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ORIGINAL ARTICLE

Evaluation of different fractionation methods for the simultaneous protein and carbohydrate extraction from microalgae

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Abstract
The production of high-value products from microalgae, one of the preferred emerging biorefineries' feedstocks, relies on the crucial step of biomass fractionation. In this work, the fractionation of *Chlorella vulgaris* and *Scenedesmus obliquus* biomass was tested for protein extraction using a wide range of physical, chemical, and enzymatic treatment combinations, including ultrasound, cell homogenizer, cellulase, and alcalase combinations in aqueous and alkali extraction conditions. The impact of these processes on biomass carbohydrates was also evaluated. Alkaline-assisted ultrasound treatments using alcalase presented the highest protein extraction yield, reaching 90 g/100 g protein on *C. vulgaris*, closely followed by the same treatment in aqueous conditions (85 g/100 g protein). The same aqueous treatment achieved the best performance on *S. obliquus*, reaching 82 g/100 g protein. All treatments on both microalgae partially solubilized the polysaccharide fraction with all alkaline treatments solubilizing over 50 g/100 g sugars for all conditions. Overall, all the treatments applied were effective methods for biomass fractionation, although they showed low selectivity regarding the individual extraction of protein or carbohydrates.

Keywords Biorefineries · Blue bioeconomy · Cell disruption · Pre-treatments · *Chlorella vulgaris* · *Scenedesmus obliquus*

1 Introduction

Microalgae are one of the most innovative and promising biomass sources that have been suggested for biorefinery applications, namely for the production of biofuels, e.g., biodiesel from microalgae oil [1]. However, microalgae potential is much wider, as they have also been reported as a viable source of bioproducts [2], namely of pigments [3–5], protein [6, 7], amino acids [6], and fatty acids [8] for food and feed applications. The microalgae sector has gained increasing relevance in the EU, as part of the European Green Deal that includes the use of microalgae as an essential facet of the Blue Bioeconomy in the European space [9].

Microalgae have a large range of potential high-value commercial applications, in sectors such as bioplastics and biomaterials [10] with nutraceutical applications, due to their high nutritional value [11]. Applications for the production of advanced biofuels have also gained increased interest, thereby granting microalgae an even more important role in climate change mitigation [12]. The first approaches to use microalgae in biorefineries were based on solvent extraction for isolation of their lipid fraction and subsequent conversion of bio-oil to biodiesel, while a protein and sugar-rich solid residue was left with little use. By itself, biofuel production from microalgae is not economically viable [13], and needs to be coupled with the production of other high-value products for the economic feasibility of the approach. Therefore, the most recent strategy is to target microalgae biomass towards the production of added-value products such as platform chemicals, biomaterials, and food and feed products, which may also be connected to biofuel production.

Microalgae have an incredible ability to accumulate different types of macromolecules, depending on their growth conditions, thereby presenting a versatility in composition that is virtually unparalleled elsewhere in the biosphere. Due to this versatility, growth conditions are very important as,

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for example, in nutrient-depleted microalgae, the protein content can drop significantly [14–16]. But this compositional diversity that grants microalgae a huge potential for the production of many bioproducts is not the only interesting aspect since they also have great wastewater treatment capabilities [17, 18]. Microalgae can be excellent bioremediators with high effectiveness in removing pollutants and heavy metals from aqueous biomes [19]. Also, as the cultivation of microalgae can be a very costly process, the use of low-cost culture media like wastewater can make microalgae more economically sustainable [20].

Microalgae are adequate to be used in feed, and chemical industries. When compared to the lignocellulosic biomasses commonly used in biorefineries, microalgae do not contain lignin, which is a technical advantage in the fractionation and handling of downstream processes. Also, they are an alternative protein source for food and feed as they can contain all essential amino acids [7, 21]. This amino acid profile is rarely found in terrestrial plants and can therefore become an integral part of non-meat diets that are increasingly popular in the Western world, providing a greener alternative to meat production, which is an important source of carbon emissions [13].

However, microalgae valorisation requires fractionation techniques to separate the most important components, mainly protein, and sugars, and the challenge is to find methodologies that are cheap and can provide selective fractionation. Simple, fast, and economical fractionation methods are required for microalgae that allow for the release and easy recovery of most protein [22], facilitate downstream processing [23], and also enable the separation and recovery of the sugar fraction.

In contrast to other molecules, such as starch and lipids that can be found in cell storage organelles, protein is present in almost every part of the cell and therefore can be easily removed by disrupting the cells and extraction with solvents. Microalgae commonly have rigid cell walls consisting of polysaccharides, mainly cellulose [24], and other polysaccharides containing xylose, mannose, and uronic acids, or trilaminar layers with pectin and glycoproteins in the middle layer. Algeanan is also a biopolymer that forms an outer layer in several microalgae species, conferring protection against degradation, hydrolysis, and mechanical disruption of the cells [25] which will make protein extraction more challenging. This compound is present in some Chlorophyta species such as *Scenedesmus* and *Chlorella* [25] that were studied in this work. In addition to algeanan, some species of the *Scenedesmus* genus also present a crystalline glycoprotein in the cell wall, which can further affect cell disruption and product recovery, and increase the difficulty in protein extraction. However, some proteins can be found freely in the cytosol like ribosomes, free enzymes, and other protein complexes, and are easily accessible through cell disruption. Although some advances have been made in the past decade, the study of algal proteins and their extraction remain a poorly researched topic, when compared to proteins from terrestrial crops [26]. Protein extraction can be a challenging endeavor, due to the complexity of the cell walls, which are in many cases interlinked with protein [27]. Therefore, protein extraction is mostly achieved in two steps, cell disruption and protein recovery [28]. This extraction may be enhanced by the use of some pre-treatments that cause cell wall degradation, and improve accessibility [29]; i.e., cell disruption techniques can be used for the extraction of intracellular components [7, 30, 31]. Many processes have been tested such as cell homogenization [32], ultrasonication [33, 34], microwave radiation [35], high-pressure cell disruption [36], and enzymatic lysis kits [37]. Some of these methods, such as high-pressure homogenization and enzymatic treatment, have been reported to achieve extraction yields of around 80–90% [38], although their commercial application for algal protein extraction remains limited, due to issues with up-scaling and operational costs [26]. Furthermore, the effectiveness of these techniques is highly dependent on the algae species, and therefore individual studies are vital to assess their application suitability for each case [26].

