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Valorization of residual biomass from the hydrocolloid industry: The role of hydrothermal treatments in the recovery of high-value compounds

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ABSTRACT

Gelidium seaweeds are recognized for their high-quality hydrocolloids, but their industrial extraction leaves behind biomass, whose available valorization approaches are not economically and/or environmentally sustainable. This work aimed to evaluate, for the first time, the effect of batch hydrothermal treatments (and their severity (S_0)) on the fractionation of carbohydrates and proteins from this residue. At $S_0 \leq 3.13$, agars with a maximum gelling strength of 185 g/cm^2 can be obtained. At $S_0 = 3.46$, galactose-based non-gelling oligosaccharides are recovered. Harsher processing conditions ($S_0 \geq 2.23$) result in higher protein solubilization and antioxidant activity. Moreover, sequential processing is a feasible approach to improve process selectivity. A novel proposed two-step hydrothermal treatment (at 200 °C and 230 °C), results in maximum oligosaccharide and protein recovery, leaving behind a cellulose-enriched solid that can be used for biofuel production, in a biorefinery approach.

1. Introduction

Red seaweeds from the *Gelidium* genus are recognized for their bacteriological grade high-quality agar. Due to high demand, and the incomplete biomass solubilization during the process, there is significant production of spent seaweed biomass. Considering it is still composed of proteins (up to 21 %) (Martínez-Sanz et al., 2020; Ester Trigueros et al., 2021) and carbohydrates (ranging from 9 % to >50 %) (Martínez-Sanz et al., 2020; Sudhakar et al., 2017), better valorization strategies are necessary for this residue.

Several approaches have been proposed in the last years for the valorization of this residue. The use of chemicals is the most traditional approach, with reports of sequential use of toluene/ethanol, $NaClO_2$ and KOH resulting in the cascading extraction of pigments, non-cellulolytic carbohydrates, and cellulose (Martínez-Sanz et al., 2020). This valorization strategy is environmentally unsustainable, due to the need for several strong chemicals, resulting in further residue streams. On the other hand, the combined use of cellulase, protease and xylanase has proved effective in the extraction of one fraction rich in phenolic compounds, carbohydrates, and proteins (Trigueros et al., 2021b), while the sequential use of β-agarase and cellulase successfully produced two oligosaccharide-enriched fractions (Li et al., 2022). Nevertheless, the use of enzymes is associated with high energy demand (due to the longer incubation hours), does not eliminate the necessity for chemical use (due to the typical use of pH buffers), and the price is often prohibitive for large-scale applications in these biomasses (due to the lack of specific enzymes). In a different approach, chemical and biological treatments have been combined, producing high-value products and biofuels, but did not prove as an ideal solution, due to the need for chemicals and high energy demands, alongside the longer processing times (Elalami et al., 2020; Sudhakar et al., 2017; Tůma et al., 2020).

On the other hand, hydrothermal treatments are based on the properties of water at high pressures and temperatures: in the range of 100 °C to 374 °C the ionization constant of water increases, allowing the autoionization of water into acidic hydronium ions and basic hydroxide ions that act as catalysts, resulting in an efficient penetration of the biomass structures and depolymerization of hemicellulose to oligomers and monomers (Ruiz et al., 2013). This green technology has been successfully employed in the recovery of agar, oligosaccharides, and fermentable sugars from the red seaweed *Gelidium corneum* (formerly known as *G. sesquipedale)* (Gomes-Dias et al., 2022, Gomes-Dias et al., 2020) but also in the valorization of its residue for residual agar and amino acids (Diop et al., 2022a, 2022b; Trigueros et al., 2021b).

Nevertheless, there is still a knowledge gap surrounding the effects of

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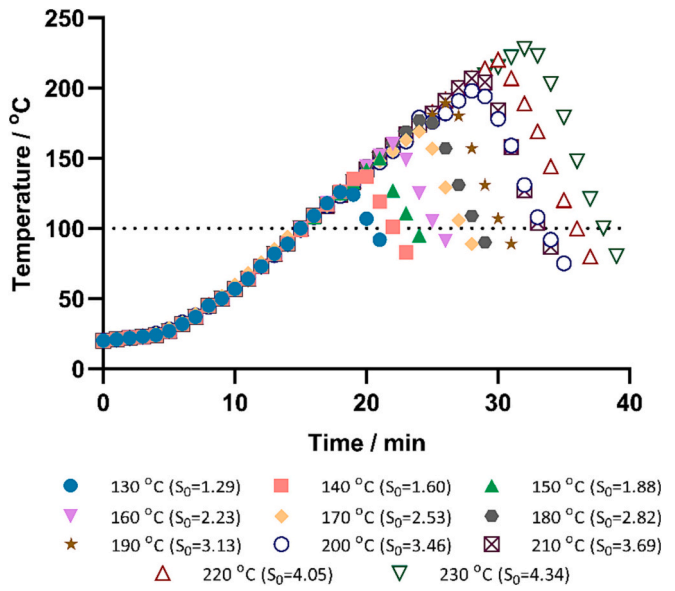

Fig. 1. Heating-cooling profiles for the hydrothermal treatments conducted at different severities.

this technology on the fractionation of this industrial residue for multiple applications. Thus, this work aimed to evaluate the effect of hydrothermal treatments on the release of carbohydrates and proteins from this biomass, as well as their possible applications. Additionally, the feasibility of sequential hydrothermal treatments aiming at maximum oligosaccharide recovery and maximum peptide recovery was assessed, in a novel approach for this kind of biomass. To find the best strategy for the whole residue valorization, the final biomass was characterized, and possible uses were proposed, taking into consideration the possible impacts of each processing step.

