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Recovery of the protein fraction with high antioxidant activity from red seaweed industrial solid residue after agar extraction by subcritical water treatment

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Abstract

In this work valorization of the underexploited industrial solid residue generated after agar extraction from *Gelidium sesquipedale* was studied by using subcritical water in a semicontinuous fix-bed reactor. First, a complete characterization of this by-product was carried out, determining up to 21% (w/w) of protein content (for a nitrogen factor of 4.9) with high content of essential amino acids, 37% (w/w) of carbohydrate fraction and high amount of ash, 22% (w/w). The effect of temperature, in the range from 129 to 200 °C, and flow rate, in the range from 2 to 6 mL min^{-1}, on protein and carbon fraction extraction/hydrolysis was studied. At constant flow rate of 2 mL min^{-1}, a maximum in the protein extraction was achieved at 185 °C. Higher temperatures led to degradation of protein or its hydrolysis products. Free amino acids release followed the same trend as the protein fraction. The most temperature sensitive amino acids, as determined by gas chromatography (EZ:faast Phenomenex), were serine and aspartic and glutamic acids. As a consequence, the selectivity towards non-polar amino acids increased by working at high severity factors. A Pearson correlation between antioxidant capacity of the collected extracts with the bioactive compounds determined in the extracts (total polyphenolic compounds –TPC-, peptides and free amino acids) was established, being stronger for TPC. The ash content in the solid residue after treatment steadily increased with temperature due to non-solubilization being possible its application in agriculture as fertilizers.

Keywords Subcritical water extraction · Biorefinery · Macroalgae residue · Protein · Amino acids · Antioxidant activity

Introduction

The industrial importance of phycocolloids from marine macroalgae is well known for food and cosmetic industries. Agar is a hydrocolloid that is dissolved in hot water and forms gel when it is cooled. Worldwide, agar production exceeds 14,500 t annually with a sale value of 246 million US$ (Porse and Rudolph 2017). *Gelidium* (Rhodophyta, Gelidiaceae) is a red alga mainly found in the Atlantic coasts of France, Spain, Portugal and Morocco, and it is the major seaweed resource in the Spanish agar industry, providing a high quality agar.

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The industrial process to produce agar generates a solid residue that is used for fodder and fertilizer although most of it is disposed of (Ferrera-Lorenzo et al. 2014a). However, this by-product still contains valuable compounds that can be recovered reducing the environmental impact by incorporating this by-product within a circular economy concept.

In the literature some studies can be found for macroalgae biomass valorization (Meillisa et al. 2013; Gereniu et al. 2017; Pangestuti et al. 2019; Bordoloi and Goosen 2020). However, only a few deal with macroalgal by-product valorization (Rajak et al. 2020). Regarding valorization of the solid waste generated in the industrial production of agar, previous studies have already been published by the Instituto Nacional del Carbon (CSIC) analysing the potential energetic uses of the product generated from the pyrolysis of this residual marine biomass and the potential as precursor of activated carbon (Ferrera-Lorenzo et al. 2014a, b).

In this work a different approach has been considered to valorize this solid waste biomass, based on the use of subcritical water (subW) to hydrolyze and fractionate the different biomass compounds, mainly the protein fraction. subW

treatment is a green technology that uses water as solvent at temperatures above its boiling point, 100 °C, and below its critical point, 374 °C, and pressure high enough to keep water at its liquid state. Water has unique properties with temperature. An increase in temperature leads to a decrease in the dielectric constant, increasing solubility of organic compounds in water. Due to the breaking of hydrogen-bonds with temperature, at these conditions, water presents low viscosity and high diffusivity that facilitate water to diffuse into the matrix. Furthermore, concentrations of H^+ and OH^- ions are higher than in its ambient liquid state. Thus, it provides an effective medium for acid- and base-catalyzed reactions (Akiya and Savage 2002).

Biomass can be hydrolyzed and fractionated into high value components such as amino acids, proteins, polysaccharides and other bioactive compounds. In this regard, it has been reported that among the three types of macroalgae, the red algae have the highest protein content containing, in general, all essential amino acids (Postma et al. 2018). The main goal of this work is to explore the subW treatment of *Gelidium sesquipedale* solid residue after agar extraction to valorize mainly its protein fraction and further obtain different bioproducts based on marine proteins, such as small bioactive peptides and free amino acids from the breakdown of the peptide bonds that could have potential applications in the field of the biotechnology, food and pharma industries. In this work, the effect of temperature and water flow rate in a semicontinuous fix-bed reactor on extraction and hydrolysis kinetics of proteins and free amino acids, as well as on the total organic fraction was determined. Furthermore, the antioxidant capacity and total polyphenol compounds released in the extracts were determined, as well as the composition of the solid residue after subW treatment that could be considered for further use.

Material and methods

Raw material

The raw material used in this work has been kindly provided by Hispanagar (Burgos, Castilla y León, Spain, https://www.hispanagar.com/es). It consisted of the solid residue from *Gelidium sesquipedale* after agar extraction. This raw material was oven dried at 45 °C until constant weight to retard microbial growth, preserve the desirable quality and reduce storage volume. This final solid residue was called macroalgae residue (MR). The final moisture content of the MR was determined gravimetrically by weighing it before and after drying in an oven at 105 °C until constant weight, 5.0 ± 2.4%. Sample moisture was used to express results per gram of dried MR (DMR).

Subcritical water equipment

subW treatment was carried in a laboratory scale semicontinuous apparatus (see Fig. 1) equipped with a fixed-bed reactor (20.6 cm length and 2.8 cm internal diameter). Degassed distilled water at room temperature was pumped into the reactor through a HPLC pump (Gilson 305, head SC-10, 10 mL min^{-1} maximum flow) and was heated to the desired working temperature by circulating it through a heat exchanger (60 cm of 1/8″ AISI 316 piping) placed inside an oven (Selecta T 204A) together with the fixed bed reactor where around 7 g of MR were placed. Two metallic filters were place at the top and the bottom of the reactor to avoid loss of solid particles and clogging of the system. A back pressure regulator (Autoclave Engineers SWB2200) allowed pressure control. In this study, a constant pressure of 50 bar was fixed to keep water in its liquid state.

subW treatment was carried out at different temperatures (T = 129, 142, 155, 171, 185 and 200 °C) and flow rates (F = 2 and 6 mL min^{-1}) for a total treatment time of 240 min. Before starting the extraction at the selected extraction temperature, the reactor was loaded with distilled water at room temperature. Temperature and pressure were steadily increased to the selected working conditions during a static holding time of 30 min, selected to improve the DMR swelling and accessibility to water. Then the pump was turned on at the selected feed water flow selecting time 0 as the time at which liquid extract was obtained at the outlet pipe. Different liquid fractions were collected with time and stored at − 18 °C until analysis.

The temperature profile from this zero time is presented in Fig. 2a. In the experiments performed at low temperature, from 129 to 171 °C, it can be observed that 30 min of static holding time was enough to reach the working temperature; however, at the highest temperatures assayed in this work (185 and 200 °C), 30–40 min were still needed to reach the selected temperature. The temperature profile along heating is usually not reported in literature. For instance, Hata et al. (2008) reported that 5–10 min were needed for a small vessel (9 mL) placed in an oven heating system, while it took 25–40 min for a 117-mL reactor when using a mantle heater to reach 260–270 °C. The heating rate and time needed to reach the working temperature must be reported to compare the subW results. A Crison GLP 22 pH-meter was used to measure the pH of the subW extracts at the outlet vessel (Fig. 2b).

Analysis

Biomass characterization

The chemical characterization of MR was performed according to the NREL protocols (https://www.nrel.gov/bioenergy/biomass-compositional-analysis.html). MR free of extractives was subjected to two acid hydrolysis steps. Carbohydrates

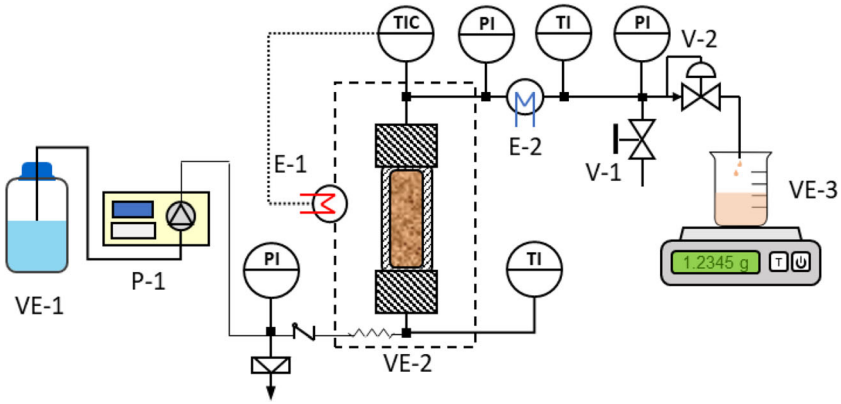

Fig. 1 Diagram of the laboratory-scale pressurized water equipment. VE-1: solvent reservoir; P-1: Gilson 305 HPLC pump; VE-2: extractor; VE-3: collector; V-1: vent valve; (V-2) back-pressure valve

were quantified by high-performance liquid chromatography (HPLC) with a Biorad Aminex-HPX-87 H column, a variable wavelength detector (VWD) and refractive index detector (RID) using a mobile phase constituted by 0.005 M sulphuric acid. Presence of galactose was confirmed by Megazyme L-Arabinose/D-Galactose assay kit. Total lipids were determined according to the Bligh and Dyer method (Bligh and Dyer 1959). Ash content was determined by placing the sample in a muffle furnace at 575 ± 25 °C for 24 ± 6 h until constant weight.

