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Protein extraction from seaweed *Saccharina latissima* with deep eutectic solvents

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ABSTRACT

Interest in seaweed as a sustainable source of protein is growing, and deep eutectic solvents (DESs) are a promising green alternative with proven efficacy in protein extraction. This work studies the selective extraction of protein towards carbohydrates from the brown algae *Saccharina latissima* using DESs. Eleven DESs based on choline chloride (ChCl) and betaine were tested for freeze-dried biomass. The four DESs with the best performance (ChCl:Oxalic acid, ChCl:2Urea, ChCl:2Levulinic acid and Betaine:2Urea:Water) were subsequently used to investigate the effect of temperature (20, 30 and 40 °C) and the addition of water (0 and 47 %) on the extraction process. Betaine:2Urea:Water at 1 h, 40 °C, and 47 % added water provided the highest protein recovery yield (10.6 %) while minimizing carbohydrate extraction (1.3 %). These results demonstrated similar protein recovery yields to a benchmark process using bead milling, but with better selectivity. Thus, this work provides a sustainable extraction method for the recovery of proteins from seaweed and open new alternatives combining both approaches.

1. Introduction

In the next decades, there will be a substantial demand for protein [1], and due to environmental concerns associated with animal protein production [2], new and more sustainable alternative sources will be needed. In this sense, seaweed is a promising option to obtain proteins in an environmentally friendly way [3] since it does not require freshwater, agricultural land, or fertilizers, it does not compete for resources with land-based feed crops, and has a smaller environmental footprint than animal protein production [4]. Although the Phaeophyceae class generally has a lower protein concentration (5 to 13 % of dw) than Rhodophyta (47 % of dw) or Chlorophyta (10 to 25 % of dw) [5], the large brown kelp species (in the order Laminariales) are currently being grown in large quantities in Europe, so they are more readily available [6]. The brown algae that is the most widely cultivated in Europe is the sugar kelp *Saccharina latissima*, due to its high productivity, well-understood life cycle and the potential to produce different compounds such as alginate or fermentable sugars [7].

Albeit in a lower proportion, these algae also contain proteins that could be recovered by applying the concept of a multiproduct biorefinery. Nevertheless, one of the bottlenecks is that the proteins produced by seaweed are mainly interlinked with polysaccharides on the cell wall and within their cells. Hence, the complex nature of seaweed cell walls is the main challenge in recovering their proteins [8], where alginate accounts for half the amount of carbohydrates [9]. The classical approaches for protein extraction include aqueous, acid and alkaline solution methods, followed by centrifugation, fractionation, and enrichment steps (like ultrafiltration, precipitation or chromatography), in combination with physical cell disruption methods (osmotic shock, ultrasonication, shearing, grinding, etc.) [10]. In general, industrial technologies employ physical methods, such as bead milling or membrane filtration, as well as chemical approaches such as alkaline treatments or organic solvents, which present several drawbacks: they are expensive, time and energy consuming and, some of them, environmentally harmful [11]. Consequently, it is crucial to develop a more sustainable extracting solvent method.

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Deep eutectic solvents (DESs) are an emerging type of solvent, liquid at room temperature, formed by the combination of two or more compounds in a specific molar ratio. Generally, DESs are formed by two or more components: a hydrogen bond donor (HBD) such as urea, glycerol, water, or metal halide, and a hydrogen bond acceptor (HBA) like betaine or choline chloride [12]. These components reach a very low eutectic point as a result of the establishment of strong hydrogen-bond interactions. DESs starting materials are generally cheap and easy to produce and the individual compounds are usually apt for human consumption, as they are metabolites naturally present in living organisms. For example, choline chloride (ChCl), which is one of the most used hydrogen bond acceptors, is a member of the B vitamin family [13]. Among the increasing applications of DESs, they have been successfully used for the extraction of many macromolecules and proteins from several matrices [14]. Yet, the application of DESs to the extraction of seaweed protein is still scarce, and most of the research is focused on polysaccharides and other related macromolecules such as alginate [15] or biologically active metabolites like fucoxanthin [16].

This article discusses for the first time the suitability of using DESs to disrupt the cell wall and extract *Saccharina latissima* proteins, studying different extraction factors such as the DESs composition, temperature, and the addition of water. Full factorial experimental designs were implemented to elucidate the significant effects of the extraction factors examined and their interactions. Furthermore, by adopting a biorefinery perspective [17], the goal will be to achieve a process that maximizes protein extraction while minimizing the carbohydrates coextraction, since they are likely to be recovered in subsequent stages and to facilitate further protein purification stages. Lipids, the other major macrocomponent, represent a small portion of the dry weight of biomass and are generally not found in the same extraction fraction as proteins and carbohydrates due to their hydrophobic nature [18]. Finally, to compare the results gathered in this study with those obtained in an established benchmark process, bead milling was applied. A bead mill is a milling device designed for large-scale production that employs beads to facilitate the reduction in particle size and cell disruption [19], so it is intended to facilitate the release of biocompounds from the cell and the cell wall [20]. Still, bead milling has not been used for the extraction of macroalgae proteins. The best results obtained with the DES method were compared with those of the bead mill to test whether DESs represent a suitable alternative in terms of cell disruption leading to significant protein recovery yield and selectivity toward coextracted carbohydrates, as well as with respect to the size of the proteins obtained. Overall, the findings of this work will contribute to the growing body of research on the use of DESs to selectively extract proteins from seaweed.

2. Materials and methods

2.1. Seaweed material

Saccharina latissima was collected from Kamperland in May 2023 and provided by The Seaweed Company (Zeeland, The Netherlands). After collection, the biomass was frozen using liquid nitrogen and stored in plastic bags at −80 °C. The moisture in the frozen biomass was 90.1 %. To obtain dry biomass, frozen biomass was mechanically mixed using a blender and then it was freeze-dried in a Sublimator 2 × 3 × 3–5 (Zirbus Technology, Germany) for 46 h, and the dry pellets obtained were ground to a powder and kept in the refrigerator at 4 °C. The biomass was lyophilized because it is more convenient in terms of sample homogeneity, stability during experimentation and ease of storage. Different methods were used to determine the main components of the biomass: water and ashes [22], proteins [23,24], and lipids [25]. The carbohydrate content was derived by subtracting the sum of all from the initial results. The major components of *Saccharina latissima* (in dry mass percentage) were as follows: 10.8 % of proteins, 8.7 % of lipids, 40.2 % of ash, and 40.3 % of carbohydrates.

2.2. Chemicals

Betaine and bovine serum albumin (BSA) were purchased from ThermoScientific. Choline chloride, 1,4-butanediol, D-glucose, levulinic acid, oxalic acid, phenol, sulfuric acid and urea were obtained from Sigma-Aldrich. DL-lactic acid and glycerol were purchased from VWR International. A standard protein mix (product number 69385, 15–670 kDa) from Merck (Darmstadt, Germany) was used for calibration in size exclusion chromatography. All chemicals were used without further purification.

2.3. DES preparation

Eleven DESs were prepared by mixing the HBA with various HBDs in different molar ratios. DESs mixtures were heated to 80 °C in a water bath until a transparent liquid was obtained and then cooled to room temperature [26]. The DESs prepared with choline chloride or betaine (as HBAs) and one or two HBDs with their molar ratios and code are shown in Table 1:

Some of the DESs prepared in this study had previously been used for protein extraction (ChCl:Oxalic acid, ChCl:1,4-butanediol:water, ChCl:2Levulinic acid, ChCl:2Urea, ChCl:2Glycerol, and Betaine:2Urea:Water) in other matrices like oilseed cakes, lobster shell, bamboo shoot tips, or oats [27–31]. To the best of current knowledge, the remaining four DESs (ChCl combinations with lactic acid and Betaine:2Lactic acid) have not been used for protein recovery, although they have been applied to other extraction purposes, like the recovery of genipin from unripe *Genipa americana L.* [32]. To assess the correct DES formation, FTIR spectra of the eleven DES were recorded with a Bruker Tensor 27 spectrometer using attenuated total reflection sampling method (ATR-FTIR) and a deuterated L-alanine doped triglycine sulfate detector with a resolution of 1 cm^{-1}. The four best performing DES (see below) were further characterized by measuring their viscosities with an Anton Paar rotational viscometer (ViscoQC 300R), and the results were compared to published values when available (see Supplementary Material).

