Strategies for extraction and recovery of the protein fraction from the macroalga *Gelidium sesquipedale*

Mariana Duarte Gordalina

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Supervisors:

Doctor Maria Teresa Ferreira Cesário Smolders

Professor Marília Clemente Velez Mateus

Examination Committee

Chairperson: Professor Helena Maria Rodrigues Vasconcelos Pinheiro

Supervisor: Professor Marília Clemente Velez Mateus

Member of the Committee: Professor Maria Ângela Cabral Garcia Taipa Meneses de Oliveira

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Declaration

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

Preface

The work presented in this thesis was performed at iBB - Institute for Bioengineering and Biosciences of Instituto Superior Técnico, Universidade de Lisboa, and developed within the scope of the Smart Valorization of Macroalgae project (FA_05_2017_033) financed by Fundo Azul - Direcção Geral da Política do Mar, during the period of February - October 2020, under the supervision of Doctor Maria Teresa Ferreira Cesário Smolders and Professor Marília Clemente Velez Mateus. Due to the COVID-19 pandemic, laboratorial work was limited.

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Aos meus avós.

Abstract

The red seaweed *Gelidium sesquipedale* is commercially explored and harvested for extraction of agar, leaving a large biomass share needing valorization. From a biorefinery concept perspective, implementing a cascading valorization of both protein and agar represents a step closer to a "no waste" agar extraction process.

Sun dried and milled *G. sesquipedale* from the coast of Portugal, composed of 92.3 ± 0.2% of total solids, and 7.7 ± 0.2% of moisture, (in dry weight: 62.4 ± 3.5% total carbohydrates, with 9.5 ± 2.0% cellulose and 52.9 ± 1.2% agar, 14.8 ± 0.2% total protein, and 19.6 ± 1.0% ash content), was used in the screening of 15 protein multi-steps extraction procedures by means of aqueous, alkaline, and acidic solutions, ultrasound-assisted, and enzyme-assisted extractions. Consecutive aqueous and alkaline extractions allowed recovering 14.7 ± 2.3% protein content. Sequential implementation of two enzyme-assisted extractions (Celluclast, followed by Alcalase) and two sequential alkaline extractions, resulted in a protein recovery of 45.5 ± 2.8%. Scaling up of this procedure (with and without the Alcalase step) led to recovery yields of 38.5 ± 3.5% and 15.8 ± 0.6%, respectively. Extraction residues revealed a loss of ~30% of the carbohydrate fraction, although agar levels were still high (~40% dw).

Protein precipitation with 85% ammonium sulfate and subsequent diafiltration and concentration using a centrifugal ultrafiltration (3 kDa MWCO) yielded overall recoveries of 14.4% and 11.8%, respectively.

Keywords: *Gelidium sesquipedale*, protein content, protein extraction, ammonium sulfate precipitation, agar, chemical composition.

Resumo

A alga vermelha *Gelidium sesquipedale* é explorada e colhida para extração de ágar. Do ponto de vista de um conceito de bio-refinaria, a implementação de um processo em cascata capaz de valorizar a fração proteica e a fração de agar seria importante para reduzir o desperdício desta extração.

G. sesquipedale seca e moída e com uma composição de 92,3 ± 0,2% de sólidos totais, e 7,7 ± 0,2% de humidade, (em massa seca: 62,4 ± 3,5% de carboidratos totais, com 9,5 ± 2,0% de celulose e 52,9 ± 1,2% de ágar, 14,8 ± 0,2% de proteína total e 19,6 ± 1,0% de cinzas), foi utilizada na triagem de 15 procedimentos de extração de proteínas recorrendo ao uso de soluções aquosas, alcalinas e ácidas, sonicação e enzimas. Uma extração aquosa seguida por uma extração alcalina recuperou 14,7 ± 2,3% da proteína total. A implementação sequencial de duas extrações assistidas por enzimas (Celluclast, seguida por Alcalase) e duas extrações alcalinas sequenciais, resultou numa recuperação de 45,5 ± 2,8% da proteína total. O scale-up deste processo (com e sem a etapa com Alcalase) gerou rendimentos de 38,5 ± 3,5% e 15,8 ± 0,6%, respetivamente. Os resíduos após extração revelaram uma perda de ~30% da fração de hidratos de carbono, embora os níveis de ágar continuem altos (~ 40% dw).

A precipitação com 85% de sulfato de amónio e a subsequente diafiltração e concentração por ultrafiltração centrífuga (3 kDa de exclusão molecular) resultaram em recuperações globais de 14,4% e 11,8% da proteína total, respetivamente.

Palavras-chave: *Gelidium sesquipedale*, conteúdo proteico, extração proteica, sulfato de amónio, agar, composição química.

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Abbreviations

AAA	Amino Acid Analysis
ACE	Angiotensin-Converting Enzyme
AEX	Anion-Exchange Chromatography
ANOVA	One-Way Analysis Of Variance
APC	Allophycocyanin
ASE	Accelerated Solvent Extraction
ATPE	Aqueous Two-Phase Extraction
CDOM	Colored Dissolved Organic Matters
DIT	Diiodotyrosine
DME	Dimethyl Ether
dw	Dry Weight
EAA	Essential Amino Acids
EAE	Enzyme-Assisted Extraction
EBA	Expanded Bed Adsorption
EGU	Endoglucanase Units
ESI	Electrospray Ionization
FBGU	Fungal Beta-Glucanase Units
FMOC	Fluorenylmethoxy Chloroformate
FTIR	Fourier Transform Infrared
GC	Gaseous Chromatography
GPs	Glycoproteins
HPAEC-PAD	High-Performance Anion-Exchange Chromatography
HPLC	High Performance Liquid Chromatography
HPSEC	High-Performance Size Exclusion Chromatography
HSD	Honest Significant Difference
IEA	International Energy Agency
ILs	Ionic Liquids
LC	Liquid Chromatography
MAE	Microwave Assisted Extraction
MALDI-TOF	Matrix Assisted Laser Desorption Ionizations/Time-Of-Flight
MF	Microfiltration
MIT	Mono-Iodotyrosine
MS	Mass Spectrometry
MW	Molecular Weight
MWCO	Molecular Weight Cut-Off
NaAc	Sodium Acetate
NAC	N-acetyl-L-cysteine
NF	Nanofiltration

NPN	Non-Protein Nitrogen
NREL	National Renewable Energy Laboratory
ODW	Oven Dry Weight
OPA	Ortho Phtalaldehyde
PBPs	Phycobiliproteins
PC	Phycocyanin
PE	Phycoerythrin
PEC	Phycoerythrocyanins
PEFE	Pulsed Electric Field
PEG	Polyethylene Glycol
PLE	Pressurized Liquid Extraction
RO	Reverse Osmosis
R-PC	R-Phycocyanin
R-PE	R-Phycoerythrin
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
RT	Room Temperature
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEC	Size Exclusion Chromatography
SFE	Supercritical Fluid Extraction
SNP	Specific Seaweed-Nitrogen-To-Protein
TRIS	Tris(Hydroxymethyl)Aminomethane
UAE	Ultrasound-Assisted Extraction
UF	Ultrafiltration
w/w	Weight/Weight

1. Introduction

1.1. Context and Biorefinery Concept

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", which includes systems that may exist as concepts, facilities, processes, plants, or clusters of facilities [1]. The main goal of the biorefinery concept is to use raw biomass in an optimal way, leading to a longer lifespan of resources. Several conversion strategies can be integrated to maximize the production of valuable components, while minimizing waste streams. Biomass processing can be separated into two fractions – a higher quality fraction which leads to high value products (e.g., chemicals, materials), and a lower quality fraction which can be used to produce biofuels. Products can have direct market applications, or they can be used as raw biomass for further manufacturing operations in a cascading approach (Figure 1.1). The concept of seaweed biorefinery can contribute to a sustainable development by adding value to the original biomass, therefore it is crucial to better understand the overall technological processes and pathways.

When it comes to protein production, macroalgae represent a promising protein source. In some cases, they can be richer in protein [up to 47% dry weight (dw)] than conventional protein-rich foods, such as soybean (40%), cereals (15%), eggs (9%), and fish (25%). They can also have higher protein yields per unit of production area (2.5-7.5 $t \cdot ha^{-1} \cdot year^{-1}$) compared to terrestrial crops, such as soybean (0.6-1.2 $t \cdot ha^{-1} \cdot year^{-1}$), vegetable seeds (1-2 $t \cdot ha^{-1} \cdot year^{-1}$), and wheat (1.1 $t \cdot ha^{-1} \cdot year^{-1}$) [2]. Implementing protein extraction operations in processes that already produce carbohydrate fractions (e.g., agar production from *Gelidium sesquipedale*) can thus be a strategy of great relevance.

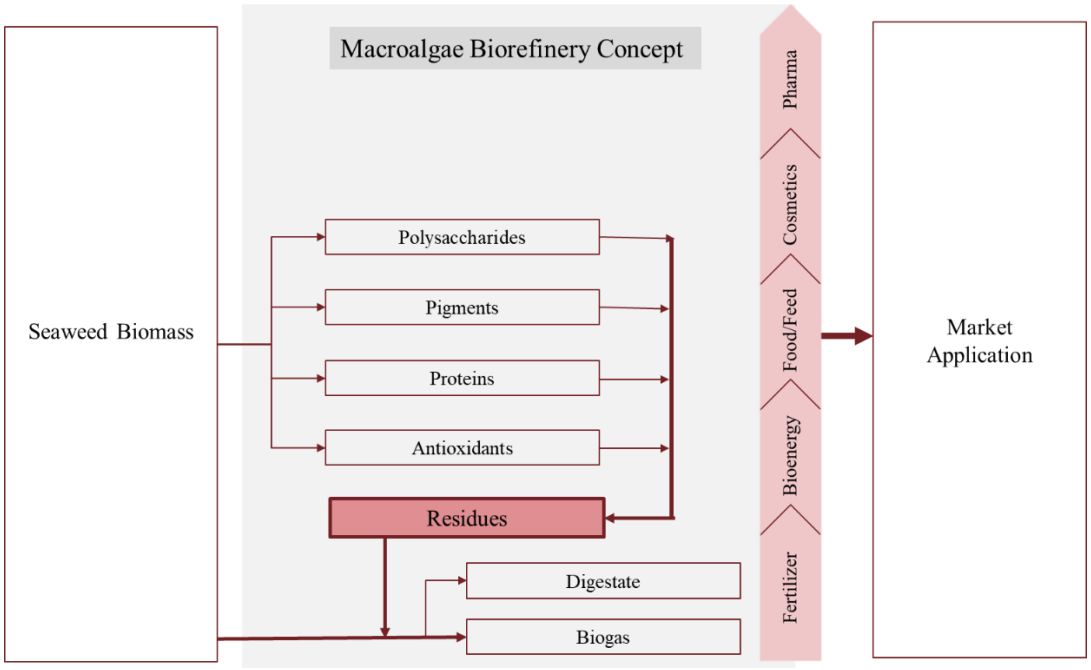

Figure 1.1: Seaweed biorefinery concept (adaptated from [3]*).*

1.2. Approach

Starting with raw *Gelidium sesquipedale*, the first approach was to apply 15 different protein extraction procedures (with sequential extraction methods). After centrifugation, all supernatants (one per extraction method) were analyzed for protein content. Two extraction procedures were chosen for scale up and followed the overall process described in Figure 1.2. The enrichment of the extracts was carried out by ammonium sulfate precipitation followed by diafiltration and concentration using centrifugal ultrafiltration. Future work includes the evaluation of the influence of the protein extraction methods in agar extraction and agar quality, and protein characterization, bioavailability, and biological activity of both the purified and the unpurified extract which is of great interest.

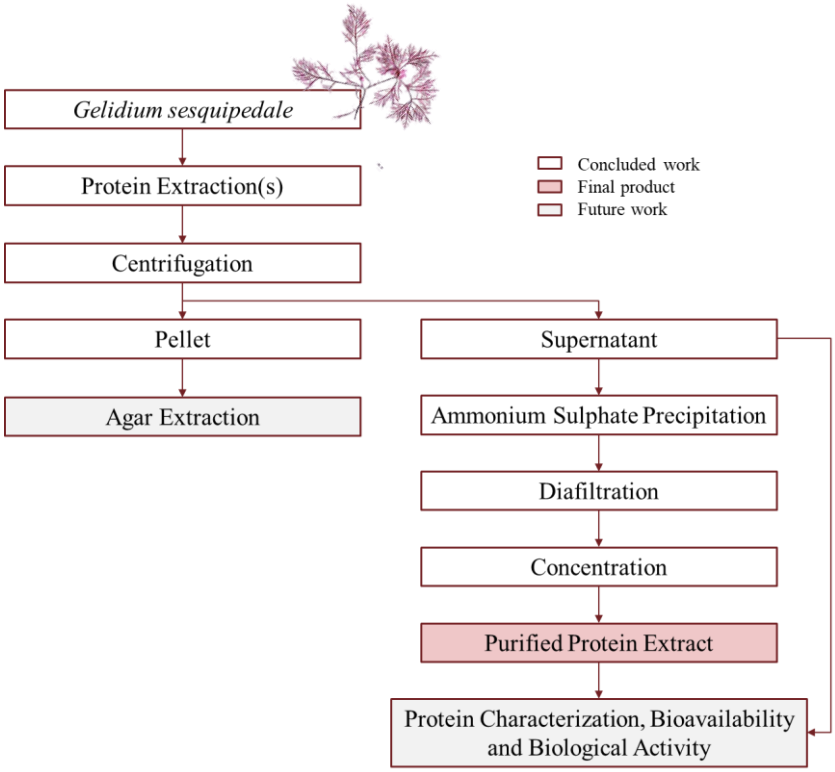

Figure 1.2: Schematic representation of the overall process, from harvested macroalgal biomass to a protein enriched extract.

1.3. Macroalgal Background

1.3.1. Definition and Characterization

The term algae refers to a large diversity of unrelated phylogenetic entities, ranging from picoplanktonic cells to macroalgae kelps [4]. In contrast to terrestrial plants which share a common ancestor, algal diversity includes several distantly related groups of mainly photoautotrophic organisms that inhabit aquatic habitats. The vast taxonomy of algal species groups and species within groups, and their chemical diversity correlated with their genetic diversity and geographical distribution are the main contributors to the richness and diversity of algal compounds. Algal chemistry is largely linked to evolution, but phenotypic modifications can also be a result of environmental and biological stimuli as individual populations exhibit phenotypic plasticity and adaptation to their environment. Because they often experience stressful conditions and highly fluctuating environments, most algae possess mechanisms to enable acclimations to stressors (e.g., UV radiation, temperature, and salinity) and are able to defend themselves against biological pressures (e.g., competitors, grazers and/or parasites). This wide range of

tolerance combined with their specific cellular structure predisposes them to growth and development under laboratory and industrial conditions. [4]

Macroalgae (or seaweed) are multicellular algae of thalli-like structure that can adhere to solid underwater substrates or float freely in water. Compared to other algal groups, such as microalgae, they occupy available space slower, but grow faster and are less vulnerable to grazing and water turbulence [5]. Macroalgae are classified as green, red, and brown algae, according to the thallus color derived from natural pigments. Green algae belong to the Chlorophyta phylum and have the same ratio of chlorophyll a to b as terrestrial plants. There are about 4500 species of green algae [6]. Red algae are included in a single class – Rhodophyceae, which consists of two subclasses: Florideophycidae and Bangiophycidae. The red color is attributed to the pigments' chlorophyll a, phycoerythrin and phycocyanin. There are about 4000–6000 red algae species in over 600 genera [7]. Brown algae are classified as Phaeophyceae and their principal photosynthetic pigments are chlorophyll a and c, β-carotene, and other xanthophylls. There are about 1500–2000 species of brown algae [8]. The classes of macroalgae are vertically distributed from the upper zone (sea surface) to the lower sublittoral zone [9]. For example, while most macroalgae are in littoral zones near the coast, some red algae like *Gelidium sp.* inhabit the deeper sea areas (over 25m below the surface) where sunlight availability is limited [10]. *Gelidium sp.* has phycoerythrin and phycocyanin pigments, which can efficiently absorb light with wavelengths of photosynthetically active radiation (PAR) which are able to penetrate seawater and reach deeper zones [10].