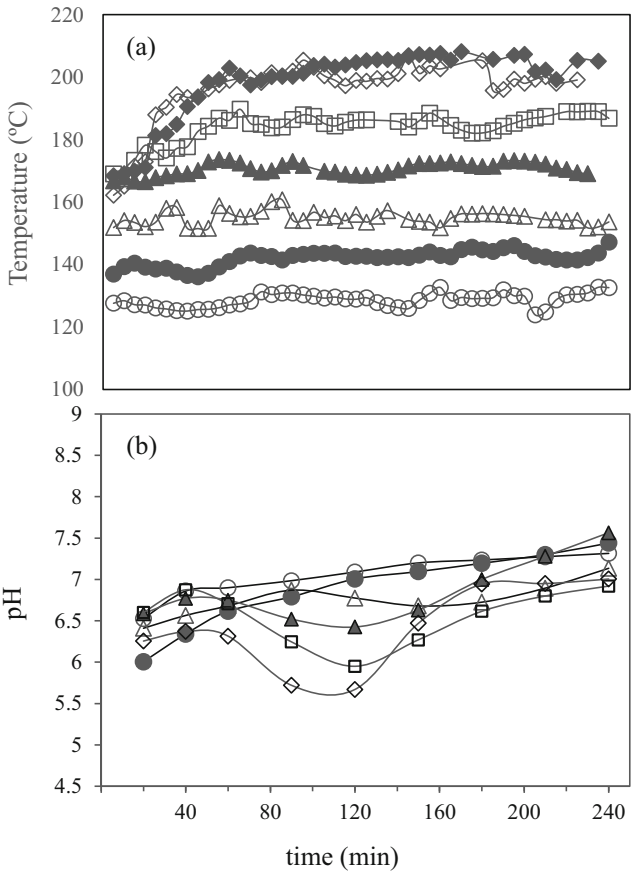

Fig. 2 **a** Extraction temperature profile. **b** pH of the liquid extracts at the outlet pipe along treatment time at different working conditions (F = 2 mL min^{-1}: ○ 129 °C, ● 142 °C, ▵ 155 °C, ▲ 171 °C, □ 185 °C, ◊ 200 °C; F = 6 mL min^{-1} ◆ 200 °C). Continuous lines are guide for the eye

Elemental and inorganic composition Elemental composition (C, H, N, S) of the MR was determined by an organic elemental microanalyzer (Thermo Scientific Model Flash 2000). Oxygen content was determined by mass balance. Inorganic composition of the MR was performed by inductively coupled plasma mass spectrometry (ICP-MS-Agilent 7500cx). Samples were digested with a HNO_3 solution (2 wt%) by using a microwave system. The digested samples were cooled and diluted with deionized water. Concentrations of inorganic elements were determined using standard solutions prepared in the same acid matrix.

Amino acid profile Protein content in the MR was obtained by the total nitrogen content as measured by the elemental analysis by using the corresponding nitrogen factor (NF). The NF was determined according to the amino acid profile of the MR determined after acid hydrolysis with 6 N HCl: 0.1–1.0 g of dried MR was weighed and 1 mL of 6 N HCl was added. The mixture was incubated for 24 h at 110 °C. Afterwards, 1 mL of a 1 N HCl and ethanol solution (1:1 v:v) was added and then filtered. It must be highlighted that during acid hydrolysis, asparagine and glutamine are quantitatively converted to aspartic and glutamic acid, respectively. Most amino acids can be determined by acid hydrolysis; however, tryptophan, cystine and cysteine were lost by acid hydrolysis, and methionine could be partially destroyed by acid hydrolysis. To quantify these amino acids, basic hydrolysis was carried out by mixing 0.1 g of MR with 7 mL of 4.2 M NaOH. The mixture was incubated at 110 °C for 24 h, cooled and neutralized with 6 M HCl in order to get a pH between 1.5 and 5.5. After hydrolysis, the amino acid profile was determined by using the EZ:faast Phenomenex procedure, consisting of a solid phase extraction followed by derivatization and a final liquid/liquid extraction. The resulting samples were analyzed by gas chromatography (Hewlett-Packard, 6890 series) with an EZ:faast AAA LC-integrated column and FID detector. Norleucine was used as internal standard.

Characterization of liquid and solid products from subcritical water treatment

Liquid extract *Protein content* in the extracts was estimated by two different methods. A colorimetric assay based in a reaction similar to the Lowry assay by using the DC kit (Bio Rad Laboratories) and bovine serum albumin as standard. Protein determination was also carried out by total nitrogen content by using a TOC/TN analyzer (Shimadzu TOC-V CSN analyzer) using KNO_3 as standard and the corresponding nitrogen to protein conversion factor (NF).

The hydrolysis degree (DH), the percentage of hydrolyzed peptide bonds, was evaluated by the ninhydrin reaction method. Most amino acids react with ninhydrin but also primary and secondary amines. The measurement was carried out according to ninhydrin Sigma Aldrich protocol. Two milliliter of sample was gently mixed with 1 mL of ninhydrin reagent solution and placed into a boiling water bath for 10 min. Samples were cooled and 5 mL of 95% ethanol were added. Absorbance was measured at 570 nm. A calibration curve was obtained by following the same protocol but substituting the 2.0 mL of sample by 2 mL of different standards of leucine solution that was prepared daily (Friedman 2004). The DH was obtained as (Adler-Nissen et al. 1983):

$$DH(\%) = (h/h_{tot}) \times 100 \quad (1)$$

where h is the number of equivalent peptide bonds hydrolyzed, expressed as $m_{eq}\ g^{-1}$ protein and h_{tot} is the total amount of millimol of individual amino acids per gram in the unhydrolyzed protein that can be evaluated from the amino acid profile.

Free amino acids in the subW extracts were determined by the EZ:faast Phenomenex procedure as described in the previous section ("Biomass characterization").

Total organic carbon (TOC) of the liquid extracts was measured with a TOC/TN analyzer by subtracting the measured inorganic carbon (IC) from the measured total carbon (TC).

Total polyphenolic compounds (TPC) were determined according to Folin-Ciocalteu procedure (Singleton et al. 1999), using gallic acid (GA) as standard. Results were expressed as mg of gallic acid equivalent (GAE) per g_{DMR}.

The reducing capacity of subW extracts was determined by the ferric reducing ability of plasma (FRAP) method according to Benzie and Strain (1996). Results were expressed as μmol of $FeSO_4$ per g_{DMR}.

Colour of the extracts. The L*, a* and b* values were obtained by a suitable program installed in a Beckman DU-650 spectrophotometer with diode-array of UV–vis (Beckman Instruments), representing brightness, red to green colour and yellow to blue colour, respectively (Illera et al. 2018). Other conditions are illuminant D65 (daylight source) and a 10° standard observer (perception of a human observer) following the CIE recommendations.

Residual agar from MR was also co-extracted and determined in the subW extract similarly to Meena et al. (2008). Frozen samples were thawed and air-dried for 24 h at ambient temperature for gel formation. The gelled material was then oven-dried at 50 °C until constant weight. Finally, agar content was determined by weighing the dry agar.

Solid residue after subcritical water treatment The solid residue after subW treatment was dried and weighed and subjected to elemental composition analysis (Thermo Scientific Model Flash 2000). The high heating value (HHV) of the solid residue was evaluated by the following equation and compared to the one evaluated for the MR (Friedl et al. 2005):

$$HHV\left(kJkg^{-1}\right) = 3.55C^2 - 232C - 2230H + 51.2C{\cdot}H + 131N + 20600 \quad (2)$$

Statistical analysis

All determinations were taken at least in duplicate and expressed as mean ± standard deviation. 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. Analyses were carried out by the Centurion Statgraphics software.

Results

Macroalgae residue characterization

Chemical characterization of *G. sesquipedale* residue after agar extraction is presented in Table 1 in a dry weight basis. Carbohydrates constituted the main class of chemical compounds, mainly glucans, 23.4 ± 0.9%, and galactans, 10.9 ± 0.5%, with small amounts of arabinans, 2.9 ± 0.2%. Galactans determined by the enzymatic kit yielded a value of 9.5 ± 0.3%, similar to the one obtained by HPLC determination. Extractives represented 11.5% of MR composition, 22.6% of the extractives were proteins, 6.8% glucuronic acid and 2.4% total polyphenols compounds (2.6, 0.78 and 0.28%, respectively when referred to global macroalgae residue composition).

