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Kinetic study of the semi-continuous extraction/hydrolysis of the protein and polysaccharide fraction of the industrial solid residue from red macroalgae by subcritical water

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ABSTRACT

The valorization of the underexploited solid residue after agar extraction from red marine algae was studied by subcritical water treatment. Experiments were carried out in two different semi-continuous fix-bed reactor configurations at 185 ºC at different subcritical water residence times. The use of a by-pass section allowed to heat the water previous contact to the biomass, avoiding the exposure of the sample to high temperatures during the heating procedure and reducing the formation of degradation products. Higher hydrolysis yields were obtained for the protein fraction (reaching 96.1%) than for the carbohydrate fraction (reaching 45.7%, 11.3%, 27.5% and 57.6% for galactans, glucans, arabinans and uronic acids, respectively). With the decrease of the residence time, by increasing the flow rate, higher initial hydrolysis rates were obtained due to enhancing diffusion of the hydrolysis products into the bulk solution. It was determined a similar dependence of the initial hydrolysis rates on the residence time for the carbohydrate oligomers and total protein fraction, but the release of free amino acids was less dependent on increasing flow rate due to higher diffusion coefficients for small molecules.

1. Introduction

Subcritical water (subW) is pressurized water in its liquid state in the temperature range from 100 ºC to 374 ºC (T_c = 374 ºC, p_c = 22 MPa). Under these conditions, water presents unique properties such as H-bonding weakening, allowing dissociation of water into hydronium ions (H_3O^+) and basic hydroxide ions (OH^-), thus leading to higher ionization constant, K_w, that confers hydrolysis properties of water as solvent [1]. At ambient pressure and temperature, water is considered a polar solvent due to its high dielectric constant of 80. At elevated temperatures in its liquid state, the dissociation of hydrogen bonding decreases the dielectric constant and water becomes a less polar solvent. For instance, when the water temperature is elevated to 250 C at 25 MPa, water possesses a dielectric constant of 25 and behaves as methanol or ethanol to dissolve different organic molecules [2]. Furthermore, under subcritical conditions, water presents lower viscosity and higher diffusivity than at ambient conditions that facilitates the penetration of water into complex structures [1]. Due to its properties, subW is an attractive alternative solvent to be integrated in the valorisation process of different sources of biomass for hydrolysing and dissolving its different components to obtain high added-value products with application in the food, cosmetic and pharma industries. The use of subW meets the green chemistry and green chemical engineering concepts, avoiding the use of harsh reagents, minimizing the environmental impact [3].

Among the different types of biomass, algae presents several advantages as feedstock since algae productivity is higher than for terrestrial plants, algae are lignin-free and are not used as a food crop [4, 5]. In literature, the use of subW in a biorefinery context has been recently reviewed to valorise different marine algae [6]. However, valorisation of industrial by-products from marine algae has been hardly considered.

Phycocolloids are the main commercial seaweed extracts. However, during processing, only 15–30% of the total dry mass of seaweed is used and important amounts of waste are generated [7]. *Gelidium* (Rhodophyta *Gelidiaceae*) is a red alga used as the major seaweed resource in the Spanish agar industry, providing a high-quality agar. The industrial extraction process generates a solid residue that is usually disposed of, although it presents a valuable chemical composition since it still contains an important fraction of polysaccharides and a high protein content with all the essential amino acids [8].

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Most of the works reported in literature have been carried out in a discontinuous mode [6]. In this operation mode, a certain amount of the biomass is charged in a batch reactor and put in contact during a certain time with water in subcritical conditions. In a previous work of our group, subW treatment at different temperatures was carried out in a semi-continuous fixed bed reactor to hydrolyse and recover the protein fraction of the industrial solid by-product generated after agar extraction from the red algae *Gelidium sesquipedale* [8]. In a semi-continuous reactor, there is a continuous supply of fresh water, enhancing the mass transfer due to high concentration gradients at the solid-liquid interface [9]. Under these conditions, more than the 70% of the protein fraction could be recovered. Experimental conditions such as temperature and flow rate determine the degree of hydrolysis as well as the formation of degradation products from biomass. However, other important variables must be also considered such as reactor size and geometry. The heating rate, has been also identified as a key parameter in the subW treatment of the biomass and it also influences the degree of hydrolysis obtained [10]. Furthermore, heating effects are also important in technical applications for the design of a heat exchanger [11]. In literature, many of the subW heating set-ups consist in a preheating coil placed inside an oven together with the subW reactor. However, the heating of water can also be reached by using a heating jacket or belt [12].

The aim of this work was to study the effect of the heating mode and residence time on the hydrolysis rate of the components of the solid residue generated after agar extraction. A detailed characterization of biomass and its hydrolysis products, including carbohydrates, uronic acids and protein fraction was carried out to help understand the effect of the above experimental conditions on the kinetics and yield of the process.

2. Material and methods

2.1. Raw material

The raw material consisted of the industrial solid residue from *Gelidium sesquipedale* after agar extraction and it was kindly provided by Hispanagar (Burgos, Castilla y León, Spain, https://www.hispanagar.com/es). It was oven dried at 45 ºC and the final moisture content of the macroalgae residue (MR) was 5.0 ± 2.4%. Sample moisture was used to express the results per gram of dried MR (DMR).

A complete chemical characterization of the MR was performed according to the National Renewable Energy Laboratory (NREL, https://www.nrel.gov/bioenergy/biomass-compositional-analysis.html). Uronic acids were determined according to the method of Filisetti-Cozzi [13,14].

