
Functional protein extraction from brown seaweed from the North Sea

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Summary

The aim of this project was to investigate whether it is possible to extract functional proteins for human application from brown seaweed from the Dutch North Sea. To achieve this, knowledge about the molecular cell structures, location and function of proteins in seaweeds is essential. In brown seaweed, the cell wall mainly consists of a carbohydrate network of alginate, fucoidan and cellulose. Proteins have various functions and are located in different places in the cell. Roughly speaking, we can distinguish between glycoproteins, enzymes and proteins built into photosystems. Glycoproteins are protein-sugar compounds that are located in the cell walls and give the cell wall rigidity. Photosystems are complex structures for capturing light which are located in the cell membranes. The enzymes are located freely in the cells, are water-soluble and in this way relatively easy to isolate. To recover all these proteins the carbohydrate network in the cell wall must be broken open.

It was investigated whether it is possible to extract proteins from the seaweed species *Saccharina* and *Undaria* by means of a combined mechanical and enzymatic treatment. After only mechanical treatment, the protein yield in the liquid fraction was comparable for both seaweeds (~35% (protein/total protein)). The protein content in *Undaria* was higher (14 vs 10% (protein/DW) in *Saccharina*) which also resulted in a higher protein content (8 vs 5% (protein/DW)) in the liquid fraction. These recoveries and compositions are insufficient for a commercially viable process (target protein recovery >80% and protein composition >40% (protein/DW)). By making a gel using these crude extracts, it was shown that the liquid fractions had gelling capacity and are therefore potentially interesting as functional ingredients for food application.

An attempt was made to open more cells by means of a combined mechanical and enzymatic treatment. Commercial carbohydrases were used for this, which are enzymes that break down carbohydrates, but leave proteins untouched. The protein yield for *Saccharina* was increased to ~40% (was 35%) and the protein content to ~7% (was 5% (protein/DW)). Microscopy showed that the majority of the cells were still intact, meaning that the combination of mechanical and enzymatic treatment did not completely break the cells open. The explanation for this limited effectiveness is that commercially available carbohydrases are suitable for breaking down complex carbohydrate compounds in land plants such as cellulose and hemicellulose. Carbohydrases that can break down complex carbohydrate compounds in seaweed are currently not commercially available. Such enzymes exist and are present in fungi that naturally grow on seaweed: seaweed biomass is broken down in the marine environment. Together with NIOZ and Westerdijk Institute, a new project was started in 2022, aiming at isolating specific enzymes from marine fungi that are able to break down the cell walls in seaweeds. This project is part of the NWO program "From Sea to Society".

1 Introduction

The Societal Innovation Program *Proseaweed*, setup by Wageningen Research and the Stichting Noordzeeboerderij (North Sea Farmers) commissioned by The Dutch ministry, focuses on the applicability of seaweed for food and feed. The government has indicated that external partners should support this program and that societal impact should be achieved. The aim of the project *Extraction of functional protein* was to develop technology for extraction of functional protein from brown seaweed living in the Dutch North Sea. The goal within the project was to test the process on lab scale with up scalable technologies and to get a first impression on the technical functionality of the protein (gelling), using two brown seaweeds (*Saccharina* and *Undaria*).

Based on literature (Annex 1), it was concluded that the strong interactions between polysaccharides and protein within seaweed limit protein extraction in terms of recovery (protein/total protein) and content (protein/DW). Therefore, the focus in this project was on opening the cell wall structure. Because of the need to extract functional proteins, only mechanical and enzymatic disruption technologies were tested. The envisaged process is schematically shown in Figure 1-1. The project focused on the first three unit operations.

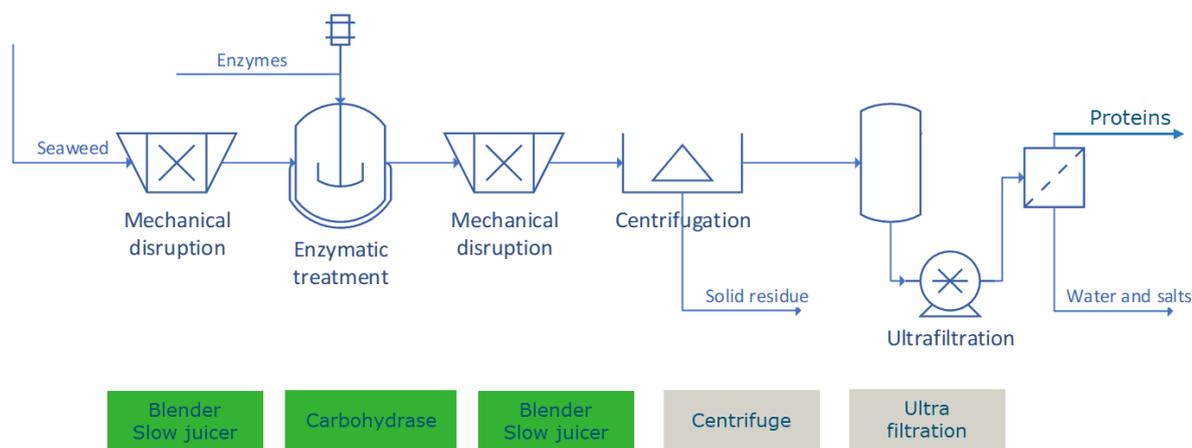


Figure 1-1 Schematic view of proposed process for functional protein extraction from seaweed.

To enhance protein recovery and content, knowledge about the molecular cell structures, location and function of proteins in seaweeds is essential (Chapter 2). Lab experiments were carried out to extract protein by mechanical and mechanical + enzymatic treatment (Chapter 3). Based on the cell wall structure, specific carbohydrases were selected and tested (Chapter 3). The functionality (gelling) of some crude extracts was tested (Chapter 4).

2 Understanding the crop seaweed

2.1 Introduction

In order to facilitate choices regarding harvesting, preservation and processing towards a high recovery of functional proteins it is important to understand how seaweed functions as a crop. Subjects important to understand include seaweed morphogenesis (how its shape develops) and its growth cycles, cell wall characteristics and seasonal nitrogen balance. Our understanding of these aspects and their implications on harvesting and processing to extract functional proteins are summarized below.

2.2 The crop seaweed

Seaweeds are algae from red, green and brown lineages that, at some stage in their life cycle, form multicellular or siphonous macrothalli (Hurd *et al.*, 2014, chapter 1). Thalli are plant bodies that are not differentiated into stems and leaves and lack true roots and vascular systems (Figure 2-1). Adult seaweed growth originates from a meristem, from which the different thalli organs originate. Usually, the meristem is located at the thickened base of the blades. New blades are generated during the growth season, which starts in spring and ends during summer (Rosenberg & Ramus, 1982; Hurd *et al.*, 2014, chapter 1). Some seaweed species, such as *Saccharina latissima* shed their old blades or let them die off during autumn (Zhang & Thomsen, 2019).

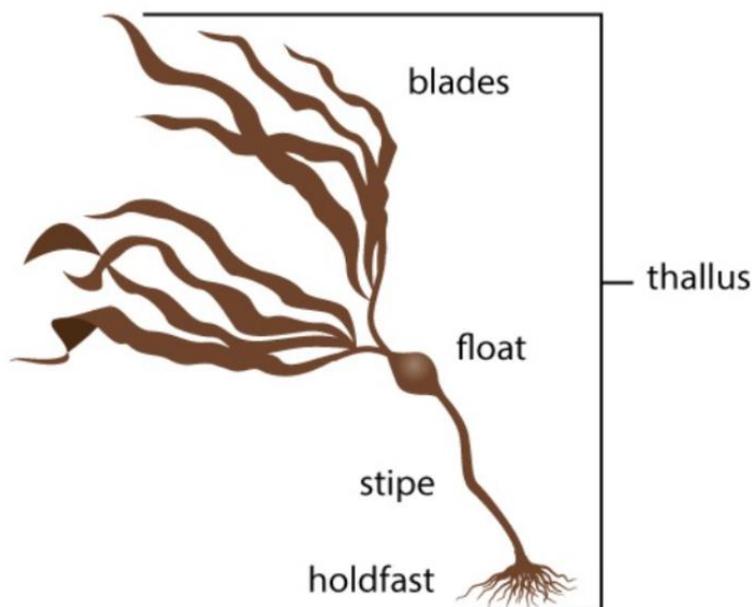


Figure 2-1 *Schematic representation of a general seaweed morphology: holdfast, stipe and several blades, accompanied by a float that helps direct the seaweed away from the seafloor. Some seaweeds will differ from this schematic by absence, shape or relative abundance of these tissues (Taken from Duncan Seraphine et al., 2021).*

While their morphology resembles that of plants, the different lineages of seaweed have different evolutionary histories in relation to plants (Cock *et al.*, 2010; Figure 2-2). Red and especially green seaweeds are relatively closely related to plants, particularly in their photosystem organization. Brown seaweeds on the other hand are evolutionary more different from plants (Cock *et al.*, 2010).

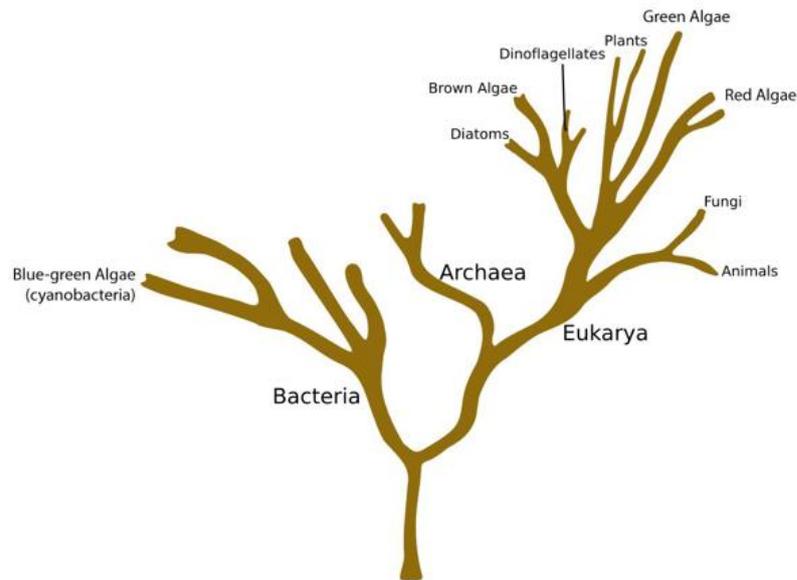


Figure 2-2 Placement of the algae groups (and seaweed lineages) within the domains of Bacteria and Eukarya. Green, red and brown seaweeds are placed among the Eukarya branches of green, red and brown algae (Taken from Duncan Seraphine et al., 2021).

There are three classes of seaweed: green, red and brown, each with their own thallus structure and light absorbing pigment composition (Hurd *et al.*, 2014, chapter 1; Table 2-1). Green seaweeds have a relatively simple thallus structure composed of delicate blades that are attached to their substrate by holdfast discs. Red seaweeds have delicate blades that are separated from the holdfast discs by filamentous stipes, which hold the blades further away from the substrate. Due to the addition of stipes, red seaweeds are more diverse in structure than green seaweeds. Brown seaweeds have the most diverse structures with blades, stipes and holdfasts, which can be accompanied by floats. Floats are specialized plant bodies filled with gas that serve to keep the thallus off the seafloor. Some brown seaweeds can relocate nutrients to different organs through sieve tubes. Due to these differences in thallus structure and pigment composition, red and brown seaweeds are able to grow at larger depths (usually < 100 m deep; 295 m is max. known depth) and rougher waters.

Like plants, seaweeds absorb light to drive photosynthesis and convert carbon dioxide and water into carbohydrates and oxygen (Hurd *et al.*, 2014, chapter 5). However, while light blue and green light can penetrate the water up to a depth of 200 m, red light only penetrates the water up to 10 m deep (Webb, 2019; Figure 2-3). Additionally, light penetration is further influenced by factors such as the light angle, cloudiness, murkiness and seasonal seaweed blooms, which are the main reasons why light penetrates less deep in coastal waters, which lie between land and oceans and are typically less than 200 m deep. While seaweed can theoretically be cultivated at larger depths in oceanic waters, this would require a submerged frame for the seaweed to attach itself and an active nutrient supply, e.g. cycling water from the ocean floor (Flannery, 2017).

In seaweeds, the photosynthetic machinery is located within the blades. Seaweeds have adapted to different depths by incorporating different pigments in their photosynthetic machinery, which also define their color (Figure 2-3; Hurd *et al.*, 2014, chapter 5; Dumay & Morancais, 2016). Green seaweeds predominantly make use of chlorophyll a and b and to a lesser extent of carotenoids in their light harvesting antenna. Chlorophylls and carotenoids mainly absorb the blue and red light penetrating the shallower waters. Red seaweeds use carotenoids and phycoerythrin, which allows them to also absorb the deeper penetrating green light. Brown seaweeds predominantly use carotenoids for light harvesting and, to a lesser extent, chlorophyll c.

Table 2-1 Overview of the differences between green (*Chlorophyta*), red (*Rhodophyta*) and brown (*Ochrophyta*) seaweed species.

Species	<i>Chlorophyta</i>	<i>Rhodophyta</i>	<i>Ochrophyta</i>
Example species¹	<i>Ulva lactuca</i>	<i>Palmaria palmata</i>	<i>Saccharina latissima</i>
			
Structure thallus	Holdfast disc w/ delicate blades	Holdfast w/ filamentous stipes and/or delicate blades	Diverse w/ holdfasts, stipes, blades and/or floats)
Growing depth	Upper sublittoral*	Sublittoral	Sublittoral
Light-absorbing pigments^{2,3}	Chlorophyll a & b Carotenoids	Phycoerythrin Carotenoids Chlorophyll a & d	Chlorophyll a & c Carotenoids
Light harvesting complexes²	LHC proteins PSI & II proteins	Phycobiliproteins PSI & II proteins	LHC proteins PSI & II proteins
Protein content (% DW)³	9 - 26	7 - 47	3 - 15
Rubisco (% TSP)⁴	2.9 - 7.6	6.6 - 9.9	17.4 - 37.3
Seasonal variation³	Yes	Yes	Yes

* Sublittoral is the permanently submerged zone where light hits seafloor (max 300 m depth).

Abbreviations: LHC, Light harvesting complex; PSI & PSII, Photosystems I and II; DW, dry weight; TSP, total soluble protein. References: ¹Lubsch, 2019; ²Hurd *et al.*, 2014; ³Dumay & Moraçais, 2016; ⁴Íñiguez *et al.*, 2018.

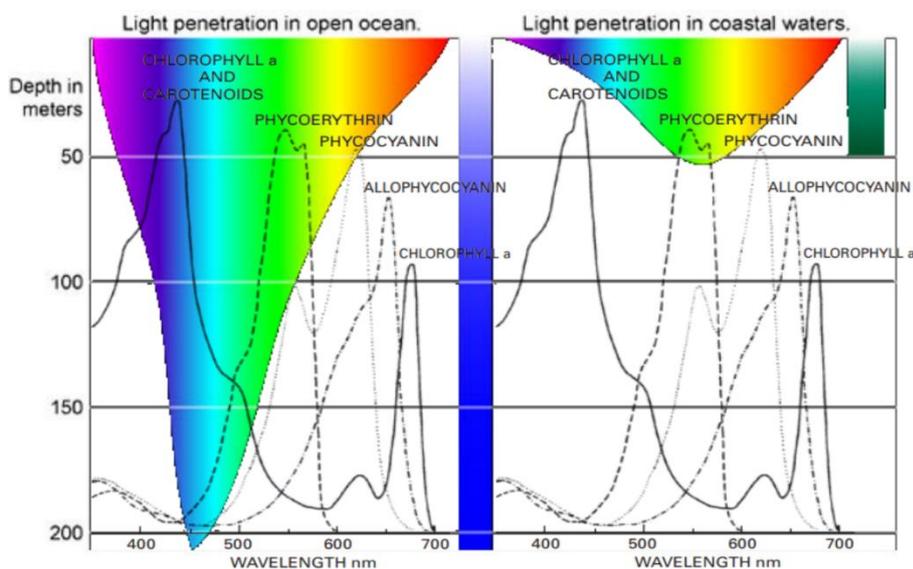


Figure 2-3 Representations of light penetration in water and light absorption by seaweed pigments. Light penetration is visualized depending on wavelength of visible light (400 – 700 nm) and water depth in either open ocean or coastal waters (adapted from Webb, 2019). Light absorption by seaweed pigments is presented as an overlay and shown as absorption depending on wavelength (adapted from Gannt, 1975).

Meristems (tissues consisting of undifferentiated cells that can grow and differentiate into other tissue types), seeds and photosynthetic machinery generally are the most nitrogen-rich parts of plants. Since, in seaweeds, both the meristem and the photosynthetic machinery are located in or at the base of the blades, the blades will be the most interesting target to harvest protein and other nitrogen-rich material.