2. Materials and methods

2.1. Raw material

Gelidium corneum industrial residues were kindly supplied by Iberagar–Sociedade Luso-Espanhola de Colóides Marinhos S.A. The biomass used in this study has a reported composition of 35.26 ± 0.87 % ash, 16.00 ± 0.94 % crude protein, 19.24 ± 0.88 % glucan, and 5.24 ± 0.06 % galactan (on a dry weight basis) (Bondar et al., 2022). Samples were stored in vacuum-sealed bags, in a dry and cold place, away from direct sunlight, until use.

2.2. Hydrothermal treatments

Biomass samples (40 g dry weight basis) were mixed with distilled water (400 g), resulting in a 10 % solid loading, in a 1.9 L 4520 Stirred Pressurized Bench Top Reactor (Parr Instruments Company, Moline, Illinois, USA) and subjected to a hydrothermal pretreatment, in a non-isothermal approach, at different maximum temperatures (from 130 °C to 230 °C), under stirring at 120 rpm. To facilitate the comparison between treatments, the severity factor (S_0) was calculated for each extraction condition (Ruiz et al., 2013).

$$S_0 = logR_0 = log\left[R_{0_{heating}} + R_{0_{cooling}}\right]$$
$$= log\left[\int_0^{t_{heating}} e^{\frac{T'(t)-T_{ref}}{\omega}}\,dt + \int_{t_{heating}}^{t_{cooling}} e^{\frac{T'(t)-T_{ref}}{\omega}}\,dt\right]$$

In the equation, $t_{heating}$ and $t_{cooling}$ represent the time (in minutes) necessary to achieve the target and final temperatures, respectively; T'(t) and T"(t) represent the temperature profiles in the heating and cooling stages; T_{ref} is the reference temperature (100 °C) and ω is an empirical parameter related to activation energy (14.75 assuming first-order kinetics for cellulosic based biomass).

After the hydrothermal pretreatment, the content of the reactor was filtered whilst still hot (80 °C) with a pure cotton filter cloth (Rocha et al., 2019). The heating-cooling profiles of each experimental condition can be observed in Fig. 1. The solid fraction was dried at 60 °C overnight in a ventilated oven. The liquid fraction was submitted to a freeze-thawing process, and the resulting solid gelling fraction (native agar) was washed with ethanol (96 % *v*/v) to facilitate syneresis and dried at 60 °C overnight in a ventilated oven, while the liquor was frozen (−20 °C) until further analysis.

2.3. Characterization of the extracted fractions

2.3.1. Yields

Solubilization yield was calculated based on the solid mass difference before and after the hydrothermal treatment, being reported as g of solid solubilized per 100 g initial raw material, on a dry weight basis.

Similarly, agar yield was calculated based on the dry agar mass recovered from the freeze-thaw process, being expressed as g of gelling fraction per 100 g of raw material, on a dry weight basis.

2.3.2. Gel strength

The solid fraction recovered after the freeze-thaw cycle (considered to be agar) was solubilized with distilled water (at 1.5 % *w*/w) at 100 °C, until complete dissolution. The hot solution (15 g per replicate) was poured into a cylindrical container and allowed to rest at room temperature for 16 h. The gel samples were penetrated (using a TA.HDplus texture analyser equipped with a 10 mm diameter cylindrical probe) for a maximum of 8 mm, at a set rate of 0.2 mm/s. Gel strength (reported in g/cm^2) was considered to be the stress required for breaking the gel surface, within the test parameters used (Rocha et al., 2019).

2.3.3. Carbohydrate profile

Liquors from the extractions were filtered through 0.22 μm membranes and directly (without any hydrolysis) analyzed by high-performance liquid chromatography (HPLC) to quantify the monosaccharides (against a calibration curve composed of galacturonic acid, glucose, galactose, arabinose, and mannose, using a RI detector) and inhibitory compounds (against a calibration curve of acetic acid, using a RI detector; and a calibration curve of furfural and hydroxymethylfurfural (HMF), using a UV detector at 210 nm). Aliquots from the same samples were subjected to quantitative post-hydrolysis (121 °C, 20 min, 4 % H_2SO_4) for oligosaccharide determination (based on the increase in monosaccharide content post-hydrolysis), according to the standardized methods established by the National Renewable Energy Laboratory (NREL). Aminex HPX-87H column at 60 °C and a mobile phase of 0.05 M H_2SO_4 at a flow rate of 0.6 mL/min were used (Sluiter et al., 2008a).

2.3.4. Molecular weight distribution

The molecular weight distribution of the liquors was evaluated by HPLC gel permeation chromatography (GPC) on a PolySep-GFC-P Linear column. Extracts were diluted with mobile phase until 1 mg/mL concentration was reached. The samples were eluted with ultrapure water, using a flow rate of 0.8 mL/min at 40 °C with RI (for oligosaccharides) and UV detection (for peptides). Molecular weight was calculated based on a calibration curve performed using a pullulan kit (P-82) within a range of 6.3–642 kDa (Castro-Ferreira et al., 2022). In these conditions, the retention time of the molecular weight standards used was as follows: 6 kDa – 12.3 min; 10 kDa – 12.1 min; 21 kDa – 11.8 min; 47 kDa – 11.4 min; 107 kDa – 11.0 min; 194 kDa – 10.6 min; 337 kDa – 10.2 min; 642 kDa – 9.7 min.