2.2. Subcritical Water Equipment

Two different fixed-bed reactor configurations were used. In the first configuration, a fixed-bed reactor (length 20.6 cm and internal diameter of 2.8 cm) was placed inside an oven (Selecta T 204 A) with ca 7 g of MR unground and unsieved. In this configuration, water and biomass were subjected to the same temperature profile during the heating procedure. A detailed description of this configuration can be found elsewhere [8]. In the second configuration, a smaller reactor was used (length 20.3 cm and internal diameter of 1.3 cm) with ca. 2.3 g of MR loaded into the reactor (Fig. 1). In this case, water was heated up by circulating through the preheating coil. A second heating jacket covering the reactor allowed to keep constant temperature during treatment. The reactor vessel loaded with the biomass was first flooded with a small amount of water and kept at around 70 ºC until water circulating through the preheating coil reached the selected temperature by-passing the reactor vessel. Then, the bypass section was closed allowing the hot and pressurized water to circulate through the reactor at the working temperature. This way, the exposure of the biomass to high temperatures during the heating procedure was avoided.

In both configurations, an HPLC pump (Gilson 305, SC-10 head with a maximum flow of 10 mL / min) was used for pressurization and water pumping. Two metallic filters were placed at the top and bottom of the reactors to avoid loss of solid particles and clogging of the system. Pressure was controlled by a pressure regulating valve (pressure Tech 6784 V962 max 414 bar). The temperature was continuously registered with type K thermocouple sensors connected to PDI systems (RS PRO) that controlled the temperature of the heating elements. The time zero was taken as the time at which liquid extract was obtained at the outlet pipe. Effluents were cooled at the exit pipe and periodically collected for further characterization. The results were calculated as accumulative values along treatment time. The yields of the different biomass components were evaluated as the ratio of the mass of the component determined in the subW extracts and the total amount of such component in the raw material.

Four experiments were carried out: one experiment in the first configuration at 185ºC and a working flow rate of 2 mL/min and three experiments in the second configuration at 185 ºC and different working flow rates in the range from 4 to 8 mL/min. The residence time for the water in the reactor, τ (min), was evaluated as:

$$\tau = \frac{V}{F_{v,o}} \frac{\rho_r}{\rho_o} \quad (1)$$

where V is the reactor volume in mL, $F_{v,o}$ the flow rate measured at ambient conditions, in mL·min^{-1}, ρ_o is the water density at ambient

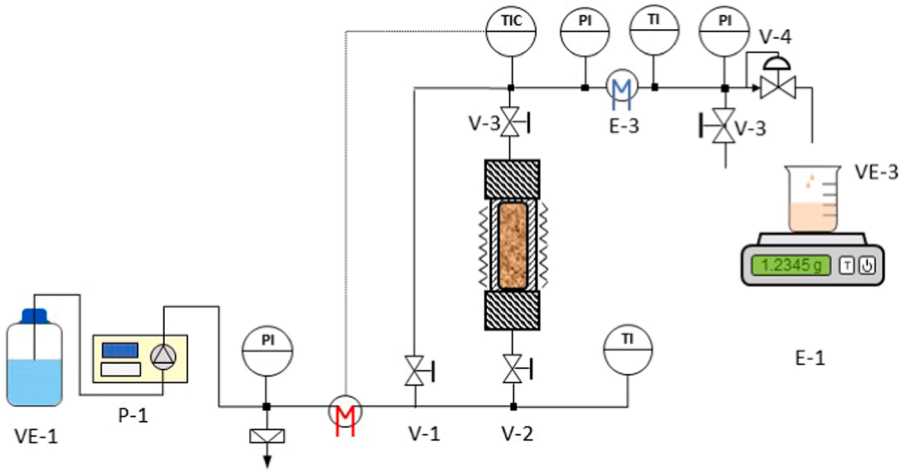

Fig. 1. Subcritical water equipment provided with a bypass system.

conditions and ρ_r the water density at the reaction conditions. The calculated residence time was 56.2 min in the first configuration and 6, 4.2 and 3 min in the second configuration, decreasing by increasing flow rate.

The solid residue that remained in the reactor after subW treatment was washed and dried in an oven at 45 °C until constant weight. The hydrolysis yield was evaluated as:

$$Hydrolysis\quad yield\quad (\%)\quad =\quad \frac{m_{o,MR}-m_{H,MR}}{m_{o,MR}}\cdot 100 \tag{2}$$

where $m_{o,MR}$ and $m_{H,MR}$ are the initial mass charged in the reactor and the mass after subW hydrolysis of the macroalgae residue, respectively.

2.3. Analytical methods

2.3.1. Polysaccharide fraction

Identification and quantification of polysaccharides and their degradation products were performed by HPLC equipped with a Biorad Aminex-HPX-87 H column (300 ×7.8 mm, Bio-Rad), and its corresponding pre-column, with two detectors, a variable wavelength detector (VWD) and a refractive index detector (RID) using 0.005 M sulfuric acid as mobile phase with a flow rate of 0.6 mL/min. The column and detectors were maintained at 40 ºC. Monosaccharides and degradation products were directly analyzed in the subW hydrolysates previously filtered through a 0.22 μm pore size syringe filter (Scharlab). Total sugars were determined after hydrolysis of the sample according to the National Laboratory Analytical Procedure, (https://www.nrel.gov/bioenergy/biomass-compositional-analysis.html) to hydrolyze all the oligomers in monomers sugars. Sugar yield was calculated as monomer and oligomer yield [15]:

$$Monomer\quad yield\quad (\%) = \frac{(Monomeric\quad sugar)_{hydrolysate}}{(Sugar)_{raw\ material}}\cdot 100 \tag{3}$$

$$Oligomer\quad yield\quad (\%) = \frac{(Total\quad sugar - Monomeric\quad sugar)_{hydrolysate}}{(Sugar)_{raw\ material}}\cdot 100 \tag{4}$$

2.3.2. Total protein content and free amino acids

Protein content in the raw material was estimated from the elemental nitrogen content present in the samples by using the corresponding N-factor of 4.9 [8]. Nitrogen in the subW extracts was determined by using a Shimadzu TOC-V CSN analyser.

The amino acid profile of the MR and the free amino acids in the subW extracts were analyzed by gas chromatography by using the EZ: faast™ kit (Phenomenex). Details of the analytical procedure can be found elsewhere [8].