Macroalgae are photoautotrophic and therefore produce and store organic carbons by using CO_2 or HCO_3^-. Most of them directly uptake HCO_3^- due to the low diffusion rate of CO_2 in seawater [11]. The photosynthetic rates of macroalgae highly depend on the species, although green and red algae usually have higher rates than their brown counterparts (1 or 2 orders of magnitude) [5]. When comparing macroalgae and terrestrial plants, significant chemical compositions can be pinpointed in addition to the physiological and morphological differences. Macroalgae have manan, ulvan, carrageenan, agar, laminarin, mannitol, alginate, and/or fucoidan which are not included in lignocellulosic biomass [7]. Like terrestrial biomass, macroalgae apart from green algae, do not have high contents of starch and oil. Their lack of lignin makes them less rigid and contributes to the flexibility of their cell wall [7].

Carbohydrate compounds are abundant in macroalgae. The carbohydrate contents of green, red, and brown algae vary between 25–50%, 30–60%, and 30–50% dry weight, respectively [12]. Their composition evidently differs between species. Green macroalgae usually have polysaccharides in the form of starch (i.e., α-1,4-glucan) and lipids although in small proportions (1–4% for starch and 0–6% for lipids) [13]. Water-soluble ulvan is a distinctive feature of the green algae's cell wall, as well as insoluble cellulose [14]. When it comes to brown algae, their major polysaccharide is alginic acid (i.e., alginate). Brown algae tend to have their respective alginate structure and proportions of mannuronic and guluronic acids in alginate. As a unique polysaccharide, brown algae have laminarin (β-1,3-glucans) which accounts for up to 35% dw. Additionally, brown algae have fucoidan, glucose and glyoxylic acid although in smaller quantities [15]. Unlike green and brown algae, red algae have floridean starch and floridoside, which are similar to general starch. Floridean starch is an α-1,4-glucosidic linked glucose homopolymer that accounts for up to 80% of the cell volume [16]. The major polysaccharide constituents of red algae are galactans such as carrageenan (up to 75% dw) and agar (up to 52% dw) [17].

It is relevant to note that the composition of macroalgae of the same species can also differ. Local conditions at the collection site such as light, salinity, nutrients, temperature, pollution, and water motion can

considerably impact metabolite levels and bioactive composition. The biological status of the algae (e.g., life cycle, development stage and thallus structure) can also have an impact on their biochemical composition [18].

1.3.2. Protein Extraction

1.3.2.1. Proteins in Seaweed

Proteins of marine origin have been the focus of several studies due to their bioactive potential. The protein content varies according to phylum. Brown algae generally have low protein content (3 to 15% dw) which contrasts with the protein content of green (9 to 26% w/w) and red algae (20 to 47% dw) [19] [20]. These concentrations are comparable to those found in high-protein vegetables – leafy greens and legumes (mint – 30.9% dw, cilantro – 22.2% dw, spinach – 26.5% dw, cauliflower – 29.9% dw, soybean – 35 to 40% dw, chickpea – 20 to 25% dw), major cereals (wheat – 8 to 15% dw, barley – 8 to 15% dw, rice – 7 to 9% dw, corn – 9 to 12% dw) [21].

The protein content of macroalgae varies with seasonal cycles. One example is the protein content of the red seaweed *Palmaria palmata* collected on the French Atlantic coast which showed fluctuations between 9 and 25% of protein content. The highest values occurred during winter and spring [19]. Seaweed may contain non-proteinic nitrogen, obtained from nitrates, pigments or nucleic acids, which results in an over-estimation of their protein content (usually estimated by the general Nitrogen-to-Protein conversion factor of 6.25). Specific Seaweed-Nitrogen-to-Protein (SNP) conversion factors for brown, red and green seaweeds have been reported [22].

1.3.2.1.1. Phycobiliproteins

Protein–pigment complexes like phycobiliproteins (PBPs) are one of the most important groups of marine proteins. In red seaweed, these complexes are the main light-harvesting pigments and the only water-soluble algal pigments, accounting for up to 50% of the total protein content [23]. They are a family of fluorescent proteins covalently linked to tetrapyrrole groups, known as bilins. They constitute a structure attached to the cytoplasmic surface of thylakoid membranes named phycobilisomes (unlike carotenoids and chlorophylls which are located in the lipid bilayer) [23]. PBPs are grouped into four classes: phycoerythrin (PE), phycocyanin (PC), phycoerythrocyanins (PEC), and allophycocyanin (APC) [18]. The most common phycobiliprotein in many red seaweeds is known as R-phycoerythrin (R-PE). Isolation of PE has been reported in many species, for instance *Gelidium pusillum* [24], *Grateloupia turuturu* [25] [26], and *R. pseudopalmata* [27].

1.3.2.1.2. Glycoproteins and Lectins

Glycoproteins (GPs) are carbohydrate-binding proteins. Glycans can be conjugated to peptide chains by N-glycosyl linkages and/or O-glycosyl linkages. Protein glycosylation can happen co- or post-translation. Glycoproteins are located on the cell wall and cell surfaces or are secreted [18]. A few seaweed glycoproteins have been isolated by hot- or cold-water extraction. In three glycoprotein-rich fractions obtained from *Ulva sp.*, two of them showed a higher content in proteins than neutral sugars [28]. Extracts of *Ulva lactuca* with high contents of both carbohydrates and protein suggests the presence of glycoproteins [29].

Phycolectins are a group of carbohydrate binding proteins in macroalgae. Lectins interact with specific glycan structures that constitute the soluble and membrane bound glycoconjugates [18]. Seaweeds are particularly good sources of novel lectins. A few examples are griffithsin, a mannose-specific lectin isolated from the red algae

Griffithsia sp. [30], SfL1 and SfL2 from *Solieria filiformis* [31], and HRL40 from *Halimeda renschii* [32]. Although macroalgae phycolectins have been characterized, very little is currently known about their structural and biochemical properties [18].

Arabinogalactan proteins which belong to the hydroxyproline-rich glycoproteins have been reported in the cell wall of few species of seaweeds, namely in the green seaweeds *C. vermilara* [33] and *C. fragile* [34].

1.3.2.1.3. Enzymes

Seaweed are rich sources of enzymes. Alkaline phosphatase (Zn-containing metalloproteinase that catalyzes the non-specific hydrolysis of phosphate monoesters) is widely distributed in seaweeds, namely *Ulva pertusa* [35]. Alternative oxidases (proteins involved in the electron flow through the electron transport chain and in the regulation of mitochondrial retrograde signaling pathway) have also been described, namely in *Caulerpa cylindracea* [36]. The fibrinolytic enzyme (trypsin-like serine protease) has also been isolated from green algae, such as *Codium fragile* and *Codium latum* [37] [38]. Rubisco (catalyzes carbon dioxide fixation and oxygenation) has been reported in *K. alvarezii* [39].

1.3.2.1.4. Peptides and Amino Acids

Although the structure and biological properties of algal proteins are still relatively poorly documented, the amino acid composition of several species of algae is known [40]. Most species contain all essential amino acids (EAA) that may represent about 50% of total amino acids. These amino acids are rich in aspartic and glutamic acid residues (22.7 g/100 g protein in several red algae) [23]. The levels of some amino acid residues, such as threonine, lysine, tryptophan, cysteine, methionine, and histidine, are higher than those found in terrestrial plants [19]. Seaweed amino acid analysis have demonstrated profiles similar to ovalbumin (52.4% EAA) and leguminous plants (41.62% EAA) [19][41]. The interest in marine proteins, however, may not be directly linked to the proteins themselves, but rather to the possibility of these originating bioactive peptides [42]. Bioactive peptides usually contain 3–40 amino acid residues, and their activities stem from both their amino acid composition and sequence. They are generated from parent proteins either through digestion processes in the gastro-intestinal tract or produced during fermentation or other processes like enzymatic hydrolysis [43] [44].

The free amino acid fraction of macroalgae is composed primarily of alanine, taurine, omithine, citrulline, and hydroxyproline. Laminine, kainoids, and mycosporine-like amino acids, have also been found in marine macroalgae [45]. The successful production of bioactive peptides originated from hydrolyzed proteins of *Palmaria palmata, Solieria chordalis, Ulva lactuca* and *Saccharina longicruris* has been reported [46].

1.3.2.2. Protein and Derivatives Applications

Marine macroalgae are rich sources of structurally diverse bioactive components with valuable pharmaceutical and biomedical potentials. They can also be used as functional health-promoting ingredients in food (e.g., animal feeds) [47]. The functional properties of proteins are mainly associated with their ability to form and/or stabilize gels and films, foams, emulsions, and sols [48]. Macroalgae or macroalgal extracts have shown effects on the immune status and intestinal health of several monogastric farm animal species including pigs [49], broiler chicken [50], and fish [51]. These bioactive properties could be related to the presence of proteins and/or peptides.

1.3.2.2.1. Bioactive Proteins

Among all macroalgal proteins, lectins and phycobiliproteins have received much attention due to their reported biological activities [52]. The main biological activity associated with lectins is their hemagglutinating activity against erythrocytes [52], whereas phycobiliproteins are used in fluorescent immunoassays, fluorescent immunohistochemistry assays, biomolecule labeling, fluorescent microscopy, and as natural colorants for food and cosmetic applications [53]. Different phycobiliproteins have exhibited antioxidant, anti-inflammatory, neuroprotective, hypocholesterolemic, hepatoprotective, antiviral, antitumor, liver-protecting, atherosclerosis treatment, serum-lipid-reducing, and lipase-inhibition activity [53]. Bioactivities of several macroalgal proteins are shown in Table 1.1.

Table 1.1: Bioactivities of several macrolgal proteins.

Species	***Type of protein***	***Bioactive Activity***	***Ref.***
Codium fragile	Lectins	Blood typing; characterization of cell-surface polysaccharides; lectinosorbent assays for cell-binding-pattern examinations.	[54]
Bryothamnion triquetrum, Bryothamnion seaforthii, and *Amansia multifida*	Lectins	Antinociceptive effects.	[52]
Eucheuma serra	ESA-2 (lectin)	Colonic carcinogenesis suppression in mice; growth inhibition of 35 human cancer cell lines.	[52]
Hypnea cervicornis and *Solieria robusta*	Lectins	Anti-inflammatory and mitogenic activities in mice spleen lymphocytes; growth inhibition of mice leukemia cells L1210 and mice FM3A tumor cells.	[52]
Eucheuma serra and *Galaxaura marginata*	Lectins	Antibacterial activity against the fish pathogen *Vibrio vulnificus.*	[54]
Hypnea cervicornis	Mucin-binding agglutinin	Antinociceptive and anti-inflammatory activity.	[52]
Griffithsia sp.	Novel lectin	Strong anti-HIV activity.	[52]
Codium intricatum, Codium latum, and *Codium divaricatum*	Fibrinolytic enzymes	Preferentially hydrolyzed fibrinogen Aa chain.	[54]

1.3.2.2.2. Bioactive Peptides

Bioactive peptides have been shown to possess properties such as opioid, immunomodulatory, antibacterial, antithrombotic, and antihypertensive activity [55] and some may exhibit multifunctional bioactivities (Table 1.2) [56].

Table 1.2: Bioactivities of several macrolgal peptides.

Species	***Peptide Sequence/Name***	***Bioactive Activity***	***Ref.***
Porphyra yezoensis	Ile-Tyr; Met-Lys-Tyr; Ala-Lys-Tyr-Ser-Tyr; Ley-Arg-Tyr	ACE-I inhibitory activity	[57]
	Ala-Lys-Tyr-Ser-Tyr		[58]
Palmaria Palmata	Val-Tyr-Arg-Thr; Leu-Asp-Tyr; Leu-Arg-Tyr; Phe-Glu-Gln-Trp-Ala-Ser		[59]
	Asn-Ile-Gly-Gln	Anti-inflammatory activity.	[60]
Undaria pinnatifida	Val-Tyr; Ile-Tyr; Phe-Tyr; Ile-Trp	ACE-I inhibitory activity	[61]
	Val-Tyr; Ile-Tyr; Ala-Trp; Phe-Tyr; Val-Trp; Ile-Trp; Leu-Trp	Anti-hypertensive	
Bryopsis sp.	Kahalalide F	Antitumoral activity.	[54]
Galaxaura filamentous	Galaxamide	Anti-proliferative activity against human epithelial cancer cell lines.	[62]

ACE: angiotensin-converting enzyme.

1.3.2.3. Challenges and Impact of Cell Structures

The successful extraction of proteins highly depends on their accessibility since most of them are located intracellularly. Therefore, the complex nature of algal cell walls is the main challenge when it comes to the development of seaweed as protein sources. The algal cell is metabolically active, possessing many intracellular enzymes and proteins [63]. Its cell wall is composed of a highly integrated network of biopolymers, mainly polysaccharides, which interact with water and metal cations, amongst other molecules [64] and it can be divided into three main domains: the fibrillar wall, the amorphous matrix, and the glycoprotein domain [23]. The fibrillar polysaccharides and the glycoproteins form a reticulated cell wall which is embedded in the gel-like amorphous matrix. The fibrous part is the most inert and resistant cell wall component, with cellulose being the most significant element amongst others, like xylan and hemicellulose. Very little is known about the glycoprotein domain, but it is constituted by glycoproteins that contain cellulose binding domains. The gel-like matrix is made of carboxylic and/or sulfated polysaccharides, like sulfated galactans, such as carrageenans and agarans and it usually extends to intercellular spaces between adjacent cells [23]. Other biopolymers like proteins and polymeric phenolics can also participate in cell wall formation. Red algae have complex cell walls made of cellulose, xylan, or mannan fibrils and sulfated galactans as the main matrix components. Sulfated galactans comprise the hot water-soluble portion of the cell wall and are the main components of marine red algae. Agarans are mainly synthesized by red seaweeds belonging to the *Pyropia*, *Gelidium*, *Gracilaria* and *Pterocladia* genera [15].

The presence of polysaccharide-bound cell wall mucilage including anionic or neutral polysaccharides, and polyphenols reduces protein extractability and requires additional steps for fractionation and purification. Polysaccharides induce strong electrostatic interactions [65], whereas polyphenols may form reversible hydrogen bonds with proteins or oxidize. Oxidized phenolic compounds can react with amino acids and form insoluble complexes [66]. The morphology of different seaweed species has also been suggested to be an important factor in protein extraction, with tougher thallus forms reported to require increased processing. The raw biomass from seaweed after harvesting must be preserved by drying or freezing or used fresh as soon as possible to avoid protein degradation [18]. The increased extractability from oven-dried biomass was suggested to have been due to the decomposition of phenolic compounds, as well as increased disruption of anionic or neutral polysaccharides found within the cell wall of the seaweed [43].

The combination of extraction methods and purification techniques is necessary to improve protein yield. The greater the scale, the bigger the challenge because methods with low-time, -cost and -energy consumption (i.e., environmentally friendly) are required.

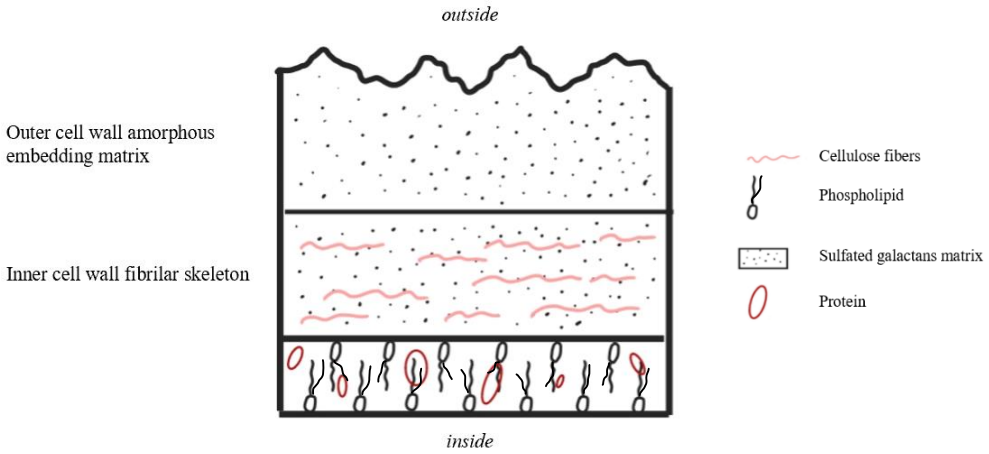

Figure 1.3: General cell wall structure of red algae (adaptaded from [67]*).*

1.3.2.4. Conventional Extraction Methods

As mentioned above (section 1.3.2.3. Challenges and Impact of Cell Structures), the extraction and utilization of algal proteins evidently depends on the disruption of the cell wall. To ensure extraction of intracellular proteins, additional stress factors are often applied which improve the extraction efficiency.

