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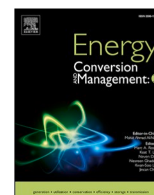

Effective fractionation of microalgae biomass as an initial step for its utilization as a bioenergy feedstock

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ABSTRACT

Scenedesmus obliquus, a biotechnologically relevant microalgae, was grown in 70 L vertical photobioreactors using non-supplemented secondary brewery wastewater as a culture medium. Upon collection, by mechanical means, the cells were subjected to hydrothermal (autohydrolysis) and dilute acid hydrolysis (0.5 % sulfuric acid) pre-treatments carried out using pressure micro-reactors under isothermal conditions up to 300 min.

Both processes enabled a high recovery of soluble sugars (~50 %) that were, in a great majority, present in the added-value oligomeric form (92 % and 90.5 % for autohydrolysis and dilute acid hydrolysis, respectively). Protein solubilization also presented relevant yields (35 % removal), with dilute acid hydrolysis allowing both higher oligosaccharides and protein productivities at a milder temperature.

As compared to the current whole microalgae biomass-based upgrade strategies, the use of these mild processes is extremely promising, as they will enable the future co-production of added-value oligosaccharides, and protein, which can be relevant co-products of a biofuels-based biorefinery.

Introduction

The use of microalgae for the production of biofuels, the so-called third-generation biofuels, and bioproducts is an increasingly relevant topic within the blue bioeconomy framework, defined as the set of economic activities associated with renewable aquatic biological resources [1]. The relevance of third-generation biofuels, such as biodiesel, bioethanol, and bio-oil, to fight climate change is indisputable [2]. Biodiesel can be produced from microalgae lipids [3]. Bioethanol can be produced from carbohydrate-rich microalgae [4], or directly from sunlight (*lato sensu* e.g. using the genetically modified cyanobacteria *Synechocystis* sp. [5]). Finally, bio-oil (and biochar) can be prepared using thermochemical treatment of microalgae biomass or residual biomass after lipid extraction, saccharification, and/or ethanol production [6]. Similarly, due to their ability to accumulate different types of macronutrients depending on the growth medium composition and culture conditions, microalgae also present an unparalleled versatility and huge potential as important sources of a wide plethora of bioproducts with different applications. These include fertilizers [7], animal feed (most noteworthy for poultry and aquaculture) [8], feed and food additives [9] as a relevant source of protein [10], biomaterials [11], pigments [12], cosmetics, antioxidants, and other pharmaceuticals [13]. Additionally, many microalgae species are robust enough to be able to grow in unconventional and cheap growth media, such as wastewater, using economic/low-tech installations, implemented at sites that do not compete for arable/agricultural land. Furthermore, it has been reported that microalgae can not only grow in wastewater but also have wastewater treatment capabilities, which is a most relevant trait from a sustainability standpoint [14] providing additional sustainability and synergistic effects. In fact, brewery wastewater is especially interesting in this context as, it is a non-toxic substrate with high organic matter and very low amount of contaminants (e.g. heavy metals). Despite this, it poses significant environmental risks, if discarded without suitable treatment (e.g. inducing eutrophization and diminishing oxygen availability in aquatic environment). But, on the other hand, its easy degradability makes it a prime candidate for microalgae growth [14]. Furthermore, depending on the source, its biochemical parameters can be highly compatible with microalgae growth [15], with little need for chemical remediation. This would be enhanced after adequate treatment, namely after secondary wastewater treatment, where it can be a potential low-cost and beneficially alternative source to be used for microalgae growth. These are important

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advantages when compared to first and second-generation biomasses, even considering the bioremediation potential of some land energy crops, and open new valorization opportunities.

Despite these significant traits and the technical developments achieved in the past few years, several techno-economic limitations still hinder the commercial production of third-generation biofuels and many of these bioproducts. As these organisms are efficient carbon fixators and metabolizers [10], the constraints are mainly linked to post-cultivation processes [16], which require the processing of large volumes of culture broth with low microalgae titers. As such, research is increasingly focusing on the development of effective downstream processes for microalgal biomass in order to have an economically sustainable overall production of biofuels together with added-value bioproducts [17], when this is technically, legally, and/or regulatorily possible under local/regional constraints.

Amongst the wide variety of microalgae, *Scenedesmus obliquus* is recognized as a fast-growing, very robust species that readily adapts to heterotrophic growth, which allows its production in a wide range of conditions, from sole autotrophic conditions to fully heterotrophic conditions using wastewater as a source of carbon and nutrients. It is also relatively easy to harvest due to the high biomass concentration that it is possible to achieve [18]. Furthermore, its typical chemical composition is well balanced regarding, carbohydrates, protein, and lipids, making it a prime subject for biorefinery studies and applications [7,19]. Nevertheless, similarly to lignocellulosic materials, *S. obliquus* upgrade is dependent on the development of effective fractionation processes, especially after lipids extraction, when it is necessary to break its thick cell wall [20].

Commonly, microalgae have rigid cell wall structures consisting of different polysaccharides, but unlike higher plants, microalgae do not contain lignin, which gives important operational advantages as lignin imposes limitations on the fractionation of polysaccharides [21]. Microalgae polysaccharides include cellulose as the main component, but also other structural components such as mannan, xylan, and uronic acids [22], and in the case of *Scenedesmus* species, they can also have trilaminar layers of algeanan and glycoproteins, chitin-like polysaccharides [22], and minerals.