The hydrolysis degree (DH) was evaluated by the ninhydrin reaction method according to the Sigma Aldrich protocol previously described in detail [8]. A daily prepared leucine solution was used as standard. The DH was obtained as [16]:

$$DH = h/h_{tot}.100\ (\%) \tag{5}$$

where h is the number of equivalent peptide bonds hydrolysed, expressed as m_{eq}/g protein and h_{tot} is the total amount of milimols of individual amino acids per gram in the unhydrolyzed protein that can be evaluated from the amino acid profile of the raw material.

2.3.3. Total organic carbon

A Total Organic Carbon (TOC) Analyzer Shimadzu (TOC-V CSN) was used to quantify the concentration of total carbon (TC) and inorganic carbon (IC). The TOC concentration was then calculated by subtracting the IC from the TC concentration.

2.3.4. Total Polyphenols Content (TPC) and Antioxidant Activity

Total polyphenols content, TPC, was determined by using the Folin–Ciocalteu reagent [17]. Results were expressed as mg of gallic acid equivalent (GAE) per gram of DMR.

The FRAP method was used to determine the antioxidant activity. It was performed according to Benzie and Strain [18]. Results were expressed in μmoles of Fe^{2+} per gram of DMR.

2.4. Statistical Analysis

All values were expressed as mean ± standard deviation of at least three replicates. To confirm significant differences, the Fisher's least significant differences (LSD) method at p-value ≤ 0.05 was applied. Correlations between variables were determined by the Pearson correlation method. Analysis were carried out by the Centurion Statgraphics software.

3. Results and discussion

3.1. Macroalgae residue characterization

Table S1 collects the chemical composition of the solid residue from the red macroalgae used in this work. The composition of fresh *Gelidium sesquipedale,* before agar extraction, was also presented for comparison. Extractives and galactans content decreased after agar extraction since agar is a mixture of two components: agarose, a linear polysaccharide made up of repeating sequences of (β-1→4) linked 3,6-anhydro-L-galactopyranose and (α-1→3) linked D-galactose, and agaropectin, a sulphated galactan with different percentages of ester sulfates, pyruvic acid and D-glucuronic acid [19]. Furthermore, extractives were also reduced in the MR as a result of soluble compounds extraction together with agar [20]. Therefore, the percentage of other compounds, such as glucans, arabinans, proteins and ashes, is higher in the MR than in fresh algae. Uronic acids mass percent in the MR was 3.8 ± 0.1% while slightly higher mass percent was determined in fresh algae (4.3 ± 0.1% (w/w). Uronic acids are highly valuable chemicals used in the pharmaceutical, cosmetic and food industries due to its potential as platform chemicals [3]. The value determined for uronic acids in this work, was lower than the value reported by Cui et al. [21] in a purified polysaccharide fraction from *Gelidium pacificum* who determined nearly 20% of uronic acids in this polysaccharide fraction. For fresh *G. sesquipedale* uronic acids accounted for 11.3% of the total carbohydrates, while this percentage decreased down to 9.0% of the total carbohydrates in the MR.

The N conversion factor of the macroalgae residue was evaluated from the amino acid profile listed in Table 2S and previously evaluated as 4.9 [8]. The low value of the N conversion factor, compared to the commonly used value of 6.25, is due to the presence in seaweeds of non-protein nitrogen compounds such as pigments and inorganic nitrogen (nitrite, nitrate and ammonia) that led to lower NF for algae species [22].

3.2. Heating rate

The temperature profile, average value between the top and the bottom of the reactor, in both reactor configurations is presented in Fig. 2. When the oven was used as the heating element, the biomass was exposed to high temperature during the heating procedure. However, in the second configuration, water entered the reactor when the working temperature was achieved by circulating it through the bypass configuration (time zero). The bypass system reduced the contact time of the biomass with water at high temperatures during the heating procedure and the heating jacket led to higher heating rates compared to the oven system due to the different heat exchange mechanism. Therefore, in the second configuration a good control of the heating procedure could be exerted.

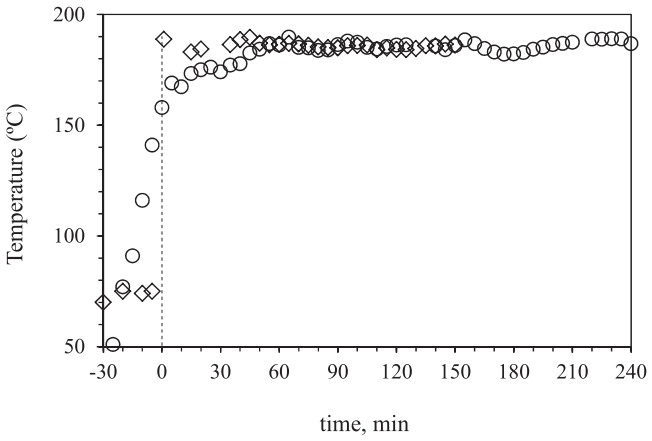

Fig. 2. Temperature profile for the different reactor configurations to reach 185 ºC. Configuration 1: ○ $V_{reactor}$ = 127 cm^3 and oven as heating element. Configuration 2: ⋄ $V_{reactor}$ = 27 cm^3 and heating jacket as heating element with a bypass configuration.