Since algal proteins are not very well described, they can generally be divided into four main classes based on their solubility - albumins which are soluble in water, globulins which are soluble in salt solutions, glutelins which are soluble in dilute acids or bases, and prolamins which are soluble in 70% alcohol [63]. Sequential extraction steps are often carried out to ensure the extraction of different types of proteins unless a specific type is being targeted.

Algal proteins are conventionally extracted by means of aqueous, acid, and alkaline methods, followed by fractionation and enrichment techniques such as centrifugation, ultrafiltration, precipitation and/or chromatography [68]. For instance, when targeting glycoproteins or lectins from Rhodophyta species, phosphate or Tris-HCl buffers are usually used [31] [69] [70] [71].

Physical methods, such as osmotic shock, freeze/thawing or grinding, can enhance the extraction in some seaweeds. Conventional pre-treatments and protein extraction methods are presented in Table 1.3.

1.3.2.5. Enzyme-Assisted Extraction

Enzyme-Assisted Extraction (EAE) is often the preferable method to extract proteins/hydrolysates from seaweed [43]. Polysaccharidases can be applied as a cell disruption treatment prior to protein extraction to increase protein yield. Several polysaccharidases (κ-carrageenase, β-agarase, xylanase, cellulase) have been used in protein extractions from red seaweed species, namely *C. crispus*, *G. verrucosa*, and *P. palmata* [72]. The use of cocktails which contain multiple hydrolytic activities (cellulase, hemicellulase and β-glucosidase) for carrageenan, agar, alginate, and cellulose is a promising option to increase extraction yields [73]. The selective degradation of structural proteins, such as glycoproteins of the cell wall, might be possible by using subtilases or serine proteases [74]. Different digestion enzymes have also been used to release bioactive peptides from parent proteins, being chymotrypsin, trypsin, and pepsin the most used [18]. Combining it with other processes (e.g., enzymatic hydrolysis combined with alkaline extraction) is usually the go-to approach (Table 1.3) [75].

Once again, the enzyme choice is highly correlated with the desired end product (e.g., intact proteins, specific proteins or bioactive peptides). When it comes to feasibility, every case needs to be looked at individually. If a high enzyme:substrate concentration is required, an enzymatic treatment might not be viable, particularly at an industrial scale.

1.3.2.6. Ultrasound-Assisted Extraction

Ultrasound-Assisted Extraction (UAE) has been shown to be an attractive technology. It acts by creating compression and decompression through sound waves at frequencies higher than 20 kHz. It can be applied to numerous food sources, particularly in the modification of plant micronutrients to improve bioavailability, simultaneous extraction, and encapsulation, quenching radical sonochemistry to avoid degradation of bioactives, and increasing bioactivity of phenolics and carotenoids by targeted hydroxylation [76]. The bioavailability of seaweed proteins can be improved by the degradative effect of radical sonochemistry which is not produced by the ultrasound waves directly, but rather by acoustic cavitation. When the pressure is higher than the tensile strength of the liquid, the formation and growth of vapor bubbles occurs. Such bubbles, under high ultrasound fields, violently collapse which leads to peeling, erosion, particle breakdown and degradation of the solid-liquid surfaces. After cell disruption, the solvent can easily penetrate the cells, releasing the intracellular compounds to the bulk solvent [77].

The application of ultrasound can be divided into two different categories: low intensity–high frequency (100 kHz–1 MHz) and high intensity–low frequency (between 20 and 100 kHz) ultrasound, the latter being the type that is typically used for the disruption of cell walls and membranes [78]. Higher extraction yields are usually achieved at lower processing times and lower temperatures, making this method suitable for the extraction of thermolabile compounds [79]. Solvent consumption is also lower which facilitates the downstream processing of the target compounds [80].

An ultrasound pre-treatment reportedly increased protein extraction in *Ascophyllum nodosum* when followed by acid or alkaline treatment by 540% and 27%, respectively, when compared with extraction performed with no pre-treatment. It also resulted in a reduced processing time (from 60 to 10 minutes). Extraction of R-phycoerythrin has been deemed effective by combining EAE and UAE in *G. turuturu* [26], due to a synergetic effect. In *G. pusillum*, UAE followed by maceration and buffers, allowed the recovery of 77% and 93% of R-PE and R-phycocyanin (R-PC), respectively [81]. Some examples are presented in Table 1.3.

Table 1.3. Conventional cell disruption and protein extraction methods for different seaweeds.

Cell disruption method	***Extraction method***	***Reagents***	***Conditions***	***Species***	***Initial protein content***	***Protein recovery yield***	***Protein quantification method***	***Ref.***
Enzymatic hydrolysis	Aqueous polysaccharidase degradation and buffer treatment (sequential)	Phosphate buffer, commercial mixture of polysaccharides containing cellulase, hemicellulose and β-glucanase, Tris HCl	***Enzymatic pre-treatment*** 10 g freeze-dried algal powder; 200 mL of enzymatic medium, pH 6 (6g of polysaccharidase powder in 200 mL of phosphate buffer 0.1 M, pH 6); 30 ºC; 2h. ***Buffer treatment*** After filtration through a nylon mesh, the residue was ground with a pestle and a mortar in 100 mL Tris HCI (0.1 M, pH 7.5); 4 °C. Supernatant collected after centrifugation (10,000 × g, 20 min, 4ºC).	*Ulva rigida*	112.0±5.8 $g \cdot kg^{-1}$ dw	18.5±2.1%[a]	Kjeldahl method (N × 6.25)	[82]
				Ulva rotundata	100.1±4.9 $g \cdot kg^{-1}$ dw	22.0±1.5%[a]		
	Aqueous polysaccharidase degradation and alkaline treatment (sequential)	Deionized water, Cellulase and xylanase (Celluclast + Shearzyme), NaOH and NAC	***Enzymatic pre-treatment*** 1:30 (w/v) of dried milled seaweed to liquid suspension at pH 5 was pre-incubated (30 min, 40ºC). Enzyme:substrate (E:S) of $48.0 \cdot 10^3$ units/100g; reaction incubated at 40 ºC; 24h. The supernatant was removed following centrifugation at 11,950 × g, room temperature. ***Alkaline treatment*** Weight:volume of 1:15; 0.12 M NaOH; 0.1% (w/v) NAC; stirred for 1h; room temperature. Supernatant collected after centrifugation (11,950 × g, 20 min, room temperature).	*Palmaria palmata*	Not specified.	11.6±0.08% dw[b]	Lowry method (modified)	[75]
	Aqueous treatment, Potter homogenization and alkaline treatment	Ultra-pure water, NaOH	***Aqueous treatment*** 50 mg of freeze-dried sample; 4 mL ultra-pure water; 12h; 4ºC. Potter homogenization for 5 min; 4ºC. Supernatant collected after centrifugation (15,000 × g, 20 min, 4ºC). ***Alkaline treatment*** Pellet resuspended in 1 mL of 0.1 N NaOH; shaking occasionally for 1h; room temperature. Supernatant collected after centrifugation (15,000 × g, 20 min, room temperature).	*Porphyra acanthophora var. acanthophora*	16.5% dw	8.9±0.7% dw[b]	Lowry method (precipitation with TCA 2.5:1)	[22]
				Sargassum vulgare	11.5% dw	6.9±0.2% dw[b]		
				Ulva fasciata	12.8% dw	7.3 ± 0.8% dw[b]		
High shear force	Aqueous and alkaline treatment (sequential) with Ultra-turrax.	Deionized water, NaOH and NAC	***Aqueous high shear force treatment*** Ultra-turrax; 24,000 rpm; 1:20 of weight:volume of deionized water. Following shearing, stirred for 1h; 4ºC. Supernatant collected after centrifugation (11,950 × g, 20 min, 4ºC). ***Alkaline treatment*** Pellet resuspended in 0.12 M NaOH; 0.1% (w/v) NAC; weight:volume of 1:15; stirred for 1h; room temperature. Supernatant collected after centrifugation (11,950 × g, 20 min, room temperature).	*Palmaria palmata*	Not specified.	6.9±0.1% dw[b]	Lowry method (modified)	[75]
	Buffer treatment with sonication	Tris HCl	10 g of algal powder; 200 mL Tris HCl (0.1M pH 7.5); suspension submitted to ultrasound for 1h (Ultrasonick 300 Ney, maximal power); 4ºC; stirred overnight. Supernatant collected after centrifugation (10,000 × g, 20 min, 4ºC).	*Ulva rigida*	112.0±5.8 $g \cdot kg^{-1}$ dw	10.4±0.8%[a]	Kjeldahl method (N × 6.25)	[82]
				Ulva rotundata	100.1±4.9 $g \cdot kg^{-1}$ dw	16.1±0.9%[a]		
	Sonication in aqueous conditions and ammonium sulfate-induced precipitation (sequential)	Ultra-pure water and ammonium-sulfate	***Sonication in aqueous conditions*** 10 g of freeze-dried and milled seaweed was suspended in 1 L of ultra-pure water; ultra-sonication for 1h; left to stir overnight; 4°C. Supernatant decanted after centrifugation (10,000 × g, 1h). Pellet suspended in 200 mL of ultra-pure water and subjected to a second extraction. ***Precipitation*** Supernatants were pooled and brought to 80% (w/v) ammonium sulfate saturation; stirred for 1h; 4°C; centrifuged (20,000 × g, 1h) to precipitate the protein fraction. The precipitates were dialyzed using 3.5- kDa MWCO dialysis tubing against Milli-Q water; overnight; 4 ºC.	*Ulva lactuca*	Not specified.	19.6±0.6%[a]	Lowry method (modified)	[83]

[a]Total protein yield expressed as % of total protein (Protein extracted/Total protein x 100); [b]dw expressed as algal dry weight; NAC: N-acetyl-L-cysteine.

Table 1.3. Conventional cell disruption and protein extraction methods for different seaweeds.

Cell disruption method	***Extraction method***	***Reagents***	***Conditions***	***Species***	***Initial protein content***	***Protein recovery yield***	***Protein quantification method***	***Ref.***
High shear force (cont.)	Aqueous treatment, alkaline solubilization and isoelectric precipitation (sequential)	Deionized water, NaOH and HCl	***Aqueous treatment*** Dry-milled seaweed in distilled water in a 1:6 (w/v) ratio, based on the original wet weight of each species. Homogenization using Ultra-Turrax; 2 min; 18 000 rpm. Milling with beads; 2 min; 1/30 s. Homogenized sample was stirred for 1h at 8 ºC. ***Alkaline solubilization and isoelectric precipitation*** pH adjusted to 12; sample kept in ice. Supernatant collected after centrifugation (at 8,000 × g, 10 min). pH adjusted to 2 and frozen overnight; -20 °C. After thawing and a second centrifugation (8,000 × g, 10 min), the pellet was collected and freeze dried.	*Saccharina latissima*	Not specified	25.1±0.9%[a]	Lowry method (modified)	[83]
				Porphyra umbilicalis	Not specified.	22.6±7.3%[a]		
Osmotic shock	Aqueous treatment	Deionized water	10 g of algal powder; 200 mL deionized water; 4ºC; stirred overnight. Supernatant collected after centrifugation (10,000 × g, 20 min, 4ºC).	*Ulva rigida*	112.0±5.8 $g \cdot kg^{-1}$ dw	9.7±0.6%[a]	Kjeldahl method (N × 6.25)	[82]
				Ulva rotundata	100.1±4.9 $g \cdot kg^{-1}$ dw	14.0±1.8%[a]		
	Aqueous and alkaline treatment (sequential)	Deionized water and NaOH	***Aqueous treatment*** 10 g of algal powder; 200 mL deionized water; 4ºC; stirred overnight. ***Alkaline treatment*** After centrifugation (10 000 × g, 20 min, 4ºC), the pellet was treated with NaOH (0.1M) and mercaptoethanol (0.5% v/v); stirred for 1h; room temperature. Supernatant collected after centrifugation (10 000 × g, 20 min, room temperature).	*Ulva rigida*	112.0±5.8 $g \cdot kg^{-1}$ dw	26.8±1.3%[a]	Kjeldahl method (N × 6.25)	[82]
				Ulva rotundata	100.1±4.9 $g \cdot kg^{-1}$ dw	36.1±1.4%[a]		
		Deionized water, NaOH and NAC	***Aqueous treatment*** Dried milled seaweed suspended in deionized water (1:20 (w/v); stirred for 16h; 4 ºC. Supernatant collected after centrifugation (11,950 × g, 20 min, 4 ºC). ***Alkaline treatment*** Pellet resuspended in 0.12 M NaOH; 0.1% (w/v) NAC; weight:volume of 1:15; stirred for 1h; room temperature. Supernatant collected after centrifugation (11,950 × g, 20 min, room temperature).	*Palmaria palmata*	Not specified.	6.7±0.2% dw[b]	Lowry method (modified)	[75]
None	Buffer treatment	Tris HCl	10 g of algal powder; 200 mL Tris HCl (0.1M pH 7.5); 4ºC; stirred overnight. Supernatant collected after centrifugation (10 000 × g, 20 min, 4ºC).	*Ulva rigida*	112.0±5.8 $g \cdot kg^{-1}$ dw	9.4±1.6%[a]	Kjeldahl method (N × 6.25)	[82]
				Ulva rotundata	100.1±4.9 $g \cdot kg^{-1}$ dw	13.8±1.2%[a]		
	Buffer and alkaline treatment (sequential)	Tris HCl and NaOH	***Buffer treatment*** 10 g of algal powder; 200 mL Tris HCl (0.1M pH 7.5); 4ºC; stirred overnight. ***Alkaline treatment*** After centrifugation (10 000 × g, 20 min, 4ºC), the pellet was treated with NaOH (0.1M) and mercaptoethanol (0.5% v/v); stirred for 1h; room temperature. Supernatant collected after centrifugation (10 000 × g, 20 min, room temperature).	*Ulva rigida*	112.0±5.8 $g \cdot kg^{-1}$ dw	17.5±1.3%[a]	Kjeldahl method (N × 6.25)	[82]
				Ulva rotundata	100.1±4.9 $g \cdot kg^{-1}$ dw	25.2±1.9%[a]		
	Aqueous biphasic system	PEG/ K_2CO_3	PEG 1550 (10% w/v) and K_2CO_3 (15% w/v). Algal powder (30 g/L) was mixed with PEG, suspended in Milli-Q water (15 min), extracted 15 min after the addition of the salt solution and centrifuged (4,500 × g, 25 min, 23°C). Top phase-I was separated and stirred with the same volume of salt solution for 15 min and centrifuged (5000 × g, 5 min, 23°C). Anionic polysaccharides present a high affinity for the salt saturated aqueous phase and proteins show affinity for the polyethylene glycol phase.	*Ulva rigida*	112.0±5.8 $g \cdot kg^{-1}$ dw	19.1±1.1%[a]	Kjeldahl method (N × 6.25)	[82]
				Ulva rotundata	100.1±4.9 $g \cdot kg^{-1}$ dw	31.6±2.1%[a]		

[a]Total protein yield expressed as % of total protein (Protein extracted/Total protein x 100); [b]dw expressed as algal dry weight; NAC: N-acetyl-L-cysteine.

1.3.2.7. Novel Methods

1.3.2.7.1. Pulsed Electric Field Extraction

Pulsed Electric Field Extraction (PEFE) is used as a cell disruption method, particularly in microalgae [43]. It involves the application of high electric currents to perforate a cell wall or membrane. Depending on the intensity, amplitude, duration, number, and repetition frequency of the external electric pulses, reversible or irreversible pores are formed in the membranes. Irreversible pores formation is of particular importance for extraction of bioactive compounds from natural matrices [18]. Treatments of electric field strength from 0.7 to 3 kV/cm, a specific energy of 1–20 kJ/kg, a couple of hundred of pulses, and a total time duration lower than 1s are usually used for natural products extraction [78]. When applied to *U. lactuca*, a higher protein content was observed - 59 $\mu g\ mL^{-1}$ against the 23 $\mu g\ mL^{-1}$ obtained in the control [84]. When 50 pulses of 50 kV, applied at a 70.3 mm electrode gap, were used in the extraction of proteins from *Ulva sp.*, a sevenfold increase in total protein content was obtained when compared to the use of osmotic shock [85]. The conductivity and electrode gap can possibly limit this method's scale up.