Lipid content was very low, less than 1%, as it was also determined for other macroalgae species. MR presented also low insoluble lignin content, 3 ± 1%, as corresponds to third generation biomass. Ash content represented 22 ± 2%. The mineral and inorganic elements of MR have been listed in

Table 1 Chemical composition of macroalgae residue expressed as % ± standard deviation in a dry weight basis. ($n = 2$, number of independent samples and 3 technical replicates)

Compound	Composition, %
Extractives	11.5 ± 0.9
Polyphenols	0.28 ± 0.02
Proteins	2.6 ± 0.3
Glucuronic acid	0.78 ± 0.06
Carbohydrates	37 ± 2
Glucans	23.4 ± 0.9
Galactans	10.9 ± 0.5
Arabinans	2.9 ± 0.2
Lignin	12 ± 1
Soluble	8.7 ± 0.1
Insoluble	3 ± 1
Proteins*	21 ± 1
Lipids	0.87 ± 0.09
Ashes	22 ± 2

*Protein includes also the protein content in the extractive fraction

Table 2. This table also includes the composition for fresh macroalgae for comparison with the industrial solid residue generated. Fresh *G. sesquipedale* has a content of inorganic components equal to 100 ± 6 g kg^{-1} of dry algae, while in the MR, this content was reduced down to 54 ± 4 g kg^{-1} dry MR, mainly due to lower levels of Na and K in the MR, less than 3% of the levels presented in the fresh macroalgae.

The nitrogen factor was obtained according to the amino acid profile obtained for MR that has been listed in Table 3. The ammonia content from the degradation of some amino acids during acid hydrolysis was included in the calculation of protein nitrogen (Lourenço et al. 2002). According to the amino acid profile, a NF of 4.9 was determined that yielded a total protein content of 21% (Table 1).

The total amount of amino acids, as determined by gas chromatography, was 172 ± 9 mg aa g_{DMR}^{-1}. The major amino acid in the MR was aspartic acid with 20.4 ± 0.8 mg g_{DMR}^{-1} (97 ± 4 mg $g_{prot\text{-}MR}^{-1}$). Glutamic and aspartic acids were found to be the main amino acids for many algae, including red algae with mean values of 123 and 142 for aspartic and glutamic acid, respectively (Lourenço et al. 2002). Total content of essential amino acids (EAA) in the MR was 76 ± 5 mg EAA g_{DMR}^{-1} (362 ± 5 mg EAA $g_{prot\text{-}MR}^{-1}$). Therefore, valorization of this by-product is of great interest due to the high protein and essential amino acids content, which gives it a great interest as dietary supplement for humans, among other uses.

Elemental composition of MR can be found in Table 4. This table also lists the elemental composition of fresh *G. sesquipedale* for comparison with its industrial by-product. Fresh algae and its by-product presented similar content of carbon, but higher nitrogen and hydrogen content and lower oxygen content. Consequently, higher H:C molar ratio and lower O:C molar ratio were obtained in the MR compared to

Table 2 Inorganic composition of fresh *G. sesquipedale*, macroalgae residue (MR) after agar extraction and solid residue after subW extraction (200 °C—2 mL min^{-1}) in ppm ± standard deviation ($n = 3$ number of technical replicates)

Element	*G. sesquipedale*	MR	Residue-subW$^{200\ °C}$
Li	1.2 ± 0.2	13.4 ± 0.6	23.7 ± 0.4
B	260 ± 6	100 ± 7	62 ± 22
Na	34,484 ± 317	875 ± 31	3573 ± 623
Mg	12,764 ± 187	8918 ± 214	8359 ± 1976
Al	175 ± 59	8156 ± 217	17,831 ± 2102
Si	190 ± 62	109 ± 27	132 ± 28
P	956 ± 87	5786 ± 677	7709 ± 1129
S	11,711 ± 1014	4993 ± 357	1161 ± 57
K	29,352 ± 1082	863 ± 9	1624 ± 401
Ca	449 ± 93	6584 ± 221	12,742 ± 3105
Ti	0.7 ± 0.2	7.32 ± 0.03	15 ± 2
V	2.1 ± 0.1	6.23 ± 0.07	11 ± 2
Cr	1.3 ± 0.4	14 ± 1	47 ± 14
Mn	149 ± 56	386 ± 115	626 ± 206
Fe	251 ± 83	6330 ± 346	12,509 ± 404
Co	0.6 ± 0.2	2.4 ± 0.1	4.1 ± 0.9
Ni	5 ± 1	10 ± 1	25 ± 9
Cu	6 ± 1	8.8 ± 0.7	14 ± 6
Zn	246 ± 2	247 ± 26	377 ± 188
Ga	0.3 ± 0.1	10.50 ± 0.01	21 ± 1
As	5.7 ± 0.2	7.6 ± 0.1	6.8 ± 0.1
Br	3755 ± 826	4871 ± 363	334 ± 133
Rb	29.8 ± 0.4	10.2 ± 0.2	19 ± 1
Sr	35 ± 9	267 ± 110	587 ± 206
Zr	0.21 ± 0.05	2.08 ± 0.01	4 ± 1
Cd	0.53 ± 0.05	1.3 ± 0.2	2 ± 1
I	5793 ± 1926	5766 ± 1381	79 ± 39
Ba	1.1 ± 0.3	39.5 ± 0.4	81 ± 10
La	0.21 ± 0.06	5 ± 1	9.2 ± 0.3
Ce	0.3 ± 0.1	10 ± 3	18 ± 1
Nd	0.15 ± 0.05	4 ± 1	7.9 ± 0.1
Pb	4 ± 2	10.9 ± 0.3	21 ± 8
Total	100,629 ± 5815	54,417 ± 4111	68,035 ± 10,677

the fresh macroalgae. The ash content was much higher for the MR compared to the fresh raw material. Ferrera-Lorenzo et al. (2014b) reported elemental analysis composition of *G. sesquipedale* by-product. These authors obtained higher C (43.99%), N (5.21%) and S (1.02%) content compared to our results. The most important difference lies in the ash content; these authors reported 7.7%, while more than double ash content was obtained in this work. The difference in elemental composition in the *G. sesquipedale* residue can be attributed to the different source of the macroalgae, seasonality, as well as differences in the agar extraction process. With the data of the

Table 3 Amino acid profile of macroalgae residue, MR, and of the subW extracts, expressed as mg $g_{protein\text{-}MR}^{-1}$ ± standard deviation

