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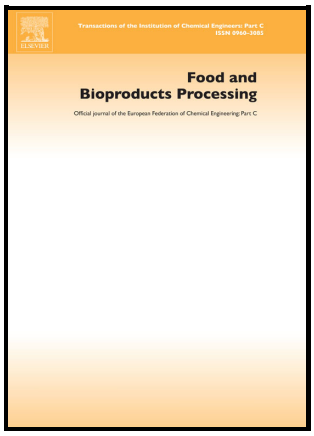

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Enzymatic approach for the extraction of bioactive fractions from red, green and brown seaweeds

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ABSTRACT

Although several enzymatic approaches have been applied with this intent, the sequential use of enzymes covering both cellulolytic and proteolytic activity has never been performed in seaweeds. Sequential use of these enzymes improved the overall extraction yield by up to 160 %, 30 % and 80 % in the different seaweeds when compared to the control condition (water extraction), use of a carbohydrases' cocktail alone and use of proteases alone, respectively. Regarding the proximate composition of extracts, it proved to be an efficient approach for the solubilization of carbohydrates (up to 28 % in *G. vermiculophylla*, 66 % in *P. dioica*, 77 % in *U. rigida* and 35 % in *F. vesiculosus*) and protein (up to 55 % in *G. vermiculophylla*, 47 % in *P. dioica*, 52 % in *U. rigida* and 42 % in *F. vesiculosus*). For all biomasses, the combination of enzymes induced a significant increase in antioxidant activity, not only by the increase of phenolic compounds but also by the hydrolysis of protein to peptides. Moreover,

extracts from red seaweeds displayed prebiotic activity which can be ascribed to their increased content in oligosaccharides and protein/peptides. Overall, the sequential use of enzymes with different activities demonstrated to be an efficient approach for extracting functional fractions to be used as functional ingredients to improve the nutritional value (e.g. in proteins) and/or to include antioxidant and prebiotic features in the food product.

Keywords: bioactive compounds carbohydrases; extraction; proteases; seaweeds

1. Introduction

Marine macroalgae, commonly known as seaweeds, are autotrophic multicellular organisms, with a great diversity of forms and sizes. They can be categorized into three broad taxonomic groups according to their main photosynthetic pigments, namely chlorophylls, fucoxanthins and phycobilins for green algae (Chlorophyceae), brown algae (Phaeophyceae) and red algae (Rhodophyceae), respectively (Jönsson *et al*., 2020; Kadam *et al*., 2013; Mohamed *et al*., 2012).

In the past years, seaweed farming has been growing rapidly worldwide, being practiced in about 50 countries (FAO, 2018). However, several legal and health requirements must be taken into consideration before selecting seaweed or seaweed extracts for human consumption purposes. Firstly, it should be noted that only 21 seaweed or seaweed extracts are currently authorized for human consumption on the European

Union, under the EU 2020/1820 and EU 2018/1023 regulations (Geada et al., 2021). Secondly, health hazards contaminants like heavy metals, pesticide residues, toxins, pharmaceuticals and the presence of pathogenic microorganisms should be according to specific regulations (Banach et al., 2020). Further, the food grade should be maintained throughout the whole chain, which is particularly relevant when considering waste-streams as algae growth medium.

The growing awareness of food as a source of functional ingredients for health promotion has aroused the food industry's interest in seaweed. They are recognized as an excellent food source for health promotion, as they are rich in dietary fibres, polysaccharides, proteins/amino acids, polyunsaturated fatty acids (PUFAs), vitamins polyphenols and pigments (Charoensiddhi *et al*., 2017a; Paiva *et al*., 2017). Though seaweeds could be used for direct consumption, the industry is focused on their processing to obtain added-value products with a wider range of potential applications (food, pharmaceuticals, nutraceuticals and hydrocolloid industries). Nevertheless, the bioavailability and bioaccessibility of some nutrients is often impaired by the complex seaweed structure and composition, and mild processing or extraction may improve its nutritional value. Despite the recognized nutritional value and their enormous potential in a wide range of industries, one of the major drawbacks to their use is the lack of protocols specially tailored to the isolation of compounds or fractions of interest from this biomass (Matos *et al*., 2021).

Seaweed cell walls are made up of rigid and chemically complex and heterogeneous biomolecules i.e., sulfated and branched polysaccharides associated with proteins and several bound ions that limit the extraction of bio compounds (Rodrigues *et al*., 2015). Traditionally, the extraction of interest compounds in seaweed is water-based. This extraction has the advantage of being inexpensive, environmentally friendly and food

compatible, however, it has low extraction efficiency and selectivity (Matos *et al*., 2021). Extractions with organic solvents such as diethyl ether, benzene and acetonitrile have higher yields and selectivity, however, these methods are non-food compatible and/or harmful to the environment (Rodrigues *et al*., 2015; Wijesinghe and Jeon, 2012). The application of cell wall degrading food-grade enzymes such as carbohydrases and proteases to seaweeds has been growing considerable interest in the scientific community due to their hydrolytic action. It has the advantage of being an eco-friendly and non-toxic technology since it alleviates the use of solvents in the process (Charoensiddhi *et al*., 2017a; Kadam *et al*., 2013; Ummat *et al*., 2021). Enzyme-assisted extraction is a high bioactive yielding technology where the rigid and heterogeneous cell wall structures are weakened or disrupted, releasing the interest biocompounds to the extraction medium. In addition, it also removes the barriers to water solubility, thus reducing the insolubility of bioactive compounds and preserving their biological value (Kadam *et al*., 2013; Wijesinghe and Jeon, 2012).

Considering the limitations of water-based extraction and aiming to increase the extraction efficiency of target compounds using food-compatible methods, the main objective of this work was to study the effect of a multi-enzyme complex containing a wide range of carbohydrases (Viscozyme® L) and two proteases (Flavourzyme® and papain), alone or in combination, in green (*Ulva rigida*), brown (*Fucus vesiculosus*) and red seaweeds (*Gracilaria vermiculophylla and Porphyra dioica*). Despite the several reports of single enzyme approaches used in seaweeds, to the best of the authors' knowledge, the extensive characterization and evaluation of the synergetic effect of enzymes in these biomasses is a novelty. To achieve this goal, the chemical characterization of the different seaweeds was performed, alongside the optimization of the extraction conditions using the different enzymes. In addition, the chemical

characterization of the extracts and the evaluation of their antioxidant and prebiotic potential were also assessed.

2. Materials and methods

2.1. Raw material

The seaweeds used in this study were supplied dried by AlgaPlus (Ílhavo, Portugal). *G. vermiculophylla* (batch G1.01119.D: B1)) was washed twice with tap water and once with distilled water to remove all the debris. Subsequently, they were dried at 60 °C for 16 h and stored in the dark at room temperature until use. Samples of *P. dioica* (batch P1.01019.M.B1), *U. rigida* (batch U1.00219.M) and *F. vesiculosus* (batch F1.00719.M.B1) were purchased ready to use (flakes < 10 mm). The same batch of all seaweeds (submitted to the same standardized procedures at AlgaPlus) was used in all tests to avoid differences in the initial biomass so that the results could be ascribed to the different treatments used.

2.2. Chemical characterization of raw material

Each seaweed was analyzed for ashes, carbohydrates, lipids, and proteins following standard protocols. The ash content (NREL/TP-510-42618) was determined by calcination in a muffle furnace at 575 °C until achieving a constant weight (Sluiter *et al*., 2008a). The nitrogen content of the biomass samples was measured by Kjeldahl digestion (NREL/TP-510-4262) (Hames *et al*., 2008) and the protein content was estimated using a nitrogen factor of 5 (Angell *et al*., 2016). The lipid content was determined by Bligh and Dyer method with chloroform and methanol (Bligh and Dyer, 1959).

To assess the carbohydrate content sequential Soxhlet extractions with (i) distilled water for 16 h and (ii) 80 % v/v ethanol for 8 h were carried out to remove water-soluble and liposoluble compounds, respectively (Sluiter *et al*., 2012, 2008b). For oligosaccharide quantification, aliquots from the extractions and residues were subjected to hydrolysis with 4 % H_2SO_4 (121 °C, 20 min). The resulting post-hydrolyzed liquid and the directly extracted liquid were filtered through 0.22 μm membranes and analyzed by high-performance liquid chromatography (HPLC) to quantify the solubilized compounds such as uronic acids (i.e., glucuronic, galacturonic and manuronic acids were used as standards) and sugars (i.e., glucose, galactose, xylose, mannose, arabinose, rhamnose, fucose and mannitol were used as standards). The conditions used in the HPLC analysis were as follows: refractive index detector; UV detector at 210 nm; Aminex HPX-87H column at 60 °C; and a mobile phase of 0.05 mol/L H_2SO_4 at a flow rate of 0.6 mL/min. Extract-free seaweed was subjected to a two-step quantitative acid hydrolysis (QAH) with: (i) 72 % H_2SO_4, 60 min, 30 °C and (ii) 4 % H_2SO_4, 60 min, 121 °C. The liquid from the QAH was also analyzed by HPLC, using the same conditions as before. Uronic acids, glucan, galactan, xylan, mannan, arabinan, rhamnan, fucoidan and mannitol groups were calculated from the concentrations of the respective monomeric sugars, using the standardized correction factor of 132/150 for pentoses and 162/180 for hexoses. The insoluble phase from the QAH was subjected to gravimetric quantitation in number 3 Gooch crucibles and reported as an acid-insoluble residue (AIR).

2.3. Optimization of enzyme-assisted extraction

The optimization of enzyme-assisted extractions was performed on *G. vermiculophylla*. The pH (4.5 for Viscozyme® L and 7.0 for papain, Flavourzyme®) and temperature (50 ºC) conditions were chosen according to the manufacturer's specifications.

Before the enzyme-assisted extractions, the cellulolytic and proteolytic activity of the enzymes was determined in filter paper units (FPU) (Miller, 1959) and tyrosine release from casein (Murado *et al*., 2009), respectively. The extraction conditions for *G. vermiculophylla* were optimized considering two steps: a pre-treatment with Viscozyme (Visc) and the treatment with Flavourzyme (Visc_Flav) or papain (Visc_Pap).

The first experimental design (pre-treatment with Viscozyme) was applied to test the effects of the independent variables, time (X1) (7.9 to 28.1 h), cellulolytic enzyme concentration (X_2) (3.1 to 61.9 FPU/g of seaweed) and solid:solvent ratio (weight:volume; w:v) (X_3) (1 to 11 % w:v).

The optimization of the subsequent treatment with proteases was made by testing the effects of the independent variables, time (X1) (1.2 to 6.8 h) and proteolytic enzyme concentration (X_2) (1378.7 to 5621.3 U/g of seaweed), considering the optimal conditions for the pre-treatment and adjusting the media to the optimal pH of the selected proteases. Results were evaluated through a 2^2 central composite design with four replicates at the central point. The extraction yield (g of extract / g of seaweed x 100) was taken as the dependent variable for both experimental designs. The correlation between dependent (Y_n) and independent variables (X_1, X_2 and X_3) for the cellulolytic enzyme and (X_1 and X_2) for the proteolytic enzyme was established by the empirical models, considering the linear, quadratic and interaction effects of all studied variables. The regression coefficients were calculated from the experimental data by multiple regression using the least-squares method.

2.4. Recovery of biocompouds using enzyme-assisted extraction

In this study, enzyme-assisted extractions were performed on four different seaweeds such as *G. vermiculophylla and P. dioica* (red seaweeds), *U. rigida* (green seaweed) and

F. vesiculosus (brown seaweed). The optimal conditions for each step obtained in section 2.3 were then applied to all seaweeds, considering different enzyme combinations, and performed in triplicate for each combination. After the extractions, the enzymatic reactions were stopped by heating the sample at 90 °C for 10 min followed by immediate cooling in an ice bath. The enzymatic aqueous solutions were then centrifuged at 5000 rpm for 20 min and the supernatant was kept at -20ºC until analysis.