2.3 Cell wall structure of seaweeds

When comparing the functions of cell walls in land plants to those of seaweeds, there are a lot of similarities. In both types of organisms, cell walls provide structure, act as defensive barriers, transfer information and aid in the environmental and developmental signaling (Sørensen *et al.*, 2010). In land plants, the structure provided by the cell wall is tailored to withstand forces from gravity and wind. To deal with these forces, shorter land plants use primary cell walls in combination with turgor pressure. Taller land plants have developed thick secondary cell walls composed largely of lignin to provide rigidity against wind and gravity. Compared to land plants, seaweed cell walls do not have to supply much stiffness, but have to withstand much larger tensile forces coming from moving water, such as currents and waves. To deal with these forces, seaweed cell walls are 100-1000 times less stiff compared to those of land plants and have a higher degree of flexibility (Charrier *et al.*, 2019). For example, while tomato meristem cells can expand and shrink by about 9% in volume, cells of the brown seaweed *Ectocarpus* can expand and shrink by up to 70 and 35%, respectively. However, the structure-strength relations are diverse among species (Swanson, 2018).

The differences between plant and seaweed cell walls are also reflected in their chemical composition. For example, primary cell walls of plants and brown seaweed chemically differ on molecular level by over 80% (Charrier *et al.*, 2019 and references therein). While the cell walls in both types of organisms contain a cross-linked cellulose microfibril primary matrix and a secondary embedded matrix, that is where the similarities end (Figure 2-4). Seaweed cell walls contain fewer and ribbon-shaped cellulose microfibrils that are crosslinked by sulfated fucans and proteins and the secondary matrix consists of an alginate gel crosslinked with phlorotannins. Sulfated fucans, proteins and alginates can comprise up to 40%, 5% and 40% of the cell wall (on dry basis), respectively. Due to these major differences, using knowledge of plant cell walls and applying it directly to seaweed cell walls is unreliable.

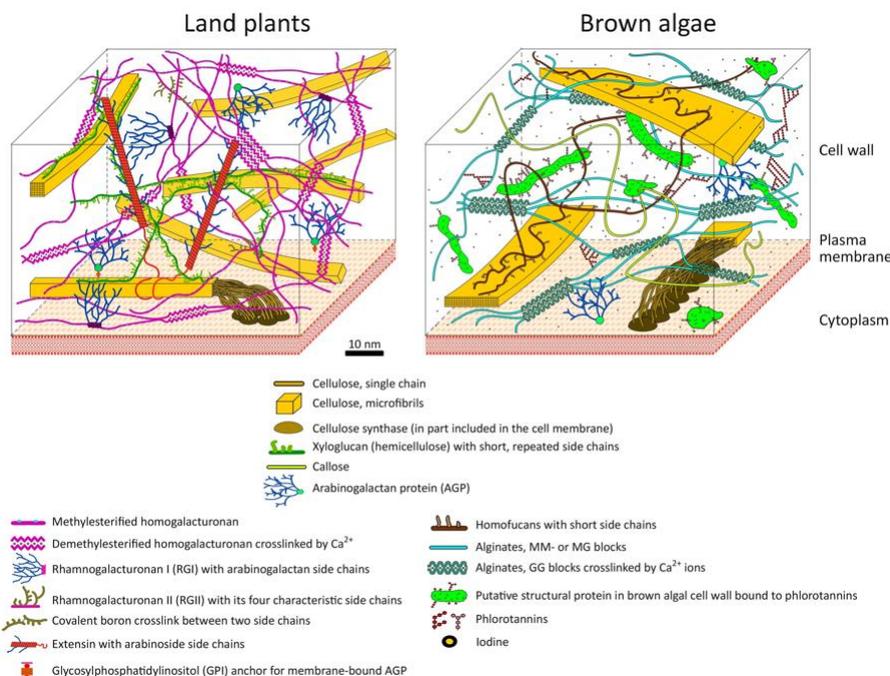


Figure 2-4 Schematic cell wall representations of land plants and brown seaweed (adapted from Charrier *et al.*, 2019).

Cell walls from green, red and brown seaweed are composed of different polysaccharides (Figure 2-5; Stiger-Pouvreau *et al.*, 2016). Green seaweed have a primary cell wall matrix composed of cellulose, xylans and mannans, whereas the secondary embedded matrix consists of ulvan and hydroxyproline rich glycoproteins. Red seaweed have a well-ordered, relatively rigid, primary matrix built up from cellulose, mannans and xylans (Joubert *et al.*, 2008; Stiger-Pouvreau 2016; Bjarnadottir *et al.*, 2018), with an embedded matrix of flexible sulphated glucans and sulphated galactans. Little is known on the types of protein in these cell walls, although in *Palmaria palmata* around 30% protein (on dry weight) is charged and covalently bound to polysaccharides (Deniaud *et al.*, 2003). In addition to primary cell walls, Corraline red seaweed also have cellulose-rich secondary walls, which contribute to increased tension strength (Martone *et al.*, 2019). Brown seaweed have a primary cell wall matrix that consists mainly of cellulose (Stiger-Pouvreau 2016; Charrier *et al.*, 2019). The embedded matrix in brown seaweed consists of alginates and homofucans (Stiger-Pouvreau, 2016).

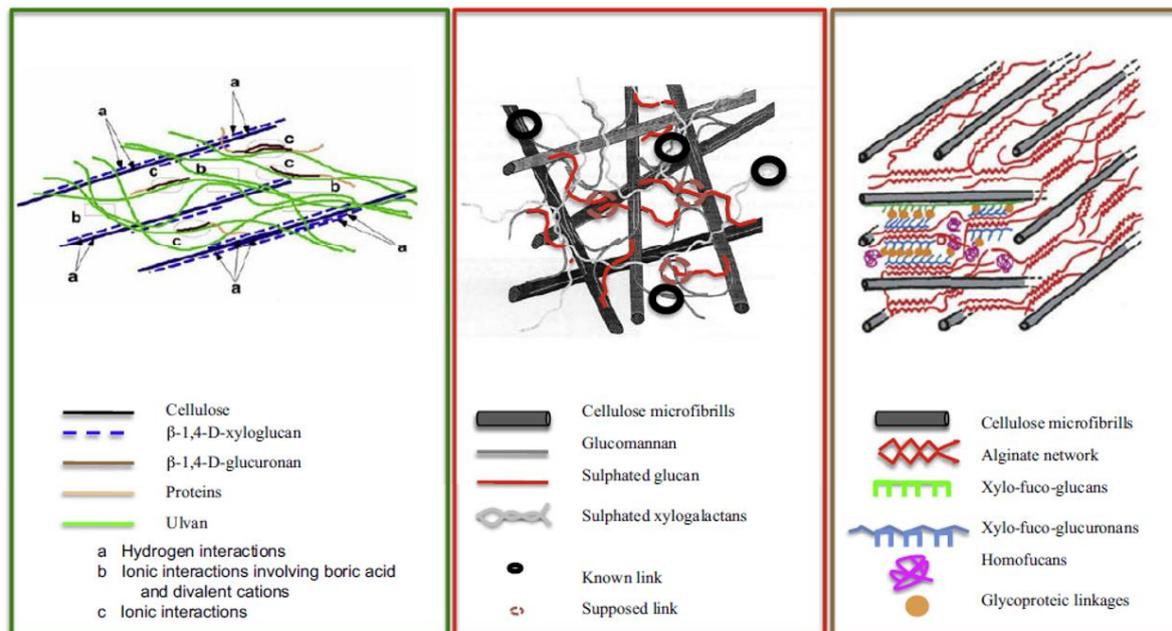


Figure 2-5 Schematic cell wall representations in green (left), red (middle) and brown (right) seaweeds including characteristic polysaccharides (adapted from Stiger-Pouvreau *et al.*, 2016).

Besides the differences between species, there are also examples of seasonal variation in cell wall composition. Overwintering tissues of *Laminaria* possess thicker cell walls and higher proportions of cellulose and fucose-containing polysaccharides, leading to increased cell wall stiffness and tensile strength (Starko *et al.*, 2018).

2.4 Osmotic acclimation

Water from oceans and sea have a relatively high salinity, which is highly variable depending on proximity of freshwater runoff from rivers and melting ice, geochemistry, tidal flows, wind, precipitation and evaporation (Karsten, 2012). High salinity can lead to salt-induced inhibition of growth and photosynthesis in unadapted seaweed species. As such, different seaweed species have adapted to different combinations of salinity and environmental factors such as solar irradiation level and temperature.

To prevent dehydration due to hypersalinity stress, some seaweeds accumulate ions such as Na^+ , K^+ and Cl^- , usually in a high K^+/Na^+ ratio (Karsten, 2012). Since these ions can inhibit protein and organelle function, concentrations of these ions are kept as low as possible in the cytosols and are instead stored within the vacuoles of the seaweed cells.

Some seaweeds, such as *Laminaria digitata*, also use NO_3^- in spring and as this ion is metabolized during summer, mannitol is accumulated (Davison & Reed, 1985). Accumulation of organic osmolytes such as mannitol and sucrose can provide osmotic protection without affecting protein and organelle function. However, generation of these osmolytes is energetically costly. Among brown seaweeds, mannitol is the predominantly present osmolyte, whereas green seaweeds usually contain sucrose instead (Karsten, 2012). In red seaweed species, many different low molecular weight carbohydrates have been identified as osmotic solutes, the main of which are floridoside and to lesser extents digeneaside and trehalose.

High salt ion contents translate to high ash contents in *Laminaria digitata* harvested in spring, which can negatively affect bio-digestibility when used for biogas production. Washing in 40°C water removed over half of the salt and improved digestibility (Tabassum *et al.*, 2017).

2.5 Nitrogen in seaweeds

In seaweed, like in plants, the majority of cellular nitrogen is invested in the photosynthetic machinery to facilitate the assimilation of carbon (Hurd *et al.*, 2014 chapter 5). As such, the blades, which contain the photosynthetic machinery, will be the most nitrogen-rich organs within seaweed. This nitrogen is divided amongst the light harvesting antennae, photosystems, photoassimilatory pathway (RuBisCO a.o.) and the photorespiratory pathway (Hurd *et al.*, 2014 chapter 5; Evans & Clarke, 2019).

In land plants, of the photosynthetic machinery, RuBisCO is generally considered to be the most abundant protein, as it has been reported to constitute up to 65% of the total soluble protein (Ellis, 1979). A more recent report, based on total cellular protein, stated that on average 54% is allocated to photosynthesis (Evans & Clarke, 2019). Proteins related to light harvesting complexes, photosystems, RuBisCO and photorespiration make up 6, 8, 20 and 3% of the total cellular protein, respectively, leaving 17% of other protein related to photosynthesis. However, these numbers are averages and can vary considerably, depending on species, presence of carbon concentrating mechanisms (CCMs), environmental conditions and seasons (Hurd *et al.*, 2014 chapter 5; Evans & Clarke, 2019).

Seaweed species show differences in the use of CCMs (Cornwall *et al.*, 2017). By increasing the local availability of CO_2 , CCMs can increase the efficiency of RuBisCO and reduce the need of photorespiration (Raven, 2013). Presence of a CCM can reduce the required amount of RuBisCO to less than half of that present in regular plants. In seaweed, 3 classes related to CCMs can be distinguished: no CCM, CCMs with low affinity for dissolved inorganic carbon (DIC) or CCMs with high affinity for DIC (Cornwall *et al.*, 2017).

Environmental conditions and seasons greatly influence the concentration, balance and subcellular distribution of the nitrogen within seaweed. During spring, protein content increases as the photosynthetic machinery is built up. As spring progresses into summer, carbohydrates are accumulated in the cell walls and stored as starch or soluble compounds such as mannitol or laminarin (Rosenberg & Ramus, 1982; Hurd *et al.*, 2014; Roleda & Hurd, 2019). Due to the accumulation of carbohydrates during summer, protein concentrations decrease as summer progresses. Consequently, young blades formed during spring generally have higher protein contents (Hurd *et al.*, 2014; Dumay & Morançais, 2016).

During autumn and winter, many land plants shed their leaves to prevent dehydration and involuntary shedding during frost in a process called leaf senescence. To recycle the nitrogen invested into the leaves, plants degrade the photosynthetic machinery in the leaves and relocate it to buds and bark, which are better protected against winter conditions. A similar protein degradation event has not been mentioned to occur in seaweed blades and it has been stated that little is known about catabolism and turnover of cellular protein (Hurd *et al.*, 2014).

The consensus from literature seems that during winter, when levels of NH_4^+ , NO_2^- , NO_3^- , urea and amino acids in seawater are high, reserves are accumulated (Chapman & Craigie, 1977; Nielsen *et al.*, 2014; Zhang & Thomsen, 2019). This nitrogen can be stored in blade tissues or meristems, depending on the species and can be transported between tissues when nitrogen is required, e.g. from source thalli to sink meristems for growth (Davison & Steward, 1983; Li *et al.*, 2009; Nielsen *et al.*, 2014). Nitrogen reserves are utilized for growth in winter through to spring and depletion of reserves follows disappearance of external nitrogen sources with a 2-months lag (Chapman & Craigie, 1977; Nielsen *et al.*, 2014).

For more details on nitrogen in seaweeds, the reader is referred to Annex 2. It can be concluded that the protein content in seaweeds is likely highest during or at the end of spring, when the photosynthetic machinery has been built up and carbon accumulation is still relatively low. The percentage of RuBisCO among the protein may be reduced as more carbon is assimilated into cell walls, as cell walls also contain a percentage of the protein. As such, the season where most of the nitrogen has been converted into protein, but has not had as much time to assimilate carbon, will likely yield the highest purity of protein.

2.6 Conclusions

Red and green seaweeds are relatively closely related to plants, brown seaweeds are evolutionary more different from plants. Cell walls in land plants and seaweeds have comparable functions. However, compared to those of land plants, seaweed cell walls have to withstand much larger tensile forces coming from moving water; seaweed cell walls are 100-1000 times less stiff. These differences are reflected in the chemical composition of the cell wall: primary cell walls of plants and brown seaweeds chemically differ by over 80%. Due to these major differences, using knowledge of plant cell walls and applying it directly to seaweed cell walls is unreliable. Furthermore, cell walls from green, red and brown seaweeds are composed of different polysaccharides. The main polysaccharides present in brown seaweeds are alginate, fucoidan and cellulose.

In seaweeds, like in plants, the majority of cellular nitrogen is invested in the photosynthetic machinery: the blades are the most nitrogen-rich organs. During winter, when NH_4^+ , NO_2^- , NO_3^- , urea and amino acids in seawater are high, reserves are accumulated. This nitrogen can be stored in blade tissues or meristems and can be transported between tissues when nitrogen is required. Nitrogen reserves are utilized for growth in winter and through spring and depletion of reserves follows disappearance of nitrogen in seawater with a 2-months lag. Due to the accumulation of carbohydrates during summer, protein content decreases as summer progresses. Consequently, young blades formed during spring generally have the highest protein content.

3 Protein extraction

3.1 Introduction

Lab experiments were carried out to recover protein by mechanical and mechanical + enzymatic (carbohydrases) treatment, using the Dutch brown seaweeds *Saccharina* and *Undaria*. A second set of experiments was carried out focusing on finding more specific carbohydrases to enhance protein release. Carbohydrases were selected based on the cell wall structure of brown seaweeds. These enzymes were tested on model substrates (the single polysaccharides present in the cell wall structure) and on *Saccharina*. The aim in both sets of experiments was to obtain a liquid fraction high in functional protein. Protein recovery target was set at >80% and the protein content >40% on dry weight basis. Protease (alcalase) was used in the experiments as a benchmark to test if protein accessibility was improved by carbohydrases.

3.2 Material and Methods

3.2.1 Mechanical and mechanical + enzymatic treatment

Two mechanical treatments were tested: slowjuicer and blender. Fresh *Saccharina* and *Undaria* were provided by The Dutch Seaweed Group. Part of the mechanically treated material was consequently treated with enzymes (carbohydrases and protease).

For the enzymatic treatment the mechanically treated seaweed was diluted (to 5% DW) and 0.5% enzyme (based on DW) was added. Enzymes used: Viscozym L, Cellic® CTec2, Pectinex Ultra SP, all 3, alcalase. Conditions: 40°C, 20 h, pH 6. At the end of the experiment the pH was increased to 8 to stop the enzyme. Centrifuge (20 min, 15,000×g, 4°C) and supernatant and pellet were stored for further analysis. A blank experiment was also carried out in which no enzyme was added. Mass balances were made of dry matter, protein and ash. SDS-page analysis was performed on the liquid fractions obtained.

Microscopy with staining (calcifluor for cell wall and rhodamine B for protein) was performed to visualize intact cell walls and proteins.

3.2.2 Testing carbohydrases to enhance protein release

The aim was to find carbohydrases that weaken or break down brown seaweed cell walls and that hereby enhance protein extraction. Based on the main components present in the cell wall of brown seaweeds (alginate, fucoidan and cellulose), carbohydrases were selected. These carbohydrases were first tested using the single carbohydrates as model substrates. The setup and results of these experiments are described in Annex 3. Follow up experiments were carried out using frozen *Saccharina* as substrate with carbohydrases showing breakdown of the model substrates. The selected enzymes, together with their optimal conditions, are given in Table 3-1. It should be noted that Alg 3 and Alg 4 are not commercially available.