Conversely, to other microalgae, whose pretreatment and saccharification have been broadly studied, the selective fractionation of [23–26] *S. obliquus* is still not fully explored. Efficient saccharification in other algae species can be achieved by either chemical or/or enzymatic means. The use of mineral acids, such as sulfuric, hydrochloric, and nitric acid is effective but the severities typically employed range similar to the ones used for land/lignocellulosic biomass, generally using temperatures between 120 and 140 °C and reaction times well above 15 min when high saccharification are required [4,23]. Previous reports for the acid hydrolysis of *S. obliquus* report conditions at 120 °C with up to 3 N sulfuric acid for 30 min to achieve sugar recoveries between 71 and 97 % [20,23]. Similarly, *S. bijugatus* (26 % carbohydrate content after lipid extraction), under acid hydrolysis with sulfuric acid up to 1.08 N) at 130 °C, 45 min, and 20 g/L solid concentration, presented a saccharified yield of 84 % of biomass sugars [21,23], which are rather severe/potentially uneconomic conditions.

Furthermore, the full conversion of microalgae cell-wall carbohydrates into monosaccharides might not be fully beneficial, especially if the production of specialty chemicals is possible [27], namely when algae are cultivated under controlled/hygienic conditions. Besides the production of biomethane/biogas [19,20] and bioethanol [28,29] carbohydrates can also have other applications, as already demonstrated for lignocellulosic biomass. Some of the most interesting of these are based on oligosaccharides, which can have applications as food and feed as additives with prebiotic, antioxidant, and other bioactive properties [30,31]. Furthermore, when associated with biofuel production, oligosaccharides can significantly contribute to the biorefinery economy [32]. Oligosaccharide production can be achieved by several fractionation methods. The most commonly used are hydrothermal processes such as autohydrolysis [7,33] and dilute acid hydrolysis [20,34], which have not yet been extensively applied to microalgae, and specifically to *S. obliquus* with this aim.

This work aims to study, compare and optimize autohydrolysis and dilute acid hydrolysis, with very low acid concentration (0.5 % w/w), for the selective fractionation of microalgae using *S. obliquus,* grown in brewery wastewater effluent as a model system for biomass pretreatment for subsequent products formation (e.g. biofuels) and to envisage the application of this microalga in future added-value applications. Both hydrolysates and processed solids obtained after pre-treatments are analyzed to provide a detailed understanding of polymers fractionation, and monomeric and oligomeric sugars recovery in hydrolyzates.

Materials and methods

Growth medium and microalgae cultivation

The microalgae *Scenedesmus obliquus*, from the Coimbra University Algae Collection (Portugal), was cultivated in a pilot cylindrical vertical acrylic bubble column photobioreactor (70 L total volume, 50 L operational volume). The growth medium was a brewery effluent recollected after a secondary treatment from a local industrial brewery. Upon collection, treated effluent was stored in closed plastic containers at 4 °C until use. No chemical remediation was used on the effluent. Table 1 presents the physical and chemical composition of the brewery effluent used as growth medium for microalgae cultivation.

Total solids were lower than previously reported for this type of effluent [15], as expected for a treated effluent. Low amounts of sugars indicate a low possibility for major growth of heterotrophic bacterial or fungal contamination. The high level of COD is in line with comparable similar substrate [15], and together with the nitrogen level, 5.6 mg /L as determined by the Kjeldahl method (i.e., 0.207 % dry weight) this effluent presented good prospects for microalgae cultivation.

Batch cultivation was carried out at room temperature, and under constant fluorescent light (43.2 μmol/m^2.s) and aeration with air (0.1 vvm) as described in [7]. Cell growth was monitored through optical density measurements at 540 nm and cell dry weight.

Microalgae harvesting and concentration

The culture broth was concentrated by an in-house developed centrifugation method based on the use of a continuous dairy centrifuge (electric cream separator, Alfa Laval, Sweden). Biomass was centrifuged, washed with water, and centrifuged again. The concentrated algae paste presented a moisture content close to 90 % and although it can be used

Table 1
Physical, and chemical composition of the brewery effluent used for the growth of *S. obliquus*.

	Component	
Physical	Total solids (mg/L)	2690
	Ash (mg/L)	1760
	Density	0.99
Chemical	pH	7.13
	Total Kjeldahl Nitrogen (mg/L)	5.6
	COD (mg/L)	5376
	GlcOS (mg/L)	2.91
	XOS (mg/L)	8.49
	Glucose (mg/L)	22.07
	Acetic acid (mg/L)	6.50
Elemental	C (% dry weight)	11.83
	H (% dry weight)	0.85
	N (% dry weight)	<0.3

GlcOS – *gluco*-oligosaccharides; XOS – xylo-oligosaccharides.

as is, in order to produce a homogeneous, reproducible lot, concentrated biomass was frozen (-18 °C) and then freeze-dried (Thermo Scientific, Hector PowerDry LL3000, USA), until further use.

Fractionation processes

Autohydrolysis

Hydrothermal assays (autohydrolysis) were conducted in pressure micro-reactors (25 mL glass tubes, ACE Glass Inc., USA) capped with Teflon screw caps using 1 g of dry biomass and water to reach a liquid-to-solid ratio (LSR) of 12 g/g. The tubes were incubated in an oil bath preheated to the desired reaction temperature (150 °C), controlled by a probe (Sigma, IKA ETS-D5, USA) and under continuous magnetic stirring. Zero-time reading corresponds to the sample (tube reactor) taken at the time when the desired bath oil temperature is reached. This corresponds, typically, to 5–7 min to reach again the set temperature of 150 °C after the addition of the glass tubes. When the desired reaction time was attained, the tubes were rapidly cooled down to room temperature [35]. The liquid and solid phases were separated by centrifugation (Ortoalresa, Digicen 21 R, Spain) at 5300 g, for 10 min, at 10 °C. The liquors were collected for analysis, and the solid pellets were washed with the same amount of distilled water and centrifuged again. The washed solids were stored until further analysis after freeze-drying. Part of the liquors was freeze-dried (Thermo Scientific, Hector PowerDry LL3000, USA), weighted for mass quantification, and analyzed for molecular weight as described below.

