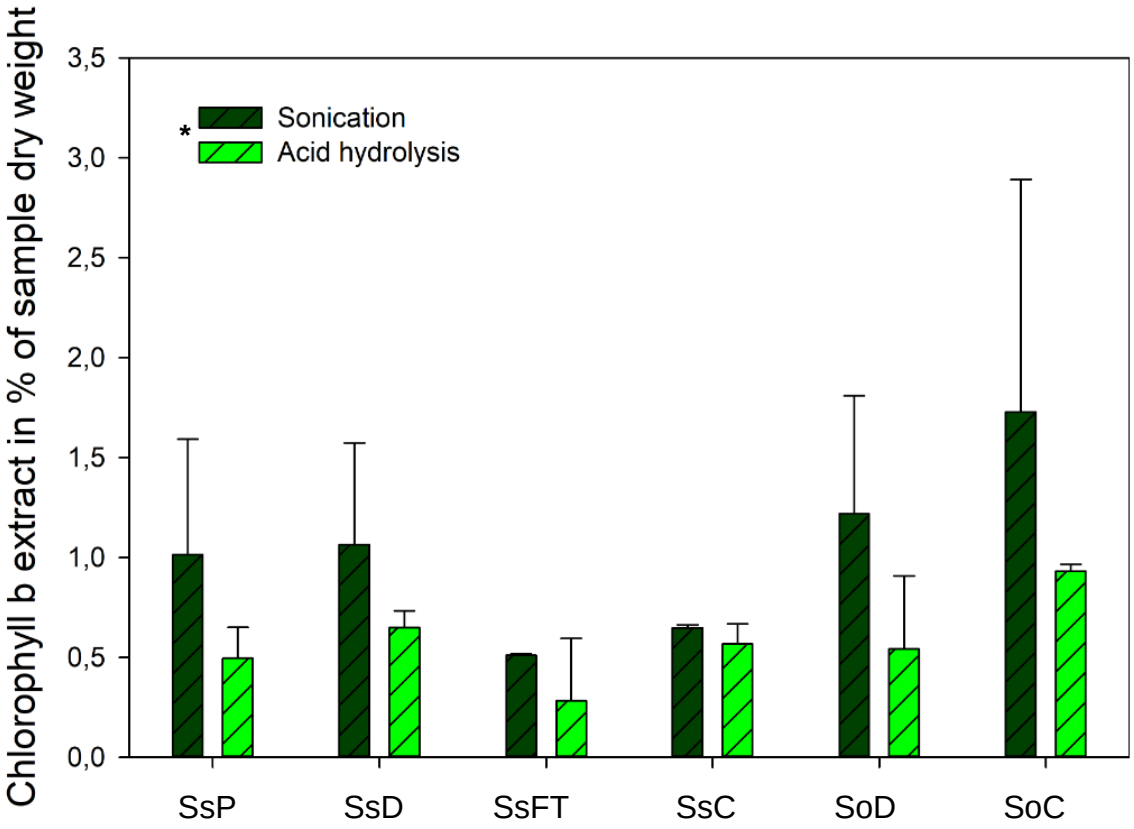

Figure 12*:* Results of Chlorophyll b quantification by the spectrophotometer method of Wellburn, (1994), (A) after 2h of acid hydrolysis, and (B) after sonication 45 minutes, of the 6 different samples, olive oil equivalent. Quantities are expressed as percentage of the algae sample in dry weight (mean ± SD). The symbol (*) among the extraction techniques, indicates significant differences $p < 0.05$.

The carotenoid quantification assay showed no significant differences in any of the groups in the test of variance (Figure 13). The sample with the highest amount of carotenoids was SoC, which showed a percentage of 0.07 ± 0.06 mg per $100\ mg^{-1}$DW using SB as the extraction method. This sample also showed the highest number of carotenoids extracted by AH, with an amount of 0.05 ± 0.02 mg of carotenoids per $100\ mg^{-1}$DW. The amounts of carotenoids extracted by the SB method were 0.02 ± 0.01 % in SsP, 0.01 ± 0.00 % in SsD, 0.01 ± 0.02 % in SsC and 0.01 ± 0.01 % in SoD, and the lowest amount was shown by the sample SsFT with 0.003 ± 0.01 %. The lowest number of carotenoids was shown by the sample SsD extracted by AH, which

showed a percentage of 0.002 ± 0.00 mg per $100\,mg^{-1}$DW. With AH the amounts of carotenoids extracted were 0.02 ± 0.00; 0.02 ± 0.00; 0.02 ± 0.00 and 0.003 ± 0.00 mg of carotenoids per $100\,mg^{-1}$DW, for SsP, SsFT, SsC and SoD, respectively.