Enzymatic treatments were done with single enzymes, enzyme cocktails and sequential enzyme treatments. The setup is given in Table 3-2. Incubation time for all experiments was 24 h. Additional treatment with alcalase to assess protein accessibility was done at pH 8, 50°C, 20 h. The liquid and solid fraction after centrifugation were analyzed for protein (nitrogen, Kjeldahl), dry matter, and ash content.

Table 3-1 Selected carbohydrases to weaken cell wall structure of brown seaweeds.

Carbohydrate	Enzyme	Conditions
Alginate	Matis (not commercial, but in-house) Alg 3 and Alg 4	Alg 3 - pH 5.5, 75°C
		Alg 4 - pH 6.5, 80°C
Fucoidan	Pectinex Ultra SP, contains various glycosidases	pH 6, 50°C
	Viscozyme, contains various glycosidases	pH 6, 50°C
	Ronozyme HisPos	pH 6, 50°C
Cellulose cellulases (only test in combination)	Cellic® CTec2 (especially cellulases and some hemicellulase)	pH 5.5, 50°C
	Cellic® HTec2 (contains especially endo-xylanases)	pH 5.5, 50°C

Table 3-2 Enzymatic treatment carbohydrases.

Enzyme 1	Conditions 1	Enzyme 2	Conditions 2
Cellic Ctec2	pH 5.5, 55°C	-	-
Viscozym	pH 6, 40°C	-	-
Pectinex Ultra P	pH 6, 40°C	-	-
Viscozym + Cellic Ctec 2 + Pectinex Ultra SP	pH 6, 40°C	-	-
Alg 3	pH 5.5, 75°C	Cellic Ctec2	pH 5.5, 55°C
Alg 4	pH 6.5, 80°C	Cellic Ctec2	pH 5.5, 55°C
Alg3 + Alg4	pH 6, 75°C	Cellic Ctec 2/ Pectinex Ultra SP/ Ronozym HiPhos	pH 6, 50°C

3.3 Results

3.3.1 Mechanical, enzymatic and combined treatment

Saccharina was rather thick with tough plant material. The leaves were covered with a slimy layer. During treatment in the slowjuicer a sticky material was formed. For an impression, see Figure 3-1. *Undaria* was thinner than *Saccharina*, much less slimy and less tough. It was treated with the blender and the slowjuicer. For an impression see Figure 3-2.

Saccharina and *Undaria* before and after treatment (blender + enzyme cocktail with three enzymes) were stained with calcifluor (colors cell walls blue) and with rhodamine B (colors protein red). Since it was tough material only the surface was stained. The results are shown in Figure 3-3. The untreated *Saccharina* and *Undaria* show intact cell structures with protein inside the cells (*Undaria* missing in Figure 3-3). The treated *Saccharina* and *Undaria* show broken and intact cell structures with empty and filled cells. It looks like that *Undaria* is cut in a different way compared to *Saccharina*.



Figure 3-1 *Impression Saccharina. From left to right: starting material, after treatment in slowjuicer (2x).*



Figure 3-2 *Impression Undaria. From left to right: starting material (twice), before and after in the blender and before and after in the slowjuicer.*

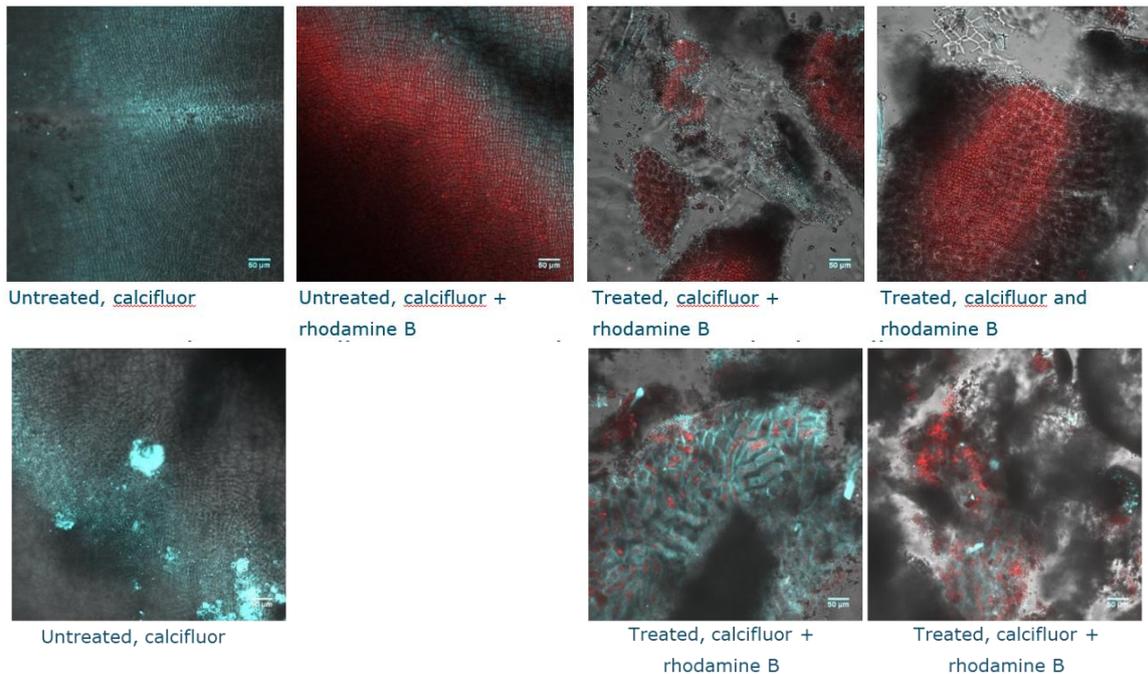


Figure 3-3 *Microscopy with staining (blue cell wall and red protein) of Saccharina (upper row) and Undaria (lower row).*

An overview of the dry matter release for *Saccharina* and *Undaria* for mechanical and combinations of mechanical + enzymatic treatments are given in Figure 3-4. For all treatments, dry matter release for *Saccharina* was between 35-50% and for *Undaria* between 30-45%. *Saccharina* had a higher dry matter release ($\pm 9\%$) with blender compared to slowjuicer. For *Undaria* a higher dry matter release ($\pm 12\%$) was found for the slowjuicer compared to blender. For both seaweeds, no increase in dry matter release due to mechanical + enzymatic treatment was observed. Only a slight increase in dry matter release with alcalase ($\pm 4\%$) for *Saccharina* was found.

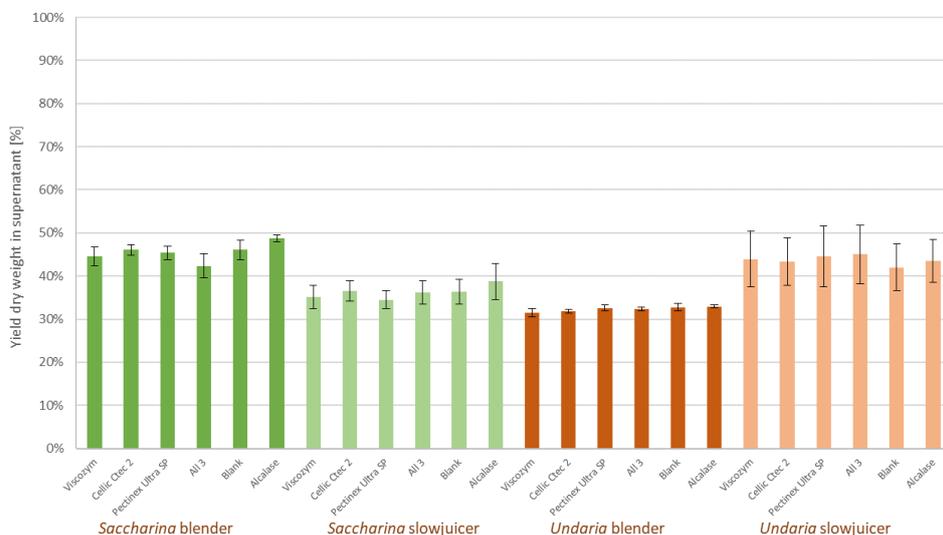


Figure 3-4 Dry matter release for *Saccharina* and *Undaria* for various combinations of mechanical and mechanical + enzymatic treatment. Duplo experiments.

An overview of the protein (peptide in case of alcalase) release for *Saccharina* and *Undaria* for various combinations of mechanical and enzymatic treatment is given in Figure 3-5. *Saccharina* had a higher protein release with blender ($\sim 45\%$) compared to slowjuicer ($\sim 35\%$). A small additional protein release ($\sim 5\%$) was found for the combined mechanical + carbohydrase treatment. *Undaria* had a higher protein release with slowjuicer ($\sim 35\%$) compared to blender ($\sim 30\%$). This difference is less pronounced due to the large deviation for the slowjuicer experiments, which may be related to lower quality of the second *Undaria* batch. No increase in protein release was observed following to mechanical + carbohydrate treatment with carbohydrases for *Undaria*. The highest increase in protein/peptide release was obtained when protease (alcalase) followed mechanical treatment of *Saccharina* ($\pm 22-28\%$). The combination of blender and alcalase treatment came close to the targeted release of 80%. However, these peptides will have limited functionality. The increase in peptide release for *Undaria* was less pronounced.

The protein content (% DW) in the liquid fraction was lower compared to the starting material for all combinations with and without carbohydrases, indicating that less protein was released compared to other cell material (Figure 3-6). For *Saccharina*, the peptide content after alcalase treatment was comparable to the protein content of the starting material. All values are far below the targeted protein content of 40%. The protein content of the final product can be increased by removal of impurities using ultrafiltration further downstream in the process (Figure 1-1).

The SDS-page analysis was performed on the liquid fractions from the blender experiments. Because there was a relatively small difference in protein release compared to the slowjuicer, similar results may be expected for the slowjuicer, although this was not confirmed. The same pattern was observed for *Saccharina* and *Undaria*: in the liquid fraction obtained after mechanical treatment with the blender, small peptides (< 4 kDa) were detected and specific bands at 20 and 63 kDa (Figure 3-7). No clear differences in the liquid fraction obtained with the different carbohydrases were observed, indicating the same size of resulting peptides and proteins. As expected, alcalase was very effective in cleaving all proteins into small peptides: all bands have disappeared and only the lower band at 3 kDa remains.

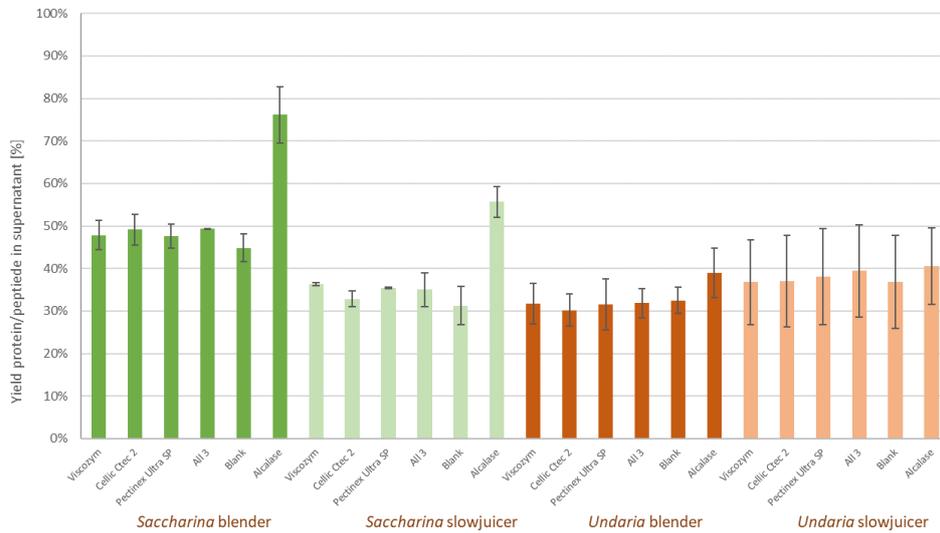


Figure 3-5 Protein and peptide (in case of alcalase) release for Saccharina and Undaria for various combinations of mechanical and enzymatic pretreatment. Duplo experiment.

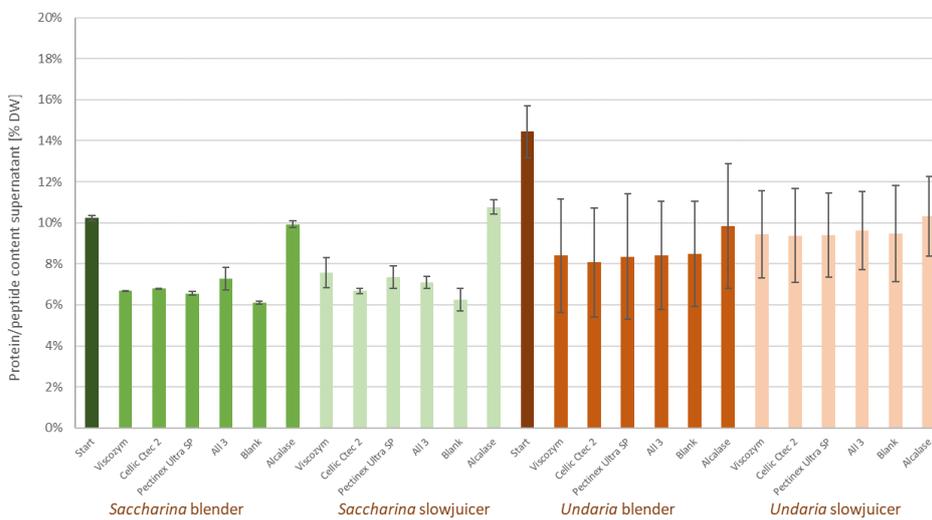


Figure 3-6 Protein and peptide (in case of alcalase) content (% DW) in supernatant for Saccharina and Undaria for various combinations of mechanical and enzymatic treatment. Duplo experiment.

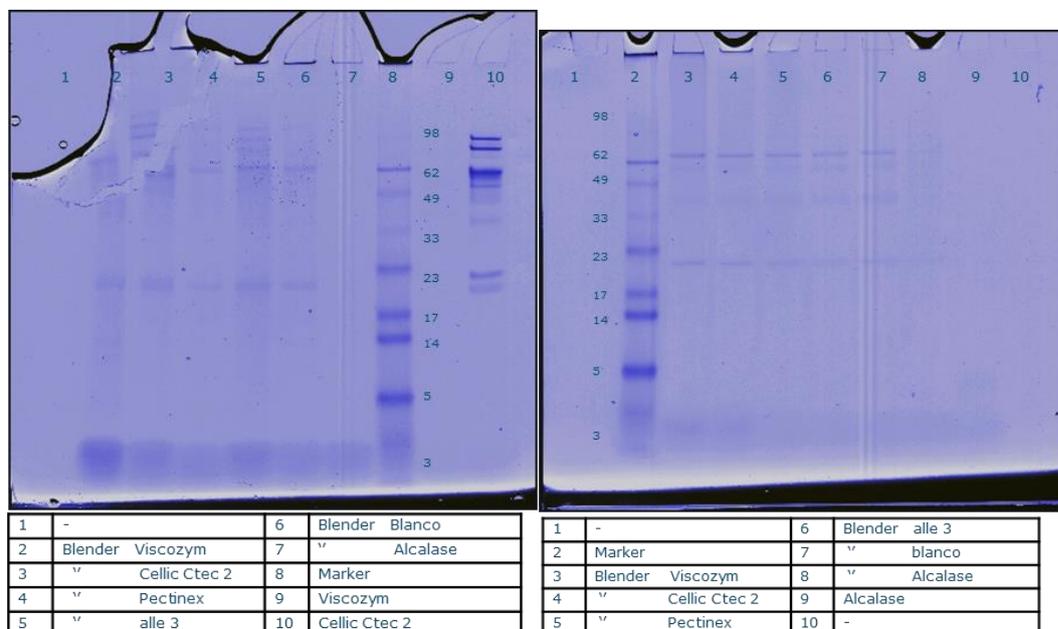


Figure 3-7 SDS-page liquid fraction *Saccharina* (left) and *Undaria* (right) after various enzymatic treatments following mechanical treatment with blender.

Mechanical treatment is required to enhance the dry matter in the liquid fraction, furthermore it enhances the surface area and potentially the accessibility for enzymatic treatment. Based on the results of the two mechanical treatments, the blender is preferable for *Saccharina* and the slowjuicer for *Undaria*. No enzymatic treatment based on carbohydrases has been identified that results in substantially higher protein release. Both protein yield (30-50% of total protein) as well as protein content (6-9% (protein/DW)) need further improvement to be commercially interesting.

