Screening of ionic liquids for the extraction of proteins from the macroalga *Ulva lactuca* aiming at an integrated biorefinery approach

Carlota Fernandes Miranda

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Supervisors:
Dr. Corjan van den Berg
Dr. Maria Teresa Ferreira Cesário Smolders

**Examination Committee**
Chairperson: Dr. Helena Maria Rodrigues Vasconcelos Pinheiro
Supervisor: Dr. Maria Teresa Ferreira Cesário Smolders
Members of the Committee: Dr. Maria Catarina Marques Dias de Almeida

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Preface

The work described in this document was performed under the framework of the curricular course Master’s Thesis in order to obtain the Master’s degree in Biotechnology at Instituto Superior Técnico.

All the research work was carried out, from February to July 2017, in the Bioprocess Engineering Group, part of the Agrotechnology and Food Sciences Group from Wageningen University and Research (Netherlands), under the supervision of Dr. Corjan van den Berg (assistant professor) and the PhD student Edgar Suarez Garcia. Dr. Mª Teresa Cesário (post-doc researcher), from the Bioengineering Department, was the internal supervisor at Instituto Superior Técnico.

This dissertation consists of a literature review of the research topic under study, description of the materials and methods used, obtained results and respective discussion. Finally, conclusions and future prospects are also reported.
Acknowledgements

The limited space of this acknowledgment section does not allow me to thank, as I should, to all of the people that, in the last months, direct or indirectly, helped me fulfilling my expectations and achieving my goals, finalizing such important phase of my academic life. Hence, I leave here only a few words, but in a very felt and thankful way.

Firstly, I would like to thank Professor Mª Manuela Fonseca and Dr. Mª Teresa Cesário for giving me the opportunity to do my master’s dissertation abroad and Dr. Corjan van den Berg for integrating me in his research group. I believe that the technical and social resources I had available in Wageningen University and Research were plenty for being able to perform an excellent work. Furthermore, the six months I spent in the Netherlands highly contributed to my personal development, and, despite not being an easy journey, I truly believe that my limits were pushed further, and that now I can do more and better.

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Last, but definitely not the least, I would like to thank to my family, especially my mother, Luisa, my father, Vasco, my brother, Tomás and my sister, Margarida. Thank you for always walking by my side - even 2000 km apart from me -, for your unconditional support and comprehension and for all the sacrifices you made for me. Thank for believing in me more than I believe in myself, for always pushing me to do better and be better, and for all life teachings. I could not end without mentioning my grandparents, my aunt Teresa and Dr. Carla, for the constant encouragement, so to successfully finish this great challenge.
Abstract

Alternative protein sources and production methods are required to fulfil the consumer needs and to meet the predicted increase of global protein demand. Although seaweeds are recognized as a valuable source of protein, new techniques for the extraction and separation of these biomolecules, under mild and stabilizing conditions, are required. Ionic liquids, due to their unique properties, have been considered promising alternatives for the recovery of value-added compounds from different sources of biomass.

The green macroalga *Ulva lactuca* was used in this study. It was composed of 96.50±0.87% of total solids, of which 22.80±0.35% was ashes, 4.95±0.35% lipids, 17.81±0.79% proteins and 60.92±14.62% carbohydrates. Three methods commonly used for macroalgal protein extraction were used as reference cases. Extraction by means of aqueous and alkaline solutions (sequential), mechanical grinding under alkaline conditions and aqueous biphasic system (PEG/Na₂CO₃) led to extraction efficiencies of 49.08±6.66%, 6.68±1.09% and 10.52±0.35%, respectively.

Twenty-five ionic liquids were screened towards the selection of the most suitable for the disintegration of *Ulva lactuca* cellular structure. 1-ethyl-3-methylimidazolium dibutyl phosphate showed the best results, allowing the recovery of 80.62±4.95% of the total protein. Aiming at an integrated biorefinery approach, an aqueous three-phase partitioning system (1-ethyl-3-methylimidazolium dibutyl phosphate/K₂HPO₃) is further proposed as a platform for the single-step cell disintegration and protein-carbohydrate fractionation. Proteins and carbohydrates are mainly concentrated in the top and inter-phase, respectively.

Finally, ultrafiltration was evaluated for the recovery of ionic liquid from macroalgal extracts, yielding recoveries of ~80% of ionic liquid and ~90% of proteins and carbohydrates.

Keywords

Seaweed | Ionic liquid | Integrated biorefinery | Protein | Carbohydrate | Aqueous biphasic system
Resumo

Para atender às necessidades dos consumidores e ao previsto aumento global da sua procura, são necessárias novas fontes de proteína e métodos para a sua produção. Apesar das macroalgas serem reconhecidas como potenciais fontes de proteína, é preciso desenvolver novas técnicas para a extração/separação destas biomoléculas, em condições suaves e estabilizadoras. As propriedades únicas dos líquidos iónicos tornam-nos alternativas promissoras para recuperar compostos de valor agregado de diferentes fontes de biomassa.

A alga verde Ulva lactuca, usada neste estudo, era composta por 96.50±0.87% de sólidos totais, dos quais 22.80±0.35% era cinzas, 4.95±0.35% lípidos, 17.81±0.79% proteínas e 60.92±14.62% carboidratos. Três métodos convencionais para a extração de proteínas das macroalgas foram usados como casos de referência. A extração através de soluções aquosas e alcalinas (sequencial), de moagem mecânica em condições alcalinas e de um sistema aquoso bifásico (PEG/Na₂CO₃) conduziram a eficiências de extração de 49.08±6.66%, 6.68±1.09% e 10.52±0.35%, respectivamente.

Rastrearam-se vinte-cinco líquidos iónicos a fim de selecionar o mais adequado para desintegrar a estrutura celular da Ulva lactuca. O 1-etil-3-metilimidazol dibutilfosfato mostrou o melhor resultado, extraíndo 80.62±4.95% da proteína total. Visando uma estratégia de biorrefinaria integrada, é ainda proposto um sistema aquoso de partição trifásico (1-etil-3-metilimidazol dibutilfosfato/K₂HPO₃) como uma plataforma de passo único para a desintegração celular e separação de proteínas-carboidratos. As proteínas e os carboidratos ficam concentrados principalmente na fase superior e interfase, respectivamente.

Finalmente, avaliou-se a recuperação do líquido iônico por ultrafiltração a partir dos extratos algáceos, alcançando-se taxas de recuperação de ~80% de líquido iônico e ~90% de proteínas/carboidratos.

Palavras-Chave

Alga marinha | Líquido iônico | Biorrefinaria integrada | Proteína | Carboidrato | Sistema aquoso bifásico
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Abbreviations

[1,4,4,4-P][MSO4] tributyl(methyl)phosphonium methylsulfate
[6,6,6,14-P][Cl] trihexyltetradecylphosphonium chloride
[6,6,6,14-P][dca] trihexyltetradecylphosphonium dicyanamide
[Bmim][Ac] 1-butyl-3-methylimidazolium acetate
[Bmim][Cl] 1-butyl-3-methylimidazolium chloride
[Bmim][dbp] 1-butyl-3-methylimidazolium dibutyl phosphate
[Bmim][dca] 1-butyl-3-methylimidazolium dicyanamide
[Ch][Ac] Choline acetate
[Ch][Cl] Choline chloride
[Emim][dbp] 1-ethyl-3-methylimidazolium dibutyl phosphate
[P]B Concentration of protein in bottom phase
[P]T Concentration of protein in top phase
AA(s) Amino Acid(s)
ACE Angiotensin Converting Enzyme
AHBB Anion Hydrogen Bond Basicity
AIDS Acquired Immune Deficiency Syndrome
ANOVA Analysis of Variance
ATPS(s) Aqueous Two-Phase System(s)
B Bottom phase
BIL Concentration of ionic liquid in the bottom phase
BPE Bioprocess Engineering
BSA Bovine Serum Albumin
Bsalt Concentration of salt in the bottom phase
CAB Concentration of component A in the bottom phase
CAT Concentration of component A in the top phase
Cf Feed concentration
Cp Permeate concentration
DEA Donor-Electron Acceptor
DW Dry Weight
EAA(s) Essential Amino Acid(s)
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<tr>
<td>FAO</td>
<td>Food and Agriculture Organization</td>
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<tr>
<td>g</td>
<td>Centrifugal force</td>
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<td>GHG</td>
<td>Greenhouse Gases</td>
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<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<tr>
<td>HSD</td>
<td>Tukey's Honest Significant Difference</td>
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<td>IEA</td>
<td>International Energy Agency</td>
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<td>IL(s)</td>
<td>Ionic Liquid(s)</td>
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<td>K</td>
<td>Partition coefficient</td>
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<td>M</td>
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<td>p</td>
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<td>PEG</td>
<td>Polyethylene Glycol</td>
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<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
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<tr>
<td>TPP</td>
<td>Three-Phase Partitioning</td>
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<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
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<td>U. lactuca</td>
<td>Ulva lactuca</td>
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<tr>
<td>ULPC</td>
<td>Ultra High-Performance Liquid Chromatography</td>
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<tr>
<td>UNU</td>
<td>United Nation University</td>
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<tr>
<td>Vf</td>
<td>Final volume</td>
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<td>Weight fraction</td>
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<td>WHO</td>
<td>World Health Organization</td>
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Introduction
1.1 Context

According to the United Nations, the current world population of 7.3 billion is expected to reach 9.7 billion in 2050 (FAO, 2017), requiring an estimated 70% increase in food production (FAO, 2009). In addition, the economic development and the rising in living standards, will result in changes in food consumption patterns.

It is undeniable that proteins take a crucial role in the human diet. Its global demand is driven not only by the population increase and socio-economic changes, but also by the growing recognition of protein role in a healthy diet, particularly for an aging population (Henchion et al., 2017).

Predictions for protein requirements show that the world demand for animal-derived protein will have to triple by 2050 (Westhoek et al., 2011), resulting in sustainability and food security concerns. It is proved that animal-based foods produce higher levels of greenhouse gases (GHG) than plant-based. These emissions come from, for instance, livestock, manure, feed production and land conversion (Westhoek et al., 2011). In particular, livestock sector accounts for 14.5% of all human induced GHG emissions (Gerber et al., 2013), being responsible for substantial emissions of nitrogen in various forms (ammonia, nitrates), which in turn leads to losses of terrestrial and aquatic biodiversity (Leip et al., 2015; Westhoek et al., 2011).

Also, the oceans, which are one of the largest sources of protein (fish and seafood), are reaching the limit of their resources. In 2013, about 89.5% of the global fish stocks were fully or over exploited (FAO, 2014a), leading to severe consequences for marine ecosystems. Farmed fish is likely to meet the needs of an ever-growing population; forecasts say it will account for 2/3 of global supply by 2030 (The World Bank, 2013). Nevertheless, it is fast reaching its limit since farmed fish is heavily dependent on wild caught fish for feed (fishmeal) (FAO, 2014a). Alternative protein sources and production methods are therefore required to fulfill the demand of consumers and to meet predicted global protein requirements.

Macroalga, the so called “seaweed”, represents a promising and novel future protein source. Some species of seaweed, like the red seaweed Porphyra sp., have been shown to contain levels of protein up to 47% dry weight (wt.) (Marsham et al., 2007). In some cases, macroalgae can be richer than some conventional protein-rich foods, such as soybean (40% protein), cereals (15% protein), eggs (9% protein) and fish (25% protein) (Harned and FitzGerald, 2013). In addition, the production of proteins from macroalgae has advantages over traditional sources in terms of productivity. Macroalgae have higher protein yield per unit area (2.5-7.5 tons.Ha\(^{-1}.\text{year}^{-1}\)) compared to terrestrial crops, such as soybean, vegetable seeds, and wheat (0.6-1.2 tons.Ha\(^{-1}.\text{year}^{-1}\), 1-2 tons.Ha\(^{-1}.\text{year}^{-1}\), and 1.1 tons.Ha\(^{-1}.\text{year}^{-1}\), respectively) (Krimpen et al., 2013).

Approximately 70% of the total global freshwater is used for agriculture (FAO, 2016) with animal-based protein production requiring 100 times more water than producing an equivalent amount of protein from plant sources (Pimentel and Pimentel, 2003). Marine algae do not require freshwater or arable land to grow, maximising resources that can be used for additional food production or other cash crops (Bleakley and Hayes, 2017).
Although seaweeds can be considered a potential source of proteins, their widespread use is limited by the availability of scalable production methods for protein isolation. The current processes are time-consuming, economically unviable and may still compromise the protein functionality due to the severe conditions applied (Wijffels and Barbosa, 2010). New scalable biorefining processes for the extraction and separation of macroalgal proteins, which ensure high recovery yields without compromising the extracts functionality, need to be developed.

Besides being promising to meet the future protein demand, macroalgae are also useful for bioenergy production based on the carbohydrate fraction. A single-unit operation for cell disruption and component fractionation – integrated biorefinery – that allows for the isolation of different macroalgal fractions, is essential for the valorisation of macroalgae as feedstocks (IEA, 2017, 2016a).

Recently, the unique properties of ionic liquids (ILs) have triggered the interest of scientific community as promising solvents for the extraction and separation of biomacromolecules from biomass, under mild and stabilizing conditions (MacFarlane and Tan, 2010). Although research on macroalga cell disruption using these novel solvents is limited to date (Malihan et al., 2014; Pezoa-Conte et al., 2015), their use for the disintegration of lignocellulosic material and algae has been already proven for several feedstocks (Brandt et al., 2013; Kim et al., 2012). The use of ILs is easily scaled up to industrial processes (Socha et al., 2014), therefore, this study can result in a new integrated biorefinery approach for macroalgal protein valorisation.
1.2 Objectives

The main goal of this experimental work was the screening of ionic liquids for the extraction of proteins from the green macroalga *Ulva lactuca*. For that, the following objectives should be met:

1. Biomass characterization in terms of total solids, ash, lipid, carbohydrate and protein content.
2. Replication of conventional methods, reported in literature, for the extraction of protein from macroalgae.
3. Screening of ionic liquids for the disintegration of *U. lactuca* cell wall and the consequent protein extraction.
4. Development of an aqueous two-phase system based on the most promising ionic liquid aiming at an integrated fractionation of biomolecules.
5. Study the recovery of ionic liquid from the macroalgal extracts;
6. Perform an economical evaluation of the proposed integrated biorefinery approach.
State of the Art
### 2.1 Biorefinery concept

In the long run, our current way of life is unsustainable. Our consumption and waste levels cannot be maintained without decreasing the possibilities for next generation. Our usage of ecosystem services is at such level that the earth is unable to regenerate them at the same pace (Eickhout, 2012).

We live in a fossil-based economy, in which fossil resources are used for all sorts of everyday applications, such as electricity, cooling and heating, fuels, materials and chemicals. They have been used at unprecedented rates (Höök and Tang, 2013) and it is becoming evident that our economies are unsustainable given that the fossil resources are dwindling and set to get more expensive. In addition to the non-renewable nature of these resources, their over-consumption also contributes to serious environmental issues. In 2016, the energy sector accounted for 68% of the GHG emissions, the main cause of global warming (IEA, 2016b).

The world needs feedstocks that are widely available, relatively inexpensive, renewable and that can be grown and processed in a sustainable manner. It is undeniable that several sorts of biomass can fulfil these requirements. The conversion of biomass to energy carriers and a range of useful products, including food and feed, can be carried out in multi-product biorefineries (IEA, 2009).

The International Energy Agency (IEA) Bioenergy Task 42 defines biorefinery as the “sustainable and synergetic processing of biomass into marketable food and feed ingredients, products (chemicals, materials) and energy (fuels, power, heat)”, which includes systems that may exist as a concept, a facility, a process, a plant, or even a cluster of facilities (IEA, 2014).

A biorefinery is the integral upstream, midstream and downstream processing of biomass into a range of products (IEA, 2009). The concept of “integrated biorefinery” describes the main goal of biorefineries, in which biomass can be used in an optimal way, leading to a longer lifespan of the resource/product, and thereby increasing its efficient and effective application. Under this system, several conversion technologies are integrated and designed to maximize the valuable components, while minimizing the waste streams and processing steps. Biomass is separated into fractions: the valuable molecules are processed into high-value products such as chemicals and materials, while the lower quality fractions – side streams – can be used to produce fuels or to energy recovery (Fernando et al., 2006).

A biorefinery can use all kinds of biomass including forest resources, agricultural crops such as switchgrass, corn and soybeans, organic residues (both plant and animal-derived, and industrial and municipal wastes) and aquatic biomass (microalga and seaweed). The major components of these biomasses include carbohydrates (cellulose and hemicellulose for crop residues, forage crops and woody crops, starch for grain, and primarily sucrose for sugar crops), lipids (fats, waxes and oils), proteins, aromatic compounds (primarily lignin), and ash (non-carbon minerals such as calcium, phosphorus, potassium, etc.) (Dale and Kim, 2008).

The development of biorefineries represents the access key to an integrated production of food, feed, chemicals, goods, and fuels to meet the future demands.
2.1.1 Aquatic biomass

The main driver for the establishment of biorefineries is sustainability, which implies that biorefinery products should be produced without impacting the economy and ecosystems from the life cycle perspective (Jung et al., 2013). Therefore, the feedstock supplies must conform to these parameters.

The use of agricultural substrates – corn, soybean, or sugar cane – to feed biorefineries (mainly to produce biofuels) is involved in controversial debates due to the “food vs energy” dilemma. Crop biomass is traditionally destined for food and feed applications and its use as feedstock competes with both purposes for land, water and other resources, which raises the question of social sustainability (Fernand et al., 2017).