2.5. Chemical characterization of the extracts

Total phenolic content (TPC) was determined by the colorimetric method of Folin−Ciocalteu according to Singleton and Rossi, (1965) and optimized to 96-well plates (Teixeira-Guedes *et al*., 2019). Results were expressed as g of GA equivalent per 100 gram of seaweed extract. Total flavonoid content (TFC) was assessed by the aluminium chloride complexation method according to Domínguez-Perles *et al*., (2014) with some modifications (Teixeira-Guedes *et al*., 2019). Results were expressed as g of catechin equivalent per 100 grams of seaweed extract.

2.6. Determination of the antioxidant activity

The antioxidant activity of the different extracts was determined using three different methods, ABTS (Domínguez-Perles *et al*., 2014) and DPPH (Blois, 1958) and ferric reducing antioxidant power (FRAP) (Bolanos de la Torre *et al*., 2015) as previously optimized by Teixeira-Guedes *et al*., (2019). Oxygen radical absorbance capacity (ORAC) assay was applied to extracts used for the evaluation of prebiotic potential. ORAC procedure was performed according to the method described by Coscueta *et al*. (2020). The antioxidant activity of the samples was determined by interpolation of the

standard curve for Trolox. In the ABTS and DPPH radical scavenging methods, the % of radical inhibition was calculated using the following Eq:

Eq1: $\%\ inhibition = \frac{Abs_{Radical} - Abs_{Sample}}{Abs_{Radical}} \times 100$

2.7. Evaluation of proteins and peptides profile by size exclusion chromatography

The molecular weight distribution was determined using the AKTA Pure 25 system (GE Healthcare Life Sciences, Freiburg, Germany) with a configuration of two pumps, a gel filtration column prepacked with Superdex 200 10/300 GL, a column Superdex Peptide 10/300 GL and an UV multiwavelength detection monitor U9-L. The column was operated at a flow rate of 0.5 mL/min with 0.025 M phosphate buffer (pH 7) with 0.15 M NaCl and 0.2 g/L NaN3. The absorbance was monitored at 280 nm. Standard proteins with known molecular weights were used to establish the molecular weight standard curve (Thyroglobulin, 669 kDa; Aldolase, 158 kDa; Conalbumin, 75 kDa; Ovalbumin, 43 kDa; Carbonic anhydrase, 29 kDa; Ribonuclease A, 13.7 kDa; Aprotinin, 6.5 kDa) (Campos *et al*., 2019) and the antihypertensive peptide KGYGGVSLPEW (1.2 kDa).

2.8. In vitro simulation of gastrointestinal digestion

In vitro simulation of the gastrointestinal (GI) tract was performed in an extract from *G. vermiculophylla* and *P. dioica* subjected to pre-treatment with Viscozyme and treatment with Flavourzyme. For each replicate, 1 g of each extract was dissolved in 20 mL of phosphate buffer. The system used aimed to simulate the conditions of each phase of the GI tract (mouth, stomach and small intestine) (Campos *et al*., 2020). For the mouth simulation, samples were mixed with simulated oral fluid, the pH was adjusted between 5.6 and 6.9 and α-amylase enzyme (100 U/mL) was added at a rate of 0.6 mL/min.

Samples were incubated for 2 min at 37 °C, at 200 rpm. In the gastric simulation, simulated gastric fluid was added and pH was adjusted to 2.0. The enzyme pepsin (25 mg/mL) was added at a rate of 0.05 mL/mL of sample. Digestion was performed for 2 h at 37 °C, 130 rpm. In the last step, a small intestine simulation was performed by adding intestinal gastric fluid and adjusting the pH to 6.0 ($NaHCO_3$, 1M). A mixture of pancreatin enzyme (2 g/L) and bile salts (12 g/L) was added at a rate of 0.25 mL/mL of sample. Intestinal digestion took place for 120 min at 37 °C, at 45 rpm.

2.9. Prebiotic potential

The prebiotic potential was tested in digested and undigested extracts of *G. vermiculophylla* and *P. dioica* at a final concentration of 1 and 2 % according to Bordiga, Montella, Travaglia, Arlorio, & Coïsson, (2019). The prebiotic strains used were *Lactobacillus acidophilus* Ki (LAS) (isolated from fermented milk, CSK, Netherlands), and *Lactobacillus casei* (*L. casei*) L26 (DSM - Moorebank, NSW, Australia), *Bifidobacterium animalis* spp. *lactis* Bb12 (Chr. Hansen, Denmark) and *Bifidobacterium animalis* Bo (CSK, Ede, Netherlands). Each culture was propagated twice in the appropriate medium at 37 ºC for 24 h. The growth of *Lactobacillus* was performed using Man-Rogosa-Sharpe (MRS) broth (Biokar Diagnostics, France), while *Bifidobacterium* was grown in MRS broth supplemented with 0.5 g/L of L-cysteine-HCl (Fluka, Switzerland). Prior to the experiment, the bacteria were grown for 24 h at 37 ºC in the mentioned mediums. The reaction was performed in 96-well-microplates.

Tested samples were essentially rich in carbohydrates and proteins/peptides. Thus, to understand how extracts affect bacterial growth, two tests were performed for each sample. In the first test, bacterial strains were grown in media with 2 % glucose and in the second test in media without a sugar source. All assays were performed in triplicate.

For each test, 2 % of bacterial inoculum was used. Fructooligosaccharide (FOS) (Orafti P 95®; Orafti, Belgium) (2 %) was used as the positive control. Wells with *Bifidobacterium* were sealed with paraffin to assure anaerobiosis conditions. The microplate was incubated for 48 h at 37 ºC in a microplate reader and bacterial growth was monitored at 660 nm, by measuring the optical density (OD) every hour.

2.10. Statistical analysis

Results were presented as mean ± standard deviation (SD) of three independent experiments. Statistical analyses were performed using Graph Pad Prism 6 (GraphPad Software, Inc., San Diego, CA). Differences between samples were tested using analysis of variance (ANOVA) followed homogeneity test and Tukey's multiple comparisons test. Results were considered as being statistically significant for values of $p<0.05$.

3. Results and discussion

3.1. Composition of raw material

Seaweeds have an exceptional combination of macro- and micronutrients that make them very interesting from a nutritional perspective. However, the concentrations of such elements are very susceptible to seasonality, environmental conditions, geographical origin and several other factors making generalizations of algal composition very difficult (Sharma *et al*., 2018). Since the chemical composition of the seaweed, such as the monomers profile, differs in nutritional value, fermentability, and biological and functional behaviour leading to different industrial applications, such as fermentation medium, biodiesel, texturizing agents, natural pigments, proteins sources, the authors considered this step to be of the foremost importance. Thus, the chemical

composition of each seaweed is presented in Table 1. The same batch of each seaweed was used through all the work to allow direct comparisons between different treatments. Higher carbohydrate content was found in *G. vermiculophylla* followed by *F. vesiculosus*, *P. dioica* and *U. rigida*. Regarding carbohydrate profiles, as can be observed in **Table 1**, values found in the present study are in concordance with the available literature.

The major storage carbohydrates and cell polymers are common in this taxonomic group: red agarophyte seaweeds are composed of glucose-based reserve storage floridean, cellulose, mannan and agar composed by galactopyranose and galactose. In the case of *P. dicoica*, the main polysaccharide is an agarose-related constituent porphyran, composed of galactosyl and 3,6-anhydro-α-l-galactosyl units, with minor 3,6-anhydrogalactosyl residues and partial methylation. On the other hand, green seaweeds' cell walls are composed of cellulose and xyloglucans, while their sulfated polysaccharides are composed by uronic acid-rich polysaccharides containing rhamnose, xylose and galactose residues. Brown seaweeds are typically composed by laminaran, mannitol, alginate/alginic acids, that are composed of mannuronic and guluronic acid units and fucoidans, a sulfated fucose-rich polysaccharides that can present galactose, mannose, xylose, glucose, glucuronic acid residues (Stiger-Pouvreau *et al*., 2016).

Although only about 80 % of the seaweed composition was identified, it is predicted that the remaining fraction is constituted by carbohydrates that, due to their particular structure, are known to react poorly to the two-step acid hydrolysis commonly used for the determination of structural carbohydrates in biomass, namely uronic acids (Manns *et al*., 2014), mannitol and fucose (Kostas *et al*., 2016) and 3,6-anhydro-L-galactose (Park

et al., 2012). However, phenolic compounds and pigments were also present but in small quantities (Peñalver *et al*., 2020).

Protein determination in seaweeds presents a very high uncertainty associated with the conversion factor, some authors use the standard nitrogen conversion factor of 6.25 for all seaweeds, while others refer to factors for specific groups or even species. Based on the lack of consensus, the authors have chosen to use the conversion factor of 5 proposed by Angell, Mata, de Nys, & Paul, (2016), as the most accurate universal seaweed nitrogen-to-protein conversion factor. *P. dioica* presented higher protein content, circa 27 % in dry weight when compared to *G. vermiculophylla* (16 %), *U. rigida* (15 %) and *F. vesiculosus* (9 %). Values found in *P. dioica* were higher than several legumes (e.g., common bean, peas chickpeas) and comparable with that of high-protein plant foods such as soybean and lupins (Rizzo and Baroni, 2018), thus, representing an excellent food source of dietary protein.

The values of ash observed in this study ranged from 20 to 35 % in the different seaweeds, *U. rigida* presenting the highest values. In all seaweeds, lipids values were low, representing below 8 % of dry matter. For all seaweeds, ash, lipids and proteins are within the reported ranges for all seaweeds (del Río *et al*., 2020; Mæhre *et al*., 2014; Neto *et al*., 2018; Silva *et al*., 2015; Varela-Álvarez *et al*., 2019).

3.2. Optimization of enzymatic hydrolysis conditions

In this work, it was considered the sequential use of two different types of enzymes. The multi-enzyme complex Viscozyme contains a wide range of carbohydrase activities, including β-glucanase, arabinase, cellulase, hemicellulase and xylanase to exert a broader action on the heterogenic cell wall of different seaweeds, as a preliminary structure disruption step or pre-treatment. The proteolytic enzymes Flavourzyme and

papain, both with endopeptidase and exopeptidase activity, were used to help further rupture the algal cell wall and release internal storage material. This may enhance the recovery of the usually inaccessible intracellular compounds, additionally increasing the biological activities of the extract, namely, the antioxidant activity. In addition, it may also enhance protein solubilization, bioaccessibility and bioactivity, by breaking down and releasing soluble bioactive peptides.

Table 2 shows the optimization of the extraction condition with Viscozyme based on the extraction yield (%) obtained in *G. vermiculophylla* seaweed. The subsequent optimization with Flavourzyme or papain in *G. vermiculophylla* was performed in carbohydrase pretreated seaweed and is presented in **Table 3**. Based on the fitting parameters listed in **Table 4**, it can be observed that the dependent variables were well interpreted by the empirical model, as can be noted by the R^2 and F values. The extraction yield was directly correlated with time and enzyme concentration and inversely correlated with the solid:solvent ratio. The higher extraction yield was observed after 24 h incubation with 50 FPU/g of seaweed using 3 % solid:solvent (run 4). On the application of Flavourzyme, a higher extraction yield was obtained between 4 to 6 h incubation and 3500 to 5621 U/g of seaweed. For papain, 4 to 6 h incubation and 5000 to 5621 U/g of seaweed showed the best results. Based on these results, for the pre-treatment with Viscozyme, the conditions of 24 h incubation with 50 FPU/g of seaweed using a solid:solvent ratio of 3 % were chosen. On the treatment with proteolytic enzymes, 5000 U/g of seaweed during 5 h for Flavourzyme and 6 h for papain were applied.