2.4. Optimization of the extraction procedure

The goal of the process was to maximize the protein extraction while achieving high selectivity towards carbohydrate coextraction, as they are the main component of *Saccharina latissima*. For that, firstly a screening of eleven DESs with different properties was carried out to make a preselection of the four best-performing DESs. After that, with those four DESs, an experimental design was implemented to study the effect of the solvent, the temperature and the occurrence of water in the biomass (if fresh biomass is used without prior drying) on the extraction of proteins from the seaweed, and to identify the optimal conditions of the factors assayed to maximize protein recovery and protein-carbohydrate separation. Full factorial designs were employed to evaluate the significant effect of the extraction parameters and their interactions. The experimental designs were completely randomized, and

Table 1
Composition and coding of the tested DESs.

DES code	Composition	Ratio
DES 1	Choline chloride:Lactic acid	1:1
DES 2	Choline chloride:Lactic acid	1:2
DES 3	Choline chloride:Lactic acid	1:3
DES 4	Choline chloride:Oxalic acid	1:1
DES 5	Choline chloride:1,4-butanediol:Water	1:1:1
DES 6	Choline chloride:Levulinic acid	1:2
DES 7	Choline chloride:Urea	1:2
DES 8	Choline chloride:Glycerol	1:2
DES 9	Betaine:Levulinic acid	1:2
DES 10	Betaine:Urea:Water	1:2:1
DES 11	Betaine:Lactic acid	1:2

each treatment was performed in duplicate.

2.4.1. Screening of DESs

The extractions were carried out in 50 mL Falcon flasks, in which adequate amounts of dry biomass and DES were mixed in a 9:1 DES: biomass ratio (w/w), as reported in other published works [33]. The mixtures were accurately weighted in an analytical balance with precision ± 0.01 mg and vortexed for 20 to 30 s to ensure homogenization. The samples were then placed in a thermostatic water bath (±0.1 °C) at 30 °C for 1 h. After extraction, the tubes were cooled to room temperature (~22 °C), centrifuged 5 min at 5000 rpm and the liquid phase was transferred to a 20 mL volumetric flask. Finally, 2 mL aliquots were filtered using cellulose acetate filters of 0.45 μm (Minisart® NML Plus) and stored in the dark at 4 °C for protein and carbohydrate quantification.

2.4.2. Effect of temperature and added water

Based on the screening results (Section 2.4.1), the four DESs that maximized protein extraction while minimizing carbohydrate coextraction were selected for further testing. The impact of different temperatures and water content levels on extraction efficiency was analyzed. The experimental setup was analogue to the one described in Section 2.4.1 but tuning the temperature and added water levels accordingly. For the added water content, two levels were tested: 0 % (no water addition) and 47 % of added water referred to the total mass of suspension. While the level 0 % water represents the extraction of dry biomass with pure DES, the 47 % level was chosen to mimic the water concentration present in the biomass-solvent mixture when using fresh biomass (90 g of water per 100 g of fresh biomass). These two levels were assayed to check if the process could be applied to fresh biomass without compromising the extraction performance of the DES. This concentration of water allows to keep the integrity of DESs, as they are supposed to lose their hydrogen bond structure when the water content exceeds 50 % [34]. The FTIR spectra of the four mixtures of DES with 47 % water were also registered to check whether hydrogen bonds are still present in the solvent systems. Regarding temperature, three levels were examined: 20 °C to determine if the recovery yield decreases at room temperature, 40 °C to investigate if higher temperatures enhance extraction without compromising protein integrity, and 30 °C to evaluate the existence of quadratic effects. Control experiments were also conducted using only water as the solvent in the same 9:1 mass ratio, resulting in five levels for the solvent factor. Extra water was added for 47 % added water experiments.

2.5. Bead milling as a benchmarked cell disruption step

To compare DES extractions with a benchmark process, bead milling was used. From frozen biomass, a suspension in water of 25 g L^{-1} was prepared and subjected for 5 min to a high-speed blender, UltraTurrax®, T 50 basic (IKA – LABORTECHNIK, Germany) at 8800 rpm. After that, a portion was introduced into a horizontal stirred mill bead mill (Dyno-Mill Research Lab, Willy A. Bachofen AF Maschinenfabrik, Switzerland) operated in batch recirculation mode. The equipment comprises a 79.6 mL milling chamber filled with 70 % (v/v) and Yttrium-stabilized Ø 0.3 mm beads. A sieve plate of 0.13 at the milling chamber outlet separates the macroalgal suspension. The heat from the collisions of beads was dissipated by an integrated cooling jacket and coil, maintaining the temperature below 30 °C to prevent protein denaturation [30]. The bead milling of the liquid suspension was carried out during 30 min at 4000 rpm. Finally, the suspension was centrifuged and the supernatant transferred to a volumetric flask for protein and carbohydrate determination.

2.6. Determination of proteins and carbohydrates by UV spectrometry

Established spectrophotometric methods were employed to assess the protein and carbohydrate content of the filtered extracts. To determine protein content, the Bradford method was followed using Merck's 96 microplate well assay protocol [35]. The total carbohydrate content was measured using the modified phenol–sulfuric acid method adapted to 96-microplate wells [36]. The equipment used for the absorbance measurements was a Tecan Infinite 200 PRO microplate reader. All measurements were made in duplicate. The two response variables monitored were expressed as protein recovery yield (PRY) and the carbohydrates recovery yield (CRY), both as a dry weight percentage and calculated using Eq. (1) and (2):

$$PRY = \frac{V_T \cdot c_P}{m_0 \cdot P} \cdot 100 \quad (1)$$

$$CRY = \frac{V_T \cdot c_C}{m_0 \cdot C} \cdot 100 \quad (2)$$

where m_0 is the initial amount of dry biomass (in mg), V_T is the total volume of the extracted solution (in L), c_P and c_C are the measured concentrations in the liquid fraction of proteins and carbohydrates, respectively (in mg L^{-1}) and P and C are the mass fraction of proteins and carbohydrates in the initial biomass, respectively.

Due to the structure and chemical properties of DESs, it is known that they interact with some spectrophotometric methods [37,38], so they could be a source of systematic error. Thus, the matrix effect was assessed for the 11 DESs adding glucose (650 mg L^{-1}) and BSA (450 mg L^{-1}) to DESs solutions (90 mg of DES per mL of solution). The relative bias in the estimation of carbohydrate and protein content was less than 10 % for both parameters in the 11 DESs tested.

2.7. Statistical analysis

The results of the experiments were evaluated using analysis of variance (ANOVA) to identify factors with a significant effect on the protein and carbohydrate recovery yields. Tukey's HSD *post hoc* test was used to find the optimum levels of the factors studied. The compact letter display (cld) methodology was used for a simple presentation of the results of multiple comparisons [39]. Data are presented as mean ± standard deviation (SD). Experiments were carried out in duplicate, since a good balance between the experimental cost and the number of residual degrees of freedom is needed to have an adequate power test. Software R (4.3.2 version) was used to perform the statistical analysis and generate the graphs of this work. A significance level of 5 % was used in all statistical calculations.