Dilute acid hydrolysis

Dilute acid hydrolysis assays were conducted in the same conditions as described for autohydrolysis, but using 1 g of sample and a sulphuric acid solution to reach 6 g H_2SO_4/100 g microalgae biomass, also using a LSR of 12 g/g. The reaction temperature was set at 140 °C. When the desired reaction time was attained, the reaction was stopped, and the liquid and solid phases were processed as described above. Zero-time was determined as described above for autohydrolysis. This corresponds to approximately 5–7 min to reach 140 °C.

Analytical methods

All of the following analytical procedures were carried out, at least, in duplicate.

Moisture, dissolved solids, and ash content of process samples

The moisture content of the biomass samples was determined by oven-drying at 105 °C, and the total ash content was determined by incineration at 550 °C for, at least, 16 h, with both procedures based on the algae-specific NREL protocol [36]. Total solids and ash content in the secondary effluent were measured using the same methodologies.

Determination of the elemental composition of process samples

The elemental composition (C, H, N) of the freeze-dried secondary brewery effluent was measured in an Elemental analyzer (Elementar –Vario Macro Cub, Germany) according to ISO 16948:2015 standard [37]. The elemental composition of biomass samples was determined using a Vario el III elemental analyzer system (GmbH, Germany), according to the procedure provided by the manufacturer. Oxygen is quantified by difference, taking into account the content of the macro-elements. Macro and micro minerals were qualitatively and quantitatively characterized using a Microwave Plasma Atomic Emission Spectrometer (MP-AES, Agilent) [38] after acid digestion of the samples for approximately 20 min in a microwave system (Discover SP - Microwave Synthesizer, CEM Corporation). Standards were prepared from certified Agilent Technologies standard solutions, and proper blanks were also performed for comparison.

Quantification of the chemical oxygen demand (COD) in the effluent

COD was determined according to the open reflux method – Method 5220-B [39].

Extractives quantification in biomass samples

The content of extractives was determined by sequential solvent extraction in a Soxhlet apparatus using a modified method based on [40], switching the solvent order. Briefly, the extractions were performed firstly with ethanol, followed by water for 18 h for each solvent. Total and partial extractives content were calculated relating to the feedstock dry mass. Lipids/fat quantification was performed according to a relevant Portuguese standard [41].

Quantification of carbohydrates

For the quantification of the carbohydrates present in the feedstock and pre-treated biomass, samples were subjected to quantitative acid hydrolysis using 72 % (w/w) H_2SO_4 (30 °C, 60 min) followed by dilution to 4 % (w/w) H_2SO_4 and hydrolysis (121 °C, 60 min) in an autoclave, according to [42]. The acid-insoluble organic residue was quantified by filtration through 1.22 μm glass fiber filters (VWR, USA), after correction for ash and protein. The monosaccharides present in the effluent used as a growth medium and in the hydrolyzates were analyzed by HPLC as described below.

Starch was enzymatically determined using a test kit supplied by Boheringer (Boheringer, Germany). The uronic acid content was determined by spectrophotometry at 520 nm, using a Jasco V-520 spectrophotometer (Jasco, Japan) according to [43] using glucuronic acid as a standard.

Monosaccharides (glucose, mannose, xylose, galactose, rhamnose, 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), using an Aminex HPX-87P column (Bio-Rad, Hercules, USA) in combination with a cation Pb^{2+}-guard column (Bio-Rad). Elution took place at 80 °C with water as eluent at a flow rate of 0.6 mL/min [44]. All samples were previously neutralized using barium hydroxide or a combination of Amberlite® MB-20 resin (Sigma-Aldrich, USA) and calcium carbonate.

Oligosaccharide concentrations were calculated by an indirect method based on the increase in sugar monomers quantified by HPLC after liquor acid hydrolysis with 4 % (w/w) H_2SO_4 (121 °C, 60 min) [45]. Acetic acid and acetyl groups in oligosaccharides were determined using an Aminex HPX-87H column (Bio-Rad, USA) in combination with a cation H^{+}-guard column (Bio-Rad) using 5 mM sulfuric acid as mobile phase at 0.6 mL/min flow rate and the column temperature of 50 °C [46].The degree of polymerization of oligosaccharides was estimated by HPSEC on BioSep-SEC-S2000 column with a BioSep-SEC-S4000 guard column (Phenomenex, USA) as previously described in Moniz et al. [47]. Elution was monitored using a refractive index and took place at 30 °C with 50 mM sodium nitrate at a flow rate of 0.7 mL min^{-1}. Calibration was performed using mannose and dextrans with molecular weight ranging between 1 kDa and 580 kDa, as standards.

All samples were filtered through 0.22 nylon membrane filters (VWR, USA) before HPLC analysis.

The percentage of the polymeric sugars was calculated from the concentration of the corresponding monomeric sugars, using an anhydro correction of 0.90 (162/180) for C-6 sugars (glucose, galactose, and mannose) and 0.88 (132/150) for C-5 sugars (xylose and arabinose). A correction factor for sugars degradation during post-hydrolysis (F) was also considered, corresponding to 1.04 for hexoses and 1.09 for pentoses. As example, the percentages of glucan and xylan can be calculated as follows (Eq. (1) and Eq. (2)).

$$\text{Glucan} = 0.9 \times F \times \frac{100}{\rho} \times \frac{\text{Glc} \times W_{sol}}{DW} \quad (1)$$

$$\text{Xylan} = 0.88 \times F \times \frac{100}{\rho} \times \frac{\text{Xyl} \times W_{sol}}{DW} \quad (2)$$

W_{sol} and DW are the weight of the solution and the sample dry weight, respectively and ρ is the volumetric mass density of the solution (g/L).

Nitrogen quantification

Nitrogen quantification in all biomass samples and in effluent was also carried out by the Kjeldahl method. For estimating the protein content in algae biomass, the N × 4.78 conversion factor [48] was used.

Chlorophyll quantification

Chlorophyll *a* and chlorophyll *b* content were determined spectrophotometrically according to the method described by Richie [49].