3.3.2 Testing carbohydrases to enhance protein release

Interesting carbohydrases were identified based on the cell wall structure of brown seaweeds. These carbohydrases were tested on model substrates (alginate and fucoidan) and the results are described in Annex 3. Based on these results, carbohydrases were selected and tested using *Saccharina*. The dry matter release using different carbohydrases is summarized in Figure 3-8. Experiments were carried out under various conditions (pH (5.5-6.5), temperature (40-80°C)) and using either a blender or a slowjuicer as pretreatment. For all enzymatic treatments, the amount of released material is compared to a run without any enzyme added. At 75-80°C (blanks for Alg 3 and Alg 4 treatments), the dry matter release increased: without enzyme addition, ~60% of the dry matter was released. At these temperatures, protein is generally known to lose functionality, but to what level is unknown as proteins may be protected by cell structures. The largest additional release of 30% of the dry matter, up to 90% of total, was obtained with the complex enzyme cocktail Alg 3 + Alg 4 + Cellic Ctec2 + Pectinex + Ronozym HiPhos.

The protein release using different carbohydrases is summarized in Figure 3-9. Experiments were carried out under various conditions (pH (5.5-6.5), temperature (25-80°C)) and using either a blender or a slowjuicer as pretreatment. For all enzymatic treatments, the amount of released material is compared to a run without any enzyme added. No clear trend for protein release was observed: the protein release was ~25-45% without enzyme treatment for all conditions studied. The largest additional protein release of 20%, up to 50% total, was obtained with the enzyme cocktail Alg 4 + Cellic Ctec2.

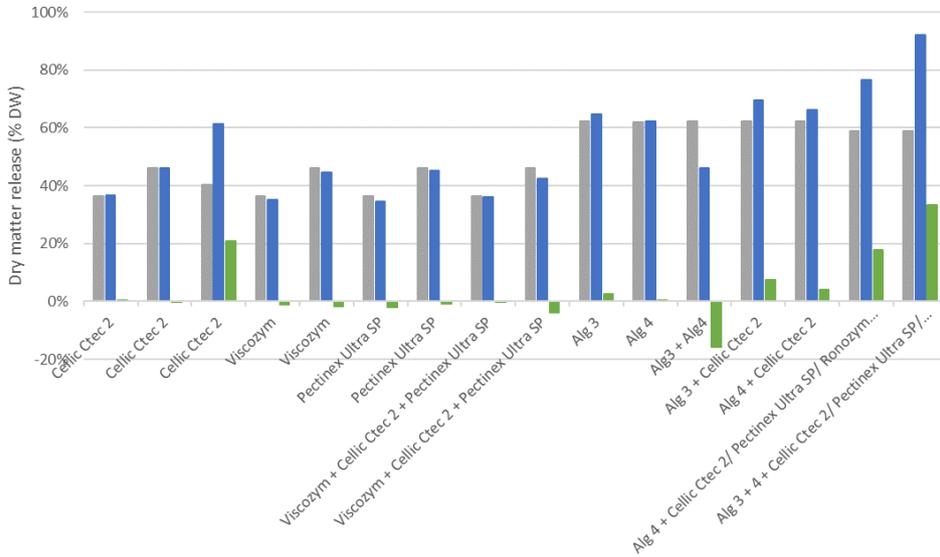


Figure 3-8 Dry matter release from Saccharina treated with carbohydrases. Grey without enzyme, blue with enzyme and green difference.

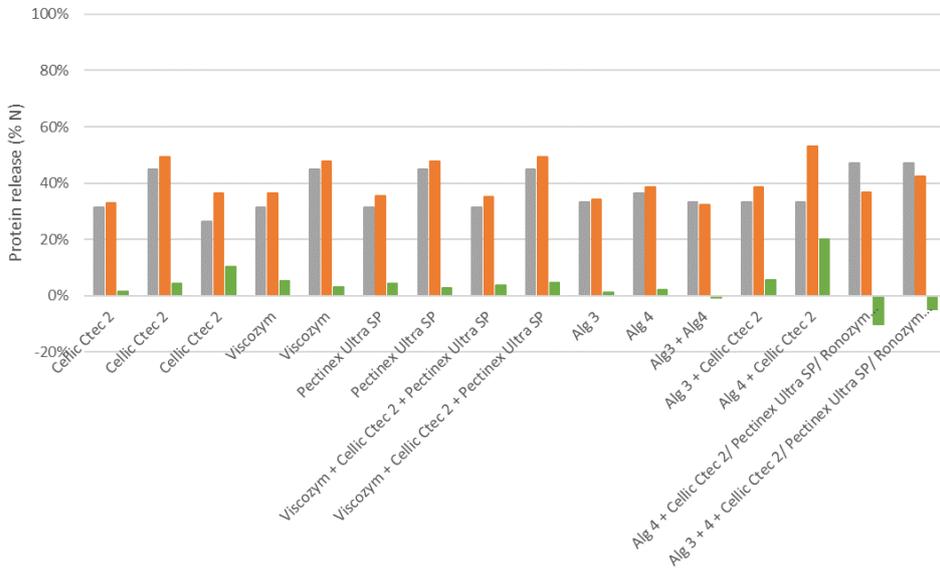


Figure 3-9 Protein release from Saccharina treated with carbohydrases. Grey without enzyme, orange with enzyme and green difference. Orange arrows indicate the most promising results.

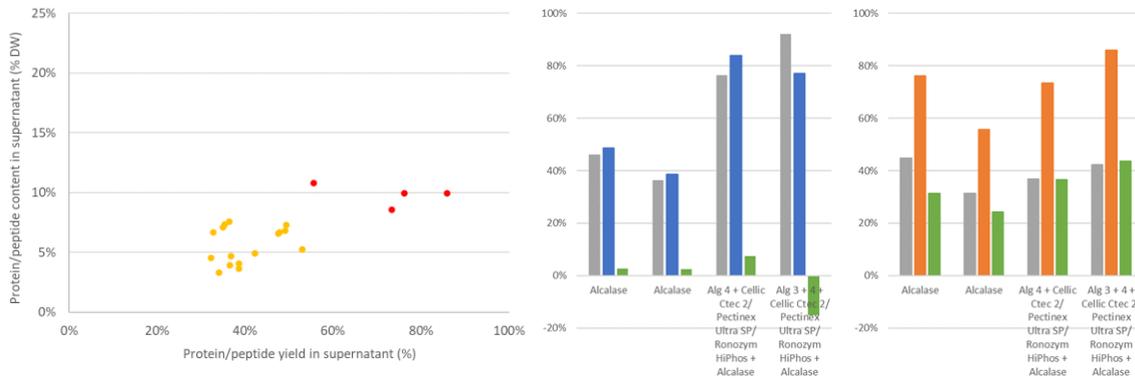


Figure 3-10 Left: protein and peptide (in case of alcalase) release after various enzymatic treatments (carbohydrases and protease). Middle: dry matter release (blank (grey), dry matter release (blue) and difference (green)). Right: peptide release (blank (grey), peptide release (orange) and the difference (green)).

The combined carbohydrase and proteases (alcalase) was also tested to determine if peptide release was enhanced by carbohydrase pretreatment. The results are given in Figure 3-10. The application of alcalase resulted in a limited increase in the total amount of material released, but with a 'preference' for peptides, leading to increased peptide content (7 to 10%). The peptide release results for when carbohydrases were applied together with alcalase were not conclusive, as the variation resulting from only the alcalase treatment was too large.

The results are summarized in Table 3-3. No substantial improvement with regard to protein release and content has been obtained compared to mechanical treatment and the previous tested carbohydrases. To increase the protein release further, without the use of proteases, a mild alkaline and temperature pretreatment prior to enzymatic treatment could be beneficial. Some preliminary experiments were carried out (Annex 4).

Table 3-3 Summary best results dry matter and protein (peptide in case of alcalase) release Saccharina using carbohydrases and proteases (+ = additional release compared to blank).

<i>Saccharina</i>	Dry matter release (%)	Protein/peptide release (%)	Protein/peptide content (%)
Cellic Ctec2 (55°C, pH5.5)	61 (+21)	37 (+10)	4
Alg4 + Cellic Ctec2 (75 & 55°C, pH6.5 & 5.5)	66 (+4)	53 (+20)	5
Alg3 + Alg4 + Cellic Ctec +Pectinex +Rohozym (75 & 50°C, pH6)	92 (+33)	42 (-5)	5
Alcalase (40°C, pH6)	49 (+3)	76 (+31)	10
Alg3 + Alg4 + Cellic Ctec +Pectinex +Rohozym + Alcalase (75 & 50°C, pH6)	77 (-15)	86 (+44)	10

3.4 Conclusions

After only mechanical treatment, the protein yield in the liquid fraction was comparable for both *Saccharina* as well as *Undaria* seaweeds (~35%). The protein content in *Undaria* was higher (14 vs 10% in *Saccharina*) which also resulted in a higher protein content (8 vs 5%) in the liquid fraction. These protein yields and protein contents are insufficient for a commercially viable recovery.

Carbohydrases were selected based on the polysaccharides present in the cell wall of brown seaweeds. However, no substantial improvement on protein yield and content were obtained. Under the microscope it appeared that the majority of the cells were still intact. An explanation may be that the complex carbohydrates in seaweeds differ from those in land plant to such an extent that commercially available carbohydrate degrading enzymes, being based on land plant complex carbohydrates, are not applicable in seaweeds. Carbohydrases that can break down complex carbohydrate compounds in seaweeds are currently not commercially available.

4 Technical functionality – WP 3

4.1 Introduction

Proteins are used in many different food products, for nutritional and functional reasons. The average nutritional requirement for adults is 0.66 g protein per kg body weight per day (EFSA, 2012; Institute of Medicine, 2005). For an individual weighing 65 kg, this amounts to 42.9 g per day. However, with the average requirement, half the population is ingesting too little protein. The recommended daily intake is therefore higher: 0.8 g per kg body weight per day. For the same individual of 65 kg, this would mean a protein intake of 52 g (EFSA, 2012; Institute of Medicine, 2005; Voedingscentrum, n.d.).

In many food products, proteins perform a functional role such as gelling, foaming or emulsifying. Gelling is important in meat products, sausages, cheese and baked goods. Foams can be found in bread, mousses, nougat and whipped cream. Emulsifying properties are essential in sauces, mayonnaise, cheese and sausages. The protein has to be soluble or at least dispersible (small protein aggregates instead of individual proteins in solution) to be able to fulfil these functional properties. Gelling is used as the functional property of choice. Gelation starts with the unfolding of a protein. This unfolding exposes hydrophobic patches that were previously in the interior of the protein. Aggregation and gelation takes place when the hydrophobic patches of different proteins connect. Since the unfolding is often heat-induced, denaturation temperature is an important parameter.

In this project, we are aiming for a functional, sustainable ingredient. The protein content of the final product (fraction) is not the primary concern, although it is expected that a higher protein content will result in better functional properties. To understand the gelation properties, solubility, denaturation temperature and gelation upon heating are measured. These functional properties are dependent on the conditions that are used. pH and salt content specifically are very important. Two pH values that are the extremes of the relevant range for food products (Figure 4-1): pH 4 (yoghurt, yoghurt-type drinks, mayonnaise) and pH 7 (eggs, cheese, fish, milk) were used.

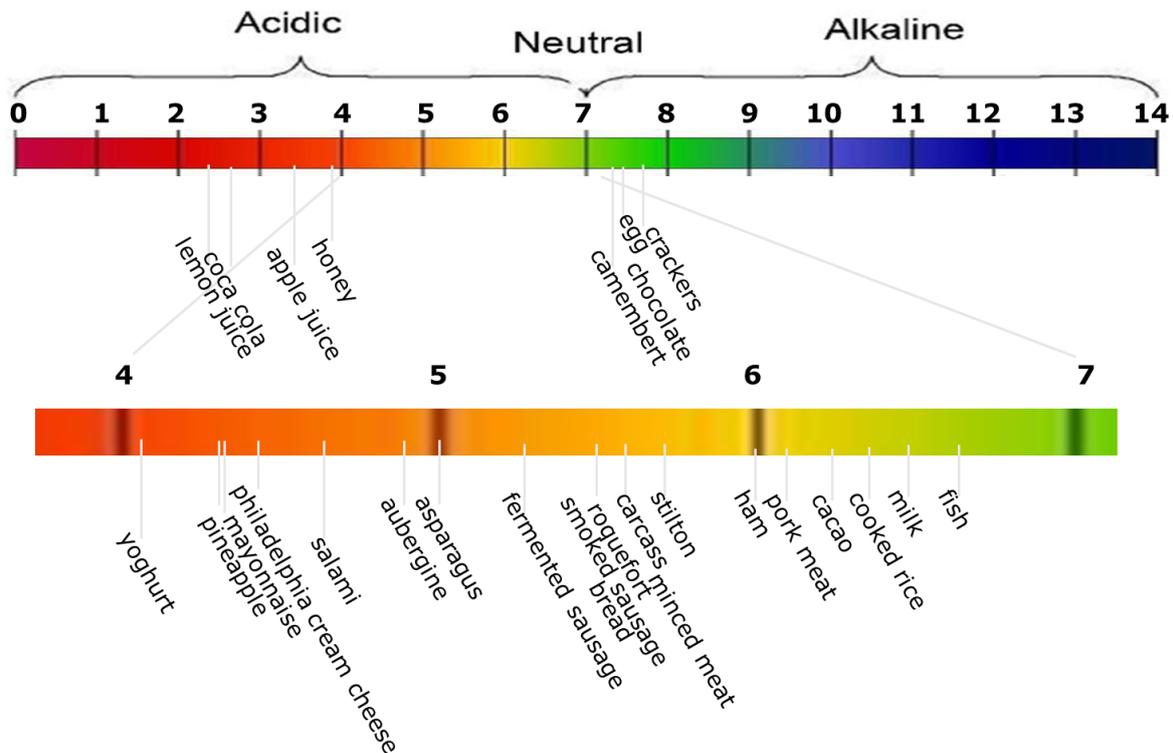


Figure 4-1 pH values of some food products (food-info.net, n.d.).

4.2 Materials and methods

Sample preparation

Crude *Saccharina latissima* extracts were prepared as described in Annex 5. 15% DW solutions were prepared using freeze-dried seaweed and set to pH 7. Penstrep was added (1.6% based on dry matter) to prevent microbial growth. The solution was split in two. Alcalase was added to one part (2.5% based on dry matter) to determine the contribution of protein to the gelling properties of the extract. Both parts were incubated for 40 h at 40°C while shaking. DMTA and DSC were determined.

Dispersibility

The 15% solutions were diluted to 2%, mixed for 2 min and centrifuged (45 min, 4000×g). Part of the supernatant was kept separate for protein solubility analysis with a Bradford assay (Bradford, 1976). The remaining supernatant was decanted in an aluminium tray. The supernatant and pellet in the Greiner tube were oven dried at 105°C. The dispersibility was calculated by dividing the amount of dry matter in the supernatant by the total amount of dry matter in the sample.

Differential scanning calorimetry

Thermal analysis was performed with a TA Instruments type Q200 (Delaware, U.S.A.) modulated differential scanning calorimeter. Samples (25 - 30 mg from the 15% samples prepared at pH 4 or 7 as described above) were weighed in high volume stainless steel cups, after which these were hermetically sealed. After an equilibration step of 5 min at 20°C, the temperature scan was performed in linear mode from 20 to 160°C with a rate of 5°C/min. During this scan the heat flow was measured. The onset of protein denaturation (T_{onset}) and peak temperature (T_{peak}) were determined using the analysis tool available in the Universal Analysis software (TA Instruments). Measurements were performed in triplicate, and results are presented as mean values.

Gelation

Gelation kinetics were studied as a function of time using a stress-controlled Discovery HR-2 rheometer TA Instruments (Delaware, U.S.A.). A concentric cylinder system with a bob diameter of 14 mm was used. A temperature range was applied in which a stabilization of 5 min at 25°C was followed by a temperature increase from 25 to 95°C with a heating rate of 5°C/min. It was kept at this temperature for 1 h (while measuring G' and G'' using a strain 10–3, frequency 1 Hz) before cooling back to 25°C. The applied strain was within a linear region. G' , G'' , and $\tan \delta$ were measured during both steps as a function of time. At the end of the measurement a strain sweep was performed from 0.001 to 1 (25°C). Samples were covered with a thin layer of paraffin oil to prevent evaporation. Because of limited amount of sample, the measurements were done only once.

4.3 Results

4.3.1 Sample properties

In Table 4-1 the composition of the starting material and crude *Saccharina* extracts are shown.

Table 4-1. Composition of *Saccharina* starting material and crude extract (Annex 5) (dry).

Material	% DW (105°C)	% Protein	% ash (550°C)	% ash (900°C)
<i>Saccharina</i> starting material	98	10	28	13
<i>Saccharina</i> crude extract	98	5	15	5

Both the starting material and the extract are low in protein. For *Saccharina*, the total minerals including carbonates (ash at 550°C) is decreased after extraction, as is the mineral content excluding carbonates (ash at 900°C). The amount of ash, associated with salt, is rather high. For that reason, it was decided not to vary the salt content and to only measure the samples without added salt.