3.3. Extraction yield

Using the operational conditions selected, different combinations of enzymatic treatments were tested for all the seaweeds. The extraction yields achieved for each extraction condition of the four different seaweeds are presented in **Fig. 1**. Higher extraction yields were observed for *U. rigida* (green algae), followed by *P. dioica*, *G. vermiculophylla* (red algae) and *F. vesiculosus* (brown algae) extracts. For all seaweeds, the use of cellulolytic enzyme cocktails resulted in a significant increase (40-80 %) in the overall extraction yield, while the use of proteases resulted in a smaller increase (10-40 %), most of the time not significant in comparison with the control (extraction with water, at the same temperature for the same extraction time). However, when combined, cellulolytic and proteolytic enzymes proved to have a synergetic effect on the red and green seaweeds. In particular, the sequential use of a carbohydrase followed by protease showed an increase in the extraction yield of around 40 to 60 % when compared to water extraction. Red seaweeds showed the greatest increase, approximately 40 % in Visc_Pap and 75 % in Visc_Flav. This phenomenon occurs due to the more intensive and/or diverse degradation of cell walls and is also linked to a different composition profile and bioactivity. This raises, once more, the need for and importance of a thorough characterization of extracted fractions. The synergistic effects have never been tested but the use of cellulolytic or proteolytic enzymes has been previously reported as having a positive effect on extraction yield (Nadar *et al*., 2018).

3.4. Proximate characterization of the extracts

The proximate composition of the extracted fractions is presented in **Table 5**. Considering the total carbohydrate yield, the combination of Viscozyme and Flavourzyme (Visc_Flav) proved to be the most efficient enzymatic approach for all four seaweeds, ranging from 25 % total carbohydrates solubilization in *G.*

vermiculophylla to a maximum of 78 % in *U. rigida.* Simultaneously, it was observed that each seaweed's depolymerization was differently influenced by this combination of enzymes, in the red seaweed *P. dioica*, the most significant sugar solubilization is associated with carbohydrates rich in xylose/mannose/galactose units (representing 85 % of total extracted carbohydrates), while *G. vermiculophylla* presents an extract composed of carbohydrates with similar amounts of glucose and xylose/mannose/galactose units (47 % and 45 % of total extracted carbohydrates). Due to the HPLC operational conditions used in this work, it is not possible to differentiate xylose, mannose and galactose, since these present the same retention time. However, galactose is expected to be the majority sugar present in red seaweeds, due to their reported composition (known to be rich in agar or porphyran, with only minor traces of other monomers) (Torres *et al*., 2019). These ratios differ from the initial biomass composition assessed, indicating a higher affinity for the enzyme mixture towards the sulfated galactan in *P. dioica* and the structural glucan (e.g., cellulose) in *G. vermiculophylla.* These results can be a consequence of the higher molar ratio of xylose:galactose units in porphyran (1:50) than in agar (1:174), causing a higher affinity towards the carbohydrase used in this study and leading to higher hydrolysis (Pereira *et al*., 2021; Qiu *et al*., 2021). This result is of high interest since marine galactose can be efficiently converted into tagatose, a low glycemic index and low caloric value equivalent to sucrose (Baptista *et al*., 2021). Similar results were obtained for the uronic acids in *F. vesiculosus*, with this component corresponding to 77 % of total extracted in the condition Visc_Flav. A higher amount of uronic acids in fucose-containing fractions (oligosaccharides) is associated with a higher antibacterial and antiviral effect and antioxidant activity (Ayrapetyan *et al*., 2021; Shen *et al*., 2018; Zhao *et al*., 2008).

Overall, the use of Viscozyme increased the extraction yield of carbohydrates and significantly changed the profile of the extracted sugars (**Table 6**). The most representative example of this change was the detection of arabinose and glucose units on *P. dioica* extracts only on the fractions obtained with Viscozyme. The same phenomena were observed for arabinose-rich carbohydrates' extraction on *G. vermiculophylla* and xylose-rich carbohydrates' extraction on *U. rigida.* Since these are structural components of the seaweed cell walls, it corroborates the increased hydrolysis yields and subsequent extraction efficiency obtained when enzymes are applied, being the synergistic effect more pronounced on the green seaweed (with carbohydrates richer in glucose units). Furthermore, the synergetic effect of protease and carbohydrases was more pronounced on the green seaweed (richer in glucose units), resulting in a 10- fold increase of carbohydrates extraction over control and use of proteolytic enzymes alone. Since the nutraceutical and bioactive properties of the carbohydrates depend not only on their composition but also on their size, the distinction between monomeric sugars and oligosaccharides/low molecular weight polysaccharides was assessed in all the extracts. This determination was performed based on the sugar content of each extract (measured by HPLC) before and after dilute acid hydrolysis. As can be observed in **Fig. 2**, the use of Viscozyme, alone or in combination with proteases, resulted in an increased size of the carbohydrates extracted in both red seaweeds. The liquid extracts of *P. dioica* only presented arabinose oligomers and/or solubilized polysaccharides, in concentrations ranging from 19 to 25 mg per g of seaweed. Fractions from *G. vermiculophylla* presented a concentration of polymerized arabinose- and galactose-based compounds more than 15-fold greater, increasing from 10 mg per g of seaweed in non-carbohydrase assays up to 215 mg per g of seaweed when these enzymes were employed. These findings can be a result of the lack of affinity of Viscozyme for the red seaweed specific

carbohydrates, aligned with their affinity for the glucose-based cell wall components. Although the lack of affinity, the use of carbohydrases led to depolymerization and weakened the cell wall. The intracellular storage polysaccharides were exposed and, despite not being subjected to complete hydrolysis, they were somewhat depolymerized and/or solubilized. The same does not apply to the remaining seaweeds, where the use of these enzymes did not change carbohydrate complexity. Since these seaweeds'carbohydrates present more glucose, xylose and rhamnose units, they are more susceptible to the enzymatic cocktail used.

Based on the knowledge that oligosaccharides and low molecular weight polysaccharides are associated with several beneficial effects (Cheong *et al*., 2018), the use of these sequential combination enzymes is recommended for health, cosmetic and nutraceutical applications, as it results in the formation of higher amounts of oligosaccharides.

Overall, the use of cellulolytic and proteolytic enzymes increased significantly the protein yield when compared to the control (water extraction). Enzymatic hydrolysis with Viscozyme followed by papain proved to be the most efficient approach to extract protein for all seaweeds, although both Flavourzyme and papain have endo- and exopeptidases activity. Specifically, the combination of Viscozyme and papain resulted in a total of 55 % of protein solubilization in G. *vermiculophylla*, 47 % in *P. dioica*, 52 % in *U. rigida*, and 42 % in *F. vesiculosus*. The combination of Viscozyme followed by Flavourzyme seems to be more efficient for the extraction of carbohydrates, although the differences were not statistically significant.

Seaweeds are a rich source of phenolic compounds, of which phenolic acids, flavonoids, tannins and particularly phlorotannins are prevalent (Kadam *et al*., 2013). In the present work, total phenolic content (TPC) and total flavonoid content (TFC) were analyzed by

colorimetric assays and are presented in **Table 5**. Among the seaweeds, *U. rigida* had the lowest values of TPC (0.087-0.390 g/100g DW) when compared to *G. vermiculophylla* (0.129-0.439 g/100g DW), *P. dioica* (0.408-0639 g/100g DW) and *F. vesiculosus* (0.371-0.599 g/100g DW). Regarding TFC, the lowest values were found in both red seaweeds, *G. vermiculophylla* (0.049-0.065 g/100g DW) and *P. dioica* (0.044-0.070 g/100g DW).

Nonetheless, for all seaweeds, the enzyme-assisted extracts presented an increase of TPC (>1.6-fold) and TFC (approximately 1.4-fold) when compared to the control water extract. Despite what was previously reported by Habeebullah *et al*., (2021), where proteases induced a higher release of TPC in comparison to carbohydrase, similar values of TPC were found in extracts obtained by Viscozyme, papain and Flavourzyme. The sequential treatment with Viscozyme and Flavourzyme and Viscozyme and papain increased significantly TPC extraction in all seaweeds in comparison with enzymes used alone. In algal cell walls, phenolic compounds are attached mainly to proteins and polysaccharide moieties (Deniaud-Bouët *et al*., 2014). Thus, the use of carbohydrases and proteases can break the links of phenolic compounds to proteins and polysaccharides, releasing and, consequently, increasing their extraction yield.

Bioactive peptides have gained interest in different fields, namely for functional foods, nutraceuticals, cosmetic or pharmaceutical applications. In the present work, proteins and peptides profiles were analyzed by size exclusion chromatography and are shown in **Fig. 3**. The peptide/protein profile shows one to two main peaks near or at 1200 Da. The profile clearly shows the protein hydrolysis during the process of extraction with the application of enzymes, demonstrated by the decrease of the main peaks and generation of lower intermediate MW species. Results demonstrate that a combination of carbohydrases and proteases proved to increase protein solubilization, protein

hydrolysis to peptides and increased antioxidant activity in extracts subjected to the combination of enzymes. This may lead to technological advances, increases in bioavailability, digestibility and bioactive potential.

3.5.Antioxidant activity

Over the past years, dietary oligomers, polyphenols and peptides from seaweeds have been widely studied for their biological activities including antioxidant activity, anti-inflammation, antibacterial, anticancer prebiotic to immuno-modulatory among others (Cheong *et al*., 2018; Cotas *et al*., 2020).

The antioxidant activity of the seaweed extracts was assessed by three different *in vitro* assays. Results have shown that antioxidant activity varied with the application of enzymes, as well as with the seaweed species. *F. vesiculosus* presented the highest antiradical scavenging activity, in all antioxidant assays, being this correlated with its higher total phenolic compounds. As shown in **Fig. 4**, both sequential treatment with Viscozyme and Flavourzyme and Viscozyme and papain exhibited a higher radical scavenging effect than when the enzymes were used separately. The increase in the antioxidant activity seems to be correlated not only with the phenolic compounds but also with the release of peptides and oligomers within the hydrolysis of substrate by both proteases and carbohydrases. The increase in the antioxidant activity has been correlated with an increase in polyphenols (Wang *et al*., 2009), protein hydrolysates (Cian *et al*., 2015b) and oligosaccharides (Cheong *et al*., 2018) from different seaweeds. Antioxidant extracts may be interesting for industrial applications, such as the development of functional foods. However, the antioxidant activity must be maintained after being ingested and submitted to the gastrointestinal (GI) tract under adverse conditions (Cunha and Pintado, 2022). Based on this, the two most promising seaweed

extracts (*G. vermiculophylla* and *P. dioica*) were submitted to an *in vitro* GI simulation. In other to understand the effect of each step of the GI digestion in the extracts, the antioxidant activity/capacity was measured by the ABTS and ORAC. In both methods, a slight increase in the antioxidant activity after the steps of the GI digestion was observed, especially after small intestine simulation (Data not shown). This could be an interesting result, since these extracts may be incorporated into functional foods without compromising their antioxidant properties after GI digestion.

3.6. Prebiotic potential

Recent studies have demonstrated the prebiotic potential of marine resources, namely, seaweeds. These effects have been attributed to its richness in complex polysaccharides that can be resistant to digestion by enzymes present in the human gastrointestinal tract, and selectively stimulate the growth of beneficial gut bacteria (Charoensiddhi *et al*., 2017a; Cian *et al*., 2015a). Likewise, ingested polyphenols, proteins/peptides can also reach the large intestine where they can be converted into beneficial bioactive metabolites by microbiota (Cian *et al*., 2015a; Echave *et al*., 2021). Nonetheless, in the literature, no information is available regarding the prebiotic potential of extracts obtained by the combination of carbohydrases and proteases targeted in this study.