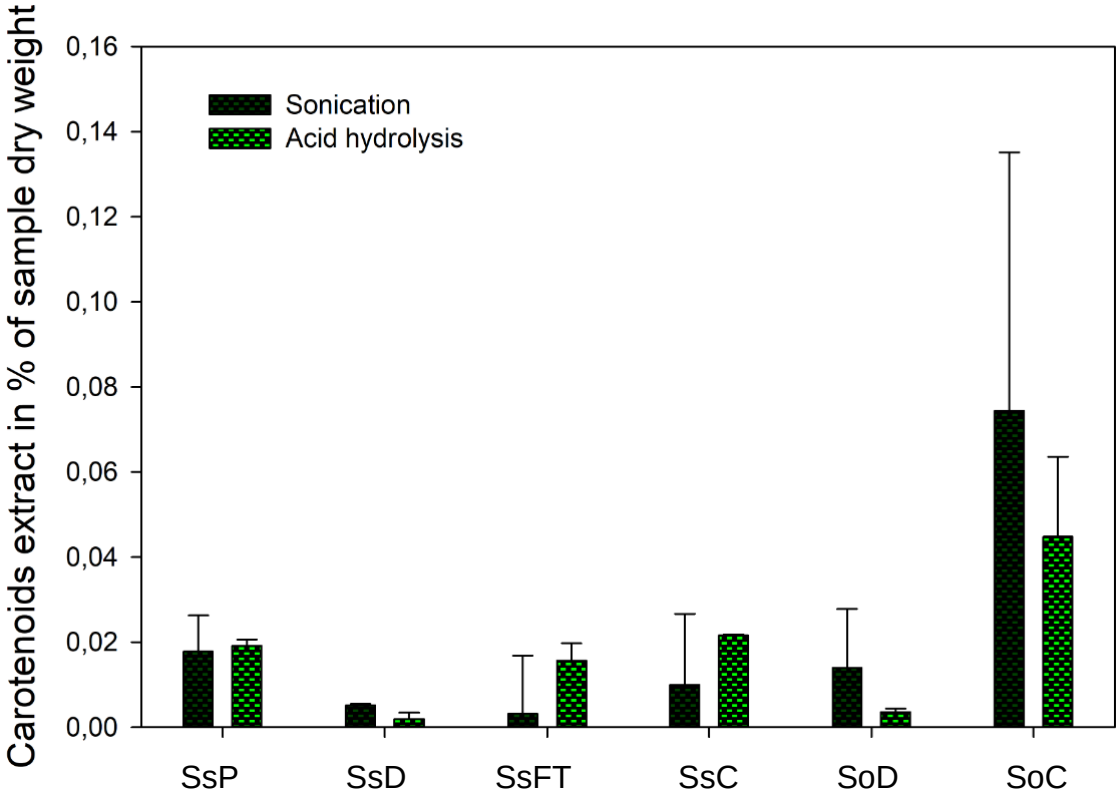

Figure 13*:* Results of total carotenoids quantification by the spectrophotometer method of Wellburn, (1994), (A) after 2h of acid hydrolysis, and (B) after sonication 45 minutes, of the 6 different samples, olive oil equivalent. Quantities are expressed as percentage of the algae sample in dry weight (mean ± SD).