4.3.2 Dispersibility and protein solubility

Dispersing the samples at 15% dry weight yielded a dark coloured, strongly smelling solution (Figure 4-2). Centrifugation (after dilution) yielded a very loose pellet.

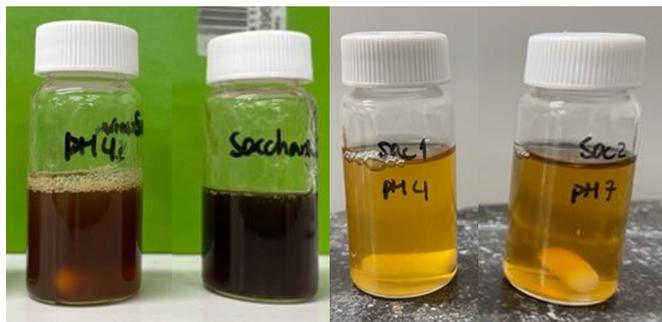


Figure 4-2 Extract dispersions. From left to right: 15% DW, pH 4; 15% DW, pH 7; 2% DW, pH 4; 2% DW, pH 7.

The dispersibility of the material was good: all was dispersed (Figure 4-3, left). The numbers slightly over 100% are probably due to the presence of a coating in the Greiner tubes where the experiments are performed in, that is removed upon heating. While only a few mg large, with the small sample size used in these experiments and the complete solubility of the samples it results in a more than 100% dispersibility. The protein solubility was better at pH 7 than at pH 4. An estimated 13% of the total protein dissolved at pH 7 (Figure 4-3, right). These numbers are rough estimates. The assay is based on a colouring reaction that is different for every protein. The reference protein, BSA, is very different from the proteins studied here. To quantify the solubility further a calibration curve is needed. Still, the numbers serve well as a first estimate.

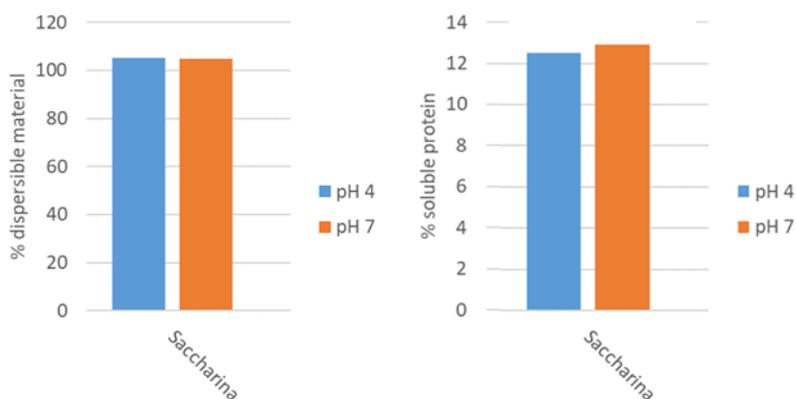


Figure 4-3 Dispersibility (left) and protein solubility (right) as % total protein at pH 4 and pH 7.

4.3.3 Protein denaturation temperature

Dynamic scanning calorimetry of the 15% protein solutions yielded no peaks (Figure 4-4).

One of the proteins that are known to be present in seaweeds and other plants is RuBisCO. RuBisCO is known to have slightly different properties between species. It is reported to have denaturation temperatures between 61°C and 68°C (Nieuwland et al., 2021), and for duckweed protein the peak disappears when setting the pH to 4, indicating denaturation of the protein under those conditions. While the absence of a peak at pH 4 may be a result of denaturation, it is more likely that a more concentrated solution is required to determine the denaturation temperature of the proteins in the crude extract, since also at pH 7 no peaks are present. The very low protein concentration of 0.75% in these samples is in line with this hypothesis.

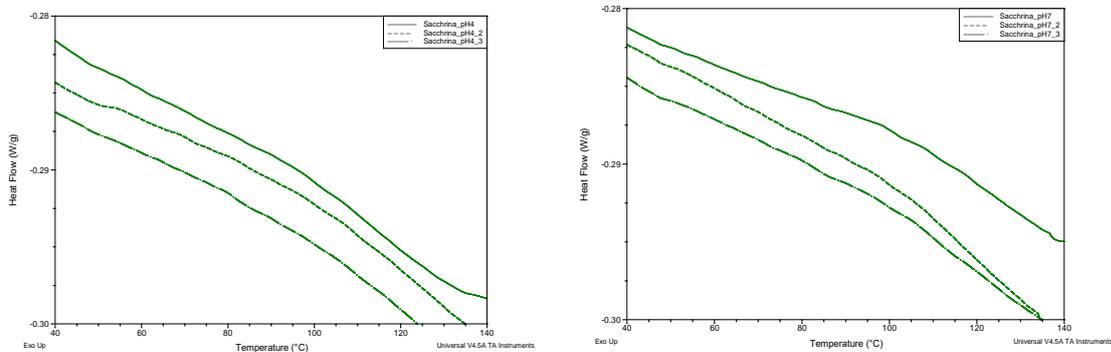


Figure 4-4 DSC results for crude Saccharina extract at pH 4 (left) and 7 (right).

4.3.4 Gelling

Two experiments were performed to measure gelling properties of the crude extract. In the first experiment, the extract was analyzed at two different pH values. In a second experiment, the protein contribution to the gel was assessed by enzymatically removing the protein.

Gelling properties were measured at pH 4 and pH 7, both on samples with a dry matter content of 15%. The storage modulus (G') of the gelling curves is shown in Figure 4-5. In this figure it is clear that the samples at pH 7 have a lower starting viscosity and a higher final gel strength than those at pH 4. However, the most important observation is that the gels are very weak. For example, duckweed protein extracts of about 15% DW (10% protein) have a final gel strength of 4 kPa at pH 4 and almost 10 kPa at pH 7 (Nieuwland et al., 2021), over 100x as much as reported for these extracts. Soy protein isolate is shown in the same article to have a final gel strength of over 45 kPa at the same dry matter content. However, both of these extracts have a much higher protein content than the seaweed extracts. To have a functional protein extract, the protein concentration thus needs to be increased.

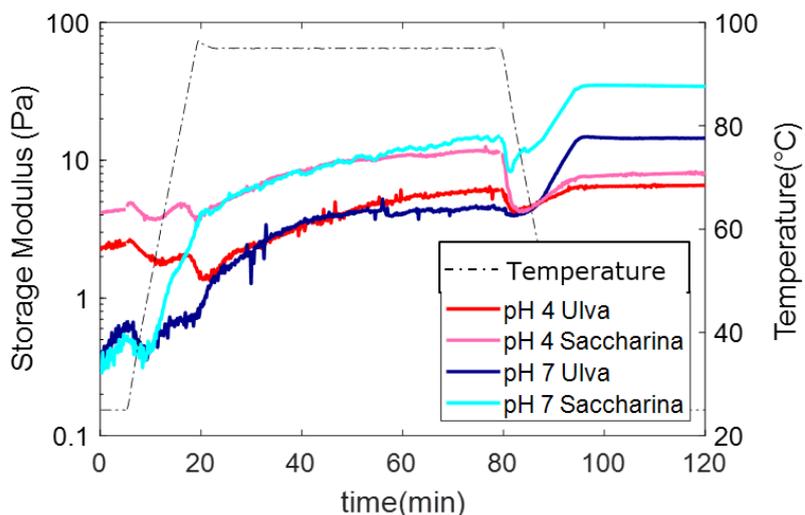


Figure 4-5 Storage modulus of the gelling experiment.

It is expected that the proteins are the components in the extract related most to the gelling. To check that assumption, the extracts were treated with alcalase to remove all protein related functionality. The difference in functionality between the original and the final extract would be attributed to the protein functionality. As a reference, the extract was treated in the same way (temperature treatment) without addition of alcalase. These experiments were performed in duplicate at pH 7. The results are shown in Figure 4-6. From these experiments, it can be concluded that the protein has an effect on the gel strength, since the gel strength decreases after alcalase treatment. These results show that the protein part of the extract has a clear influence on gelling properties. It is expected that the gelling properties will improve with increasing protein content of the extract.

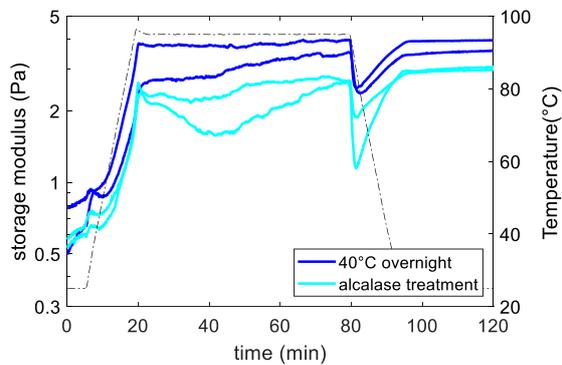


Figure 4-6 Gelling properties of Saccharina extract, without alcalase treatment (dark blue) and with alcalase treatment (light blue).

4.4 Conclusions

Crude extracts from *Saccharina latissima* were tested for technical functionality. The crude extracts were obtained by pressing *Saccharina*, removing minerals using ultrafiltration and diafiltration, followed by freeze-drying. The protein content was low (5%), the dispersibility was good (100%) and the protein solubility (12%) was low. The denaturation temperature could not be determined because of the low protein content. Gelling was observed and the gel strength increased upon heating. By converting the proteins to peptides, the contribution of proteins to the gelling properties was ascertained.

5 Conclusions and recommendations

Red and green seaweeds are relatively closely related to plants, while brown seaweed are evolutionary more different from plants. Cell walls in land plants and seaweeds have comparable functions. However, compared to those in land plants, seaweed cell walls have to withstand much larger tensile forces coming from moving water and therefore seaweed cell walls are 100-1000 times less stiff. These differences are reflected in the chemical composition of the cell wall: primary cell walls of plants and brown seaweed chemically differ by over 80% on molecular level. Furthermore, cell walls from green, red and brown seaweed are composed of different polysaccharides. The main polysaccharides present in brown seaweeds are alginate, fucoidan and cellulose.

In seaweed, like in plants, the majority of cellular nitrogen is invested in the photosynthetic machinery: the blades are the most nitrogen-rich organs. During winter, when NH_4^+ , NO_2^- , NO_3^- , urea and amino acids in seawater are high, reserves are accumulated. This nitrogen can be stored in blade tissues or meristems and can be transported between tissues when nitrogen is required. Nitrogen reserves are utilized for growth in winter and through spring and depletion of reserves follows disappearance of nitrogen in seawater with a 2-months lag. Due to the accumulation of carbohydrates during summer, protein content decreases as summer progresses. Consequently, young blades formed during spring generally have highest protein content.

After only mechanical treatment, the protein yield in the liquid fraction was comparable for both *Saccharina* as well as *Undaria* seaweeds (~35%). The protein content in *Undaria* was higher (14 vs 10% in *Saccharina*) which also resulted in a higher protein content (8 vs 5%) in the liquid fraction. These protein yields and protein contents are insufficient for a commercially viable recovery. Carbohydrases were selected based on the polysaccharides present in the cell wall of brown seaweeds. However, no substantial improvement on protein yield and content were obtained. Under the microscope it appeared that the majority of the cells were still intact. An explanation may be that the complex carbohydrates in seaweeds differ from those in land plant to such an extent that commercially available carbohydrate degrading enzymes, being based on land plant complex carbohydrates, are not applicable in seaweeds. Carbohydrases that can break down complex carbohydrate compounds in seaweeds are currently not commercially available.

Crude extracts from *Saccharina latissima* were tested for technical functionality. The crude extracts were obtained by pressing *Saccharina*, removing minerals using ultrafiltration and diafiltration, followed by freeze-drying. The protein content was low (5%), the dispersibility was good (100%) and the protein solubility (12%) was low. The denaturation temperature could not be determined because of the low protein content. Gelling was observed and the gel strength increased upon heating. By converting the proteins to peptides, the contribution of proteins to the gelling properties was ascertained.

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Annex A Literature overview protein type and concentration in seaweed

Aim of this literature overview was to collect information on the type of proteins, their concentration and if possible their location in the different type of seaweed. Seaweed of interest were *Saccharina* (brown), *Palmaria* (red), *Ulva* (green) and *Undaria* (brown).

Patent WP 2019/115671/A2 Method for cascaded processing of fresh algae

Filing date 13 December 2018
 Priority date 15 December 2017
 Applicant Sabidos B.V.
 Inventor Theo Verleun

Example 4: Large scale processing of fresh *Ulva* for isolation of protein and production of biogas in a cascaded process

Washing with seawater Excess of water, 76 kg wet seaweed, 20°C
 Low speed centrifugation Not specified
 Mixing with demineralized water 1 kg seaweed with 1 L water
 Chopping and adding water 1 mm² and 1 L water
 Within three hours after harvest.....
 Enzyme treatment 24 h (76 kg in 600 L demineralized water)
 Enzyme mixture "A", 20°C, pH as is
 Gentle agitation

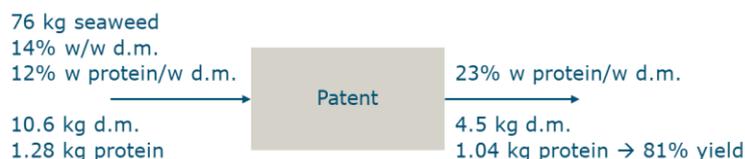
Enzyme mixture A (all from DSM)

- 2 mL cellulase (Filtrase BRX)
- 2 mL xylanase (Filtrase NLC)
- 2 mL amylase (MATS classic)
- 2 mL phytase (Phytase 5000L)
- 2 mL phospholipase A2 (Purifinae PLA2)
- 90 mL demineralised water
- 2 g β-glucanase/endo xylanase (Battonage, Oenobrands, France)

Dosage: 500 mL / 1000 kg d.m. seaweed

S/L separation 4x folded cheese cloth
 Spray drying Box dryer (inlet 180°C, outlet 94°C)

Mass balance



Protein characterization and compared to protein obtained from heat-dried *Ulva*. SDS-page showed more intact protein. The solubility was higher and was less sensitive for pH. Different iso-electric point (6 versus 8). The salt sensitivity was low.

kDa area	GOA-protein profile (ex-fresh <i>Ulva</i>)	Protein profile (ex-dried <i>Ulva</i>)	Ratio Fresh vs dried material	pH	% soluble protein from dried	% soluble protein from fresh
					<i>Ulva</i>	GOA
58-62	2.5 %	-	N.A	3	22 – 24	75 – 77
50-55	9.5 %	8 %	1.2	4	16 – 18	78 – 80
40-45	14 %	7.5 %	1.9	4.5	13 – 15	73 – 76
27-30	71%	39%	1.8	5	16 – 18	76 – 77
18-22	-	18.5 %	N.A	6	5 – 7	73 – 74
<6	3%	27%	0.1	7	11 – 13	72 – 73
				8	18 – 19	66 – 69
				9	26 – 28	75 – 77

Biochemical composition of two red seaweed species grown on the Brazilian coast (Gressler *et al.*, 2011)

Different seaweed than the one we will use: *Plocamium brasiliense* and *Ochtodes secundiramea*. Evaluated as having the same nutritional value of *Palmaria*. The total lipid contents (dry weight) were 36.3 and 35.4 g/kg; fatty acid contents were 9.3 and 12.1 g/kg; total nitrogen contents were 37.4 and 24.9 g/kg; soluble protein contents were 157.2 and 101.0 g/kg; amino acid contents were 127.5 and 91.4 g/kg; and total carbohydrate contents were 520.3 and 450.7 g/kg for *P. brasiliense* and *O. secundiramea*, respectively. Based on amino acid composition, the protein content would be more around 95 and 127 g/kg protein.

Seaweed	Protein	Amino acid	Carbohydrate	Total lipid	PUFA (% of total FA)	$\omega 3/\omega 6$ FA
	(g kg ⁻¹ dry weight)					
P.b.	157.2	127.5	520.3	36.3	20.7	0.7
O.s.	101.0	91.4	450.7	35.4	8.6	4.0
L.j.	91 ¹⁵	87 ¹⁴	680 ¹⁵	58 ⁶⁰	61.3 ⁶⁰	1.2 ⁶⁰
P.p.	135 ^{61*}	131 ⁶²	630 ^{61*}	20 ⁶³	54.2 ⁶³	9.6 ⁶³

* Results chosen in the same period of algal collection.

Fatty acids, total lipid, protein and ash contents of processed edible seaweeds (Sanchez-Machado *et al.*, 2004)

Protein content calculated with N x 6.25.