For the evaluation of the prebiotic effect the two red seaweeds, *G. vermiculophylla* and *P. dioica* were chosen in the treatment condition that induced the highest carbohydrate and proteins/peptides release that was the pre-treatment with Viscozyme followed by treatment with Flavourzyme.

With regard to media with a carbon source (**Fig. 5**), in Bb12, significantly higher values ($p < 0.05$) of growth were observed for media enriched with 2 % of digested and non-digested *G. vermiculophylla* extract and 2 % of non-digested and 1 % of digested *P.*

dioica extract in comparison with negative control. In Bo, growth was significantly increased in media supplemented with 1 % of non-digested *G. vermiculophylla* extract and 2 % of digested *P. dioica* extract. Whereas in the LAS strain, a significant inhibition in cell growth was observed for both extracts at the different concentrations tested. The opposite was observed in the *L. casei* strain where a strong prebiotic effect was observed in both extracts for all concentrations, apart from 2 % of digested *P. dioica*.

Results of incorporation of extracts in MRS medium without glucose supplementation are presented in **Fig. 6**. In Bb12, the incorporation of extracts showed a positive effect in the conditions of 1 % non-digested and digested *G. vermiculophylla* extract and 1 and 2 % non-digested *P. dioica*. Similar results were observed in the Bo strain after incubation with non-digested extracts of *G. vermiculophylla* and *P. dioica* and 2 % of digested *P. dioica*. Greater results were observed in *L. casei*, where the growth was significantly enhanced in all media incorporated with extract in comparison with MRS without sugar source (negative control). By opposite, LAS growth was not positively affected by the presence of any of the tested extracts; in fact, the growth of this bacterium seems to be decreased by the presence of the extracts. This confirms how the impact of prebiotic ingredients is strain-dependent.

Concerning the incorporation of FOS in MRS without a carbon source, a significant increase in bacterial growth was observed in all strains, except for the LAS strain. Overall, a similar bifidogenic effect with FOS was observed in *G. vermiculophylla* and *P. dioica* extracts. This effect was maintained in 1 % of *G. vermiculophylla* extract after digestion. A greater prebiotic effect was obtained in *L. casei* strain in *G. vermiculophylla* extracts in both digested and non-digested samples, in comparison with the positive control of FOS. In *P. dioica*, the prebiotic effect was like those obtained in

FOS. This effect is probably due to polysaccharides and oligosaccharides derived from hydrocolloids present in each seaweed that can act as sources of soluble fibers exerting a prebiotic effect.

Prebiotic effects were reported in *B. animalis* and *L. acidophilus* La-5 when incubated with extracts obtained by ultrasound-assisted extraction with Viscozyme in *Sargassum muticum*, *Osmundea pinnatifida*, and *Codium tomentosum* (Rodrigues *et al*., 2015). Polysaccharide extracts of *Gracilaria sp*. extracted with Cellulase R-10 and Macerozyme R-10 also showed bifidogenic effects by increasing colony formation of *B. longum* BCRC 11847 (Shao-Chi *et al*., 2012). In both studies, the investigation of the effect of GI digestion was not performed.

In vitro studies using an anaerobic human fecal fermentation model for gut fermentation processes also reported that laminarin could stimulate the production of short-chain fatty acids (SCFA), metabolites produced by a healthy microbiota (Devillé *et al*., 2007). Low molecular weight polysaccharides from the red seaweed *Gelidium sesquipedale* induced a significant increase in populations of *Bifidobacterium*, as well as an increase in acetate and propionate (Ramnani *et al*., 2012). A similar result using an extract from the brown seaweed *Osmundea pinnatifida* was reported by Rodrigues *et al*. (2016). Charoensiddhi, Conlon, Vuaran, Franco, & Zhang, (2016, 2017) also demonstrated that extracts from the brown seaweed *Ecklonia radiate* stimulated the growth of beneficial microbes such as *Bifidobacterium* and *Lactobacilli* and SCFA production.

4. Conclusions

Seaweeds are a valuable source of bioactive compounds, mainly poly-, oligo- and monosaccharides, proteins and peptides and phenolic compounds. The potential of these compounds for several industries, particularly in the food industry, has been increasingly recognized.

A significant improvement in the extraction yield of carbohydrates, proteins, and phenolic compounds was here reported with a sequential treatment with the use of carbohydrase followed by proteases. Particularly, the combination of Viscozyme and papain proved to be more efficient for the extraction of protein, whereas Viscozyme and Flavourzyme in the extraction of carbohydrates. In addition, an increase in polyphenols, protein hydrolysates and oligosaccharides in the different enzyme-assisted extracts was observed. This increased the antioxidant activity of extracts, being maintained even after gastrointestinal digestion simulation. Extracts from red seaweeds also demonstrated prebiotic potential showing an increase in cell growth of *Lactobacillus* and *Bifidobacterium* strains.

Overall, this study demonstrates that these edible seaweeds are natural sources of valuable compounds with important biological interest. The combination of cellulolytic and proteolytic enzymes had a synergistic effect on the extraction of the different classes of compounds and was revealed to be a promising tool to obtain extracts with attractive biological properties. These results envisage that advances in enzymatic processes are a key trend for efficient extraction of added-value bioactive compounds from seaweeds. However, future studies should be performed regarding the development of functional food and nutraceutical.

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References

Angell, A.R., Mata, L., de Nys, R., Paul, N.A., 2016. The protein content of seaweeds: a universal nitrogen-to-protein conversion factor of five. J. Appl. Phycol. 28, 511–524. https://doi.org/10.1007/s10811-015-0650-1

Ayrapetyan, O.N., Obluchinskaya, E.D., Zhurishkina, E. V, Skorik, Y.A., Lebedev, D. V, Kulminskaya, A.A., Lapina, I.M., 2021. Antibacterial properties of fucoidans from the brown algae Fucus vesiculosus L . of the Barents Sea. Biology (Basel). 10. https://doi.org/doi.org/10.3390/biology10010067

Banach, J.L., Hoek-van den Hil, E.F., van der Fels-Klerx, H.J., 2020. Food safety hazards in the European seaweed chain. Compr. Rev. Food Sci. Food Saf. 19, 332–364. https://doi.org/10.1111/1541-4337.12523

Baptista, S.L., Romaní, A., Oliveira, C., Ferreira, S., Rocha, C.M.R., Domingues, L., 2021. Galactose to tagatose isomerization by the L -arabinose isomerase from Bacillus subtilis : A biorefinery approach for Gelidium sesquipedale valorisation. LWT 151, 112199. https://doi.org/10.1016/j.lwt.2021.112199

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total extration and purification. Can. J. Biochem. Physiol. 37, 911–917. https://doi.org/10.1139/o59-099

Blois, M.S., 1958. Antioxidant Determinations by the Use of a Stable Free Radical. Nature 181, 1199–1200. https://doi.org/10.1038/1811199a0

Bolanos de la Torre, A.A.S., Henderson, T., Nigam, P.S., Owusu-Apenten, R.K., 2015. A universally calibrated microplate ferric reducing antioxidant power (FRAP)

assay for foods and applications to Manuka honey, Food Chemistry. https://doi.org/10.1016/j.foodchem.2014.11.009

Bordiga, M., Montella, R., Travaglia, F., Arlorio, M., Coïsson, J., 2019. Characterization of polyphenolic and oligosaccharidic fractions extracted from grape seeds followed by the evaluation of prebiotic activity related to oligosaccharides. Int. J. Food Sci. Technol. 54. https://doi.org/10.1111/ijfs.14109

Campos, D.A., Coscueta, E.R., Valetti, N.W., Pastrana-Castro, L.M., Teixeira, J.A., Picó, G.A., Pintado, M.M., 2019. Optimization of bromelain isolation from pineapple byproducts by polysaccharide complex formation. Food Hydrocoll. 87, 792–804. https://doi.org/10.1016/j.foodhyd.2018.09.009

Campos, D.A., Coscueta, E.R., Vilas-Boas, A.A., Silva, S., Teixeira, J.A., Pastrana, L.M., Pintado, M.M., 2020. Impact of functional flours from pineapple by-products on human intestinal microbiota. J. Funct. Foods 67, 103830. https://doi.org/10.1016/J.JFF.2020.103830

Charoensiddhi, S., Conlon, M.A., Franco, C.M.M., Zhang, W., 2017a. The development of seaweed-derived bioactive compounds for use as prebiotics and nutraceuticals using enzyme technologies. Trends Food Sci. Technol. 70, 20–33. https://doi.org/10.1016/j.tifs.2017.10.002

Charoensiddhi, S., Conlon, M.A., Vuaran, M.S., Franco, C.M.M., Zhang, W., 2017b. Polysaccharide and phlorotannin-enriched extracts of the brown seaweed Ecklonia radiata influence human gut microbiota and fermentation in vitro. J. Appl. Phycol. 29, 2407–2416. https://doi.org/10.1007/s10811-017-1146-y

Charoensiddhi, S., Conlon, M.A., Vuaran, M.S., Franco, C.M.M., Zhang, W., 2016. Impact of extraction processes on prebiotic potential of the brown seaweed Ecklonia radiata by in vitro human gut bacteria fermentation. J. Funct. Foods 24, 221–230. https://doi.org/10.1016/J.JFF.2016.04.016

Cheong, K.L., Qiu, H.M., Du, H., Liu, Y., Khan, B.M., 2018. Oligosaccharides derived from red seaweed: Production, properties, and potential health and cosmetic applications. Molecules 23. https://doi.org/10.3390/molecules23102451

Cian, R.E., Drago, S.R., De Medina, F.S., Martínez-Augustin, O., 2015a. Proteins and carbohydrates from red seaweeds: Evidence for beneficial effects on gut function and microbiota. Mar. Drugs 13, 5358–5383. https://doi.org/10.3390/md13085358

Cian, R.E., Garzón, A.G., Ancona, D.B., Guerrero, L.C., Drago, S.R., 2015b. Hydrolyzates from Pyropia columbina seaweed have antiplatelet aggregation,

antioxidant and ACE I inhibitory peptides which maintain bioactivity after simulated gastrointestinal digestion. LWT - Food Sci. Technol. 64, 881–888. https://doi.org/10.1016/j.lwt.2015.06.043

Coscueta, E.R., Reis, C.A., Pintado, M., 2020. Phenylethyl isothiocyanate extracted from watercress by-Ppproducts with aqueous micellar systems: Development and optimisation. Antioxidants 9. https://doi.org/10.3390/antiox9080698

Cotas, J., Leandro, A., Monteiro, P., Pacheco, D., Figueirinha, A., Goncąlves, A.M.M., Da Silva, G.J., Pereira, L., 2020. Seaweed phenolics: From extraction to applications. Mar. Drugs 18. https://doi.org/10.3390/MD18080384

Cunha, S.A., Pintado, M.E., 2022. Bioactive peptides derived from marine sources: Biological and functional properties. Trends Food Sci. Technol. 119, 348–370. https://doi.org/https://doi.org/10.1016/j.tifs.2021.08.017

del Río, P.G., Gomes-Dias, J.S., Rocha, C.M.R., Romaní, A., Garrote, G., Domingues, L., 2020. Recent trends on seaweed fractionation for liquid biofuels production. Bioresour. Technol. 299. https://doi.org/10.1016/j.biortech.2019.122613

Deniaud-Bouët, E., Kervarec, N., Michel, G., Tonon, T., Kloareg, B., Hervé, C., 2014. Chemical and enzymatic fractionation of cell walls from Fucales: insights into the structure of the extracellular matrix of brown algae. Ann. Bot. 114, 1203–1216. https://doi.org/10.1093/aob/mcu096

Devillé, C., Gharbi, M., Dandrifosse, G., Peulen, O., 2007. Study on the effects of laminarin, a polysaccharide from seaweed, on gut characteristics. J. Sci. Food Agric. 87, 1717–1725. https://doi.org/10.1002/jsfa.2901

Domínguez-Perles, R., Teixeira, A.I., Rosa, E., Barros, A.I., 2014. Assessment of (poly)phenols in grape (Vitis vinifera L.) stems by using food/pharma industry compatible solvents and Response Surface Methodology. Food Chem. 164, 339–346. https://doi.org/10.1016/j.foodchem.2014.05.020

Echave, J., Fraga-Corral, M., Garcia-Perez, P., Popović-Djordjević, J., Avdović, E.H., Radulović, M., Xiao, J., Prieto, M.A., Simal-Gandara, J., 2021. Seaweed protein hydrolysates and bioactive peptides: Extraction, purification, and applications. Mar. Drugs 19, 1–22. https://doi.org/10.3390/md19090500

FAO, 2018. The global status of seaweed production, trade and utilization, Globefish Research Programme.