2.8. Molecular size profiles of extracted proteins

The protein extracts of the experimental design described in Section 2.4.2 and Section 2.5 were analyzed by size exclusion chromatography (SEC), using HPLC (Infinity 1290, Agilent Technologies) equipped with an Advance Bio SEC column, 300 pore size, 2.7 μm particles (300 mm x 7.8 mm). Injection volumes of 20 μL of each sample were analyzed with UV detection at 280 nm. The isocratic elution mode was applied using a mobile phase composed of 0.1 M sodium phosphate, 0.1 M sodium sulfate and 0.05 % sodium azide, with the chromatographic column thermostatted at 30 °C. The apparent molecular weights of the major protein fractions were estimated by interpolation on a calibration curve prepared from the standard protein mix described in Section 2.2. The concentration (g L^{-1}), molecular weight (kDa), and retention times (min) of the components of the standard solution were: thyroglobulin bovine (0.5, 670, 6.499), γ-globulins from bovine blood (1, 150, 8.333), ovalbumin (Albumin chicken egg grade V) (1.0, 44.3, 10.282); Ribonuclease A type I-A from bovine pancreas (1, 13.7, 11.493); p-aminobenzoic acid (pABA) (0.01, 0.137, 15.250). Calibration was established by plotting the decimal logarithm of the molecular weight (MW) of a standard solute against the respective retention time using the same

conditions according to a third-order polynomial model [40]. From the chromatograms, a mass percent of the protein range was estimated with Eq. (3) by averaging the area of the peaks of independent injections ($n = 2$).

$$RA_j(\%) = \frac{\sum A_{ij}}{\sum A_i} \cdot 100 \quad (3)$$

where RA_j is the relative abundance of the protein size category j, A_{ij} are the SEC peaks found in the j molecular weight range, and A_i are all the SEC peaks within the calibration range. The three protein size categories considered in this work were: large proteins (650–100 kDa), medium proteins (99.9–10.0 kDa), and polypeptides (9.99–0.15 kDa).

3. Results and discussion

3.1. Screening of DESs

The recovery yields of proteins (PRY) and carbohydrates (CRY) for each DES are shown in Fig. 1.

An ANOVA of the experimental results indicated that the DES factor was significant for both responses ($p - value < 0.05$). The maximum PRY value (8.76 ± 0.05 %) was achieved using DES 10 (Betaine:2Urea: Water), although no significant differences were detected when compared to DES 7 (ChCl:2Urea). The minimum value of CRY (3.57 ± 0.34 %) was found with DES 9 (Betaine:2Levulinic acid), but similar yields were achieved with DES 4 (ChCl:Oxalic acid), DES 6 (ChCl:2Levulinic acid), and DES 10. If the goal was to maximize the carbohydrates recovery, DESs based on lactic acid (DES 1, DES 2, DES 3, and DES 11) provided the highest CRY. Attending to the data, DES 4, DES 6, DES 7, DES 9, and DES 10 (Betaine:2Urea:Water) were the ones that showed higher PRY than CRY. However, the PRY for DES 9 was significantly lower than for the others (letter *d* versus *bc*, *c*, *ab* and *a*).

Apparently, these DESs do not have much in common. For instance, DES 4 and DES 6 are acidic, whereas DES 7 and DES 10 are alkaline. Notably, although only two alkaline DESs were examined, both were identified as among the most effective DESs, despite their distinct hydrogen bond acceptors (one comprising choline chloride and the other incorporating betaine). Contrary to claims in other studies that high viscosity of DES hinders mass transfer, viscosity seems not to play a significant role in our experiments. For example, DES 4 presents a considerably higher viscosity than DES 6 [41,42] (see Supplementary Material), yet its performance was significantly better. DES 4 and DES 6 were chosen not due to their high PRY, which was significantly lower than DES 7 or DES 10, but because they exhibited a lower recovery of carbohydrates compared to the other solvents studied. Thus, DES 4, DES 6, DES 7, and DES 10 were selected to evaluate the effect of temperature and the presence of water if fresh instead dry seaweed is used as starting biomass.

3.2. Effect of temperature and added water

The PRY and CRY values for the different levels of solvents (DES 4, DES 6, DES 7, DES 10, and water) at three different temperatures and two levels of added water (no added water simulating dry biomass-DES mixtures; 47 % water simulating fresh biomass-DES mixtures) are presented in Fig. 2a and b, respectively. An ANOVA of the results from the experimental design (see Supplementary Material) revealed that for PRY and CRY the three assayed factors were statistically significant ($p - value < 0.05$). Regarding the interactions, in the case of PRY only the solvent:temperature interaction was weakly significant ($p - value = 0.025$), while for CRY all interactions were strongly significant ($p - value < 0.001$). In terms of factor and interaction contribution, for PRY, solvent and temperature contribution to total variance accounted for 49.0 % and 35.3 %, respectively, indicating that the influence of added water (3.64 %), although significant, is less important.

From the mean plot results and the Tukey HSD *post hoc* test (Fig. 3a and 3b), it can be concluded that DES 10 yielded the highest PRY, while the worst solvents for protein recovery were DES 6 and pure water, without significant differences between them, indicating that these solvents have a similar affinity and behavior toward proteins. Concerning CRY, DES 4 and DES 10 provided the lowest values, while the highest results were achieved using DES 6. Thus, DES 10 maximized the selectivity towards proteins, but DES 6 and water presented the opposite behavior. For its part, the use of temperature at 40 °C did also account for higher PRY compared to 30 or 20 °C, but higher temperatures reduced the carbohydrates yield. In both responses, the linear effect prevails over the quadratic effect for the temperature factor (see Fig. 3). This indicates that for PRY higher temperatures could lead to higher yields. However, temperatures over 40 °C were not assayed to prevent protein denaturation. Finally, the 47 % of added water gave slightly larger PRY and CRY, although its effect was much less significant than that of temperature and DES. Due to statistically significant interactions,

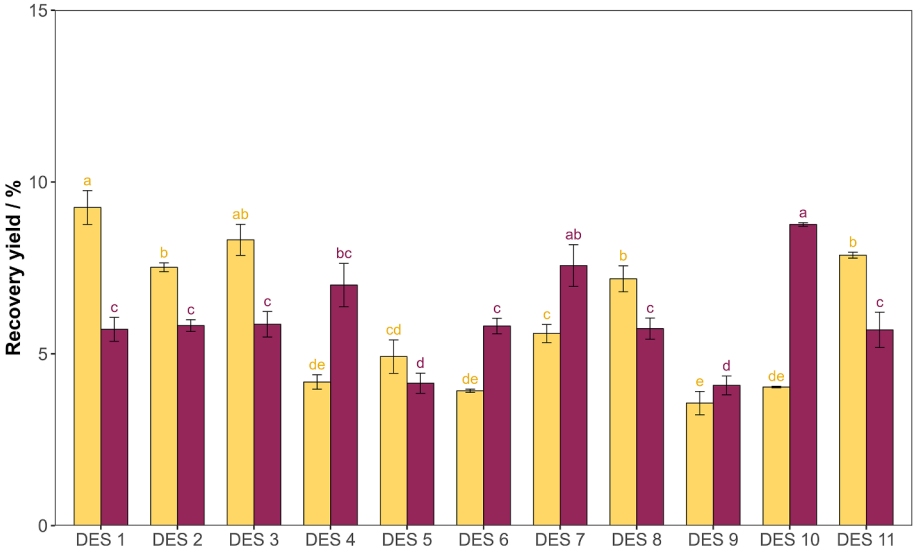

Fig. 1. Effect of DESs on the recovery yield of carbohydrates (yellow) and proteins (magenta). Experimental data are shown as mean ± SD (n = 2). The letters on top of the error bars are displayed according to the compact letter display methodology using the Tukeý*s post hoc* test. Means with a common letter and the same color are not significantly different. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