	MR mg g_{DMR}^{-1}	129 °C, 2 mL min^{-1}		142 °C, 2 mL min^{-1}		155 °C, 2 mL min^{-1}		171 °C, 2 mL min^{-1}		185 °C, 2 mL min^{-1}		200 °C, 2 mL min^{-1}		200 °C, 6 mL min^{-1}	
		mg g_{prot}^{-1}	Yield, %	mg g_{prot}^{-1}	Yield, %	mg g_{prot}^{-1}	Yield, %	mg g_{prot}^{-1}	Yield, %	mg g_{prot}^{-1}	Yield, %	mg g_{prot}^{-1}	Yield, %	mg g_{prot}^{-1}	Yield, %
Ala	19.8 ± 0.7	0.81 ± 0.01	0.86 ± 0.08	0.95 ± 0.01	1.0 ± 0.1	2.36 ± 0.01	2.5 ± 0.2	4.19 ± 0.01	4.4 ± 0.4	6.9 ± 0.2	7.3 ± 0.8	6.22 ± 0.02	6.6 ± 0.1	6.94 ± 0.02	7.4 ± 0.6
Gly	8.6 ± 0.5	0.91 ± 0.01	2.2 ± 0.3	1.44 ± 0.02	3.5 ± 0.4	3.43 ± 0.01	8.4 ± 0.9	6.92 ± 0.01	17 ± 2	10.04 ± 0.04	25 ± 3	7.38 ± 0.05	18 ± 2	8.37 ± 0.05	20 ± 2
Val	14.0 ± 0.9	0.90 ± 0.01	1.3 ± 0.2	0.52 ± 0.01	0.8 ± 0.1	1.04 ± 0.01	1.6 ± 0.2	3.07 ± 0.03	4.6 ± 0.6	6.85 ± 0.07	10 ± 1	6.74 ± 0.02	10 ± 1	6.20 ± 0.03	9 ± 1
Leu	16.3 ± 0.8	0.85 ± 0.01	1.1 ± 0.1	0.73 ± 0.01	0.9 ± 0.1	1.18 ± 0.01	1.5 ± 0.2	2.16 ± 0.01	2.8 ± 0.3	3.83 ± 0.01	4.9 ± 0.5	2.95 ± 0.01	3.8 ± 0.4	3.80 ± 0.02	4.9 ± 0.5
Ile	9.4 ± 0.7	0.57 ± 0.01	1.3 ± 0.2	0.34 ± 0.01	0.8 ± 0.1	0.60 ± 0.01	1.3 ± 0.2	125 ± 0.01	2.8 ± 0.4	2.97 ± 0.02	6.6 ± 0.9	2.50 ± 0.01	5.6 ± 0.7	2.35 ± 0.02	5.3 ± 0.7
Thr	7.5 ± 0.1	0.31 ± 0.02	0.9 ± 0.1	0.29 ± 0.02	0.8 ± 0.1	0.50 ± 0.02	1.4 ± 0.1	0.87 ± 0.05	2.4 ± 0.3	1.03 ± 0.09	2.9 ± 0.4	0.80 ± 0.05	2.2 ± 0.3	1.19 ± 0.03	3.3 ± 0.3
Ser	8.0 ± 0.9	0.74 ± 0.01	1.9 ± 0.3	0.97 ± 0.01	2.5 ± 0.4	1.78 ± 0.01	4.7 ± 0.8	2.47 ± 0.04	6.5 ± 0.9	3.55 ± 0.05	9 ± 2	1.80 ± 0.04	4.7 ± 0.9	2.21 ± 0.09	6 ± 1
Pro	15.4 ± 0.1	0.64 ± 0.01	0.88 ± 0.06	0.97 ± 0.01	1.3 ± 0.1	1.57 ± 0.01	2.1 ± 0.1	2.51 ± 0.01	3.4 ± 0.2	3.67 ± 0.02	5.0 ± 0.3	3.05 ± 0.01	4.2 ± 0.2	4.27 ± 0.02	5.8 ± 0.3
Asp	20.4 ± 0.8	0.40 ± 0.01	0.41 ± 0.05	0.93 ± 0.13	1.0 ± 0.2	5.37 ± 0.01	5.5 ± 0.5	10.19 ± 0.02	10.5 ± 0.9	10.3 ± 0.2	11 ± 1	4.5 ± 0.1	4.6 ± 0.5	18.3 ± 0.1	19 ± 2
Met	1.7 ± 0.2	0.12 ± 0.01	1.5 ± 0.04	0.15 ± 0.01	1.9 ± 0.4	0.52 ± 0.06	7 ± 2	0.84 ± 0.01	10 ± 2	1.30 ± 0.01	16 ± 3	0.97 ± 0.01	12 ± 2	1.9 ± 0.3	24 ± 7
Glu	16.6 ± 0.2	1.47 ± 0.02	1.9 ± 0.1	1.04 ± 0.06	1.3 ± 0.2	0.89 ± 0.04	1.1 ± 0.1	1.15 ± 0.04	1.5 ± 0.1	2.48 ± 0.02	3.1 ± 0.2	1.19 ± 0.04	1.5 ± 0.1	3.3 ± 0.5	4.2 ± 0.8
Phe	10.6 ± 0.8	0.51 ± 0.01	1.0 ± 0.1	0.45 ± 0.01	0.9 ± 0.1	0.79 ± 0.08	1.6 ± 0.3	1.67 ± 0.01	3.3 ± 0.4	2.99 ± 0.04	5.9 ± 0.8	2.34 ± 0.06	4.6 ± 0.7	2.88 ± 0.03	5.7 ± 0.8
Lys	12.0 ± 0.8	1.26 ± 0.08	2.2 ± 0.4	1.26 ± 0.02	2.2 ± 0.3	1.42 ± 0.09	2.5 ± 0.4	1.71 ± 0.02	3.0 ± 0.4	2.85 ± 0.04	5.0 ± 0.6	1.92 ± 0.08	3.4 ± 0.5	6.9 ± 0.5	12 ± 2
His	3.4 ± 0.2	0.26 ± 0.05	1.6 ± 0.5	0.44 ± 0.02	2.7 ± 0.4	0.80 ± 0.15	5 ± 1	1.37 ± 0.01	9 ± 1	2.29 ± 0.06	14 ± 2	1.9 ± 0.2	11 ± 2	5.4 ± 0.4	34 ± 6
Tyr	7.3 ± 0.9	1.33 ± 0.05	3.8 ± 0.8	1.9 ± 0.2	5 ± 1	2.33 ± 0.03	7 ± 1	2.46 ± 0.02	7 ± 1	3.32 ± 0.06	10 ± 2	2.93 ± 0.09	8 ± 1	5.47 ± 0.05	16 ± 3
Trp	0.60 ± 0.02	–	–	–	–	–	–	0.55 ± 0.01	20 ± 2	2.22 ± 0.09	78 ± 10	1.50 ± 0.01	53 ± 5	8.13 ± 0.01	> 100
EAA	76 ± 5	4.8 ± 0.2	1.3 ± 0.2	4.2 ± 0.1	1.2 ± 0.2	6.8 ± 0.4	1.9 ± 0.3	13.5 ± 0.1	3.8 ± 0.4	26.3 ± 0.4	7.3 ± 0.9	21.6 ± 0.5	6.0 ± 0.8	39 ± 1	11 ± 2
TAA	172 ± 9	11.1 ± 0.3	1.4 ± 0.2	12.4 ± 0.6	1.5 ± 0.2	24.5 ± 0.6	3.0 ± 0.4	43.4 ± 0.3	5.3 ± 0.6	67 ± 1	8.1 ± 0.9	48.7 ± 0.8	6.0 ± 0.7	88 ± 2	11 ± 1
NPS	0.6 ± 0.1	0.55 ± 0.02		0.53 ± 0.03		0.55 ± 0.01		0.60 ± 0.01		0.67 ± 0.02		0.75 ± 0.01		0.56 ± 0.02	

1.80 mg NH_3 g_{DMR}^{-1} liberated during acid hydrolysis due to amino acid degradation

Extraction yield percentage (yield, % ± error associated to the ratio of two variables) of individual amino acid in the subW extracts. (*EAA* essential amino acids, *TAA* total amino acids, *NPS* non-polar selectivity, *NP/TAA* molar ratio of non-polar amino acids to total amino acids ($n = 3$ technical replicates, in case of raw material the number of independent samples were 2)

Table 4 Ash content, elemental analysis and estimated heating value (HHV) of original fresh macroalgae, macroalgae residue (MR) and the solid residue after subW treatment (F = 2 mL min^{-1})

Sample	C (%, w/w)	H (%, w/w)	N (%, w/w)	S (%, w/w)	O (%, w/w)	Ashes (%, w/w)	H:C	O:C	HHV (kJ kg^{-1})
G. sesquipedale	36.0 ± 0.3^{d}	5.4 ± 0.2^{e}	3.5 ± 0.3^{d}	0.26 ± 0.07^{b}	40 ± 1^{e}	14.9 ± 0.9^{a}	1.80 ± 0.08^{d}	0.83 ± 0.03^{f}	$15{,}219 \pm 73^{b}$
DMR	35.6 ± 1.2^{d}	5.9 ± 0.2^{f}	4.2 ± 0.4^{e}	0.21 ± 0.05^{b}	32.3 ± 0.3^{d}	21.8 ± 1.1^{b}	2.0 ± 0.1^{e}	0.68 ± 0.03^{e}	$14{,}987 \pm 466^{a,b}$
Residue-SW-129 °C	$33.5 \pm 0.9^{b,c}$	5.2 ± 0.3^{e}	4.2 ± 0.3^{e}	0.20 ± 0.03^{b}	29 ± 3^{c}	29.5 ± 0.8^{c}	$1.9 \pm 0.2^{d,e}$	0.61 ± 0.08^{d}	$14{,}685 \pm 149^{a,b}$
Residue-SW-142 °C	$33.3 \pm 0.6^{a,b,c}$	$4.5 \pm 0.4^{c,d}$	$3.5 \pm 0.5^{c,d}$	0.04 ± 0.03^{a}	$27 \pm 2^{b,c}$	$31.9 \pm 1.8^{c,d}$	1.6 ± 0.2^{c}	$0.61 \pm 0.06^{c,d}$	$14{,}907 \pm 136^{a,b}$
Residue-SW-155 °C	$32.8 \pm 1.6^{a,b}$	4.7 ± 0.1^{d}	3.0 ± 0.2^{c}	n.d.	$21 \pm 4^{a,b}$	$33.4 \pm 2.4^{d,e}$	$1.7 \pm 0.1^{c,d}$	$0.54 \pm 0.05^{b,c,d}$	$14{,}615 \pm 133^{a}$
Residue-SW-171 °C	$34.9 \pm 1.1^{c,d}$	4.0 ± 0.2^{b}	2.2 ± 0.1^{b}	n.d.	$22 \pm 2^{a,b}$	$36.2 \pm 2.6^{e,f}$	$1.4 \pm 0.1^{a,b}$	$0.51 \pm 0.02^{a,b}$	$15{,}343 \pm 188^{b}$
Residue-SW-185 °C	$34.6 \pm 0.9^{b,c,d}$	$4.0 \pm 0.3^{b,c}$	1.8 ± 0.1^{b}	n.d.	$22 \pm 2^{a,}$	37.9 ± 1.9^{f}	1.4 ± 0.1^{b}	0.47 ± 0.04^{a}	$15{,}225 \pm 336^{b}$
Residue-SW-200 °C	31.4 ± 1.6^{a}	3.1 ± 0.3^{a}	1.0 ± 0.2^{a}	n.d.	22 ± 3^{a}	42.7 ± 2.1^{g}	1.2 ± 0.2^{a}	$0.52 \pm 0.06^{b,c}$	$15{,}017 \pm 387^{a,b}$

n.d. not detected. Values with different letters in each column are significantly different when applying the Fisher's least significant differences (LSD) method at p value ≤ 0.05

Errors in the calculated variables have been estimated using the theory of error propagation

(*C* carbon; *H* hydrogen; *N* nitrogen; *S* sulphur; *O* oxygen) expressed as % (w/w) ± standard deviation (n = 3 technical replicates)

elemental composition, the HHV was evaluated according to Eq. 2. Similar values were obtained for fresh *G. sesquipedale* and the MR, 15219 ± 73 and 14,987 ± 466 kJ kg^{-1} values, respectively.