Table 1
Effect of extraction conditions (maximum temperature and subsequent severity factor) on the solubilization yield, agar yield and gelling strength, and carbohydrate profile (monosaccharide, inhibitory compound, and oligosaccharide content) and protein content of the liquors obtained. For each variable, different letters represent statistically significant differences ($p < 0.05$). Conditions marked as n.d. represent concentrations below the detection limit of the methods used.

Fraction	**Experimental conditions**											
	T (°C)	130	140	150	160	170	180	190	200	210	220	230
	S_0	1.29	1.60	1.88	2.23	2.53	3.82	3.13	3.46	3.69	4.05	4.34
Solid	SY (%)	18.1 ± 0.4^{a}	21.2 ± 0.2^{a,b}	22.4 ± 0.1^{b}	22.9 ± 0.6^{b,c}	24.7 ± 1.1^{c}	28.8 ± 1.4^{d}	31.2 ± 2.1^{d,e}	32.2 ± 3.3^{e}	33.5 ± 2.6^{e}	36.7 ± 1.4^{e}	41.0 ± 1.6^{f}
Gel	Agar yield (%)	1.77 ± 0.31^{a}	1.85 ± 0.03^{a}	2.38 ± 0.23^{a,b}	2.41 ± 0.26^{a,b}	2.62 ± 0.28^{b}	3.73 ± 0.05^{c}	3.22 ± 0.31^{b,c}	n.d.	n.d.	n.d.	n.d.
	Gelling strength (g/cm^2)	182 ± 25^{d}	196 ± 5^{d}	163 ± 16^{d}	167 ± 21^{d}	106 ± 1^{c}	26 ± 2^{b}	<20^{a}	–	–	–	–
Liquid	Uronic Acids (g/L)	n.d.	n.d.	n.d.	n.d.	0.10 ± 0.01^{a}	0.15 ± 0.03^{a,b}	0.20 ± 0.02^{b}	0.31 ± 0.01^{c}	0.43 ± 0.02^{d}	0.50 ± 0.01^{d}	0.50 ± 0.01^{d}
	Glucose (g/L)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.12 ± 0.01^{a}	0.18 ± 0.01^{a}	0.23 ± 0.01^{b}	0.28 ± 0.01^{b}	0.31 ± 0.02^{b}
	Galactose (g/L)	n.d.	n.d.	n.d.	n.d.	n.d.	0.11 ± 0.01^{a}	0.15 ± 0.01^{a}	0.27 ± 0.01^{b}	0.37 ± 0.01^{c}	0.63 ± 0.02^{d}	0.56 ± 0.03^{d}
	Total monosaccharides	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**0.10 ± 0.01^{a}**	**0.26 ± 0.03^{b}**	**0.47 ± 0.02^{c}**	**0.76 ± 0.01^{d}**	**1.03 ± 0.02^{e}**	**1.41 ± 0.02 ^{f}**	**1.37 ± 0.04^{f}**
	Acetic/Levulinic Acid (g/L)	n.d.	n.d.	n.d.	0.10 ± 0.03^{a}	0.10 ± 0.04^{a}	0.14 ± 0.03^{a}	0.17 ± 0.05^{a,b}	0.26 ± 0.04^{b}	0.42 ± 0.07^{c}	0.69 ± 0.08^{d}	1.00 ± 0.05^{e}
	HMF (g/L)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.06 ± 0.02^{a}	0.13 ± 0.03^{a}	0.28 ± 0.01^{b}	0.33 ± 0.03^{b}
	Furfural (g/L)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.10 ± 0.01^{a}	0.12 ± 0.01^{a,b}	0.15 ± 0.01^{a,b}	0.21 ± 0.01^{b}
	Total inhibitory compounds	n.d.	n.d.	n.d.	**0.10 ± 0.03^{a}**	**0.10 ± 0.04^{a}**	**0.14 ± 0.03^{a}**	**0.17 ± 0.05^{a}**	**0.41 ± 0.05^{b}**	**0.67 ± 0.08^{c}**	**1.12 ± 0.08^{d}**	**1.54 ± 0.05^{e}**
	Glucooligosaccharides (g/L)	0.97 ± 0.09^{a}	1.04 ± 0.03^{a}	1.08 ± 0.02^{a}	1.10 ± 0.02^{a}	1.10 ± 0.05^{a}	1.29 ± 0.08^{b}	1.51 ± 0.06^{c}	1.86 ± 0.06^{d}	1.87 ± 0.07^{d}	1.80 ± 0.04^{d}	1.51 ± 0.05^{c}
	Galactooligosaccharides (g/L)	0.89 ± 0.06^{a}	0.99 ± 0.03^{a}	1.05 ± 0.11^{b,c}	1.22 ± 0.07^{c}	1.39 ± 0.04^{d}	1.76 ± 0.13^{e}	2.43 ± 0.27^{f}	4.15 ± 0.10^{h}	3.54 ± 0.09^{g}	2.24 ± 0.14^{f}	0.99 ± 0.15^{a}
	Total oligosaccharides	**1.86 ± 0.10 ^{a}**	**2.03 ± 0.05^{a}**	**2.13 ± 0.11^{a}**	**2.33 ± 0.07^{b}**	**2.49 ± 0.07^{b}**	**3.05 ± 0.16^{c}**	**3.94 ± 0.28^{d}**	**6.01 ± 0.11^{g}**	**5.41 ± 0.12^{f}**	**4.04 ± 0.15^{e}**	**2.49 ± 0.16^{b}**
	Protein (g_{BSAeq}/L)	n.d.	n.d.	n.d.	0.07 ± 0.01^{a}	0.07 ± 0.01^{a}	0.09 ± 0.03^{a}	0.14 ± 0.04^{a,b}	0.27 ± 0.07^{b}	0.36 ± 0.06^{b}	0.55 ± 0.08^{c}	0.80 ± 0.05^{d}

2.3.5. Protein content

The soluble protein content was analyzed using the Bradford method (Bradford, 1976), with some modifications. To 20 μL of sample or standard, 200 μL of Bradford dye reagent was added and the absorbance was measured at 595 nm following 30 min of incubation at room temperature, in the dark. Bovine albumin serum (BSA) was used to perform the standard calibration curve and the results were expressed as milligrams of BSA equivalents.