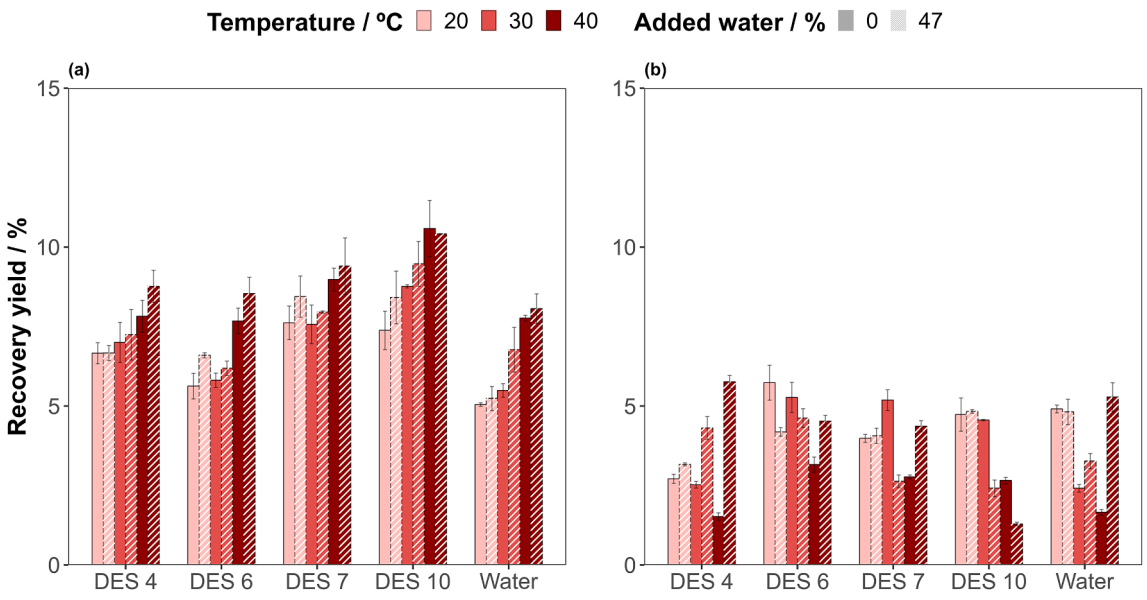

Fig. 2. Effect of solvent (x-axis), temperature (different colors), and water (different patterns) on the recovery yield of (a) proteins and (b) carbohydrates. Experimental data are shown as mean ± SD (n = 2).

to clarify the influence of added water and temperature on each solvent, two-factor ANOVAs were computed individually for each solvent (see Supplementary Material). The Tukey HSD *post hoc* test demonstrated that there were no significant differences in PRY between the levels of added water when looking for each solvent. However, for CRY, significant differences were observed with no clear pattern. No significant differences were found among the three temperatures tested with DES 4, DES 7 and DES 10, but water and DES 6 at 40 °C provided significantly higher PRY.

The maximum observed PRY of 10.6 ± 0.9 % was achieved using DES 10 without added water at 40 °C. This indicates that DES 10 is more effective in extracting proteins than the other solvents tested under the specific conditions studied. There were no significant differences in PRY across different temperatures and water content levels, except at 20 °C with 0 % added water, which resulted in a lower protein recovery. When compared to other solvents used, DES 10 and DES 7 (both urea-based DES) showed the highest PRY values, indicating that an alkaline pH favors protein extraction. Proteins are generally more soluble under alkaline conditions, which likely explains this observation [43]. The relatively low PRY values (below 20 %) could be attributed to the presence of alginate in *Saccharina latissima*, as alginate can bind water or DES, rendering them unavailable to dissolve proteins [44].

Regarding CRY, the lowest value of 1.29 ± 0.06 % was obtained using DES 10 at 40 °C with 47 % added water (fresh biomass). This suggests that DES 10 is selective for protein extraction under these conditions, achieving high selectivity with an eightfold higher protein extraction compared to carbohydrate recovery. Additionally, the presence of added water improves the DESs handling by reducing notably its viscosity (see Supplementary Material). When water alone was used as solvent, the lowest PRY values were observed (5.05 ± 0.06 %), and water coextracted more carbohydrates than DES 4 and DES 10, but less than DES 6 and DES 7. In view of the results, it can be concluded that water is less selective and efficient than DES 7 and DES 10. The behavior of CRY is more complex due to significant interaction effects, with overall interaction contribution exceeding 73 % compared to 24 % for all factor contributions (see Supplementary Material). Consequently, no clear trend or set of factor levels reliably predicts its low performance. Nevertheless, DES 6 exhibited a higher affinity for carbohydrates than other DESs, as also observed in Section 3.1, where lactic acid-based DESs had the highest CRY (Fig. 1). Therefore, DES 6 would be the best option from the ones studied to recover carbohydrates.

Overall, DES 10 emerged as the most effective solvent for selective protein extraction under the tested conditions, particularly at 40 °C with 47 % added water (fresh biomass). Its high selectivity and handling benefits make it a promising choice for protein extraction from *Saccharina latissima*. In contrast, DES 6 showed a higher carbohydrate affinity, indicating its potential use as extracting agent for this biomolecules.

Since no studies have been published to date dealing with protein extraction from seaweed using DES, the results of this work were compared to other methodologies followed so far, such as enzyme-assisted extraction or alkaline extraction (Table 2). For example, Kulshreshtha et al. extracted 2.9 % protein from *Codium fragile* through enzyme-assisted extraction using neutrase, and 1.4 % using osmotic shock [45]. Regarding the study of protein extraction from *Saccharina lattisima,* Trigo et al. tried other extraction methods, such as blanching and soaking after pH-shift, achieving a maximum protein recovery yield of 20.6 ± 0.1 % using pH-shift with dialysis of supernatant at pH 12 with freeze-thawing [46]. This result is higher than the maximum obtained in this work, but the other combinations assayed by these authors provided comparatively lower yields, such as when using a pH shift and no freeze-thawing (7.7 ± 1.8 %). Moreover, the carbohydrates were not quantified in the work by Trigo et al., so the selectivity of the process is unknown.

In case purification is required, separation of the carbohydrates from the proteins could be done applying different approaches, such as membrane separation methods [48,49], capillary electrophoresis [50], solid-phase extraction [51], or chromatographic techniques [52–54].

3.3. Size of the extracted proteins

The molecular weight of the protein/peptides extracted using the five assayed solvents (DES 4, DES 6, DES 7, DES 10 and water) were estimated from HPLC-SEC measurements. The relative abundance of each size category and its variation with temperature is shown in Fig. 4a. Samples with different levels of added water were also measured, but no significant differences between 0 and 47 % were found.

For DES 7, DES 10 and water, the protein size distribution was not altered when temperature increased, being the small fraction (sizes smaller than 9.9 kDa) the most abundant. However, for DES 4 and DES 6 a larger fraction of medium and large proteins was obtained for extractions at 30 °C. In the case of DES 6, the 650–100 kDa fraction accounted for the 11 %, while for DES 4 it grew to 24 %, although a