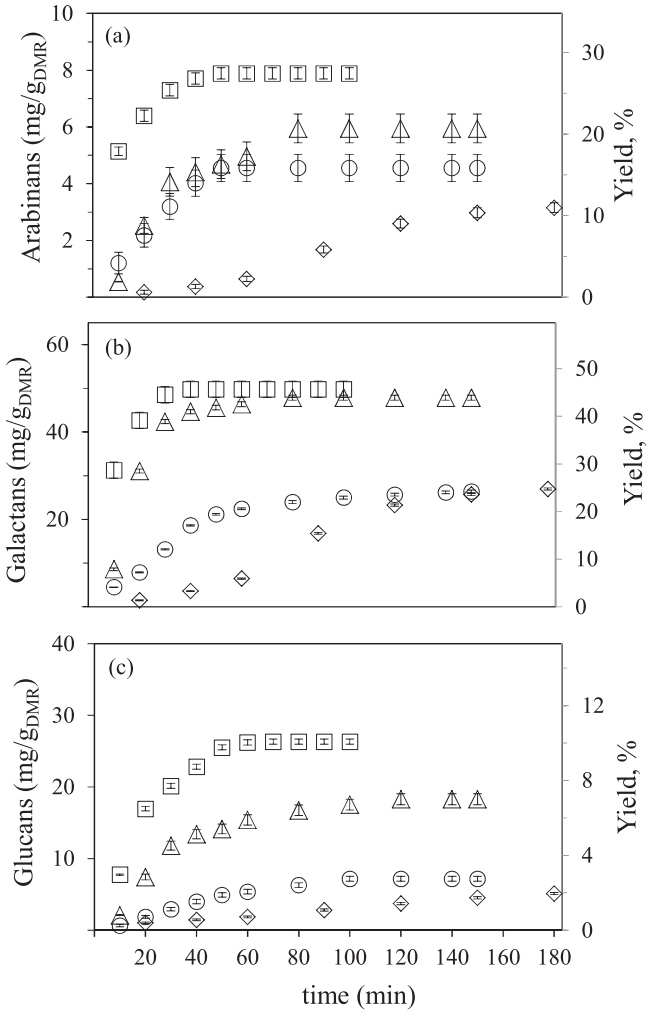

Fig. 3. Extraction/hydrolysis yield by subW treatment (a) arabinans (b) galactans (c) glucans. Configuration 1: (⋄) $V_{reactor}$ = 127 cm^3, 56.2 min of residence time. Configuration 2: $V_{reactor}$ = 27 cm^3 and different residence times, 3 min (□), 4.2 min (△), 6 min (○). T = 185 ºC.

3.3. Polysaccharide fraction hydrolysis

Fig. 3 shows the accumulated hydrolysis curves for arabinans, galactans and glucans expressed as hydrolysis yield, mg/g_{DMR} and percentage yield. At the working conditions selected in this work, the carbohydrate fraction was recovered as sugar oligomers, since no monomers were observed in the subW extracts collected. For all the experiments carried out, preferential hydrolysis was observed for galactans followed by arabinans, with the lowest yield obtained for glucans.

The hydrolysis curves indicated that for configuration 2, the maximum yield was reached at approximately 40 min of subW treatment. However, by using configuration 1, the maximum yield was not reached until 120–140 min. The difference in the extraction/hydrolysis rate determined between both configurations can be also observed in Fig. S1 that shows the concentration elution profile of the oligomers in the different subW extracts collected with time. The maximum oligomer concentration was reached earlier, at around 10–20 min (see Fig. S1), in the experiments performed in configuration 2. On the other hand, by using configuration 1, the maximum level of oligomer concentration in the extracts was reached much later, at around 90 min. It must be highlighted that the high concentration determined for glucans after 20 min of treatment by using the oven as heating element was probably due to the presence of soluble glucans in the extractives that would have been easily extracted during the heating period in the oven (see Fig. S1) since, after this first point, glucans concentration in the extract sharply decreased due to the lower extraction rate in the configuration 1.

It was observed an increase of the initial slope of the extraction/hydrolysis curve by decreasing residence time (increasing flow rate, see Fig. 3). The increase of the initial rate for the different polysaccharide oligomers by decreasing residence time indicates that external mass transfer limitations can play an important role in the transport of the soluble oligomer carbohydrates from biomass surface to the bulk of the solvent. Liu et al. [23] studied the effect of flow rate of subW on hemicellulose removal from corn stover and proposed that water will hydrolyze long-chain oligomers more slowly than short chain oligomers. As a result, long chain oligomers could build up on the biomass surface creating an “icelike” layer, due to slower dissolution and diffusion than shorter oligomers, that slows the access of water to carbohydrates. An increase in flow rate would decrease the thickness of this “icelike” layer enhancing diffusion of oligomers into the bulk solution. This effect led to an increase in carbohydrate solubilisation, as can be observed in Fig. 3. The initial extraction rate of oligomers at the different flow rates (or residence time) essayed in this work were evaluated through the initial slope of the accumulated curves expressed as percentage yield, for a better comparison between the different carbohydrates. A linear relationship was determined between the napierian logarithm of the initial rate and the inverse of the residence time of water inside the reactor:

$$\ln r_o = a + b/\tau \quad (6)$$

where r_o is the initial extraction rate and τ is the residence time. The linear regressions for the different polysaccharide oligomers are shown in Fig. 4a. Table 1 shows the linear parameters and the quality of the fittings together with statistical significance of the terms in the model to compare the different regression lines. There were not statistically significant differences among the slopes at the 90% or higher confidence level while there were statistically significant differences among the intercepts at the 99% confidence level. The higher the value of the intercept, the higher the extraction/hydrolysis rate for the oligomer, following the order galactans, arabinans and glucans. However, the similar values of the slopes of the linear regression showed that the effect of increasing flow rate (decreasing residence time) on oligomer hydrolysis/solubilisation was of the same order regardless the type of oligomer.