The use of non-food biomass, such as crop residues (corn stover, manure, straw, waste wood) and energy crops cultivated on non-arable lands (miscanthus, poplar, willow, switchgrass), is not in confrontation with food and feed availability. However, their use is not cost effective due to the abundance of lignin in the composition of these biomasses. Several expensive pre-treatment steps are required to disintegrate these matrices in order to convert such substrates into biofuels and bioproducts (Sambusiti et al., 2015).

Although woody biomass is considered a potential feedstock option for biorefineries, its composition is similar to agricultural residues, meaning that the expensive pre-treatment steps are still required. In addition, getting this kind of biomass from forests, which are rich in biodiversity and delicate ecosystems, go against the idea of sustainability (Ghatak, 2011).

Aquatic biomass – microalgae and seaweeds – seems to circumvent the concerns related with terrestrial biomass. Algae production does not affect the conventional agriculture because these organisms do not need land or fresh water once they have the ability to grow under harsh conditions like saline, brackish and coastal seawater (Behera et al., 2015). In addition, aquatic biomass has a lower risk for the competition for food than other crops, since this application is important in few East Asia countries (Jung et al., 2013). Other macroalgal uses are for hydrocolloid extraction, application as fertilizer, and animal feed. It should be noted that macroalgae do not include lignin in their composition (Saqib et al., 2013) because they do not need to stand rigidly in the water. This allows the processing of algae in relatively mild conditions comparing with lignocellulosic biomass. Lower temperatures, less severe acid conditions, and shorter reaction times are generally needed (Van Hal et al., 2014).

Algae can grow through the year, consequently they have a short harvesting cycle in comparison to other conventional crops, which have harvesting cycles of once or twice in a year due to the seasonal variations (Chisti, 2008; Schenk et al., 2008). Moreover, they can convert solar energy into chemical energy with higher photosynthetic efficiency (6-8%) than terrestrial biomass (1.8-2.2%) (Ross et al., 2009), therefore they can generate and store higher amounts of carbon and lipid resources, which can be exploited in biomaterial and bioenergy production.

Amongst aquatic biomass, the use of macroalgae in a bio-based economy is more advantageous than the use of microalgae because seaweeds show higher volumetric production rates (biomass per volume per time) and biomass densities (Van Hal et al., 2014).
2.2 Macroalgal backgrounds

Seaweeds are the most important organisms in marine ecosystem for the preservation of marine bio-resources and seawater quality by preventing pollution and eutrophication, as well as absorbing and fixating CO$_2$ aided by solar energy (Notoya, 2010).

Macroalgae are multicellular organisms and belong to the lower plants, consisting of a leaf-like thallus instead of roots, stems, and leaves (Cavalier-Smith, 2007; Lobban and Wynne, 1983). They are photoautotrophic organisms, therefore, produce and store organic carbon by the conversion of CO$_2$ and HCO$_3^-$ (Gao and McKinley, 1994). Their photosynthetic rates vary according to the species but are usually higher than those of terrestrial biomass, like corn and switchgrass (Chung et al., 2011).

The pigment content, growth rates, and chemical composition of macroalgae are significantly affected by their habitat conditions such as light, temperature, salinity, nutrient, pollution and even water motion (Lobban and Wynne, 1983). Light represents the most important factor affecting pigmentation, allowing to classify macroalgae into Phaeophyta (brown), Rhodophyta (red) and Chlorophyta (green) classes (Chan et al., 2006). According to the respective pigments, algae are able to selectively absorb light with specific wavelengths. For instance, some red algae are able to grow in the deep sea (over 25 m below the surface) (Santelices, 1991).

In terms of chemical composition, macroalgae have high contents of carbohydrates (20-60%) (Jung et al., 2013), minerals (10-50% dry wt.) (Ross et al., 2008) and proteins (7-47% dry wt.) (Jensen, 1993; Marsham et al., 2007); lipids are also present but in very small quantities (1-5% dry wt) (Jensen, 1993).

The content and specific carbohydrate composition vary significantly among macroalgal classes. As recently reviewed by Jung et al. (Jung et al., 2013), green algae (25-50% dry wt. carbohydrates) are mainly composed of mannan, ulvan, starch (i.e., α-1,4-glucan) and cellulose. The proportion of starch is very small (1-4%) and ulvan, which major components are D-glucoronic acid, D-xylose, L-rhamnose, and sulfate, is a distinctive feature of green algae.

The main polysaccharides of red algae are carrageenan (up to 75% dry wt.), which consists of repeating D-galactose units and anhydrogalactose, and agar (up to 52% dry wt.), made up of alternating β-D-galactose and α-L-galactose, with scarce sulfations (Jung et al., 2013). Purified carrageenan from red algae is generally used to form thick solutions or gels, for instance, it is used in air freshener gels, toothpaste, firefighting foam, shampoo, cosmetic creams, as food additives for dairy products, and also in biotechnology as a gel to immobilise cells/enzymes (Necas and Bartosikova, 2013). In turn, agar is applied in food, pharmaceutical and biological industries (e.g. it is used as food ingredients, as laxative in pharmaceutical industry, and as solid media for microbial growth) (McHugh, 2003).

Finally, brown algae are mainly composed of alginic acid (i.e., alginate), laminarin, fucoidan, cellulose and mannitol. Alginate, composed of mannuronic and guluronic acid blocks, is the principal constituent of the cell wall (up to 40% dry wt.) and is mostly used in textile (50%)
and food (30%) industries (McHugh, 2003). Laminarin, composed of β-1,3-glucan units, accounts to 35% dry wt. of brown algae (Jung et al., 2013).

Almost 95% of seaweeds used by humans is a result of cultivation activities (FAO, 2014b, 2014c). Seaweed farming is practised in about 50 countries and it expanded 8% per year in the past decade (FAO, 2014a). Indonesia is the major contributor for aquatic plant production growth, with an increase in annual farmed seaweeds output by more than 10 times, from less than a million tonnes in 2005 to 10 million tonnes in 2014 (FAO, 2014a). This impressive expansion is mainly due to the vast areas of sunlit shallow sea, which are suitable for culture sites of the tropical seaweed *Kappaphycus alvarezi* and *Eucheuma sp.* (FAO, 2014a). In addition to these species, *Laminaria japonica*, *Gracilaria sp.*, *Undaria pinnatifida* and *Porphyra sp* are also the most commonly farmed. (FAO, 2014a).

In 2014, 28.5 million tonnes of seaweeds were harvested for direct consumption or further processing for food (traditionally in Japan, the Republic of Korea and China), fertilizers, pharmaceuticals, cosmetics and other purposes (FAO, 2014a).

Although the new developments in which seaweeds are considered potential feedstocks for bioenergy production, it is shown that no commercial process can be developed only based on the generation of bioenergy (IEA, 2017). However, the maximization of the inherent value of all macroalgal components in integrated biorefinery approaches could make the process economically viable.

Growing attention is also focused on the nutritional value of several seaweed species due to the high content of natural vitamins, minerals, and plant-based proteins. Macroalgae have shown to provide a rich source of natural bioactive compounds with anti-viral, anti-fungal, anti-bacterial, anti-oxidant, anti-inflammatory, hypercholesterolemia, hypolipidemic and anti-neoplastic properties (Plaza et al., 2008).

Recently, different food and drink products containing seaweed flavours have been commercialized. The majority of seaweed-flavoured food and drink products are currently launched in the Asia Pacific region, accounting for 88% of global product launched between 2011 and 2015, Europe launched 7% in this time, outpacing both North America (4%) and Latin America (1%) (Mintel, 2016).

### 2.2.1 Macroalgae as a source of protein

The protein content and amino acid (AA) composition in seaweeds vary with species and season (Harnedy and FitzGerald, 2013). The highest protein levels are commonly found during the period of winter-early spring and the lowest during summer-early autumn (Galland-Irmouli et al., 1999; Khairy and El-Shafay, 2013; Rouxel et al., 2001). Such fluctuations have been related to several variables, including nutrient supply, temperature, available light and salinity (Harnedy and Fitzgerald, 2011). Generally, the protein content is low in brown seaweeds (3-25% dry wt.), moderate in green algae (9-26% dry wt.) and high in red seaweeds (<47% dry wt.) (Fleurence, 2004).

The bioactive properties of a protein can be determined essentially by the content, proportion and availability of its amino acids (Millward et al., 2008). The World Health Organization
(WHO), Food and Agricultural Organization of the United Nations (FAO) and the United Nation University (UNU) have been focused on the energy and nutritional needs of the world population and have produced recommendations on fundamental nutritional requirements, including the estimation of indispensable amino acid demand (reference pattern of protein in diet) (WHO/FAO/UNU, 2007).

The comparison between the amino acid composition in seaweeds, reference pattern (WHO/FAO/UNU, 2007) and other food-proteins, give us a first estimation about the macroalga protein quality (based only on chemical analysis). However, a biological evaluation including human and animal feeding studies (to assess in vivo protein digestibility and bioavailability of essential amino acids) is required to establish the complete nutritional value of macroalgae (Kumar and Kaladharan, 2007).

The digestibility in vivo of algal proteins is not well studied, and available studies about their assimilation by humans and animals have not provided conclusive results (Conde et al., 2013). However, a high rate of algal protein degradation in vitro by proteolytic enzymes such as pepsin, pancreatin and pronase has been described (e.g. 78%, 87% and 95% for Pyropia tenera, Ulva pinnatifida and Ulva pertusa, respectively) (Fleurence, 1999).

Despite seasonal and interspecies variability, seaweeds contain all the essential amino acids (EAAs) and seem to be able to contribute with the adequate levels of EAAs, meeting the requirements reported in reference pattern (Kumar and Kaladharan, 2007; Matanjun et al., 2009). Moreover, macroalgae show higher concentrations of EAAs than those commonly present in other protein sources, such as soy beans and eggs (Bleakley and Hayes, 2017; Lourenço et al., 2002).

Concerning the EAAs, lysine and tryptophan are often in short supply, in relation to metabolic requirements, in most alga species (Bleakley and Hayes, 2017). The most frequent EAA in brown, green and red macroalgae is phenylalanine (Matanjun et al., 2009). Cysteine, methionine, leucine and isoleucine typically occur at low levels in seaweeds (Bleakley and Hayes, 2017; Cerna, 2011).

In the case of non-essential amino acids, all three classes of seaweeds show similar profiles (Matanjun et al., 2009), with glutamic acid and aspartic acid representing the highest fraction. Both AAs reach together up to 23% of total AAs in red algae, 28% in green algae, and 30% in brown algae. They are responsible for the distinctive ‘umami’ taste associated to these organisms (Biancarosa et al., 2017; Wells et al., 2017).

2.3 Extraction of macroalgal proteins

In general, most of macroalga proteins are located intracellularly, surrounded by a cell wall. The disintegration of the cell wall and the consequent release of intercellular proteins are very important to ensure the commercial application of such biomolecules. For instance, seaweeds have poor protein digestibility in their raw, unprocessed form (Bleakley and Hayes, 2017), which limits their use in food and feed applications; the extraction of macroalgal proteins will improve their bioavailability, increasing their commercial value.
In addition, as mentioned in section 2.2, although seaweeds are considered potential feedstocks to produce bioenergy, the economic viability of the process is dependent on the valorisation of the seaweed remaining components, such as proteins, through integrated biorefinery approaches – Figure 2.1.

Algal proteins are conventionally extracted by means of aqueous, acidic, and alkaline methods, followed by several fractionation techniques such as centrifugation, ultrafiltration, precipitation and/or chromatography (Kadam et al., 2017). However, one of the most important factors influencing the successful extraction of proteins is the accessibility of such molecules, which can be substantially hindered by macromolecular cell wall assemblies, cross-linked via disulphide bonds to polysaccharides (Harnedy and Fitzgerald, 2015). Therefore, cell-disruption methods and specific chemical reagents (e.g. reducing agents) are often used to improve the efficiency of protein extraction.

To date osmotic shock, mechanical grinding, ultrasonic treatment and enzyme aided digestion are the most common methods used for macroalga cell-disruption - Table 2.1. In general, after cell disintegration, aqueous protein extracts are first obtained. A second extraction step under strong alkaline conditions (pH~12) is usually performed to recover the fraction of insoluble proteins (Harnedy and FitzGerald, 2013).
<table>
<thead>
<tr>
<th>Cell-disruption method</th>
<th>Extraction Process</th>
<th>Macroalga species</th>
<th>Protein Recovery Yield</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osmotic shock</td>
<td>Aqueous &amp; alkaline (sequential)</td>
<td><em>Palmaria Palmata</em></td>
<td>6.77±0.22% dry wt.</td>
<td>(Harnedy and FitzGerald, 2013)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Ulva rigida</em></td>
<td>26.80±1.3%*</td>
<td>(Fleurence et al., 1995)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Ulva rotundata</em></td>
<td>36.10±1.4%*</td>
<td>(Fleurence et al., 1995)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Ulva rigida</em></td>
<td>9.73±0.6%*</td>
<td>(Fleurence et al., 1995)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Ulva rotundata</em></td>
<td>14.00±1.8%*</td>
<td>(Fleurence et al., 1995)</td>
</tr>
<tr>
<td>High shear force</td>
<td>Aqueous &amp; alkaline (Sequential)</td>
<td><em>Palmaria Palmata</em></td>
<td>6.92±0.12% dry wt.</td>
<td>(Harnedy and FitzGerald, 2013)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Porphyra acanthophora var. acanthophora</em></td>
<td>8.94±0.53% dry wt.</td>
<td>(Barbarino and Lourenço, 2005)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Sargassum vulgare</em></td>
<td>6.91±0.15% dry wt.</td>
<td>(Barbarino and Lourenço, 2005)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Ulva fasciata</em></td>
<td>7.30±0.84% dry wt.</td>
<td>(Barbarino and Lourenço, 2005)</td>
</tr>
<tr>
<td>Enzyme hydrolysis</td>
<td>Aqueous/protein released by action of enzyme activity &amp; alkaline (sequential)</td>
<td><em>Palmaria Palmata</em></td>
<td>11.57±0.08% dry wt.</td>
<td>(Harnedy and FitzGerald, 2013)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Ulva rigida</em></td>
<td>18.48±2.10%*</td>
<td>(Fleurence et al., 1995)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Ulva rotundata</em></td>
<td>22.00±1.20%*</td>
<td>(Fleurence et al., 1995)</td>
</tr>
<tr>
<td>Sonication</td>
<td>Acidic</td>
<td><em>Ulva rigida</em></td>
<td>10.35±0.80%*</td>
<td>(Fleurence et al., 1995)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Ulva rotundata</em></td>
<td>16.08±0.90%*</td>
<td>(Fleurence et al., 1995)</td>
</tr>
<tr>
<td></td>
<td>Acidic</td>
<td><em>Ulva rigida</em></td>
<td>9.37±1.60%*</td>
<td>(Fleurence et al., 1995)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Ulva rotundata</em></td>
<td>13.80±1.20%*</td>
<td>(Fleurence et al., 1995)</td>
</tr>
<tr>
<td>None - direct</td>
<td>Acidic &amp; alkaline (sequential)</td>
<td><em>Ulva rigida</em></td>
<td>17.50±1.30%*</td>
<td>(Fleurence et al., 1995)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Ulva rotundata</em></td>
<td>25.17±1.90%*</td>
<td>(Fleurence et al., 1995)</td>
</tr>
<tr>
<td></td>
<td>Aqueous biphasic system (PEG/K2CO3)</td>
<td><em>Ulva rigida</em></td>
<td>19.10±1.10%*</td>
<td>(Fleurence et al., 1995)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Ulva rotundata</em></td>
<td>31.56±2.10%*</td>
<td>(Fleurence et al., 1995)</td>
</tr>
</tbody>
</table>

Little or no studies to date have looked at industrial significant protocols for the extraction of macroalga proteins. Most of the conventional methods described above are laborious and time-consuming, and there are also scalability concerns associated to some of them. However, the
main drawback is the use of alkaline and acidic solutions to mediate the protein extraction in the majority of these conventional methods. The feasibility of a method for protein extraction depends mainly on the type of product and the intended downstream application. The use of severe conditions (extreme pH) can affect the integrity and the consequent functionality of the extracted proteins.

Novel methods for cell-disruption and extraction of proteins from macroalgae are required. Since the value of isolated proteins depends on their functional properties, mild operation conditions for their recovery and separation are desirable. It is important to note that, in addition to ensure protein functionality, the extractive performance (high protein recovery yields) is also a crucial parameter to take into account when attempting the development of novel extraction processes (Harnedy and Fitzgerald, 2015).

### 2.4 Ionic liquids as new solvents

In the last decades, the world “green” acquired a new meaning in chemistry-related fields, changing the way by which academia and industry design chemical processes. “Green chemistry” has demonstrated how fundamental scientific methodologies can protect human health and the environment in an economically beneficial manner (Anastas and Kirchhoff, 2002). In this context, besides the development of renewable feedstocks, the design of safer chemicals and environmentally benign solvents became a priority (Capello et al., 2007). Ionic liquids have triggered the attention of scientific community as a possible alternative to meet the requirements of “green chemistry”. At the same time, ILs have been proposed as new solvents for the extraction and separation of value-added compounds from biomass (Passos et al., 2014), being able to maintain the stability of extracted molecules, namely, proteins, enzymes and nucleic acids (Naushad et al., 2012; Vijayaraghavan et al., 2010). These evidences make the use of ILs promising for the extraction and isolation of proteins from macroalgae.

Ionic liquids are organic salts, with melting points below 100°C, composed by cations and anions with low symmetry and low charge density (Seddon, 1997). The choice of these components determines the distinct physicochemical properties of each IL (melting point, polarity, viscosity, chemical and thermal stability, solvation properties) (Stark, 2011). They are described as “designer solvents” since there is a large degree of cation/anion combinations, allowing the possibility of tuning their characteristics, such as thermo-physical properties, biodegradation ability or toxicological issues, as well as their hydrophobicity and solution behaviour (Passos et al., 2014).