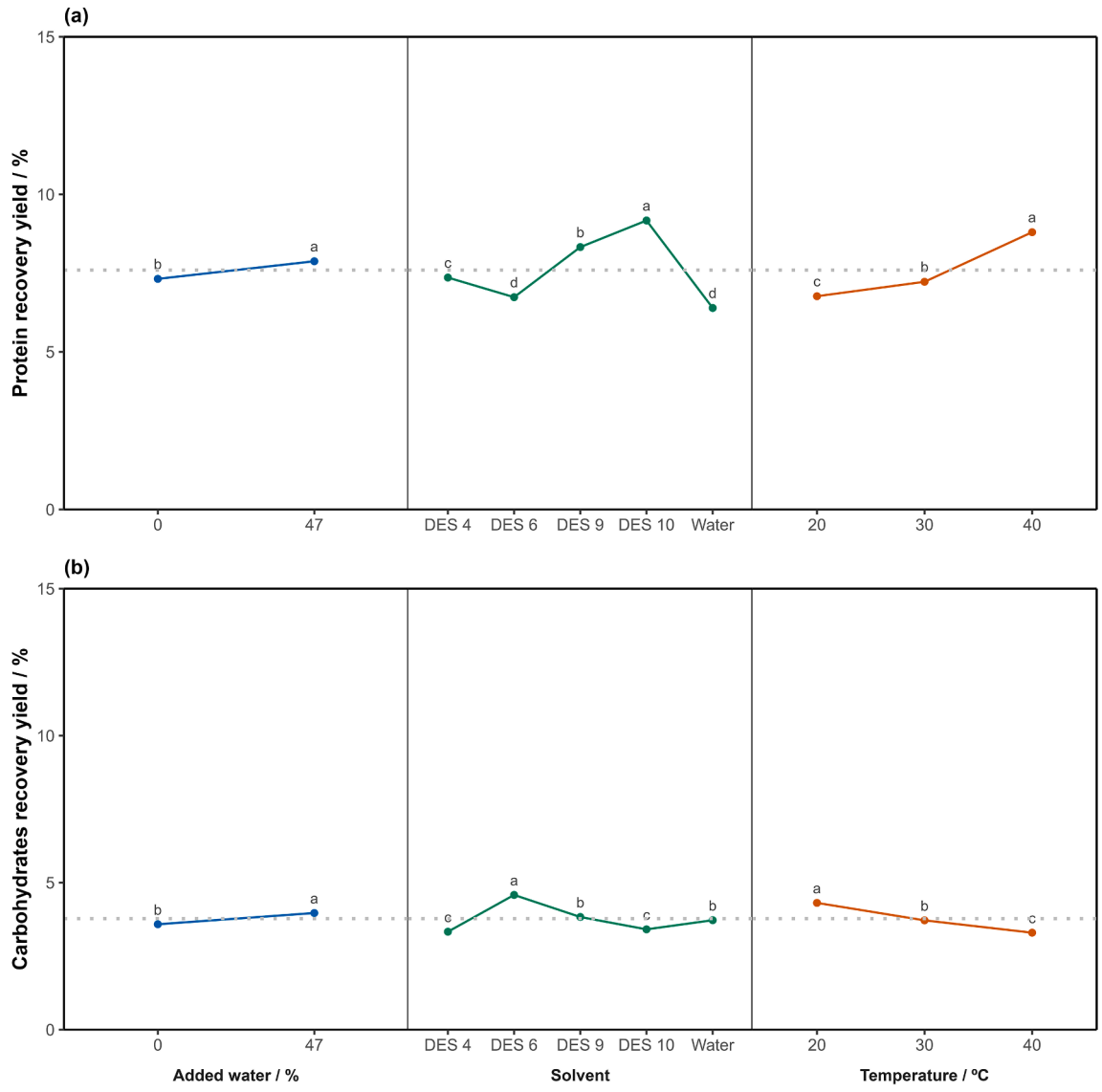

Fig. 3. Mean plots for the different levels of added water, solvent, and temperature for (a) proteins and (b) carbohydrates. The letters on top of each point are displayed according to the compact letter display methodology using Tukey's post hoc test. Means with a common letter within the same factor are not significantly different. The horizontal dotted line indicates the great mean of the response.

Table 2
Protein recovery yields from different macroalgae using different methods.

Seaweed	Extraction methodology	Protein recovery yield / %	Reference
Codium fragile	Enzyme-assisted extraction	2.9	[45]
	Osmotic shock	1.4	
Saccharina latissima	pH shift with freeze-thawing	20.6	[46]
	pH shift without freeze-thawing	7.7	
	DES 10	10.6	This work

noticeable protein fraction was also detected in the range of 10–99.9 (17 %). This could be explained by the fact that temperature aids in the formation of protein aggregates, as it promotes hydrophobic interactions between proteins [55]. Nonetheless, higher temperatures might also disrupt those interactions, as results were not obtained at 40 °C, indicating that 30 °C is the optimal temperature for the conditions tested in this work.

These results suggest that DES could affect the protein structure, thereby reducing its size. Nonetheless, this is not in agreement with other authors who have pointed out the potential of DESs to stabilize proteins, and in particular Betaine:Urea-based DESs [56]. Furthermore, Trigo et al. studied the protein size of unprocessed *Saccharina latissima*, consisting of 7 % proteins larger than 670 kDa, 5 % in the range of 670–100 kDa, 3 % in 10–100 kDa, the major fraction consisted in proteins within the range of 5–10 kDa [46]. Thus, with DES extraction, only small-medium protein/peptides are being recovered because large proteins were not readily available from the beginning. This is in connection with the fact that the cell wall of brown algae is generally composed of sulfated fucans and alginates (≈ 45 % of dw), cellulose (1–8 % of dw), but a small portion of proteins and phenolic compounds [57]. Therefore, if the cell wall is not disrupted, it would be difficult to obtain large proteins, which are generally interlinked within the cell wall with carbohydrates or lipids, providing a rigid structure to cells [58]. However, obtaining polypeptides from seaweed could also be a source of interest. For instance, carnosine and glutathione, two antioxidant peptides commonly present in animals, have also been found in seaweed [59].

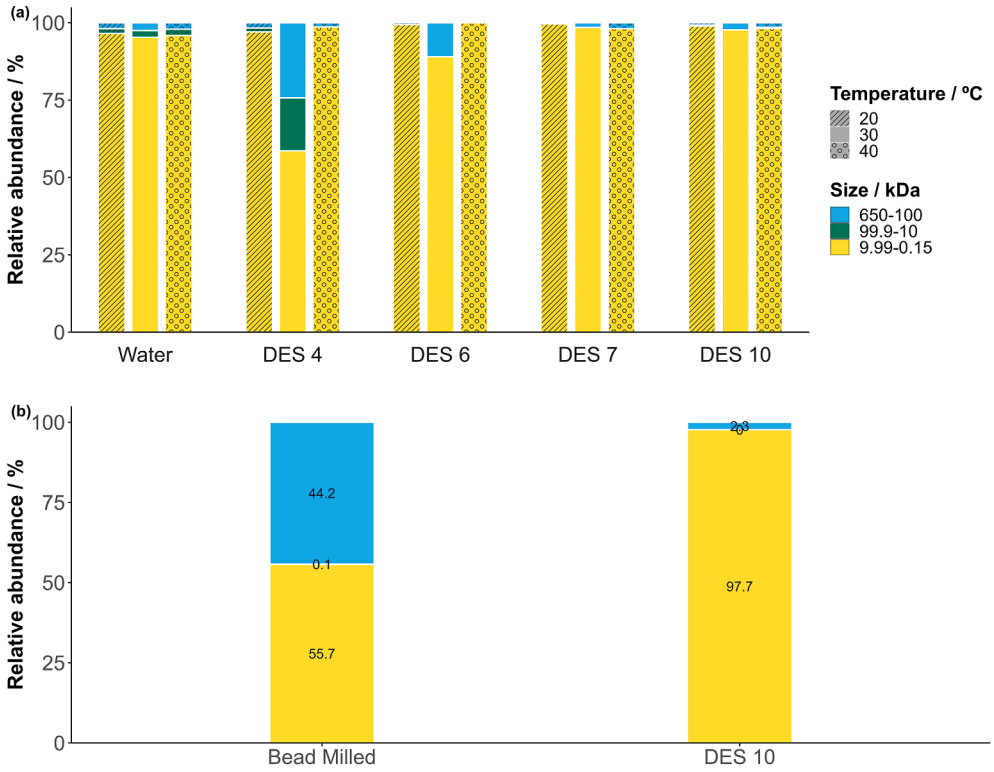

Fig. 4. Relative abundance of proteins in liquid extracts according to their apparent molecular weight range for (a) the extracts with the five solvents assayed in the optimization design at the three temperatures tested, and (b) bead milling versus DES 10 (at 30 °C). The three molecular size intervals are indicated by different colors, while the different temperature values have distinct patterns.

Still, more efforts must be made to analyze the peptides and bioactive compounds that can be derived from *Saccharina latissima*.