In subW treatment resulting products are usually defined in two categories: (a) hydrolysis products as the combination of oligomers, and

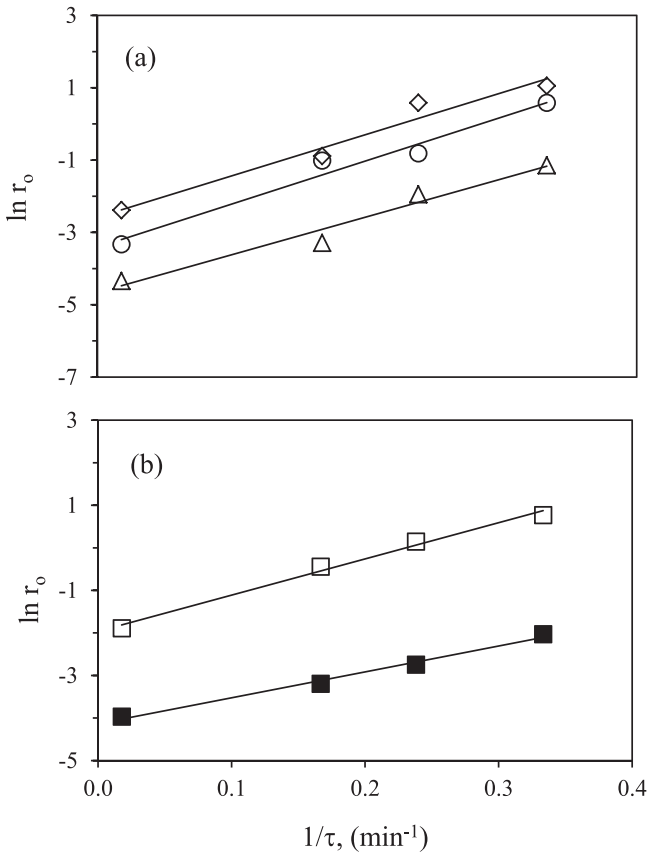

Fig. 4. Linear relationship of the initial extraction/hydrolysis rate of the different biocompounds: (a) glucans (△), arabinans (○), galactans (◇), (b) total protein fraction (□), free amino acids (■). T = 185 ºC.

Table 1
Linear regression parameters from the linear relationship ($\ln r_o = a + b/\tau$) for the different chemical compounds.

Compounds	Slope, b	Intercept, a	R^2
Galactans	11.5 ± 1.6^{b,c}	-2.58 ± 0.35^{d}	0.9628
Arabinans	12.0 ± 1.5^{c}	-3.41 ± 0.33^{c}	0.9692
Glucans	10.5 ± 1.4^{b,c}	-4.66 ± 0.31^{a}	0.9658
Protein fraction	8.5 ± 0.6^{b}	-1.96 ± 0.13^{d}	0.9912
Amino acids	6.1 ± 0.4^{a}	-4.14 ± 0.09^{b}	0.9906

Different letters in each column indicate that there are statistically significant differences among the slopes at the 90% of higher confidence level and among the intercepts at 95% confidence level.

monomers of polysaccharides (b) degradation products such as organic acids, hydroxymethyl furfural (HMF) and furfural and retroaldol condensation products of C6 sugars such as glycoaldehyde or glyceraldehyde among others [24]. Fig. 5 shows the final extraction/hydrolysis yield for oligomers and the total production of the degradation products identified in this work as a function of the severity factor (log R_o). Severity factor, a common factor used when working with pressurized fluids, was evaluated according to:

$$\log R_o = log\left(\tau \cdot exp^{\left((T-T_{ref})/14.75\right)}\right) \quad (7)$$

where τ is the residence time (min), T is the operating temperature (°C) and T_{ref} is equal to 100 °C. The higher the residence time and temperature, the higher the severity factor. According to Fig. 5, oligomers yield decreased by increasing the severity factor (higher residence times at constant temperature). Higher severity factors led to higher concentration of degradation products. The final accumulative yield profile of the

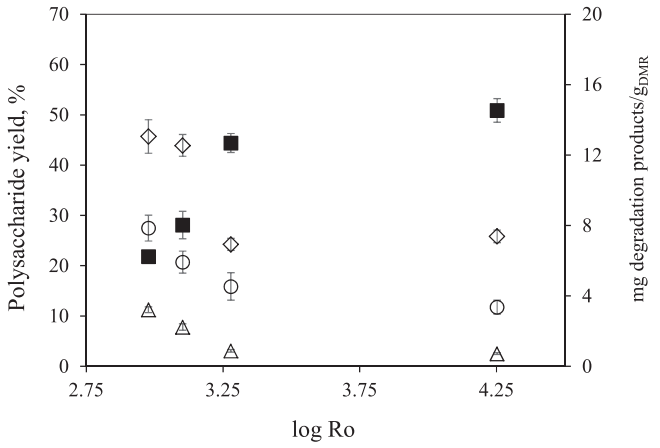

Fig. 5. Final extraction/hydrolysis yield for the different polysaccharide oligomers as a function of the severity factor by varying the residence time: glucans (△), arabinans (○), galactans (◇). Final yield of sugars degradation products (■). T = 185 ºC.

different degradation products has been plotted in Fig. S2 as a function of the severity factor. Degradation products identified in the extracts of configuration 2 included mainly small organic acids such as acetic and formic acids. Acetic acid final accumulative profile varied in the range from 2.3 ± 0.2–5.0 ± 0.2 mg/g_{DMR} with increasing values by increasing the severity factor. However, for formic acid, values in the range from 3.5 ± 0.2–4.6 ± 0.5 mg/g_{DMR} were obtained but no trend with the severity factor was observed. Dehydration degradation products from sugars, hydroxymethylfurfural (HMF) and furfural from glucose and pentoses degradation, respectively, were obtained in much lower amounts. Final accumulative values of HMF varied in the range from 0.010 ± 0.001–0.86 ± 0.02 mg/g_{DMR}, increasing with the severity factor value. Due to the low amount of pentoses in the raw material, the amount of furfural detected in the subW extracts was much lower than the amount of HMF, being less than 15% of the total amount of HMF. For the experiment carried out in configuration 1, other organic acids were also determined in the extracts such as succinic, 3.1 ± 0.1 mg/g_{DMR}, lactic, 2.2 ± 0.2 mg/g_{DMR}, and levulinic, 0.08 ± 0.01 mg/g_{DMR}, acids. In this work, products from retroaldol condensation of C6 sugars such as glycoaldehyde or glyceraldehyde were not detected in the subW extracts.