2.3.6. Bioactive (antioxidant) potential

The antioxidant activity of the different extracts was determined using three different methods.

DPPH radical scavenging activity assay was carried out according to the protocol of Blois (1958), as modified by Teixeira-Guedes et al. (2019). ABTS radical inhibition was carried out according to the protocol developed by Miller and Rice-Evans (1996) and modified by Teixeira-Guedes et al. (2019). The ferric-reducing antioxidant power (FRAP) assay was performed according to Benzie and Strain (1996), with the modifications proposed by Teixeira-Guedes et al. (2019). All results were expressed in μM Trolox equivalents.

2.3.7. Proximate composition of residual spent biomass

The solids obtained from the best conditions were analyzed for the same parameters as the original biomass. Thus, ash, carbohydrate and protein content were calculated according to NREL Technical Reports 510-42622, NREL/TP-510-42625 and NREL/TP-510-42618, respectively (Hames et al., 2008; Sluiter et al., 2012, Sluiter et al., 2008b).

2.4. Statistical analysis

All extractions and experimental analyses were carried out at least in triplicate ($n \geq 3$), with the data being presented as mean value with standard deviation. The statistical analyses were performed using GraphPad Prism® software (version 8.0.1) at $p \leq 0.05$, based on the analysis of variance (ANOVA), with homogeneity test and Tukey's multiple comparisons test for correction. For each condition, different superscript letters represent significant (p-values <0.05) differences.

3. Results and discussion

3.1. Effect of hydrothermal treatment conditions on the fractionation of residual biomass from the hydrocolloid industry: extract composition and functional and bioactive properties

To assess the influence of autohydrolysis extraction conditions on the yield, composition and bioactive properties of extracts produced from industrial agar extraction residues from *Gelidium corneum* biomass, a series of non-isothermal treatments were performed.

The results referring to the influence of processing conditions on the overall extraction efficiency can be observed in Table 1.

Overall, as previously reported for raw seaweed biomasses, as the temperature increases, the solubilization yield increases too (Gomes-Dias et al., 2022, Gomes-Dias et al., 2020), reaching a maximum of 41 %. Furthermore, the effects of the temperature applied (and its corresponding severity factor) can be observed on the polymerization degree of the extracted compounds, and the functional and bioactive properties that originate thereof.

For temperatures up to 190 °C a gel fraction was recovered, with yields averaging 2.6 ± 0.7 %. Considering that the galactan content of the used seaweed residue was slightly over 5 %, this is a significant recovery yield. The range of temperatures where agar can be obtained is by the available literature reports, stating a maximum working severity inferior to 3.2 to avoid the depolymerization of this hydrocolloid (Gomes-Dias et al., 2022, Gomes-Dias et al., 2020). Since agar's economic value is intrinsically correlated with its texturizing behaviour, this parameter was also analyzed. The gelling strength of the extracted

agars decreases drastically for temperatures superior to 160 °C, with the fraction obtained at 190 °C presenting almost no detectable gelling capacity. These results can be explained by the depolymerization of galactans into lower molecular weight fractions and/or by the presence of other compounds present in the biomass (Fig. S1). Nevertheless, all the gelling capacities obtained are less than a third of the necessary for high-grade agars (Lee et al., 2017), but within the range reported for non-pretreated agars directly extracted from lower-grade sources such as *Gracilaria* seaweeds (Pereira et al., 2021).

Thus, for the valorization of this industrial residue towards a gelling fraction, extraction conditions with severities leading up to 2.2 are recommended, since they produce agars with 177 ± 15 g/cm^2 of gelling strength. Due to the lower processing temperatures (and subsequent lower total processing time and energy consumption), hydrothermal treatments in the severity range of 1.44 ± 0.22 are more promising for large-scale applications, producing 1.81 ± 0.06 g of agar per 100 g of biomass, with a gelling strength of 189 ± 10 g/cm^2. However, these results are still below the threshold values for most applications, strongly hindering the commercial potential of this fraction, which, allied to the low extraction yield, can render the process unviable. Furthermore, industrial agar's gel strength is usually measured by the Nikan-Sui method (or Kobe test), which measures the necessary load to break, in 20 s, a 1 % gel using a cylindrical piston with 1 cm^2 area (Zucca et al., 2016). Due to the difference in the method, it is not possible to determine with precision which applications are in line with the extracted agars' texturizing potential. Still, these results are within the range reported in the literature, with values from 25 to 350 g/cm^2 being obtained for pressurized hot water-assisted agar recovery from this residue at temperatures between 80 and 130 °C (Diop et al., 2022b). However, the maximum gelling strength obtained was achieved at 87.9 °C, 1 bar, 150 min, resulting in a much longer processing condition than the ones used in this work (corresponding to a total treatment of 20–30 min in agar-producing conditions, due to the heating and cooling periods). Based on the results obtained in this work, an optimal condition of 130 °C under non-isothermal processing is proposed, due to the lower temperature (thus faster heating) and equally effective yield and gelling strength. This reinforces the use of autohydrolysis as a rapid and efficient alternative for the valorization of seaweed hydrocolloids.