ILs’ features and all related advantages make them competitive against conventional organic solvents (Canales and Brennecke, 2016). They stand out by their negligible vapour pressure, non-flammability, wide electrochemical window, high chemical and thermal stability and ability to dissolve a wide range of organic and inorganic compounds, facilitating diverse types of chemical transformations and separation processes (Bennett and Leo, 2004). The replacement of volatile organic solvents by non-volatile ILs eliminates solvent losses to the atmosphere,
decreasing the environmental footprint. This is the main reason by which ionic liquids are considered “green” (Zhao et al., 2005).

2.4.1 Ionic liquids for macroalga cell disintegration

The basis of the extraction mechanism proposed in this dissertation lies on the interaction of ionic liquids with the macroalga cell wall. It is expected that such interaction leads to the complete or partial cell wall disintegration, allowing access to the desired protein molecules.

The core component of plant cell walls are cellulose microfibrils, accounting to roughly one-third of the total mass of many plants (Somerville, 2006). Although the proportion of cellulose in marine algae varies more than in higher plants, cellulose remains a significant fraction in the majority of seaweeds. In most green algae, the cellulose content can reach up to 70% of the dry wt. (e.g. Cladophorales and Ulvales) (Baldan et al., 2001). However, in red and brown algae, the cellulose content is lower and can be lacking in some species, being replaced by xylans and mannans, which also perform structural function, although with less rigidity than cellulose microfibrils.

Cellulose consists of a polydisperse linear glucose polymer chain which forms hydrogen-bonded supramolecular structures (Finkenstadt and Millane, 1998). Glucose units are linked by 1,4-β glycosidic bonds, which stability is reinforced by intrachain hydrogen bonds (Hinterstoisser and Salmén, 2000).

Although the monomer glucose and short oligomers are water-soluble, cellulose is not. Reasons for this are its high molecular weight (solubility is usually inversely related to polymer length) and the comparatively low flexibility of cellulose polymer chains (Arneniades and Baer, 1977), which arises from the strong glycosidic bonds and the huge degree of both intra- and intermolecular hydrogen bonds (Figure 2.2) (Pinker et al., 2009).

![Figure 2.2 | Intra- and intermolecular hydrogen bonds in cellulose. Adapted from (Pinker et al., 2009).](image)

To separate the polymeric structure of cellulose, an excellent solvent must be employed in order to disrupt the intermolecular H-bond interactions, leading to the polymer dissolution (Mohd et al., 2017).

In 2002, it was found that several ionic liquids can dissolve large amounts of cellulose (Swatloski et al., 2002), which opened up the way to the development of new cellulose solvent systems. The most successful cations for cellulose dissolution are based on methylimidazolium
and methylpyridinium cores, with allyl-, ethyl-, or butyl- side chains and the most promising anions are chloride, acetate, and formate (Pinker et al., 2009).

It is thought that both anions and cations are involved in the cellulose dissolution process. Figure 2.3 shows the proposed dissolution mechanism of cellulose by ionic liquids, in which electron donor-electron acceptor (EDA) complexes are formed between the hydroxyl groups of cellulose and the ionic liquids. In these complexes, the oxygen atoms serve as electron pair donors and the hydrogen atoms act as electron acceptors. On the other hand, the cations of the ionic liquid act as electron acceptor centres and the anions as electron donor centres. Both centres must be located close enough in space to allow the interaction and the formation of EDA complexes. Upon interaction of the cellulose-OH with the ionic liquid, the oxygen and hydrogen atoms from hydroxyl groups are separated, resulting in the opening of the hydrogen bonds between the molecular chains, and their dissolution (Feng and Chen, 2008).

![Figure 2.3 | Proposed dissolution mechanism of cellulose in ionic liquid 1-Butyl-3-methylimidazolium chloride ([BMIM][Cl]). Adapted from (Pinker et al., 2009).](image)

It has been demonstrated that the capability of ILs to dissolve cellulose are directly correlated to their strong hydrogen bond basicity and lower viscosity (Zhang et al., 2017). The hydrogen bond basicity describes the ability of a molecule to act as a hydrogen bond acceptor (Oliferenko et al., 2004), a role played by ionic liquid anions according to the proposed mechanism. In this sense, the better the hydrogen bond acceptor ability of the IL anion, the higher is its ability to solubilize cellulose (Stark et al., 2012). On the other hand, the chemical structure of cations also has an important effect on the cellulose dissolution. In general, when the chain of alkyl groups or the symmetry of the cations increases, the dissolution rate of cellulose in ILs decreases, due to the rise of the viscosity and/or decrease of cation hydrogen bond acidity (Zhang et al., 2017).

### 2.4.2 Ionic liquid-based aqueous biphasic systems for biomolecules fractionation

Aqueous two-phase systems (ATPSs) have been used for the recovery of a broad array of biomolecules (e.g. proteins, polysaccharides, nucleic acids) through their partition between the two aqueous liquid phases (Liu et al., 2012), which makes its use a promising strategy for refining other seaweed components beyond the desired proteins. This allows the maximization of the inherent value of several components from seaweed, which may be crucial for the profitable use of seaweeds as raw-materials (Van Hal et al., 2014).
ATPS consists of two immiscible aqueous-rich phases based on polymer-polymer, polymer-salt or salt-salt combinations. Although the most investigated ATPSs are the polymer-based systems, in the last decade, ionic liquids were proposed as alternative phase-forming components (Gutowski et al., 2003).

In an ATPS, both solutes that make up the system are water soluble, they separate into two coexisting phases above their critical concentration, in which each phase will contain predominantly only one type of solute (Freire et al., 2012). The coexisting phases will offer different physicochemical environments for the biomolecules, driving them more extremely to one phase, according to the biomolecule properties (e.g. hydrophobicity, charge, size) (Andrews and Asenjo, 1989).

The phase diagram can be considered as a fingerprint of an ATPS under specific conditions (e.g. temperature and pH). It is unique and shows the potential working area of the ATPS, providing information about the required concentration of phase-forming components for phase splitting, the concentration of phase-components in the top and bottom phases, and the ratio of phase volumes (Raja et al., 2012). Figure 2.4 represents a phase diagram for a hypothetical system, in which the binodal curve (TCB) indicates the boundaries of monophasic- and biphasic regions. For mixture compositions above the binodal curve, there is the formation of a two-phase system, while mixture compositions below this curve fit into the monophasic region and will not phase separate. The three systems X, Y and Z differ in their initial compositions and in volume ratios. However, in equilibrium, all the systems have the same top phase composition ($T_{IL}, T_{salt}$) and the same bottom phase composition ($B_{IL}, B_{salt}$). This is because they are lying on the same tie-line (TL) – TB in Figure 2.4 –, whose end points determine the equilibrium phase composition. Point C on the binodal curve is called critical point, just above this point the volume of both phases is theoretically equal, and the tie-line length (TLL) is 0.

![Figure 2.4 | Phase diagram for a hypothetical system composed of ionic liquid + salt + water (weight fraction percentage). TCB: Binodal curve; C: Critical point; TB: Tie-line; T: Composition of the top phase; B: Composition of the bottom phase; X, Y and Z: total composition of the ATPS. Adapted from (Raja et al., 2012).](image-url)
The units of TLL are % w/w, same as the component concentrations, and it can be estimated by using the ratio of the mass compositions (Equation 2.1) (Freire, 2016a):

\[
\frac{V_T \rho_T}{V_B \rho_B} = \frac{XB}{XT}
\]

Equation 2.1

in which \( V \) and \( \rho \) stands for volumes and densities of top (T) and bottom (B) phases while \( XB \) and \( XT \) are the segment lengths of the tie-line as shown in Figure 2.4.

Equation 2.2 describes an alternative and more accurate way for the TLL determination based on the equilibrium phase compositions (Freire, 2016a):

\[
TLL = \sqrt{[B_{salt} - T_{salt}]^2 + [T_{IL} - B_{IL}]^2}
\]

Equation 2.2

\( B_{salt} \) and \( T_{salt} \) are the concentrations of salt in the bottom and top phases, respectively, whereas \( T_{IL} \) is the concentration of IL in the top phase, and \( B_{IL} \) is the concentration of ionic liquid in the bottom phase.

The tie-lines are commonly parallel, and their slope (STL) can be determined by Equation 2.3 (Freire, 2016a), facilitating the construction of further TLs.

\[
STL = \frac{[T_{IL} - B_{IL}]}{[B_{salt} - T_{salt}]} = \frac{\Delta IL}{\Delta salt}
\]

Equation 2.3

The knowledge of the binodal curve is essential when working with ATPS because the composition of the two phases must be known in order to understand the partition of biomolecules. The binodal curve can be determined by three methods: turbidimetric titration, cloud-point and node determination methods (Iqbal et al., 2016).

In most of IL-based ATPS studies, the binodal experimental data is usually fitted according to Equation 2.4 (Merchuk et al., 1998), in which Y and X are the mass fraction percentages of the ionic liquid and the salting-out agent, respectively. \( a, b \) and \( c \) are fitting parameters obtained by least squares regression.

\[
Y = aexp[(b \times X^{0.5}) - (c \times X^3)]
\]

Equation 2.4

The fitting of the binodal data allows the determination of TLs by a weight balance relationship. The compositions of the top and bottom phases and the overall system composition are calculated by the lever-arm rule. Commonly, the following system of four unknown constants is applied to fit the tie-line data (Equation 2.5 to Equation 2.8) (Merchuk et al., 1998): \( T, B \) and \( M \), designate the top phase, the bottom phase and the mixture, respectively; \( Y \) represents the IL weight fraction percentage, whereas \( X \) represents the salting-out agent; \( \alpha \) is the ratio between the mass of the top phase and the total mass of the mixture; and \( A, B \) and \( C \) are the fitted constants obtained by the application of Equation 2.4.
\[ Y_T = a \exp[(b \times X_T^{0.5}) - (c \times X_T^3)] \]  
Equation 2.5

\[ Y_B = A \exp[(B \times X_B^{0.5}) - (C \times X_B^3)] \]  
Equation 2.6

\[ Y_T = \frac{Y_M}{\alpha} - \frac{1 - \alpha}{\alpha} \times Y_B \]  
Equation 2.7

\[ Y_T = \frac{X_M}{\alpha} - \frac{1 - \alpha}{\alpha} \times X_B \]  
Equation 2.8

The ratio of equilibrium compositions in the ATPS top and the bottom phases determines the partition coefficient \((K)\) of biomolecules, which is given by Equation 2.9.

\[ K = \frac{C_{AT}}{C_{AB}} \]  
Equation 2.9

where \(C_{AT}\) is the equilibrium concentration of component A in the top phase and \(C_{AB}\) is the equilibrium concentration of A in the bottom phase. The partition coefficient is often used to evaluate the extension of biomolecules separation in the ATPS. The more different the \(K\) of a target biomolecule from the \(K\) of the remaining biomolecules present in the system, the better the extraction. In other words, \(K\) values greater than one indicate the effectiveness of partitioning in the ATPS (Mazzola et al., 2008).

IL-based ATPSs have been firstly proposed in 2003 (Gutowski et al., 2003), and have been widely used in biological separation and purification fields (Chen et al., 2014). The system is usually formed by water + ionic liquid + inorganic salt, owing to the high ability of salt ions to induce the salting-out of IL. This way, the phase formation is due to the preferential hydration of the high charge-density salt ions, over the low-symmetry and charge delocalized IL ions, which are only able of weak directional intermolecular interactions (and hence weakly hydrated as compared to the common salting-out inducing salts) (Freire, 2016b).

Obviously, the nature of the inorganic salt used as salting-out agent plays a crucial role in the definition of any ATPS. The ATPS formation trend follows closely the well-known Hofmeister series, which ranks the relative influence of ions on the physical behaviour of a wide variety of aqueous processes (Zhang and Cremer, 2006). Generally, this influence is more pronounced for anions than for cations (Schneider et al., 2011), and the typical ordering of the ions and some of its related properties are shown in Figure 2.5. The species in the green region are described as kosmotropes, while those in red region are chaotropes. These terms refer to the ion ability to alter the hydrogen bonding network of water (Collins and Washabaugh, 1985). The kosmotropes, which are believed to be “water structure makers”, are strongly hydrated and have stabilizing and salting-out effects on proteins. On the other hand, chaotropes (“water structure breakers”) are known to destabilize folded proteins and give rise to salting-in behaviour.
Several studies have reported the successful application of IL-based ATPS for protein partition. The proteins were mainly concentrated in the IL-rich phase (top phase) and, although the partition mechanism involved in the ATPS is still poorly understood, hydrophobic and electrostatic interactions, as well as salting-out effects are suggested to be the main driving forces responsible for such behaviour (Ventura et al., 2017). The formation of IL aggregate-protein complexes has also been proposed as the driving force for the selective separation of proteins (Pei et al., 2010).

From an engineering point of view, the extraction and separation methods should be connected, preferably merged and conducted in a single-step. Therefore, high expectations are placed on the use of ionic liquids as novel solvents once they can provide a promising platform, by means of an ATPS, for the simultaneous extraction and selective separation of compounds from macroalgae.

### 2.4.3 Ionic liquid recovery

Ionic liquids remain to date the most expensive research-grade solvents, under investigation, for the dissolution of biomass, which is one of the major drawbacks when envisaging their large-scale application (George et al., 2015). In addition, when considering their application at an industrial scale, the possible environmental impact must be considered. Although the lack of volatility of ionic liquids does not contribute to the release of harmful vapour into the atmosphere, there still exist concerns about their toxicity and biodegradability, which can cause severe water contamination upon the release to aquatic environments (Frade and Afonso, 2010).

In this sense, it is of the most importance the recovery of ionic liquids from the algal extracts, so that they can be reused in further extractions/separations steps, saving the overall costs of the process and avoiding the associated environmental impact. Depending on the nature of the ILs and the target molecules to extract, a wide range of recovery methods have been proposed in literature (Mai et al., 2014). However, barrier processes stand out from competitive techniques because, in addition to their high-performance (Kuzmina, 2016), membrane separations are a well-known procedure, commercially available to industrial deployment and considered a low-
energy demanding process, in which no chemicals are added, decreasing the costs (Haerens et al., 2010; Wu et al., 2009).

A membrane process performs a certain separation by means of a membrane capable of retaining specific compounds, while allowing the passage of others. In pressure-driven membrane processes (reverse osmosis, nanofiltration, ultrafiltration and microfiltration), when a force is exerted across the membrane, the feed stream goes through it and is split into permeate and retentate. Both fractions contain the same solvent, permeate is rich in lower size molecules whereas the retentate is concentrated with larger size molecules (Beier, 2015).

Particles and dissolved components are (partially) retained based on the properties of the membrane such as size, shape and charge. The separation efficiency is expressed by the rejection coefficient \( R \) of a given compound (Equation 2.10):

\[
R = 1 - \frac{C_p}{C_f}
\]

where \( C_p \) stands for permeate concentration and \( C_f \) stands for feed concentration of the specific compound. The rejection coefficient ranges from 0 for complete permeation to 1 for complete rejection (Van Der Bruggen et al., 2003).

Pressure-driven membrane processes can be classified by several criteria: the characteristics of the membrane (pore size), size and charge of the retained particles or molecules, and pressure exerted on the membrane (Beier, 2015). This classification distinguishes microfiltration, ultrafiltration, nanofiltration, and reverse osmosis. In the specific case of this master’s dissertation, ultrafiltration was performed in order to recover the ionic liquid from the macroalga extracts (Figure 2.6). Ultrafiltration membranes have pores with 2-100 nm that allow the retention of macromolecules (e.g. proteins) while it is permeable to salts (e.g. ionic liquid) (Van Der Bruggen et al., 2003).

![Figure 2.6 | Schematic representation of ultrafiltration. Adapted from (Van Der Bruggen et al., 2003).](image-url)
2.5 Macroalgal proteins applications

Seaweeds are rich sources of proteins and contain all the essential amino acids at various concentrations (Kumar and Kaladharan, 2007; Matanjun et al., 2009), which suggest the high-quality of macroalgal proteins. Although further studies in in vivo digestibility of seaweed proteins are necessary to evaluate their functional properties, macroalga proteins seem to be suitable for the formulation of rich-protein supplements for human and animal dietary or to enhance the protein content of foods and feed, which will be useful to fulfil the future needs. In addition, it is also important to note that proteins are an essential nutritional component in the diet of athletes, required to repair and build muscle during exercise (Kerksick et al., 2006). The American College of Sports Medicine recommends a protein intake of 1.2-1.7 g.kg⁻¹.day⁻¹ for endurance- and resistance-trained athletes (Gerovasili et al., 2009). Once again, algae could represent a valuable resource for athletes requiring high levels of protein, especially for vegan athletes for whom eggs and dairy whey protein may not be suitable (Bleakley and Hayes, 2017).

Macroalgal protein extracts can also be a potential source for valuable amino acids for food and feed supplements. The presence of essential amino acids in right quantities is crucial to make quality proteins and they cannot be replaced by other “less-valuable” building-blocks (Holdt and Kraan, 2011).

Besides the nutritional purposes in food, proteins can also provide and/or stabilize the characteristic structure of individual foods. The functional properties of proteins are mainly associated with their ability to form and/or stabilize networks (gels and films), foams, emulsions and sols, which is often revealed by their tertiary structure (Foegeding and Davis, 2011). For instance, in milk proteins, the intrinsically unfolded structure of caseins, in contrast to the compact globular structure of whey proteins, alters their film forming ability and thereby foaming properties (Marinova et al., 2009).