Seaweed	Protein ^a	Ash ^a	Lipid ^a
Canned seaweeds			
<i>Himanthalia elongata</i>	10.95 ± 0.27	22.98 ± 0.60	0.93 ± 0.05
<i>Saccorhiza polyschides</i>	13.10 ± 0.12	26.58 ± 0.65	0.70 ± 0.09
Dried seaweeds			
<i>Himanthalia elongata</i>	5.46 ± 0.16	26.78 ± 0.24	0.97 ± 0.07
<i>Laminaria ochroleuca</i>	7.49 ± 0.12	29.47 ± 1.05	0.92 ± 0.01
<i>Undaria pinnatifida</i>	18.00 ± 1.46	31.24 ± 0.22	1.05 ± 0.01
<i>Porphyra</i> sp.	24.11 ± 1.03	19.07 ± 0.61	1.03 ± 0.04
<i>Palmaria</i> sp.	13.87 ± 0.28	34.00 ± 0.11	1.80 ± 0.14

Nutritional value of proteins from edible seaweed *Palmaria palmata* (Dulse) (Galland-Irmouli *et al.*, 1999)

Protein content calculated with N x 6.25. They also investigated the seasonal variation. Spring showed the highest amount of protein.

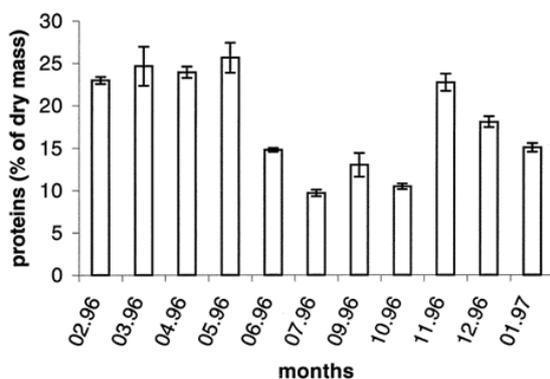


Table 2 Protein content of some algae compared with *Palmaria palmata**

Algae	Protein (% of frond)
<i>Porphyra tenera</i>	47.5
<i>Analipus japonicus</i>	23.7
<i>Grateloupia turuturu</i>	20.0
<i>Palmaria palmata</i>	18.3 ± 5.9
<i>Ulva pertusa</i>	17.5
<i>Laminaria japonica</i>	15.6
<i>Codium fragile</i>	15.6
<i>Eisenia bicyclis</i>	13.1
<i>Undaria pinnatifida</i>	12.5

Heat treatment increases the protein bioaccessibility in the red seaweed dulse (*Palmaria palmata*), but not in the brown seaweed winged kelp (*Alaria esculenta*) (Maehre et al., 2016)

The dried seaweed samples (n=5 for each species) were cut into pieces of 2×2 cm and divided into four different batches. One of the batches remained raw, while the other three were subjected to boiling in distilled water (1:20 w/v) for 15, 30, and 60 min. After boiling, the samples were transferred to a sieve for removal of excess water, and following cooling, they were weighed in order to define the uptake of water during boiling. Maybe an idea to try different heat treatment to increase the protein extractability.

Extraction of protein from the macroalga *Palmaria palmata* (Harnedy & Fitzgerald, 2013)

The most prominent area of staining being observed around 20 kDa. This region may contain subunits of phycoerythrin (240 kDa) or other phycobiliproteins such as phycocyanin. Phycoerythrin, which is a highly pigmented protein found in Rhodophyta, is composed of three subunits (a, b and g) with molecular masses of 20 and 21 and 31 kDa, respectively. The former two subunits (a and b) which were observed at high intensity on the SDS-PAGE gel are considered in the literature as being the major subunits in phycoerythrin (lane 2). A less intense protein band with an approximate molecular mass of 31 kDa (presumably due to the g subunit) was also seen on the gel (lane 2). Protein band(s) with molecular weights of approximately 16-18 kDa were also shown to be present in abundant quantities in the alkaline soluble protein extracts (lanes 3 & 4). Protein bands with apparent molecular weights of 14.7, 35.0 and 55 kDa were also seen in the alkaline extract (lanes 3 & 4). The position of these bands is indicated in Fig. 2. However, the identity of these protein bands is unknown: this actually could be RuBisCO 15 and 55 kDa), but in this case it seems to be present in a very small amount.

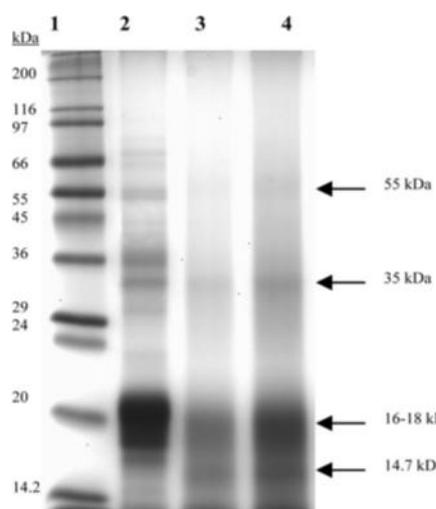


Table 5
Comparison of the concentration of aqueous, alkaline and total protein extracted from milled oven-dried *Palmaria palmata* by osmotic shock, shear force and polysaccharidase aided macroalgal cell disruption methods.

Method of cell disruption	Extraction process	Protein recovery (g protein/100 g dry weight)
Osmotic shock	Aqueous	1.02 ± 0.07
Osmotic shock	Aqueous & alkaline (sequential)	6.77 ± 0.22
Osmotic shock	Aqueous & alkaline (simultaneous)	4.30 ± 0.14
High shear force	Aqueous	0.74 ± 0.02
High shear force	Aqueous & alkaline (sequential)	6.92 ± 0.12
High shear force	Aqueous & alkaline (simultaneous)	3.83 ± 0.22
Polysaccharidase	Aqueous/protein released by action of polysaccharidase activity	3.18 ± 0.29
Polysaccharidase	Aqueous/protein released by action of polysaccharidase activity & alkaline (sequential)	11.57 ± 0.08
None – direct	Aqueous & alkaline (simultaneous)	4.16 ± 0.17

Simultaneous extraction of proteins and DNA by an enzymatic treatment of the cell wall of *Palmaria palmata* (Rhodophyta) (Joubert & Fleurence, 2008)

Fig 1. As the cell wall contains cellulose and xylans, we used one cellulase (Celluclast®) and one xylanase (Shearzyme®) for enzymatic digestion. Fig 3. Spectrophotometry and phycoerythrin content evaluation. The spectral analysis of the supernatants was performed with a spectrophotometer at wavelengths between 400 and 600 nm. Using Beer Lambert’s law, the phycoerythrin extinction coefficient of 2.106 M⁻¹.cm⁻¹ at 565 nm and the phycoerythrin molecular weight of 240,000 Da, the amount of phycoerythrin in the supernatant was estimated. 17-20% protein was extracted.

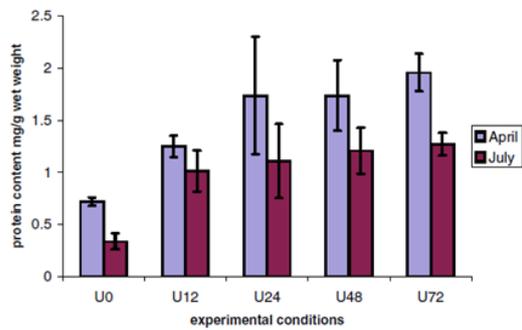


Fig. 1 Protein content in control (U₀) and experimental samples following the enzymatic procedure (U₁₂=12 U Celluclast+12 U Shearzyme, U₂₄, U₄₈, U₇₂). Means ± confidence interval (95%)

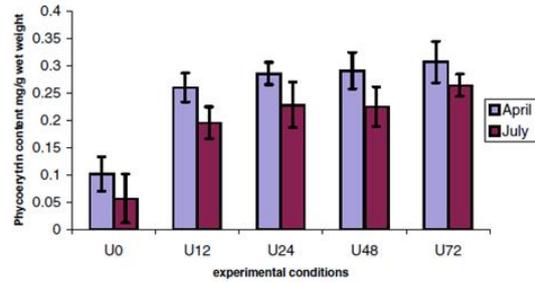


Fig. 3 Phycoerythrin content in control (U₀) and experimental samples following the enzymatic procedure (U₁₂=12 U Celluclast+12 U Shearzyme, U₂₄, U₄₈, U₇₂). Means ± confidence interval (95%)

Optimization of hydrolysis conditions of *Palmaria palmata* to enhance R-phycoerythrin extraction (Dumay *et al.*, 2013)

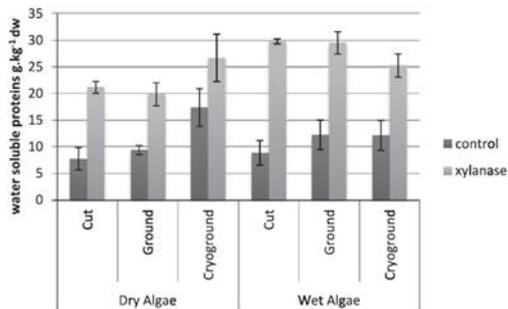


Fig. 3. Effect of pretreatment methods on water-soluble protein content of *Palmaria palmata*.

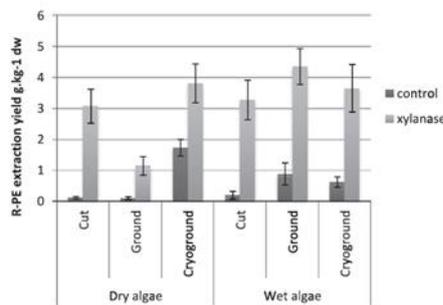


Fig. 2. Effect of pretreatment methods on R-PE extraction yield of *Palmaria palmata*.

R-PE concentration and purity were determined spectrometrically using the Beer and Eshel equation (1) (Beer and Eshel, 1985) and the $A_{565\text{nm}}/A_{280\text{nm}}$ ratio (=R-PE Purity Index or PI), respectively (Galland-Irmouli *et al.*, 2000; Liu *et al.*, 2005). R-PE yield was expressed as $\text{mg g}^{-1} \text{ dw}$.

$$[\text{R-PE}] = [(A_{565} - A_{592}) - (A_{455} - A_{592}) \times 0.20] \times 0.12 \quad (1)$$

The R-PE absorption spectra displayed three peaks: two at 495 and 545 nm and one main peak at 565 nm; 455 and 592 nm constituted the lower wavelengths. The spectral profile is commonly used to indicate the non-degradation of R-PE.

Roughly cut wet algae provides the most interesting results in terms of extract quality and economic cost. Optimization of hydrolysis conditions led to less time and enzyme-consuming process.

Palmaria palmata as an alternative protein source: enzymatic protein extraction, amino acid composition, and nitrogen-to-protein conversion factor (Bjarnadóttir *et al.*, 2018).

The red seaweed *Palmaria palmata* has previously been reported to have high protein content high in essential amino acids. To extract the proteins a rigid cell wall consisting mainly of β -(1→4)/ β -(1→3)-D-xylans must be disrupted. The conversion factor was 4.7 for the untreated sample.

Evaluation of the *in vitro* biological activity of protein hydrolysates of the edible red alga, *Palmaria palmata* (dulse) harvested from the Gaspé coast and cultivated in tanks (Beaulieu *et al.*, 2016)

Using liquid chromatography-tandem mass spectrometry (LC-MS/MS) with database searches, protein precursors such as protein ribulose-1,5-biphosphate carboxylase/oxygenase (RuBisCo) enzyme, allophycocyanins and phycocyanins, were identified in <10 kDa HF.

Extraction and quantification of phycobiliproteins from the red alga *Furcellaria lumbricalis* (Saluri et al., 2019)

At different photosynthetic molecule ratios, the colour of the algae may vary from purple to green or black. The red colour of these algae results when chlorophyll and other photosynthetic pigments are masked by phycobiliproteins (PBPs), mainly phycoerythrin and phycocyanin. PBPs are proteinic pigments comprising of α , β and occasionally γ subunits with a proteinic part, energy transferring tetrapyrrolic open-chain chromophores (phycobilins) and other non-protein moieties. These hydrophilic accessory pigments (red-coloured phycoerythrins and blue-coloured phycocyanins or allophycocyanins) are connected via linker-polypeptides to macromolecular structures – phycobilisomes – which are covalently bound to the stromal surface of the photosystem II core in the thylakoid membrane. B-phycoerythrin (B-PE, first extracted from red algal order Bangiales), R-phycoerythrin (R-PE, first extracted from Rhodophyceae), C-phycocyanin (C-PC, first extracted from cyanobacteria) and allophycocyanin (APC) are among the brightest fluorescent pigments ever. However, phycobiliproteins lose their fluorescence gradually at elevated temperatures, partially limiting their uses. The pigment-protein complexes are stable at temperatures ranging from 4°C up to 40°C, but show a significant reduction of absorption after heating above 60°C for 1 h. Also, PBPs are most stable at pH 6–7 with significant absorption reduction at pH levels lower than 4 or higher than 10. The most common pretreatment method used for the extraction of R-PE, to date, is based on mechanical cell wall breakage, which is usually done with the aid of liquid N₂. However, the yield can be further increased with different techniques, such as ultrasonication in combination with different enzymes.

Current knowledge and challenges in extraction, characterization and bioactivity of seaweed protein and seaweed-derived proteins (Pliego-Cortes et al., 2020)

The **protein content** in seaweeds can reach up to 50% of dry weight. For example, in the human edible red seaweeds *Porphyra sp.* 44%, *Palmaria palmata* 35%, and *Chondrus crispus* 19.5%, while the green *Ulva sp.* 23%, and the brown *Undaria pinnatifida* 19% DW. Generally, the protein content is higher in red species (20-47% DW) than green (9%-6% DW) and brown (3%-15% DW). However, seaweeds may contain non-proteinic nitrogen, obtained from nitrates, pigments or nucleic acids, and resulting in an over-estimation of their protein content, which usually is estimated by the general Nitrogen-to-Protein conversion factor of 6.25. Therefore, a Seaweed-Nitrogen-to-Protein (SNP) conversion factors of 5.0 is proposed based on the analysis of 103 species of seaweeds.

Type of enzymes: (i) Alkaline phosphatase is a Zn-containing metalloproteinase that catalyses the non-specific hydrolysis of phosphate monoesters. This enzyme is widely distributed in seaweeds. (ii) Alternative oxidases (AOX) proteins has been described for electron flow through electron transport chain and regulation of mitochondrial retrograde signalling pathway. (iii) The fibrinolytic enzymes has been isolated from the green alga, *Codium latum*, and *Codium fragile*, which is a trypsin-like serine protease with a high substrate specificity. (iv) The bifunctional enzyme RuBisCO, which is known to catalyse carbon dioxide fixation and oxygenation, was reported in *S. chordalis* and *K. alvarezii*. Both red algae.

Glycoprotein. The high-mannose type N-glycans are linked to GP expressed in different species of seaweeds. GP are located on the cell wall, on the cell surfaces or they are secreted, and their functions in plants include recognition, intercellular interactions, and adhesion. Currently, a few GP have been isolated from seaweeds, and their structures and functional roles are yet to be investigated. In *Ulva sp.* three glycoprotein-rich fractions were obtained, UvGP-1 (hot water extraction), and UvGP-2-DA and UvGP-2-DS (cold-water extraction). Lectins are proteins or glycoproteins of non-immune origin, containing at least one non-catalytic domain that binds reversibly to specific mono- or oligo-saccharides without modify the carbohydrates that they bind. Lectins precipitate glycoproteins and agglutinate cells. In seaweeds, lectins are involved in gamete recognition and reproductive cell fusion. Seaweeds are good sources of novel lectins, such as *Griffithsin*, a mannose-specific lectin isolated from the red alga *Griffithsia sp.*, or Sfl-1 and Sfl2 from *Solieria filiformis*, or HRL40 from *Halimeda renshii*.

Cell wall attached protein. Arabinogalactan proteins (AGPs) belong to the hydroxyproline-rich glycoproteins (HRGP), they are highly glycosylated proteins reported in the cell wall of few species of seaweeds. AGP are implicated in terrestrial plant developmental process but their roles in seaweed has been yet poorly known. The presence of HRGP was confirmed by immunolabeling and by the b- Glc

Yariv reactive in the green seaweeds *Codium vermilara* and *C. fragile*, in which an arabinose-rich fraction showed a furanose a-arabinosyl structure.

Phycobiliproteins (PBPs) are the main light-harvesting pigments in red seaweeds and the only water-soluble algal pigments, representing up to 20% DW. PBPs are grouped into four classes: phycoerythrin (PE), phycocyanin (PC), phycoerythrocyanins (PEC), and allophycocyanin (APC). PE is the main pigment, and it is divided into R- for Rhodophyta (R-PE) and B- for Bangiales (B-PE). Biliproteins are ensemble of a and b subunits, and only R-PE possess an extra g subunit. Isolation of PE has been reported in many species, for example *Gelidium pusillum*, *Grateloupia turuturu* and *R. pseudopalmeta*.