Extraction/hydrolysis of the protein fraction

The extraction/hydrolysis kinetics of the protein fraction by subW has been plotted in Fig. 3a, as determined by TOC/TN analyzer by using the NF of 4.9. Total protein content has also been determined by a spectrophotometric method by using the DC kit. A linear correlation was stablished between both methods:

mg protein g_{DMR}^{-1} N-kit = 1.139 mg protein g_{DMR}^{-1} TOC/TN, $R^2 = 0.9862$.

The slope of this linear correlation was higher than the unit, indicating that protein determination by spectrophotometric methods was overestimated. In this work, TOC/TN analyzer was considered to evaluate the protein fraction extraction since it is reliable and easy.

At a constant flow rate of 2 mL min^{-1}, accumulative protein content in the liquid extracts increased with temperature in the range from 129 to 185 °C. At 185 °C, the protein yield accounted for nearly 70% of the protein content in the MR. However, at the highest temperature studied in this work, 200 °C, the accumulative protein fraction in the extracts was lower than at 185 °C (see Fig. 3a).

To assess the effect of the residence time, Fig. 3a also shows the protein fraction hydrolysis at 200 °C but at higher water flow rate, 6 mL min^{-1}. It can be clearly observed that faster extraction/hydrolysis kinetic was achieved by working at higher flow rate at the same operating temperature, 200 °C. By working with pressurized water, a common variable is the severity factor that combines temperature and the treatment time:

$$logR_o = log\left(t \cdot exp^{((T-T_{ref})/14.75)}\right) \quad (3)$$

where t is the treatment time (min), T is the operating temperature (°C) and T_{ref} is equal to 100 °C. To evaluate a similar parameter in a semicontinuous fixed bed reactor, the treatment time has been replaced by the residence time of the water in the reactor. The residence time for the water in the reactor was evaluated as:

$$\tau = \frac{V}{F_v} = \frac{V}{F_{v,o}}\frac{\rho_r}{\rho_o} \quad (4)$$

where V is the reactor volume in m^3 calculated with the dimensions of the reactor, $F_{v,o}$ the flow rate measured at ambient conditions, in m^3 s^{-1}, ρ_o is the water density at ambient conditions and ρ_r the water density at the reaction conditions. According to Fig. 3a, a maximum around values of severity factor of 4.22–4.25 that corresponds to the experiments performed at 185 °C and 2 mL min^{-1} and at 200 °C and 6 mL min^{-1}, respectively, was achieved for protein fraction release.

Free amino acids production

The accumulative content of the free amino acids, expressed as mg free aa $g_{protein\text{-}MR}^{-1}$ obtained in the subW extracts are plotted in Fig. 3b. The total content of free amino acids has been calculated as the sum of the individual amino acids determined by gas chromatography. A similar trend as the total protein-fraction was observed. At a constant flow rate of 2 mL min^{-1}, a maximum at 185 °C for free amino acids was observed. At 200 °C (F = 2 mL min^{-1}) lower content of free amino acids was determined due to the degradation of amino acids at this temperature and such long residence time.

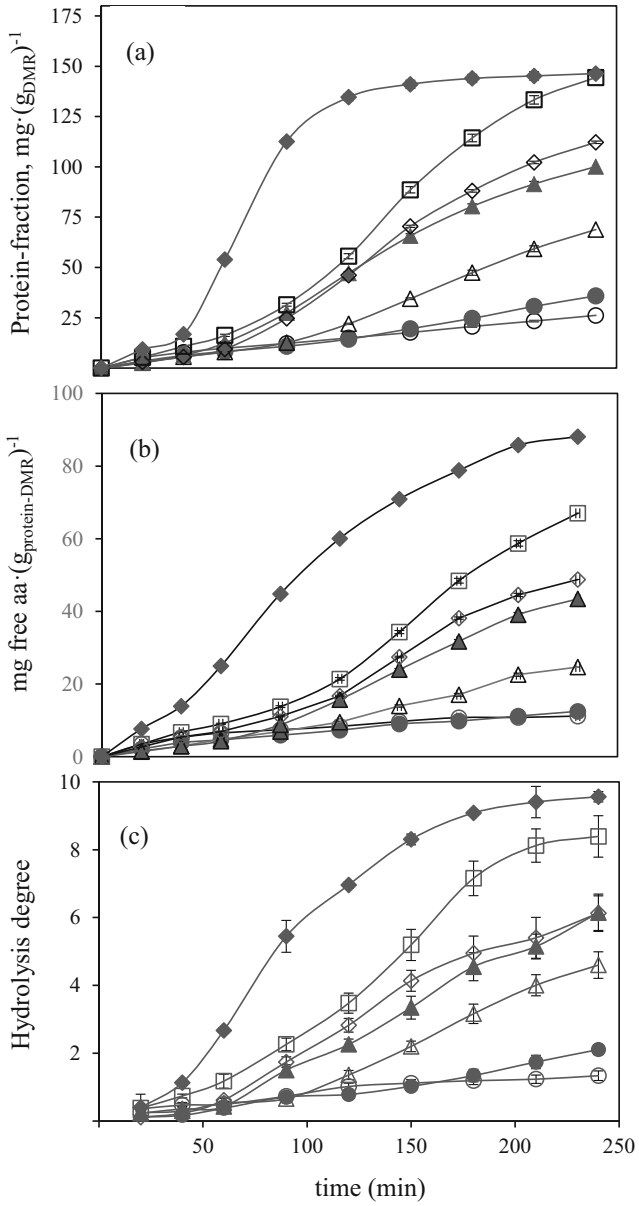

Fig. 3 **a** Accumulative protein (mg·g_{DMR}^{-1}). **b** Accumulative free amino acid content (mg·$g_{protein\text{-}MR}^{-1}$). **c** Hydrolysis degree in subW extracts from macroalgae solid residue. F = 2 mL min^{-1} (○ 129 °C, ● 142 °C, ▵ 155 °C, ▲ 171 °C, □ 185 °C, ◇ 200 °C), F = 6 mL min^{-1} and 200 °C (⬥) (Experimental data includes standard deviations, $n = 3$ technical replicates)

Decreasing the residence time by working at higher flow rate, 6 mL min^{-1}, led to faster and higher amino acid yield.

As an example, Fig. 4a-d show the individual amino acid profile along extraction time for the experiments performed at 185 °C and 2 mL min^{-1} and at 200 °C and 6 mL min^{-1}, grouped into polar and non-polar amino acids. A slower release at 185 °C and 2 mL min^{-1} compared to the formation rate at 200 °C and 6 mL min^{-1} was also observed for individual free amino acids, obtaining the highest content for small amino acids, such as valine, alanine and glycine, as well as for aspartic acid (+ asparagine).

Table 3 lists the final free amino acid yield, evaluated as the ratio of the free amino acid content in the subW extracts to the initial amount of the amino acid in the MR. At a constant flow rate of 2 mL min^{-1}, for most of the amino acids, a maximum was observed at 185 °C. At this temperature, the total content of essential amino acids was 26.3 ± 0.4 mg of essential aa $g_{protein}^{-1}$. By increasing temperature up to 200 °C (F = 2 mL min^{-1}), the highest decrease in the accumulative amino acid fraction in the extracts was observed for polar amino acids such as serine and glutamic and aspartic acids.

Table 3 also lists the results for the production yield of the individual amino acids at 200 °C and 6 mL min^{-1}. By increasing flow rate, the water residence time in the reactor decreased, leading to lower severity factors than at 2 mL min^{-1}, and the formation yield for all the individual amino acids increased. This increase was especially noticeable for aspartic acid, methionine, lysine and histidine. Therefore the selectivity towards non-polar amino acids decreased (see Table 3).

Figure 3c shows the hydrolysis degree for the different experiments carried out. According to the protein fraction release, the highest DH was obtained at 6 mL min^{-1} and 200 °C. The total content of free amino acids, expressed as mmol free amino acids $g_{protein\text{-}MR}^{-1}$, obtained as the sum of the individual amino acids as determined by gas chromatography, have been compared with the meq–NH_2 $g_{protein\text{-}MR}^{-1}$obtained by the ninhydrin assay. For that, a linear correlation between both values has been established and any statistically significant differences in the slopes from unity at the 90% or higher confidence level have been stablished (Table 5). Although the ninhydrin assay determines all primary amines, be they amines, amino acids, peptides, proteins and even ammonia (Friedman 2004), no statistically significance difference between both analytical techniques was determined, except for 142 and 155 °C (see Table 5). From these data sets, it can be concluded that formation of small peptides could not be satisfactorily distinguished with the ninhydrin assay compared to the total amount of free amino acids detected by gas chromatography. According to Friedman (2004), the ninhydrin assay of amino groups in proteins was not as satisfactory as for free amino acids since it does not always produce the theoretical yield.