During the last decade, consumer requirements in the field of food production have changed considerably (Síro et al., 2008). Consumers increasingly believe that food contribute directly to their health and, today, foods are no more intended to only satisfy hunger and to provide the necessary nutrients, but also and specially to prevent nutrition-related diseases and to improve physical and mental well-being (Bigliardi and Galati, 2013). In this regard, there is a market trend in the food industry towards the development and manufacturing of functional products¹ (Plaza et al., 2010). The general contribution of proteins/peptides with specific bioactivities is of increasing importance prompting the development of food products based on protein-related health issues (Kitts and Weiler, 2003).

¹ Functional products are considered to be fortified, enriched, altered or enhanced foods that provide an additional health or well-being benefit besides the energetic and nutritional aspects that every food must confer (Hasler, 2002).
Some macroalgal proteins (lectins and phycobiliproteins) are considered bioactive compounds\(^2\) and therefore, they have great potential to be used as ingredients for functional foods, in addition to be useful for pharmacologic proposes (Samarakoon and Jeon, 2012). Seaweed proteins are also sources of bioactive peptides that, although inactive within the parent proteins, can be released through microbial fermentation or enzymatic hydrolysis (Daliri et al., 2017; Jiménez-Escrig et al., 2011). These protein fragments have useful properties for human health, such as anti-microbial, anti-fungal, anti-viral, anti-oxidative, anti-thrombotic, anti-hypertensive, anti-tumor, immunomodulatory and appetite suppression activities (Korhonen and Pihlanto, 2006; Perez Espitia et al., 2012).

Algal proteins and derivatives are also suitable for several cosmetic applications. For instance, these biomolecules are important for moisture retention on hair and skin. On the other hand, they also show a strong affinity for both, improving their nourishments. In the sense that cosmeceuticals are supposed to be involved in healing and repairing damage skin, not only by moisturizing, but also maintaining the nourishment, proteins and peptides derived from algae can be applied in cosmeceutical products (Hagino and Salto, 2004).

The anti-oxidative marine algal peptides might be an interesting source of primary ingredients for the formulation of future cosmeceuticals due to its protective effect from the reactive oxygen species damaging activities (Samarakoon and Jeon, 2012).

### 2.5.1 Macroalgal bioactive proteins

#### 2.5.1.1 Lectins

Lectins are glycoproteins that selectively recognize and reversibly bind to carbohydrates without initiating their further modifications through the associated enzymatic activity (Weis and Drickamer, 1996). They are involved in many biological processes, such as host-pathogen interactions, cell-to-cell recognition and signalling, induction of apoptosis, cancer metastasis, cell growth and differentiation (Ziółkowska and Wlodawer, 2006). Furthermore, due to their high-specific carbohydrate binding capacity, lectins have been found to increase the agglutination of blood cells (erythrocytes). They are also useful as cancer biomarkers or as targets for drug delivery and in the detection of disease-related alterations of glycan synthesis, including infectious agents such as viruses, bacteria, fungi and parasites (Holdt and Kraan, 2011; Naeem et al., 2007). Nevertheless, other bioactive properties exhibited by macroalgal lectins include antibiotic, mitogenic, cytotoxic, anti-nociceptive, anti-inflammatory, anti-adhesion, anti-human immunodeficiency virus (HIV), and human platelet aggregation inhibition activities (Harnedy and Fitzgerald, 2011) – Table 2.2.

---

\(^2\) Bioactive compounds are essential and non-essential compounds that occur in nature, are part of the food chain, and have an effect on human health (Biesalski et al., 2009).
Table 2.2 | Macroalgal source species and bioactivity of lectins.

<table>
<thead>
<tr>
<th>Macroalgal source species</th>
<th>Bioactivity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Caulerpa cupressoides</em></td>
<td>Anti-nociceptive and anti-inflammatory activity</td>
<td>(Vanderlei et al., 2010)</td>
</tr>
<tr>
<td><em>Codium fragile</em></td>
<td>Host-pathogen interactions</td>
<td>(Hori et al., 2000; Smit, 2004)</td>
</tr>
<tr>
<td><em>Eucheuma amakusaensis</em></td>
<td>Induction of apoptosis, metastasis and differentiation</td>
<td>(Hori et al., 2000)</td>
</tr>
<tr>
<td><em>Eucheuma cottonii</em></td>
<td>Recognition and binding of carbohydrates, including virus, bacteria, fungi and parasites</td>
<td>(Bird et al., 1993; Cardozo et al., 2007; Hori et al., 2000)</td>
</tr>
<tr>
<td><em>Eucheuma serra</em></td>
<td>Induction of apoptosis in several cancer cell-lines (including Colo201 and HeLa)</td>
<td>(Sugahara et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>Suppression of colonic carcinogenesis in mice</td>
<td>(Hori et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>Growth inhibition of 35 human cancer cell-lines</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Antibacterial against fish pathogen <em>Vibrio vulnificus</em></td>
<td>(Liao et al., 2003)</td>
</tr>
<tr>
<td><em>Galaxaura marginata</em></td>
<td>Anti-HIV</td>
<td>(Mori et al., 2005; Smit, 2004)</td>
</tr>
<tr>
<td><em>Gracilaria sp.</em></td>
<td>Antibacterial against fish pathogen <em>Vibrio vulnificus</em></td>
<td>(Liao et al., 2003)</td>
</tr>
<tr>
<td><em>Griffithsia sp.</em></td>
<td>Anti-adhesion</td>
<td>(Smit, 2004)</td>
</tr>
<tr>
<td><em>Hypnea japonica</em></td>
<td>Inhibition of human platelet aggregation</td>
<td>(Matsubara et al., 1996)</td>
</tr>
<tr>
<td></td>
<td>Cytotoxic</td>
<td>(Smit, 2004)</td>
</tr>
<tr>
<td><em>Solieria robusta</em></td>
<td>Mitogenic activity on mouse spleen lymphocytes</td>
<td>(Hori et al., 1988)</td>
</tr>
<tr>
<td><em>Ulva sp.</em></td>
<td>Antibiotic</td>
<td>(Smit, 2004)</td>
</tr>
</tbody>
</table>

**2.5.1.2 Phycobiliproteins**

Phycobiliproteins are water-soluble, coloured and fluorescent proteins with an important role in photosynthesis (Glazer, 1994). These molecules can constitute a major proportion of red alga cell proteins, with reported values of 12% (w/w) dry wt. in *Palmaria palmata* (Guangce et al., 2002). There are four main groups of phycobiliproteins – phycoerythrin, phycocyanin, allophycocyanin and phycoerythrocyanin – which are classified based on their colour and absorption characteristics (Sekar and Chandramohan, 2008).

Phycobiliprotein pigment molecules spontaneously fluoresce *in vivo* and *in vitro* (Aneiros and Garateix, 2004; Fleurence, 2004) and, therefore, they are used in fluorescent immunoassays, fluorescent immunohistochemistry assays, biomolecule (proteins, antibody, nucleic acid) labelling, and fluorescent microscopy (Harnedy and Fitzgerald, 2011). They are also currently used as natural pigments for food (chewing gums and dairy products) and in cosmetic applications (Sekar and Chandramohan, 2008; Viskari and Colyer, 2003). Niohan Siber Hegner Ltd. and Dainippon Ink & Chemical Inc., two companies in Tokyo, market the phycobiliprotein phycocyanin as a natural food dye (Houghton, 1996). Nevertheless, seventeen patents for the therapeutic application of phycobiliproteins have been submitted worldwide due to the anti-oxidant, anti-
inflammatory, neuroprotective, hypocholesterolaemic, hepatoprotective, anti-viral, anti-tumour, liver-protecting, atherosclerosis treatment, serum lipid-reducing and lipase inhibition activities that these biomolecules have shown (Sekar and Chandramohan, 2008).

2.5.2 Macroalgal bioactive peptides

Several protein hydrolysates and associated peptides exhibiting bioactive properties have been found in marine macroalgae - Table 2.3 and Table 2.4. Therefore, they have potential to be applied as functional food ingredients (Harnedy and Fitzgerald, 2011). It is important to note that several studies have reported that alga-derived peptides are able to resist gastrointestinal digestion from enzymes such as trypsin, pepsin, and chymotrypsin. This is an essential trait for bioactive peptides in order to achieve their physiological effect at their site of action (Bleakley and Hayes, 2017).

Table 2.3 | Examples of macroalgal peptides exhibiting biological activity.

<table>
<thead>
<tr>
<th>Macroalga Species</th>
<th>Sequence/Name</th>
<th>Biological Activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galaxaura filamentous</td>
<td>Galaxamide</td>
<td>Anti-Cancer</td>
<td>(Xu et al., 2008)</td>
</tr>
<tr>
<td>Hizikia fusiformis</td>
<td>Gly-Lys-Tyr; Ser-Val-Tyr; Ser-Lys-Thr-Tyr</td>
<td>Anti-hypertensive</td>
<td>(Suetsuna, 1998a)</td>
</tr>
<tr>
<td>Palmaria palmata (dulse)</td>
<td>Val-Tyr-Arg-Thr; Leu-Asp-Tyr; Leu-Arg-Tyr; Phe-Glu-Gln-Trp-Ala-Ser</td>
<td>Anti-hypertensive</td>
<td>(Furuta et al., 2016)</td>
</tr>
<tr>
<td></td>
<td>Asn-Ile-Gly-Gln</td>
<td>Anti-inflammatory</td>
<td>(Fitzgerald et al., 2013)</td>
</tr>
<tr>
<td>Porphyra yezoensis</td>
<td>Ile-Tyr; Met-Lys-Tyr; Ala-Lys-Tyr-Ser-Tyr; Ley-Arg-Tyr</td>
<td>Anti-hypertensive</td>
<td>(Cha et al., 2006; Suetsuna, 1998b)</td>
</tr>
<tr>
<td></td>
<td>Ala-Lys-Tyr-Ser-Tyr</td>
<td>Anti-hypertensive</td>
<td>(Saito and Hagino, 2005)</td>
</tr>
<tr>
<td>Ulva sp.</td>
<td>Glu-Asp-Arg-Ley-Lys-Pro</td>
<td>Mitogenic in skin fibroblasts</td>
<td>(Ennamany et al., 1998)</td>
</tr>
<tr>
<td>Undaria pinnatifida</td>
<td>Val-Tyr; Ile-Tyr; Phe-Tyr; Ile-Trp</td>
<td>Reduction of blood pressure in spontaneously hypertensive rats</td>
<td>(Sato et al., 2002)</td>
</tr>
<tr>
<td>Undaria pinnatifida</td>
<td>Val-Tyr; Ile-Tyr; Ala-Trp; Phe-Tyr; Val-Trp; Ile-Trp; Leu-Trp</td>
<td>Anti-hypertensive</td>
<td>(Sato et al., 2002)</td>
</tr>
</tbody>
</table>
Table 2.4 | Examples of macroalgal protein hydrolysates exhibiting biological activity.

<table>
<thead>
<tr>
<th>Macroalga species</th>
<th>Biological activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Caulerpa racemosa</em></td>
<td>Anti-oxidant, anti-cancer</td>
<td>(Lakmal et al., 2014)</td>
</tr>
<tr>
<td><em>Costaria costata</em></td>
<td>Anti-oxidant, anti-tumour, ACE (angiotensin converting enzyme)-inhibitory</td>
<td>(Lee et al., 2005)</td>
</tr>
<tr>
<td><em>Ecklonia cava</em></td>
<td>Anti-hypertensive</td>
<td>(Cha et al., 2006)</td>
</tr>
<tr>
<td></td>
<td>Antioxidant</td>
<td>(Heo et al., 2003)</td>
</tr>
<tr>
<td><em>Enteromorpha prolifera</em></td>
<td>Anti-hypertensive, anti-oxidant, anti-tumour</td>
<td>(Lee et al., 2005)</td>
</tr>
<tr>
<td><em>Grateloupia filicina</em></td>
<td>Anti-hypertensive, anti-oxidant, anti-tumour</td>
<td>(Lee et al., 2005)</td>
</tr>
<tr>
<td><em>Hizikia fusiformis</em></td>
<td>Anti-hypertensive</td>
<td>(Suetsuna, 1998a)</td>
</tr>
<tr>
<td><em>Ishige okamurae</em></td>
<td>Anti-oxidant</td>
<td>(Heo et al., 2003)</td>
</tr>
<tr>
<td><em>Sargassum coreanum</em></td>
<td>Anti-oxidant</td>
<td>(Heo et al., 2003)</td>
</tr>
<tr>
<td><em>Sargassum fulvelum</em></td>
<td>Anti-oxidant</td>
<td>(Heo et al., 2003)</td>
</tr>
</tbody>
</table>
3

Aim of studies
Although seaweeds show great potential as sources of proteins for both human and animal nutrition, to date there is no feasible procedure for the recovery of these proteins (Bleakley and Hayes, 2017). In order to meet this gap, this master’s dissertation aims to study a new integrated biorefinery approach for the single-step extraction and separation of proteins from macroalga Ulva lactuca, using ionic liquids as new solvents.

Among the seaweed species available in European Atlantic waters, Ulva sp. (Figure 3.1) is extensively characterised (Fleurence et al., 1995), having a high protein content (up to 44% dry wt.) (Holdt and Kraan, 2011). In addition, this species is successfully cultivated in integrated multitrophic aquaculture systems, enabling not only scalable and controllable cultivation conditions (Marinho et al., 2013; Robertson-Andersson et al., 2008) but also the removal of N- and P-rich compounds in waste water from land-based aquaculture (Lawton et al., 2013). On the other hand, since Ulva sp. is the world’s most abundant seaweed, this can have a negative impact on the environment and tourism in the coastline, making its harvesting essential (Briand and Morand, 1997).

![Figure 3.1 | Seaweed Ulva lactuca (Krisp, 2011).](image)

One of the limitations in protein extraction from seaweed is the presence of polysaccharides as structural components of macroalga cell walls (Harnedy and Fitzgerald, 2015). The main polysaccharides in U. lactuca cell wall are ulvan and cellulose, which have a structural behaviour as described in Figure 3.2.

![Figure 3.2 | Distribution of the different Ulva sp. cell wall polysaccharides in a schematic cross section of a thallus (A) and the proposed associations between the different cell wall polysaccharides (B). Adapted from: (Lahaye and Robic, 2007).](image)
Ulvan is a water-soluble polysaccharide, which main constituents are sulphate, rhamnose, xylose, and glucoronic acid (Lahaye and Robic, 2007). On the other hand, cellulose is insoluble in water and in most common organic liquids, mainly due to the many intermolecular hydrogen bonds present in its structure (Swatloski et al., 2002). The insoluble character of cellulose is a big challenge in cell disruption methods, especially when the desired molecules are proteins, which functionality can be easily compromised by aggressive pre-treatment and extraction processes. Fortunately, ionic liquids have demonstrated a good performance in the complete dissolution of a wide range of biomass matrices mainly due to their ability to dissolve cellulose (Brandt et al., 2013). Moreover, they have also been proposed as promising alternative solvents for the extraction and separation of added-value compounds from biomass. Ionic liquids can provide a non-denaturing environment for biomolecules, maintaining the protein structure and, if applicable, ensuring their enzymatic activity (Passos et al., 2014).

In this perspective, this thesis focuses on the development of a process in which *U. lactuca* biomass is processed for protein extraction in the presence of a suitable ionic liquid. An aqueous biphasic system aiming to separate the proteins from the carbohydrates is also studied. Hereby, the proposed biorefinery approach would allow not only the valorisation of seaweed proteins but also of carbohydrates for further use, for instance, for bioenergy generation. Finally, the recovery of the ionic liquid from the macroalga extracts is evaluated by means of ultrafiltration.
Materials and Methods
4.1 Sample collection and preparation

*Ulva lactuca* was kindly provided by Dr. Willem de Visser from Wageningen Plant Research. In short, cultures were maintained in 1 m³ tanks containing filtered sea water in a greenhouse property of Wageningen University and Research (Nergena, Wageningen – The Netherlands). Samples were collected every month in the period June-August 2016. After biomass collection, the excess water was removed and the samples were freeze dried in a sublimator 2x3x3 (Zirbus Technology GmbH, Germany) for 72 hours. Dried samples were stored in sealed bags and maintained in the dark, at room temperature, until further use. Before starting the experiments, the dried alga was milled in a coffee grinder to obtain a fine and homogeneous powder, which was stored under the same conditions and regarded as starting biomass (sB).

4.2 Biomass characterization

The algal batch under study was characterized in terms of ash and moisture content. The quantification of total lipids, carbohydrates and proteins was also performed.