Although effective for protein extraction, the impact of these technologies on the sugar fraction is usually not mentioned, and it is still largely unknown, despite its importance. Also, studies focusing on carbohydrate fractionation are few and only achieve sugar recovery rates above 50% when using very high concentrations of acid catalysts and severe and potentially uneconomic conditions [39].

In this work, a new approach to microalgae valorisation was studied to attempt an effective protein and carbohydrate separation. For this purpose, multiple physical, chemical, and enzymatic methods were tested, both *per se* and in combination, including ultrasonication, cell homogenization, alkaline treatments, and cellulase and alcalase treatments for protein extraction from commercial *Chlorella vulgaris*. The most effective methods were selected and further validated on the microalgae *Scenedesmus obliquus* that was previously grown on secondary wastewater brewery's effluent. The impact of protein extraction methods on the carbohydrate fraction in both algae was also evaluated, thereby providing a first insight into the effect of these pre-treatments in the sugar extraction from algae.

2 Materials and methods

2.1 Microalgae cultivation and harvesting

Chlorella vulgaris biomass was obtained from Allmicroalgae (Pataias, Portugal), in freeze-dried form. *Scenedesmus obliquus* (ACOI 204/07, Coimbra University Culture

Collection, Portugal), obtained from the LNEG culture bank, was cultivated in 70-L vertical acrylic column photobioreactors. This microalga was grown in brewery secondary effluent (Table 1) provided by Central de Cervejas, SA (Vialonga, Portugal) in batch mode, as described [39]. Microalgae were harvested in the exponential growth phase and concentrated using an in-house developed centrifugation methodology with a continuous dairy centrifuge (electric cream separator, Alfa Laval, Sweden) [39]. The centrifuged biomass was washed with distilled water and centrifuged again to obtain a concentrated paste with 90% moisture content. This paste was frozen (−18°C) and then freeze-dried for further use (Thermo Scientific Heto PowerDry LL3000, USA).

2.2 Pre-treatment processes

All pre-treatments and their combinations were carried out in duplicate.

2.2.1 Aqueous extraction

For aqueous protein extraction, 0.5 g of freeze-dried microalgae (*C. vulgaris* and *S. obliquus*) were weighted to 50-mL closed flasks and suspended in 20 mL of distilled water (physical extraction) or buffer (enzymatic extraction) and incubated in an orbital shaker (Comecta; Spain) at 30 °C, 250 rpm for 2 h. After incubation, the obtained suspension was subjected to further treatments of ultrasonication or cell homogenization, as listed below.

2.2.2 Ultrasonication

Sample ultrasonication was carried out using an ultrasound probe (Sonics vibracell, USA) with a treatment time of 2 min in cycles of 5-s pulse and 15-s resting time. These conditions were chosen after an optimization of ultrasonication conditions determined by chlorophyll extraction yield. During the treatment, an ice bath was used to prevent sample overheating and degradation that can normally occur in this process [37].

Table 1 Physical and chemical composition of the brewery effluent used for the growth of *Scenedesmus obliquus* (adapted from [39])

	Parameter	
Chemical	pH	7.13
	Total solids (mg/L)	2690
	Ash (mg/L)	1760
	Total Kjeldahl nitrogen (mg/L)	5.6
	COD (mg/L)	5376
	Total oligosaccharides (mg/L)	11.40
	Glucose (mg/L)	22.07
Elemental composition of dry solids	C (% dry weight)	11.83
	H (% dry weight)	0.85
	N (% dry weight)	<0.3

COD chemical oxygen demand

2.2.3 Cell homogenization

Cell homogenization was carried out using a Rotor Stator disperser homogenizer (Turrax-type homogenizer, Atomixmill, Germany) for 30 s.

2.2.4 Enzymatic treatment

Sample preparation for enzymatic treatment was similar to the aqueous process, except that water was replaced by an adequate buffer. For samples treated with cellulase mixture (i), sample hydrolysis was carried out using 0.1M citrate buffer (pH 5.0). A Cellic® CTec2 (Novozymes, Denmark) enzyme cocktail (199 FPU/ml) was used in a ratio of 0.5 mg/g biomass, and samples were incubated at 50°C and 250 rpm for 16 h.

For samples tested with alcalase (ii), the treatment was carried out using 0.1 M phosphate buffer (pH 8.0). Alcalase 2.4L (Sigma-Aldrich, Germany) was used in a ratio of 0.5 mg/g biomass, and samples were incubated at 60°C and 150 rpm for 16 h. These enzymatic processes were tested both before (for *C. vulgaris*) and after physical treatment (for *C. vulgaris* and *S. obliquus*).