Geada, P., Moreira, C., Silva, M., Nunes, R., Madureira, L., Rocha, C.M.R., Pereira, R.N., Vicente, A.A., Teixeira, J.A., 2021. Algal proteins: Production strategies and

nutritional and functional properties. Bioresour. Technol. 332. https://doi.org/10.1016/j.biortech.2021.125125

Gomes-Dias, J.S., Romaní, A., Teixeira, J.A., Rocha, C.M.R., 2020. Valorization of seaweed carbohydrates: Autohydrolysis as a selective and sustainable pretreatment. ACS Sustain. Chem. Eng. https://doi.org/10.1021/acssuschemeng.0c05396

Habeebullah, S.F.K., Alagarsamy, S., Sattari, Z., Al-Haddad, S., Fakhraldeen, S., Al-Ghunaim, A., Al-Yamani, F., 2021. Effect of enzymatic hydrolysis on the antioxidant activity of red and green seaweeds and characterization of the active extracts. JAOCS, J. Am. Oil Chem. Soc. 98, 185–200. https://doi.org/10.1002/aocs.12463

Hames, B., Scarlata, C., Sluiter, A., 2008. Determination of protein content in biomass laboratory. Technical Report NREL/TP-510-42625. NERL - Natl. Renew. Energy Lab. https://doi.org/10.1177/004051757604600715

Jönsson, M., Allahgholi, L., Sardari, R.R.R., Hreggviosson, G.O., Karlsson, E.N., 2020. Extraction and modification of macroalgal polysaccharides for current and next-generation applications. Molecules 25. https://doi.org/10.3390/molecules25040930

Kadam, S.U., Tiwari, B.K., O'Donnell, C.P., 2013. Application of novel extraction technologies for bioactives from marine algae. J. Agric. Food Chem. 61, 4667–4675. https://doi.org/10.1021/jf400819p

Kostas, E.T., Wilkinson, S.J., White, D.A., Cook, D.J., 2016. Optimization of a total acid hydrolysis based protocol for the quantification of carbohydrate in macroalgae. J. Algal Biomass Utln 7, 21–36.

Lorbeer, A.J., Tham, R., Zhang, W., 2013. Potential products from the highly diverse and endemic macroalgae of Southern Australia and pathways for their sustainable production. J. Appl. Phycol. 25, 717–732. https://doi.org/10.1007/s10811-013-0003-x

Mæhre, H.K., Malde, M.K., Eilertsen, K.E., Elvevoll, E.O., 2014. Characterization of protein, lipid and mineral contents in common Norwegian seaweeds and evaluation of their potential as food and feed. J. Sci. Food Agric. 94, 3281–3290. https://doi.org/10.1002/jsfa.6681

Manns, D., Deutschle, A.L., Saake, B., Meyer, A.S., 2014. Methodology for quantitative determination of the carbohydrate composition of brown seaweeds (Laminariaceae). RSC Adv. 4, 25736–25746. https://doi.org/10.1039/c4ra03537b

Matos, G.S., Pereira, S.G., Genisheva, Z.A., Gomes, A.M., Teixeira, J.A., Rocha,

C.M.R., 2021. Advances in extraction methods to recover added-value compounds from seaweeds: Sustainability and functionality. Foods 10. https://doi.org/10.3390/FOODS10030516

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31, 426–428. https://doi.org/10.1021/ac60147a030

Mohamed, S., Hashim, S.N., Rahman, H.A., 2012. Seaweeds: A sustainable functional food for complementary and alternative therapy. Trends Food Sci. Technol. 23, 83–96. https://doi.org/10.1016/j.tifs.2011.09.001

Murado, M.A., González, M.D.P., Vázquez, J.A., 2009. Recovery of proteolytic and collagenolytic activities from viscera by-products of rayfish (Raja clavata). Mar. Drugs 7, 803–815. https://doi.org/10.3390/md7040803

Nadar, S.S., Rao, P., Rathod, V.K., 2018. Enzyme assisted extraction of biomolecules as an approach to novel extraction technology: A review. Food Res. Int. 108, 309–330. https://doi.org/10.1016/j.foodres.2018.03.006

Neto, R.T., Marçal, C., Queirós, A.S., Abreu, H., Silva, A.M.S., Cardoso, S.M., 2018. Screening of Ulva rigida, Gracilaria sp., Fucus vesiculosus and Saccharina latissima as functional ingredients. Int. J. Mol. Sci. 19. https://doi.org/10.3390/ijms19102987

Paiva, L., Lima, E., Neto, A.I., Marcone, M., Baptista, J., 2017. Nutritional and functional bioactivity value of selected Azorean macroalgae: Ulva compressa, Ulva rigida, Gelidium microdon, and Pterocladiella capillacea. J. Food Sci. 82, 1757–1764. https://doi.org/10.1111/1750-3841.13778

Park, J.H., Hong, J.Y., Jang, H.C., Oh, S.G., Kim, S.H., Yoon, J.J., Kim, Y.J., 2012. Use of Gelidium amansii as a promising resource for bioethanol: A practical approach for continuous dilute-acid hydrolysis and fermentation. Bioresour. Technol. 108, 83–88. https://doi.org/10.1016/j.biortech.2011.12.065

Peñalver, R., Lorenzo, J.M., Ros, G., Amarowicz, R., Pateiro, M., Nieto, G., 2020. Seaweeds as a functional ingredient for a healthy diet. Mar. Drugs 18, 1–27. https://doi.org/10.3390/md18060301

Pereira, S.G., Teixeira-Guedes, C., Souza-Matos, G., Maricato, É., Nunes, C., Coimbra, M.A., Teixeira, J.A., Pereira, R.N., Rocha, C.M.R., 2021. Influence of ohmic heating in the composition of extracts from Gracilaria vermiculophylla. Algal Res. 58, 102360. https://doi.org/10.1016/J.ALGAL.2021.102360

Qiu, Y., Jiang, H., Fu, L., Ci, F., Mao, X., 2021. Porphyran and oligo-porphyran

originating from red algae Porphyra: Preparation, biological activities, and potential applications. Food Chem. 349, 129209. https://doi.org/10.1016/J.FOODCHEM.2021.129209

Ramnani, P., Chitarrari, R., Tuohy, K., Grant, J., Hotchkiss, S., Philp, K., Campbell, R., Gill, C., Rowland, I., 2012. In vitro fermentation and prebiotic potential of novel low molecular weight polysaccharides derived from agar and alginate seaweeds. Anaerobe 18, 1–6. https://doi.org/10.1016/J.ANAEROBE.2011.08.003

Rhein-Knudsen, N., Ale, M.T., Meyer, A.S., 2015. Seaweed hydrocolloid production: An update on enzyme assisted extraction and modification technologies. Mar. Drugs 13, 3340–3359. https://doi.org/10.3390/md13063340

Rizzo, G., Baroni, L., 2018. Soy, soy foods and their role in vegetarian diets, Nutrients. https://doi.org/10.3390/nu10010043

Rodrigues, D., Sousa, S., Silva, A., Amorim, M., Pereira, L., Rocha-Santos, T.A.P., Gomes, A.M.P., Duarte, A.C., Freitas, A.C., 2015. Impact of enzyme- and ultrasound-assisted extraction methods on biological properties of red, brown, and green seaweeds from the Central West Coast of Portugal. J. Agric. Food Chem. 63, 3177–3188. https://doi.org/10.1021/jf504220e

Rodrigues, D., Walton, G., Sousa, S., Rocha-Santos, T.A.P., Duarte, A.C., Freitas, A.C., Gomes, A.M.P., 2016. In vitro fermentation and prebiotic potential of selected extracts from seaweeds and mushrooms. LWT 73, 131–139. https://doi.org/10.1016/J.LWT.2016.06.004

Shao-Chi, W., Sheen-Kye, K., Bartosz, K., Chang-Jer, K., Chorng-Liang, P., 2012. Antivirus and prebiotic properties of seaweed-oligosaccharide lysates derived from agarase AS-II. J. Fish. Soc. Taiwan 39, 11–21.

Sharma, S., Neves, L., Funderud, J., Torunn, L., Øverland, M., 2018. Seasonal and depth variations in the chemical composition of cultivated Saccharina latissima. Algal Res. 32, 107–112. https://doi.org/10.1016/j.algal.2018.03.012

Shen, P., Yin, Z., Qu, G., Wang, C., 2018. Fucoidan and its health benefits, in: Bioactive Seaweeds for Food Applications. Elsevier Inc., pp. 223–238. https://doi.org/10.1016/B978-0-12-813312-5.00011-X

Silva, D.M., Valente, L.M.P., Sousa-Pinto, I., Pereira, R., Pires, M.A., Seixas, F., Rema, P., 2015. Evaluation of IMTA-produced seaweeds (Gracilaria, Porphyra, and Ulva) as dietary ingredients in Nile tilapia, Oreochromis niloticus L., juveniles. Effects on growth performance and gut histology. J. Appl. Phycol. 27, 1671–1680.

https://doi.org/10.1007/s10811-014-0453-9

Singleton, V.L., Rossi, J.A., 1965. Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. Am. J. Enol. Vitic. 16, 144 LP – 158.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., 2008a. Determination of ash in biomass. Technical Report NREL/TP-510-42622. NERL - Natl. Renew. Energy Lab.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., 2008b. Determination of Sugars , Byproducts , and Degradation Products in Liquid Fraction Process Samples Laboratory Analytical Procedure (LAP) Issue Date : 12 / 08 / 2006 Determination of Sugars , Byproducts , and Degradation Products in Liquid Fraction Proce.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D., 2012. NREL/TP-510-42618 analytical procedure - Determination of structural carbohydrates and lignin in Biomass. Lab. Anal. Proced. 17.