4.2.1 Total solids and ash

The dry weight concentration and ash content were determined according to NREL (National Renewable Energy Laboratory) analytical procedure, specific for algal biomass (Van Wychen and Laurens, 2013). For total solids determination, three aluminum trays were weighed in an analytical balance (ME 23 SP, Santorius, Germany), and the weight of each aluminum tray was recorded. An amount of 100 mg of sB was weighted in the pre-weighed trays (experiment performed in triplicate); the weight of each tray and sample was then recorded. Samples were placed in a convection drying oven (Nabertherm, Germany), and dried for 18 hours at 105°C and atmospheric pressure. Afterwards, trays were removed and cooled to room temperature in a desiccator. Trays and dried samples were weighed and the respective weights recorded. In order to determine the percentage of total solids and moisture content, Equation 4.1 and Equation 4.2 were, respectively, used. All the results reported in this dissertation are expressed on dry wt. basis (DW), defined as the weight of biomass mathematically corrected for the amount of moisture in the sample (Equation 4.3).

\[ % \text{Total Solids} = \frac{\text{Weight}_{\text{aluminum tray + dry sample}} - \text{Weight}_{\text{aluminum tray}}}{\text{Weight}_{\text{sB}}} \times 100 \]

Equation 4.1

\[ % \text{Moisture} = 100 - % \text{Total solids} \]

Equation 4.2

\[ \text{DW}_{\text{sample}} = \frac{\text{Weight}_{\text{sB}} \times % \text{Total Solids}}{100} \]

Equation 4.3

To access the ash content, two glass tubes (VWR International, USA) were weighed in an analytical balance, and each glass tube weight was recorded. An amount of 100 mg of sB was
weighted in the pre-weighted glass tube (experiment performed in duplicate). Samples were ignited and incinerated using an ash-oven (muffle furnace L 24/11, Nabertherm, Germany) equipped with a ramping program: temperature was ramped to 105°C and maintained at this temperature for 30 minutes; temperature was ramped again until 575°C in 4 hours and maintained at this temperature for more 4 hours; temperature was finally dropped until 105°C in 4 hours. Samples were removed and cooled down to room temperature in a desiccator. The weight of glass tubes and respective ashes was recorded. The ashes were expressed as a percentage of dry matter according to Equation 4.4.

\[
\% Ash = \frac{Weight_{glass\ tube+ash} - Weight_{glass\ tube}}{DW_{sample}} \times 100
\]

Equation 4.4

4.2.2 Total lipid content

The total lipid content was determined according to Folch et al. (Folch et al., 1987). An amount of 100 mg of sB was weighed in a glass tube, using an analytical balance (experiment performed in duplicate). The total lipid extraction began with the addition of 1 mL of chloroform (HPLC grade, Biosolve Chimie SARL, France), followed by the addition of 1 mL of methanol (HPLC supra gradient, Biosolve Chimie SARL, France). The mixture was vortexed for 3 minutes, after which 1 mL of chloroform was added; the mixture was again vortexed for 3 minutes. A volume of 0.8 mL of phosphate buffer saline (prepared according to Table 4.1) was added and the resulting suspension was mixed for 45 minutes in an orbital shaker (LD79, Labinco, Netherlands) at 150 rpm.

<table>
<thead>
<tr>
<th>Components</th>
<th>Purity</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.21 g KH₂PO₄</td>
<td>≥ 99%</td>
<td>Merck Millipore, Germany</td>
</tr>
<tr>
<td>0.48 g Na₂HPO₄.2H₂O</td>
<td>≥ 98%</td>
<td>Merck Millipore, Germany</td>
</tr>
<tr>
<td>9.00 g NaCl</td>
<td>≥ 99%</td>
<td>Merck Millipore, Germany</td>
</tr>
<tr>
<td>1 L distilled water</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.1 | Composition of 1 L phosphate buffer saline.

Samples were then centrifuged at 1000xg (Allegra X-30R Centrifuge, Beckman Coulter, USA) for 1 minute to accelerate phase separation, and the bottom layer was collected to a pre-weighted glass tube. The steps described before were performed four times, using the middle and upper layers as starting material. The bottom layers of the following four extractions were also collected and the solvent was evaporated using a rotational vacuum concentrator for 45 minutes (RVC 2-25 CDplus, Martin Christ Freeze Dryers, Germany). Samples were left overnight, at room temperature, under N₂ flow to remove any remaining traces of solvent. The weight of glass tubes and respective lipids was recorded, and the total lipid content was determined by Equation 4.5.

\[
\% Total\ lipids = \frac{Weight_{glass\ tube+lipids} - Weight_{glass\ tube}}{DW_{sample}} \times 100
\]

Equation 4.5
4.2.3 Total carbohydrate content

Concerning the determination of total carbohydrate content in macroalgae, 1 mg of sB was weighed in lysing matrix D tubes (MP Biomedicals, USA), followed by the addition of 1 mL milliQ water (experiment performed in triplicate). In order to get a homogeneous suspension, the tubes were placed in a tissue homogenizer (Precellys 24 Homogenizer, Bertin Instruments, France) and bead beaten for 3 cycles of 60 seconds at 6500 rpm, with 120 seconds break between cycles. Samples were diluted 10x with milliQ water and analyzed for carbohydrates as described in section 4.6.2.

4.2.4 Total protein content

To determine the total protein content, two different colorimetric procedures were used, namely the Lowry’s method and the Bradford assay, each one requiring a particular sample preparation (experiment performed in triplicate and quadruplicate, respectively). For the first method, 1 mg of sB was resuspended in 1 mL of lysis buffer (60 mM Tris pH 9, 2% SDS – Table 4.2) in lysing matrix D tubes, whereas for the second assay, the biomass was resuspended in 0.1 M NaOH (Sigma Aldrich, Germany). Tubes were placed in the tissue homogenizer and bead beaten for 3 cycles of 60 seconds at 6500 rpm, with 120 seconds break between cycles, followed by 30 minutes of incubation at 100ºC in a block heater (SBH200D/3, Stuart, UK). Then, samples were centrifuged for 10 minutes at 3500 rpm (Centrifuge 5424R, Eppendorf, Germany), and analyzed for protein quantification as described in section 4.6.1.

Table 4.2 | Reagents and respective purity and supplier used in lysis buffer preparation.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Purity</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris(hydroxymethyl)aminomethane (Tris)</td>
<td>≥ 99.9%</td>
<td>Sigma Aldrich, Germany</td>
</tr>
<tr>
<td>Sodium dodecyl sulphate (SDS)</td>
<td>≥ 98.5%</td>
<td>Sigma Aldrich, Germany</td>
</tr>
</tbody>
</table>

4.3 Conventional methods for protein extraction

After literature review for the most common methods applied in protein extraction from macroalgae, three conventional procedures were replicated to be used as reference cases.

4.3.1 Aqueous and alkaline extraction

Aqueous and alkaline extraction (sequential) was performed according to Fleurence et al. (Fleurence et al., 1995). 1 g of sB was resuspended in 20 mL of milliQ water, and gently stirred overnight at 4ºC (experiment performed in triplicate). The suspension was centrifuged for 20 minutes at 10000xg. The supernatant was collected for further analysis (aqueous extract) whereas the pellet was resuspended in 10 mL of NaOH 0.1 M. The resulting suspension was gently stirred, at room temperature, for 1 hour in a tube shaker (Multi Reax, Heidolph, Germany) and subsequently centrifuged at 10000xg for 20 minutes. The supernatant, corresponding to alkaline extract, was collected for further analysis.
Both aqueous and alkaline extracts were analyzed for protein by Bradford assay, as described in section 4.6.1.

The pH was measured in alkaline extract using a pH electrode (SI-600, Sentron, Netherlands).

4.3.2 Extraction by high-shear force under alkaline conditions

High-shear disruption of macroalgal cells was performed based on Harnedy and FitzGerald (Harnedy and FitzGerald, 2013) (experiment performed in duplicate). An amount of 2.5 g of sB was resuspended in 50 mL of a pH 8 solution, and the biomass was processed using an Ultra-turrax (Ultra-Turrax® T25, IKA®, Germany), at 6400 rpm, for 10 minutes, at room temperature. The beaker was placed in an ice bath to avoid sudden temperature fluctuations, which were controlled with a thermometer along the experiment.

After mechanical grinding, the suspension was centrifuged at 10000xg for 20 minutes and the supernatant was collected for protein analysis. The concentration of protein in the resulting extract as determined by Bradford assay as described in section 4.6.1.

4.3.3 Aqueous biphasic system PEG/Na₂CO₃

Aqueous two-phase system was created based on the binodal curves reported by Snyder et al. (Snyder et al., 1992). The system was prepared at room temperature by mixing the required amount of 9.90% (w/w) Polyethylene Glycol 1000 (PEG) (Sigma Aldrich, Germany), 10.90% (w/w) Na₂CO₃ (≥ 99%, Sigma Aldrich, Germany), and milliQ water in microtubes (Eppendorf, Germany) to obtain the final weight of 1.5 mg (experiment performed in duplicate). Afterwards, 45 mg of sB was added to the tube, which was thoroughly stirred for 20 minutes in the tube shaker. Then it was centrifuged at 4500xg for 20 minutes to speed up the phase formation. The volumes of each phase were determined, and the content of both phases was analysed for protein and carbohydrate concentration. A system composed by 9.90% (w/w) PEG 1000, 10.90% (w/w) Na₂CO₃, and milliQ water was prepared as negative control, as well as a mixture composed by 45 mg of algal biomass and 1.5 mL of milliQ water as a positive control. The concentration of protein in each phase was determined by Lowry’s method (section 4.6.1) requiring a 2x dilution with lysis buffer (120 mM TRIS pH 9, 4% SDS). For carbohydrate quantification (section 4.6.2), samples were diluted 20x with milliQ water. The negative control was used as blank in both analytical methods.

The partition coefficient of protein in the formed system was determined by Equation 4.6

\[ K = \frac{[P]_T}{[P]_B} \]

Equation 4.6

where \([P]_T\) and \([P]_B\) are the protein concentration in the top phase and bottom phase, respectively.
4.4 Assays with ionic liquids

4.4.1 Ionic liquid selection

The screening of ionic liquids for protein extraction and fractionation comprised three stages. Firstly, using the physicochemical properties of the ionic liquids to predict their ability to interact with the macroalgae cell wall, ten ionic liquids were selected among the twenty-five available in Bioprocess Engineering (BPE) group from Wageningen University and Research. Several reports have identified features of ionic liquids associated with cellulose dissolution. For instance, the anion hydrogen bond basicity (AHBB) is one of the properties that most influence the ability of certain ionic liquids to dissolve cellulose (i.e. the better the hydrogen bond acceptor ability of the anion, the higher the solubility of cellulose) (Stark et al., 2012). In this perspective, based on a BPE group’s database comprising different physicochemical properties of several ionic liquids and respective cations and anions, the ten ILs selected were those with the highest values of anion hydrogen bond basicity.

A second selection allowed to reduce the number of candidates from ten to five. This selection was based on a qualitative analysis of ionic liquid-macroalgae interaction, IL costs (information from suppliers) and toxicity (information from literature). In order to evaluate qualitatively the interaction of ionic liquids and macroalgae, solutions of 40% (w/w) IL were prepared for each of the 10 ILs (Table 4.3) in 2 mL-microtubes and 10 mg of sB was added to all of them. Positive controls, composed by sB and milliQ, and negative controls, composed by 40% (w/w) IL and milliQ water, were also prepared and the samples were then vortexed for 2 minutes – the experiment was performed in triplicate for each ionic liquid.

Under this experimental setting, each suspension had a particular behaviour, and by its observation (e.g. phase formation; supernatant colour development), the interaction of the tested ionic liquids and alga was evaluated.

<table>
<thead>
<tr>
<th>Ionic Liquid</th>
<th>Purity</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Choline acetate ([Ch][Ac])</td>
<td>98%</td>
<td>Iolitec, Germany</td>
</tr>
<tr>
<td>1-butyl-3-methylimidazolium acetate ([Bmim][Ac])</td>
<td>95%</td>
<td>Iolitec, Germany</td>
</tr>
<tr>
<td>1-ethyl-3-methyl-imidazolium dibutyl phosphate ([Emim][dbp])</td>
<td>97%</td>
<td>Iolitec, Germany</td>
</tr>
<tr>
<td>1-butyl-3-methylimidazolium dibutyl phosphate ([Bmim][dbp])</td>
<td>98%</td>
<td>Iolitec, Germany</td>
</tr>
<tr>
<td>1-butyl-3-methylimidazolium chloride ([Bmim][Cl])</td>
<td>98%</td>
<td>Iolitec, Germany</td>
</tr>
<tr>
<td>Choline chloride ([Ch][Cl])</td>
<td>98%</td>
<td>Sigma Aldrich, Germany</td>
</tr>
<tr>
<td>trihexyltetradeylphosphonium chloride ([6,6,6,14-P][Cl])</td>
<td>97%</td>
<td>Iolitec, Germany</td>
</tr>
<tr>
<td>tributyl(methyl)phosphonium methysulfate ([1,4,4,4-P][MSO4])</td>
<td>98%</td>
<td>Sigma Aldrich, Germany</td>
</tr>
<tr>
<td>1-butyl-3-methylimidazolium dicyanamide ([Bmim][dca])</td>
<td>95%</td>
<td>Iolitec, Germany</td>
</tr>
<tr>
<td>trihexyltetradeylphosphonium dicyanamide ([6,6,6,14-P][dca])</td>
<td>95%</td>
<td>Iolitec, Germany</td>
</tr>
</tbody>
</table>

3 The databased was made by Edgar Suarez, member of BPE group of Wageningen University and Research, based on Cho et al. (Cho et al., 2012, 2011).
Finally, the third stage of the screening was carried out by quantitative analysis, and allowed the selection the most suitable ionic liquid, among the five previously chosen, for the following experiments. A mixture of 40% (w/w) IL and 10 mg of sB was prepared in lysing matrix D tubes for each of the five ionic liquids (positive controls, composed by sB and milliQ water, and negative controls, composed by 40% (w/w) IL and milliQ water, were also prepared) – the experiment was performed in duplicate for each ILs and quadruplicate for positive controls. Suspensions were placed in the tissue homogenizer and processed under a program composed by three cycles of 60 seconds at 6500 rpm, with 120 seconds break between cycles. Samples were then centrifuged for 10 minutes at 3500 rpm and analyzed for proteins by Bradford assay (section 4.6.1), requiring a dilution of 5x with milliQ water. The ionic liquid that led to the highest fraction of released proteins was selected to the following experiments.

4.4.2 Aqueous biphasic system: binodal curve

After the selection of the most suitable IL for the extraction of proteins, an aqueous biphasic system composed by ionic liquid and salt was created for the extraction and separation of proteins, requiring the construction of a binodal curve. Aqueous solutions of 50% (w/w) K$_2$HPO$_4$ (≥99%, Merck Millipore, Germany) and 60% (w/w) IL were prepared for the determination of the corresponding binodal curve, which was established at room temperature and at atmospheric pressure through the cloud point titration method (Kaul, 2000). Repetitive dropwise addition of the aqueous salt solution to the ionic liquid aqueous solution (with known weight), was followed by vortexing and time to settle. Upon settling, if a cloudy solution was formed (i.e. biphasic region), the cloud point is considered reached and the concentration of phase-forming components was determined based on the mass of aqueous salt solution added. The mixture was then diluted with milliQ water until the formation of a clear solution (i.e. monophasic region), the weight of milliQ water added was recorded and the above process was repeated until enough points were measured for an accurate curve. All the measurements were taken as weight percentage.

The experimental binodal curve was fitted by least-squares regression according to Equation 4.7 (Merchuk et al., 1998) (correlation shown in Appendix B).

$$[IL] = a \exp[(b \times [K_2HPO_4]^{0.5}) - (c \times [K_2HPO_4]^3)]$$

Equation 4.7

4.4.3 Bead mill experimental setup

The DYNO-Mill Research Lab (Willy A. Bachofen AG Maschinenfabrik, Switzerland) was used to conduct simultaneous disintegration, extraction and fractionation of compounds from macroalgae. The DYNO-mill (Figure 4.1) consists of a horizontal milling chamber ($V_{chamber} = 80$ mL), with the engine connected over a central shaft. 65% (v/v) of the milling chamber volume was filled with 1 mm ZrO$_2$ beads (YTZ® beads, Tosoh, Japan) and a suspension composed of 1% (w/w) sB, 11% (w/w) IL and 24% (w/w) K$_2$HPO$_4$ ($V_l = 200$ mL) was fed to the system. The beads were accelerated in the opposite direction of the shaft (the average of agitator shaft speed was 2039 rpm) by a single DNYO-accelerator (Ø 56.20 mm). Different layers of beads were formed
moving at different speeds causing a grinding effect responsible for cell disintegration. The major part of the energy introduced (energy input ~0.04 kWh) was converted to heat due to friction and, therefore, a cooling jacket integrated in the milling chamber and a cooling coil in the feed funnel were used to remove the heat, avoiding temperatures that could lead to protein denaturation (the average temperature was ~24°C). The algal suspension and grinding beads were separated by a sieve plate at the outlet, and the algal suspension was fed back to the feed funnel. The bead mill was operated under batch recirculation mode (i.e. the algal suspension was recirculated, passing multiple times through milling chamber) for 1 hour. In the end of the experiment, the processed mixture was removed from the bead mill to 50 mL-centrifuge tubes (Sarstedt, Germany). After time to settle and phase formation, the weight of each phase was noted down and the top and bottom fractions were analysed for proteins and carbohydrates, requiring a 2x dilution with milliQ water for the former and a 100x dilution for the latter. A negative control composed by 11% (w/w) IL, 24% (w/w) K₂HPO₄ and water was used as blank in both analytical methods.

![Figure 4.1 | DYNO-mill equipment and respective components.](image)

**4.5 Recovery of ionic liquid**

A bead mill experiment was run under the same conditions described in section 4.4.3, however the feed suspension was composed by 15% (w/w) IL and 10% (w/w) sB. The resulting mixture was centrifuged, at 4700 rpm, for 20 minutes (Alegra X-30R, Beckman Coulter, USA) and aliquots of supernatant were analysed for protein (sample previously diluted 3x with milliQ water).

An ultrafiltration process was carried out in order to recover the ionic liquid from the algal extract (previously diluted 2x with milliQ water). Two different setups were tested: a stirred ultrafiltration cell (model 8050, EMD MilliPore Amicon™ Bioseparations, USA) – Figure 4.2 – and a centrifugal filter unit (Amicon Ultra-4 centrifugal filter with Ultracel-3 membrane, Millipore, USA) – both setups were performed in duplicate.

In the first setup, a Biomax polyethersulfone membrane (EMD MilliPore Amicon™ Bioseparations, USA) with a pore size of 10 kDa was used. The stirred cell was filled with 10 mL
of supernatant, and the stir bar/pressure cap assembly was fitted onto the cell. The stirred cell was placed on a magnetic stirrer plate with the stirring speed set to 600 rpm, preventing the build-up of a biomass layer at the membrane surface. The pressure inside the chamber was maintained at 32.50 psi by nitrogen gas, which provided the driving force for the filtration. The separation process was carried out at a constant flow rate (0.10 mL.min⁻¹) until the retentate volume is reduced to approximately 2 mL.