2.2.5 Alkaline pre-treatment

The samples that were treated as above described were subjected to chemical protein extraction. For this, sodium hydroxide (2 M) was added until pH 12 was reached. Samples were then incubated in similar conditions as described in Section 2.2.1, except that temperature was 40°C.

2.2.6 Pre-treated biomass separation and protein precipitation

Pre-treated samples were centrifuged (Ortoalresa digicen 21 R, Spain) at 5300 *g*, and 15°C for 10 min to separate the pre-treated biomass biomass pellet from the supernatant. The supernatant was treated with an HCl 0.1 M solution to decrease pH to 2.5. The protein precipitate obtained was separated by centrifugation, in the same conditions as described above. The biomass pellets and protein precipitates were freeze-dried for further analysis.

2.3 Analytical methods

Throughout the work, for all experiments, deviations were typically lower than 5%.

2.3.1 Quantification of extractives

Extractives content of microalgae (*C. vulgaris* and S. *obliquus*) was determined by sequential solvent extraction in Soxhlet using dichloromethane, ethanol, and water, using a modified method based on TAPPI-T204 cm-97 [40] and NREL/TP-510-42619 protocols for lignocellulosic biomass [41]. For this determination, 2 g of sample was weighted into extraction cartridges, inserted into a 125-mL Soxhlet apparatus, and extracted with 190 mL of solvent. The extraction times were 6 h for dichloromethane and 18 h for both ethanol and water. Extractives solubilized by each solvent were calculated by solvent evaporation in relation to the total dry mass.

2.3.2 Moisture and ash content

The moisture content was determined by oven-drying at 105°C to constant weight and the ash content was determined by incineration at 550°C for 16 h using NREL/TP-510-42622 protocol [42].

2.3.3 Protein quantification

Nitrogen content of microalgae biomass and of both supernatant and pellet obtained after protein extraction was determined using the Kjeldahl method [43] and a semiautomatic protein analyzer (Tecator, Sweden). Protein content was calculated using a conversion factor of $N \times 4.78$, as indicated for algae [44].

2.3.4 Carbohydrate analysis

Extracted and non-extracted biomass samples were subjected to quantitative acid hydrolysis using 72% (w/w) H_2SO_4 followed by hydrolysis with 4% (w/w) H_2SO_4 at 121°C in an autoclave for 1 h, according to NREL protocol for algae [45]. The acid-insoluble residue was determined by filtration using 1.22 µm glass fiber filters (VWR, USA), after correction for ash (incineration at 550°C for 16 h).

The monosaccharides in the hydrolysis liquor (glucose, mannose, xylose, galactose, and arabinose) were analyzed in an HPLC system (Agilent 1100 Series, Waldbronn, Germany), equipped with a refractive index (RI) detector and a diode array detector (DAD). An Aminex HPX-87P column (Bio-Rad, Hercules, USA) in combination with a cation Pb^{2+}-guard column (Bio-Rad) was used. The column temperature was 80°C, and water was used as eluent at a flow rate of 0.6 mL/min [46]. Samples were previously neutralized, when needed, using barium hydroxide or a combination of Amberlite® MB-20 resin (Sigma-Aldrich, USA) and calcium carbonate [39]. All samples were filtered through 0.22 nylon membrane filters (VWR, USA) before HPLC analysis. The percentage of polymeric sugars was calculated according to the methodology described in [39].

Sugars' solubilization was calculated based on the composition of the residual solid obtained after the protein extraction treatments, according to the equation:

$$\text{Solubilized Sugars} = \frac{S_I - \left(SY \times S_{sol}\right)}{S_{sol} \times 100} \quad (1)$$

where S_I corresponds to the biomass initial sugars, S_{sol} to the sugars in treated solids, and SY to the solid yield obtained with the treatment.

3 Results and discussion

3.1 Biomass composition

Two microalgae were used to evaluate the effects of different treatments on the protein and carbohydrate fractions of the biomass. *Chlorella vulgaris* was used as benchmarking due to its commercial relevance and availability, and for being a widely studied species. Therefore, it was used as a model in a more extensive set of test conditions, allowing screening to provide optimization to be further applied to *Scenedesmus obliquus*.

Table 2 shows the chemical composition of the microalgae used in this study. The protein content found on *C. vulgaris* is high, similar to those previously reported for this species [34, 47, 48], and in agreement with the commercial analysis certificate (provided by Allmicroalgae). The protein content found is only slightly lower than that of other species like the marine microalgae *Dunaliella salina* (57%) and the cyanobacteria *Spirulina platensis* (55.8%) [38, 48].

The high protein content of *C. vulgaris* suggests that this species is a highly viable source of this nutrient that can be potentially fractionated and further upgraded. Therefore, it is a suitable biomass for an extended battery of protein extraction tests.

S. obliquus protein content was 25.4%, a lower value than that previously reported for this [38] and other microalgal genera, e.g., *Chlorella*, *Dunalliella*, and *Porphyridium* [14, 21, 34, 48]. This depends on the microalgae species but can also be attributed to the low nitrogen content of the culture medium [49]. In contrast to *C. vulgaris*, which has a quite low sugar content (6.9%), *S. obliquus* contains a much higher sugar content (16.0%), making it an interesting source of both protein and sugars.