Results and discussion

Chemical composition of Scenedesmus obliquus

The chemical composition of *S. obliquus* is shown in Table 2. Protein is the most relevant component, presenting a value of 25.4 %, similar to the values previously reported for this species [50,51] and also for *Chlorella vulgaris* [52], one of the most studied Chlorophyceae species. This value is also close to those reported for another effluent-grown *S. obliquus* (27–36 %) [53,54]. As the protein content found in microalgae is highly variable and also dependent on culture media composition, the content found is still lower than others reported for *S. obliquus* [7,21,50].

Carbohydrate is also a relevant group of biomass constituents, and the total content found, 16.30 %, is in the range of the previously reported for this species [21,51,52] although slightly lower than preferred for this type of study. Glucose, expressed as glucan was the main sugar found, representing 53 % of total sugars, with galactose being the second most prevalent sugar. However, due to limitations of the HPLC column used in this study, both galactose and rhamnose signals appear overlapped, and as galactose is known to be more relevant in these microalgae, galactose and rhamnose will be henceforth referred to as galactose.

As compared to lipid accumulating microalgae, this microalga has a very low lipid content, reaching only 3.09 % which is a consequence of the growth conditions [55].

Total extractive content of 29.15 % was found. The analysis of water extractives showed an 8.05 % content of protein and 4.81 % of carbohydrates, demonstrating that an important part of these components can be easily removed by the conventional Soxhlet extraction. The ash content found was quite high, close to the values reported for some brown and green macroalgal species [56] and higher than the described for some species from the *Chlorella* genus [23,57–59]. However, it is still lower than previously reported in other studies with *S. obliquus* [51]. It is important to highlight that the ash content of biomass, may also depend on the downstream processing, namely the washing of biomass during the harvest process. In this study, a 10 % decrease in ash content was obtained after washing biomass with distilled water. This value could potentially be further decreased with additional washings, especially with pH 4 buffer solution, as a way to solubilize salts, with emphasis on phosphates and calcium, although this was not done to prevent biomass losses, which can occur due to the washing process.

Besides these major components, *S. obliquus* also contains a not negligible amount of uronic acids (3.24 %) and acetyl groups (1.99 %). The value of these parameters is not often measured/reported in the literature for microalgae biomass; however, it is similar to the data found in ambient air-grown species of the Chlorella genus [60], in the range of the values previously reported for *Phaeodactylum tricornutum* and *Chlorella vulgaris* [61] but lower than those found for some red microalgae [62].

Similarly, the values for acid-insoluble residue are also not usually analyzed, although they account for a significant part of the biomass composition. The chlorophyll content is quite similar to previously reported [7].

This microalga was also analyzed for elemental composition and this data is shown in Fig. 1. Carbon is the most relevant element, comprising 45 % of all elements quantified, with hydrogen and nitrogen representing 7 % each. The values for these elements are in the range of some previously reported for *S. obliquus* [51,63] but lower than those described for *Chlorella vulgaris* [58,63]. However, when compared to some lignocellulosic biomasses, the nitrogen content found was much higher [51]. Oxygen (O) and Sulfur (S) were calculated by difference and represented 34 % of the measured elements. The C/N ratio of 6.6 found in this microalgae also makes it a possible candidate for anaerobic co-digestion for the production of biogas [64,65].

Regarding the elemental minerals, calcium and phosphorous were the most important found, representing 62 % and 24 % of minerals detected, respectively. Magnesium accounted for 6.7 % and potassium for 4 % of total minerals. Iron and all other heavy metals accounted for only 2 % of all minerals and less than 0.01 % of all elements quantified. This composition is an interesting and important characteristic of this biomass, especially being a microalga grown in a wastewater-treated effluent, pointing to its low toxicity and potential for usage in high-valued applications and as a biofertilizer.

Fractionation of Scenedesmus obliquus by autohydrolysis

Fig. 2 shows the composition of liquors obtained after autohydrolysis of *S. obliquus* at 150 °C for different reaction times. For all conditions, most sugars solubilized were obtained in the oligomeric form (Fig. 2a), with monomeric sugars representing about 10 % of total sugars (Fig. 2b), on average. This result was not completely unexpected, as autohydrolysis is a very mild pre-treatment, that mainly promotes the hydrolysis of the carbohydrate chains through the acetic of H^+ ions (both from acetyl groups and water auto-ionization), resulting in a high yield of oligomers [66].

Overall, sugar solubilization increased with time, although after 120

Table 2
Chemical characterization of microalgae *Scenedesmus obliquus* (g/100 g biomass).

Component		Water Extractable	Structural	g/100 g biomass
Protein		8.05	17.35	25.40
Carbohydrates		4.81	11.49	16.30
	Glucan[a]	2.01	6.68	8.69
	Xylan	0.55	1.24	1.79
	Arabinan	n.d.	0.71	0.71
	Galactan[b]	2.25	1.32	3.57
	Mannan	n.d.	1.55	1.55
Acetyl groups				1.99
Uronic acids				3.24
Chlorophylls[c]				0.10
Lipids				3.09
Acid insoluble residue[d]				13.87
Ash				15.78
Extractives	Ethanol			11.75
	Water			17.45

n.d.: Not detected.
[a] From which 1.32 g/100 g biomass corresponds to starch.
[b] May also include rhamnose.
[c] Chlorophylls *a* + Chlorophyll *b*.
[d] Protein and ash-free.