All things considered, despite being a viable valorization approach, the authors believe that the depolymerization of carbohydrates into oligosaccharides might provide a more commercially appealing strategy. Thus, the gelling fraction was not subjected to further characterizations.

The liquor monosaccharide contents (and inherent valorization options) are negligible when considerable agar recovery occurs. The monosaccharide content of this fraction only surpasses 1 g/L for treatments performed in the 210 °C to 230 °C range. At its maximum value (1.4 g/L obtained at 220 °C), this represents a conversion of slightly over 5 % of the original carbohydrates present in the industrial residue. On the other hand, at this severity, the content of inhibitory compounds reaches 1 g/L, which is enough to delay the growth of several microorganisms, not making it impossible to use this extract as fermentation media but strongly impacting the time necessary for this step (Benemir Erkan et al., 2022). This, in alliance with the low monosaccharide content, makes the hydrothermally produced extracts of *Gelidium corneum* industrial residue not desirable to be used as a fermentation substrate directly. Nonetheless, these liquors can be used for other applications, and the residue obtained can be saccharified to produce said fermentation media, not excluding the use of the industrial residue for this application.

In contrast, the oligosaccharide content (and possible residual polysaccharide content not recovered during the freeze-thaw cycle) of the extracts does not follow a direct relationship with the treatment's severity. The content of these compounds starts from 2 g/L at the lowest temperatures, reaching a maximum of 6 g/L at 200 °C, decreasing again with temperature increase, to values below 3 g/L at the highest studied temperature. At the optimal operating conditions, this represents a solubilization and depolymerization of nearly 25 % of the original carbohydrates present in the biomass. Furthermore, most of the oligosaccharides obtained are derived from galactose units, and not glucose units. Since galactooligosaccharides have long been recognized for their prebiotic potential, with several reports of the beneficial effects of these compounds obtained from red seaweeds (de Jesus Raposo et al., 2016). In fact, at this condition, over 80 % of the galactose content present in the biomass is solubilized. This reinforces the possible use of this fraction for food and nutraceutical applications. To the best of the authors' knowledge, there are no reports of autohydrolysis for the extraction of oligosaccharides from seaweed residues. Nevertheless, the optimal temperature identified in this study lies in the range reported (non-isothermal processing from 150 °C to 220 °C) for the direct extraction of oligosaccharides from seaweeds using this technology (Andrade et al., 2022; Flórez-Fernández et al., 2022, Flórez-Fernández et al., 2019; Pérez-Larrán et al., 2020). Furthermore, it is described that the molecular weight of seaweed polysaccharides is a key factor in their prebiotic potential, with a decrease from 201 kDa to 65 kDa making the difference between a non-significant to a significant increase in bifidobacterial populations, alongside a five-fold increase in short-chain fatty acid production (Ramnani et al., 2012). When it comes to the RI spectra (Fig. S1), more associated with the carbohydrate fraction of the samples, it is possible to observe that the chromatograms obtained in the range of 130 °C to 180 °C all show the same profile, with a first peak corresponding to weights superior to 642 kDa, expected to be from the aggregation of remaining agar extracted at these temperatures. This peak decreases in intensity and fades as the treatments become more severe, not being detected for treatments above 200 °C. Generally, all samples present the main peak within the range of the pullulan standards used, with the highest molecular weight of 40 ± 2 kDa being recorded for the treatment performed at 130 °C, decreasing up to 10 ± 1 kDa in the treatment performed at 200 °C and reaching values below 6 kDa in the highest temperature. Unfortunately, to the best of the authors' knowledge, there is no complete literature consensus on the effect of oligosaccharide type, molecular weight and bioactive potential in this range. Thus, the extract produced at 200 °C is still considered to be the most promising in terms of prebiotic activity, due to its higher oligosaccharide concentration and molecular weight within the ranges reported for this bioactivity. Nevertheless, a full in vitro evaluation of prebiotic potential and cytotoxicity should be performed before considering the use of this fraction.

The protein content is another element to consider when analyzing the possible valorization strategies for this industrial residue. This compound is indetectable in the lowest conditions, with the method used, but follows a trend with the increase in temperature, reaching its maximum of 0.8 g/L at the highest temperature studied (230 °C). This value represents a solubilization of 5 % of the initial protein content of the biomass. Furthermore, bioactive size-dependent effects have been reported for the peptides extracted from seaweeds, with smaller peptides (<10 kDa) producing higher Angiotensin-Converting Enzyme inhibition than larger peptides (>10 kDa), but the inverse is observed for other activities such as antimicrobial behaviour (Cermeño et al., 2020). When it comes to the molecular weight UV spectra (Fig. S1) there is a strong indication of depolymerization of compounds as the temperature increases. At the highest temperature studied, 230 °C, a main peak in the range of 30 kDa with two shoulders in the range of 45 kDa and 22 kDa can be observed.

Nevertheless, the low recovery yield of protein indicates that, despite being possible to use the technology, more efforts should be made to increase protein content in the extracts. For example, the use of moderate electric fields as an alternative heating source for subcritical water processing of proteins has shown promising results, substantially increasing solubilization yields and energy efficiency, and ultimately decreasing energy consumption (Kadem et al., 2023).