The lipid fraction as given by the dichloromethane extractives [50, 51] was 2.8% and 5.3% for *C. vulgaris* and *S. obliquus*, respectively. For protein production and to facilitate downstream purification, this lipid content could be lowered with a small nitrogen supplementation, since

Table 2 Chemical characterization of the microalgae *Chlorella vulgaris* and *Scenedesmus obliquus* used in this work

Biomass component (g/100 g dry weight)			*C. vulgaris* (this work)	*S. obliquus* (adapted from [39])
Protein			44.73	25.40
Carbohydrates	Glucose*		2.13	8.69
	Xylose		ND	1.79
	Arabinose		0.74	0.71
	Galactose		2.97	3.57
	Mannose		1.05	1.55
	Total sugars		6.88	16.30
Extractives	Dichloromethane		2.79	5.33
	Ethanol		8.61	11.75
	Water		27.74	17.45
		Protein**	12.53	8.05
		Carbohydrates**	2.29	5.50
Ash			11.36	15.78

ND not detected

*From which, 0.74% for *C. vulgaris* and 1.32% for *S. obliquus* correspond to starch

**Determined in water extractives

nitrogen-limited environments boosted the production and accumulation of lipids in other microalgal species [15].

Although ash content is not often reported for microalgae, the values found for both biomasses were slightly higher than the desired values. However, the ash content of *S. obliquus* was obtained after washing with distilled water during the harvest process which led to a 10% reduction in ash content. This value could be further lowered by using additional washing steps or by the use of acid buffer solutions (around pH 4), such as citrate buffer.

3.2 Protein extraction

Different fractionation methods mainly targeted for protein extraction including ultrasound, homogenization (dispersion), alkali, and enzymatic (cellulase and alcalase) treatments were used to break cell walls and release protein. Some combinations of these treatments were also tested to evaluate their effect on protein extraction.

Figure 1 shows the effect of the extraction process or combination of processes on the protein extraction yield from *C. vulgaris*, under aqueous conditions only (panel A), and in aqueous conditions followed by alkali treatment (panel B). Data are shown as the percentage of protein in relation to the initial biomass protein obtained in the precipitate and in the corresponding liquid fraction.

Overall, alkaline-assisted treatments had a better performance on protein extraction, as protein recovery yield can be enhanced in alkaline conditions [47].

In aqueous conditions (panel A), the treatments were compared with an aqueous control. It has been reported that in microalgae, a significant fraction of the protein can be found free inside the cell walls [14]. Moreover, it has been documented that water can be an excellent solvent for protein extraction when compared with other solvents such as ethanol and methanol [23, 52], which can cause protein denaturation [53], and facilitate its downstream processing. Therefore, the aqueous treatment only (control) can be a good benchmark for comparing with other treatments. It amounted to 34%, slightly higher than the water-extractable fraction determined in the chemical characterization (Table 2). The extraction yield above this value suggests the impact of each treatment on cell disruption and in the desegregation of protein from the cell structure and other macromolecules, making the protein available for recovery.

This protein out-diffusion from the cells by aqueous extraction was higher than that previously reported for this microalga [34]. This difference could be attributed to cell fragility derived from the manufacturing process, since the sample was from an industrial source, and commonly employed processes like freeze-drying have been reported to have some positive effect on protein extraction [54].

Overall, when comparing the aqueous treatments, only 5 of the assays had yields close to the control, with a maximum increase of 15%. All the others amounted to yield increases ranging from 34 to 52%. Regarding the physical non-combined treatments, the turrax-type homogenizer yielded the lowest protein recovery, only 10% higher than the aqueous control. This physical method is based on the

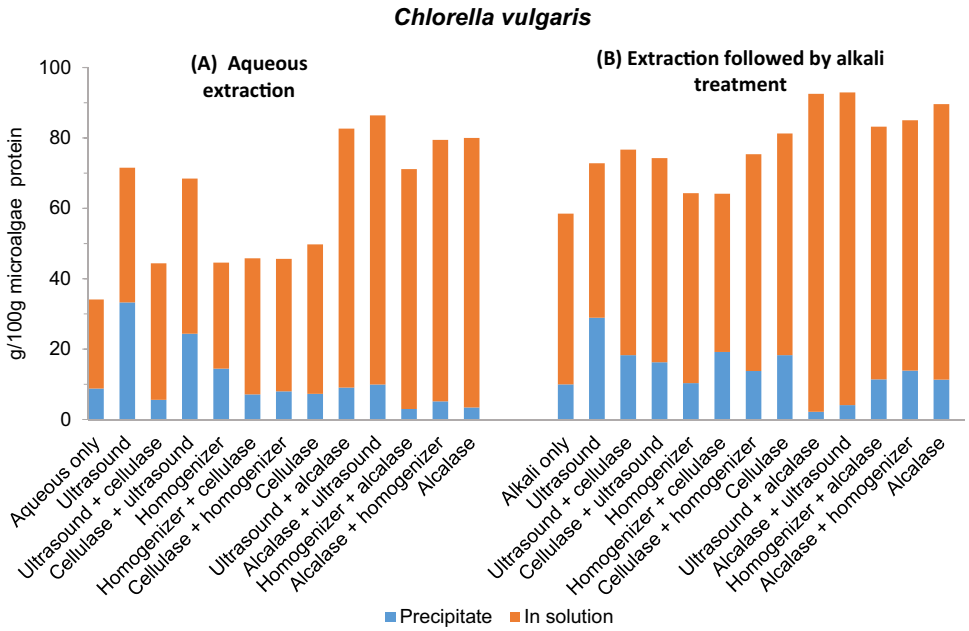

Fig. 1 Protein extraction yield in the precipitate and in solution obtained with all the different process conditions applied to *Chlorella vulgaris*

rotor-stator principle and is commonly used in disrupting microalgae for lipid extraction [55] or carotenoids [56]. In contrast, the application of ultrasound treatment resulted in one of the highest aqueous protein recoveries, with 71% of total protein. This process does not rely on mechanical aspects but on the application of ultrasonic waves that causes cavitation of the cell walls. As such, it can easily affect a wider number of cells and effectively disrupt cell walls and cell membranes, causing the release of intracellular components [57]. This method has been reported as effective for cell disruption and protein recovery [58].