1.3.2.7.2. Microwave Assisted Extraction

Microwave Assisted Extraction (MAE) is another procedure used to enhance protein extraction. When energy is transferred to the solution, the vibration/oscillation of polar molecules occurs, causing inter- and intra-molecular friction. This effect combined with the movement and collision of a large number of charged particles leads to the heating of matrices. Intracellular heating ultimately leads to pressurized effects that induce the breakdown of cell walls and membranes, in addition to electroporation effects [78]. In the literature, MAE's results focus on the reducing of extraction times rather than on its effects on parameters like protein functionality since it is mostly used for analytical purposes (sample preparation) [78].

This method can be carried out in open vessels (atmospheric pressure) or closed ones, under controlled pressure and temperature. In closed vessels, the solvent can be heated above its normal boiling point by manipulating the pressure, which accelerates the mass transfer of the compounds from the natural matrix to the bulk solvent [86]. The higher the solvent's dielectric constant (ε'), the greater the energy absorbed by the molecules and the faster the extraction temperature is reached [87] [88], which makes polar solvents the preferred choice.

1.3.2.7.3. Pressurized Liquid Extraction

Pressurized Liquid Extraction (PLE), or Accelerated Solvent Extraction (ASE), combines temperatures that range from 50 to 200 ºC and pressure that ranges from 35 to 200 bar. These parameters are set for values lower than the solvent critical temperature and pressure, which means it stays in its liquid state [18]. Both temperature and pressure increase the mass transfer rate. High pressures cause the solvent to reach temperatures higher than its boiling point and higher temperatures enhance solubility and reduce the viscosity and surface tension [78]. Water is the most widely used solvent but other solvents, such as propane and dimethyl ether (DME) can also be used. Since DME is partially miscible with water, it allows the simultaneous extraction of non-polar target metabolites and the removal of water from wet matrices [89] [68] [90] [91].

1.3.2.7.4. Supercritical Fluid Extraction

Supercritical Fluid Extraction (SFE) is an alternative extraction technique that produces extracts with very few polar impurities [92]. It is a green technology, since a concentration step is most often skipped after the

extraction process [93]. Experimental studies using SFE are usually limited to the region of $P_c < P \leq 6P_c$ and $T_c < T \leq 1.4T_c$ [94]. When the fluid is heated to a temperature above its critical point, it becomes supercritical. Under supercritical conditions, the properties of the fluid become indistinguishable from its gaseous state, with a density similar to a fluid, but diffusivity and viscosity matching those of a gas. This makes supercritical fluids capable of a faster and deeper penetration into the solid particles [92]. The fluid must be chosen carefully, especially when dealing with thermolabile compounds. CO_2 with T_C and P_C values of 31.1ºC and 73.9 bar is the most used [95]. In addition to being ideal for the extraction of thermolabile compounds, it has low viscosity, low surface tension, high diffusivity, is non-toxic, non-flammable, widely available and chemically inert under several conditions. The fact that it is gaseous at normal pressure and temperature eliminates the need for a solvent evaporation step after extraction [95]. The greatest limitation of supercritical CO_2 is that it is not suitable for extraction of polar compounds [96].

The addition of an organic modifier, such as EtOH or MeOH, can greatly improve extraction efficiency [95]. Other solvents, like water, MeOH, EtOH, acetone, chloroform, ethyl acetate, and toluene, are usually avoided when extracting bioactive compounds, since their T_C is above 200 °C [78].

1.3.2.7.5. Switchable or Smart Solvents

Switchable solvents are a new class of smart extraction solvents which can switch from a non-ionic form to an ionic liquid by bubbling CO_2. Exposure to N_2 allows them to return to their non-ionic form [18]. Ionic liquids (ILs) are composed of large asymmetric organic cations (imidazolium, pyrrolidinium, pyridinium, ammonium or phosphonium) and different inorganic or organic anions, such as BF^{4-}, PF^{6-}, Cl^-, and Br^- anions [78]. They are very versatile, since their polarity, hydrophobicity, viscosity, and other properties highly depend on the cationic or anionic constituents selected, hence being referred to as “designer solvents”. They have low-melting points (below 100ºC), and their non-flammable and non-volatile nature makes them an attractive choice for the development of safer processes [78].

The basis of the extraction mechanism lies on the interaction of ionic liquids with macroalgal cell walls. As mentioned, cellulose is a core component of algae cell walls, although its proportion varies between species. The use of a solvent capable of disrupting the intermolecular H-bond interactions leads to cellulose dissolution [97]. Indeed, several ILs have been found to dissolve large amounts of cellulose [98]. These interactions are relevant because they ultimately lead to the complete or partial disruption of cell walls [99].

1.3.2.8. Protein Enrichment Methods

Using seaweed as protein resources requires the processing of biomass to deliver a concentrated form of high-quality protein. Protein concentration from plant materials has traditionally been achieved directly by extracting and isolating the protein, or indirectly by extracting non-protein components. In contrast with other plant materials, isolating and concentrating proteins from seaweed is relatively unexplored and most protocols tend to focus solely on the extraction methods [100]. Purification of extracted proteins represents a challenge, especially for novel proteins because of their unknown physicochemical properties. The selection of the method also depends on the final application of the product and the scale of production. The extraction method used also influences the purification method chosen. For example, reducing agents, like N-acetyl-L-cysteine which are used to improve cell wall-associated protein extraction, might have to be removed depending on the applications of the extracted proteins.

Single or combined methods can be employed. These methods include chromatography, membrane technologies, and precipitation [18]. A combination of membrane technologies could be used to isolate seaweed proteins using the same principals used in the dairy industry. Microfiltration (MF) could be used to remove cell wall components, ultrafiltration (UF) could be used to isolate proteins with a molecular weight between 1 and 200 kDa, nanofiltration (NF) could be used to remove monovalent salts, and reverse osmosis (RO) to reduce volume [101]. Ultrafiltration has been used after supercritical CO_2 and ultrasonic-assisted extraction to isolate polysaccharides from *Sargassum pallidum* [102] and after hot water extraction in *Ulva fasciata* [103]. Most protocols described in the literature often describe methods for the purification and isolation of specific proteins, namely R-PE. Some examples are presented in Table 1.4.

Table 1.4: Algae species and methods used for protein purification.

Aim	***Extraction method***	***Enrichment Method***	***Conditions***	***Species***	***Results***	***Ref.***
Concentrate R-PE; pre-purify by eliminating proteins other than R-PE and polysaccharides.	Centrifugation of a suspension of algal powder in distilled water.	Ultrafiltration	Polyethersulfone membrane; MWCO of 25-30 kDa; surface area of 0.033 m^2; 20 ºC; 4 bar; volume reduction factor of 5.	*Grateloupia turuturu*	100% of R-PE recovered; 32.9% of other proteins and 64.6% of sugars passed through the membrane.	[104]
Separate internal cell components (starch and sugars) from proteins.	High-pressure homogenization of the microalgal suspension in distilled water.	Two-stage ultrafiltration	Polyethersulfone membranes; MWCOs of 100 kDa (retention of starch) and 10 kDa (separation of proteins from sugars); surface area of 50 cm^2; 2.07 bar; volumetric concentration ratio of 2.32.	*Tetraselmis suecica*	1st ultrafiltration Complete retention of starch and pigments. 2nt ultrafiltration 100% of proteins and 65% of sugars were retained.	[105]
Concentrate phycobiliproteins (R-PE and allophycocyanin).	Mixing of algal powder in 50 mM citrate buffer (pH 6) for 24h and centrifugation of the suspension.	Microfiltration; Ultrafiltration and Size–Exclusion Chromatography (SEC)	***Microfiltration and Ultrafiltration*** Regenerated cellulose; 0.45 μm; Polyethersulfone membrane; MWCO of 50 kDa. ***SEC*** Mobile phase: Phosphate buffer (pH 7.2, 50 mM sodium phosphate and 150 mM NaCl); room temperature; average particle size of 34 μm.	*Furcellaria lumbricalis*	60–75% of R-PE and allophycocyanin were recovered, dependent on the detector used (fluorescence or photodiode array).	[106]

Table 1.4: Algae species and methods used for protein purification.

Aim	***Extraction method***	***Enrichment Method***	***Conditions***	***Species***	***Results***	***Ref.***
Isolate R-PE.	Mixing of fresh thallus in 0.02 mM phosphate buffer (pH 7.2); pulverization of the mixture.	Isoelectric Precipitation and Anion-Exchange Chromatography (AEX)	Filtration (cheese cloth); repeated freezing and thawing; centrifugation; supernatant precipitation with 35% saturated ammonium sulfate; supernatant precipitation with 55% saturated ammonium sulfate; centrifugation; dialysis against 50 mM phosphate buffer (pH 7.2). Q-Sepharose column; flow rate of 2.0 mL/min; elution with 50 mM phosphate buffer (pH 7.2) with an increasing gradient of NaCl (0 to 200 mM); elution of the active fraction occurs at a concentration of NaCl of 200 mM.	*Portieria hornemannii*	R-PE recovery of 64.8% with a purity of 5.2%.	[107]
Isolate R-PE.	Dried algal hydration with deionized water overnight; slurry filtration through gaze; supernatant precipitation with ammonium sulfate (final concentration of 0.5 M).	Expanded Bed Adsorption (EBA) and Anion-Exchange Chromatography (AEX)	***EBA*** Streamline™ column; supernatant injected with the crude extracts; eluates pooled and dialyzed against distilled water overnight at 4 ºC. ***AEX*** DEAE-Sepharose column; flow rate of 2.5 mL/min; 4 mM sodium acetate (NaAc) buffer (pH 4.5) eliminates phycocyanin contaminants; 1 mM NaAc buffer (pH 4.5) and 50 mM phosphate buffer (pH 6.8) eliminates other contaminant proteins; elution with 30 mM phosphate buffer (pH 6.8) using an increasing gradient of NaCl from 0 to 200 mM;	*Gracilaria lemaneiformis*	R-PE recovery of 21% with a purity ratio >3.2.	[108]

1.3.2.9. Protein Characterization

Characterization and/or identification of isolated proteins is usually carried out by direct comparison with standard molecules and/or data collected from the available literature. This is successful until unknown compounds are brought into consideration for which standards are not available [18].

1.3.2.9.1. SDS-PAGE

SDS-PAGE is used to identify the molecular weight of dominant protein subunits' bands (Table 1.5). This method has been used to identify protein profiles of different seaweeds, such as *Ulva sp.* [109], *Gracilaria changii* [110], *Caulerpa lentillifera*, *Caulerpa racemose* and *Kappaphycus sp.*, amongst others [111].

Table 1.5: Protein isolation and characterization using SDS-PAGE methods in different seaweed species.

Species	***Identified proteins***	***Molecular Weight***	***Method***	***Ref.***
Himantalia elongata	5 proteins	71.6, 53.7, 43.3, 36.4, 27.1 kDa	Tris-Tricine-SDS-PAGE using 10–20% Mini-Protean® Tris-Tricine Precast Gel.	[112]
Furcellaria lumbricalis	R-PE	~25 kDa	SDS-PAGE using 4–15% Mini-Protean® TGX Stain Free Precast Gel.	[106]
P. palmata	One prominent area of staining (suspected of being subunits of phycoerythrin or other phycobiliproteins).	~20 kDa	SDS-PAGE using a Mini-Protean® II electrophoresis system with a 4 g/100 ml acrylamide stacking gel and a 12.5 g/100 ml acrylamide resolving gel.	[75]
L. japonica	LJGP (*Laminaria japonica* novel glycoprotein)	~10 kDa	SDS-PAGE on 15% gels; periodic acid-Schiff (PAS) staining for glycoprotein bands.	[113]
E. bicyclis	EHEP (Eisenia hydrolysis enhancing protein)	25 kDa	SDS-PAGE and 2D-PAGE (Two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis).	[114]
Pyropia yezoensis	2 proteins: PYP1 and PYP2 (*Pyropia yezoensis* porphyran 1 and 2)	PYP1: 10 kDa, SDS-resistant dimer; PYP2: 10 kDa.	SDS-PAGE using a 18% acrylamide gel.	[115]

1.3.2.9.2. Chromatography

As mentioned in section 1.3.2.8. Protein Enrichment Methods, chromatographic methods are widely used for separation and purification of seaweed proteins. High Performance Liquid Chromatography (HPLC) is often used coupled with other chromatographic methods, like Size Exclusion Chromatography (HPSEC) [106]. Amino acids can be analyzed with gaseous or liquid chromatography (GC or LC) with a derivatization step, which includes treatment with Ortho Phtalaldehyde (OPA) or Fluorenylmethoxy Chloroformate (FMOC) [116] [117]. However, possibility of analyzing the amino acid profile without derivatization using anion exchange (HPAEC-PAD) has

been reported [118]. Examples of chromatography methods used for seaweed protein isolation and characterization are present in Table 1.6.

Table 1.6: Protein isolation and characterization using chromatographic methods in different seaweed species.

Species	***Identified proteins***	***Molecular Weight***	***Method***	***Ref.***
A. nodosum	Protein profile	From 2.6 to 3.8 kDa	HPLC and SEC (HPSEC); particle size of 4-6 µm and pore size of 150-300 Å; macroporous HPLC column.	[106]
S. latissima	Trypanothione reductase and ATP synthase subunit beta (chloroplastic); actin-1; elongation factor Tu; glyceraldehyde-3-phosphate dehydrogenase.	51, 41, 40, 39 kDa (respectively)	HPSEC and SDS-PAGE; 4–20% Precast Mini-Protean® linear gel; two serially connected columns: one with 5 µm particle size, 150 Å pore size, one with 5 µm particle size and 300 Å pore size.	[119]

1.3.2.9.3. Spectrometry

Fourier Transform Infrared (FTIR) spectroscopy is used to find information about the structural composition of proteins, especially when it comes to their secondary structural composition [27]. Mass spectrometry (MS) can be an accurate protein identification tool and electrospray ionization (ESI) and matrix assisted laser desorption ionizations/time-of-flight (MALDI-TOF) can also be important tools [27]. Examples are shown in Table 1.7.

Table 1.7: Protein characterization using spectrometry methods in different seaweed species.

Species	***Structural composition identified and respective bands, or identified proteins/peptides***	***Method***	***Ref.***
M. pyrifera and *C. chamissoi*	3281 cm^{-1} and 3274 cm^{-1}: N—H vibrations. 1637 cm^{-1} and 1544 cm^{-1}: C=O vibrations. 1220 cm^{-1} and 1243 cm^{-1}: S=O vibrations.	FTIR of dried seaweed and protein extracts of seaweeds.	[73]
Kappaphycus alvarezzi	704 cm^{-1}: N—H bending. 616 cm^{-1}: phosphate group.	The lyophilized protein concentrate was ground with potassium (1/100 ratio w/w) and the spectral analysis was carried out using FTIR.	[120]
P. palmata, P. umbilicalis, U. rigida, U. pinnatifida and *L. zchroleuca*	Mono-iodotyrosine (MIT) and diiodotyrosine (DIT)	Reverse phase high performance liquid chromatography (RP-HPLC) with inductively coupled plasma mass spectrometry (ICP-MS).	[121]
P. palmata and *S. chordalis*	Biactive peptides	Peptide samples were separated by online reversed-phase (RP) nanoscale capillary liquid chromatography (nanoLC) and analyzed by electrospray mass spectrometry (ES MS/MS).	[46]
C. fragile	Codiase	Fibrin(ogen)olytic activity of codiase was examined by FTIR spectroscopy and the molecular weight of codiase was determined by MALDI-TOF mass spectrometry in linear mode.	[122]

1.3.2.10. Protein Quantification Methods

Determination of protein content of algae can provide important information on the chemical characteristics of algal biomass. Protein extraction yields are generally low for seaweeds due to the presence of cell wall and phenolic compounds [22].