3.4. Bead milling benchmark comparison

The bead milling results were compared to those of DESs extraction using DES 10. As bead milling was used with a maximum operating temperature of 30 °C, the DES results used for comparison were those carried out at the same temperature of 30 °C. Interestingly, there were no significant differences between their PRY values at the 0.05 significance level (10.62 vs 9.47 %). However, the carbohydrates obtained by bead milling were almost threefold the amount recovered with the DES (6.38 vs 2.41 %). Thus, the benchmark process exhibited a lower selectivity for protein extraction compared to DES extraction, and even a lower compared to the optimal conditions found with DES 10 at 40 °C (1.29 % carbohydrate extraction). No studies have been conducted regarding seaweed protein extraction using bead milling, although Firdayanti et al. studied the extraction of carrageenan from *Kappaphycopsis cottonii* [60]. However, the bead mill has been widely applied in microalgae protein recocovery, proving to be efficient with respect to not only the quantity but also the size of the recovered proteins. For example, Mear et al. recovered between 11 and 32 % of the initial proteins from *Traselmis chui* [61]. For their part, Schwenzfeier et al. reported a protein yield of 21 % using the green microalgae *Tetraselmis sp* [62]. These values are considerably larger than those obtained in the present work, although it is worth mentioning that the goal of those papers was to find the best conditions to bead mill the biomass. Moreover, the cell wall composition of the microalgae is different from that of seaweed. Regarding protein sizes when using bead milling, larger protein fractions were obtained compared to the use of DES 10 (Fig. 4b), indicating that cell disruption was more efficient when using the bead mill, as larger proteins were released (almost half of the proteins have sizes greater than 100 kDa). This is supported by Alavijeh et al., who found protein sizes greater than 10 kDa when applying bead milling to *Chlorella vulgaris* [20].

When contrasting the bead milling with the steps of the DES extraction process, which are schematized in Fig. 5, the DES approach stands out as a much simpler alternative, because even though both processes share the first and final stages (blending and centrifugation), the bead milling requires an additional step. Moreover, the extraction process using DES could be further simplified by removing step A.2, when freshly collected biomass is directly extracted. In addition, reducing the particle size using Ultra-Turrax is needed, and the bead milling itself is more energy-consuming than simply using DESs, as the biomass could be sun-dried without additional energetic costs [21]. Thus, the DES extraction methodology using freeze-dried biomass can provide similar protein recovery yields while reducing the carbohydrates extracted, although the protein size is compromised. These findings suggest that an interesting approach could be to use the bead mill with an aqueous DES solution. This could have a cooperative effect on the protein extraction process and could allow not only to obtain higher protein recovery yields than the ones found in this work, but also with a larger portion of medium and large proteins while maintaining the selectivity of the process. Other alternatives for future work could be studying the DES extraction under alkaline conditions (for example, adding NaOH), as well as combining it with bead milling. Finally, since this work has utilized freeze-dried biomass, it would be advisable to verify whether similar results are obtained using sun-dried biomass as well as directly employing fresh biomass, to reduce the energy consumption associated to the drying step.

4. Conclusions

DES extraction was explored to recover proteins from lyophilized biomass of *Saccharina latissima*. Different DESs, temperatures, and added water levels were investigated to evaluate their influence on selective

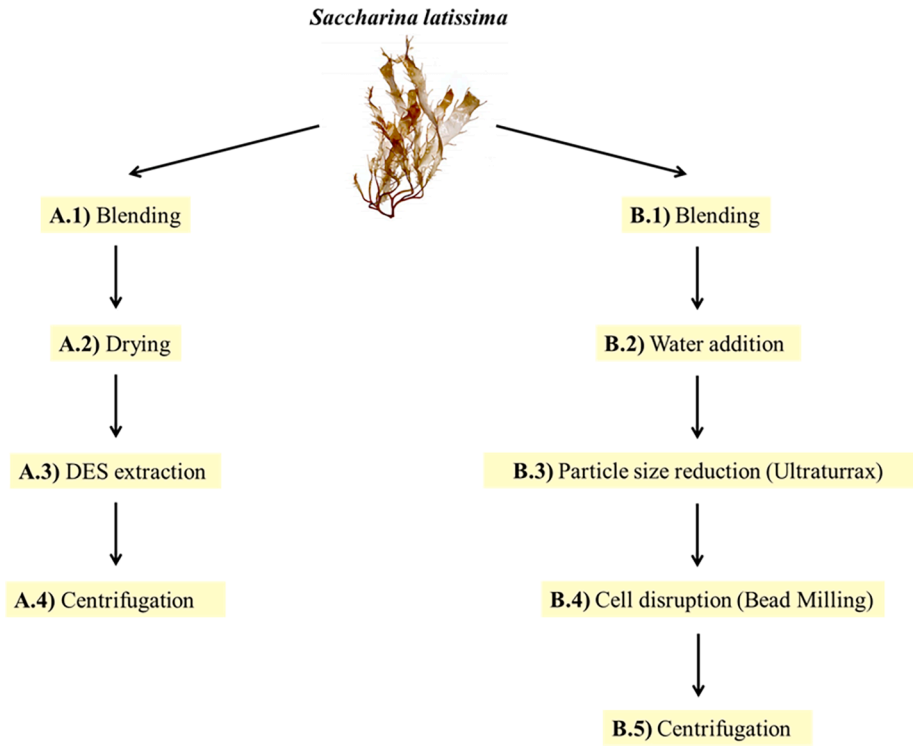

Fig. 5. Schematic comparison of the main steps for the DES extraction process (A) and the bead mill procedure (B) to obtain a protein rich liquid extract.

and efficient protein recovery. DES 10 (Betaine:2Urea:Water) at 40 °C yielded the highest protein recovery (10.6 %) with minimal carbohydrate coextraction (1.29 %). Added water factor was not significant. Compared to bead milling procedure, extraction with DES reduced protein sizes and improved selectivity, while the protein recovery yields were comparable. Therefore, the DES extraction method offers potential for selective protein recovery under mild conditions, minimizing energy consumption, and avoiding toxic chemicals. Future research should explore combining DES with bead milling to improve yield and protein sizes.

CRediT authorship contribution statement

David Moldes: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Patricia F. Requejo:** Writing – review & editing, Supervision. **Marisol Vega:** Writing – review & editing, Supervision, Funding acquisition. **Silvia Bolado:** Writing – review & editing, Supervision, Funding acquisition. **René H. Wijffels:** Writing – review & editing. **Antoinette Kazbar:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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References

[1] H. Aiking, J. de Boer, The next protein transition, Trends Food Sci Technol 105 (2020), https://doi.org/10.1016/j.tifs.2018.07.008.
[2] J. Poore, T. Nemecek, Reducing food's environmental impacts through producers and consumers, Science 360 (2018) (1979) 987–992, https://doi.org/10.1126/science.aaq0216.
[3] G. de Souza Celente, Y. Sui, P. Acharya, Seaweed as an alternative protein source: prospective protein extraction technologies, Innov. Food Sci. Emerg. Technol. 86 (2023) 103374, https://doi.org/10.1016/j.ifset.2023.103374.
[4] E. Röös, B. Bajželj, P. Smith, M. Patel, D. Little, T. Garnett, Protein futures for Western Europe: potential land use and climate impacts in 2050, Reg Environ Change 17 (2017) 367–377, https://doi.org/10.1007/s10113-016-1013-4.
[5] J. Fleurence, M. Morançais, J. Dumay, Seaweed proteins, in: Proteins in Food Processing, Second Edition, 2018: pp. 245–262. https://doi.org/10.1016/B978-0-08-100722-8.00010-3.
[6] U.G. Bak, C.W. Nielsen, G.S. Marinho, Ó. Gregersen, R. Jónsdóttir, S.L. Holdt, The seasonal variation in nitrogen, amino acid, protein and nitrogen-to-protein conversion factors of commercially cultivated Faroese Saccharina latissima, Algal Res 42 (2019) 101576, https://doi.org/10.1016/j.algal.2019.101576.
[7] S. Forbord, J. Skjermo, J. Arff, A. Handå, K.I. Reitan, R. Bjerregaard, K. Lüning, Development of Saccharina latissima (Phaeophyceae) kelp hatcheries with year-round production of zoospores and juvenile sporophytes on culture ropes for kelp aquaculture, J Appl Phycol 24 (2012) 393–399, https://doi.org/10.1007/s10811-011-9784-y.
[8] M. Gordalina, H.M. Pinheiro, M. Mateus, M.M.R. da Fonseca, M.T. Cesário, Macroalgae as protein sources—a review on protein bioactivity, extraction, purification and characterization, Applied Sciences (switzerland) 11 (2021) 7969, https://doi.org/10.3390/app11177969.
[9] M. Sterner, U. Edlund, Multicomponent fractionation of Saccharina latissima brown algae using chelating salt solutions, J Appl Phycol 28 (2016) 2561–2574, https://doi.org/10.1007/s10811-015-0785-0.