Total phenolic content and antioxidant activity of subW extracts

Figure 5 a shows the accumulative curve of the total polyphenolic compounds (TPC), as determined by the Folin-Ciocalteu assay. At 2 mL min^{-1}, TPC release increased by increasing temperature. Higher flow rate, 6 mL min^{-1}, led to faster release of TPC, at the same operating temperature of 200 °C, but similar TPC was obtained after 240 min, 25.9–26.5 mg GAE g_{DMR}^{-1}.

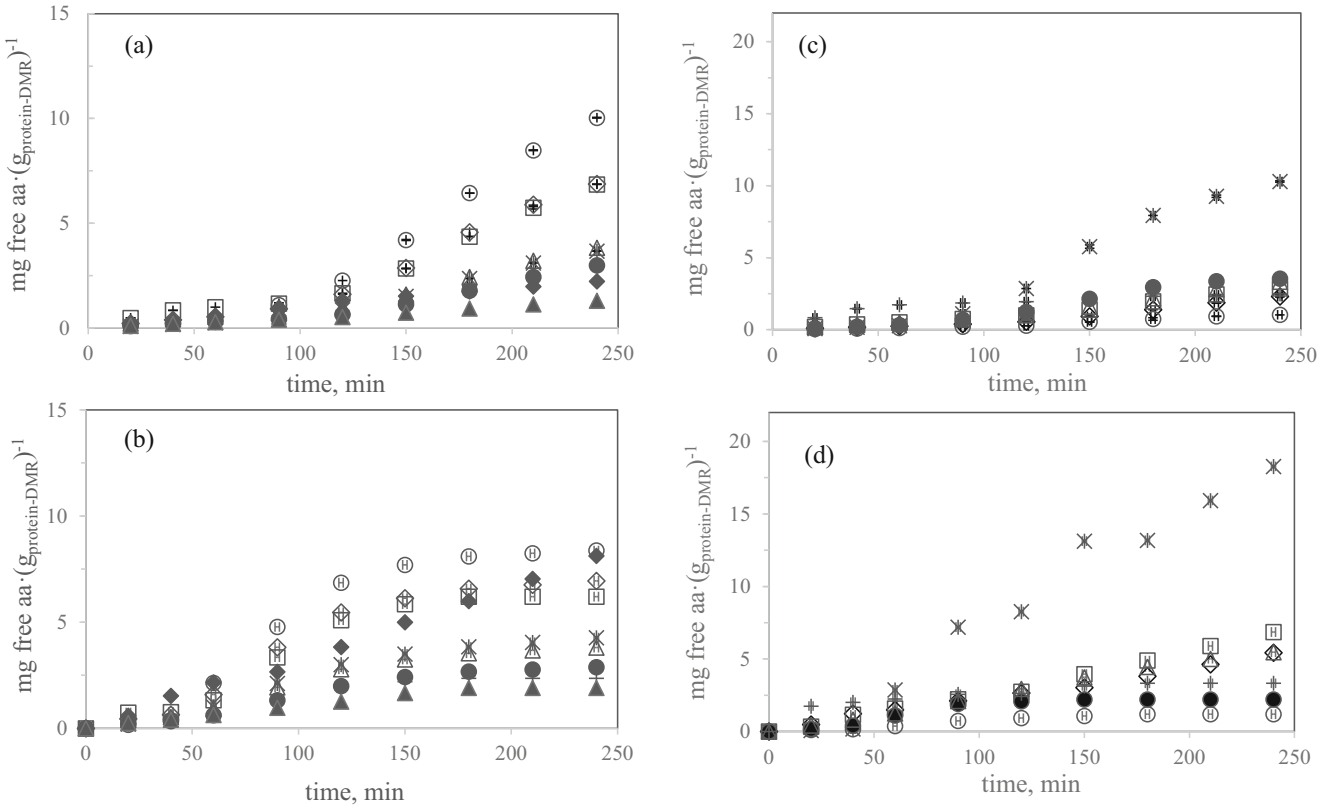

Fig. 4 Accumulative formation of individual amino acids. Non-polar amino acids (**a**) at 185 °C and 2 mL min^{-1} (**b**) at 200 °C and 6 mL min^{-1} (◇ alanine, ○ glycine, □ valine, △ leucine, + isoleucine, ∗ proline, ● phenylalanine, ◆ tryptophan, ▲ methionine). Polar amino acids (**c**) at 185 °C and 2 mL min^{-1} (**d**) 200 °C and 6 mL min^{-1} (◇ hystidine, ○ threonine, □ lysine, △ tyrosine, + glutamic acid, ∗ aspartic acid, ● serine). (experimental data includes standard deviations, $n = 3$ technical replicates)

The antioxidant capacity of the extracts collected at the different time intervals was determined by the FRAP assay and results are presented in Fig. 5b. Antioxidant capacity increased with temperature and flow rate. The maximum antioxidant capacity in the collected extracts when working at 6 mL min^{-1} was obtained at 40–60 min, while at 2 mL min^{-1}, the maximum was reached later at 60–90 min.

Table 5 Linear relationship between meq–NH_2 determined by the ninhydrin assay and free amino acid, FAA, (mmol aa $g_{protein\text{-}MR}{}^{-1}$), determined by the sum of individual amino acids by gas chromatography: meq-NH_2 = (intercept ± standard error) + (slope ± standard error) × FAA_{GC}

Experiment	slope	intercept	R^2
129 °C, 2 mL min^{-1}	1.08 ± 0.10	− 0.006 ± 0.007	0.940
142 °C, 2 mL min^{-1}	1.49 ± 0.06*	− 0.023 ± 0.004*	0.987
155 °C, 2 mL min^{-1}	1.53 ± 0.05*	− 0.009 ± 0.006*	0.992
171 °C, 2 mL min^{-1}	1.15 ± 0.07	0.022 ± 0.015*	0.974
185 °C, 2 mL min^{-1}	1.09 ± 0.07	0.023 ± 0.021	0.975
200 °C, 2 mL min^{-1}	0.96 ± 0.05	0.006 ± 0.011	0.984
200 °C, 6 mL min^{-1}	0.94 ± 0.04	0.015 ± 0.020	0.988

*Significant difference from slope unity or 0 intercept. (p values ≥0.1)

These results agree with others obtained for different fresh red algae in literature that determined a greater antioxidant activity as temperature increased with a maximum at 210 °C (Park et al. 2019). In that study, the TPC obtained after subW treatment varied in the range from 4.70 ± 0.08 (at 120 °C) to 14.2 9 ± 0.33 (210 °C) mg GAE $g_{dry\ algae}{}^{-1}$ (Park et al. 2019).

Correlation coefficients between the antioxidant capacity and the components determined in the extracts (TPC, protein fraction and free amino acids) have been determined according to the Pearson product moment correlation. Analysis indicated a statistically significant non-zero correlations at the 95.0% confidence level and positive correlation coefficients between antioxidant capacity and the TPC, solubilized protein and free amino acids in the extracts with values of the correlation coefficients of 0.882, 0.771 and 0.381, respectively ($n = 63$, the number of pairs of data values used to compute each coefficient).

Browning process along extraction could be clearly observed at first sight. To quantify the colour of the extract, the evolution of the colour of the collected extracts at the different time intervals, when working at 6 mL min^{-1} and 200 °C, was evaluated and colour parameters were compiled in Table 6. It can be observed that the lowest values of the luminosity and yellowness correspond to the highest value of the antioxidant