5. Discussion

In the face of the growing need for renewable energy, microalgae present great potential, due to their rapid growth, high biomass productivity and their ability to use sunlight as an energy source. However, commercial production is hindered mainly by two well-known bottlenecks: a) harvesting the algae and b) extracting high-value compounds from the cells (Canelli et al., 2021). The *Scenedesmus sp.* UCV identified in our laboratory presented advantages for the first bottleneck; a) algal harvesting; because of its ability to self-aggregate. Similarly, some *Scenedesmus* strains have been previously reported to change their behavior by showing a tendency to self-flocculate (Chen et al., 2020a; Dias et al., 2021; Kumar et al., 2021). However, further exploration is needed to identify the specific genes responsible for the self-flocculation process in microalgal cells. Understanding the genes involved in this trait is crucial for the development of genetically improved strains that exhibit controlled flocculation, which will ultimately benefit the microalgal biorefinery. The advancement of microalgal genetic engineering holds promise for achieving this goal (Fayyaz et al., 2020). Some of the compounds that may influence this self-flocculation are found inside the cell and may be bound to membranes and/or the cell wall.

Cell wall structure varies greatly between species, as well as being influenced by environmental conditions and life cycle stage (Bernaerts et al., 2018). An important criterion when selecting strains for mass culture is a robust cell wall in order to survive the strong stresses associated with mixing in open ponds and photobioreactors (Stirk et al., 2020). Nevertheless, the presence of a resistant cell wall poses a challenge to extract compounds from microalgae, as it hinders the penetration of organic solvents into the cell and restricts the extraction of intracellular compounds (Bernaerts et al., 2018). Typically, the thickness of microalgal cell walls can vary depending on the growth phases and environmental conditions. As microalgae enter the stationary growth phase and experience increased stress, they appear to respond by producing thicker cell walls. Previous studies have documented the capacity of *Scenedesmus sp*. to thicken its cell wall as the culture matures (Spain & Funk, 2022). They observed that on day 5 of exponential growth, it showed a mean cell wall thickness of 0.11 μm, while on day 10, it reached a mean cell wall thickness of 0.20 μm. In comparison, other microalgae, such as *Chlorella vulgaris*, only reached a thickness of 0.11 μm on day 10 of growth. Bensalem et al. (2018) observed that, during the exponential growth phase, *Chlamydomonas reinhardtii* microalgal cells that are not under stress typically have single-layer cell walls. However, when exposed to prolonged stress, most microalgal cells develop cell walls consisting of up to three layers. However, in spite of thicker cell

walls are generally expected to present challenges in subsequent processing, it was observed that older algal cultures with multiple-layered cell walls exhibited a higher capacity for lipid extraction compared to younger cultures with fewer layers in their cell walls. The occurrence mentioned implies that the cell wall of microalgae may become more susceptible during the stationary phase. Interestingly, the microalga *Tetraselmis sp.* DS3, characterized by a thinner cell wall, proved to be more susceptible to breakage by high-pressure gases compared to the microalga *Desmodesmus sp.* F2, which had a thicker cell wall (Yong et al., 2020). Knowledge of the components of these layers will allow us to target these compounds and break the cell wall, thus facilitating the extraction process (Mayeli et al., 2005). *Scenedesmus* species, which belong to the order *Chlorococcales*, possess a unique cell wall compared to other orders. These microalgae can exist as individual cells or form coenobia consisting of 4 to 16 cells during later stages of their life cycle. To facilitate coalescence within the coenobium, the cell walls develop a pectic layer that separates each individual cell. Interestingly, the outer surface of this pectic layer may exhibit occasional ornamentations such as spines, teeth, and bristles. It has been proposed that these spines could serve as a defense mechanism against grazing by zooplankton. (Spain & Funk, 2022).

Scenedesmus sp. typically exhibits a cell surface composition characterized by elevated protein levels, relatively lower carbohydrate content, and a higher proportion of lipids compared to polysaccharides. The increased carbohydrate content in this species can be attributed to the presence of tooth-like structures and spines on the outer surface of the cell, which are embedded within an apectic layer. Studies have revealed that this apectic layer is composed of glycoproteins, contributing to the carbohydrate component that generally is observed in the cell surface of *Scenedesmus sp.* (Spain & Funk, 2022). During a period of nitrogen starvation, such as that occurring in two-stage cultivation, protein content decreased, while lipid and carbohydrate content increased, because microalgae carbon is allocated to energy-rich compounds, generating competition between lipid synthesis metabolism and starch synthesis metabolism (Ho et al., 2012). The cell wall of green microalgae can be divided into two groups, namely a low resistant cell wall or a highly chemical resistant cell wall. *Chlorella* and *Scenedesmus* species have a highly resistant cell wall (Dunker & Wilhelm, 2018). The more resistant the cell wall of the algae, the less components will be extracted, or the more energy will be needed to extract them (Afify et al., 2018). The observed lack of significant differences in the culture system despite the successful color change from green to orange in the microalgal samples subjected to the two-