Among the different polysaccharides determined in the raw material, uronic acids present a great attractive due to its potential as platform chemicals. Galacturonic acid was the most abundant uronic acid

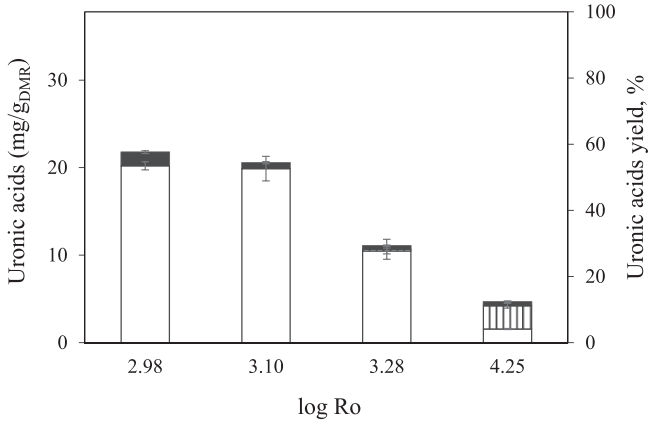

Fig. 6. Uronic acids yield as a function of the severity factor by varying the residence time: galacturonic acid as monomer (□) and oligomer(▥); glucuronic acid as monomer (■). T = 185 ºC.

determined in the subW extracts with small amounts of glucuronic acid. The final extraction yield obtained from the sum of the individual uronic acid content in all the subW extracts collected along the extraction time has been represented in Fig. 6, expressed as mg/g_{DMR} and percentage yield, as a function of the severity factor. A decrease in the severity factor (lower residence times due to higher flow rates), led to an increase in uronic acids yield due to an enhancement of external mass transfer limitation, as previously explained, but also to a reduction in the degradation of the already solubilized uronic acids due to shorter residence times inside the reactor at high temperatures. Some characteristic products of thermal degradation of uronic acids that have been reported in literature are 2-furancarboxylic and 5-formyl-2-furancarboxylic acids but these compounds were not measured in this work [25]. In Fig. 6, it can be also observed that by decreasing the severity factor, as a consequence of a decreasing in the residence time, galacturonic acid was mainly released as monomer.

3.4. Protein fraction

The solubilisation of the protein fraction of the MR is presented in Fig. 7a. The solubilized protein fraction in the subW extracts was evaluated from the nitrogen content in the extracts, by applying the same N conversion factor as for the raw material, although the N-factor could have varied in the protein hydrolysates compared to the raw material.

By working at the lowest residence time essayed in this work, nearly 100% of the protein content was recovered in the subW extracts. A comparison of Figs. 3 and 7 showed higher hydrolysis yields of the protein fraction than that of the carbohydrate fraction for all the experiments carried out. This could be attributed to the fact that proteins were not bonded as strongly as the carbohydrate fraction to the algae structure and they were more easily extracted [25].

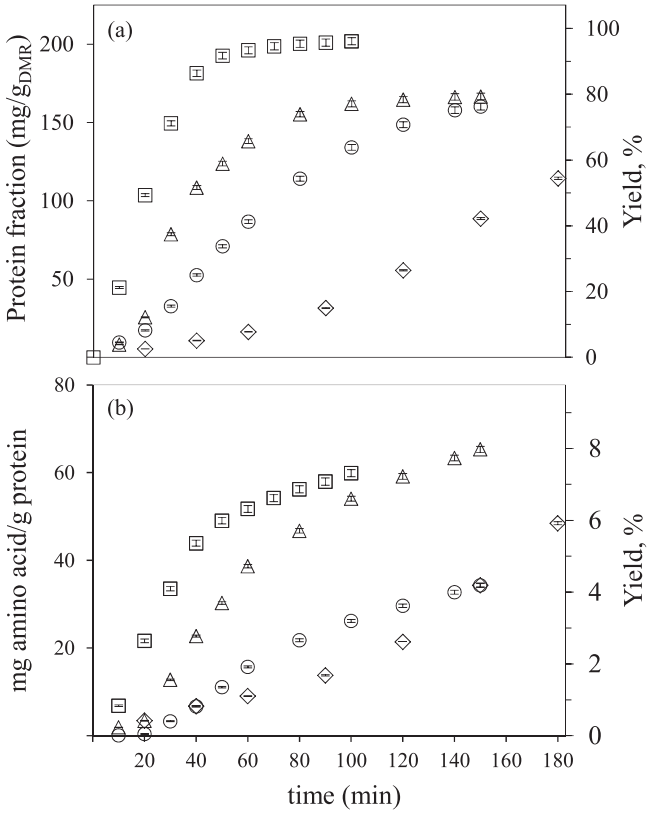

Fig. 7. Extraction/hydrolysis yield by subW treatment of (a) the protein fraction and (b) free amino acids. Configuration 1: (⋄), $V_{reactor}$ = 127 cm^3, 56.2 min of residence time. Configuration 2: $V_{reactor}$ = 27 cm^3 and different residence times, 3 min (□), 4.2 min (△), 6 min (○). T = 185 °C.

A small fraction of the protein content was hydrolysed as free amino acids in the subW extracts (see Fig. 7b). The free amino acid yield was evaluated as the ratio of the sum of all the individual free amino acids determined by gas chromatography in the collected subW extracts and the sum of individual amino acids determined in the raw material. The highest free amino acid yield, around 8%, was obtained from the experiment carried out in the second configuration at the lowest residence time. As an example, Fig. S3 shows the accumulative yield of the individual free amino acids for the experiment carried out at the lowest residence time. Some of the amino acids present in the raw material were not detected in the subW extracts as free amino acids, specifically threonine, methionine, glutamic acid, tryptophan and lysine. In this regard, polar amino acid side groups have been reported to present a high tendency to undergo Maillard reactions with carbonyl groups or reducing carbohydrates generated during subW treatment [26]. This could justify the low or null yield obtained for threonine, lysine, glutamic acid and serine. The highest free amino acid yield was obtained for the non-polar amino acid glycine, which is the smallest amino acid. High molecular weight amino acids are more susceptible to degradation not only to ammonia, organic acids and amines but also to other amino acids of lower molecular weight, which could compensate the degradation of these amino acids [27]. Similar results were obtained in a previous work where the effect of temperature on free amino acid profile and yield [8] was studied in the subW extracts.