[10] S.U. Kadam, B.K. Tiwari, C.P. O'Donnell, Application of novel extraction technologies for bioactives from marine algae, J Agric Food Chem 61 (2013) 4667–4675, https://doi.org/10.1021/jf400819p.
[11] M. Garcia-Vaquero, G. Rajauria, B. Tiwari, Conventional extraction techniques: Solvent extraction, in: Sustainable Seaweed Technologies: Cultivation, Biorefinery, and Applications, 2020: pp. 171–189. https://doi.org/10.1016/B978-0-12-817943-7.00006-8.
[12] E.L. Smith, A.P. Abbott, K.S. Ryder, Deep eutectic solvents (DESs) and their applications, Chem Rev 114 (2014) 11060–11082.
[13] F. Saadatpour, F. Mohammadipanah, Enhancement of bactericidal effect of Chlorhexidine using choline augmentation as a natural additive, Am J Infect Control 50 (2022) 39–48, https://doi.org/10.1016/j.ajic.2021.05.012.
[14] Q. Zeng, Y. Wang, Y. Huang, X. Ding, J. Chen, K. Xu, Deep eutectic solvents as novel extraction media for protein partitioning, Analyst 139 (2014) 2565–2573, https://doi.org/10.1039/C3AN02235H.
[15] P.S. Saravana, Y.N. Cho, H.C. Woo, B.S. Chun, Green and efficient extraction of polysaccharides from brown seaweed by adding deep eutectic solvent in subcritical water hydrolysis, J Clean Prod 198 (2018) 1474–1484, https://doi.org/10.1016/j.jclepro.2018.07.151.
[16] E.D. Obluchinskaya, O.N. Pozharitskaya, L.V. Zakharova, A.V. Daurtseva, E. V. Flisyuk, A.N. Shikov, Efficacy of natural deep eutectic solvents for extraction of hydrophilic and lipophilic compounds from fucus vesiculosus, Molecules 26 (2021) 4198, https://doi.org/10.3390/molecules26144198.
[17] K.A. Jung, S.R. Lim, Y. Kim, J.M. Park, Potentials of macroalgae as feedstocks for biorefinery, Bioresour Technol 135 (2013) 182–190, https://doi.org/10.1016/j.biortech.2012.10.025.
[18] M.S. Prabhu, A. Israel, R.R. Palatnik, D. Zilberman, A. Golberg, Integrated biorefinery process for sustainable fractionation of Ulva ohnoi (Chlorophyta): process optimization and revenue analysis, J Appl Phycol 32 (2020) 2271–2282, https://doi.org/10.1007/s10811-020-02044-0.
[19] H. Schütte, M.-R. Kula, Bead Mill Disruption, Separation Processes in Biotechnol. (2020), https://doi.org/10.1201/9781003066392-7.
[20] R.S. Alavijeh, K. Karimi, R.H. Wijffels, C. van den Berg, M. Eppink, Combined bead milling and enzymatic hydrolysis for efficient fractionation of lipids, proteins, and carbohydrates of Chlorella vulgaris microalgae, Bioresour Technol 309 (2020) 123321, https://doi.org/10.1016/j.biortech.2020.123321.
[21] A. Santiago, R. Moreira, Drying of edible seaweeds, Sustainable Seaweed Technologies: Cultivation, Biorefinery, and Applications (2020) 131–154. https://doi.org/10.1016/B978-0-12-817943-7.00004-4.
[22] Official Methods of Analysis, in: Official Methods of Analysis of AOAC INTERNATIONAL, 2023. https://doi.org/10.1093/9780197610145.002.001.
[23] K.C. Rhee, Determination of total nitrogen, Curr. Protocol Food Anal. Chem. 00 (2001), https://doi.org/10.1002/0471142913.fab0102s00.
[24] A.R. Angell, L. Mata, R. de Nys, N.A. Paul, The protein content of seaweeds: a universal nitrogen-to-protein conversion factor of five, J Appl Phycol 28 (2016), https://doi.org/10.1007/s10811-015-0650-1.
[25] J. Folch, M. Lees, G.H. Sloane Stanley, A simple method for the isolation and purification of total lipides from animal tissues, J Biol Chem 226 (1957), https://doi.org/10.1016/s0021-9258(18)64849-5.
[26] A.P. Abbott, G. Capper, D.L. Davies, R.K. Rasheed, V. Tambyrajah, Novel solvent properties of choline chloride/urea mixtures, Chem. Commun. (2003) 70–71, https://doi.org/10.1039/B210714G.
[27] A. Grudniewska, E.M. De Melo, A. Chan, R. Gniłka, F. Boratyński, A.S. Matharu, Enhanced protein extraction from oilseed cakes using glycerol-choline chloride deep eutectic solvents: a biorefinery approach, ACS Sustain Chem Eng 6 (2018) 15791–15800, https://doi.org/10.1021/acssuschemeng.8b04359.
[28] S. Hong, Y. Yuan, Q. Yang, P. Zhu, H. Lian, Versatile acid base sustainable solvent for fast extraction of various molecular weight chitin from lobster shell, Carbohydr Polym 201 (2018) 211–217, https://doi.org/10.1016/j.carbpol.2018.08.059.
[29] N. Li, Y. Wang, K. Xu, Y. Huang, Q. Wen, X. Ding, Development of green betaine-based deep eutectic solvent aqueous two-phase system for the extraction of protein, Talanta 152 (2016) 23–32.
[30] A. Patra, V. Arun Prasath, R. Pandiselvam, Deep eutectic solvent: an emerging trend for extraction of plant proteins, J Mol Liq 389 (2023) 122887, https://doi.org/10.1016/j.molliq.2023.122887.
[31] J. Yue, Z. Zhu, J. Yi, Y. Lan, B. Chen, J. Rao, Structure and functionality of oat protein extracted by choline chloride-dihydric alcohol deep eutectic solvent and its water binary mixtures, Food Hydrocoll 112 (2021) 106330, https://doi.org/10.1016/j.foodhyd.2020.106330.
[32] M. Isabel Landim Neves, B. Socas-Rodríguez, A. Valdés, E. Keven Silva, A. Cifuentes, M. Angela, A. Meireles, E. Ibáñez, Synergic effect of natural deep eutectic solvent and high-intensity ultrasound on obtaining a ready-to-use genipin extract: crosslinking and anti-neurodegenerative properties, Food Chem X 16 (2022) 100489, https://doi.org/10.1016/j.fochx.2022.100489.
[33] S. Ali, D. Ahmed, Comparing DES-mediated ultrasound- and heat-assisted extraction of bioactive metabolites from Saussurea lappa and optimization by RSM and validation studies, Green Analyt. Chem. 7 (2023), https://doi.org/10.1016/j.greeac.2023.100080.
[34] P.J. Smith, C.B. Arroyo, F. Lopez Hernandez, J.C. Goeltz, Ternary deep eutectic solvent behavior of water and urea choline chloride mixtures, J. Phys. Chem. B 123 (2019) 5302–5306, https://doi.org/10.1021/acs.jpcb.8b12322.
[35] A. Jain, Protein measurement using bradford assay and photopette ®, Tip Biosystems (2017) 1–3.
[36] W. Chen, L. Gao, L. Song, M. Sommerfeld, Q. Hu, An improved phenol-sulfuric acid method for the quantitative measurement of total carbohydrates in algal biomass, Algal Res 70 (2023) 102986, https://doi.org/10.1016/j.algal.2023.102986.
[37] A. Elik, N. Altunay, Chemometric approach for the spectrophotometric determination of chloramphenicol in various food matrices: using natural deep eutectic solvents, Spectrochim Acta A Mol Biomol Spectrosc 276 (2022), https://doi.org/10.1016/j.saa.2022.121198.