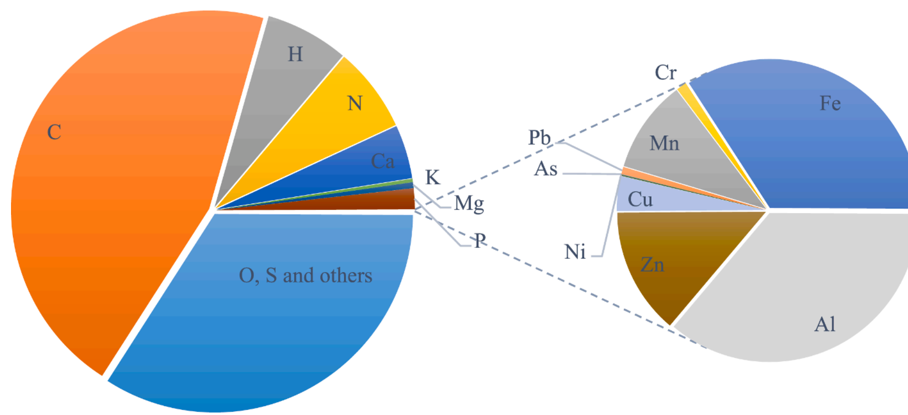

Fig. 1. Elemental composition of secondary brewery effluent-grown microalgae *Scenedesmus obliquus*.

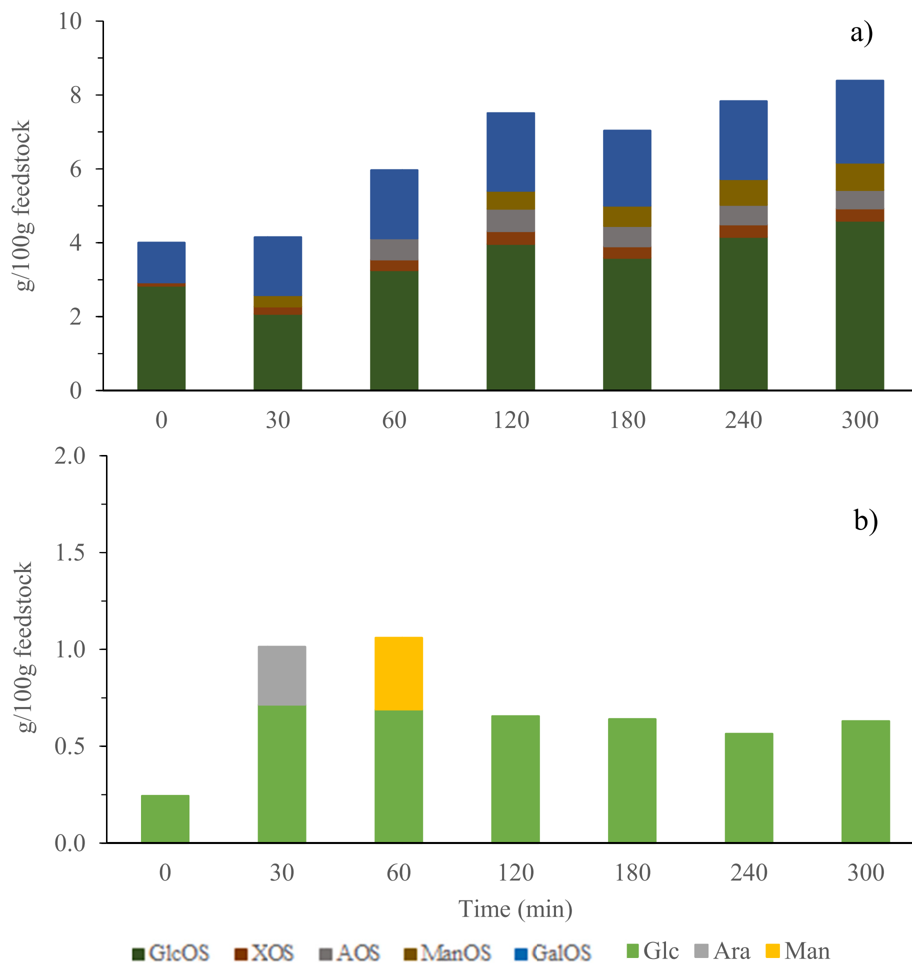

Fig. 2. Time course of oligosaccharide (a) and monosaccharide (b) yields obtained after hydrothermal treatment of *S. obliquus* at 150 °C.

min, only slight increases in solubilization were obtained. In the mildest condition, the total yield of sugars was 4.25 g/100 g of initial biomass (92 % in oligomeric form), corresponding to 26 % of initial sugars. This fact suggests an initial high reactivity and relatively easy solubilization of these biomass carbohydrates, which is in agreement with the previously reported for the composition of extractives (see above). At 120 min, the sugar yield was 8.17 g/ 100 g biomass, corresponding to 50.1 % of initial sugars, only surpassed by 51.5 and 55.3 % for 240 min and 300 min of treatment, respectively. Considering the long reaction times to obtain only a slight increase in sugar recovery, the best reaction time for this experiment was considered to be 120 min, as this leads to a significant saving in energy costs. It is important to note that the reaction pH, which is an important parameter for hydrolysis, is quite high in the case of this biomass (7.76–6.09) and much higher than the values usually obtained for lignocellulosic biomass. In this case, the presence of a catalyst that can produce a drop in pH values may have a positive effect on hydrolysis.

Few studies of autohydrolysis were made to date on microalgae and

almost all use autohydrolysis as a preliminary step for other processes, e.g., enzymatic hydrolysis or to pave the path for the application of microalgal biomass as a source of proteins [10,67]. These yields obtained are higher than the ones reported in studies conducted at lower temperatures and much higher reaction times (50 °C, 48 h) [33]. In these, the highest carbohydrate yields were 14.9 % and 31 % for *Chlorella* and *Scenedesmus* sp., respectively. Even compared with alkaline-assisted treatment, the yields obtained above 120 min were roughly 10 % higher than those obtained for the later species [33]. Although the autohydrolysis studies of microalgae are very scarce, [67] studied the effect of autohydrolysis on *Scenedesmus* sp. using temperatures of 160 °C and above. Under these conditions, a concentration of total sugars of 13.4 g/L with a liquid-to-solid ratio (LSR) of 15 was reported. However, the starting biomass had a carbohydrate content of 48 %, which is much higher than the species of this study. For an initial carbohydrate content of 16.3 %, the results obtained are very encouraging and show some possible routes to boost sugar yields.