![Diagram of stirred cell ultrafiltration assembly](image)

Figure 4.2 | Schematic diagram of stirred cell ultrafiltration assembly used for recovering the ionic liquid from the supernatant. Adapted from (Merck Millipore, 2015).

In the second setup, a centrifugal filter unit equipped with an ultracel-3 membrane (pore size of 3 kDa) was filled with 4 mL of supernatant and spun, at 4000xg, for 10 minutes (Allegra X-30R Centrifuge, Beckman Coulter, USA). The centrifugation step was repeated 4 times until the filtrate volume reached 3 mL.

The concentration of ionic liquid, protein and carbohydrates in retentates and filtrates were determined. Proteins were quantified by Bradford assay (section 4.6.1), whereas carbohydrates and ionic liquid were quantified according to section 4.6.2 and section 4.6.3, respectively. The required dilutions for each assay are described in Table 4.4.

<table>
<thead>
<tr>
<th>Protein quantification</th>
<th>Carbohydrate quantification</th>
<th>IL quantification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retentates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3x</td>
<td>500x</td>
<td>50x</td>
</tr>
<tr>
<td>Filtrates</td>
<td>Without dilution</td>
<td>50x</td>
</tr>
</tbody>
</table>

The rejection coefficients of proteins and carbohydrates in both membranes were determined according to Equation 4.8, in which \( C_p \) and \( C_f \) stand for the concentration of protein/carbohydrate in the permeate and in the feed, respectively.

\[
R = 1 - \frac{C_p}{C_f}
\]

Equation 4.8
4.6 Analytical methods

4.6.1 Protein quantification

Lowry’s method or Bradford assay were used for protein quantification depending on the assay. For both methods, a calibration curve was prepared using bovine serum albumin (BSA) (Sigma Aldrich, Germany) as standard, for a working range of 0–1.40 mg.mL\(^{-1}\) for Lowry’s method and 0–2 mg.mL\(^{-1}\) for Bradford assay.

Lowry’s method was performed, with a commercial kit (DC\(^{TM}\) Protein Assay, Bio-Rad, USA), following the microplate assay protocol. 10 µL of each standard and unknown samples were transferred into a 96-well plate (Greiner Bio-One International, Austria), and after the addition of Lowry’s reagents, as described in the protocol, the microplate was covered with aluminium foil and incubated, at room temperature, for 30 minutes. The absorbance was then measured at 750 nm using a microplate reader (Infinite M200, Tecan, Switzerland).

The Bradford assay was also performed following the microplate assay protocol of a commercial kit (Pierce\(^{TM}\) Coomassie protein assay, Thermo Fisher Scientific, USA). 5 µL of standards and unknown samples were added to a 96-well microplate and after the addition of 250 µL of Coomassie reagent, the microplate was shaken for 30 seconds and incubated 10 minutes at room temperature. The absorbance was measured at 595 nm in the microplate reader.

According to the experiment, the concentration of protein was determined by direct interpolation from the calibration curves described in Appendix A.1. The total protein content in macroalgae and the fraction of released proteins in each extraction procedure were determined by Equation 4.9 and Equation 4.10, respectively.

\[
\% \text{Total protein} = \frac{[\text{Protein}] (\text{mg.mL}^{-1}) \times Volume_{\text{sample}} (\text{mL})}{DW_{\text{sample}} (\text{mg})} \times 100
\]

Equation 4.9

\[
\% \text{Released protein} = \frac{[\text{Protein}]_{\text{extract}} (\text{mg.mL}^{-1}) \times Volume_{\text{extract}} (\text{mL})}{DW_{\text{sample}} (\text{mg}) \times \frac{\% \text{Total Protein}}{100}} \times 100
\]

Equation 4.10

4.6.2 Carbohydrate quantification

The quantification of carbohydrates was carried out according to Dubois et al. (Dubois et al., 1956). Calibration curves were prepared using glucose (Sigma Aldrich, Germany) as standard, for a working range of 0-0.10 mg.mL\(^{-1}\). 40 µL of standard and unknown samples were transferred to a 96-well microplate, followed by the addition of 40 µL of 5% (w/w) phenol solution (molecular biology grade, Sigma Aldrich, Germany), and 200 µL of concentrated H\(_2\)SO\(_4\) (95-98%, Sigma Aldrich, Germany). The microplate was covered by aluminum foil and incubated, at room temperature, for 10 minutes, followed by an incubation step for 35 minutes, at 35°C. The absorbance was measured at 483 nm in the plate reader.

The concentration of carbohydrates was determined, according to the experiment, by direct interpolation from the calibration curves described in Appendix A.2. The percentage of total
carbohydrate content in macroalgae and the fraction of released carbohydrates in each extraction procedure were determined by Equation 4.11 and Equation 4.12, respectively.

\[
\% \text{Total carbohydrates} = \frac{[\text{Carbohydrates} \text{(mg.mL}^{-1}) \times \text{Volume}_{\text{sample}}]}{\text{DW}_{\text{sample}} \text{(mg)}} \times 100
\]

Equation 4.11

\[
\% \text{Released carbohydrates} = \frac{[\text{Carbohydrates}_{\text{extract}} \text{(mg.mL}^{-1}) \times \text{Volume}_{\text{extract(mL)}}]}{\text{DW}_{\text{sample}} \text{(mg)} \times \% \text{Total Carbohydrates}} \times 100
\]

Equation 4.12

4.6.3 Ionic liquid quantification

The concentration of ionic liquid in the fractions resulting from the ultrafiltration experiment was estimated using an Ultra High-Performance Liquid Chromatography (UPLC) apparatus (Nexera X2, Shimadzu, USA) equipped with a RezexTM ROA-Organic Acid H+ 8% (300mm x 7.8mm) column (Phenomenex, USA), coupled with a security guard (Phenomenex, USA), a pump (LC-30AD, Shimadzu, USA), an autosampler (SIL-30AC, Shimadzu, USA) and a refractive index detector (RID-20A, Shimadzu, USA). The injection volume was 20 µL and the isocratic elution was performed with 0.005 N H₂SO₄ solution, as mobile phase. The column was kept at 60°C, under a pressure of 55 bar, and the pump operated at a flow rate of 0.60 mL.min⁻¹.

The concentration of ionic liquid was determined by direct interpolation of the calibration curve described in Appendix A.3.

The ionic liquid recovery was calculated according to Equation 4.13.

\[
IL_{\text{recovery}}(\%) = \frac{[IL]_{\text{filtrate}} \text{(mg.mL}^{-1}) \times \text{Volume}_{\text{filtrate(mL)}}}{[IL]_{\text{feed}} \text{(mg.mL}^{-1}) \times \text{Volume}_{\text{feed(mL)}}} \times 100
\]

Equation 4.13

4.6.4 Native-PAGE gel electrophoresis

Native-PAGE electrophoresis was performed to study how the conditions used in aqueous and alkaline extraction and mechanical grinding extraction affect the protein conformation. Aliquots from extracts were diluted with milliQ water in order to obtain solutions with the same concentration of protein. Afterwards, those samples were diluted with native sample buffer (BioRad, USA) in a proportion of 1:2. After mixing, 25 µL of each sample were transferred into a 4-20% Criterion Tris-Glycine eXtended (TGX) precast gel (BioRad, USA), as well as 5 µL of NativeMark™ Unstained Protein Ladder (Invitrogen, EUA). Samples were run in 10x Tris/Glycine buffer (BioRad, USA), at 125 V, for 75 minutes.

Native gel was stained using the Pierce® silver stain kit (Thermo Fisher Scientific, USA), following the respective user guide. The image of native gel were acquired with LabScan 6.0 software, using the ImageScanner III (GE Healthcare, UK).
4.7 Statistical Analysis

R-studio was used to carry out the statistical analysis of results. Replicates were performed for almost all experiments and data are presented as mean values ±SD (standard deviation). Experimental data were analysed by one-way analysis of variance (ANOVA) with the significance level of $p=0.05$. When significant differences were found (i.e. $p<0.05$), the Tukey’s Honest Significant Difference (HSD) test was used to detect significant differences among treatments.
5

Results and Discussion
5.1 Chemical composition of *Ulva lactuca*

The freeze-dried *Ulva lactuca* was analysed and its composition determined. The average composition is shown in Table 5.1.

Table 5.1 | Chemical characteristics of macroalga *U. lactuca* (% w/w on dry basis).

<table>
<thead>
<tr>
<th>Components</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Solids (%)</td>
<td>96.50±0.87</td>
</tr>
<tr>
<td>Ash</td>
<td>22.80±0.35% dry wt.</td>
</tr>
<tr>
<td>Protein</td>
<td>17.81±0.79% dry wt. (Lowry’s method) 3.70±0.30% dry wt. (Bradford assay)</td>
</tr>
<tr>
<td>Lipid</td>
<td>4.95±0.35% dry wt.</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>60.92±14.62% dry wt.</td>
</tr>
</tbody>
</table>

The moisture content (3.50±0.87% dry wt.) is very low because the starting algal biomass has been lyophilized previously. Lyophilization (freeze-drying) is a gentle dehydration process that allows the removal of cellular water, leading to a residual moisture contents in the biomass (Dejoye et al., 2011; Wessman et al., 2013).

The ash content (22.80±0.35% dry wt.) is comparable with values published in previous studies. For instance, Wong and Cheung measured 21.30±2.78% dry wt. of ash in *U. lactuca*, whereas Yaich et al. determined 19.59±0.51% dry wt. of ash in the same species (Wong and Cheung, 2000; Yaich et al., 2015).

The lipid content (4.95±0.35% dry wt.) is found to be significantly higher than that usually found in *U. lactuca*, which varies between 0.30% dry wt. and 1.79% dry wt. (Ortiz et al., 2006; Tabarsa et al., 2012; Wong and Cheung, 2000). This difference could be attributed to factors such as the conditions for seaweed growth such as climate and geography, as well as the timing for harvest or the method used for lipid extraction (Yaich et al., 2015).

The carbohydrate content determined in this study (60.92±14.62% dry wt.) agrees with values reported in literature for *U. lactuca* (De Pádua et al., 2004; Ortiz et al., 2006). However, although the value is within expectations, it is important to note the large standard deviation obtained, which indicates some variability in the value of carbohydrate content determined between the different biological replicates. In order to validate this analysis, the experiment should be performed again.

The total protein content in *U. lactuca* varies considerably between published studies (Table 5.2). In addition to season’s related variability, the method applied for protein quantification strongly affects the reliability of the determined value (Cerna, 2011).
Table 5.2 | Protein content in *Ulva lactuca*.

<table>
<thead>
<tr>
<th>Protein content in <em>U. lactuca</em></th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>% (w/w) dry wt.</td>
<td></td>
</tr>
<tr>
<td>8.46±0.01</td>
<td>(Yaich et al., 2015)</td>
</tr>
<tr>
<td>10.69±0.67</td>
<td>(Tabarsa et al., 2012)</td>
</tr>
<tr>
<td>7.06±0.06</td>
<td>(Wong and Cheung, 2000)</td>
</tr>
<tr>
<td>27.02±1.10</td>
<td>(Ortiz et al., 2006)</td>
</tr>
<tr>
<td>18.35</td>
<td>(De Pádua et al., 2004)</td>
</tr>
<tr>
<td>18.10</td>
<td>(Shuuluka et al., 2013)</td>
</tr>
<tr>
<td>17.88±0.10</td>
<td>(Khairy and El-Shafay, 2013)</td>
</tr>
</tbody>
</table>

The determination of total protein content in this study was carried out by two different colorimetric methods: the Lowry's method and the Bradford assay. The total protein content determined by Lowry's method is substantially higher than that determined by Bradford assay – 17.81±0.79% dry wt. and 3.70±0.30% dry wt., respectively. Several authors have reported that Bradford assay generates lower protein values for a large number of organisms compared to Lowry's method (Berges et al., 1993; Clayton Jr. et al., 1988; Eze and Dumbroff, 1982). The comparison between both methods was already performed in other macroalgal species and the results confirmed the general trends found in this study, with a Lowry:Bradford ratio varying from 1.50 to 3.20 (Barbarino and Lourenço, 2005). Actually, in this study, the Lowry:Bradford ratio is found to be slightly higher (4.80), which might be due to the procedure followed for sample preparation. In the Lowry's method, the biomass was resuspended in lysis buffer containing SDS, an anionic surfactant/detergent that assists in the dissolution of membranes by disrupting the hydrophobic-hydrophilic interactions among their molecules (Brown and Audet, 2008). Nevertheless, the interference of ionic detergents with Coomassie reagent, prevents the use of SDS in samples prepared for Bradford assay (Marshall and Williams, 2004). In order to ensure that all the proteins present in macroalgae were quantified, as it is assumed to happen with the Lowry's quantification, the biomass was suspended in 0.1 M NaOH, assuming that such alkaline environment was sufficiently harsh for algal cells as SDS. However, it might not be and cell membranes were not completely disintegrated, avoiding the release of all the proteins present in seaweeds. In consequence, the value of total protein measured by Bradford may be even more underestimated than expected, explaining the higher Lowry:Bradford ratio.

The difference between the alga protein content measured by Bradford assay and Lowry's method could be also explained by the interaction of respective reagents with proteins. The development of colour in Bradford protein assay is associated with the binding of Coomassie dye to basic and aromatic amino acids (Compton and Jones, 1985). Most of seaweeds show relatively low concentrations of basic amino acids, such as lysine and histidine, as well as aromatic amino acids, tyrosine and tryptophan (Bleakley and Hayes, 2017). Therefore, the binding with proteins occurs mainly with arginine and phenylalanine residues, a fact that may contribute to an underestimation of protein content. On the other hand, the Folin-Ciocalteu reagent used in the Lowry's method interacts with all peptide bonds and the colour development can still be enhanced.
by the presence of certain amino acids (e.g. tyrosine, tryptophan, cysteine and asparagine) (Legler et al., 1985). Therefore, the quantification of protein tends to be greater.

According to Barbarino and Lourenço, the Lowry’s method is the most reliable way to determine the protein content in algae since they found out that results generated with Lowry’s method were more similar to the data obtained from the sum of the amino acid residues (Barbarino and Lourenço, 2005). In this sense, it is assumed that the total protein content in Ulva lactuca is 17.81±0.79% dry wt., the value determined by Lowry’s method. The total protein content is comparable with certain values reported in literature (Table 5.2). In particular, with the value determined by Khairy and El-Shafay (Khairy and El-Shafay, 2013), which results from the analysis of U. lactuca collected during the summer, as the samples used in this study.

The Bradford analysis is nevertheless needed for the determination of the protein recovery yield when using ionic liquids. This is because of the interference between Lowry’s reagents and ionic liquids, which prevents the use of this method in IL-based experiments. Since the fraction of protein released by each extraction procedure is calculated using the total protein content in macroalga (Equation 4.10), for accurate and reliable results, the quantification of protein in the extracts should be performed with the same method used for total protein quantification.

The composition of the algal batch under study was also evaluated by thermogravimetric analysis and the results are presented in Table 5.3. The protein content is similar to the one obtained by the Lowry’s method. The amount of carbohydrates determined by thermogravimetric analysis is slightly lower than the one determined by the Dubois method. However, besides the inherent differences between the principles of each method and the absence of thermogravimetric analysis replicates to validate the results, one must consider that Dubois’ quantification has a large SD associated, which leads to concerns about the reliability of results. The lipid and ash contents also present some variation. Replicates of thermogravimetric analysis are recommended to confirm the results obtained.

Table 5.3 | Ulva lactuca composition determined by thermogravimetric analysis.

<table>
<thead>
<tr>
<th>Component</th>
<th>% (w/w) dry wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ash</td>
<td>19.36</td>
</tr>
<tr>
<td>Protein</td>
<td>16.00</td>
</tr>
<tr>
<td>Lipid</td>
<td>7.60</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>53.80</td>
</tr>
</tbody>
</table>

To conclude, it is important to note that, with the percentages obtained for ashes, proteins, carbohydrates and lipids, it is possible to close the mass balance of U. lactuca composition, suggesting the reliability and accuracy of the experiments performed for biomass characterization.

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4 Experiment carried out at Instituto Superior Técnico, Department of Chemical Engineering, by Dr. Ana Paula Vieira Soares Pereira Dias (Ferreira et al., 2016).
5.2 Reference Cases

As mentioned in Materials and Methods (section 4.3), three conventional methods used for macroalga protein extraction were replicated in this study. Extractions by means of aqueous and alkaline solutions (sequential), high-shear force and aqueous biphasic system (PEG/Na₂CO₃) were used as reference cases because, among the protein extraction techniques reported in literature, these methods have led to higher yields of protein recovery in Ulva species (Table 2.1).

Aqueous and alkaline extraction (sequential) showed better performance compared to high-shear force and ATPS extractions (Figure 5.1).

Figure 5.1 | Percentage of protein recovery by high-shear force, aqueous and alkaline extraction (sequential) and aqueous biphasic system PEG/Na₂CO₃ (sum of protein extracted into top and bottom phases). Protein yield expressed as % of total protein (Protein extracted/Total proteins x 100). Results are the means of triplicate determination for aqueous and alkaline extraction and duplicate determinations for high-shear force extraction and aqueous biphasic system ±SD; (*) - means are statistically equal (p>0.05).

The overall fraction of protein extracted by aqueous and alkaline extraction (sequential) (49.08±6.66%) seems to be significantly higher than that obtained by the same method for Ulva rigida (26.80±1.3%) and Ulva rotundata (36.10±1.4%) (Fleurence et al., 1995). Although these authors used the same procedure to extract the protein fraction from both Ulva species, the recovery yields varied considerably between species. One can thus conclude that the protein recovery yield attained using this procedure is a function of the green alga species.