Enzymatic treatments are highly dependent on the composition and complexity of the cell wall [59]. Therefore, two different enzyme cocktails were tested, with cellulase affecting cell integrity by breaking down cellulose, and alcalase disrupting by targeting the cell wall proteins. Compared to cellulase, alcalase was much more effective, extracting 80% of the initial protein, mostly in the non-acid precipitable form. Furthermore, due to the proteolytic capabilities of alcalase, these will most likely be in peptide form [59]. These peptides can have interesting high-value applications as protein supplements and in nutraceutical applications [59], making this process not only an effective method for protein extraction but also for simultaneous protein processing. On the other hand, if the functional properties of the protein are important for its further application, this method is not adequate [59]. The use of cellulase resulted in only 15% higher extraction than the aqueous control. This can be explained by the presence of carbohydrate polymers like algeanan, which provide cell wall stability and protection [25], making them more resilient against non-specific enzymes [60].

Several combinations of these treatments were also tested. In cellulase-assisted treatments, the protein yields obtained were quite similar compared to their non-enzymatic counterparts. In alcalase-assisted treatments, the enzyme impact was much higher, leading to an increase of 11–15% in ultrasound treatments and 27–35% in homogenizer treatments. It has been suggested that combining ultrasound with other treatments makes a strong boost in protein extraction for some microalgal species [61]. However, the boosting effect in ultrasound-based treatments was low, suggesting that adding enzymes to the ultrasound treatment might not be cost-effective. This is even more pronounced when comparing the ultrasound combined with the pure alcalase treatment, where the yield increase was just 2–6%.

The application of the enzyme cocktails either before or after the physical treatment was also tested. It only had a slight boosting effect (5%) when the enzyme was applied before the physical treatment. Overall, the highest recovery yield was 86 g/100 g total protein when using alcalase followed by ultrasound.

After all the treatments under aqueous conditions, alkaline conditions were also tested (Fig. 1(B)). By itself, the alkaline extraction resulted in a 24% boosting effect, when compared to the aqueous control, with a protein extraction of 58.5% of the total protein. This was higher than previously reported for this microalga [34]. In the case of alkali treatments, six were similar to the alkali control, with a maximum yield increase of 18%. All the other conditions yielded a 23 to 35% higher protein increase. Although the alkaline treatment only led to better results than the aqueous treatments using only homogenizer and cellulase, it still did not

outperform the use of alcalase, which was suggested as a cheaper method [62].

When the alkali process is applied after other treatments, the protein yield was increased in all of the conditions, between 12 and 30%, and more notorious for the least effective aqueous treatments (cellulase and homogenizer). This boosting effect was milder in the other treatments. Overall, the application of alkaline extraction decreased the difference between the extraction yields obtained (59% in the lowest and 92% in the highest), compared with the yields verified in the aqueous extractions (between 34% and 86%). Overall, the highest protein recovery was obtained for the ultrasound treatments using alcalase, followed by an alkali treatment, by which a recovery of 93 g/100 g total protein was obtained. These results are in agreement with previous findings stating that this chemical treatment is effective for protein recovery [34, 54].

Most of the protein recovered was obtained in solubilized form. The highest yield of protein recovered as precipitate was 33.3% of the total protein obtained for the aqueous ultrasound treatment, higher than the 29% obtained with its alkali counterpart. The applied acid precipitation method was selected as it is a cheap and simple method for protein recovery [20], although it was not very effective in this case.

A better option for protein recovery could be membrane separation avoiding the precipitation step, which would increase the protein extract purity [63]. Nevertheless, acid precipitation has several advantages as compared to other methods such as salts or ethanol, as these could bring difficulties in the downstream process or contamination by undesired compounds in the precipitate [64]. The purity of the precipitates was also determined (% of protein in the total precipitate) and found to be up to 60% for most conditions. On average, the purities were 10% higher on aqueous treatments than on their alkali counterparts. These values indicate protein fragmentation into small peptides, inhibiting their precipitation by acid. Also, the isoelectric point of the protein from this particular case can differ from the pH 2.5 applied, which has been suggested for similar species [65].

The most effective treatments for protein extraction from *C. vulgaris* were then applied to *S. obliquus.* These included both enzymatic and ultrasound treatments. The results obtained are shown in Fig. 2. As alkaline treatments in *C. vulgaris* had a masking effect on the impact of the physical treatments when used in combinations, only ultrasound was tested for comparison. The control aqueous extraction yielded 11% of total protein extraction, a lower value than that obtained for *C. vulgaris.* This might indicate a higher resistance of this microalga, not reaching the 32% water-extractable protein found in the chemical characterization (Table 2). This could be attributed to the duration of the treatment (2 h) when compared to the 16 h of water extraction for chemical characterization. Alkaline extraction yielded an increase of 30% in relation to the aqueous counterpart, higher than for *C. vulgaris* but still only reaching 42% of total protein recovery. Ultrasound treatment provided a yield of 38.6 g/100 g total protein, with the precipitated fraction representing 58% of the total extracted protein, the highest value found for all treatments. However, this result was nearly half the reported in other studies [37], for protein-richer *Scenedesmus* strains. The alkaline version of this treatment provided a very mild yield increase of 7%, but the precipitated fraction dropped significantly to 22.6%, indicating a severe impact of the alkaline conditions on the structure of protein and its acid precipitation capabilities.