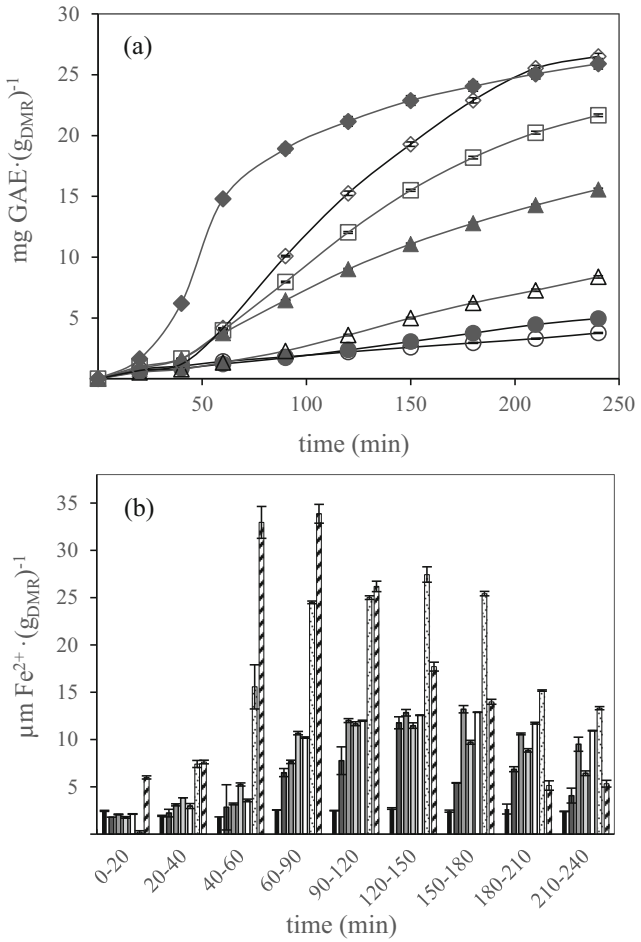

Fig. 5 **a** Accumulative total phenolic compounds, as determined by Folin-Ciocalteu, (mg GAE·g_{DMR}^{-1}). **b** Total antioxidant activity (μm $FeSO_4$·g_{DMR}^{-1}) collected at the different time intervals in subW extracts from macroalgae residue: F = 2 mL min^{-1} (○, ■ 129 °C; ●, ■ 142 °C; △, ■ 155 °C; ▲, ■ 171 °C; □, □ 185 °C; ◇, ▩ 200 °C). F = 6 mL min^{-1} and 200 °C. (◆, ▨). (Experimental data includes standard deviations, $n = 3$ technical replicates)

capacity and the TPC of the extracts (60–90 min, see Fig. 5b). Pearson correlation coefficients between these two variables and the colour parameters showed significant non-zero correlations at the 95.0% confidence level. Negative correlations for L* and b* and positive correlation for a* were obtained and stronger correlations were determined for the antioxidant capacity than for TPC (see Table 6).

Total organic carbon in the subcritical water extracts

Accumulative total organic carbon (TOC) of the different extracts collected has been plotted in Fig. 6. At 2 mL min^{-1}, TOC in the extracts increased with temperature, although no differences were observed between 185 and 200 °C.

Regarding the effect of flow rate, faster and higher release of TOC was observed by working at high flow rate. High flow rate led to lower residence time and higher yields of TOC, similar to proteins and amino acids.

Waste solid analysis

The solid residue was weighed after each extraction and the hydrolysis yield was estimated according to the equation:

$$Hydrolysisyield(\%) = \frac{W - W_1}{W} \times 100 \quad (5)$$

where W is the weight of the dry sample introduced in the reactor and W_1 is the weight of the dried residue after subW treatment. The values of the hydrolysis yield (%) are listed in Table 7 where it can be observed that the hydrolysis yield increased by increasing the working temperature. For instance, at a flow rate of 2 mL min^{-1}, the hydrolysis yield increased from 21 to 51% when varying temperature from 129 to 200 °C due to a greater solubilization of the extracted compounds at high temperatures.

Elemental composition of the residue collected in the different experiments carried out at F = 2 mL min^{-1} has been listed in Table 4. A significant decrease in C, H and especially in N was observed by increasing operating temperature, due to a higher organic and protein fraction solubilization. S content also decreased probably due to the extraction of the residual agar in the by-product. Ash content steadily increased with temperature, proving that ashes are not solubilized at the experimental conditions of this work.

Elemental composition of the MR before and after subW treatment and the accumulative content of C and N in the collected extracts help to perform the carbon and nitrogen balance:

$$C\ (or\ N)\ balance = \frac{C\ (or\ N)_{outlet}}{C(or\ N)_{inlet}} \cdot 100 \quad (6)$$

Deviations for C and N mass balances are collected in Table 7. Deviations ranged from 4 to 12% for C and from 6.8 to 28.9% for N. The greatest deviations for the mass balance were observed at the highest temperature studied. Some of the solid may also be converted to volatile compounds (Yoshida and Tavakoli 2004) that were not measured in this work. Mass balance for the ashes was also performed, considering that the ashes were only present in the solid residue after subW treatment. For ashes, mass balance deviations ranged from 3.7 to 8.1%. The inorganic content of the solid residue obtained at 2 mL min^{-1} and 200 °C was listed in Table 2. Compared to the macroalgae industrial residue, the concentration of most of the metals increased after subW treatment. For instance, Na concentration increased by a factor of 4, Cr by a factor of 3, while the concentration of K, Al, Ca, Fe, Ti and Ni was doubled. From Table 2 it can be also observed the reduction in S content after subW treatment as determined in

Table 6 Colour parameters of the extracts collected at 200 °C (F = 6 mL·min^{-1}) at different time intervals. CIElab parameters ± standard deviation (n = 3 technical replicates) Pearson correlation parameters for colour parameters and TPC and antioxidant capacity

Time interval	L*	a*	b*
0–20 min	31.6 ± 0.3^{h}	5.2 ± 0.7^{a}	32.9 ± 0.4^{f}
20–40 min	24.8 ± 0.5^{f}	9.4 ± 0.2^{d}	32.5 ± 0.5^{f}
40–60 min	3.0 ± 0.1^{a}	12.1 ± 0.3^{e}	5.1 ± 0.2^{a}
60–90 min	4.8 ± 0.2^{b}	16.9 ± 0.6^{h}	8.1 ± 0.3^{b}
90–120 min	8.1 ± 2.0^{c}	17.2 ± 0.9^{h}	13.7 ± 1.7^{c}
120–150 min	12.0 ± 0.1^{d}	15.3 ± 0.3^{g}	20.3 ± 0.1^{d}
150–180 min	17.8 ± 0.6^{e}	13.03 ± 0.08^{f}	29.1 ± 0.8^{e}
180–210 min	28.8 ± 1.3^{g}	6.2 ± 0.5^{b}	28.8 ± 3.1^{e}
210–240 min	25.3 ± 1.3^{f}	7.1 ± 0.3^{c}	30.0 ± 1.2^{e}
Correlation coefficients ($n = 9$)	TPC-L* = − 0.8327 Fe^{2+}-L* = − 0.9671	TPC-a* = 0.7392 Fe^{2+}-a* = 0.8108	TPC-b* = − 0.8770 Fe^{2+}-b* = − 0.9701

Values with different letters in each column are significantly different when applying the Fisher's least significant differences (LSD) method at p value ≤ 0.05

*$p < 0.05$ for all pair of variables

Table 4 by ICP-MS. HHV for the residues after subW treatment was also evaluated (see Table 4), but not a trend in these values was observed in the solid residue after subW treatment.

Discussion

The benefits of different components of marine algae have been well documented and described in literature. However, algae by-products generated during industrial activity have been hardly discussed. Macroalgae industry generates a large amount of by-products that still contain different valuable compounds being worthy their valorization. The solid residue generated from red algae after agar extraction still contains important amounts of carbohydrates (37%, w/w) and proteins, 21% (w/w). The NF determined for MR was 4.9, lower than the value of 6.25 commonly used (Park et al. 2019). However, for different types of macroalgae, lower values for NF have also been reported in literature. Lourenço et al. (2002) determined an average value of 4.6 ± 0.5 for nine red algae species, with an overall average NF factor of 4.9 ± 0.6 for different macroalgae species including red, brown and green. The lower values of the NF for seaweeds are due to the presence of non-protein nitrogen compounds such as pigments and inorganic nitrogen (nitrite, nitrate and ammonia) that led to lower NF for algae species (Lourenço et al. 2004).

The low lipid content agreed with the range reported in the literature, between 0.3 and 3.6 g (100 g)$^{-1}$ dry seaweed for different red, brown and green algae (Rodrigues et al. 2015). The elemental composition of the MR led to a HHV of 14,987 ± 466 kJ kg^{-1}. The HHV for MR was slightly lower than the values reported for other types of biomass. Friedl et al. 2005) reported HHV in the range from 15,974 to 20,321 kJ kg^{-1}for various types of wastes (including compost, waste from sugar, oil, and brewering industries, poultry litter and sewage sludge).