stage culture could be attributed to the excessively thickened cell wall of cultures exposed to stress factors like nutrient deficiency. This thickened cell wall may have hindered the effectiveness of lysis methods, making it challenging to extract the various cellular components from these samples. As a result, the extraction process may have been less efficient, leading to similar results in terms of the culture system, despite the noticeable color change.

Therefore, faced with a very thick microalgal cell wall, it is important to strike a balance between the effectiveness of the extraction method to reach all cellular components by crossing the barrier posed by the cell wall, and a non-aggressive extraction method that does not cause degradation or undesirable chemical changes in the products of interest. In this way, with *Scenedesmus sp.* UCV, a protocol for downstream process of biochemical compounds extraction tried to be optimized, aiming to address the second bottleneck; b) extraction of high value compounds from cells. At the beginning of the trials with this microalga, great difficulties were encountered in breaking the cell wall and thus accessing intracellular compounds. The most used technique for the *Scenedesmus* genus is enzymatic hydrolysis (Afify et al., 2018; Liyanaarachchi et al., 2021); Nevertheless, due to the availability of means in our laboratory, the techniques shown in the Table 3 were tested. Acid hydrolysis (AH) and sonication bath (SB) demonstrated the higher *Scenedesmus sp*. UCV cell wall rupture capacity. However, despite the efficacy of these techniques, subsequent quantification of the cellular components of proteins, lipids, phenolic compounds and carotenoids showed no significant differences between the two methods. This observation raised the possibility that we had reached a compromise between employing an aggressive extraction method for a very resistant cell wall and avoiding degradation or undesirable chemical changes in the extracted products. Kapoore et al. (2018) previously has reported alterations in bioactives compounds stereochemistry by the excess light, heat and oxygen to which they had been exposed due to the use of toxic solvents, such as hydrochloric acid,

Marine microalgae have a high protein content giving them a high nutritional value, specifically, they can have between 6% and 70% of their dry weight, and most of them have around 50% (Wang et al., 2021). For this reason, microalgae are acquiring a great interest in research for their use as a protein supplement in food, especially in developing countries. Some species have relatively low levels of protein, especially those selected for oil and biodiesel production. However, the genus *Scenedesmus* usually contains around 40.69% protein (Afify et al., 2018). The results of the composition of *Scenedesmus sp.* UCV are shown in Table 4. These results are shown

decreased compared to the values described in the literature for this order. Ultrasonication is a proven and popular method for microalgal cell disruption and may be the preferred method of disruption for protein extraction (Axelsson & Gentili, 2014). According with El-Chaghaby et al. (2019), the total protein content of *Scenedesmus* extracted by 2 h SB, corresponds to about 31.07%. Similarly, Kumar et al. (2021), reported a total protein content of 30 % dry weight (DW) in the self-flocculating microalgal strain *Scenedesmus sp*. NC1, estimated by the Lowry method from freeze-dried microalgal biomass, and with 30 min SB. These values are much higher compared to those shown by *Scenedesmus sp.* UCV strain, which only showed up to 14.59 ± 10.44 % for the SoC sample (Table 4). The limitation of nitrogen and sulfur in the medium causes a change in photosynthetic carbon partitioning, in which the synthesis of carbohydrates and starch is favored over the synthesis of proteins and chlorophylls (Liyanaarachchi et al., 2021). For this reason, in crops subjected to a metabolism.

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metabolism change protocol to favor lipid production, they may have a lower protein content.