Fig. 8 shows the relationship between the hydrolysis degree and the content of total free amino acids obtained as the sum of the individual amino acids determined by GC in the subW extracts. A good correlation has been established between both variables. Person's correlation coefficient between the hydrolysis degree and the content of total free amino acids indicated a statistically non-zero correlation at the 95.0% confidence level and positive and strong correlation between both parameters (n = 41, R^2 = 0.958).

The effect of increasing flow rate (decreasing the residence time) on the protein fraction hydrolysis, as small peptides or free amino acids, was similar to the one described for the polysaccharide fraction. The initial extraction/hydrolysis rate increased with flowrate (decreasing residence time), which support the fact that external mass transfer limitations could play an important role, since higher flow rates would help to enhance diffusion of hydrolysed peptides and amino acids from the biomass surface to the water bulk, similar to the theory proposed by Liu et al. [23] for hemicellulose dissolution from corn stover in subcritical water. On the other hand, lower residence times would lead to a decrease of the degradation of peptides and amino acids, thus

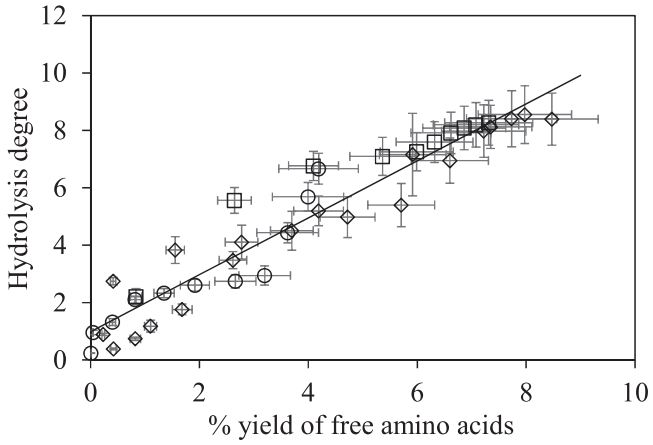

Fig. 8. Hydrolysis degree as a function of yield percentage of free amino acids determined by GC (DH = 0.9925(free amino acids) + 0.984; R^2 = 0.9175). Configuration 1: (⋄) $V_{reactor}$ = 127 cm^3, 56.2 min of residence time. Configuration 2: $V_{reactor}$ = 27 cm^3 and different residence times, 3 min (□),4.2 min (△), 6 min (○). T = 185 °C.

obtaining higher hydrolysis yields. The initial extraction/hydrolysis rate of peptides and free amino acids were evaluated through the initial slope of the accumulated curves expressed as percentage yield. As for carbohydrates, a linear relationship was established between the neperian logarithm of the initial rate and the inverse of the residence time according to Eq. (6) (see Fig. 4b). Parameters of the linear model have been listed in Table 1. Statistical analyses of the slopes and intercepts for carbohydrates, protein and free amino acid solubilisation showed that there were not statistically significant differences among the slopes at the 90% of higher confidence level for carbohydrates and total protein fraction, except for proteins and arabinans, but there were statistically significant differences in the value of the slope for the free amino acid initial release. The lowest value of the slope for free amino acid release indicates its lower dependence on increasing flow rate due to higher diffusion coefficients in water for small molecules. Longsworth [28] carried out diffusion measurements at 25 °C of amino acids, peptides and sugars in aqueous solutions, finding that an increase in molar volume led to lower diffusion coefficients. In subcritical conditions, higher values of diffusion coefficients can be expected due to lower density and viscosity of water at these conditions, but the order in which components would diffuse would not probably change. These authors also found a progressively diminishing effect in reducing the diffusion coefficient with molar volume; that is, the difference in the diffusion coefficient was greater for the monomers and dimers than for the dimers and trimers. This could explain the similar effect found for carbohydrates (released as oligomers) and peptides when increasing flow rate, while the release of free amino acids (lower slope value) was less affected by increasing flow rate, due to higher diffusivity coefficients.

3.5. Total phenolic content and antioxidant activity of SubW extracts

The elution profiles of TPC in the different experiments and the corresponding reducing power, determined according to the FRAP assay, have been plotted in Fig. 9. The maximum TPC release, as well as the maximum reducing capacity, reached higher levels and appeared sooner by increasing flow rate (decreasing residence time). The analysis of the Pearson product moment correlation indicated a statistically significant non-zero correlation at the 95.0% confidence level and strong and positive correlation between reducing capacity and TPC ($n = 41$, $R^2 = 0.8137$). It must be highlighted that, products from browning reactions such as protein-carbohydrate conjugates, melanoidins, and heterocyclic compounds may interfere in the TPC determination by the Folin-Ciocalteu assay and may also contribute to the antioxidant capacity of the collected extracts, as it has been suggested in different publications [8,29,30].

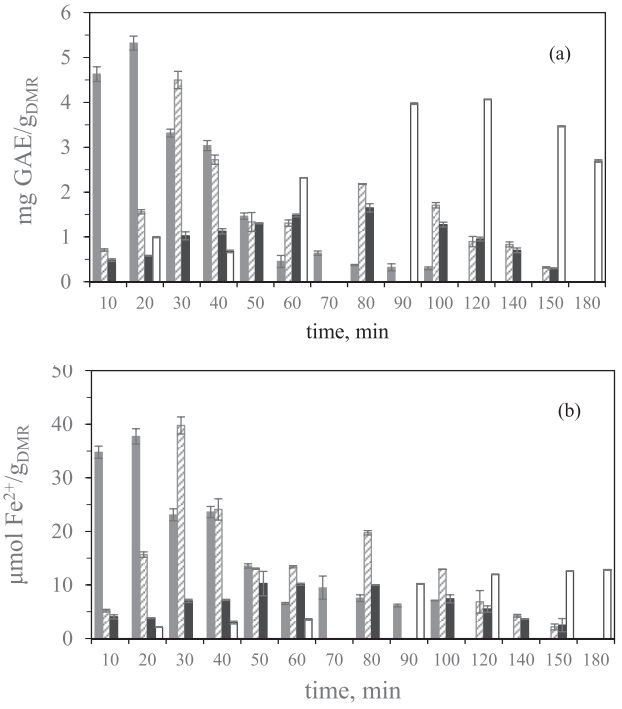

Fig. 9. (a) TPC and (b) reducing power of subW extracts collected along the extraction time. Configuration 1: (□) $V_{reactor} = 127$ cm^3, 56.2 min of residence time. Configuration 2: $V_{reactor} = 27$ cm^3 and different residence times, 3 min (■), 4.2 min (▨), 6 min (■).