When using enzymes, the combination of ultrasound treatment and cellulase is virtually ineffective, providing a similar extraction yield as the ultrasound treatment only. This does not happen for alcalase, which is highly effective both by itself and in combination with ultrasound, reaching yields of 78% and 82%, respectively. These results suggest that contrary to *C. vulgaris*, *S. obliquus* is more resistant to

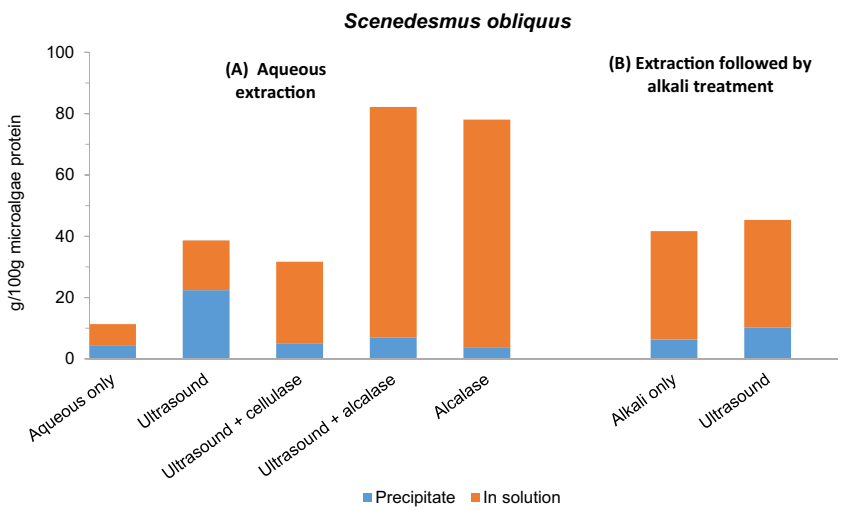

Fig. 2 Protein extraction yield in the precipitate and in solution obtained for all the different process conditions applied to *Scenedesmus obliquus*

physical treatments, but it is highly vulnerable to proteolytic enzymes which can be a powerful tool in the fractionation of this biomass.

3.3 Carbohydrate extraction

The effect of protein extraction processes on carbohydrate fraction is generally neglected in studies dealing with protein extraction from algal biomass [29, 34]. In this work, the effect of the different protein extraction processes on sugar solubilization was also evaluated. Figure 3 shows the data obtained regarding the potential total sugar extraction and the corresponding monomeric sugar composition is given in Figs. 4 and 5. Aqueous control resulted in a sugar extraction of 45% of the total algae carbohydrates, 12% more than the 33% total water-soluble sugars measured by Soxhlet extraction, in the biomass characterization (Table 2). In general, aqueous treatments had a lower effect on sugar solubilization than treatments followed by alkaline conditions. These showed a very pronounced effect, with a minimum sugar solubilization of 59.8% for alkali treatment only, and a maximum of 91.4% for the ultrasound and alcalase treatment with the same alkaline step. This was expected as alkaline treatments are known to have an important impact on hemicellulose solubilization in lignocellulosic materials [66].

The sugar yields obtained for alkali and aqueous extractions exceed both hydrothermal and alkaline pre-treatment results previously reported for this microalgae species [67]. Also, the sugar solubilization obtained in these mild conditions was similar [68] or much higher [69] than those reported for dilute acid hydrolysis of macroalgae in much more severe conditions. In microalgae, carbohydrates can be found mainly in starch grains, as glycolipids in intracellular membranes, and in cell walls as part of the structural matrix

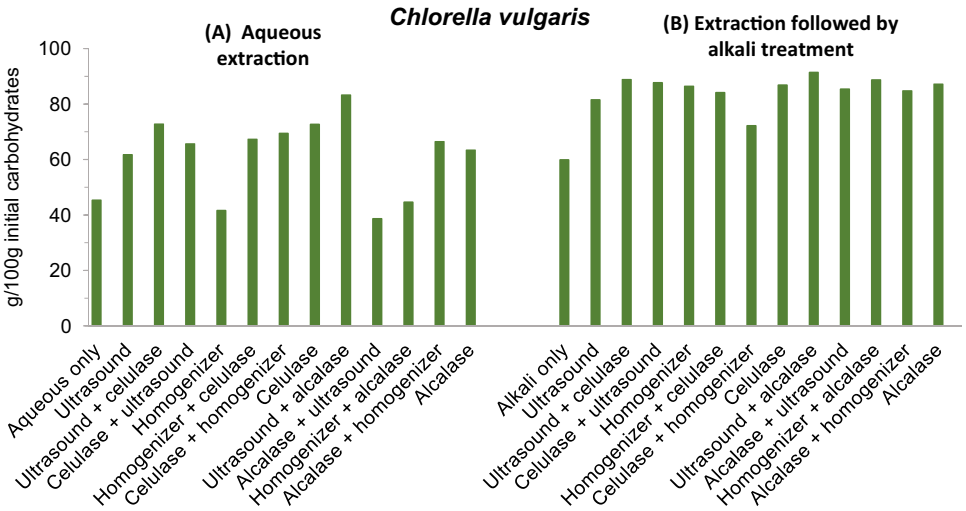

Fig. 3 Total sugar solubilization yield (in relation to the initial carbohydrates in the algae) obtained for all the protein extraction processes applied to *Chlorella vulgaris*