Separately, osmotic shock (aqueous extraction) led to a protein recovery yield of 5.04±0.95%, whereas the alkaline extraction led to 45.42±8.81%, suggesting that the majority of macroalgal proteins are insoluble in water. At high pH (~12), most of the proteins are negatively charged due to the deprotonation of amine groups, which enhances the protein-solvent interaction, thereby increasing the protein solubility (Valenzuela et al., 2013). In addition, since many water-insoluble polysaccharides are solubilized under basic pH (Hostettermann, 2014; Knill and Kennedy, 2003), the alkaline conditions may also have aided in cell wall disruption, leading to a higher protein recovery yield (Zhang et al., 2015).
Concerning the extraction by high-shear force, Barbarino and Lourenço extracted protein from *Ulva fasciata* with a recovery yield of 7.30±0.84 g protein/100 g dry wt. (Barbarino and Lourenço, 2005). On the other hand, the same method was used to extract proteins from the red seaweed *Palmaria Palmata*, obtaining a recovery yield of 6.92±0.12 g protein/100 g dry wt. (Harnedy and FitzGerald, 2013). In this study, only 0.25±0.04 g of protein was extracted per 100 g alga dry wt. (6.68±1.09%), which is an extremely low yield compared to the literature. The procedure used in this study was adapted from both published works (Barbarino and Lourenço, 2005; Harnedy and FitzGerald, 2013), in which the mechanical grinding was followed by extraction under strong alkaline conditions (pH~12), known to lead to higher recovery yields. In order to avoid the expected protein denaturation due to the extremely basic pH (Marambe and Wanasundara, 2017), the procedure followed in this study did not include the strong alkaline conditions. Alternatively, the algae were previously resuspended in a pH~8 solution, providing mild alkaline conditions, which could improve the protein solubility without compromising their functionality, and subsequently processed by mechanical grinding, saving time and costs due to a single-step process. However, the modified procedure fell short of expectations, displaying much lower yields, which suggests that the pH was not high enough to solubilize the proteins. It should also be noted that the high-shear homogenization is supposed to damage the alga cells inducing the protein release (e.g. by reducing the particle size, enhancing mass transfer between solvent and algae and/or fractionate large agglomerates), however when comparing the recovery yield obtained by mechanical grinding (6.68±1.09%) and aqueous extraction (5.04±0.95%), there is not a significant difference between both procedures (p=0.17). This fact suggests that only the effect of the solvent was playing a role while Ultra-Turrax homogenizer worked exclusively as a mixing device. A reasonable explanation for this unexpected result is the coffee grinder use in alga sample preparation, which may have reduced the algae to the minimum size, making the Ultra-Turrax action useless.

The overall fraction of protein extracted by ATPS was 10.52±0.35% (sum of protein quantified in both formed phases (9.11±0.32% in the top phase and 1.42±0.03% in the bottom phase). The same procedure was used by Fleurence et al. to extract proteins from *Ulva rigida* and *Ulva rotundata*, achieving recovery yields of 19.10±1.1% and 31.56±2.1%, respectively (Fleurence et al., 1995). In Figure 5.2, it is possible to observe that, in addition to the top and bottom phases formed, there was also the formation of an interphase in sample tubes. The interphase was solid and, although it was not analysed for proteins, these molecules may be part of its composition. In this sense, besides the influence of the species on the protein recovery yield, the overall fraction of protein extracted may be underestimated because the contribution of interphase proteins was not considered.
In addition to extract proteins from algae, the ATPS can still separate them from other components, for instance, carbohydrates. The phase separation results from the salting-out of the hydrophilic polymer PEG and leads to two phases of different hydrophilic character. Protein partitioning is known to be highly selective in PEG/salt phase systems (i.e. the partition coefficients are high \((K>>1)\)) (Iqbal et al., 2016). Generally, the proteins concentrate in the PEG-rich phase, with a more hydrophobic character, while most of the polysaccharides remains in the salt-rich phase (Jordan and Vilter, 1991). This trend was confirmed in this study, as displayed in Figure 5.3, which describes the distribution of the extracted proteins and carbohydrates between the top and bottom phases. Most of the proteins were concentrated in the top phase: i.e. the PEG-rich phase \((K=11.25)\), whereas the carbohydrates had higher affinity to the bottom phase, the salt-rich phase.

The proteins were quantified in the supernatant of positive controls. The protein recovery yield of \(9.98\pm0.83\%\) means that the presence of PEG or salt in the solution did not significantly increase the percentage of protein released, since the overall recovery yield in the ATPS was...
$10.52 \pm 0.35\% \ (p=0.46)$. In this perspective, the importance of the PEG/Na$_2$CO$_3$ ATPS lies mainly in its ability to separate the proteins from carbohydrates rather than in its use for protein extraction.

To evaluate how the conditions used in high-shear force extraction and aqueous and alkaline extraction affect the protein conformation, a Native-PAGE electrophoresis was performed, and the results are presented in Figure 5.4.

![Electrophoresis Gel](image)

Figure 5.4 | Electrophoresis gel of protein extracts under non-denaturing conditions. Lane 1: Protein ladder; Lane 2: Aqueous extract (positive control); Lane 3: Alkaline extract; Lane 4: Mechanical grinding extract.

In the native-PAGE electrophoresis, the proteins are separated according to the net charge, size, and shape of their native structure. The electrophoretic migration occurs because most proteins carry a net negative charge in alkaline running buffers (the higher the negative charge density, the faster a protein will migrate). At the same time, the gel matrix acts as a molecular sieve, regulating the movement of proteins according to their size and three-dimensional shape (small proteins face only a small frictional force, migrating more, while higher proteins face a large frictional force, migrating less) (Nelson and Cox, 2008).

Considering the protein pattern of aqueous extract (lane 2) as a positive control (performed under mild conditions in order to ensure protein conformation), it is possible to draw conclusions about how the extraction under strong alkaline conditions (pH~12) and the mechanical grinding under mild alkaline conditions (pH~8) can affect the algal proteins conformation. The protein pattern of alkaline extract (lane 3) is clearly different from the positive control, suggesting that proteins lose their native structure during the extraction. Although the alkaline extraction leads to the highest recovery yields (~45%), there are strong evidences that the functionality of proteins may be compromised, making this method unfeasible for applications in which the native structure of proteins is needed.

On the other hand, the mild alkaline conditions used in mechanical grinding did not compromise the native structure of the proteins since the protein pattern (lane 4) is similar to positive control. However, this method leads to low yields of protein recovery (~7%) as previously discussed.
5.3 Experiments with ionic liquids

5.3.1 Ionic liquid selection

The selection of the most suitable ionic liquid, among those available to test, comprised three steps. Table 5.4 shows the output of the first selection, in which the 10 ILs selected and the respective anion hydrogen bond basicity are enumerated.

Table 5.4 | Ionic liquids selected and respective anion hydrogen bond basicity.

<table>
<thead>
<tr>
<th>Ionic Liquid</th>
<th>Anion hydrogen bond basicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Ch][Ac]</td>
<td>4.84</td>
</tr>
<tr>
<td>[Bmim][Ac]</td>
<td>4.84</td>
</tr>
<tr>
<td>[Emim][dbp]</td>
<td>4.36</td>
</tr>
<tr>
<td>[Bmim][dbp]</td>
<td>4.36</td>
</tr>
<tr>
<td>[Bmim][Cl]</td>
<td>4.25</td>
</tr>
<tr>
<td>[Ch][Cl]</td>
<td>4.25</td>
</tr>
<tr>
<td>[6,6,6,14-P][Cl]</td>
<td>4.25</td>
</tr>
<tr>
<td>[1,4,4,4-P][MSO4]</td>
<td>2.81</td>
</tr>
<tr>
<td>[Bmim][dca]</td>
<td>1.84</td>
</tr>
<tr>
<td>[6,6,6,14-P][dca]</td>
<td>1.84</td>
</tr>
</tbody>
</table>

The second selection included the qualitative analysis of the IL-alga interaction, the cost and toxicity of each ionic liquid. As described in Materials and Methods (section 4.4.1), solutions of 40% of ionic liquid were prepared for the experiments because the use of pure ILs is only viable when they are liquid at room temperature and have low viscosities. Otherwise, the extraction efficiencies will be low due to the limited mass transfer coefficients and the poor penetration of ILs into the biomass structure (Passos et al., 2014). Although high temperatures could be applied when working with pure ILs, either to overcome their melting points or to reduce their viscosities, they might lead to the thermal degradation of target molecules as well as represent further economic concerns. Therefore, to overcome these issues, the ionic liquids were diluted in water, reducing their viscosity and allowing the extraction process to occur at room temperature. Besides the lower energy consumption, there is also a reduction on the overall solvent cost.

The suspensions obtained after mixing IL + water + algae are shown in Figure 5.5. After visual inspection it was possible to infer about the IL-alga interaction. Certain pigments located inside the cells of Ulva lactuca are responsible for its green colour. Therefore, the development of such colour in the supernatants suggests the release of those pigments, implying the destabilization of the cell wall. In this sense, it is possible to conclude that the greener the supernatant, the larger the pigment release, and the greater the interaction of the IL with the cell structures. Reminding the first selection in which the ability of the IL anion to act as hydrogen bond acceptor was used to predict the IL-macroalga interaction, it is expected that ionic liquids with higher AHBB (Table 5.4) lead to a greener supernatant.
Figure 5.5 | Suspensions obtained for each ionic liquid after mixing IL + water + algae. A positive control (+ Ctrl) – algae and water – is placed next to all the samples.

[Choline][Ac] and [Bmim][Ac] are the ionic liquids with the highest AHBB (Table 5.4), however their supernatants were almost uncoloured. On the other hand, [1,4,4,4-P][MSO4] had one of the greenest supernatants and has a low AHBB. These evidences contradict what was expected and, although ILs with higher AHBB are the most effective in cellulose dissolution, it is not possible to predict their interaction with macroalgae based only on this parameter. However, there are some relations that can be made based on the data reported in literature and the results obtained in this study. For instance, it is recognized that the choice of the IL cation also affects the cellulose solubility and, although data are not always consistent, it is generally accepted that the length of the cation’s alkyl chains progressively reduces the dissolution of cellulose (Pinker et al., 2009). The [Emim][dbp] supernatant is greener than the [Bmim][dbp] supernatant, suggesting a greater interaction of macroalgae and [Emim][dbp]. This behaviour can be easily explained by the shorter alkyl chain of [Emim] cation in comparison to [Bmim], increasing the ability of the IL to solubilize cellulose.

Comparing two ILs with the same cation and different anions, for instance [Bmim][dbp] and [Bmim][Cl], it is evident how the AHBB affects the interaction of both ILs with macroalgae. Although the slight difference between the AHBB of [Bmim][dbp] and [Bmim][Cl] (4.36 and 4.25, respectively), it is sufficient for the [Bmim][dbp] supernatant to be much greener than [Bmim][Cl] supernatant.
There was phase formation in the mixtures composed by [6,6,6,14-P][Cl] and [6,6,6,14-P][dca], indicating the highly hydrophobic character of both ionic liquids. Their immiscibility in water coupled with the high viscosity arising from the long alkyl chain (Zhang et al., 2017), makes difficult the use of both ionic liquids in macroalga cell disintegration, which may only be achieved under intensive mixing, for instance, using Ultra-Turrax. Considering an IL-based ATPS, only ILs miscible in water can be used for its formation because, when dealing with highly hydrophobic ILs, two phases already exist before the addition of any salting-out agent, and one of the phases is far from being aqueous due to these ILs’ low solubility in water and vice-versa (Freire et al., 2007). Moreover, experimental studies have clearly documented the correlation between increased hydrophobicity and increased cytotoxicity (Ranke et al., 2007). Hydrophobic ionic liquids have a greater ability to interact with hydrophobic patches on proteins and cell membranes in living cells, which facilitates the uptake of ILs into organisms (Matzke et al., 2010), and a consequent inhibition of the enzyme activity (Jing et al., 2016). All the mentioned issues contributed to the exclusion of both ILs from the list of suitable candidates.

[Bmim][dca] and [Choline][Ac] were excluded due to their high cost (Table 5.5) and lack of colour development in the supernatants. On the other hand, and although its supernatant was one of the greenest, [1,4,4,4-P][MSO4] was also excluded because it is recognized that phosphonium-based ionic liquids are generally more toxic than imidazolium-based ILs (Ventura et al., 2012).

Table 5.5 | Ionic liquids and estimated costs.

<table>
<thead>
<tr>
<th>Ionic Liquid</th>
<th>Cost (€.g-1)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Ch][Cl]</td>
<td>0.06</td>
<td>(Sigma Aldrich, 2017a)</td>
</tr>
<tr>
<td>[Bmim][Ac]</td>
<td>0.78</td>
<td>(Sigma Aldrich, 2017b)</td>
</tr>
<tr>
<td>[Bmim][Cl]</td>
<td>1.31</td>
<td>(Sigma Aldrich, 2017c)</td>
</tr>
<tr>
<td>[6,6,6,14-P][Cl]</td>
<td>1.63</td>
<td>(Sigma Aldrich, 2017d)</td>
</tr>
<tr>
<td>[1,4,4,4-P][MSO4]</td>
<td>2.15</td>
<td>(Gute chemie abcr, 2017)</td>
</tr>
<tr>
<td>[6,6,6,14-P][dca]</td>
<td>3.22</td>
<td>(Sigma Aldrich, 2017e)</td>
</tr>
<tr>
<td>[Ch][Ac]</td>
<td>7.04</td>
<td>(Sigma Aldrich, 2017f)</td>
</tr>
<tr>
<td>[Emim][dbp]</td>
<td>8.72</td>
<td>(Sigma Aldrich, 2017g)</td>
</tr>
<tr>
<td>[Bmim][dca]</td>
<td>16.22</td>
<td>(Sigma Aldrich, 2017h)</td>
</tr>
</tbody>
</table>

Therefore, the 5 ILs selected for the following analysis were [Ch][Cl], [Emim][dbp], [Bmim][dbp], [Bmim][Ac] and [Bmim][Cl]. There was no colour formation in the [Ch][Cl] supernatant, however it is the cheapest IL and choline-based ILs are considered biodegradable and non-toxic (Gadilohar and Shankarling, 2017), reasons why it was considered for the following analysis. The supernatants of [Emim][dcp] and [Bmim][dcp] were the greenest ones, hence they were considered promising candidates. In turn, the supernatants of [Bmim][Ac] and [Bmim][Cl] were colourless, but both ILs have already been applied in food and bioproduct industries (Toledo Hijo et al., 2016). This is an indication of the applicability of these ILs in the food sector, the same area of application of the proteins that will be extracted from macroalgae to be used as functional ingredients in food.
Table 5.6 | Output of the third selection. Features of the five ionic liquids selected.

<table>
<thead>
<tr>
<th></th>
<th>[Emim][dbp]</th>
<th>[Bmim][Ac]</th>
<th>[Bmim][Cl]</th>
<th>[Bmim][dbp]</th>
<th>[Ch][Cl]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrophilic</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>Lowest Cost</td>
<td>✔</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-toxic</td>
<td>✔</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biodegradable</td>
<td>✔</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Applied in food industry</td>
<td>✔</td>
<td>✔</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Greenest supernatants</td>
<td>✔</td>
<td>✔</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The ability of the selected ILs to extract macroalgal proteins was then evaluated. According to Figure 5.6, [Emim][dbp] appears to be the most suitable candidate, extracting 80.62±4.95% of the total proteins present in Ulva lactuca.

![Figure 5.6](image)

Figure 5.6 | Percentage of protein recovery by each of the 5 ILs tested (+ control – algae and water). Protein yield expressed as % of total protein (Protein extracted/Total proteins x 100). Results are the means of duplicates for each IL and quadruplicates for positive control ±SD. a, b, c – means in columns with the same letter are significantly equal (p>0.05).

No significant difference is observed in terms of protein recovery yield between [Bmim][Cl], [Bmim][dbp] and [Emim][dbp]. However, the cost of [Bmim][dbp] is higher than [Emim][dbp] (Table 5.5) and the larger alkyl chains of both [Bmim][Cl] and [Bmim][dbp] increase their toxicity, facts that strengthen the potential of [Emim][dbp] as the most suitable ionic liquid.

In turn, [Ch][Cl] and [Bmim][Ac] extract almost the same amount of protein as the positive control – no significant differences were observed between the protein recovery yields of [Ch][Cl] vs positive control (p=0.99) and [Bmim][Ac] vs positive control (p=0.87) – indicating the ineffectiveness of these ILs for U. lactuca protein extraction.

Concerning the reference cases, the extraction efficiency obtained by [Emim][dbp] is significantly higher than that obtained by any of the conventional methods tested. Moreover, although the effect of the ionic liquid in protein conformation has not been evaluated in this
published studies have shown that certain ILs improve the stability and half-lives of various proteins and enzymes and allow some enzymes to display superactivity (Ventura et al., 2017), which support the applicability of ionic liquids for the purpose under study.

A recent economic analysis assessment demonstrates that the application of ILs for the extraction of compounds from biomass is only viable when the concentration of extracted compounds is considerably higher (<5% dry wt.) or when they are truly value-added compounds (Passos et al., 2014). In the case under study, the use of ILs seems to outweigh the requirements, since, in addition to the concentration of extracted components ranges from 3 to 14% dry wt., such components are proteins with a high potential for downstream applications.