The methods most used to quantify protein are: (i) the alkaline copper method (Lowry method [123]); (ii) the Coomassie Brilliant Blue dye method (Bradford method [124]); or (iii) determination of crude protein (obtained by total nitrogen quantification, N×6.25) [22]. The bicinchoninic acid assay (BCA) and UV absorption methods are alternate spectrophotometric methods [125]. Protein determination can also be achieved by multiplying the total nitrogen content by a unique factor determined specifically for seaweeds (N×X) or by subtracting the non-protein nitrogen (NPN) [126]. Overall, N×6.25 is the most used method (52% out of 95% of studies on seaweed), followed by the Lowry and Bradford methods which were used 42% of times. Amino Acid Analysis (AAA) makes up for the remaining 6% [126].

There is no standardized method for protein extraction, and they vary between studies. Some factors that cause protein extraction variability are the pre-treatment of the algal sample, the volume of water and buffers used, the exposure time, whether the protein is precipitated, the method of precipitation, etc. [22]. The extraction procedures differ in efficiency and there is also additional influence of the chemical and morphological features of the seaweed themselves [126]. The main methods for quantifying protein in extracts are colorimetric (these always require an extraction step). Bovine serum albumin (BSA) is the most used protein standard for calibration curves in spectrophotometry. Good linearity can also be obtained using casein, but it has a slightly smaller reactivity than BSA resulting in a smaller quantification of protein [22], [126].

When it comes to extracted protein, most was quantified using the Bradford method (55 %), followed by the Lowry method (31 %) and the BCA method (11 %), with other methods making up less than 5% [126]. Total nitrogen in tissue is determined mainly using either the Kjeldahl method [127] (or a variation) or through combustion using CHN analyzers [126], like the Dumas method. Amino acid analysis involves a series of chromatographic techniques that can be used to measure protein levels or to identify proteins as a complementary approach to peptide mass finger printing or MS/MS sequencing [128].

The differences in the principles of the methods contribute to making comparison of results available in the literature even more difficult, since the choice of method is arbitrary. Examples of quantification methods, respective advantages and disadvantages, and specific examples are presented in Table 1.8. Studies focusing on protein quantification in algae are relatively uncommon and it is also very important to develop a simple and inexpensive protocol, using low-cost equipment and consumables.

Table 1.8: Advantages, disadvantages, and practical examples of the most common protein quantification methods in seaweeds.

Quantification Method	***Advantages***	***Disadvantages***	***Detection range***	**Examples**		
				Species	**Method**	**Ref.**
Lowry	Widely used due to its simplicity and availability [125]. 100-fold more sensitive than determining absorbance at 280 nm [22].	Reactivity is strongly influenced by the amino acid composition [22]. Several substances cause interferences (e.g., phenol and phenolases, glucosamine, detergents, flavonoids, Tris-HCl; carbohydrates, EDTA, reducing agents) and affect analyses by either over or underestimating. That can be avoided by precipitation of the protein sample with trichloroacetic acid (TCA) which also avoids the quantification of small peptides and free amino acids [125]. The Folin reagent is reactive for a short period of time after addition (30 min) [125]. Requires more steps and reagents than the BCA or Bradford assays [125]. Destructive method [126]. Requires an extraction step [125].	5–100 µg [123]	*Porphyra umbilicalis*, *Ulva lactuca* and *Saccharina latissima*	Determination according to the Lowry assay [129] modified by Markwell [83]; no precipitation.	[123]
				Fifteen species of seaweed	Protein precipitation with TCA:homogenate (2.5:1 v/v); suspension of the precipitated protein in 2 mL of 0.1 M NaOH; determination according to the Lowry assay [22].	[130]
				Palmaria palmata	Determination using the Bensadoun and Weinstein modification [123] of the Lowry assay [75]. It involves precipitation by adding 100 mL of 0.15 g/100 mL sodium deoxycholate and 100 mL of 72 g/100 mL TCA.	[125]
Bradford	Easiest and fastest method [125]. The Coomassie brilliant blue G-250 is stable for long periods of time [125]. Useful as a general, sensitive, semiquantitative assay [22].	Reactivity is strongly influenced by the amino acid composition [131]. Interferences happen when reagents that may change the pH and detergents are present [22]. Lower concentrations of protein are obtained which can be related to the binding of the dye to both basic and aromatic amino acid residues. Most algae show relatively low concentrations of tyrosine and tryptophan as well as lysine and histidine and the binding of the dye occurs mainly with arginine and phenylalanine, which contributes to lower protein measurements [125]. Concentrated protein solutions can form a precipitate upon contact with the dye reagent [125]. Destructive method [126]. Requires an extraction step [125].	0.2–20 µg [124]	Fifteen species of seaweed	Protein precipitation with TCA:homogenate (2.5:1 v/v); suspension of the precipitated protein in 0.5 mL 1.0 M NaOH; determination according to the Bradford assay [22].	[124]
				Fifteen species of seaweed	Determination using the Bradford assay [111] in microplates.	[125]
BCA	Compatible with detergents [125]. Less protein/protein variability than the Bradford assay [125].	Phospholipids, reducing, and chelating reagents cause interferences [125]. Destructive method [126]. Requires an extraction step [125].	0.2–50 µg [132]	*Palmaria palmata*	Protein concentration with bicinchoninic acid (BCA) protein reagent assay according to the manufacturer's instructions [133].	[134]

Table 1.8: Advantages, disadvantages, and practical examples of the most common protein quantification methods in seaweeds.

Quantification Method		***Advantages***	***Disadvantages***	***Detection range***	**Examples**		
					Species	**Method**	**Ref.**
AAA		Direct method [134]. Only protein analysis method where interfering substances do not affect the results [128].	Unstable derivatives, incomplete reactions, inadequate chromatographic separations, and the lack of a single hydrolysis method with sufficient recovery of all amino acids hinder precise quantitation [135]. Complicated by the variable susceptibility of amino acids and peptide bonds to acid hydrolysis, variable levels of background contamination, and potentially variable instrument and human performance [135]. Labor-intensive [135]. Peptide bonds and amino acids vary widely in their stability/lability to acid hydrolysis [22].	-	Fifteen species of seaweed	After acid hydrolysis, samples were left in a desiccator containing NaOH pellets under vacuum until dry. The residue was dissolved in a suitable Na–S, filtered (0.22 μm pore size) and analyzed by ion-exchange chromatography.	[136]
					Ten species of seaweed	Hydrolysis of total protein was performed according to the protocol described by Malmer and Schroeder [117]; membrane-filtration using 0.45 μm regenerated cellulose membranes) and analysis using RP-HPLC with fluorescence detection.	[137]
Multiplying tissue nitrogen content by a Nitrogen-to-Protein conversion factor	Kjeldahl NItrogen	Official method for food protein determination by the AOAC International due to its universality, high precision, and good reproducibility [126]. Does not require an extraction step [126].	Algae commonly have high concentrations of non-protein nitrogenous substances such as pigments, nucleic acids, free amino acids, and inorganic nitrogen whose presence makes the general conversion factor of 6.25 unsuitable, since it overestimates the actual protein content [138]. Requires specialized equipment and is time consuming [139].	-	Fifteen species of seaweed	Protein content determined according to the method described by AOAC (2000) [111] with slight modifications as recommended for a Kjeltec 2300 apparatus.	[138]
	Dumas Combustion	Easy and cost-effective alternative [138]. Faster than the Kjeldahl method [138]. Does not require toxic chemicals or catalysts [138]. Many samples can be measured automatically [126]. Does not require an extraction step [138].	Usually provides higher results than the Kjeldahl method (by about 1.5%) probably due to the near-complete conversion of non-protein forms of nitrogen into elemental nitrogen [138]. It is necessary to determine whether currently accepted nitrogen factors can be used [138]. High initial cost [138]. Small sample size makes it difficult to obtain a representative sample [140].	-	*Ascophyllum nodosum*	Protein content determined using a LECO FP628 protein analyzer based on the Dumas method according to the AOAC method 992.15 (1990) [141]. A sample extract of 0.25 g was used for protein estimation.	[15]

1.3.3. Polysaccharide Extraction

The phycocolloids alginate, agar and carrageenan are the main commercial polysaccharides derived from seaweed, that are widely used in the food, pharmaceutical and biotechnological industry [142]. They are water-extracted polysaccharides sourced from brown and red seaweeds [143]. The use of phycocolloids in these industries is largely based on their ability to form gels with unique properties. Agar and carrageenan form thermoreversible gels while alginates form ionic non-thermoreversible gels. *Laminaria* (Europe and Asia), *Ascophyllum* (Europe) and *Lessonia* (Chile and Peru) are the main sources for alginate production, *Gracilaria* and *Gelidium* are the preferred seaweed for agar production, and *Kappaphycus*, *Eucheuma* and *Chondrus* are used for carrageenan production. Agar is the most expensive colloid, followed by alginates and then carrageenan, however agar production is the lowest out of the three (Figure 1.4) [142].

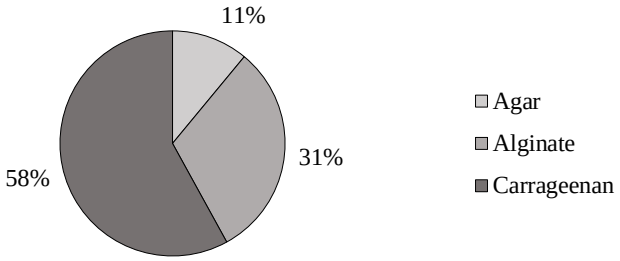

Figure 1.4: Agar, alginate, and carrageenan production (%) in 2009. Total volume of phycocolloid production was 86 100 tons. [142]

1.3.3.1. Sulfated Galactans

Sulfated galactans are polymers of partially sulfated β-D- and/or α-D- galactopyranose units and are common cell wall matrix polysaccharides of green and red algae [15].

In green algae (genera *Codium* and *Bryopsis*) polysaccharides were reported to be both homo and heteropolymers, depending on the species [15]. Red algae cell walls contain sulfated galactans which usually have a linear backbone composed of alternating 3-linked β-D-galactopyranose and 4-linked α-galactopyranose residues (unit A and B in Figure 1.5, respectively). The latter have the L-configuration in agar and the D-configuration in carrageenans. 4-linked residues may be present partially or completely as 3,6-anhydro derivatives [144]. Sulfate hemiesters, methyl ethers, and pyruvic acid are commonly bound to these polysaccharides. Sulfate and/or occasionally methyl groups may appear at the *O*-2 and/or *O*-4 position(s) of the 3-linked β-D- galactopyranose units and at *O*-2, *O*-3-, and/or *O*-6 position(s) of the 4-linked α-galactopyranose units [15].

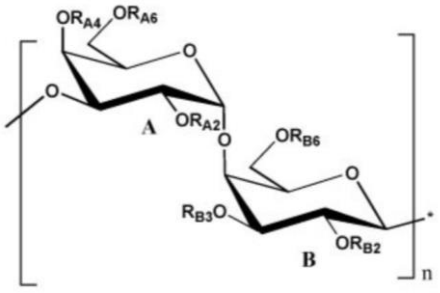

Figure 1.5: Schematic representation of sulfated galactans from red algae. B unit is in D configuration. R_{A2}*: H,* SO_3^-*;* R_{A4}*: H,* SO_3^-*, pyruvic acid (cyclic ketal with* O_6*);* R_{A6}*: H,* SO_3^-*,* CH_3*, pyruvic acid (cyclic ketal with* O_4*);* R_{B2}*: H,* SO_3*,* CH_3*;* R_{B3}*: H;* R_{B6}*: H,* SO_3^-*.* [145]

1.3.3.1.1. Agar

Agar is composed of a heterogeneous mixture of molecules, with a backbone of neoagarobiose and agarobiose – disaccharide repeating units of 3-linked β-D-galactopyranose and 4-linked 3,6-anhydro-α-L-galactopyranose residues that differ in the level of substitution of hydroxyl groups (e.g., ester sulfate, methoxyl) [144] (Figure 1.6). Substitution with sulfate hemiesters, methyl ethers and/or pyruvate ketals can occur at various sites in the polysaccharide chain [142].

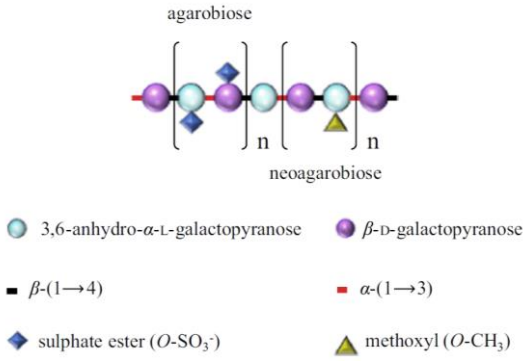

Figure 1.6: Backbone structure of agarose. The repeating disaccharide units are agarobiose and neoagarabiose. 3,6-anhydro-galactose residues are L-enantiomers [140].

Agar is commonly defined as a mixture of two polysaccharide fractions (Figure 1.7). The neutral, low sulfate/methoxyl substituted polysaccharide fraction of agar is named agarose and exhibits high gelling capacity. The charged, heterogeneous mixture of smaller molecules, highly substituted polysaccharide fraction is agaropectin and exhibits low gelling capacity. The pattern of substitution and the ratio of agarose to agaropectin depend on several aspects: environmental factors, such as hydrodynamic conditions, availability and quality of light and nutrients; physiological factors such reproductive stage and nutritional state; and the extraction and isolation conditions of agar. Agar is insoluble in cold water and requires heating at temperatures of above 85 ºC to dissolve [143].

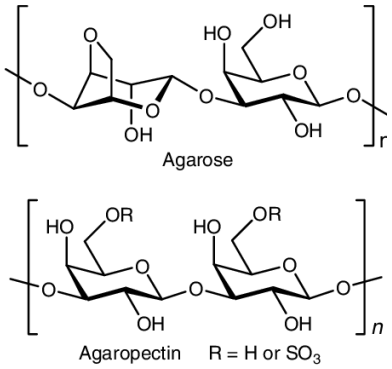

Figure 1.7: Structural features of agar – agarose and agaropectin. [146]

Agar polysaccharides isolated from *Gracilaria* are typically more sulfated than those obtained from *Gelidium* and *Pterocladia*, with the pattern of sulfation dominated by the esterification of C-6 of the linked galactose L-unit [142]. The extraction method employed could promote desulfation which results in an increase in agar quality. In *Gelidium* and *Pterocladia*, desulfation occurs as a natural internal transformation through an enzymatic process. Before agar is extracted from *Gracilaria*, it is necessary to chemically promote desulfation.

The conventional agar production follows the key stages present in the flow diagram of Figure 1.8. After being washed to remove sand, salts and other foreign matter, algae suffer a pre-treatment which depends on their genus [142].

The pre-treatment of *Gelidium* consists of a treatment with a mild alkaline solution (e.g., Na_2CO_3) to remove pigments (phycoerythrin) and to macerate the seaweed. For *Gracilaria*, alkali treatment is performed to promote desulfation and increase gel strength. This is usually performed at 85-90 ºC in a NaOH solution with a concentration ranging from 0.5-7%, for 1-2 hours. Seaweed are then washed with water or a weak acid to neutralize residual alkali. [142]

Agar extraction necessarily involves cooking the seaweeds in an excess of water at boiling point. To promote a good extraction, adjusting the pH to 6.3–6.5 is generally required. Extraction under pressure reduces processing time and increases extraction yields. The dissolved agar is then filtered to remove residual seaweed and the hot filtrate is cooled to form a gel. The gel may be bleached (e.g., sodium hypochlorite) to reduce any color. [142]

When the bleach is washed off, a gel with about 1% of agar is obtained. The remaining 99% which consists of water is removed by freeze-thawing or by squeezing it out using pressure. After thawing and straining, there is usually a 10-fold increase in concentration of agar. The eluted water carries oligomers, salts, and proteins, including phycoerythrins. Syneresis is an alternative method to reduce water and it consists of placing the agar gel between porous filter cloths and squeezing it in a hydraulic press [142]. A greater quantity of water and soluble impurities is removed, and less energy is consumed. Isolated agar is dried in hot-air oven and milled to the desired particle size [143].