In relation to the results provided in the quantification of carbohydrates, Kumar et al. (2021) previously reported a carbohydrate extraction of 21% with the self-flocculant microalgae strain *Scenedesmus sp.* NC1 subjected to 30 minutes in a sonication bath (SB) and quantified by the Dubois spectrophotometric method, which agrees with our SB results, which maintain a range of polysaccharides from 14.07 ± 8.52 to 21.04 ± 5.79 % (Table 4). Vieira et al. (2021) observed an extraction of 26.2 % of carbohydrates with the strain *Scenedesmus obliquus* BR003, extracted with 4 mol L^{-1} AH at 90 °C for 90 min. Nevertheless, with AH method, we were only able to extract up to 7.45 ± 4.51 % of the SsC sample, with the lowest amount of carbohydrate extracted being 3.24 ± 2.31 % of SoD (Table 4). The change in photosynthetic carbon partitioning due to nutrient limitation in crops subjected to the two-stage cultivation method favors carbohydrate and starch synthesis over protein and chlorophyll synthesis (Liyanaarachchi et al., 2021), so a higher number of polysaccharides should be found in orange crops (SsD, SsFT and SoD) compared to green crops (SsC and SoC). However, the results of the different cultivation systems employed showed no significant differences between the different cultivation systems used for each sample (Figure 8). On the other hand, the statistic did show significant differences between extraction methods, and also individually among the orange samples SsD, SsFT and SoD. This indicates that the difference in carbohydrate extraction between the two methods could be related to the composition of the cell wall of our variant strain and the changes that AH method may have caused in it. Members of the *Scenedesmus* family, such as *Scenedesmus obliquus,* are known to contain cellulose in the inner layers of the cell wall. However, no cellulose has been observed in the outer trilaminar layers of the cell wall that appear around the algal cells towards the stationary phase of growth. The cellulose content with advancing culture age could decrease at the expense of other polysaccharides or glycoproteins that contribute to the formation of rigid outer layers. For example, mannose formation, has been suggested as a self-protective mechanism that would help algal cells to survive and adapt to stressful environmental conditions (Voigt et al., 2014). A higher amount of polysaccharides in the cell wall of *Scenedesmus sp.* UCV in the orange cultures, and the sensitivity of these to AH, would explain the large decrease of glucose in the SsP, SsD, SsFT and SoD cultures extracted with AH method, with respect to the carbohydrates extracted by SB method. It would also explain that, contrary to SB, in AH method the highest extraction values are the green algae SsC and SoC (Figure 8). Although concentrated AH is often

employed as a method to extract carbohydrates because it allows the production of monosaccharides through depolymerization of storage (e.g., starch) and structural (e.g., cellulose and hemicellulose) polysaccharides, Vieira et al. (2021), already reported that harsh sulfuric acid hydrolysis conditions of microalgal biomass could degrade monosaccharides leading to undesirable by-products such as 5-(hydroxymethyl)furfural and acetic acid. This author recommends using a more dilute acid, such as 1.5 mol L^{-1} of sulfuric acid solution, at a less temperature.

The most commonly used method to quantify total lipids in microalgae is the gravimetric method of Bligh & Dyer, (1959); however, being a gravimetric method, it requires a larger amount of biomass to be reliable. Since sufficient biomass was not available, the spectrophotometric method of Chabrol & Charonnat, (1937) was used. Quantification of total lipids using the sulfo-phospho-vanillin reaction has been shown to result in a determination of lipid content in microalgae cells, similar to gravimetric analysis/gas chromatography (Byreddy et al., 2016). Kumar et al. (2021) reported a substantial lipid content of 28.3 % of the total DW, from the autoflocculating microalgal strain *Scenedesmus sp*. Our results by AH method showed 34.06 ± 19.36 % lipids extracted from SsC sample, and 32.45 ± 15.48 % lipids extracted from SoD sample by SB method (Table 4), suggesting that this strain shows a great potential to generate lipids. As seen, the two-stage culture method (Liyanaarachchi et al., 2021) produces a metabolism change in the algae with the main objective of increasing lipid production. That is not agree with our results, in which any significant differences were observed between the orange samples (SsP, SsD, SsFT, and SoD) and the green samples (SsC and SoC) in lipid quantification, as shown in Figure 9. It is worth noting that certain *Scenedesmus* strains have the ability to develop a rigid outer layer composed of a trilamellar alga-mannan structure. This structure contains algaenan, a highly aliphatic and non-hydrolyzable biomacromolecule found in microalgae. Algaenan plays a crucial role in protecting microalgae from water loss and abiotic stress, particularly in extreme environments (Permann et al., 2021). This highly acid- and base-resistant biopolymer containing high molecular weight long-chain saturated fats (Alhattab et al., 2019). The results observed with the HA method could be related to the development in this strain of a rigid external layer of algaenan, which could be interfering in the processes of cell lysis with HA, especially in the orange samples, in which cell wall supposed to be more thickness. Furthermore, Zych et al., (2022) showed that microalgae lacking algaenan accumulated significantly more protein than strains containing this biopolymer, so, the fact that our variant has an algaenan layer would also explain the low amount of protein shown by *Scenedesmus sp.* UCV with Lowry quantification.