The LSR used these autohydrolysis studies, along with the initial sugar concentration, and their solubility resulted in less concentrated solutions. In all conditions tested, glucooligosaccharides (GlcOS) were the main sugars found in the liquors followed by galactooligosaccharides (GalOS). This is consistent with the composition of the initial biomass, also showing that apparently, there is no selectivity according to the type of sugar. Overall, the oligosaccharides (OS) concentration tends to increase with reaction time, in particular in the case of GlcOS. The highest total OS concentration was 6.72 g/L at 300 min (7.28 g/L total sugars), and 6.03 g/L (6.61 g/L total sugars) were obtained for the 120 min reaction time. This concentration can however be theoretically increased by lowering the LSR used in other types of reactors that facilitate a better mechanical agitation of the paste-like medium formed by the highly hygroscopic microalgae. The distribution of the soluble saccharides obtained at 120 min of reaction time was also analyzed by HPSEC for the estimation of their molecular weight and polymerization degree (DP) and it was found to present a bimodal distribution between soluble polysaccharides (in lower amount) and oligosaccharides with a typical average DP around 6, that are putatively the main saccharide products present (data not shown).

Regarding the impact of these treatments on the protein fraction, it is clear that protein removal increased to reach 36.78 % after 180 min (Fig. 3). After that, it remained quite stable reaching 37.15 % (300 min). Together with protein removal, there was also a decrease in solid yield which reached 60.45 % as the lowest value. The results obtained show that autohydrolysis treatments lead to both solubilization of sugars and protein, following both a similar trend and which is inverse to the solid yield.

The analysis of pre-treated solids (Table 3) shows a stable protein content of around 30 % for all conditions and composition of

Table 3
Composition of autohydrolysis treated *S. obliquus* biomass at 150 °C (g/100 g treated biomass).

	Time (min)						
Component	0	30	60	120	180	240	300
Glucan	6.45	5.02	6.05	5.73	5.62	4.81	2.58
Xylan	0.62	0.45	0.26	0.55	0.51	0.28	0.33
Mannan	2.17	1.66	1.41	1.60	1.69	1.88	2.12
Galactan	2.70	1.78	1.51	1.59	1.55	1.20	1.14
Protein	33.15	29.50	28.99	28.65	27.40	28.38	28.31
Acid insoluble residue*	3.37	6.41	17.97	20.38	19.94	20.29	29.59
Acid insoluble ash	0.00	0.00	1.49	1.49	0.74	0.37	3.06
Others (by difference)	51.55	55.18	42.26	40.01	42.55	42.79	32.88

* Protein and ash-free.

carbohydrates that tend to decrease with reaction time, being glucose the main sugar present. However, the drop in carbohydrate content of the solids above 120 min, when compared to the small increase in sugar concentration in the liquors, suggests some possible sugar degradation under these harsher conditions. The acid-insoluble residue increased over time, indicating the formation of non-soluble products.

Overall, the autohydrolysis process enabled relevant hydrolysis of carbohydrates, producing OS as the main sugars, together with an important removal of protein. Nevertheless, the residual solids obtained still contain a protein content similar to the original microalgae biomass. Furthermore, the carbohydrate content of this processed biomass is still relevant showing that it is suitable for other applications.

Fractionation of Scenedesmus obliquus by dilute acid hydrolysis

To assess the effect of a catalyst on the hydrolysis of *S. obliquus* polysaccharides, dilute acid hydrolysis experiments, with a very low catalyst concentration (0.5 % sulfuric acid) were carried out. Fig. 4 shows the yield of mono- and oligosaccharides obtained for different reaction times. As occurred for autohydrolysis, most sugars in all treatments were recovered in oligomeric form (Fig. 4a), although the monomeric sugar fractions are higher in this case (Fig. 4b). The increase in monomeric sugars was expected because of the catalytic effect of the acid. Nevertheless, it should be noted that the OS concentration was much higher than expected and that is usually reported for dilute acid hydrolysis. However, as this dilute acid process was carried out under very mild conditions, the production of OS is favored. This is a result that, to our knowledge, has not been reported yet, and can probably be achieved under these very dilute acid conditions and due to the nature of this feedstock. Total sugar release to the liquid fraction increased with

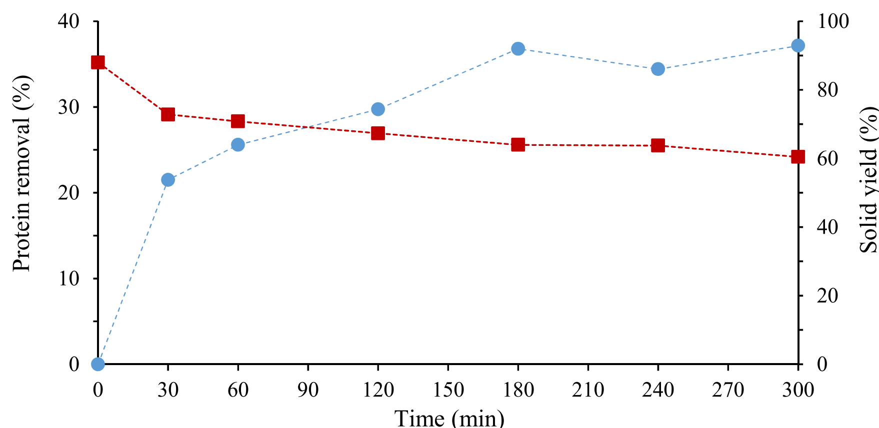

Fig. 3. Protein removal (•) and solid yield (■) obtained under hydrothermal treatment of *S. obliquus* at 150 °C.