[38] W. Reynaga-Navarro, R.H. Wijffels, M.H.M. Eppink, A. Kazbar, Isolation and quantification of alginate in choline chloride-based deep eutectic solvents, Int J Biol Macromol 262 (2024) 130103, https://doi.org/10.1016/J.IJBIOMAC.2024.130103.
[39] H.P. Piepho, Letters in mean comparisons: what they do and don't mean, Agron J 110 (2018), https://doi.org/10.2134/agronj2017.10.0580.
[40] P. Hong, S. Koza, E.S.P. Bouvier, A review size-exclusion chromatography for the analysis of protein biotherapeutics and their aggregates, J Liq Chromatogr Relat Technol 35 (2012) 2923–2950, https://doi.org/10.1080/10826076.2012.743724.
[41] E.A. Crespo, J.M.L. Costa, A.M. Palma, B. Soares, M.C. Martín, J.J. Segovia, P. J. Carvalho, J.A.P. Coutinho, Thermodynamic characterization of deep eutectic solvents at high pressures, Fluid Phase Equilib 500 (2019) 112249, https://doi.org/10.1016/j.fluid.2019.112249.
[42] C. Florindo, F.S. Oliveira, L.P.N. Rebelo, A.M. Fernandes, I.M. Marrucho, Insights into the synthesis and properties of deep eutectic solvents based on cholinium chloride and carboxylic acids, ACS Sustain Chem Eng 2 (2014) 2416–2425, https://doi.org/10.1021/sc500439w.
[43] L. Juul, S.K. Haue, A. Bruhn, T. Boderskov, T. Kastrup Dalsgaard, Alkaline pH increases protein extraction yield and solubility of the extracted protein from sugar kelp (Saccharina latissima), in: Food and Bioproducts Processing 140, 2023, pp. 144–150, https://doi.org/10.1016/j.fbp.2023.05.008.
[44] I.M. Aasen, I.S. Sandbakken, B. Toldnes, M.Y. Roleda, R. Slizyte, Enrichment of the protein content of the macroalgae Saccharina latissima and Palmaria palmata, Algal Res 65 (2022), https://doi.org/10.1016/j.algal.2022.102727.
[45] G. Kulshreshtha, A.S. Burlot, C. Marty, A. Critchley, J. Hafting, G. Bedoux, N. Bourgougnon, B. Prithiviraj, Enzyme-assisted extraction of bioactive material from Chondrus crispus and codium fragile and its effect on Herpes simplex virus (HSV-1), Mar Drugs 13 (2015) 558–580, https://doi.org/10.3390/md13010558.
[46] J.P. Trigo, K. Stedt, A.E.M. Schmidt, B. Kollander, U. Edlund, G. Nylund, H. Pavia, M. Abdollahi, I. Undeland, Mild blanching prior to pH-shift processing of Saccharina latissima retains protein extraction yields and amino acid levels of extracts while minimizing iodine content, Food Chem 404 (2023) 134576, https://doi.org/10.1016/j.foodchem.2022.134576.
[48] R. Balti, N. Zayoud, F. Hubert, L. Beaulieu, A. Massé, Fractionation of Arthrospira platensis (Spirulina) water soluble proteins by membrane diafiltration, Sep Purif Technol 256 (2021) 117756, https://doi.org/10.1016/j.seppur.2020.117756.
[49] A. Saxena, B.P. Tripathi, M. Kumar, V.K. Shahi, Membrane-based techniques for the separation and purification of proteins: an overview, Adv Colloid Interface Sci 145 (2009) 1–22.
[50] Z. Geng, Q. Song, B. Yu, H. Cong, Using ZIF-8 as stationary phase for capillary electrophoresis separation of proteins, Talanta 188 (2018), https://doi.org/10.1016/j.talanta.2018.06.027.
[51] K. Okubo, K. Ikeda, A. Oaku, Y. Hiruta, K. Nagase, H. Kanazawa, Protein purification using solid-phase extraction on temperature-responsive hydrogel-modified silica beads, J Chromatogr A 1568 (2018), https://doi.org/10.1016/j.chroma.2018.07.027.
[52] M. Kaspereit, A. Seidel-Morgenstern, Process concepts in preparative chromatography, in: Liquid Chromatography: Fundamentals and Instrumentation: Volume 1, Third Edition, 2023. https://doi.org/10.1016/B978-0-323-99968-7.00018-7.
[53] R. Bennett, M. Biba, J. Liu, I.A. Haidar Ahmad, M.B. Hicks, E.L. Regalado, Enhanced fluidity liquid chromatography: a guide to scaling up from analytical to preparative separations, J Chromatogr A (1595 (2019).), https://doi.org/10.1016/j.chroma.2019.02.017.
[54] N.M. Schiavone, R. Bennett, M.B. Hicks, G.F. Pirrone, E.L. Regalado, I. Mangion, A. A. Makarov, Evaluation of global conformational changes in peptides and proteins following purification by supercritical fluid chromatography, J Chromatogr B Analyt Technol Biomed Life Sci 1110–1111 (2019), https://doi.org/10.1016/j.jchromb.2019.02.012.
[55] W. Wang, S. Nema, D. Teagarden, Protein aggregation-pathways and influencing factors, Int J Pharm 390 (2010) 89–99, https://doi.org/10.1016/j.ijpharm.2010.02.025.
[56] M.F. Nava-Ocampo, L. Al Fuhaid, A. Santana, S.S. Bucs, R. Verpoorte, Y. Hae Choi, G.J. Witkamp, J.S. Vrouwenvelder, A.S.F. Farinha, Structural properties and stability of the Betaine-Urea natural deep eutectic solvent, J Mol Liq 343 (2021) 117655, https://doi.org/10.1016/j.molliq.2021.117655.
[57] E. Deniaud-Bouët, N. Kervarec, G. Michel, T. Tonon, B. Kloareg, C. Hervé, Chemical and enzymatic fractionation of cell walls from Fucales: insights into the structure of the extracellular matrix of brown algae, Ann Bot 114 (2014), https://doi.org/10.1093/aob/mcu096.
[58] X. Zhang, M. Thomsen, Biomolecular composition and revenue explained by interactions between extrinsic factors and endogenous rhythms of saccharina latissima, Mar Drugs 17 (2019) 107, https://doi.org/10.3390/md17020107.
[59] T. Lafarga, F.G. Acién-Fernández, M. Garcia-Vaquero, Bioactive peptides and carbohydrates from seaweed for food applications: natural occurrence, isolation, purification, and identification, Algal Res 48 (2020) 101909, https://doi.org/10.1016/j.algal.2020.101909.

[60] L. Firdayanti, R. Yanti, E.S. Rahayu, C. Hidayat, Carrageenan extraction from red seaweed (Kappaphycopsis cottonii) using the bead mill method, Algal Res 69 (2023), https://doi.org/10.1016/j.algal.2022.102906.
[61] H. Mear, P. Gillon, I. Gifuni, L. Lavenant, A. Poidevin, E. Couallier, Extraction of soluble proteins by bead milling from Tetraselmis chui in two different physiological states, Algal Res 74 (2023) 103180, https://doi.org/10.1016/j.algal.2023.103180.
[62] A. Schwenzfeier, P.A. Wierenga, H. Gruppen, Isolation and characterization of soluble protein from the green microalgae Tetraselmis sp, Bioresour Technol 102 (2011) 9121–9127, https://doi.org/10.1016/j.biortech.2011.07.046.