Protein extraction. Extraction with alkaline solubilization, isoelectric precipitation and frozen/thawing showed higher protein concentration in *Porphyra umbilicalis* (71%), *S. latissima* (51%), and *U. lactuca* (40%) compared to water extraction. Precipitation of solubilized proteins at pH 2 yielded 34.5% of proteins, and osmo shocking with 60 vol of water gave 59% yield in *S. latissima*. Glycoproteins (GP) and lectins are commonly extracted using water or buffers, followed by ethanolic, acidic or ammonium sulphate precipitation, such as the GP from *Laminaria japonica*. Enzyme-Assisted Extraction (EAE) has been applied by using enzymes to assist and to extract proteins, and hydrolysates from seaweeds. The use of commercially enzymatic preparations is frequently used with limited practices using specific enzyme on seaweeds, such as κ -carragenase or b-agarase. In a recent study, protein extraction from *Chondracanthus chamissoi* and *Macrocystis pyrifera* was enhanced using a cellulose cocktail (Cellic CTec3, Novozymes), producing 452 and 616 mg/g DW of protein. This cocktail contained multiple hydrolytic activities (cellulase, hemicellulase and b-glucosidase) for carrageenan, agar, alginate and cellulose. Ultrasound assisted extraction protein extraction using Ultrasound-Assisted Extraction (UAE) during 2 h followed by ion exchange purification yielded a protein content of 70% in *Ulva sp.* and 86% in *Gracilaria sp.* This procedure was up-scalable and suitable to obtain a 'food-grade' product. The effect of UAE can be attributed to bubble cavitation, facilitating the degradation of biological matrices. The Pulsed Electric Field (PEF) extraction uses electric potential generated from an electric field using high voltage (kV) and different duration time in the range of microseconds to milliseconds, to disrupt cell membranes. PEF treatments in *U. lactuca* showed that the higher content of protein (15% DW) was achieved at an electric field strength of 7.5 kV/cm, with two pulses of 0.05 ms, and a specific energy input of 6.6 kWh/kg protein.

Protein characterization. SDS-PAGE is performed to identify the molecular weight of dominant protein sub-units bands. The Tris-Glycine SDS-PAGE stained with silver nitrate allowed to identify that the protein profile of *Ulva sp.* varies with seasons, showing sharper protein bands during October and November than September and March. SDS method was effective to recognize five proteins bands in *Himantalia elongata* with molecular weights of 71, 53, 43, 36 and 27 kDa. R-PE purified in protein extracted from *Furcellaria lumbricalis* analysed by denaturing SDS-PAGE, allowed to identify a band near the 25 kDa region. The periodic acid- Schiff (PAS) staining after SDS-PAGE was used to detect glycoproteins in *L. japonica*.

Annex B Nitrogen in seaweeds

In seaweed, like in plants, the majority of cellular nitrogen is invested in the photosynthetic machinery to facilitate the assimilation of carbon (Hurd *et al.*, 2014 chapter 5). As such, the blades, which contain the photosynthetic machinery, will be the most nitrogen-rich organs within seaweed. This nitrogen is divided amongst the light harvesting antennae, photosystems, photoassimilatory pathway (RuBisCO a.o.) and the photorespiratory pathway (Hurd *et al.*, 2014 chapter 5; Evans & Clarke, 2019). Light harvesting antennae contain the light-absorbing pigments that upon excitation transfer electrons to the photosystems, which convert this light energy into non-storable chemical energy. This chemical energy is subsequently used by the photoassimilatory pathway, which includes ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO), to assimilate CO₂ into sugars that can subsequently be used to construct storable carbohydrate structures. However, RuBisCO can bind both O₂ and CO₂ (Hurd *et al.*, 2014 chapter 5; Hagemann & Bauwe, 2016). To recycle the carbon structures that assimilated O₂ instead of CO₂, plants, green and red seaweed make use of the photorespiratory pathway.

In land plants, of the photosynthetic machinery, RuBisCO is generally considered to be the most abundant protein, as it has been reported to constitute up to 65% of the total soluble protein (Ellis, 1979). A more recent report, based on total cellular protein, stated that on average 54% is allocated to photosynthesis (Evans & Clarke 2019). Proteins related to light harvesting complexes, photosystems, RuBisCO and photorespiration make up 6, 8, 20 and 3% of the total cellular protein, respectively, leaving 17% of other protein related to photosynthesis. However, these numbers are averages and can vary considerably, depending on species, presence of carbon concentrating mechanisms (CCMs), environmental conditions and seasons (Hurd *et al.*, 2014 chapter 5; Evans & Clarke, 2019).

Regarding seaweed species, there are major differences in the pigments associated to the light harvesting complex. Green seaweeds make use of chlorophylls and carotenoids (Hurd *et al.*, 2014; Dumay & Moranças, 2016). Chlorophylls and carotenoids have a long fatty acid tail and, as such, are membrane soluble. They bind to membrane proteins that form the light harvesting complexes (Barros & Kühlbrandt, 2009). While chlorophyll contains nitrogen in its molecular structure, this nitrogen is not readily available. In land plants, during senescence (the active process of biological aging or deterioration), after removal of the long fatty acid tail, the reactive core is deactivated and transported to the vacuole (Schelbert *et al.*, 2009). Red seaweeds make use of phycobiliproteins, a family of protein-pigment complexes that are water-soluble, stabilized by linker peptides and bound to membranes (Hurd *et al.*, 2014; Dumay & Moranças, 2016). Phycobiliproteins are structured into large light harvesting antennae complexes called phycobilisomes that in red seaweeds are the predominant source of protein, varying from 20-60% of the TSP (Jahn *et al.*, 1984). Phycobiliproteins have various documented extraction possibilities (Sekar & Chandramohan, 2008). Brown seaweeds predominantly use carotenoids and chlorophylls, which are organized in light harvesting complexes presumably similarly to those in green seaweeds (Hurd *et al.*, 2014, Dumay & Moranças, 2016).

In addition to pigments, seaweed species also show differences in the use of CCMs (Cornwall *et al.*, 2017). By increasing the local availability of CO₂, CCMs can increase the efficiency of RuBisCO and reduce the need of photorespiration (Raven, 2013). Presence of a CCM can reduce the required amount of RuBisCO to less than half of that present in regular plants. In seaweed, 3 classes related to CCMs can be distinguished: no CCM, CCMs with low affinity for dissolved inorganic carbon (DIC) or CCMs with high affinity for DIC (Cornwall *et al.*, 2017).

Environmental conditions also have a major impact on nitrogen distribution. At larger depths, seaweeds adapt to the reduced amount of light by increasing their light harvesting apparatus relative to the remaining photosynthetic machinery (Ramus *et al.*, 1976; Jahn *et al.*, 1984). Furthermore, seaweeds can adjust the ratios of their photosynthetic pigments to account for the wavelengths of light that reach the deeper waters, although seaweed species from intertidal habitats, which are above water at low tide and underwater at high tide, do not do this (Ramus *et al.*, 1976).

Seasons greatly influence the concentration, balance and subcellular distribution of the nitrogen within seaweed. Under natural growth conditions, nitrogen is available to seaweed as nitrate (NO_3^-), ammonium (NH_4^+) and urea (Roleda & Hurd, 2019). During autumn and winter nitrogen is relatively abundant in the seawater and is accumulated as soluble inorganic nitrogen (NO_3^-) in the vacuole or as soluble organic nitrogen, e.g. specialized amino acids or proteins depending on species. During spring and summer seawater nitrogen is directly assimilated into protein for photosynthesis and growth, with limitations being supplemented by the nitrogen reserves accumulated during autumn and winter. Additionally, as nitrogen availability in the seawater decreases, expression of NO_3^- , NH_4^+ and urea transporter genes is elicited (Takahashi *et al.*, 2020). Seaweed growth rates depend on the combination of light, nutrients and temperature (Rosenberg & Ramus, 1982; Hay *et al.*, 1988). However, growth can already start in anticipation of these factors, e.g. before the increases in temperature and light during spring (Rosenberg & Ramus, 1982) or prior to sunrise (Hay *et al.*, 1988).

Protein content varies strongly over the seasons (Hurd *et al.*, 2014; Kumar *et al.*, 2015). During spring, protein content increases as the photosynthetic machinery is built up. As spring progresses into summer, carbohydrates are accumulated in the cell walls and stored as starch or soluble compounds such as mannitol or laminarin (Rosenberg & Ramus, 1982; Hurd *et al.*, 2014; Roleda & Hurd, 2019). Due to the accumulation of carbohydrates during summer, protein concentrations decrease as summer progresses. Consequently, young blades formed during spring generally have higher protein contents (Hurd *et al.*, 2014; Dumay & Morancais, 2016).

During autumn and winter, many land plants shed their leaves to prevent dehydration and involuntary shedding during frost in a process called leaf senescence. To recycle the nitrogen invested into the leaves, plants degrade the photosynthetic machinery in the leaves and relocate it to buds and bark, which are better protected against winter conditions. A similar protein degradation event has not been mentioned to occur in seaweed blades and it has been stated that little is known about catabolism and turnover of cellular protein (Hurd *et al.*, 2014). Similar to plants, light, temperature and nitrogen limitation drive biosynthesis of the photosynthetic machinery and protein concentrations decrease towards autumn and winter (Rosenberg & Ramus, 1982; Hurd *et al.*, 2014). However, seaweed does not risk enhanced dehydration and freezing during this times and seawater nitrogen concentrations increase during winter (Chapman & Craigie, 1977; Kumar, 1995; Nielsen *et al.*, 2014; Zhang & Thomsen, 2019). These variations between summer and winter range from 1.8 - 4.2% DW (Chapman & Craigie, 1977) 1.3 - 3.2% DW (Nielsen *et al.*, 2014) for nitrogen and from 8 - 12% DW (Kumar, 1995) and 5 - 20% DW for protein contents (Zhang & Thomsen, 2019). Furthermore, decreased light intensities in land plants cause leaf yellowing by reducing the transcription of genes related to chlorophyll biosynthesis, rather than enhancing those related to chlorophyll degradation (Brouwer *et al.*, 2014). Finally, the reported decreases in protein concentration overlap with the period of biomass accumulation for cell wall fortification and solute storage (Rosenberg & Ramus, 1982; Hurd *et al.*, 2014; Roleda & Hurd, 2019; Zhang & Thomsen, 2019).

This leads to another variable, which is the way by which the protein concentration has been determined. Some reports use determining protein through total nitrogen and a conversion factor. Due to the high potential amino acid composition, a lower protein conversion factor is required compared to land plants and which differs per seaweed species (Shuuluka *et al.*, 2013; Loureno *et al.*, 2002). These conversion factors range between 4.52 - 5.59, 4.55 - 5.72 and 3.73 - 5.40 for green, red and brown seaweed, respectively. However, seasonal changes in the nitrogen-protein ratio and in the nitrogen availability in the seawater likely affect the conversion factors as well (acknowledged in Loureno *et al.*, 2002).

Nitrogen physiology, storage and transport in Saccharina latissima

In *Saccharina latissima*, results on seasonal effects of growth, nitrogen and protein tend to vary, with results obtained from different years, places and methods to determine protein (Schiener *et al.*, 2014; Marinho *et al.*, 2015; Manns *et al.*, 2017). For example, nitrogen and protein assay-based protein contents increased to 1.8% DW during autumn and winter, peaked at the onset of spring at 2.2% DW and decreased to 0.8% DW during summer on the Isle of Seil, Scotland between 2010 and 2011 (Schiener *et al.*, 2014). Similarly, in seaweed harvested from Kattegat, Denmark, between 2012 and 2014, nitrogen-based protein content decreased down to 2.3% DW during summer and increased to

about 20% DW during autumn and winter, but decreased during spring (Manns *et al.*, 2017). In this last example, the drop in protein during summer was complementary to the increase in carbohydrates due to photosynthesis in summer. Quite differently, protein calculated from total amino acid mass in seaweed harvested from Horsens Fjord, Denmark, between 2011 and 2013, increased during summer and autumn to 10% DW, decreased to 2% DW during autumn and winter and increased to 5% DW during spring (Marinho *et al.*, 2015). Some of these data, together with data from other reports have been concatenated into a single overview, which nicely compares the spatial and temporal variation over the year (Zhang & Thomsen, 2019). This comparison illustrates a general increase in protein content (% DW) during autumn and winter and a decrease during spring and summer.

Nevertheless, the consensus from other literature seems that during winter, when NH_4^+ , NO_2^- , NO_3^- , urea and amino acids in seawater are high, reserves are accumulated (Chapman & Craigie, 1977; Nielsen *et al.*, 2014; Zhang & Thomsen, 2019). This nitrogen can be stored in blade tissues or meristems, depending on the species and can be transported between tissues when nitrogen is required, e.g. from source thalli to sink meristems for growth (Davison & Steward, 1983; Li *et al.*, 2009; Nielsen *et al.*, 2014). Nitrogen reserves are utilized for growth in winter and through spring and depletion of reserves follows disappearance of external nitrogen sources with a 2-months lag (Chapman & Craigie, 1977; Nielsen *et al.*, 2014).

Cultivation depth has an influence on the total nitrogen content, with cultivation at 8 m deep showing around 10% DW increased nitrogen levels compared to 3 m deep, particularly as summer progressed (Sharma *et al.*, 2018). Exposure to currents and waves and shelter from these elements have no significant effect on nitrate and protein contents (Mols-Mortensen *et al.*, 2017). Growth of epiphytes on the blades during summer did not significantly alter protein contents, although it may affect the processing (Marinho *et al.*, 2015).

Nitrogen physiology, storage and transport in Ulva sp.

Ulva species accumulate nitrogen reserves in winter up to 630 $\mu\text{g-at N/g DW}$ (Rosenberg & Ramus, 1982; Hurd *et al.*, 2014). They deplete these reserves during spring and summer, proportional to their growth rate, which in turn depends on the available light, among other factors. During late spring and summer, peaks in tissue nitrogen follow peaks in seawater nitrogen (Rosenberg & Ramus, 1982). Nitrogen storage can vary considerably in closely related seaweeds and in a given species growing in different localities (Hurd *et al.*, 2014).

Annex C Enzymatic treatment model substrates cell membrane

Enzyme selection

- Alginate --> Alginate lyase
 - o Matis (not commercial, but in-house) – Alg 3 (pH 5.5, 75°C) and Alg 4 (pH 6.5, 80°C)
- Fucoidan --> Fucoidanases
 - o Pectinex Ultra SP, contains various glycosidases (pH 6, 50°C)
 - o Viscozyme, contains various glycosidases (pH 6, 50°C)
 - o Ronozyme HisPos (pH 6, 50°C)
- Cellulose --> Cellulases (only tested in combination)
 - o Cellic® CTec2, especially cellulases and some hemicellulose (pH 5.5, 50°C)
 - o Cellic® HTec2, contains especially endo-xylanases (pH 5.5, 50°C)

Setup experiments

- 1 mL buffer containing 50 mM citric acid/sodium citrate pH 4.5 or 6
- 50 mg substrate, incubation for 20 h at 50°C
- Inactivation enzyme for 10 min at 98°C, centrifugation
- Analyses supernatant: HPSEC and HPAEC

Overview experiments with alginate lyases is given in Table C-1 and for fucoidanases in Table C-2, *Saccharina* and fucoidan were used as substrates, respectively.

Table C-1 Overview experiments alginate lyases with Saccharina.

Enzyme	pH	Time (h)	T (°C)	2' enzym	pH	Time (h)	T (°C)
Blank	5.5	24	75	-	-	-	-
Blank	6.5	24	80	-	-	-	-
Alg 3	5.5	24	75	-	-	-	-
Alg 4	6.5	24	80	-	-	-	-
Cellic Ctec 2	5.5	24	55	-	-	-	-
Alg 3	5.5	24	75	Cellic Ctec 2	5.5	24	55
Alg 4	6.5	24	80	Cellic Ctec 2	5.5	24	55

Table C-2 Overview experiments fucoidanases with model substrate fucoidan.

Enzyme	Fucoidan	None
Viscozym L, pH 6	X	X
Pectinex Ultra SP, pH 6	X	X
Ronozyme HisPos, pH 6	X	X
Blank pH 6	X	

Results

The results are presented in Figure C-1, Figure C-2 and Figure C-3 and summarized in Table C-3.