Soler-Vila, A., Coughlan, S., Guiry, M.D., Kraan, S., 2009. The red alga Porphyra dioica as a fish-feed ingredient for rainbow trout (Oncorhynchus mykiss): Effects on growth, feed efficiency, and carcass composition. J. Appl. Phycol. 21, 617–624. https://doi.org/10.1007/s10811-009-9423-z

Stiger-Pouvreau, V., Bourgougnon, N., Deslandes, E., 2016. Carbohydrates from seaweeds, in: Seaweed in Health and Disease Prevention. Elsevier Inc., pp. 223–274. https://doi.org/10.1016/B978-0-12-802772-1.00008-7

Teixeira-Guedes, C.I., Oppolzer, D., Barros, A.I., Pereira-Wilson, C., 2019. Impact of cooking method on phenolic composition and antioxidant potential of four varieties of Phaseolus vulgaris L . and Glycine max L . LWT-Food Sci. Technol. 103, 238–246. https://doi.org/10.1016/j.lwt.2019.01.010

Torres, M.D., Flórez-Fernández, N., Domínguez, H., 2019. Integral utilization of red seaweed for bioactive production. Mar. Drugs 17. https://doi.org/10.3390/MD17060314

Ummat, V., Sivagnanam, S.P., Rajauria, G., O'Donnell, C., Tiwari, B.K., 2021. Advances in pre-treatment techniques and green extraction technologies for bioactives from seaweeds. Trends Food Sci. Technol. 110, 90–106. https://doi.org/10.1016/J.TIFS.2021.01.018

Varela-Álvarez, E., Tobin, P.R., Guihéneuf, F., FitzGerald, R.J., Stengel, D.B., 2019.

Phycobiliproteins, nitrogenous compounds and fatty acid contents in field-collected and cultured gametophytes of Porphyra dioica, a red sea vegetable. J. Appl. Phycol. 31, 3849–3860. https://doi.org/10.1007/s10811-019-01841-6

Wahlström, N., Nylander, F., Malmhäll-Bah, E., Sjövold, K., Edlund, U., Westman, G., Albers, E., 2020. Composition and structure of cell wall ulvans recovered from Ulva spp. along the Swedish west coast. Carbohydr. Polym. 233, 115852. https://doi.org/10.1016/j.carbpol.2020.115852

Wang, T., Jónsdóttir, R., Ólafsdóttir, G., 2009. Total phenolic compounds, radical scavenging and metal chelation of extracts from Icelandic seaweeds. Food Chem. 116, 240–248. https://doi.org/10.1016/J.FOODCHEM.2009.02.041

Wijesinghe, W.A.J.P., Jeon, Y.J., 2012. Enzyme-assistant extraction (EAE) of bioactive components: A useful approach for recovery of industrially important metabolites from seaweeds: A review. Fitoterapia 83, 6–12. https://doi.org/10.1016/j.fitote.2011.10.016

Zhao, X., Xue, C., Li, B., 2008. Study of antioxidant activities of sulfated polysaccharides from Laminaria japonica. J. Appl. Phycol. 20, 431–436. https://doi.org/10.1007/s10811-007-9282-4

Table 1. Chemical characterization of raw material

Component g/100g DW	*G. vermiculophylla*	*P. dioica*	*U. rigida*	*F. vesiculosus*
Ash	28.3 ± 0.18^{c}	20.8 ± 0.55^{a}	35.3 ± 0.13^{d}	25.9 ± 0.91^{b}
Crude Protein	15.9 ± 0.07^{c}	26.7 ± 0.07^{d}	14.8 ± 0.13^{b}	9.24 ± 0.16^{a}
Crude Lipid	1.24 ± 0.06^{a}	2.00 ± 0.14^{b}	3.52 ± 0.34^{c}	7.90 ± 0.54^{d}
Water extractives	32.5 ± 4.20^{a}	31.8 ± 0.42^{a}	37.0 ± 0.86^{a}	35.6 ± 1.42^{a}
Ethanol extractives	3.74 ± 0.40^{a}	5.86 ± 0.33^{b}	4.23 ± 0.11^{a}	7.28 ± 0.14^{c}
Carbohydrates	39.1 ± 2.02^{c}	28.8 ± 0.48^{b}	21.8 ± 1.22^{a}	36.5 ± 1.01^{c}
% Uronic acids	5.0 ± 0.03^{a}	5.06 ± 0.47^{a}	15.0 ± 0.27^{b}	30.2 ± 0.25^{c}
% Glucose units	15.7 ± 0.67^{c}	3.30 ± 0.70^{a}	43.9 ± 0.58^{d}	13.9 ± 0.25^{b}
% XMG units	67.2 ± 0.77^{b}	75.7 ± 0.72^{c}	9.17 ± 0.24^{a}	9.23 ± 0.60^{a}
% Rhamnose units	-	-	32.0 ± 0.52	-
% Arabinose units	12.1 ± 0.13^{b}	15.93 ± 0.08^{c}	-	0.33 ± 0.05^{a}
% Fucose units	-	-	-	23.7 ± 0.34
% Mannitol units	-	-	-	22.6 ± 0.38
AIR	0.92 ± 0.15^{a}	0.92 ± 0.17^{a}	11.7 ± 0.22^{b}	14.7 ± 0.83^{c}

DW – dry weight; XMG - xylose, mannose, galactose; AIR – acid insoluble residues

Table 2. Conditions applied on the extraction with Viscozyme for *G. vermiculophylla*

Run	Time X_1 (h)	Viscozyme X_2 (FPU/g)	Solid:solvent ratio X_3 (%)	Yield Y_1 (%)
5	12.0 (-1.00)	15.0 (-1.00)	9.00 (1.00)	19.59
14	18.0 (0.00)	61.9 (1.41)	6.00 (0.00)	36.50
15	18.0 (0.00)	32.5 (0.00)	6.00 (0.00)	34.01
2	12.0 (-1.00)	50.0 (1.00)	3.00 (-1.00)	34.85
5	12.0 (-1.00)	50.0 (1.00)	9.00 (1.00)	22.41
18	18.0 (0.00)	32.5 (0.00)	6.00 (0.00)	35.49
1	12.0 (-1.00)	15.0 (-1.00)	3.00 (-1.00)	22.93
9	18.0 (0.00)	32.5 (0.00)	1.00 (-1.41)	38.20
4	24.0 (1.00)	50.0 (1.00)	3.00 (-1.00)	42.44
13	18.0 (0.00)	3.07 (-1.41)	6.00 (0.00)	17.61
3	24.0 (1.00)	15.0 (-1.00)	3.00 (-1.00)	30.54
11	7.9 (-1.41)	32.5 (0.00)	6.00 (0.00)	18.82
17	18.0 (0.00)	32.5 (0.00)	6.00 (0.00)	34.96
8	24.0 (1.00)	50.0 (1.00)	9.00 (1.00)	38.36
16	18.0 (0.00)	32.5 (0.00)	6.00 (0.00)	35.98
7	24.0 1.00)	15.0 (-1.00)	9.00 (1.00)	21.47
10	18.0 (0.00)	32.5 (0.00)	11.1 (1.41)	19.52
12	28.1 (1.41)	32.5 (0.00)	6.00 (0.00)	33.80

Table 3. Conditions applied on the extraction with Flavourzyme and Papain after pre-treatment with Viscozyme for *G. vermiculophylla*

Run	Time X_1 (h)	Proteolytic enzymes X_2 (U/g)	Yield Flavourzyme Y_2 (%)	Yield Papain Y_3 (%)
8	6.80 (1.41)	3500 (0.00)	47.38	50.99
1	2.00 (-1.00)	2000 (-1.00)	40.48	42.21
10	4.00 (0.00)	3500 (0.00)	47.31	51.40
11	4.00 (0.00)	3500 (0.00)	47.87	51.04
2	6.00 (1.00)	2000 (-1.00)	42.68	44.48
3	2.00 (-1.00)	5000 (1.00)	42.99	43.06
7	1.20 (-1.41)	3500 (0.00)	36.93	38.25
9	4.00 (0.00)	3500 (0.00)	48.52	50.21
6	4.00 (0.00)	5621 (1.41)	48.89	51.79
4	6.00 (1.00)	5000 (1.00)	48.43	51.80
12	4.00 (0.00)	3500 (0.00)	47.56	50.55
5	4.00 (0.00)	1379 (-1.41)	40.61	42.84

Table 4. Correlation coefficients for the selected responses and the corresponding significance level

Run	Y_1	Y_2	Y_3
a_0	35.006	47.819*	50.802*
a_1	-4.419	2.497*	2.606*
a_2	-1.748	-1.485*	-2.011*
a_3	4.263	2.803*	3.983*
a_4	-2.649	-2.782*	-2.860*
a_5	5.513	0.809	1.623
a_6	-2.385	-	-
a_7	0.330	-	-
a_8	-0.516	-	-
a_9	1.757	-	-
R^2	0.953	0.951	0.936
F	8.092	22.823	17.857

Table 5. Characterization of extracts on carbohydrates, protein and total phenolic content (TPC) and total flavonoid content (TFC) in the different seaweeds

Seaweed	Carbohydrates (g/100g DW)	Protein (g/100g DW)	TPC (g/100g DW)	TFC (g/100g DW)
G. vermiculophylla				
Visc	9.88 ± 0.83 ^{b}	5.40 ± 0.30 ^{a,b}	0.246 ± 0.018 ^{b,c}	0.046 ± 0.004 ^{a}
Visc_Flav	10.57 ± 0.78 ^{b}	7.97 ± 0.49 ^{d}	0.439 ± 0.034 ^{d}	0.059 ± 0.004 ^{a,b}
Visc_Pap	8.23 ± 1.57 ^{b}	9.03 ± 0.52 ^{d}	0.433 ± 0.038 ^{d}	0.065 ± 0.006 ^{b}
Flav	5.72 ± 0.81 ^{a}	6.07 ± 0.30 ^{b,c}	0.194 ± 0.009 ^{b}	0.041 ± 0.004 ^{a}
Pap	6.37 ± 0.39 ^{a}	6.88 ± 0.64 ^{b,c}	0.289 ± 0.003 ^{c}	0.037 ± 0.003 ^{a}
Control	6.03 ± 0.59 ^{a}	4.65 ± 0.38 ^{a}	0.129 ± 0.009 ^{a}	0.049 ± 0.004 ^{a}
P. dioica				
Visc	14.30 ± 0.95 ^{b}	6.98 ± 0.67 ^{b}	0.427 ± 0.015 ^{a}	0.044 ± 0.001 ^{a}
Visc_Flav	18.27 ± 1.87 ^{c}	10.1 ± 0.68 ^{c}	0.567 ± 0.024 ^{b}	0.053 ± 0.005 ^{a,b}
Visc_Pap	17.02 ± 2.25 ^{c}	14.1 ± 1.80 ^{d}	0.639 ± 0.049 ^{c}	0.070 ± 0.006 ^{b}
Flav	10.73 ± 1.41 ^{a}	6.76 ± 0.62 ^{b}	0.435 ± 0.034 ^{a}	0.044 ± 0.004 ^{a}
Pap	10.43 ± 0.79 ^{a}	12.3 ± 0.54 ^{d}	0.627 ± 0.014 ^{c}	0.056 ± 0.004 ^{a}
Control	10.68 ± 0.21 ^{a}	5.22 ± 0.49 ^{a}	0.408 ± 0.011 ^{a}	0.046 ± 0.004 ^{a}
U. rigida				
Visc	9.74 ± 1.72 ^{b}	4.56 ± 0.43 ^{c}	0.241 ± 0.013 ^{b}	0.130 ± 0.015 ^{b}
Visc_Flav	17.35 ± 0.16 ^{c}	6.56 ± 0.64 ^{d}	0.360 ± 0.023 ^{c}	0.150 ± 0.006 ^{b}
Visc_Pap	16.56 ± 1.33 ^{c}	8.16 ± 0.31 ^{e}	0.390 ± 0.032 ^{c}	0.142 ± 0.018 ^{b}
Flav	1.97 ± 0.10 ^{a}	3.27 ± 0.32 ^{b}	0.125 ± 0.005 ^{a}	0.090 ± 0.011 ^{a}
Pap	1.71 ± 0.63 ^{a}	5.65 ± 0.24 ^{d}	0.211 ± 0.006 ^{b}	0.096 ± 0.007 ^{a}
Control	1.76 ± 0.05 ^{a}	2.00 ± 0.17 ^{a}	0.087 ± 0.003 ^{a}	0.095 ± 0.010 ^{a}
F. vesiculosus				
Visc	14.07 ± 0.33 ^{c}	1.80 ± 0.08 ^{c}	0.514 ± 0.044 ^{b}	0.161 ± 0.014 ^{b,c}
Visc_Flav	13.11 ± 0.61 ^{c}	2.94 ± 0.13 ^{d}	0.590 ± 0.024 ^{c}	0.175 ± 0.007 ^{c}
Visc_Pap	10.58 ± 1.29 ^{a}	3.67 ± 0.06 ^{e}	0.599 ± 0.026 ^{c}	0.163 ± 0.011 ^{b,c}
Flav	10.47 ± 1.16 ^{a}	1.44 ± 0.05 ^{b}	0.511 ± 0.015 ^{b}	0.144 ± 0.016 ^{b}
Pap	12.10 ± 0.22 ^{a}	1.65 ± 0.06 ^{c}	0.486 ± 0.006 ^{b}	0.128 ± 0.011 ^{a}
Control	11.92 ± 0.30 ^{b}	0.88 ± 0.08 ^{a}	0.371 ± 0.014 ^{a}	0.128 ± 0.007 ^{a}

Results obtained by enzyme-assisted extraction with Viscozyme® L (Visc), Flavourzyme® (Flav) and papain (Pap) and presented as mean ± SD. Different letters indicate significant differences between extracts for each seaweed at $p<0.05$.