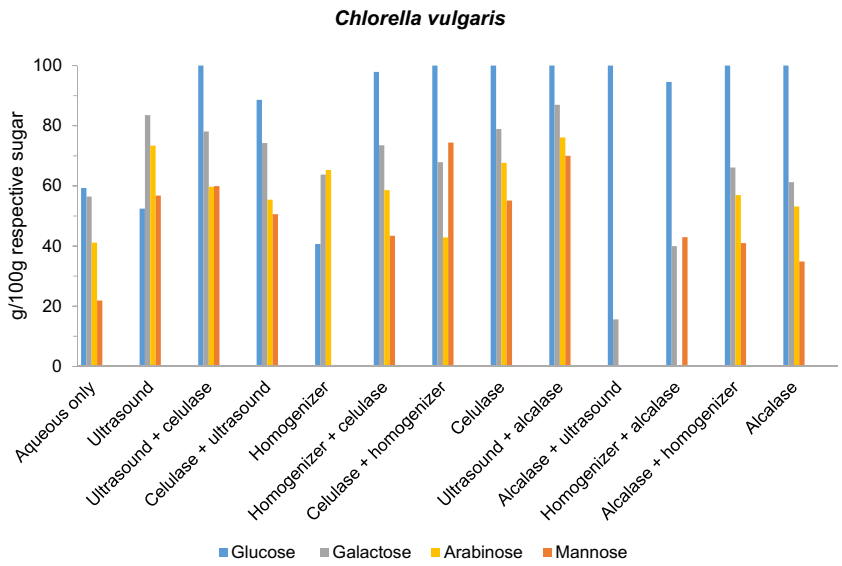

Fig. 4 Individual solubilization yield obtained for different sugars for all aqueous process conditions applied to *Chlorella vulgaris*

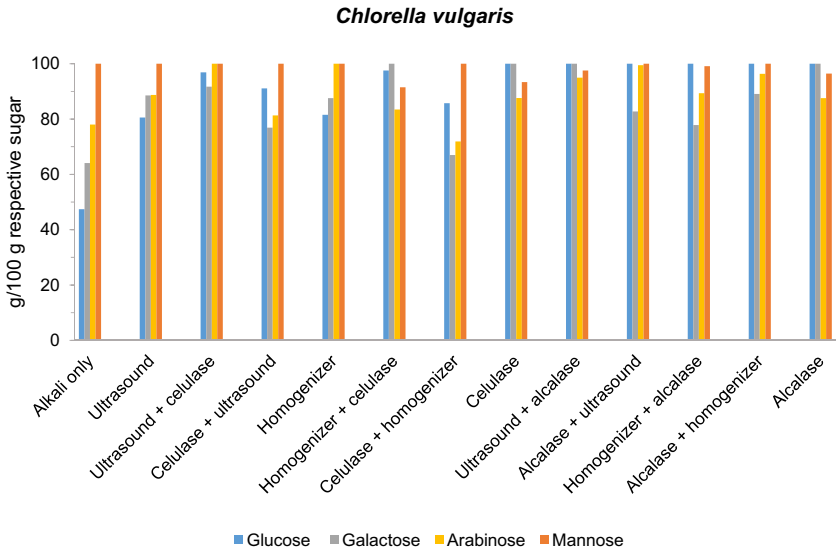

Fig. 5 Individual solubilization yield obtained for different sugars for all process conditions, followed by alkaline treatment, applied to *Chlorella vulgaris*

[70]. As both species had very low starch content (Table 2), most of the solubilized carbohydrates must derive from cell wall structural components. Therefore, it shows that treatments like ultrasound, rarely used in more conventional lignocellulosic biomasses [71], can be extremely effective for sugar solubilization in microalgae, opening up the possibility for alternative treatments to provide an innovative valorization of microalgae sugars.

Treatments using cellulase did not significantly increase sugar solubilization. This is likely due to the great variability of polysaccharides that compose the microalgae cell walls [72] and the other long-chain protective polymers [60] that may not be susceptible to enzymes that specifically target the glycosidic linkages in cellulose.

Figures 4 and 5 show the impact of the tested treatments on individual sugar removal. Glucose removal was above 40% of the total glucose in all treatments, both alkali and aqueous, although alkali treatments showed a higher impact on other sugars. Mannose solubilization could reach 100% removal in the majority of treatments that were followed by the alkaline step. The same solubilization boosting effect was verified for galactose, but to a lesser extent. Arabinose solubilization was also higher in alkali treatments.

Figure 6 illustrates the effects of the tested treatments on the sugar fraction of *S. obliquus.* All treatments strongly impacted the sugar fraction of *S. obliquus*, with the lowest solubilization of 56.6% obtained for the aqueous control. This contrasts with previous reports where the recovered carbohydrates were significantly less [67]. Solubilization exceeded 75% for all the other treatments and achieved higher or similar recovery yields for sugar and protein. As verified on *C. vulgaris*, these types of processes showed low selectivity for extraction of *S. obliquus* protein. The impact of the extraction methods on the sugar fraction is more significant for *S. obliquus* since it has a higher amount of sugar than *C. vulgaris.* Regarding the individual sugars (Fig. 7), the results obtained were quite similar to those found for *C. vulgaris*, with mannose being also the most affected sugar.