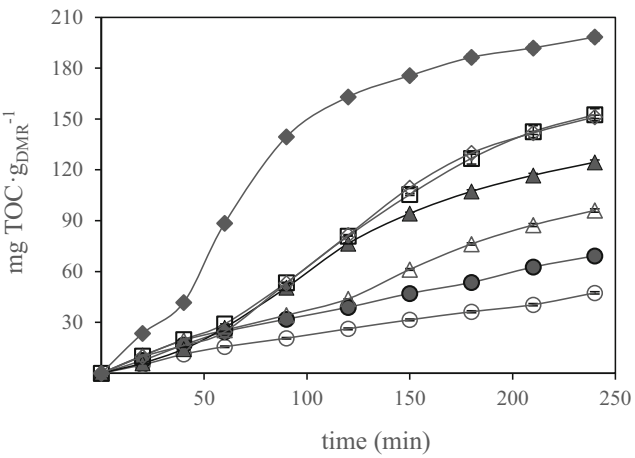

Fig. 6 Accumulative total organic carbon (mg TOC g_{DMR}^{-1} obtained by subW treatment from macroalgae residue at F = 2 mL min^{-1} (○ 129 °C, ● 142 °C, ▵ 155 °C, ▲ 171 °C, □ 185 °C, ◇ 200 °C). F = 6 mL min^{-1} and 200 °C (♦). (Experimental data includes standard deviations, $n = 3$ technical replicates)

Extraction/hydrolysis of the protein fraction

Protein fraction in the extracts has been evaluated by the TOC/TN analyzer. In algae, spectrophotometric methods such as Lowry et al. (1951) and Bradford (1976) can be subjected to some interferences, such as degradation products of proteins, saccharides, amino acids and phenolic compounds (Hata et al. 2008), but also amino acid composition affects the results obtained by these two methods. According to literature (Lourenço et al. 2004), the Cu^{2+} ion present in the reagent is overly sensitive to some amino acids such as tryptophan and tyrosine. These could be the reasons that justify the difference

Table 7 Hydrolysis yield for the experiments carried out at F = 2 mL min^{-1}. Mass balances (MB) for C, N and ash

T, °C	Yield (%)	C_{inlet} (g)	C_{outlet} (g)	MB, C (%)	N_{inlet} (g)	N_{outlet} (g)	MB, N (%)	ash_{inlet}	ash_{outlet}	MB, ash (%)
129	21.2	3.359	3.021	89.9	0.396	0.363	91.7	2.057	2.194	106.7
142	26.2	2.352	2.118	90.1	0.278	0.223	80.2	1.441	1.557	108.1
155	31.5	2.353	2.220	94.3	0.278	0.229	82.4	1.441	1.512	104.9
171	40.7	2.349	2.220	94.5	0.277	0.221	79.8	1.438	1.415	98.4
185	44.3	2.371	2.313	97.6	0.280	0.261	93.2	1.452	1.406	96.8
200	50.7	2.356	2.066	88.0	0.277	0.197	71.1	1.437	1.388	96.3

7 g of DMR were charged into the reactor except for run at 129 °C that 10 g were charged

Mass or % (w/w) ± standard deviation ($n = 3$ technical replicates)

in the protein fraction content obtained by the spectrophotometric method and the TOC analyzer determined in this work. The effect of subW treatment on hydrolysis and extraction of the protein fraction of the MR has been described. Both, temperature and solvent flow rate, have been determined as key parameters when dealing with protein hydrolysis. At constant flow rate of 2 mL min^{-1}, a maximum at 185 °C in the protein fraction hydrolysis was obtained. At subW conditions, the ionic product of water, K_w, increases with temperature that induced lower pH values in the medium due to the high levels of hydronium (H_3O^+) and hydroxide (OH^-) ions derived from autoionization of water that favours biomass hydrolysis (see Fig. 2b) (Toor et al. 2011). At these conditions, the protein fraction would be probably hydrolyzed into valuable peptides and free amino acids (Marcet et al. 2016). Furthermore, an increase in temperature could cause the algae cell wall to swell and rupture enhancing diffusion of intracellular components and water interaction (Zainan et al. 2020). Additionally, insoluble proteins have a high surface hydrophobicity that can become hydrated when the dielectric constant of water decreases with temperature (Lamp et al. 2020). By increasing temperature up to 200 °C, the residence time of water with the soluble products in the fixed bed reactor was long enough to cause protein or its hydrolysis products to degrade.

An increase in flow rate at 200 °C led to better results in terms of extraction/hydrolysis rate and yield. To explain these results, a similar mechanism as the one proposed by Liu and Wyman (2003) for hemicellulose dissolution from corn stover, can be proposed. That is, water will hydrolyze long-chain peptides slower than short-chain peptides and therefore long-chain peptides could be accumulated on the solid surface; an increase in the flow rate would reduce the thickness of the stagnant fluid layer surrounding the solid, facilitating water penetration into the solids and therefore hydrolyze and remove the protein fraction. The mass transfer limitation on protein extraction can be observed in the shape of the extraction accumulative curves, since protein release was quite slow at 2 mL min^{-1} but was much faster at 6 mL min^{-1}.

Free amino acids production

High severity factors (high temperature and high residence time) led to lower free amino acids due to amino acids decomposition. Toor et al. (2011) reported that primary mechanisms of degradation of the amino acids were decarboxylation and deamination with loss of NH_3. Rogalinski et al. (2005) studied the production and decomposition of amino acids from bovine serum albumin (BSA) by continuous subW. These authors determined the main degradation products of BSA carboxylic acids such as acetic acid, propionic acid, n-butyric acid, isobutyric acid and isovaleric acid with only two nitrogen containing compounds, ethanolamine and the non-proteinogenic amino acid ornithine. Unfortunately, no results on NH_3 release were presented.

The results obtained in this work agree with literature that also reported serine and aspartic and glutamic acids as one of the most temperature-sensitive amino acids that decomposed faster than lower molecular weight amino acids such as glycine and alanine (Ahmed and Chun 2018). A high yield loss by increasing temperature form 185 to 200 °C was also observed for lysine. Lysine and (other) polar amino acid side groups have a high tendency to undergo Maillard reactions with carbonyl groups or reducing carbohydrates (Lamp et al. 2020) generated during subW treatment. On the contrary, simple amino acids such as alanine and glycine presented higher stability under subW conditions, as it has also been reported by Rogalinski et al. (2005) in the study of amino acid production from bovine serum albumin by continuous subW. High stability was also observed by valine, but possible transformation of lysine to valine has also been reported (Zainan et al. 2020). As a consequence, the selectivity towards non-polar amino acids defined as the amount of free non-polar amino acids released in the subW extracts relative to total amount of free amino acids on molar base increased by working on more severe conditions (higher severity factor) since polar amino acids seem to be more sensitive under subW conditions (see Table 3).

In general, amino acids with high molecular weight are more susceptible to degradation not only to ammonia, organic acids and amines but also to other amino acids with lower molecular weight that could compensate the degradation of these amino acids (Esteban et al. 2010). In the present study glycine, the smallest amino acid presented the highest formation yield. The high values of tryptophan yields are probably due to the error associated during its determination by basic hydrolysis. In any case, as reported by Esteban et al. 2010), it is difficult to stablish any relation between the type of side chain of the amino acid and the hydrolysis yield since for a specific type chain, not all amino acids are obtained in proportional amounts.

Total phenolic content and antioxidant

TPC release and antioxidant capacity increased by increasing flow rate and temperature. According to these results, it could be concluded that the temperature needed for phenolic compounds degradation present in the macroalgae residue was not yet reached. However, as reported in literature, Maillard reactions could take place between sugars and amino acids and new compounds can be obtained that contribute to the antioxidant capacity of the collected extracts (Plaza and Turner 2017). Marcet et al. (2016) reported that antioxidant capacity increased when small peptides are obtained. However, the strength of the correlation between antioxidant capacity and TPC was the highest, concluding that TPC contributed in a larger extent to the determined antioxidant capacity than protein fraction or free amino acids.

Total organic carbon and waste solid

The effect of temperature and flow rate on TOC in subcritical water extracts was similar to the protein fraction. TOC would include hydrolysis products of carbohydrates such as oligomers, monomers and their degradation products, but also protein degradation products such as different organic acids (Kang et al. 2001). For instance, residual agar was analyzed in the extracts obtained at 200 °C and 6 mL min^{-1} and accounted for 5.0% ± 1.4 probably due to a non-complete industrial extraction. Degradation of carbohydrates and proteins can release NH_3 and CO_2 or other gas products that would have led to similar TOC yield at 200 °C and 185 °C, due to a higher degradation degree of the macroalgae residue components. Although gas product formation has not been studied in this work, it has been reported previously (Kang and Chun 2004; Sereewatthanawut et al. 2008).

The greater solubilization of the carbon and nitrogen fraction of the MR with temperature led to an increase of the hydrolysis yield and lower N and C content of the solid residue after subW treatment. Ash content increased in the solid residue as temperature increased due to non-solubilization. Toor et al. (2011) reported that the decrease of the dielectric constant of water with temperature led to an increase of the solubility of non-polar organic compounds, but the solubility of salts significantly decreased. Thiruvenkadam et al. (2015) in their review concluded that the solid residue after subW treatment had a high concentration of ash and low concentrations of hydrogen, nitrogen and sulphur. This can be also appreciated in Table 4 where the experiment carried out at 200 °C, and 2 mL min^{-1} presented the lowest H and N content and the highest ash content.

Conclusions

SubW treatment led to an efficient extraction/hydrolysis of the protein fraction of the macroalgae industrial solid residue after agar extraction. The best experimental conditions in the semicontinuous fix-bed reactor used in this work were 200 °C and 6 mL min^{-1} with nearly 70% of the solubilized protein content. The highest content of individual amino acids was obtained for small amino acids such as valine, alanine and glycine as well as aspartic acid. Therefore, an increase in the non-polar selectivity was observed by working at high severity factors. Positive and strong correlation was obtained for TPC and the reducing capacity of the subW extracts. The solid residue after subW treatment presented lower H, N and O content, but higher ash content due to non-solubilization of this fraction, being possible its application in agriculture as fertilizers.

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