Table 6. Carbohydrate profile of the extracts in the different seaweeds

Seaweed	Uronic acid units (g/L)	Glucose units (g/L)	XMG units (g/L)	Rhamnose units (g/L)	Arabinose units (g/L)	Fucose units (g/L)
G. vermiculophylla						
Visc	0.252 ± 0.018 [a]	1.350 ± 0.021 [c]	1.385 ± 0.063 [b]	-	0.174 ± 0.018 [a]	-
Visc_Flav	0.209 ± 0.064 [a]	1.452 ± 0.027 [c]	1.512 ± 0.107 [b]	-	0.187 ± 0.007 [a]	-
Visc_Pap	0.267 ± 0.013 [a]	1.039 ± 0.241 [b]	1.068 ± 0.178 [a]	-	0.146 ± 0.021 [a]	-
Flav	0.620 ± 0.098 [b]	0.091 ± 0.007 [a]	1.029 ± 0.148 [a]	-	-	-
Pap	0.569 ± 0.079 [b]	0.089 ± 0.021 [a]	1.296 ± 0.043 [a,b]	-	-	-
Control	0.541 ± 0.107 [b]	0.111 ± 0.008 [a]	1.182 ± 0.082 [a,b]	-	-	-
P. dioica						
Visc	0.278 ± 0.093 [a]	0.222 ± 0.059 [a]	3.484 ± 0.356 [b]	-	0.385 ± 0.050 [a]	-
Visc_Flav	0.395 ± 0.056 [a]	0.117 ± 0.003 [a]	4.551 ± 0.527 [c]	-	0.348 ± 0.012 [a]	-
Visc_Pap	0.422 ± 0.021 [a]	0.139 ± 0.001 [a]	4.133 ± 0.644 [c]	-	0.356 ± 0.008 [a]	-
Flav	0.391 ± 0.032 [a]	-	2.762 ± 0.383 [a]	-	-	-
Pap	0.364 ± 0.061 [a]	-	2.701 ± 0.239 [a]	-	-	-
Control	0.459 ± 0.052 [a]	-	2.681 ± 0.079 [a]	-	-	-
U. rigida						
Visc	0.349 ± 0.172 [a]	0.815 ± 0.022 [b]	0.191 ± 0.032 [a]	1.410 ± 0.308 [b]	-	-
Visc_Flav	0.204 ± 0.004 [a]	2.542 ± 0.170 [c]	0.266 ± 0.008 [a]	1.916 ± 0.122 [c]	-	-
Visc_Pap	0.209 ± 0.018 [a]	2.346 ± 0.233 [c]	0.206 ± 0.010 [a]	1.943 ± 0.138 [c]	-	-
Flav	0.153 ± 0.006 [a]	0.258 ± 0.016 [a]	-	0.148 ± 0.031 [a]	-	-
Pap	0.147 ± 0.025 [a]	0.237 ± 0.068 [a]	-	0.180 ± 0.014 [a]	-	-
Control	0.115 ± 0.029 [a]	0.231 ± 0.013 [a]	-	0.155 ± 0.028 [a]	-	-
F. vesiculosus						
Visc	3.189 ± 0.033 [b]	0.081 ± 0.009 [a]	0.383 ± 0.042 [a]	-	-	0.485 ± 0.028 [a]
Visc_Flav	2.954 ± 0.136 [b]	0.086 ± 0.017 [a]	0.323 ± 0.062 [a]	-	-	0.493 ± 0.012 [a]

Visc_Pap	3.111 ± 0.274 [a,b]	0.081 ± 0.010 [a]	0.328 ± 0.048 [a]	-	-	0.488 ± 0.061 [a]
Flav	2.406 ± 0.194 [a]	0.124 ± 0.075 [a]	0.322 ± 0.011 [a]	-	-	0.333 ± 0.066 [a]
Pap	2.659 ± 0.035 [a]	0.101 ± 0.004 [a]	0.342 ± 0.011 [a]	-	-	0.457 ± 0.015 [a]
Control	2.726 ± 0.121 [a]	0.083 ± 0.023 [a]	0.294 ± 0.023 [a]	-	-	0.402 ± 0.055 [a]

Results obtained by enzyme-assisted extraction with Viscozyme® L (Visc), Flavourzyme® (Flav) and papain (Pap) and presented as mean ± SD. Different letters indicate significant differences between extracts for each seaweed at $p<0.05$.

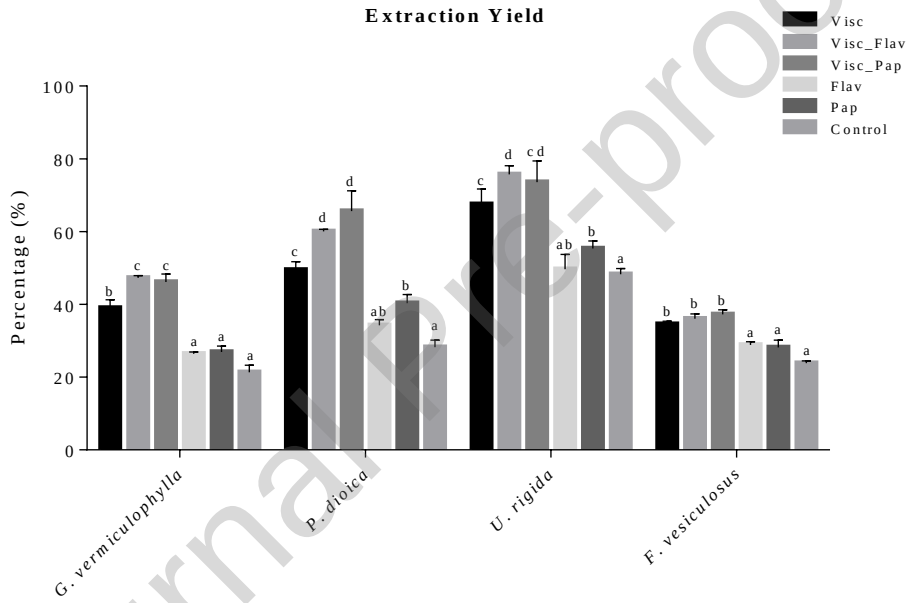

Fig. 1. Extraction yields of *G. vermiculophylla*, *P. dioica*, *U. rigida* and *F. vesiculosus* extracts obtained by enzyme-assisted extraction with Viscozyme® L (Visc), Flavourzyme® (Flav) and papain (Pap). Different letters indicate significant differences between extracts for each seaweed at $p<0.05$.

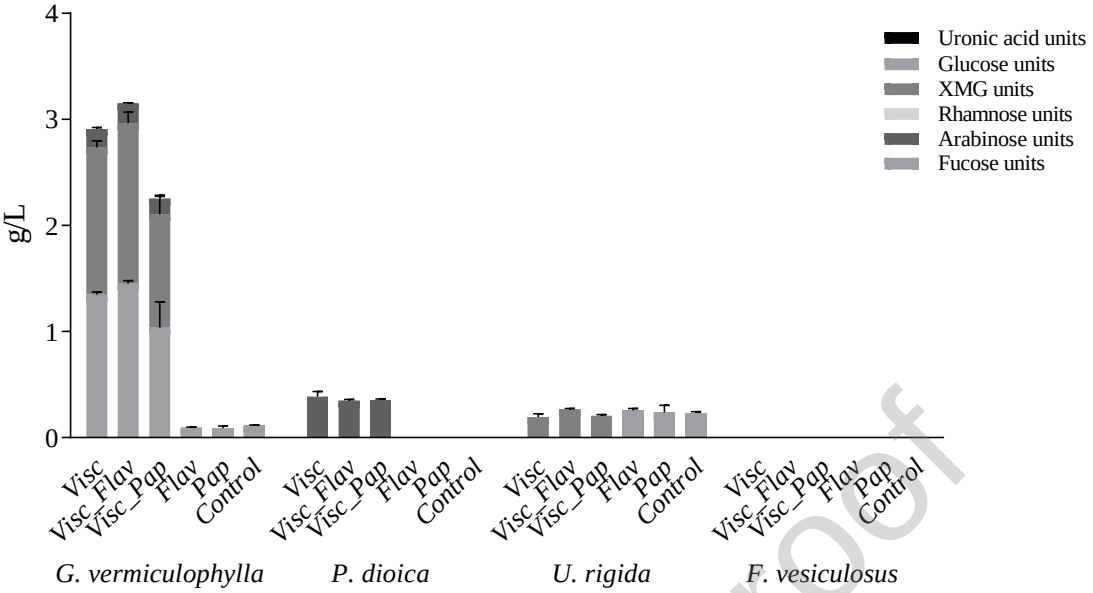

Fig. 2. Oligosaccharide/low molecular weight polysaccharide profile of *G. vermiculophylla*, *P. dioica*, *U. rigida* and *F. vesiculosus* extracts obtained by enzyme-assisted extraction.