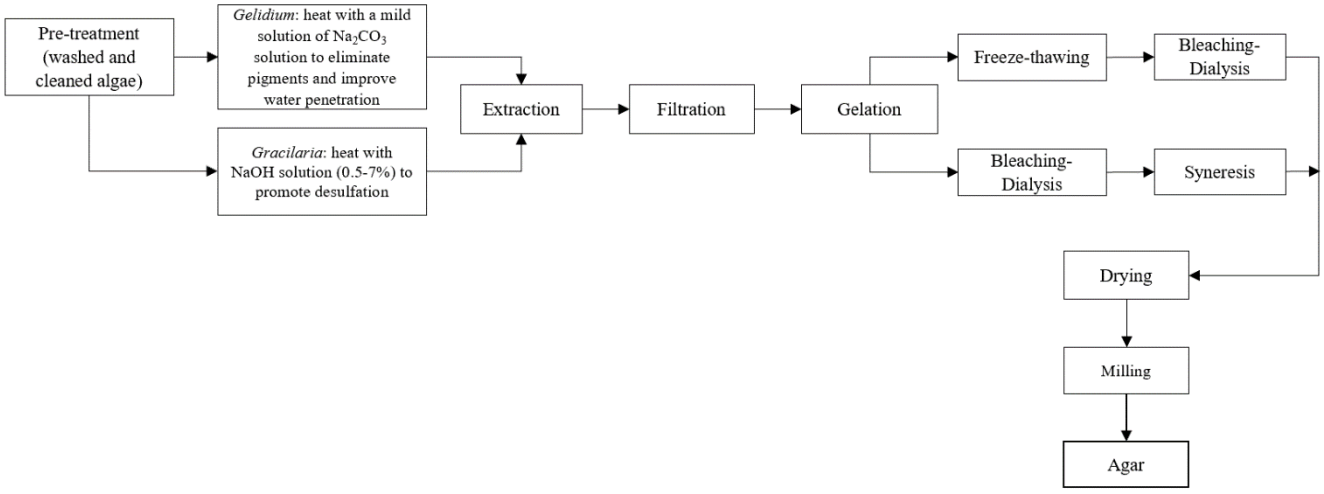

Figure 1.8: Flow diagram for agar production (adaptaed from [142] *and* [143]*).*

Basic processes may not have changed throughout the years and improvements in presses and freezing equipment are being implemented to improve efficiency and reduce energy requirements. However, current processes still require large quantities of solvents. Since the effluents produced during the bleaching process constitute a pollution problem, a photobleaching extraction process has been developed. It exploits sunlight and is based on the photochemical degradation of colored dissolved organic matters (CDOM). Microwave-assisted extraction (MAE) results in rapid internal heating of algal matrices and release of the analytes into the cold solvent.

The main advantages of the proposed procedure are the reduced consumption of solvents, the lower level of energy required and the reduced extraction time [142].

1.3.4. Gelidium sp.

Gelidium sp., is a genus of thalloid red algae which are important agarophytes. The gel-forming ability and solubility of agar polysaccharides rely on the relative hydrophobicity of the repeating unit mentioned (Figure 1.6). Natural populations of *Gelidium* are exploited worldwide for the extraction of technical agars (e.g., bacteriological agar and agarose) and constitute the most important source of raw material for the industry as *Gelidium* aquaculture has not been feasible at larger scales [147]. The agar extracted from *Gelidium* currently represents about 1.6% of the world production [148], however its natural high gelling strength and low gelling temperatures make it attractive. Consequently, higher grade, purified agars used in pharmacological, biomedical, and biotechnological industries are extracted solely from *Gelidium* [149].

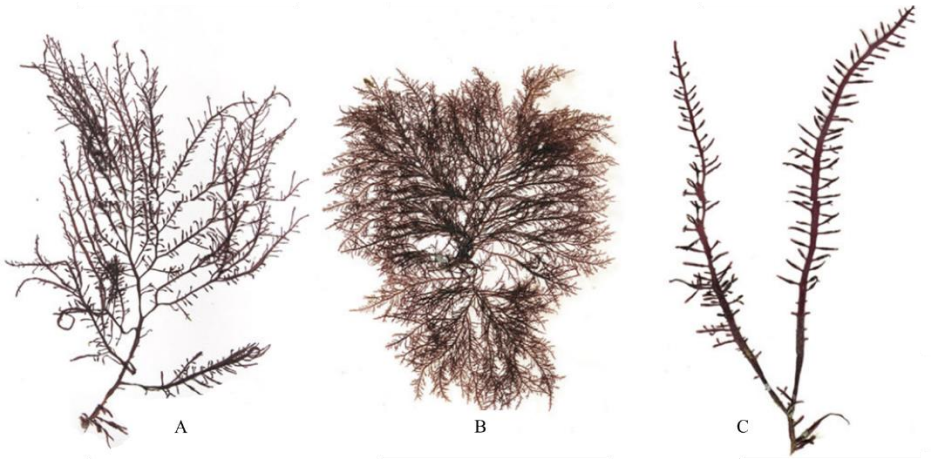

Figure 1.9: A - Gelidium johnstonii; B – Gelidium allanii; C – Gelidium koshikianum. [150]

1.3.4.1. Harvesting

The demand for bacteriological agar and agarose from *Gelidium* has increased from 250 and 15 t [151] to about 700 and 50 t [152], respectively. Technical agars are currently in short supply [153].

In 1991, about 50% of the world's *Gelidium* landings originated from Spain, Portugal, and Morocco. Japan and South Korea each contributed with around 14%, while Mexico and Indonesia contributed 10% and 7%, respectively [149]. Significant changes in the relative contribution of producers happened during the 1990s, when harvest zones decreased in countries like Portugal, Japan and Mexico, and the global production switched its focus to Morocco [149]. Morocco's contribution has increased from 23% in the 1960s to the current 82% [149]. In the 2010s, the global production of *Gelidium* reached considerably low values (25,000 t/year) [149] having collapsed in some countries, namely Japan, Korea, Spain, and Portugal. Only 4000 t of agar was extracted during this period which contrasts with the 1980s peak of almost 10,000 t [153]. Socio-economic reasons have been deemed as the main factor for this shift in production. Moroccan *Gelidium* is of better quality (and lower price) [154][155]. The scarcity of agar can be an opportunity to resume the *Gelidium* harvesting in countries where this activity crashed. There is already an increased interest in new *Gelidium*-based agarophyte resources in countries like Tunisia [156], Egypt [157] and Argentina [75].

1.3.4.2. Protein Extraction

Protein contents reported for several *Gelidium* species and protein extraction strategies described in the literature are presented in Table 1.9 and Table 1.10, respectively.

Table 1.9: Protein content in some Gelidium species.

Species	***Protein Content***	***Quantitative Method***	***Ref.***
Gelidium amansii	18.5% dw	Semi-micro Kjeldahl method [158].	[159]
	10.5 ± 0.1% dw	Kjeldahl method (conversion factor of 6.25).	[160]
Gelidium pusillum	11.3 ± 1.0% dw	Kjeldahl method (conversion factor of 6.25).	[161]
Gelidium microdon	23.4 ± 0.7% dw	Kjeldahl method (conversion factor of 6.25).	[162]
Gelidium sp.	13.2 ± 1.1% dw	Kjeldahl method (conversion factor of 6.25).	[163]
Gelidium corneum	16.5 ± 0.2 % dw 9.6% dw 13.4% dw	Kjeldahl method (conversion factor not specified).	[164]

Table 1.10: Examples of strategies used for protein extraction from Gelidium.

Source	*Extraction method*	*Reagents*	*Conditions*	*Initial protein content*	*Protein recovery yield*	*Protein quantification method*	*Ref.*
Gelidium corneum (crude)	Aqueous extraction (hot)	Distilled water	1g of macroalga; 50 mL of distilled water; Soxhlet extractor (T>100°C; 4 h).	13.4% dw	26.7% dw[a]	Kjeldahl method (conversion factor not specified).	[164]
	Aqueous extraction (RT)		1g of macroalga; 50 mL of distilled water; stirring for 4 h, RT.		19.9% dw[a]		
	Aqueous extraction (re-extracted, RT)				5.3% dw[a]		
	Ethanolic extraction	Ethanol	1g of macroalga; 10 mL of ethanol (100%); stirring for 8h, RT; rotary evaporator for 8h		0.93% dw[a]		
	Ethanolic extraction (re-extracted)				2.4% dw[a]		
Gelidium sesquipedale (crude)	Alkaline solubilization; acid precipitation	Sodium sulfate; NaOH; HCl	Suspension in 0.3% sodium sulfate (ratio of algal powder:solution = 1:10); adjustment to pH 12 using NaOH 1N; agitation for 1 h. Centrifugation; adjustment of the supernatant's pH to 4.5 using HCl 1N. Centrifugation, washing, neutralization (pH 7) and filtration of the precipitate. Lyophilization.	30% dw	75.6% dw[a]	Kjeldahl method (conversion factor of 6.25).	[165]
	Solubilization in distilled water; ammonium sulfate precipitation.	Distilled water; ammonium sulfate	Suspension in distilled water; agitation for 1h; pH 7-8. Two sequential centrifugations. Addition of 1 volume of ammonium sulfate 50% to 1 volume of the combined supernatants; centrifugation. Washing of the pellet with distilled water; dissolution of the pellet in distilled water at pH 7; dialysis against distilled water at pH 7.		51% dw[a]		
Gelidium sesquipedale (agar extraction residues)	Alkaline solubilization; acid precipitation	Sodium sulfate; NaOH; HCl	Suspension in 0.3% sodium sulfate (ratio of algal powder:solution = 1:10); adjustment to pH 12 using NaOH 1N; agitation for 1h. Centrifugation (10 min at 2,000 rpm); adjustment of the supernatant's pH to 4.5 using HCl 1N. Centrifugation, washing, neutralization (pH 7) and filtration of the precipitate. Lyophilization.	25% dw	63% dw[a]	Kjeldahl method (conversion factor of 6.25).	[165]
	Solubilization in distilled water; ammonium sulfate precipitation.	Distilled water; ammonium sulfate	Suspension in distilled water; agitation for 1h; pH 7-8. Two sequential centrifugations. Addition of 1 volume of ammonium sulfate 50% to 1 volume of the combined supernatants; centrifugation. Washing of the pellet with distilled water; dissolution of the pellet in distilled water at pH 7; dialysis against distilled water at pH 7.		40% dw[a]		
Gelidium pusillum	Primary aqueous extraction; ammonium sulfate precipitation; aqueous two-phase extraction (ATPE); ultrafiltration.	Phosphate buffer; PEG; potassium phosphate	Primary extraction with phosphate buffer (0.1 M, pH 6.8). Biomass subjected to maceration followed by ultrasonication (20 kHz, 120 µm; 'on and off' cycle of 1/1 s). Precipitation with ammonium sulfate 70%. ATPE with PEG 1450/potassium phosphate (12.26% tie line length, 0.29 volume ratio, pH 6); ultrafiltration with a 30 kDa PES membranes in a stirred cell module (50 mL, 300 rpm, 1 bar).	Not specified.	57% w/w[b]	Absorbance values at A_{564} (maximum absorbance of R-PE).	[24]

[a]dw expressed as algal dry weight; [b]R-PE extraction yield.

1.3.4.3. Carbohydrate Extraction

Carbohydrate contents reported for several *Gelidium* species are presented in Table 1.11. Agar extraction strategies described in the literature are presented in Table 1.12.

Table 1.11: Carbohydrate content of some Gelidium species.

Species	***Carbohydrate Content***	***Quantitative Method***	***Ref.***
Gelidium amansii	75.2% dw (58.6% of agar)	Glucose and galactose determined using HPLC after saccharification.	[159]
	71.4 ± 0.1% dw	Weight difference using crude protein, lipid, fiber, moisture and ash content data.	[160]
Gelidium pusillum	40.6 ± 2.2% dw	Weight difference using crude protein, lipid, fiber, moisture and ash content data.	[161]
Gelidium microdon	17.6 ± 0.3% dw	Phenol-sulphuric acid colorimetric method [166].	[162]
Gelidium sp.	53.7 ± 1.2% dw	Reductive hydrolysis [167]; gas chromatography	[163]
Gelidium sesquipedale	Agar content of ~40% dw	Phenol-sulphuric acid colorimetric method [166]; reducing sugars quantified by the 2,5-dinotrosalicylic acid reagent Miller.	[168]

Table 1.12: Examples of strategies used for agar extraction from Gelidium.

Species	***Reagents***	***Conditions***	***Carbohydrate recovery yield***	***Carbohydrate quantification method***	***Ref.***
Gelidium amansii	Distilled water	Seaweed to distilled water ratio of 1:20 w/v, pH 6-6.5; sonication (time interval of 30 min, power intensity of 30%, frequency of 35 Hz, RT); autoclave (120°C, 1h); filtration with filter cloth; cellulose nitrate membrane filtration (3 μm).	49.1% dw.	Not specified.	[169]
		Seaweed to water ratio of 1:20 w/v, pH 6-6.5; sonication (time interval of 30 min, power intensity of 30%, frequency of 35 Hz, RT); direct heating in a hotplate stirrer (99°C, 1.5h); filtration with filter cloth; cellulose nitrate membrane filtration (3 μm).	13.3% dw.		
Gelidium sesquipedale	Distilled water	Immersion of 50 g of dry seaweed powder in 500 mL of distilled water and heated up to 90 °C for 2 h. Filtration using muslin cloth.	11.9 ± 1.4%	Sulphuric acid hydrolysis; high performance anion exchange chromatography.	[170]
Gelidium serrulatum	Distilled water	3g of seaweed in 150 mL of distilled water and soaked overnight (RT); adjustment of the pH to 6-6.5; autoclave (121°C, 3h); filtration using a cheesecloth into a pressure filter; residue washing with hot water (once); pressure filtration (celite, 10 μm filter).	40.8%	Not specified.	[171]
	0.25 N NaOH	3g of seaweed in 150 mL of 0.25 N NaOH and soaked overnight; autoclave (121°C, 3h); filtration using a cheesecloth into a pressure filter; residue washing with hot water (once); pressure filtration (celite, 10 μm filter).	36.2%		
	1.0 N NaOH; distilled water	3g of seaweed in 1.0 N NaOH (80°C, 1h); filtration through a cheesecloth; washing with tap water; suspension in 150 mL of distilled water (pH 6-7) and soaked overnight (RT); adjustment of the pH to 6-6.5; autoclave (121°C, 3h); filtration using a cheesecloth into a pressure filter; residue washing with hot water (once); pressure filtration (celite, 10 μm filter).	12.2%		
	Acetone, distilled water, 100% ethanol, 40% ethanol.	Extraction with 5g of seaweed in 200 mL of acetone (stirred, 24h); filtration; 150 mL of distilled water (stirred, 24h); filtration; washing with 50 mL of distilled water. Samples were refluxed twice for 1.5h in 250 mL of boiling 100% ethanol; then twice in boiling 40% ethanol. Autoclaving in 250 mL of distilled water (pH 6-6.5); pressure filtration. Partial drying of the 40% ethanol fractions; lyophilization of autoclaved fractions.	18.7% (40% ethanol fractions)		
			28.5% (autoclaved fractions)		

2. Materials and Methods

2.1. Biomass collection and preparation

Gelidium sesquipedale was kindly provided by Iberagar - Sociedade Luso-Espanhola de Colóides Marinhos, S.A., Portugal. *Gelidium sesquipedale* is usually collected throughout Portugal's mainland and island areas, until the 10m bathymetric, but it is mostly concentrated in two areas – from south of the Mondego river until north of Foz do Arelho, and from south of Foz do Arelho until north of Cabo da Roca. Annual harvesting occurs between July the 15th and November the 15th. Right after harvesting, the fresh seaweed is washed with water, and sun dried in the summer, until a moisture content of around 20% w/w is reached. If these conditions are enforced, it can be stored at room temperature up to 2 years. The dried alga was milled to obtain a fine powder with an average granulometry of 0.25mm.

2.2. Chemicals and solutions preparation

An AG245 digital analytical laboratory scale (Mettler Toledo) was used for solutions preparation. pH measurements were performed using a FiveEasy F20 pH/mV meter (Mettler Toledo).