3.6. Total organic carbon in subcritical water extracts and hydrolysis yield

The analysis of TOC in the subW extracts showed faster release of the organic carbon fraction by increasing flow rate as well as higher yields (see Fig. 10), according to the results previously presented. TOC would include solubilized polysaccharides and proteins, as well as their degradation products. Long residence times could have led to degradation of the solubilized products that could also release CO_2 or other gas products, as it has been reported in other literature works [31,32]. Unfortunately, gas products were not determined in this work.

The final hydrolysis yield was evaluated according to Eq. (2) and showed that the residence time did not have any significant effect on the final hydrolysis yield with values of 63%, 61%, 59% and 60% for a residence time of 3, 4.2, 6 and 56 min, respectively. Similar results were obtained by other authors in the study of hydrothermal treatment of triticale straw, where flow rate had little effect on dissolved mass [25]. On the contrary, other authors observed that by working at higher flow rates, the reactor load could become more compact, hindering the mass transfer kinetics [33]. Previous work on the same raw material showed an increase on the hydrolysis yield with operating temperature in the range from 125 to 200 °C and a residence time of 56.2 min [8].

By using subW different compounds have been hydrolysed and extracted from an industrial residue contributing to the circular economy concept. As a future work, the determination of a cost-effective application of all these potential products, polysaccharides, peptides and uronic acids, will contribute to asses the economic feasibility performance of the process together with a techno-economic approach of the subcritical water process before the industrial implementation. Some studies have been found in literature about the economic aspects of subW technology. Most of them are focused on water as extracting and

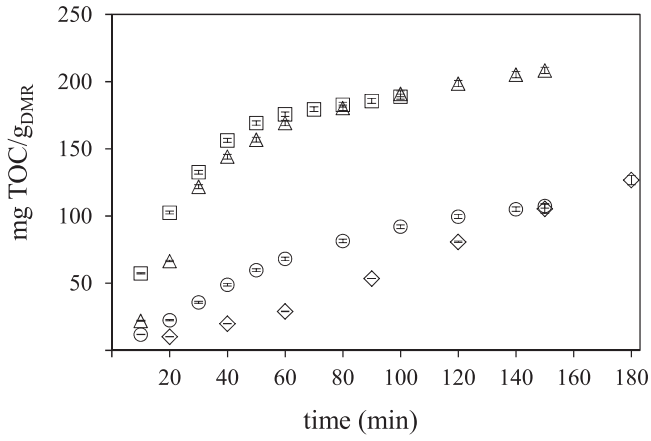

Fig. 10. Accumulative total organic carbon in the subW extracts. Configuration 1: (◇) $V_{reactor} = 127$ cm^3, 56.2 min of residence time. Configuration 2: $V_{reactor} = 27$ cm^3 and different residence times, 3 min (□), 4.2 min (△), 6 min (○). T = 185 °C.

hydrolytic agent to recover bioactive compounds. Todd et al. [34], concluded that subW was very energy-intensive compared to organic solvent extraction which counteracts the environmental advantages of using water as solvent in the study of the extraction of bioactives compounds from grape marc. On the other hand, Essien et al. [35] performed a techno-economic analysis together with the environmental impact assessment and technology readiness for the recovery of bioactive compounds from kanuka leaves. These authors found that the estimated energy consumption when considering the overall process was higher for conventional ethanol extraction than for subW with a higher carbon footprint for ethanol extraction (0.5 kg $CO_2/kg_{kanuka\ extract}$) than for subW (0.13 kg $CO_2/kg_{kanuka\ extract}$). Further research into the energy efficiency of the process would contribute to a more practical feasibility of the process. Additionally, it must be highlighted that subW allows a complete valorisation of the biomass by conveniently fractionation into its individual building blocks [1]. Lachos et al. [36] performed a sequential process to extract and hydrolyse flavanones and sugar from orange peel with optimum temperatures of 150 ºC and 200ºC, respectively. These authors performed the economic evaluation at three different scales, from laboratory, pilot plant and industrial scale determining that the different cost contribution to the process depends on the scale process. From these studies it can be conclude that the scale up process at industrial scale for subW process seems to be economically viable and offers an opportunity to face society's demands of zero residues.

4. Conclusions

The technology of subcritical water allowed hydrolysis of the solid residue generated after agar extraction from red algae. A bypass section before subcritical reactor allowed reaching the operating temperature, avoiding the exposure of the sample to high temperatures during the heating procedure. By working at low residence times, higher flow rates, led to higher hydrolysis rates due to enhancing diffusion of hydrolysed biocompounds from the biomass surface into the bulk solution. Small molecules release, such as amino acids, showed a less dependence on increasing flow rate due to higher diffusion coefficients. It was shown no dependence on residence time for the final hydrolysis yield.

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CRediT authorship contribution statement

Esther Trigueros: Investigation, Data curation, Writing – original draft. **P. Alonso-Riaño**: Investigation, Data curation. **C. Ramos:** Resources, Validation. **C. I. K. Diop**: Writing – review & editing, Visualization. **S. Beltrán:** Conceptualization, Visualization, Funding acquisition. **M. T. Sanz**: Writing – original draft, Supervision, Project administration, Funding acquisition.

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jece.2021.106768.

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