Alongside the composition of the extracts, their bioactive potential is

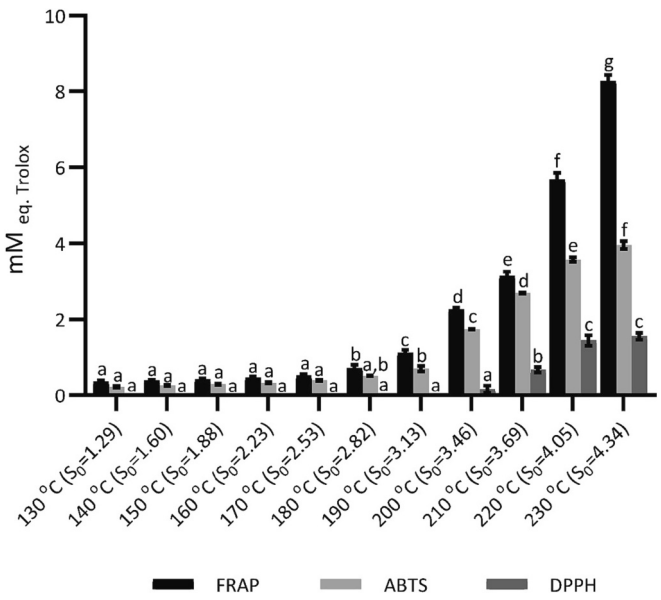

Fig. 2. Effect of extraction conditions (maximum temperature and subsequent severity factor) on the antioxidant activity (measured by three different methods) of the liquid fractions obtained. For each variable, different letters represent statistically significant differences ($p < 0.05$).

one major factor to take into consideration when assessing their possible uses. Antioxidants are compounds that inhibit oxidation, prevent the production of free radicals and are a key factor in the deterrence of cell damage, abnormal cell growth, senescence induction and high production of inflammatory cytokines (Salehi et al., 2018). The antioxidant potential of the samples, indicating their possible bioactivity, is represented in Fig. 2. An increase in this activity is recorded for temperatures higher than 180 °C when FRAP and ABTS are considered, while for DPPH a significant increase is only observed above 210 °C. Nevertheless, the results obtained in all antioxidant methods follow the same trend, increasing as the treatments become more severe. Thus, the antioxidant activity determined is expected to be derived from the protein fraction solubilized since both parameters follow the same trend. There are reports of agars and agar-derived extracts produced from this genre of seaweeds to present antioxidant activity, but the values are in the range of 8–24 μmol of Trolox equivalents per gram of extract, while the ones reported in this study are in the mmol range (Martínez-Sanz et al., 2021). Moreover, a correlation coefficient superior to 0.7 has been obtained for the relationship between the antioxidant capacity and phenolic and protein fraction from pressurized semicontinuous subcritical water hydrolysis of red seaweed industrial solid residue (Trigueros et al., 2021a). In all cases, the values obtained by the FRAP method are higher, followed by the ones obtained by ABTS and DPPH. This can be explained by the selectivity of DPPH towards Cys and Cys-containing peptides, making other types of proteins react with the remaining antioxidant methods and not this one (Zheng et al., 2015). This information also reinforces the hypothesis that the antioxidant activity measured is due to the protein content.

Overall, it was possible to conclude that this industrial residue can be further valorized for the production of agar with moderate gelling strength (at the optimal temperature of 130 °C), oligosaccharides with possible prebiotic potential (at the optimal temperature of 200 °C) or antioxidant protein fractions (at the optimal temperature of 230 °C). This information is summarized on Fig. 3.

Based on the severity differences between strategies for carbohydrate and protein valorization, a new sequential treatment was performed, to assess the feasibility of residue fractionation and multiple product recovery from this biomass, targeting maximum added value in a zero-waste approach. Since, when it comes to carbohydrate valorization, oligosaccharide production presents as a more promising approach due to the low industrial relevance of the residual agar fraction (low agar yield and gel strength and high processing costs), the 200 °C treatment was chosen as the first step of sequential processing.

3.2. Feasibility of sequential processing

Before conducting the sequential treatments, the solid remaining after the hydrothermal treatment was characterized, to assess its suitability for further processing. An ash content of 27.48 ± 0.03 %, protein content of 19.68 ± 0.58 %, carbohydrate content of 19.46 ± 0.44 % and acid-insoluble residue of 24.29 ± 3.42 % were obtained. As expected by the low protein values detected in the liquor, this solid is still rich in protein.

The comparison of the liquor composition obtained at 230 °C using a direct extraction or a sequential extraction can be observed in Table 2.

The use of sequential processing does not interfere with the amount of protein extracted, resulting in a significant increase in the protein content of the liquor. On the other hand, depending on the antioxidant method used, there is a significant loss of activity. The results obtained by the DPPH and FRAP methods are reduced to half, whereas no difference is observed when the ABTS method is used. Since neither the colourimetric protocol used for protein determination nor the HPLC molecular weight column used are capable of detecting peptides below 6 kDa (Lucarini and Kilikian, 1999), a possible explanation is the presence of smaller peptides. Small differences can be observed in the chromatograms of both fractions obtained at 230 °C, indicating a slight (i.e. 10–15 kDa) increase in the molecular weight of the fractions obtained by sequential processing (Fig. S2). Furthermore, since a great portion of the carbohydrate fraction has already been solubilized in the