The chemicals used were sulfuric acid 96% solution in water (ACROS Organics), sodium hydroxide pellets 98% (Thermo Fisher Scientific), hydrochloric acid 37% solution in water (Honeywell Fluka), calcium carbonate ≥ 99% (Merck, Germany), ammonium sulfate ≥ 99% (Panreac, USA), TRIS base ≥ 99.8% (Thermo Fisher Scientific), Tween 20 (Sigma-Aldrich, Germany), sodium carbonate ≥ 99.5% (Farma-quimica Sur Sl, Spain), potassium sodium tartrate tetrahydrate ≥ 99% (Panreac, USA), copper(II) sulfate pentahydrate ≥ 99.5% (Panreac, USA), Folin & Ciocalteu's phenol reagent 2N (Sigma-Aldrich, Germany), bovine serum albumin lyophilized powder ≥ 96% (Sigma-Aldrich, Germany), D(+) glucose anhydrous 99.5% (Thermo Fisher Scientific), and D(+) galactose ≥ 98% (Carl Roth Chemicals, Germany).

2.3. Biomass characterization

2.3.1. Total Solids, Moisture and Ash Content

Total solids (solids remaining after heating the sample as described in this section until a constant weight is achieved), moisture (water and other volatile compounds) and ash (inorganic residue remaining after dry oxidation) were determined following the National Renewable Energy Laboratory's (NREL) "Determination of Total Solids and Ash in Algal Biomass" analytical procedure [172].

Crucibles were pre-conditioned in a 575ºC muffle furnace overnight and cooled to room temperature in a desiccator. Using gloves, each crucible was weighted to the nearest 0.1 mg. 100 mg of the as-received algal biomass was added to each crucible (n=3) which were placed into a convection drying oven at 60ºC and atmospheric pressure for 18h (D 06058, Modell 200, Memmert, Germany). Samples were allowed to cool to room temperature in a desiccator. The crucibles and oven-dried samples were weighted. The percentage of total solids, moisture and oven dry weight (ODW) were calculated using Equation 2.1, Equation 2.2 and Equation 2.3, respectively.

$$Total\ Solids\ (\%) = \frac{(weight_{crucibles+dried\ sample} - weight_{crucibles})}{weight_{biomass\ as-received}} \cdot 100 \qquad \textit{Equation 2.1}$$

$$Moisture\ (\%) = 100 - Total\ Solids\ (\%)$$ *Equation 2.2*

$$ODW_{sample}\ (\%) = \frac{weight_{air\ dried\ sample} \cdot Total\ Solids\ (\%)}{100}$$ *Equation 2.3*

After oven-drying, ashing the samples was performed using a muffle furnace (L 24/11 Nabertherm, Germany) equipped with a ramping program: ramp from RT to 105ºC, hold at 105ºC for 12 min, ramp to 250ºC at 10 ºC/min, hold at 250ºC for 30 min, ramp to 600 ºC at 20ºC/min, hold at 600ºC for 16h, temperature drop to 105ºC. Samples were removed and allowed to cool until RT in a desiccator. The crucibles with the ashed samples were weighted. Ash content was determined using Equation 2.4.

$$Ash\ (\%) = \frac{(weight_{crucibles+ash} - weight_{crucibles})}{ODW_{sample}} \cdot 100$$ *Equation 2.4*

All weightings were performed using a Mettler Toledo AG245 digital analytical laboratory scale.

2.3.2. Total Carbohydrate Content

Total carbohydrates were determined following the NREL's "Determination of Total Carbohydrates in Algal Biomass" analytical procedure [173]. It was considered that a two-step sulfuric acid hydrolysis completely hydrolyzes the structural polysaccharides (cellulose and agar) into their monomeric subunits, D-glucose and D-galactose. Algal biomass (0.5 g) was weighted (AG245 digital analytical laboratory scale, Mettler Toledo) into a 250 mL Schott Duran® laboratory glass bottle (n=3). 5 mL of 72% (w/w) sulfuric acid was added to each bottle. The bottles were then placed in an orbital agitator (Agitorb 160E, Aralab, Portugal) at 30ºC, 300 rpm, for 1h. The sulfuric acid concentration was brought down to 4% (w/w) by adding 138.5 mL of distilled water. The bottles were autoclaved for 1h at 121ºC using the liquids setting. Prior to removing them from the autoclave, the bottles were allowed to cool for 15 min with the lid opened. Once removed, they were allowed to cool for 1h. After vortexing (laboratory shaker 444-1378, VWR), aliquots of 3 mL were placed in 50 mL Falcon conical centrifuge tubes. The aliquots were neutralized to a pH between 6 and 8 with calcium carbonate. The neutralized samples were centrifuged for 5 min, 10,000 × g (Centrifuge 5810 R with a A-4-62 swing-bucket rotor, Eppendorf, Germany) so that all solids were removed. Samples were analyzed for carbohydrates as described in section 2.7.2.

The amount of cellulose and agar in the biomass were calculated using Equation 2.5 and Equation 2.6, respectively, where 162 is the MW of glucose and galactose monomeric units in polymeric glucan and galactan, 180 is the MW of glucose and galactose and 1.27 is the weight ratio between L-3,6-anhydro galactose (AHG) and D-galactose in agar [174].

$$Cellulose + starch\ (\%) = \frac{c_{glucose} \cdot \frac{162}{180}}{c_{biomass} \cdot ODW} \cdot 100$$ *Equation 2.5*

$$Agar\ (\%) = \frac{(c_{galactose} + 1.27 \cdot c_{galactose}) \cdot \frac{162}{180}}{c_{biomass} \cdot ODW} \cdot 100$$ *Equation 2.6*

2.3.3. Total Protein Content

Total protein content was determined at IPMA - Instituto Português do Mar e da Atmosfera, using a nitrogen analyzer FP-528 DSP LECO (LECO, St. Joseph, USA) calibrated with EDTA ($y = 8.4 \cdot 10^{-1}x - 2.3 \cdot 10^{-3}$) according to the Dumas method [175], using a nitrogen-to-protein conversion factor of 4.59 [176]. Samples of 0.1 g were used (n=3).

2.4. Protein Extraction

A list of the extraction procedures is presented in Table 2.1. Each extraction sequence started with 10 g of algal powder (PB3001-S precision scale, Mettler Toledo). The supernatants collected for protein assay were stored at -20ºC. pH measurements were performed using a FiveEasy F20 pH/mV meter (Mettler Toledo).

Table 2.1: List of extraction procedures performed.

Extraction Procedure	***Extraction Conditions***
I	Aqueous extraction and alkaline extraction (sequential)
II	Aqueous extraction, alkaline extraction, and alkaline re-extraction (sequential)
III	Aqueous extraction, aqueous re-extraction, and alkaline extraction (sequential)
IV	Aqueous extraction, aqueous re-extraction, alkaline extraction, and alkaline re-extraction (sequential)
V	Sonication-assisted aqueous extraction and alkaline extraction (sequential)
VI	Sonication-assisted aqueous extraction and sonication-assisted alkaline extraction (sequential)
VII	Aqueous extraction and acid extraction (sequential)
VIII	Aqueous extraction, acid extraction, and acid re-extraction (sequential)
IX	Aqueous extraction, acid extraction, and alkaline extraction (sequential)
X	Hot aqueous extraction and alkaline extraction (sequential)
XI	Enzyme-assisted aqueous extraction using Viscozyme and alkaline extraction (sequential)
XII	Enzyme-assisted aqueous extraction using Celluclast and alkaline extraction (sequential)
XIII	Enzyme-assisted aqueous extraction using Viscozyme and Celluclast and alkaline extraction (sequential)
XIV	Enzyme-assisted aqueous extraction using Alcalase and alkaline extraction (sequential)
XV	Enzyme-assisted aqueous extraction using Celluclast, enzyme-assisted aqueous extraction using Alcalase, and alkaline extraction (sequential)

2.4.1. Aqueous Extraction

The algal powder was suspended in 200 mL of deionized water in 500 mL Thermo Scientific™ Nalgene™ PPCO centrifuge bottles (n=3). The suspension was stirred at 600 rpm (7×30mm cylindrical magnetic stirrer; RO 5 Power IKAMAG magnetic stirrer, IKA Werke, Germany) for 16h at 4ºC. After incubation, the suspension was centrifuged (Sorvall™ RC 6 centrifuge with a SLC-3000 rotor, Thermo Fisher Scientific, USA) at 10,000 × g, for 20 min at 4ºC. The supernatant was collected for protein assay.

Hot aqueous extraction was performed identically but at 50ºC using a MIXdrive 15 stirring drive (2mag AG, Germany) coupled with a digital telemodul 20 C controller (Thermo Fisher Scientific, USA).

2.4.2. Alkaline Extraction

Alkaline extraction was never used as a first extraction method therefore it was always applied to the pellet that resulted from the centrifugation of the preceding extraction method. The pellet was resuspended in 100 mL of 0.1M NaOH, and stirred at 600 rpm (7×30mm cylindrical magnetic stirrer; RO 5 Power IKAMAG magnetic stirrer, IKA Werke, Germany) for 1h at RT. The suspension was centrifuged (Sorvall™ RC 6 centrifuge with a SLC-3000 rotor, Thermo Fisher Scientific, USA) at 10,000 × g, for 20 min at 4ºC. The supernatant was collected for protein assay.

2.4.3. Acid Extraction

Since acid extractions were never used as a first extraction method, they were always applied to the pellet that resulted from the centrifugation of the preceding extraction method. The pellet was resuspended in 100 mL of 0.1M HCl, and stirred at 600 rpm (7×30mm cylindrical magnetic stir bar; RO 5 Power IKAMAG magnetic stirrer, IKA Werke, Germany) for 1h at RT. The suspension was centrifuged (Sorvall™ RC 6 centrifuge with a SLC-3000 rotor, Thermo Fisher Scientific, USA) at 10,000 × g, for 20 min at 4ºC. The supernatant was collected for protein assay.

2.4.4. Ultrasound-Assisted Extraction

Sonication was performed either before an aqueous extraction, or both before an aqueous extraction and the subsequent alkaline extraction. The algal powder (or pellet) was suspended in either 100 mL of deionized water or 50 mL of 0.1M NaOH in a glass beaker (n=3). The algal cells were disrupted using an ultrasonic cell disruptor (TT 13 probe, Bandelin Sonoplus), for 10 min, 50W and a 5s/10s on and off cycle. The glass beaker was kept in ice and the temperature control was set so that 15ºC were not surpassed. After sonication, the suspension was transferred to a 500 mL Thermo Scientific™ Nalgene™ PPCO centrifuge bottle and 100 mL of deionized water or 50 mL of 0.1M NaOH were added. The extraction procedure continued as described in *Aqueous Extraction* or *Alkaline Extraction.*

2.4.5. Enzyme-Assisted Extraction

The enzymes were added to 200 mL of deionized water in 500 mL Thermo Scientific™ Nalgene™ PPCO centrifuge bottles (n=3) and left under agitation. A sample was withdrawn for protein assay to account for the protein content derived from the addition of the enzyme. The algal powder was added to the enzyme solution and the pH was adjusted accordingly to the enzyme being used. The enzyme's concentration and the solution's pH are presented in Table 2.2. The suspension was stirred at 600 rpm (7×30mm cylindrical magnetic stir bar; MIXdrive 15 stirring drive, 2mag AG, Germany, coupled with a digital telemodul 20 C controller, Thermo Fisher Scientific, USA) for 16h at 50ºC. After incubation, the suspension was centrifuged (Sorvall™ RC 6 centrifuge with a SLC-3000 rotor, Thermo Fisher Scientific, USA) at 10,000 × g, for 20 min at 4ºC. The supernatant was collected for protein assay.

Table 2.2: Enzyme name, description, concentration, working pH and supplier used in extraction procedures XI, XII, XIII, XIV and XV.

Extraction Procedure	***Enzyme***	***Description***	***Concentration (% g_{enz}/g_{alga})***	***Concentration (U/mL)***	***pH***	***Supplier***
XI	Viscozyme	Mixture of arabanase, cellulase, β-glucanase, hemicellulase, and xylanase.	0.2	1.92 FBGU/mL [177]	4.5	Merck, Germany
XII, XV	Celluclast	Cellulase		$7 \cdot 10^{-2}$ EGU/mL [178]		Novozymes
XIII	Celluclast + Viscozyme		0.2 (each)	$7 \cdot 10^{-2}$ EGU/mL + 1.92 FBGU/mL		Novozymes/ Merck, Germany
XIV, XV	Alcalase	Endopeptidase	0.2	$1.2 \cdot 10^{-2}$ Anson units/mL [179]	8.0	Merck, Germany

FBGU: Fungal Beta-Glucanase Units
EGU: Endoglucanase Units

2.4.6. Scale-up

Scale-up was performed in 2L Erlenmeyer flasks (n=1). The starting biomass was 50 g of algal powder which resulted in a 5 times volume increase (1L or 0.5L for enzyme-assisted extractions or alkaline extractions, respectively). The extraction procedures followed what has been previously described but with a 10×70mm cylindrical magnetic stir bar using a IKAMAG REO Drehzahl Electronic magnetic stirrer, IKA Werke, Germany. The resulting pellet was dried for 4 days at 40ºC in a convection drying oven (D 06058, Modell 200, Memmert, Germany). The dried extraction residues were analyzed for carbohydrate content. The supernatants were collected and combined for protein quantification and precipitation.

2.5. Protein Precipitation using Ammonium Sulfate

After the scale-up extraction, 30 mL of the combined supernatants were transferred to 50 mL Falcon conical centrifuge tubes (n=4), with a 4.5×15mm cylindrical magnetic stir bar in a RO 5 Power IKAMAG magnetic stirrer, IKA Werke, Germany. A sample was withdrawn for protein assay (initial protein concentration). The amount of ammonium sulfate necessary to obtain the desired saturation (70%, 75%, 80% and 85%) was slowly added while stirring. Once the total mass of ammonium sulfate was added, the tubes were kept at 4ºC for 16h with stirring. The samples were then centrifuged at 15,000 × g for 30 min (Centrifuge 5810 R with a fixed-angle rotor, Eppendorf, Germany). The supernatants were collected for protein assay and the pellet was stored at -20ºC. Solutions with the same concentrations of ammonium sulfate were prepared with distilled water to assess whether the salt's presence interfered with the protein quantification method. The mass of protein in the pellet was determined using Equation 2.7. and the precipitation yield was determined using Equation 2.8. Precipitation with 85% of ammonium sulfate was performed in triplicate.

$$Protein\ Mass_{pellet}(g) = [Protein]_{initial} \cdot V_{initial} - [Protein]_{supernatant} \cdot V_{supernatant} \quad \textit{Equation 2.7}$$

$$Precipitation\ Yield\ (\%) = \frac{Protein\ Mass_{pellet}}{[Protein]_{initial} \cdot V_{initial}} \cdot 100 \qquad \textit{Equation 2.8}$$

The mass of ammonium sulfate necessary to attain the target saturations was calculated using Equation 2.9, which accounts for the volume increase upon salt addition. G_{sat} (g/L) are the grams of $(NH_4)_2SO_4$ in 1 liter of saturated solution, S_1 and S_2 are the initial and final fractions of complete saturation, respectively, $P = (specific\ volume\ (mL/g) \cdot G_{sat})/1000$, and $V_{initial}$ is the initial sample volume in liters.

$$Mass\ of\ (NH_4)_2SO_4\ (g) = \frac{G_{sat} \cdot (S_2 - S_1)}{1 - P \cdot S_2} \cdot V_{initial} \qquad \textit{Equation 2.9}$$

G_{sat} was determined considering the molarity of a saturated solution at 4ºC (3.93 M [180]) and the MW of ammonium sulfate (132.14 g/mol). At 4ºC the specific volume was estimated to be 0.53 mL/g [180].

Table 2.3: Parameters used in Equation 2.9, mass of ammonium sulfate added and the expected final volume after addition.

G_{sat} (g/L)	P	S_2 (%)	$V_{initial}$ (mL)	Mass of $(NH_4)_2SO_4$ (g)	V_{final} (mL)
519.1	0.275	70	30	13.51	37.2
		75		14.72	37.8
		80		15.98	38.5
		85		17.29	39.2

2.6. Diafiltration and Concentration

After precipitation with ammonium sulfate, the pellet was resuspended in 15 mL of TRIS HCl 20 mM, pH 7 (buffer). A sample was withdrawn for protein assay.