### 5.3.2 Extraction and fractioning of protein by IL based-ATPS

The economic and environmental feasibility of the use of ionic liquids to extract proteins as a single product from seaweeds needs to be assessed. Cascade biorefinery approaches aiming at the maximization of the inherent value of all the components present in the biomass would certainly improve the overall economics of the biorefinery. An integrated biorefinery process where the extracted proteins are the high-value components but also the carbohydrate fraction is used, for instance, for the production of biofuels, chemicals or materials, might meet the expectations for using seaweed as a viable feedstock for biorefineries. In this perspective, an aqueous biphasic system composed by [Emim][dbp], K$_2$HPO$_4$ and water was studied for the protein-carbohydrate separation in macroalgae extracts. K$_2$HPO$_4$ was the inorganic salt selected as salting-out agent because it is the most used in IL based-ABSs for protein extraction (Desai et al., 2016).

The binodal curve obtained for the ATPS composed by [Emim][dbp] and K$_2$HPO$_4$ is shown in Figure 5.7. Although different ATPS compositions could be selected, a low concentration of ionic liquid (11% (w/w) [Emim][dbp]) and a high concentration of salt (24% (w/w) K$_2$HPO$_4$) were used for the ATPS formation in the bead-mil experiment because the stock of [Emim][dbp] available was lower than that of K$_2$HPO$_4$.

![Figure 5.7 | Binodal curve of [Emim][dbp] and K$_2$HPO$_4$ at 298.15 K.](image)
As described in Materials and Methods (section 4.4.3), the [Emim][dbp] based-ATPS creation (Figure 5.8) was a single-step process, in which all the reagents were added at the same time (alga, ionic liquid, water and salt) and the biomass was processed inside a milling equipment.

In Figure 5.8 it is possible to distinguish two aqueous phases (top phase and bottom phase) and a solid pellet and interphase. While both top and bottom phases were collected for protein and carbohydrate analysis, the consistency of the interphase (solid gum) did not allow the quantification of both biomolecules by the available methods. Therefore, taking the starting amount of protein and carbohydrates as 100%, the extraction of both molecules in the interphase was calculated subtracting the mass of protein quantified in the top and bottom phases. On the other hand, the pellet was also not analysed because it was most probably precipitated salt.
In the several published studies in which IL based-ATPSs were applied as fractionating strategy to separate proteins from polysaccharides, the proteins were preferentially concentrated in the IL-rich phase, whereas the polysaccharides were accumulated into the bottom phase (Ventura et al., 2017).

As displayed in Figure 5.9, although the distribution of proteins agrees with literature (preferentially concentrated in the top phase), most of the carbohydrates, instead of being concentrated in the bottom phase, were enriched into the interphase.

Like inorganic salts, polysaccharides are also kosmotropic (Wu et al., 2008) and, although their presence is not sufficient to form an ATPS in macroalga extracts, they assist the K$_2$HPO$_4$ in the phase separation. The interaction between water and saccharides are strong enough to enrich the sugars in the bottom phase (Pei et al., 2010), however it has been shown that the extraction efficiency of carbohydrates into the bottom phase decreases with increasing concentrations of salt (Tan et al., 2012). This may be attributed to the salting-out effect, in which the increase in salt concentration, decreases the solubility of carbohydrates due to the competition of salt molecules for intermolecular hydrogen bonds (McKee and McKee, 1999), meaning less free water available to dissolve saccharides. This phenomenon may explain the separation of carbohydrates into the additional interphase, which should hold insoluble material with high water content, but not dense enough to settle. Instead, the water activity of this material is such that it is trapped between the water activities of the top and bottom phases (i.e. the water activity in the bottom phase was lower because the water molecules were bound to the salt molecules – density of bound water is greater than that of free water (Vaclavik and Christian, 2008)). The formation of the interphase turns the ATPS into a three-partitioning phase (TPP) system and several TPP systems have already been reported in literature for the extraction of carbohydrates into the interfacial layer (Coimbra et al., 2010; Sharma and Gupta, 2002; Tan et al., 2015).

The ATPS negative control was used as blank in protein and carbohydrate quantification to avoid interferences from the top and bottom phases, however, concerns about the quantification of such molecules in the interphase arise from its unknown composition in terms of ionic liquid and salt. Therefore, sophisticated analytical methods for the quantification of protein and carbohydrates in the three-phases formed should be attempted to a more accurate and reliable results. Moreover, replicates of the system should be performed to validate the experiment.

It is also important to keep in mind that the stability of proteins in this specific ionic liquid is a prime requirement to ensure the viability of the proposed process. Techniques such as infrared spectroscopy, NMR spectroscopy, dynamic light scattering, and circular dichroism yield important information on protein stability, which makes them suitable to evaluate the effect of the IL based-ATPS in the extracted proteins.

Once the aforementioned issues are assessed, the optimization of separation conditions is crucial to increase the extraction efficiency of protein in the top phase. Parameters such as the concentration of phase-forming components, pH and temperature affect the partition coefficient of protein in IL based-systems (Pei et al., 2010; Yan et al., 2014). Thus, a screening of the suitable
conditions for this particular separation process should be carried out. The biomass loading has important repercussions for the throughput and economics of the process as there would be cost saving if less of the solvent can be used and more biomass processed per batch. In this sense, the maximum amount of biomass that can be processed without compromising the protein extraction efficiency should be evaluated.

### 5.4 Ionic liquid recovery

As planned, the recovery of IL was further studied by ultrafiltration. The aim was to recover the IL in the filtrate, while molecules, such as proteins and carbohydrates, would be rejected by the membrane. Retentate and filtrate mass balances allowed the evaluation of each membrane performance in terms of [Emim][dbp] recovery. The results showed that both membranes are permeable to IL, allowing the recovery of 80-85% [Emim][dbp] (Figure 5.10).

![Figure 5.10](image-url)  
**Figure 5.10** | Recovery of [Emim][dbp] in filtrates and retentates, after ultrafiltration through stirred ultrafiltration cell and centrifugal filter unit. Results are the means of duplicates ±SD.

On the other hand, the separation efficiency of proteins/carbohydrates from the IL solution was analysed by the rejection coefficient. As it is shown in Table 5.7, the rejection coefficients for both filtration setups were close to 1, meaning that the proteins and the carbohydrates were effectively retained by the membranes.

**Table 5.7** | Rejection coefficients of proteins and carbohydrates for each ultrafiltration setup. Results are means of duplicates ±SD.

<table>
<thead>
<tr>
<th>Ultrafiltration setup</th>
<th>Protein rejection coefficient</th>
<th>Carbohydrate rejection coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stirred ultrafiltration cell</td>
<td>0.91±0.04</td>
<td>0.89±0.01</td>
</tr>
<tr>
<td>Centrifugal filter unit</td>
<td>0.99±0.01</td>
<td>0.99±0.02</td>
</tr>
</tbody>
</table>

Based on these results, one should conclude that ultrafiltration can effectively separate ionic liquids from other compounds in mixture, as far as the selection of the membrane considers the size of the target molecule. Improved recoveries can be achieved by optimizing the operation parameters (e.g. flow rate, concentration ratio, temperature) (Liu and Wu, 1998). Since the
efficiency of IL recovery by both ultrafiltration setups was not significantly different ($p=0.36$), considering the possible scale-up of the process, stirred ultrafiltration cell setup might be more suitable in terms of equipment requirements and time-consumption.
Conclusions and future prospects
The commercialization of macroalgal proteins depends on the development of new techniques for the extraction and separation of these biomolecules, under mild and stabilizing conditions. The work developed in this thesis aimed at using ionic liquids to mediate the extraction of macroalgal proteins. After screening twenty-five different ILs, [Emim][dbp] was selected as the most suitable for the disintegration of _U. lactuca_ cells and subsequent protein extraction. A protein extraction efficiency of 80.62±4.95% was obtained which is significantly higher than that obtained by reference cases: extraction by high-shear force under alkaline conditions, ATPS (PEG/Na_2CO_3) and sequential aqueous and alkaline extraction, with protein recovery yields of 6.68±1.09%, 10.52±0.35% and 49.08±6.66%, respectively.

Aiming at an integrated biorefinery approach and the valorisation of the different algal components, a strategy was proposed that involved the single-step processing of algae under mechanical grinding and in the presence of an aqueous solution of [Emim][dbp] and a certain amount of K_2HPO_4 capable to induce phase formation. A TPP system was created, allowing the selective separation of proteins and polysaccharides, which were mainly concentrated in the top phase (IL-rich phase) and interfacial layer, respectively. Despite this, only ~50% of the extracted proteins were enriched into the top phase, meaning that, although [Emim][dbp] extracts ~80% of the total protein content of _U. lactuca_, the addition of an aqueous solution of K_2HPO_4 permitted the recovery of only ~40% of the total proteins. Therefore, in order to improve the performance of the system under study, it is of utmost importance to increase the extraction efficiency of the proteins into the top phase by optimizing the conditions used for the TPP system formation.

For an economically sustainable process, it is crucial that the IL can be easily separated from the product and recycled for further use. Ultrafiltration showed great potential for the recovery of ionic liquids from macroalgal extracts, with IL recovery yields of almost 80% in the filtrate while allowing the concentration of proteins and carbohydrates in the retentate. In this sense, one should conclude that, if ultrafiltration would be applied only to the top phase of the proposed TPP system (rich in IL and proteins), it should lead to the isolation of the desired proteins while making the IL available for reuse.

The results attained with the TPP system for cell disintegration and protein/carbohydrate fractionation, along with ultrafiltration for IL recovery, are encouraging for a large-scale application. However, several items must be addressed in addition to the experimental issues discussed in section 5.

The purity levels required for biocompounds or refined extracts in cosmetic, food and mainly pharmaceutical and medical sectors are high. This means that the requirements of industry and markets are crucial when attempting the design and development of separation and purification processes based on ILs. The scarce information on the stability and bioactivity of ILs limits their application in such target industries, so further studies in this area are needed.

The development of reliable models that predict the partitioning behaviour of proteins or polysaccharides in TPP systems would be a major breakthrough for the rational design of these separation processes and would help the establishment of TPP systems as a standard and large-
scale unit operation. In addition, special attention should also be paid to the environmental concerns that could arise from phase-forming component waste streams.

Although ILs have been claimed as “green” solvents due to their negligible volatility, there are several studies reporting their negative impact in water ecosystems (Frade and Afonso, 2010). Therefore, a detailed life-cycle assessment on [Emim][dbp] is necessary in order to claim its sustainability from a “green” point of view. However, the integration of an additional ultrafiltration unit operation is a promising option to recovering and recycling [Emim][dbp], minimizing the environmental concerns of its disposal. Moreover, the high salt concentrations used in TPP systems can also create waste disposal concerns, for instance due to enhancing eutrophicaton phenomenon in water (Ratanapongleka, 2010). This limitation may be overcome by recycling the salts, using biodegradable salts (e.g. sodium citrate) or gentler salting-out species, such as amino acids, carbohydrates and polymers (Freire et al., 2012; Raja et al., 2012). Nevertheless, the ability of these alternative components to induce the phase-formation in [Emim][dbp]-based ATPSs needs to be evaluated because they suffer the drawback on only being able to form ATPS with a limited number of ILs due to their low salting-out ability (carbohydrates, amino acids, and polymers versus salts) (Desai et al., 2016).

Finally, it is of the utmost importance to ascertain the economic viability of the proposed biorefinery approach, which was one of the objectives of this research project. However, the obtained results did not allow the accurate establishment of the process mass and energy balances. Due to the impossibility of protein quantification in the interphase, the non-optimized partition conditions in the ATPS, the lack of replicates and the unknown amount of biomass that can be processed at the same time, a proper economic appraisal of the project was deemed not feasible.

Upon the optimization of the proposed biorefinery strategy and with reliable data available for the establishment of mass and energy balances, it will be possible to estimate the costs of raw materials (biomass, [Emim][dbp] and K₂HPO₄), as well as utilities (water supply, energy consumption and nitrogen gas/compressed air). Concerning the required equipment (bead mill, settler, filtration unit), it should be properly scaled up in order to serve the needs of the bioprocess maximizing its performance while also managing to do so in the most cost-effective manner.

Once the process equipment and utility requirements are determined, the costs associated towards the implementation of the projected plant design should be defined. In this sense, one must look at the benefits and impacts of the process through provisional demonstrations of expected costs and monetary yield. The economic outlook should be based on parameters such as capital investment, estimate production costs, provisional balances and ultimately, profitability analysis. This study should determine the success or downfall of the downstream process under development.

As final remarks, although not all the objectives of the study proposed in the beginning of this thesis were fully met, the outcome of the present work presents important insights toward the valorisation of seaweeds as feedstocks for integrated biorefineries. [Emim][dbp] seems to play a
remarkable role in the extraction of macroalgal proteins, which widespread use is envisaged in the near future to boost the quality of modern society.
References


Protein, carbohydrate and ionic liquid quantification
A.1. Protein Quantification

The calibration curves used in protein quantification were prepared with BSA for a working range of 0–1.4 mg.mL$^{-1}$ for Lowry’s method and 0–2 mg.mL$^{-1}$ for Bradford assay. Equation A. 1 to Equation A. 8 describe the calibration curves, and respective correlation factors, used in each experiment for protein determination.

**Total protein content**

*Lowry’s method*

\[
\text{Abs}_{750 \text{nm}} = 0.20[\text{Protein}] \text{mg.mL}^{-1} - 1.47 \times 10^{-2} \quad (r^2 = 0.9938)
\]

*Bradford assay*

\[
[\text{Protein}] \text{mg.mL}^{-1} = 1.50 \text{Abs}_{595 \text{nm}}^2 + 0.61 \text{Abs}_{595 \text{nm}} + 0.04 \quad (r^2 = 0.9870)
\]

**Aqueous and alkaline extraction**

*Bradford assay*

\[
[\text{Protein}] \text{mg.mL}^{-1} = 1.83 \text{Abs}_{595 \text{nm}}^2 - 1.05 \text{Abs}_{595 \text{nm}} + 0.16 \quad (r^2 = 0.9898)
\]

**Extraction by high-shear force**

*Bradford assay*

\[
[\text{Protein}] \text{mg.mL}^{-1} = 1.83 \text{Abs}_{595 \text{nm}}^2 - 1.05 \text{Abs}_{595 \text{nm}} + 0.16 \quad (r^2 = 0.9898)
\]

**Aqueous biphasic system PEG/Na$_2$CO$_3$**

*Lowry’s method*

\[
\text{Abs}_{750 \text{nm}} = 0.17[\text{Protein}] \text{mg.mL}^{-1} + 0.14 \quad (r^2 = 0.9831)
\]

**Ionic liquid selection**

*Bradford assay*

\[
[\text{Protein}] \text{mg.mL}^{-1} = 1.50 \text{Abs}_{595 \text{nm}}^2 + 0.61 \text{Abs}_{595 \text{nm}} + 0.04 \quad (r^2 = 0.9870)
\]

**Aqueous three-phase partitioning system [Emim][dbp]/K$_2$HPO$_4$**

*Bradford assay*

\[
[\text{Protein}] \text{mg.mL}^{-1} = -0.20 \text{Abs}_{595 \text{nm}}^2 + 0.84 \text{Abs}_{595 \text{nm}} + 0.02 \quad (r^2 = 0.9966)
\]
Ultrafiltration

Bradford assay

\[
[\text{Protein}] \text{mg.mL}^{-1} = 1.82 \text{Abs}_{595nm}^2 - 0.39 \text{Abs}_{595nm} + 0.04 \ (r^2 = 0.9873)
\]

Equation A. 8

A.2. Carbohydrates Quantification

The calibration curves used in carbohydrate quantification were prepared with glucose for a working range of 0–0.1 mg.mL\(^{-1}\). Equation A. 9 to Equation A. 12 describe the calibration curves, and respective correlation factors, used in each experiment for carbohydrate determination.

**Total carbohydrate content**

\[
\text{Abs}_{483nm} = 4.37[\text{Carbohydrate}] \text{mg.mL}^{-1} + 2.48 \times 10^{-3} \ (r^2 = 0.9703)
\]

Equation A. 9

**Aqueous biphasic system PEG/Na\(_2\)CO\(_3\)**

\[
\text{Abs}_{483nm} = 4.37[\text{Carbohydrate}] \text{mg.mL}^{-1} + 6.33 \times 10^{-2} \ (r^2 = 0.9703)
\]

Equation A. 10

**Aqueous three-phase partitioning system [Emim][dbp]/K\(_2\)HPO\(_4\)**

\[
\text{Abs}_{483nm} = 7.02[\text{Carbohydrate}] \text{mg.mL}^{-1} + 7.21 \times 10^{-2} \ (r^2 = 0.9981)
\]

Equation A. 11

**Ultrafiltration**

\[
\text{Abs}_{483nm} = 6.32[\text{Carbohydrate}] \text{mg.mL}^{-1} + 6.38 \times 10^{-3} \ (r^2 = 0.9985)
\]

Equation A. 12

A.3. Ionic liquid Quantification

The calibration curve and respective correlation factor used for ionic liquid quantification, in ultrafiltration experiment, was prepared with [Emim][dbp] for a working range of 100–400 mg.mL\(^{-1}\) (Equation A. 13).

\[
\text{Area}_{peak} = 3.54 \times 10^2[\text{IL}] \text{mg.mL}^{-1} - 1.56 \times 10^3 \ (r^2 = 0.9977)
\]

Equation A. 13
Binodal Curve
B.1. Binodal curve correlation

The binodal experimental data obtained for the ATPS composed by [Emim][dbp] an K₂HPO₄ and respective correlation factor are described in Equation B. 1. The r-squared statistical measure is quite far from 1, suggesting that the model does not fit the experimental data so as one should not use it for the determination of component concentrations. Due to time concerns the experimental procedure was not repeated and the concentrations of K₂HPO₄ and IL used for the ATPS formation were estimated by visual observation of the Figure 5.7.

\[
[\text{Emim}][\text{dbp}] = 420.25 \times \exp(-1.32 \times [\text{K}_2\text{HPO}_4]^3) \quad (r^2 = 0.7964)
\]

Equation B. 1