Phenolic compounds act as free radical oxidation terminators and are recognized for their physiological, biological, and medicinal activity, receiving increasing attention in the areas of health, biology and food, especially in the search for natural antioxidants that can prevent degenerative diseases (Rojas & Buitrago, 2019). Among the solvents frequently used for polyphenol extraction are: ethanol/water, acetone, ethyl acetate, hexane or water. Nihal et al. (2006) showed that the best solvent for polyphenol extraction, and also, capable of preserving the biological activity of polyphenols showing better results in the subsequent antioxidant capacity assay, was 50% acetone solvent. Even though, later, Bulut et al. (2019), obtained the highest total phenolic content in *Scenedesmus sp.* with ethanol/water, with which an amount of 0.54 mg gallic acid 100 mg^{-1} DW was obtained. More recently, Silva et al., (2020) extracted a phenolic compound content of 1.96% from freeze-dried and sonicated *Scenedesmus obliquus* microalgae, using 80% acetone as extraction solvent. Subsequently, the same microalgae showed 1.12 g of total phenolic compounds in 100 g DW, using ethanol as extraction solvent (Silva et al., 2021). In comparison, our results of the polyphenols quantification with 50% acetone as extraction solvent (Figure 6), show a very high concentration of polyphenols, with concentrations of up to 6.05 ± 4.56 % in SoC with SB, and 5.47 ± 3.07 % with AH (Table 4). Therefore, it would be interesting to test the biomass for antioxidant capacity (e.g. with a DPPH assay), in order to verify the potential of this variant.

Studies have found that chlorophylls have physiological effects like antimutagenics and anticancer (Wu et al., 2021). The main photosynthetic pigment present in the biomass of *Scenedesmus obliquus* is chlorophyll a (Wiltshire et al., 2000). Previously, Vieira et al. (2021) reported an amount of chlorophylls a and b, in the biomass of *S. obliquus* BR003 of 0.71 % and 0.32 %, respectively, which agrees with our results, in which we observed a very efficient extraction and quantification of chlorophyll a and chlorophyll b, which showed up to 1.31 ± 0.89 % and 1.73 ± 5.79 %, respectively (Table 5). Especially better extraction was achieved with SB, since in all samples it shows a higher result of extracted product (Figure 11 and Figure 12), probably because of their susceptible to degradation by external agents, such as heat, low pH (acidic) and exposure to light, that AH could have caused. As mentioned above, nitrate limitation in the culture medium induces a change in photosynthetic carbon partitioning, favoring carbohydrate synthesis over chlorophyll synthesis (Liyanaarachchi et al., 2021). Even so, green samples SoC and SsC, showed no significant difference in terms of containing a higher amount of chlorophylls than orange algae (Figure 11 and Figure 12).