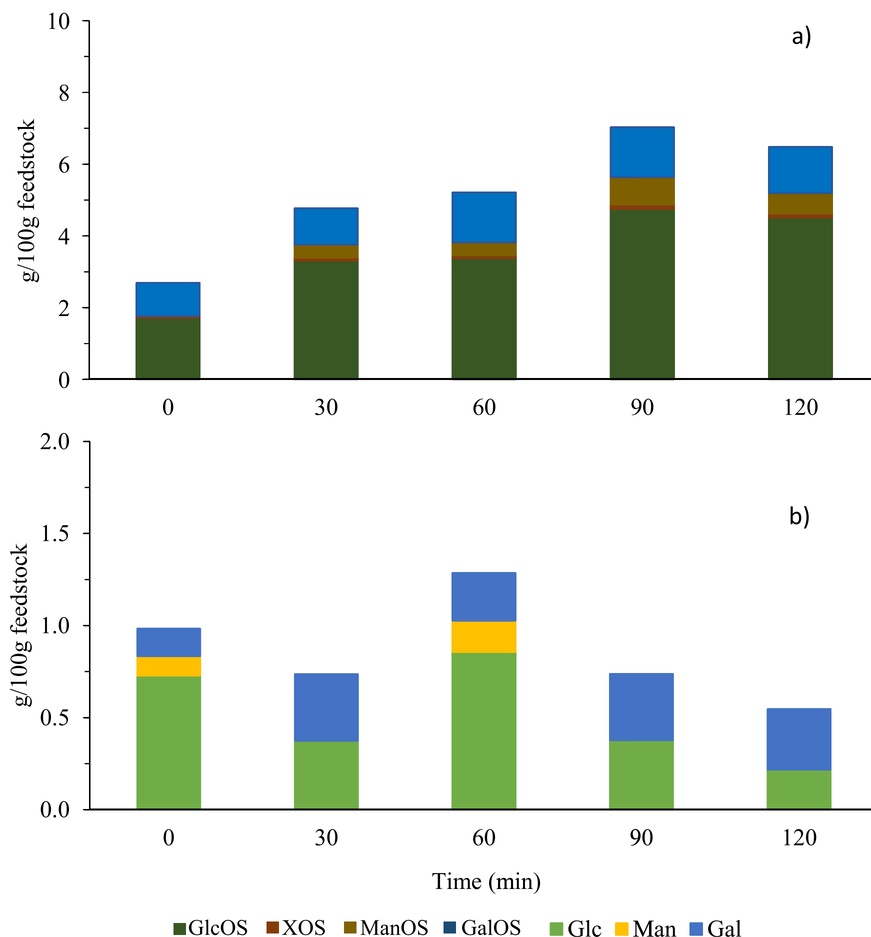

Fig. 4. Time course of oligosaccharide (a) and monosaccharide yields (b) (g/100 g feedstock) obtained after dilute acid hydrolysis of *S. obliquus* at 140 °C.

treatment time, reaching a maximum at 90 min, corresponding to a yield of 7.75 g sugars/100 g biomass, i.e., 47.58 % of total initial sugars. After 120 min treatment, there was a slight decrease in sugar yield that might suggest a possible sugar degradation.

The results obtained indicate some boosting effect of the added acid to sugar solubilization. At 90 min, the sugar solubilization was 47.5 %, which was close to the obtained in autohydrolysis at 120 min (50.1 %). Therefore, it was possible to reach an approximate sugar recovery in 30 min less time, with only a minimal addition of sulfuric acid. The catalytic effect is also due to the reaction pH, which is lower in the case of dilute acid hydrolysis. However, in the conditions tested, pH ranged from 4.79 to 4.25, which is also very mild and where further neutralization can be avoided.

The total sugar yield is a relevant parameter to assess the efficient hydrolysis of polysaccharides. But the relative amount of OS to monosaccharides is also very important. The previous data obtained for lignocellulosic materials show that, typically, autohydrolysis leads to high production of OS whereas in dilute acid hydrolysis processes monosaccharides production is favored. In this case, and as occurred for autohydrolysis, the OS content surpasses the monosaccharides. Again, GlcOS were the most relevant OS followed by GalOS (Fig. 4a). The concentration of all OS tends to increase with reaction time, reaching a maximum at 90 min of treatment with a concentration of 4.93 g/L. This value is lower than the obtained for autohydrolysis and it should occur due to the catalytic effect of the acid. At 120 min a slight drop in OS concentrations was observed, suggesting a possible degradation as this does not correspond to an increase of monosaccharides concentration. The acid treatment also showed an impact on the saccharide chains obtained. HPSEC analysis of the samples obtained at 90 min of reaction also resulted in two main groups of saccharides. The smaller, and more abundant, also averaged a DP of 6, similar to the described above for autohydrolysis, but the high DP fraction is actually smaller than the obtained from autohydrolysis in the best condition (approximately 25 % lower). These results support a more severe effect on sugar fractionation under acid hydrolysis, even considering that it was carried out for a shorter reaction time. Although some potential difficulties may arise with the use of these oligosaccharides in higher animals due to the growth medium used, the produced oligosaccharides are in the range that can be useful as supplements with prebiotic effects [68,69]. These processes can also be considered as hygienic/sterilizing, thus enabling the potential recovery of stabilized products and their true potential should be evaluated carefully in the future. Especially, these types of supplements can be recovered by applying the same technology, perhaps adjusting the growth medium, but keeping with approved safety regulations for food/feed applications [70].

Dilute acid hydrolysis of *S. obliquus* has been seldom reported. Previous reports [71] present 18 g sugars/100 g feedstock, although using higher acid concentration, i.e., 1.5 M H_2SO_4, far higher than the 50 mM used in these experiments. In the most similar conditions, the 7.75 g/100 g feedstock obtained in this study was far higher than the 3.54 g/100 g feedstock described by the previous authors [71]. In another report for *S. obliquus,* even higher concentrations of H_2SO_4 and HCl (3 N) were used for dilute acid hydrolysis in an autoclave (120 °C), resulting in around 13 g/100 g biomass sugar recovery [21]. In the same report, 2 N of sulfuric acid in the same conditions resulted in the recovery of 95.8 % of initial sugars but in monomeric form and with the production of degradation products [21]. Therefore, the present study shows that for the recovery of oligomeric sugars the acid concentrations need to be

quite low to provide high yields. Yields rounding 90 % total sugars can also be found in reports at temperatures ranging between 110 and 130 °C for *Chlorella vulgaris*. Again, the acid concentrations tested were very high (3–5 %), resulting in the recovery of mostly monomeric sugars [23,59].