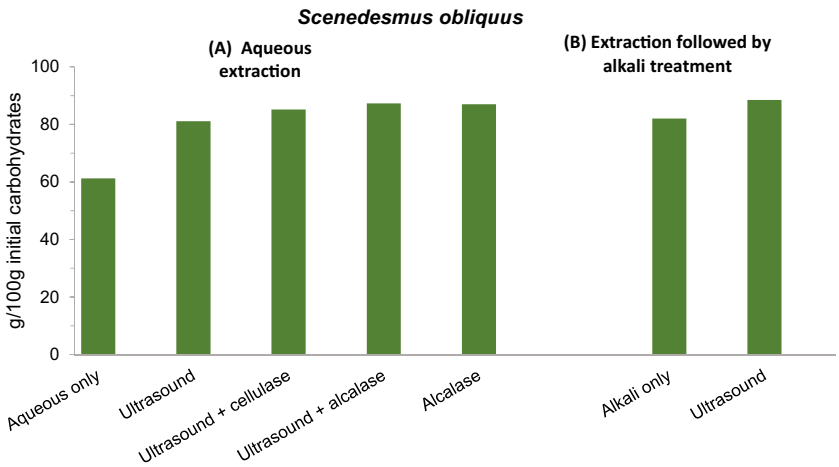

Fig. 6 Total sugar solubilization yield (in relation to the initial carbohydrates in the algae) obtained for all the protein extraction processes applied to *Scenedesmus obliquus*

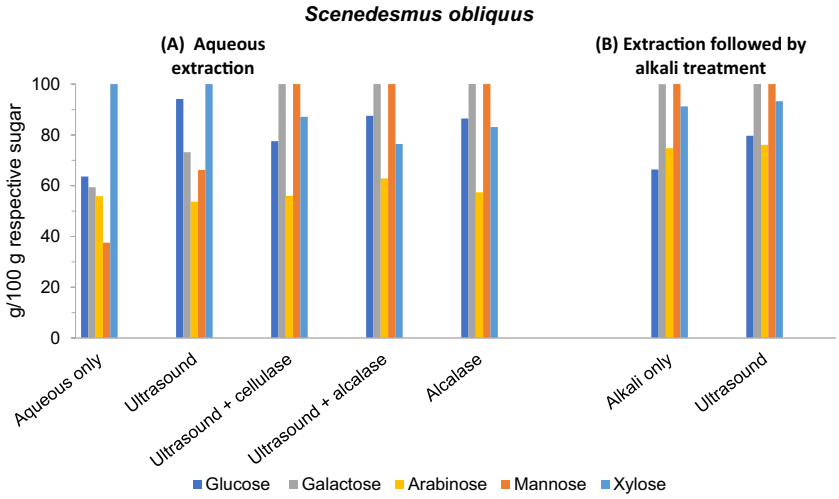

Fig. 7 Individual solubilization yield obtained for different sugars for all conditions applied to *Scenedesmus obliquus*

Overall, all the treatments tested for protein extraction also had an important impact on the sugar fraction, making these treatments a viable tool for further carbohydrate valorization. Their low selectivity towards the removal of only the protein fraction of the microalgae is a limitation that can be intrinsic to algae biomass. However, they can be a cheap, mild, and effective alternative for biomass fractionation since they require low temperatures (between 30°C and 60°C), and can prevent the protein and sugar degradation that can occur in other hydrothermal pre-treatments at higher temperatures [73]. Therefore, these processes can be effective for microalgae biomass fractionation if an efficient process for protein recovery, e.g., membrane separation, can be implemented.

4 Conclusions

Overall, all the treatments applied to *C. vulgaris* and *S. obliquus* were effective for the extraction of both protein and carbohydrates. Aqueous ultrasound treatment seems to be the most effective among the physical methods tested. Furthermore, all treatments resulted in higher extraction yields than those of the control, indicating their effectiveness in disrupting cell wall integrity and enhancing protein and sugar availability for solubilization and extraction.

Regarding the enzymatic treatments, only alcalase produced high protein extraction yields while also resulting in high sugar solubilizations, indicating that it can be viable, although non-selective alternative to physical methods. The aqueous ultrasound treatment was one of the best for protein extraction of both microalgae, with a lower impact on the carbohydrates, which prevents some potential downstream issues when compared to alkaline treatments, also being a simpler and cheaper alternative to enzymatic treatments.

These types of processes may be applied to microalgae for the production of low-purification supplements of protein or carbohydrates that can be used in food and feed applications, or as a complete growth medium or supplement for fermentation purposes for further valorization. Furthermore, the residual solid fractions obtained after extractions still present a relevant potential for further applications, e.g., anaerobic co-digestion to produce biogas or as fertilizer.

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Author contribution Pedro L. Martins: formal analysis; investigation; methodology; writing—original draft; review and editing; Alberto Reis: funding acquisition; supervision, resources; review and editing; Luís C. Duarte: formal analysis; methodology; validation; visualization; review and editing; Helena Pereira: formal analysis; supervision; validation; visualization; review and editing; Florbela Carvalheiro: conceptualization; formal analysis; funding acquisition; methodology; supervision; validation; visualization; writing—original draft; review and editing.

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Data availability The authors authorize the publication of the data and results shown in the paper.

Declarations

Ethical approval This declaration is not applicable.

Competing interests The authors declare no competing interests.

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