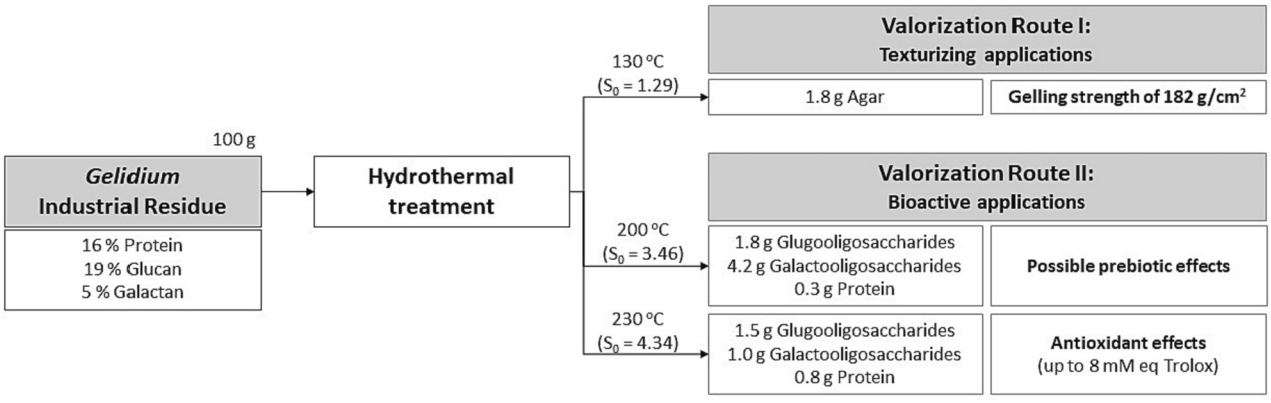

Fig. 3. Schematic representation of possible valorization strategies for residual Gelidium biomass from the hydrocolloid industry, using hydrothermal treatments.

Table 2
Effect of direct or sequential extraction at 230 °C conditions on the overall extraction yield, carbohydrate profile (monosaccharide, inhibitory compound, and oligosaccharide content), protein content and antioxidant potential of the liquid fractions obtained. For each variable, different letters represent statistically significant differences ($p < 0.05$). * - Calculated based on the solid remaining from the 200 °C treatment. ** - Calculated based on the initial biomass weight. Conditions marked as n.d. represent concentrations below the detection limit of the methods used.

Extraction condition	Direct extraction at 230 °C	Sequential extraction (230 °C after 200 °C)
SY (%)	41.0 ± 1.6[b]	*28.6 ± 1.5[a] **51.7 ± 2.0[c]
Uronic Acids (g/L)	0.50 ± 0.01[b]	0.23 ± 0.01[a]
Glucose (g/L)	0.31 ± 0.02[a]	0.40 ± 0.01[b]
Galactose (g/L)	0.56 ± 0.03[a]	0.72 ± 0.02[b]
Total monosaccharides	**1.37 ± 0.04[a]**	**1.35 ± 0.03[a]**
Acetic/Levulinic Acid (g/L)	1.00 ± 0.05[b]	0.76 ± 0.02[a]
HMF (g/L)	0.33 ± 0.03[b]	0.25 ± 0.01[a]
Furfural (g/L)	0.21 ± 0.01[b]	0.09 ± 0.01[a]
Total inhibitory compounds	**1.54 ± 0.05[b]**	**1.10 ± 0.02[a]**
Glucooligosaccharides (g/L)	1.51 ± 0.05[b]	0.39 ± 0.03[a]
Galactooligosaccharides (g/L)	0.99 ± 0.15[a]	n.d.
Total oligosaccharides	**2.49 ± 0.16[b]**	**0.39 ± 0.03[a]**
Protein (gBSAeq/L)	0.80 ± 0.05[a]	0.99 ± 0.08[b]
DPPH (mM eq Trolox)	1.56 ± 0.09[b]	0.70 ± 0.16[a]
ABTS (mM eq Trolox)	3.96 ± 0.10[a]	4.17 ± 0.27[a]
FRAP (mM eq Trolox)	8.27 ± 0.18[b]	4.88 ± 0.31[a]

previous treatment, the carbohydrate content of this liquor is significantly lower, with total oligosaccharide content decreasing to a sixth and no significant change in total monosaccharide content. The content of inhibitory compounds is also reduced.

Overall, these results prove that a two-step hydrothermal treatment can be applied to the industrial residue of *Gelidium corneum* seaweed, retrieving in the first approach a potentially prebiotic galactooligosaccharide-enriched fraction and, in a second step, a protein-enriched fraction with antioxidant activity, increasing the process selectivity. Moreover, with the use of this approach, the overall solubilization yield is increased to over 50 %, considerably reducing the amount of solid left over at the end of the extractions.

3.3. Proposed optimal biorefinery scheme

When it comes to the residue after agar extraction, the sequential hydrothermal treatment aimed at carbohydrate and protein recovery proposed in this work is considered a promising strategy. Nonetheless, the authors consider that even after two valorization steps, the remaining solid fraction can still serve a better purpose than being discarded (Fig. 4).

In fact, it has already been proven that the use of this biomass as a source for a fermentation medium to produce polyhydroxyalkanoates and other chemicals is still possible (Bondar et al., 2022). This is by the residue composition determined, composed mainly of glucans, indicating that nearly 64 % of the original content is still maintained in the solid after the two-step treatment. Furthermore, fermentation products such as ethanol present economic and environmental advantages over methane production (Fasahati et al., 2022), being a promising alternative for a biorefinery approach.

When it comes to biomass valorization, the authors believe that the entire process, from seaweed growth to the final product should be assessed. Thus, the proposed biorefinery scheme should not focus only on the industrial residue but also on the raw seaweed before hydrocolloid extraction.