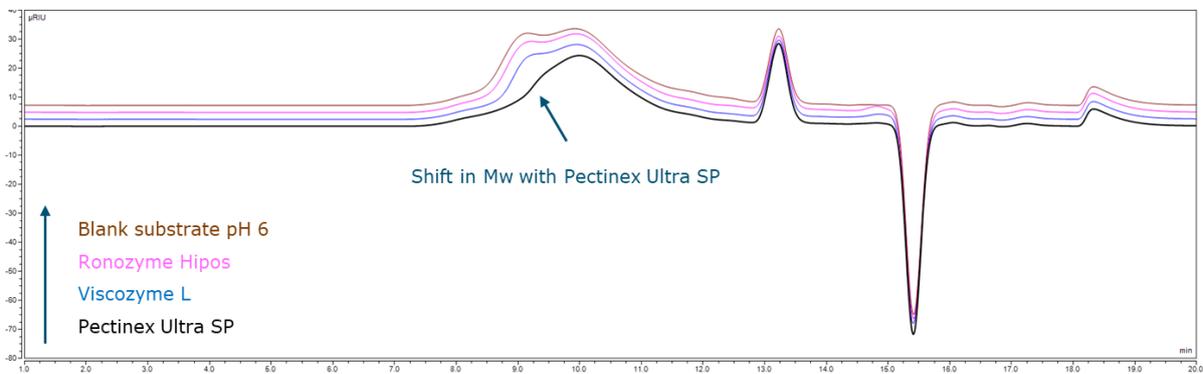


Figure C-1 *Enzymatic breakdown model substrate fucoidan, size exclusion chromatography.*

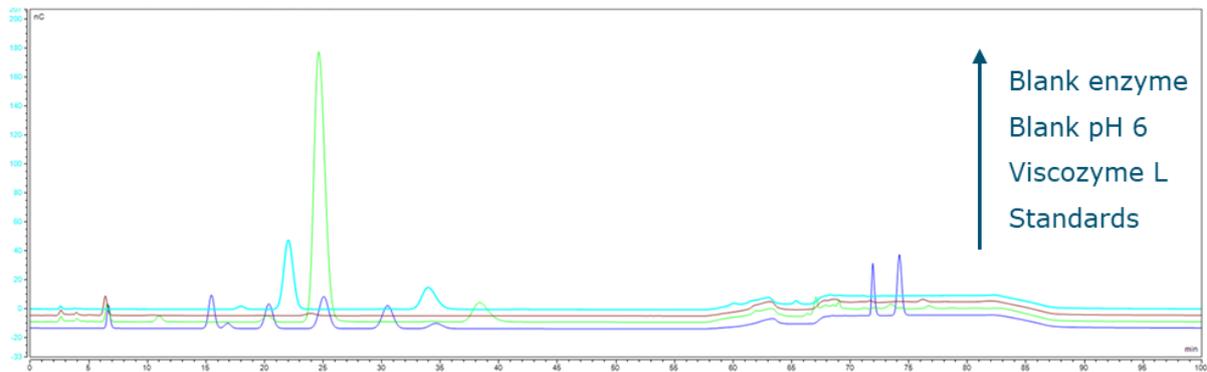
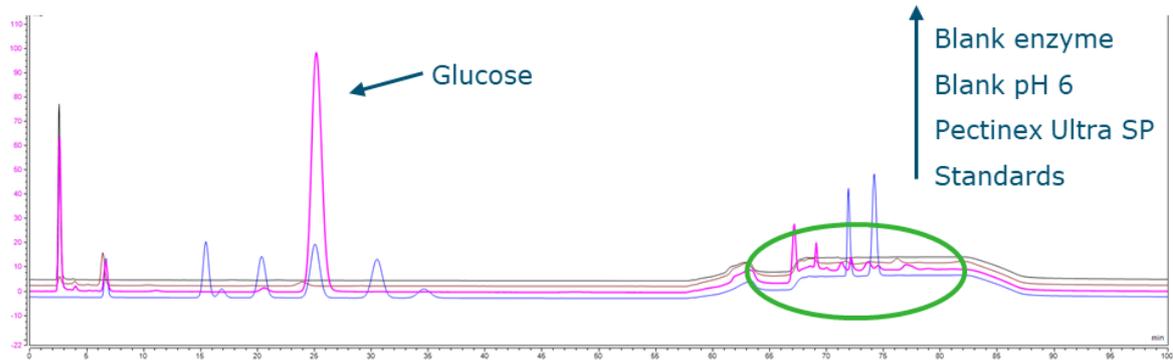


Figure C-2 *Enzymatic breakdown model substrate fucoidan, HPAEC.*

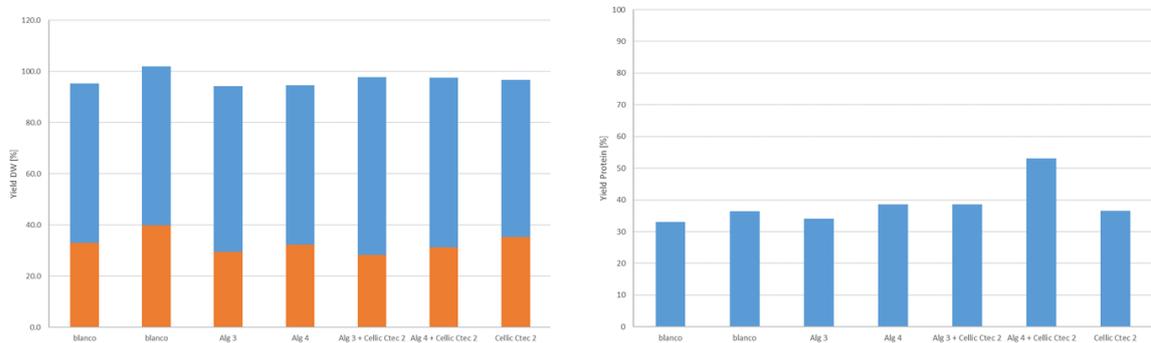


Figure C-3 *Enzymatic breakdown Saccharina treated with alginate lyase.*

Table C-3 *Summary of the results obtained with various carbohydrases on the model substrates fucoidan and Saccharina.*

	Fucoidan		Saccharina (alginate)
	HPSEC	HPAEC	
Pectinex Ultra SP	++	++	
Viscozym L	-	++	
Ronozyme HisPos	-	+	
Alg3			-
Alg4			+

Based on these results it was concluded:

- Pectinex Ultra SP showed a decrease in Mw on the HPSEC and a large amount of glucose and some oligomers on HPAEC;
- Viscozym L showed a large amount of glucose and small amount of oligomers on HPAEC;
- Ronozyme HisPos showed one extra peak (oligomer or charged monomer) on the HPAEC;
- Alg4, in combination with a cellulase, resulted in a higher protein release.

Annex D Alkali pretreatment to improve protein yield

Aim: to weaken cell wall prior to protease treatment with the objective to recover more intact protein.

Method: *Saccharina*, batch fresh/frozen 19 May 2020, blender pre-treatment. Dry matter content 10%, volume 100 mL, add amount of 5 M NaOH, reaction time 4 h, measure pH at end, followed by cooling down on ice. 16 temperature (30, 50, 60 and 80°C) and pH (8, 10, 11 and 12) combinations were tested. Analyses of dry matter, protein content and SDS-page.

Results: an impression of the liquid fraction is given in Figure D-1. The dry matter and protein/peptide release increased with pH. By increasing the temperature, the pH can be decreased to obtain the same dry matter and protein/peptide release (Figure D-2). Figure D-3 clearly shows the presence of proteins in some of the studied conditions.

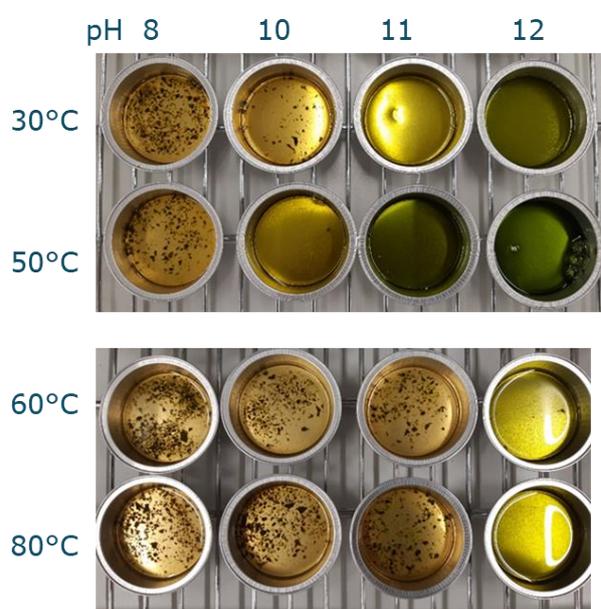


Figure D-1 Supernatant after various temperature and pH conditions.

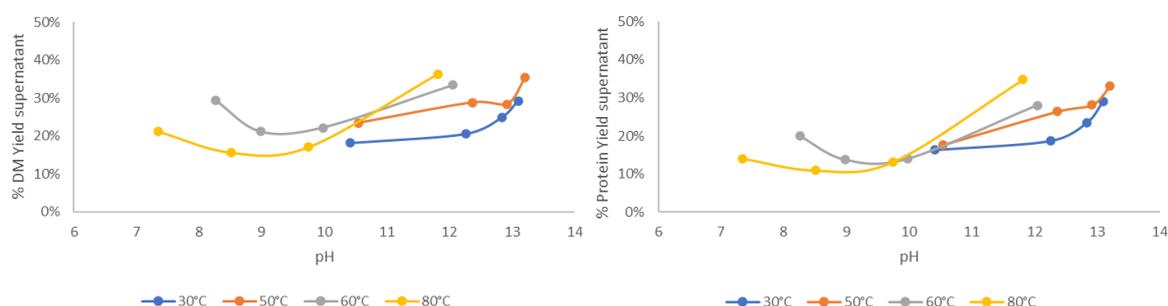


Figure D-2 Dry matter (left) and protein/peptide (right) release after 4 h incubation at various temperature and pH conditions.

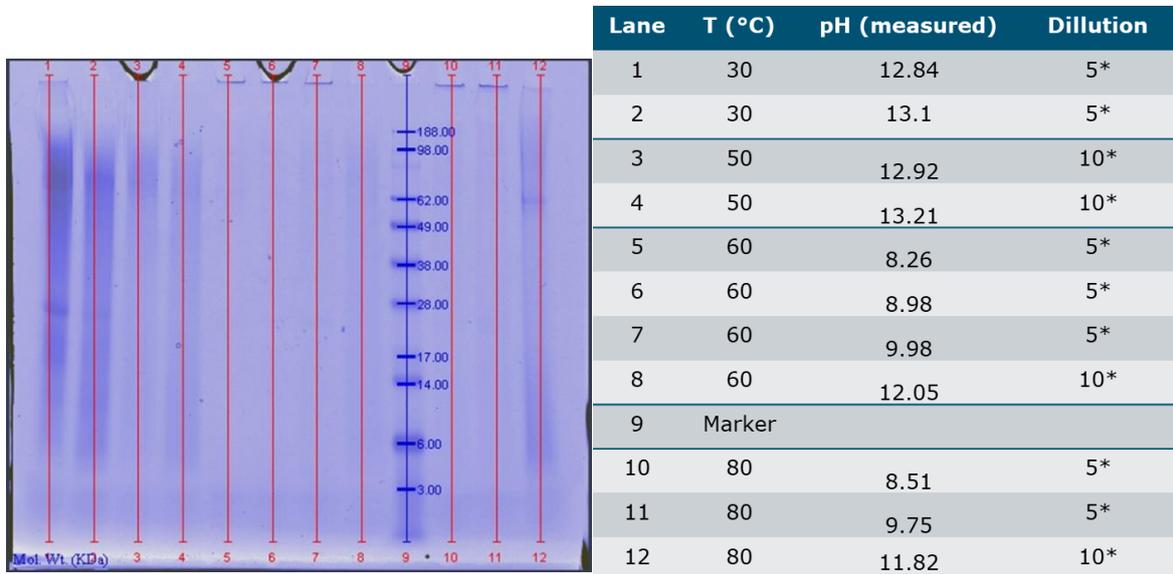


Figure D-3 SDS page liquid fractions Saccharina after alkaline treatment at various pH and temperature combinations.

Next steps

Determine the effect of the alkaline pretreatment on the release of protein/peptide by a carbohydrase and/or protease and the effect on the functionality of the liquid fraction.

Annex E Crude extracts for technical functionality

Aim: to make crude *Saccharina latissima* extracts for technical functionality.

Method: mixing with water in a blender (pH 6.5), stirring at room, separating solids by centrifugation (high speed), clarifying and desalting by ultrafiltration (0.1 m², 2 kDa) and diafiltration, freeze drying retentate to obtain product.

Results

The *Saccharina* slurry had a pH of 6.7, was viscous and slimy. After centrifugation there was a solid pellet and a clear liquid fraction. The permeate flux during ultrafiltration of the liquid fraction was 1.2 L/h at maximum pressure. The conductivity at was 12.9 mS and after diafiltration it was 0.7 mS. Mass balances of ash, wet and dry weight were made (Table E-1 and Table E-2). The extract was characterized using SDS-page (Figure E-1) and size exclusion (Figure E-2).

Table E-1 Mass balance wet and dry weight.

Extraction:	Wet and Dry weight:			Protein:		recovery
	wet (kg)	DW (%)	DW (g)	(%)	(g)	
seaweed	1.9	16.6	316	13	42	100%
water	2					
slurry	3.9	5.6	219	13	29	
Centrifugation:						
pellet	0.9	12	102			
supernatant	2.2	3.6	77			
Filtration:						
UF retentate	0.9	2.7	24	5	1	4%
UF Permeate	2	2	41			
Diafiltrate (DF3)	1.6	0.6	9			

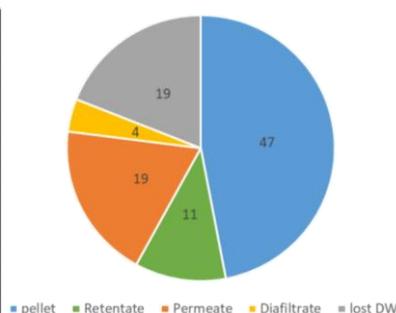


Table E-2 Mass balance ash.

Extraction:	DW (g)	ash 550°C:			ash 900°C:			
		(%)	(g)	balance	(%)	(g)	balance	balance
seaweed	316	28	90	100%	13	42	100%	
water								
slurry	219	27	60		6	12		100%
Centrifugation:								
pellet	102	23	24	40%	15	14.9	35%	123%
supernatant	77	35	27	45%	7	5.4	13%	44%
Filtration:								
UF retentate	24	15	3.6	6%	5	1.1	3%	10%
UF Permeate	41	40	16.7	28%	14	5.9	14%	49%
Diafiltrate (DF3)	9	34	3.3	5%	7	0.7	2%	6%

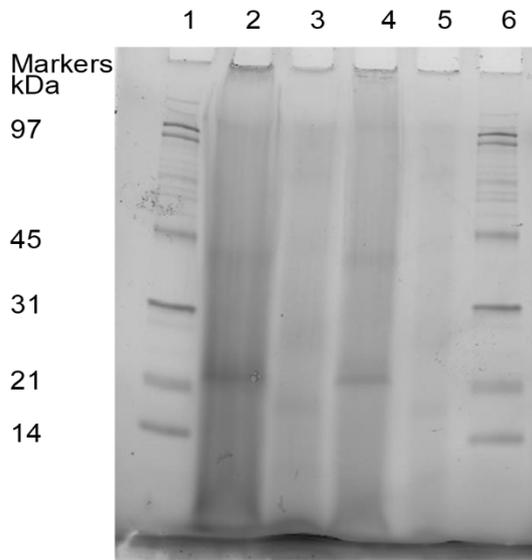


Figure E-1 *SDS-page (stain free) on Saccharina crude extract at 2% solution. Lane 1 marker, lane 3 Saccharina 30 μ L, lane 5 Saccharina 10 μ L, lane 6 marker.*

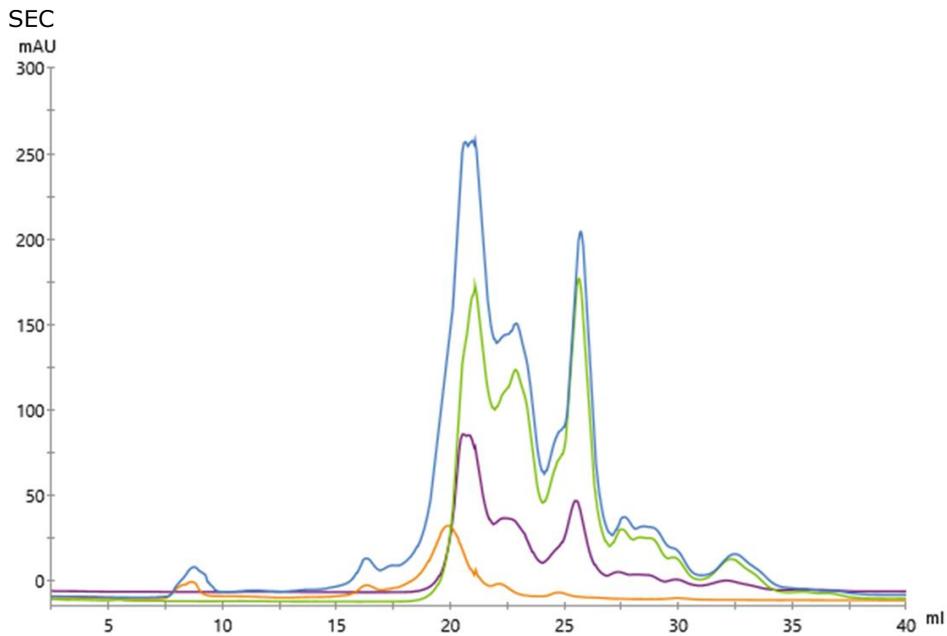


Figure E-2 *Size-exclusion chromatography on Saccharina. Blue = supernatant, green = permeate, orange = UF concentrated and 5x diluted, purple = diafiltrate.*

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