Beta-carotene is a compound that serves as a precursor to vitamin A and possesses antioxidant properties. It finds applications in the food industry as a natural colorant. Studies suggest that consuming beta-carotene through the diet may lower the risk of chronic and age-related diseases (Lin et al., 2018). In this way, Vieira et al. (2021) reported a number of carotenoids in *Scenedesmus obliquus* of 0.24 % quantified by the method of Wellburn (1994), after having subjected the biomass to SB. In contrast, our results of carotenoid quantification are very low, with both methods, AH and SB (Figure 13). Do Nascimento et al. (2021) observed that a limitation in the extraction of carotenoids, in addition to the barrier posed by the cell wall, is the association of some of these pigments, especially xanthophylls, with cell membrane proteins. Moreover, it is well known that carotenoids, as chlorophylls, are very susceptible to degradation by external agents, such as heat, low pH (acidic) and exposure to light, promoting color changes due to rearrangement or formation of degradation compounds such as cis-isomers, epoxides, short-chain products and, in some cases, volatile compounds (Zepka & Mercadante, 2009). Suggesting that either; 1) the cultivation method or, 2) the extraction methods used; are not suitable for obtaining carotenoids. Considering the lack of significant differences observed between the extraction methods and culture systems (Figure 13), it is advisable to explore alternative, more specific techniques for enhancing carotenoid production in *Scenedesmus sp.* UCV. In this respect, salinity is often used to increase carotenoid production (Figure 3). Although the compromise in biomass production due to a decrease in the growth rate of the microalgae, the application of this strategy, together with a two-stage culture approach, ensures the highest possible beta-carotene productivity. In the case of *Scenedesmus sp.* laboratory cultures for 7-days showed carotenoid values of 0.68%, while cultures subjected to salt-stress for 14-days showed carotenoid levels of 2.08% (Elloumi et al., 2020).

In summary, the economic viability of commercial cultivation of microalgae for bioactive compounds and biofuels is hampered by high production costs and low product yields, limiting cultivation to a few species capable of synthesizing valuable compounds that offset costs, or to robust strains that can thrive in low-cost, low-pollution culture systems. *Scenedesmus sp.* UCV studied under different growth conditions and extraction methods revealed its high lipid storage capacity and antioxidant potential. However, surprisingly, the two-stage culture system did not seem to be effective. This could be attributed to the lower efficiency of the lysis methods employed due to the higher cell wall thickness observed in the samples subjected to stress factors during their cultivation. It is possible that this particular strain has developed an algaenan polymer, which could explain its resistance to extreme culture conditions and

contamination, making it suitable for industrial cultivation in open-air tanks. The presence of this biopolymer in the cell wall also contributes to the difficulties encountered in cell wall disruption, so, it would also be interesting to analyze to what extent the time and energy required to break a very thick cell wall is interesting to work at an industrial level. Further research on the composition of the cell wall layers is recommended to better understand the *Scenedesmus sp.* UCV cell wall composition, in order to achieve more effective cell disruption techniques. In addition, the identification of cell wall genes encoding proteins responsible for self-flocculation would provide valuable information on the molecular mechanisms underlying this phenotype, allowing better control and selection of strains for specific biotechnological applications.

6. Conclusion

1. The two-step culture method used for *Scenedesmus sp.* UCV produced a phenotypic change (from green to orange color) indicating a possible biochemical change, however, the results showed no significant differences between any of the samples tested. This may be due to increased cell wall thickening in the stressed orange cultures, which has hindered the entry of extraction solvents, and thus the access and quantification of target compounds.

2. Analysis of different cell lysis techniques for being selected as pretreatment prior to the quantification of components, showed only three techniques that were able to break the cell wall of *Scenedesmus sp.* UCV, showing the best results for the acid hydrolysis (AH) method in 1N HCl, for 2h at 90 ºC, followed by the ultrasonic bath (SB) in ultra-pure water during 45 min, and finally the microwave in ultra-pure water, during 20 min, at 130 ºC.

3. Cellular components quantification in *Scenedesmus sp.* UCV show high extraction efficiency of carbohydrates, chlorophyll a and chlorophyll b components by SB method, while no significant differences were observed for proteins, lipids, polyphenols and carotenoids. Both methods, SB and AH, allowed an efficient extraction of chlorophyll a and b pigments, which showed up to 1.31 ± 0.89 % and 1.73 ± 5.79 %, respectively. However, they did not do so with carotenoids, of which it was only able to extract up to 0.07 ± 0.06 %. On the other hand, the strain shows low amounts of carbohydrates and proteins, which presented up to 21.04 ± 5.79 % and 14.59 ± 10.44 % respectively. In addition, it has a high capacity to store lipids and antioxidant potential, presenting up to 34.06 ± 19.36 % of lipids, and up to 6.05 ± 4.56 % of polyphenols.

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