The first drawback of *Gelidium* sp. used for high-quality agar is that this seaweed cannot be cultivated with competitive yields in an economically viable approach, with the entire market being dependent on the harvest of wild biomass, presenting possible habitat and ecosystem protection issues (Mouga and Fernandes, 2022). Since seaweed harvest locations are restricted by the natural prevalence of this biomass, transportation and storage conditions also represent significant contributions to the overall impact of the process, with reports of seaweed drying, often necessary to preserve the biomass until further processing, requiring up to 10 MJ of energy per kg of seaweed (van Oirschot et al., 2017). The drying step can be shortened, decreasing energy consumption, if a preliminary step of water removal (by pressing and/or filtration is applied), resulting in a water stream that can be reintegrated in the process or used as a plant growth stimulant (Álvarez-Viñas et al., 2019), or substituted for low-cost approaches such as sun-drying, when the conditions are favourable. Hence, both steps are inevitable but require proper optimization.

The main product obtained from *Gelidium* sp. is agar. This hydrocolloid is traditionally obtained after an alkaline pretreatment to increase purity and remove sulphate groups, increasing its gelling strength

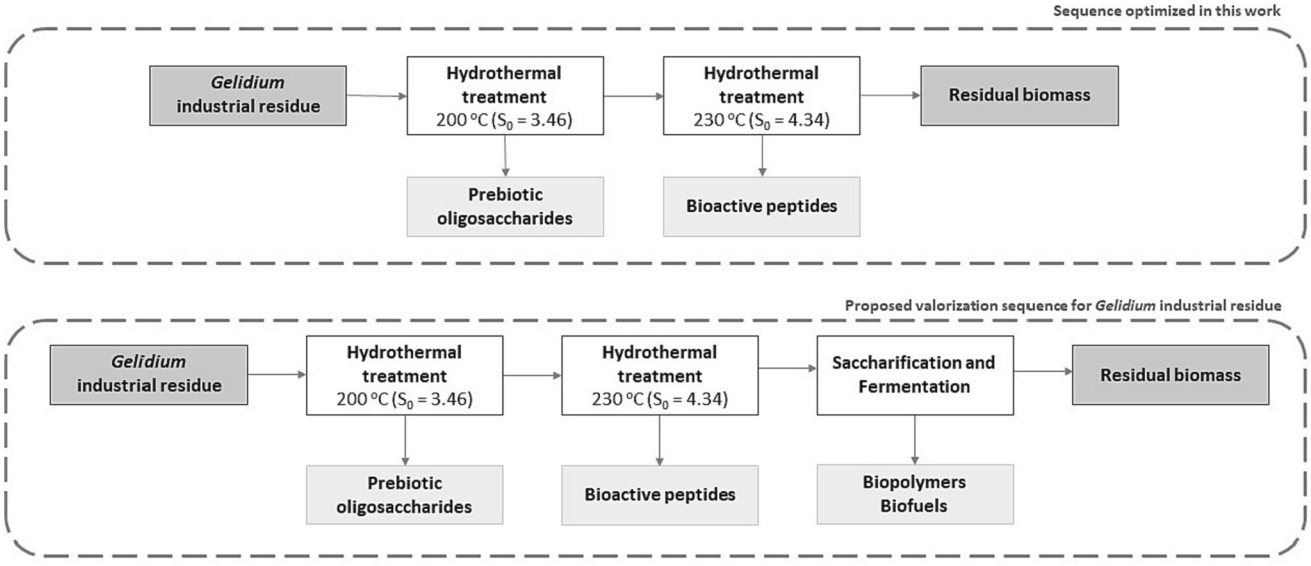

Fig. 4. Schematic representation of proposed valorization sequences for *Gelidium* industrial residues.

and market value. However, recently, greener processing alternatives have been presented, such as ultrasound-assisted extraction and auto-hydrolysis, that present the same agar yield and texturizing capacity without requiring chemical pretreatment (Gomes-Dias et al., 2022; Martínez-Sanz et al., 2021), reducing the environmental impact of the process. Moreover, several thermolabile compounds (pigments and phycobiliproteins) can be recovered before agar extraction, without compromising the market value of the main product (Zhao et al., 2020).

Ultimately, the life cycle assessment of each possible processing methodology should be employed, considering all process variables such as seaweed batch and composition, location of the processing plant, sequential processing protocol and source of the energy used in the method, to determine the ideal biorefinery approach for this biomass.

4. Conclusions

In this work, the effects of hydrothermal processing, on *Gelidium corneum* industrial residue were studied. It was proven that the process can be tuned according to the desired product: processing at 130 °C is proposed for agar recovery; 200 °C is the optimized condition for oligosaccharide recovery; harsher processing conditions result in better protein solubilization, with an optimum occurring at 230 °C. Moreover, a proposed approach of 200 °C followed by 230 °C results in higher extraction yields than a single-step approach. Overall, these results demonstrate the feasibility of using this green technology on multi-product valorization from this low-value residue.

CRediT authorship contribution statement

Joana S. Gomes-Dias: Methodology, Investigation, Formal analysis, Validation, Data curation, Visualization, Writing – original draft, Writing – review & editing. **José A. Teixeira:** Conceptualization, Writing – review & editing, Resources, Funding acquisition, Supervision. **Cristina M.R. Rocha:** Conceptualization, Methodology, Investigation, Resources, Formal analysis, Validation, Writing – review & editing, Supervision.

Declaration of competing interest

The authors have no competing interests to declare.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biteb.2023.101720.

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