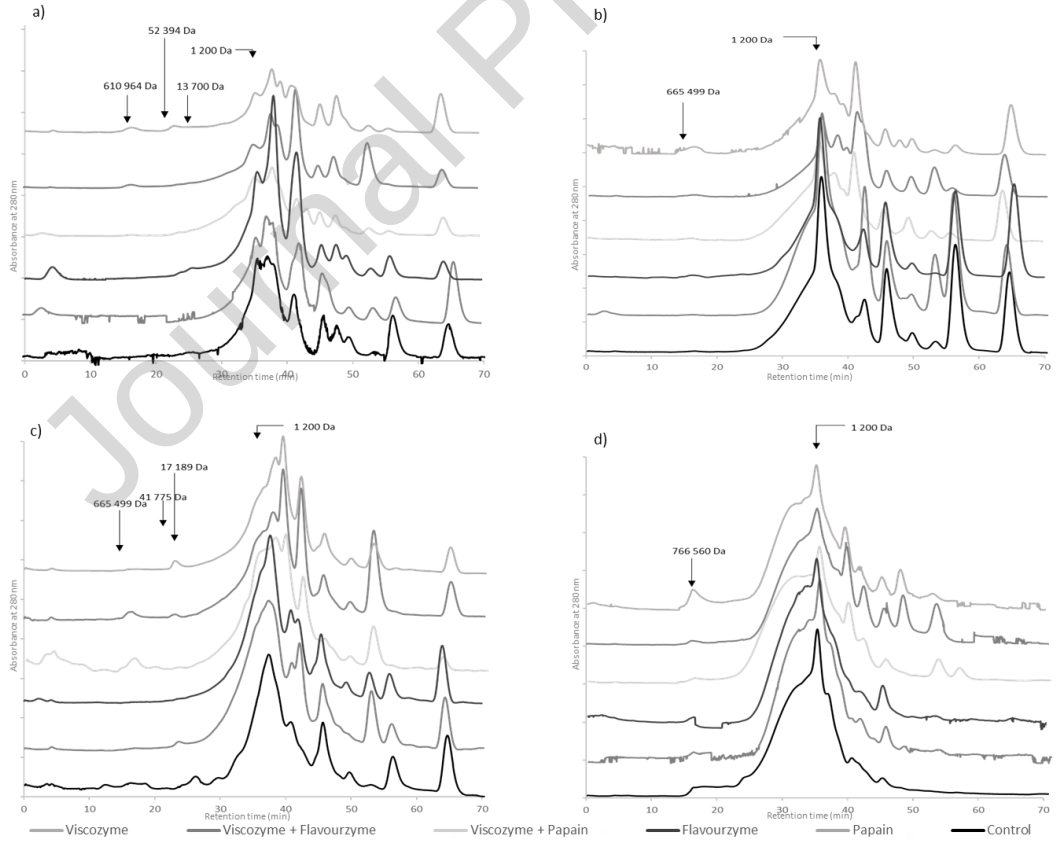

Fig. 3. Peptide profile analysed by FPLC of *G. vermiculophylla (a)*, *P. dioica (b)*, *U. rigida* (c) and *F. vesiculosus* (d) extracts obtained by enzyme-assisted extraction.

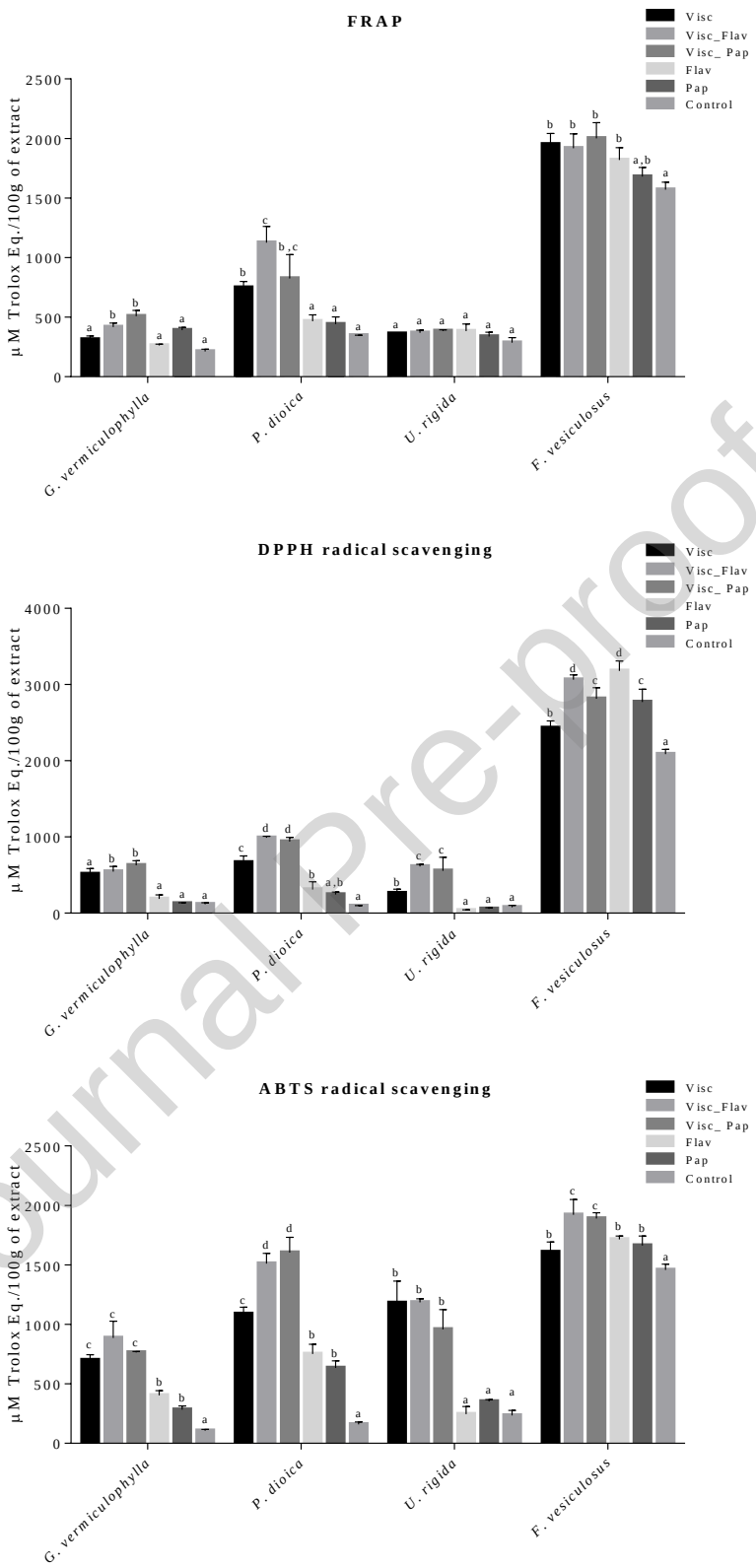

Fig. 4. Antioxidant activity assessed by FRAP, DPPH and ABTS radical scavenging of *G. vermiculophylla*, *P. dioica*, *U. rigida* and *F. vesiculosus* extracts obtained by enzyme-assisted extraction. Different letters indicate significant differences between extracts for each seaweed at $p<0.05$

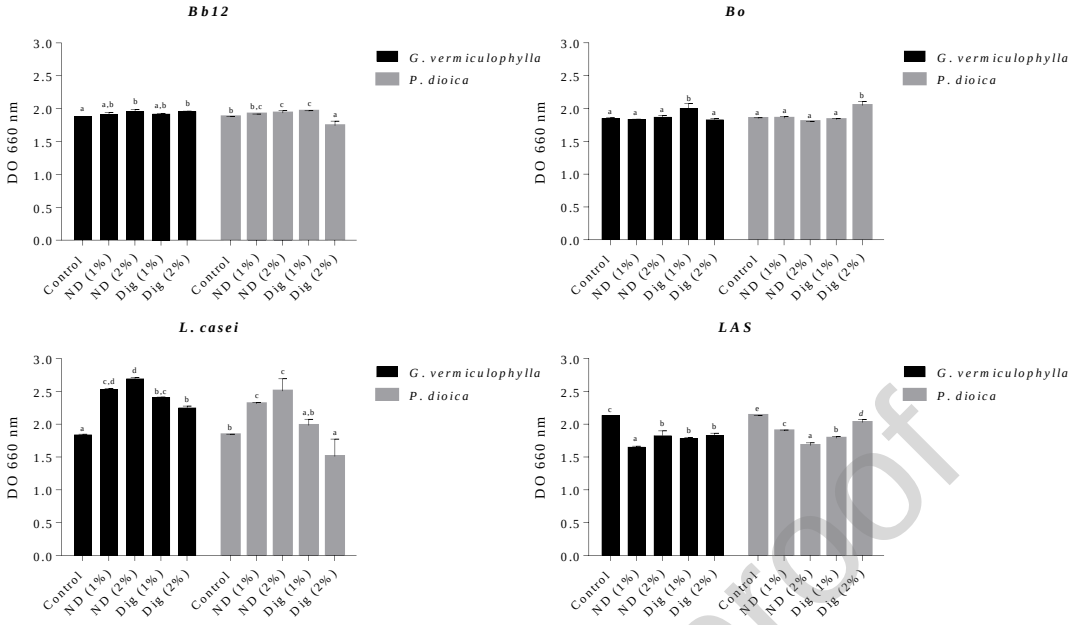

Fig. 5. Maximum growth obtained for *Bifidobacterium animalis* spp. *lactis* (Bb12), *Bifidobacterium animalis* (Bo), *Lactobacillus casei* (*L. casei*) L26 and *Lactobacillus acidophilus* Ki (LAS) strains in the presence of glucose (Glc) (growth control), *G. vermiculophylla* digested (Dig) and non-digested (ND) (1 and 2 %), and *P. dioica* digested and non-digested (1 and 2 %). Tests with the samples were performed in MRS with 2 % of glucose. The analyses were performed in triplicate and data are expressed as mean ± SD of the replicates.

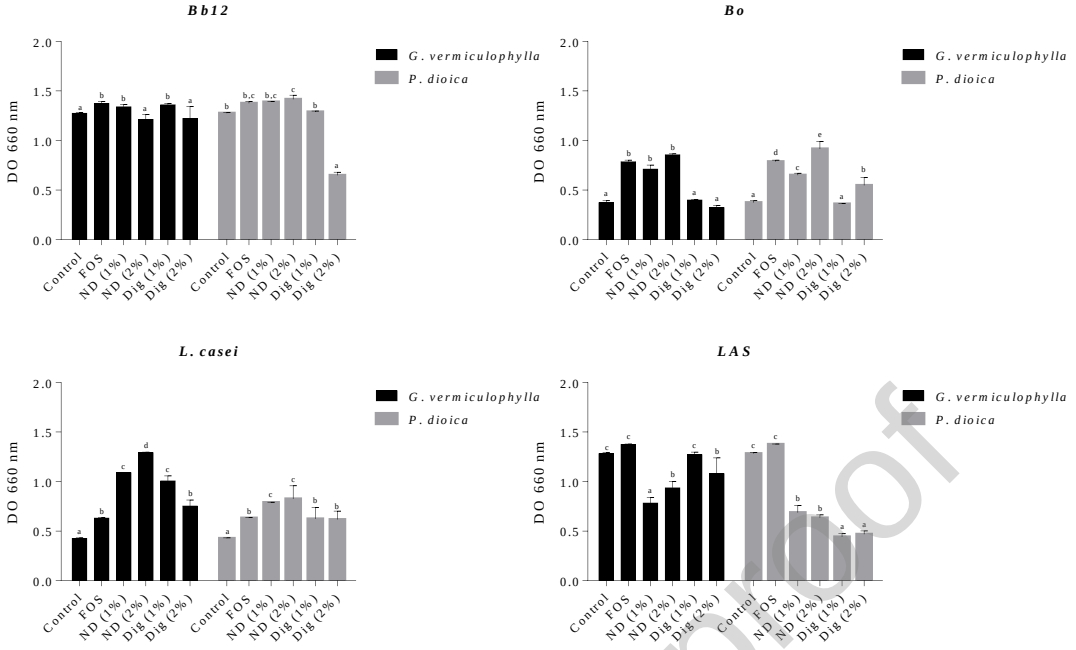

Fig. 6. Maximum growth obtained for *Bifidobacterium animalis* spp. *lactis* (Bb12), *Bifidobacterium animalis* (Bo), *Lactobacillus casei* (*L. casei*) L26 and *Lactobacillus acidophilus* Ki (LAS) strains in MRS without a sugar source (growth control), FOS (positive control), Gracilaria digested (Dig) and non-digested (ND) (1 and 2 %), and Porphyra digested and non-digested (1 and 2 %). The tests with the samples were performed in MRS without a sugar source.

Declaration of interests

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

Highlights:

- Synergetic effect of carbohydrases and proteases on four seaweeds was observed
- Combination of Viscozyme and Flavourzyme proved to be optimal for red seaweeds
- The selected extracts presented significant antioxidant and prebiotic potential