All reaction conditions affected the protein fraction of the biomass, increasing the protein solubilization over time to reach the higher protein removal (35 %) under the harshest condition (Fig. 5). Comparing the same reaction times with the autohydrolysis treatments, and although the reaction temperature is lower for dilute acid hydrolysis, a slight increase in protein removal was verified for the same reaction times, suggesting an effect of the acid catalyst on protein removal. The *Chlorophyceae* green algae, like *S. obliquus*, have a wide array of different cell wall compositions but a big part of them is highly rich in cellulose-pectin complexes and glycoproteins [72], which may explain the simultaneous glucan and protein solubilization in both treatments tested. If this can occur, the recovery of glycoprotein oligosaccharides may be of great interest for added value applications.

As discussed for autohydrolysis, dilute acid hydrolysis has a similar trend for polysaccharides and protein solubilization. Solid yield for all treatments was also measured, as shown in Fig. 5, decreasing with treatment time, reflecting the increases of sugar and protein solubilizations mentioned before.

As mentioned before for autohydrolysis, all dilute acid hydrolysis pre-treated solids, shown in Table 4, contain a final protein content of around 30 %. Again, the carbohydrate content decreased with time, with glucan being the most prevalent polysaccharide. However, at 120 min, the carbohydrate decrease in pre-treated solids is not accomplished by an increase in sugars recovery in the liquors (Fig. 4), suggesting some possible degradation. Acid insoluble residue and ash also increased over time.

As obtained for autohydrolysis, dilute acid hydrolysis under these very mild conditions enabled considerable hydrolysis of carbohydrates, still producing OS as the main sugars, but to a lesser extent, with also a relevant protein removal yield. Even so, the resulting solids still contain a protein content similar to the untreated biomass, and a relevant carbohydrate content making it a suitable feedstock for other applications, e.g., feed applications, biofertilizers and biofuels production.

Conclusion

The two studied fractionation methods, autohydrolysis and dilute acid hydrolysis, enabled the effective extraction of carbohydrates from *S. obliquus* biomass being the sugars obtained mainly in the oligomeric form. Autohydrolysis enabled a 50 % sugar recovery yield after 120 min treatment, and dilute acid hydrolysis also showed faster kinetics, reaching the maximum total sugar yield (also close to 50 %) after only 90 min, demonstrating that dilute acid hydrolysis allowed a similar sugar recovery in a shorter timeframe and slightly lower temperature. Protein solubilization was also assessed with a maximum removal yield of 35 % achieved for both processes. As compared to other currently available strategies these two approaches are considered extremely promising for microalgae fractionation enabling the production of added-value products, i.e., potentially marketable oligosaccharides, and processed solids with a potentially improved upgradability in the biorefinery framework, especially taking into account that these processes can also be considered as hygienic/sterilizing, thus enabling the potential recovery of stabilized products.

Table 4
Composition of dilute acid hydrolysis treated *S. obliquus* biomass at 140 °C (g/100 g treated biomass).

Component	Time (min) 0	30	60	90	120
Glucan	8.48	5.60	6.16	6.06	4.57
Xylan	0.43	0.00	0.22	0.10	0.09
Mannan	1.86	1.83	1.88	1.62	1.71
Galactan	2.30	1.34	1.52	1.36	2.00
Protein	31.94	29.60	30.58	30.29	29.67
Acid Insoluble residue*	4.77	30.76	24.44	29.22	33.41
Acid insoluble ash	0.00	2.22	2.05	4.54	6.72
Others (by difference)	50.21	28.65	33.15	26.81	21.84

* Protein free.

CRediT authorship contribution statement

Pedro L. Martins: Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Alberto Reis:** Funding acquisition, Project administration, Supervision, Resources, Data curation, Investigation, Writing – review & editing. **Luís C. Duarte:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Florbela Carvalheiro:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

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

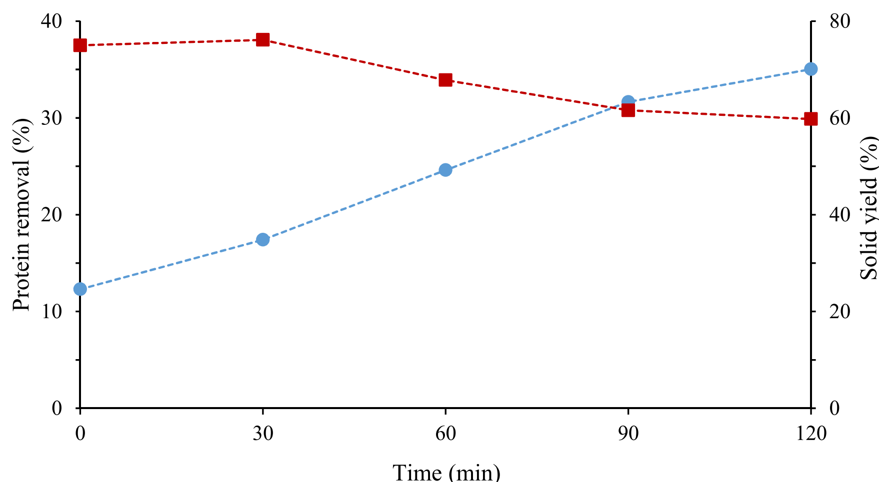

Fig. 5. Protein removal (•) and solid yield (■) obtained after dilute acid hydrolysis of *S. obliquus* at 140 °C.

Data availability

Data will be made available on request.

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