Diafiltration was performed using an Amicon Ultra-15 Centrifugal Filter Unit (MWCO of 3 kDa, regenerated cellulose, 15 mL, 7.6 cm^2, Merck, Germany). All centrifugations were carried out at 3220 × g using a centrifuge 5810 R with a A-4-62 swing-bucket rotor, Eppendorf, Germany. Centrifugation time was dependent on the permeate volume collected. Firstly, the filter was washed with 5% Tween 20 (10 mL, 10 min), then with MilliQ water (2x, 10 mL, 10 min) and finally with buffer (10 mL, 10 min). 10 mL of the resuspended pellet were loaded into the device. 5 mL of buffer were added to dilute the sample to avoid precipitation. A centrifugation run was carried out until ~5 mL of permeate was collected. The same amount of buffer was added to bring the retentate volume back to 15 mL. This was repeated until about 40 mL of cumulative permeate volume were collected. The filter was washed with 5% Tween and left in 0.1M NaOH until its next use. Samples of the final retentate and of each permeate were withdrawn for protein assay. Equation 2.10 is the mass balance equation. The protein mass in each retentate was calculated by applying Equation 2.10 to each centrifugal step. The diafiltration yield, the number of diavolumes (n_D) and the rejection coefficient (σ) for each step were calculated using Equation 2.11, Equation 2.12 and Equation 2.13, respectively.

$$(Protein\ Mass)_{load} = (Protein\ Mass)_{\ retentate} + (Cumulative\ Protein\ Mass)_{permeate} \qquad \textit{Equation 2.10}$$

$$Yield\ (\%) = \frac{(Protein\ Mass)_{retentate}}{(Protein\ Mass)_{load}} \cdot 100 \qquad \textit{Equation 2.11}$$

$$n_D = \frac{(Cumulative\ Volume)_{permeate}}{Volume_{load}} \qquad \textit{Equation 2.12}$$

$$\sigma = \frac{[Protein]_{permeate}}{[Protein]_{retentate}} \qquad \textit{Equation 2.13}$$

In concentration mode, the retentate volume that resulted from the diafiltration was reduced until about 6 mL in a single centrifugation (~2 min). Samples of the final retentate and permeate were withdrawn for protein assay. The mass balance, yield and rejection coefficient were calculated using Equation 2.10, Equation 2.11 and Equation 2.13, respectively. The volumetric concentration factor (VCF) was calculated using Equation 2.14.

$$VCF = \frac{Volume_{retentate}}{Volume_{load}} \cdot 100 \qquad \textit{Equation 2.14}$$

2.7. Analytical Methods

2.7.1. Protein Quantification

The Lowry method was used to determine protein concentration in the extracts [181]. 0.1 mL of 2N NaOH were added to 0.1 mL of sample or standard. Hydrolyzation took place at 100ºC in a digital heating block (Biotrace International) for 10 min. The hydrolysate was left to cool until RT and 1 mL of freshly prepared complex-forming reagent [2% (w/v) Na_2CO_3 in distilled water + 1% (w/v) $CuSO_4{\cdot}5H_20$ in distilled water + 2% (w/v) sodium potassium tartrate in distilled water, in a 100:1:1 (v:v:v) proportion] was added. The solution was left for 10 min at RT, after which 0.1 mL of a 1N Folin solution was added. The mixture was homogenized using a vortex and left to incubate at RT for 30 min. The absorbance was read at 750 nm in a DR3900 spectrophotometer (Hach Lange, USA), using 104-QS 10mm Hellma Analytics cuvettes. A stock solution of standard protein with bovine serum albumin containing 2 mg/mL of protein in distilled water was used to obtain a calibration curve that ranges from 0 – 0.5 mg/mL (linear correlation was lost for higher concentrations). The calibration curve is presented in *Appendix A*. Equation 2.15 and Equation 2.16 were used to calculate the protein extracted and the protein extraction yield, respectively.

$$[Protein](g/100g\ dw) = \frac{[Protein]_{supernatant}(g/L) \cdot V_{supernatant}(L) \cdot dilution\ factor}{ODW\ (g)} \cdot 100 \qquad \textit{Equation 2.15}$$

$$Protein\ Extraction\ Yield\ (\%) = \frac{[Protein]_{supernatant}(g/L) \cdot V_{supernatant}(L) \cdot dilution\ factor}{ODW\ (g) \cdot \frac{Total\ Protein\ (\%)}{100}} \cdot 100 \qquad \textit{Equation 2.16}$$

2.7.2. Carbohydrate Quantification

After a two-step sulfuric acid hydrolysis, the quantification of monosaccharides in the raw biomass and solid extraction residues was performed by High-Performance Liquid Chromatography (Hitachi LaChrom Elite), using a Rezex ROA Organic acid H + 8% (30mm × 7.8mm) column, a Hitachi LaChrom Elite L-2200 autosampler, a Hitachi LaChrom Elite L-2130 pump, and a Hitachi L-2490 refraction index detector. The injection volume was 20 μL and elution was achieved using a 5 mM solution of H_2SO_4. The pump was operated at a flow rate of 0.5 mL/min. The column was kept at 65 ºC (column heater for large columns connected externally to the HPLC system, Croco-CIL 100-040-220 P, 40cm × 8cm × 8 cm, 30–99ºC).

HPLC vials were prepared using samples after centrifugation (115 P microcentrifuge, Sigma-Aldrich, Germany) for 5 min at 9,167 × g. 200 µL of the supernatant were diluted with 200 µL of 50 mM H_2SO_4. The mixture was vortexed and centrifuged again. HPLC vials were prepared with 100 µL of the second supernatant and 900 µL of 50 mM H_2SO_4. Prior to analyses, calibration curves for glucose and galactose in the adequate concentration ranges were obtained. Standards were prepared following the same methodology. Calibration curves are presented in *Appendix A*.

2.8. Statistical Analysis

The statistical analysis of results was carried out using MS Excel. Most experiments were carried out in triplicate therefore data are presented as mean values ± standard deviation (SD). When comparing more than two sets of experimental data, one-way analysis of variance (ANOVA) was performed with a significance level of $p=0.05$. When ANOVA indicated statistical significance ($p<0.05$), pairwise post-hoc Tukey's Honest Significant Difference (HSD) tests were performed. When comparing only two extraction procedures, Student's t-test was used instead.

3. Results and Discussion

3.1. Biomass Characterization

The chemical composition of the biomass is presented in Table 3.1. The high content in total solids and, subsequently, the low moisture content is coherent with the drying treatment applied to the alga.

Table 3.1: Chemical composition of Gelidium sesquipedale (dry weight basis). Values are expressed as mean ± standard deviation, n = 3.

Component	***Composition***
Total Solids	92.3 ± 0.2%
Moisture	7.7 ± 0.2%
Total Carbohydrates	62.4 ± 3.5% dw
Cellulose + starch	9.5 ± 2.0% dw
Agar	52.9 ± 1.2% dw
Protein	14.8 ± 0.2% dw
Ash	19.6 ± 1.0% dw

The total protein content obtained (14.8 ± 0.2% dw) is comparable to the values presented for *Gelidium* in Table 1.9, which vary between 9.6 and 23.4% dw. Even though the values reported in the literature were also determined using total nitrogen quantification, a nitrogen-to-protein conversion factor of 6.25 was used. As mentioned in section 1.3.2.10. Protein Quantification Methods, the use of this general factor is often unsuitable since it overestimates the protein content, hence the use of a conversion factor of 4.59 in this work. Although a good estimate, this is an average factor calculated for 9 different strains of red algae (none of them being *Gelidium sp.*) over a range of different growth conditions [176], thus the actual protein content determined will vary. A study conducted on 34 algae strains revealed an average protein content of 18.8 ± 7.0% dw which is also consistent with but higher than value obtained [182]. The small sample size used in the quantification method (0.1 g) makes it difficult to obtain a representative sample. Moreover, carbohydrate synthesis in macroalgae may affect protein levels – lower protein levels were detected when carbohydrate synthesis was at its highest and vice versa [183]. While the protein content obtained is not comparable to the protein content of some leafy greens and legumes (20 to 40% dw), it can be compared to protein contents from major cereals (7 to 15% dw) (see section 1.3.2.1. Proteins in Seaweed).

The total carbohydrate content (62.4 ± 3.5% dw) is also in agreement with some values reported for *Gelidium* in the literature (Table 1.11). More specifically, a value of 75.2% dw (with 58.6% of agar) was obtained for *Gelidium amansii* using a similar quantification method (HPLC after saccharification). Additionally, it has been stated that carbohydrates comprise 50 to 60% of the dry weight of algae [184]. However, one study conducted on 34 algae strains revealed an average carbohydrate content of 36.3 ± 17.3 % dw which is lower than the value reported here [182]. Other values reported in Table 1.11 are indeed lower than the one obtained. Samples with ash content >10% may not be suited for HPLC quantification as some ash components may cause side reactions during hydrolysis [173]. Quantification of total carbohydrates by phenol-sulfuric acid (method used in most cases reported in Table 1.11) often fails to match HPLC quantification of even simple mixtures of sugars [173]. Although the

phenol-sulfuric acid procedure is rapid and straightforward, it is also highly dependent on the sugar used for calibration – not all carbohydrates exhibit a similar colorimetric response and some derivatives do not exhibit any response – leading to an over- or underestimation of the actual carbohydrate content [185]. Both procedures account for sugars regardless of their origin, so fractions of glycolipids and/or glycoproteins can be accounted for in both carbohydrates and lipids or proteins assays. Nonetheless, performing a colorimetric assay would be a good way of confirming the value obtained. The L-3,6-anhydro galactose (AHG) content was indirectly calculated, thus a Galactose Assay Kit could also be used. The cellulosic content obtained matches the ones reported for some Gelidiales like *Gelidiella acerosa* and *Gelidium pusillum* with 13.7% and 9.3%, respectively [186].

The ash content ($19.6 \pm 1.0\%$ dw) is also comparable to the values reported in the literature. Contents of 20.7% dw, 21.2% dw, and 26.5% dw have been reported for *Gelidium microdon* [162], *Gelidium pusillum* [161], and *Gelidium sp.* [163], respectively. 34 algae strains revealed an average of $22.9 \pm 11\%$ dw [182].

The lipid fraction was not quantified but it usually accounts for only 2.2% dw, 0.7% dw, 2.4% dw, and 1.2% dw in *Gelidium pusillum* [161], *Gelidium latijohum* [187], *Gelidium microdon* [162], and *Gelidium sp.* [163], respectively.

The mass balance can be closed considering the protein, carbohydrate, and ash content taking into account that the lipid fraction is missing.

3.2. Protein Extraction

3.2.1. Aqueous and Alkaline Extractions

As mentioned in Materials and Methods, sequential aqueous and alkaline extractions were performed (procedures I to IV). Aqueous extractions were performed at 4ºC for 16h, while alkaline extractions were carried out at RT for 1h. During aqueous extractions, the pH of the algal suspension reached values of around 7 ± 0.02, while during alkaline extractions, it reached values of 12.8 ± 0.02. Alkaline re-extractions resulted in pH values of 13.1 ± 0.04. Other extraction parameters including volume, extraction time, protein mass extracted, concentration and recovery yield are presented in Table 3.2. The total protein extracted in grams of protein per 100 grams of alga (dry weight) is presented in Figure 3.1.

Table 3.2: Initial volume, volume collected, total protein mass extracted, protein concentration in the extracts, actual protein recovery yield (considering the volume collected), maximum protein recovery yield (considering the volume used) and duration time of procedures I (one aqueous extraction and one alkaline extraction), II (one aqueous extraction, one alkaline extraction and one alkaline re-extraction), III (one aqueous extraction, one aqueous re-extraction and one alkaline extraction), and IV (one aqueous extraction, one aqueous re-extraction, one alkaline extraction and one alkeline re-extraction). Protein recovery yields are expressed in % of total protein (Protein extracted/Total protein · 100). Values are expressed as mean ± standard deviation, n = 3. Lowry method was performed in triplicate.

Procedure	***V_{used} (mL)***	***$V_{collected}$ (mL)***	***Total Mass (g)***	***Concentration (g/L)***	***Actual Protein Recovery Yield (%)***	***Maximum Protein Recovery Yield (%)***	***Time (h)***
I	300	245	$0.20 \pm 3 \cdot 10^{-2}$	$0.82 \pm 1 \cdot 10^{-4}$	14.7 ± 2.3	18.0 ± 2.3	17
II	400	343	$0.25 \pm 3 \cdot 10^{-2}$	$0.72 \pm 9 \cdot 10^{-5}$	17.9 ± 0.9	21.3 ± 0.9	18
III	500	448	$0.26 \pm 3 \cdot 10^{-3}$	$0.59 \pm 5 \cdot 10^{-6}$	19.2 ± 0.3	22.7 ± 0.1	33
IV	600	546	$0.31 \pm 1 \cdot 10^{-3}$	$0.56 \pm 5 \cdot 10^{-6}$	22.4 ± 0.1	26.0 ± 0.3	34

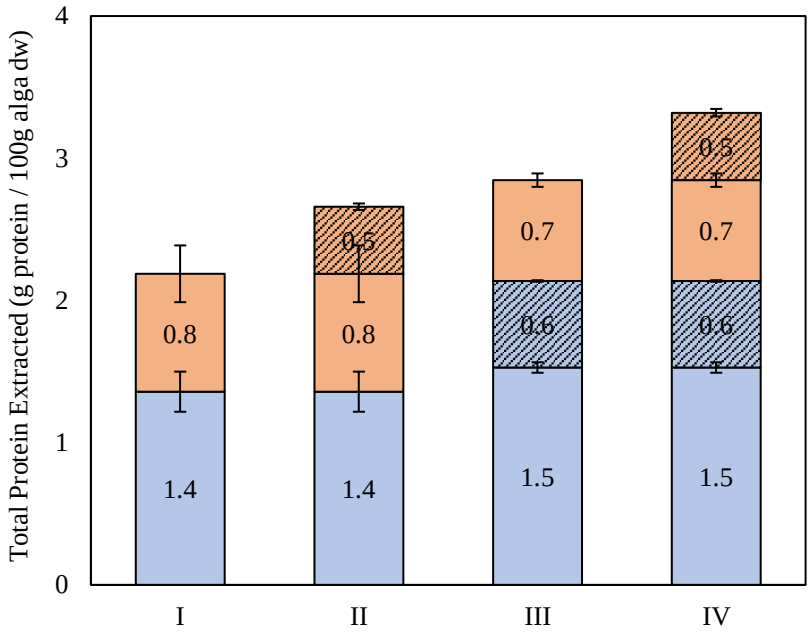

Figure 3.1: Total protein extracted in procedures I (one aqueous extraction and one alkaline extraction), II (one aqueous extraction, one alkaline extraction and one alkaline re-extraction), III (one aqueous extraction, one aqueous re-extraction and one alkaline extraction), and IV (one aqueous extraction, one aqueous re-extraction, one alkaline extraction and one alkeline re-extraction), in grams of protein per 100 grams of alga (dry weight), using the Lowry method (n=3). Values are expressed as mean ± standard deviation, n = 3.
(□ - aqueous extraction; ▨ - aqueous re-extraction; □ - alkaline extraction; ▨ - alkaline re-extraction)

Sequential aqueous and alkaline extraction led to the extraction of 1.4 ± 0.1 g/100 g alga dw (9.2 ± 1% of protein recovery yield) and 0.8 ± 0.2 g/100 g alga dw (5.6 ± 1.3% of protein recovery yield). These values are lower but comparable to those reported in the literature. For *Ulva rigida* and *Ulva rotundata*, protein recovery yields of 26.8 ± 1.3% and 36.1 ± 1.4% were reported, respectively, using an aqueous extraction with deionized water followed by an alkaline extraction with NaOH 0.1M [82]. A similar extraction procedure rendered 6.7 ± 0.2% dw of recovered protein in the red alga *Palmaria palmata* [75], which is higher but comparable to the 2.2 ± 0.2 g/100 g alga dw obtained. The authors did not report the initial protein content, so a comparison of protein recovery yield is not possible. Evidently, the arbitrary choice of protocol used for protein quantification deems comparisons almost impossible. Yields vary greatly between algae from the same species, let alone between algae from different groups.

When comparing the water-soluble and the alkaline-soluble protein fractions, the protein extracted during the aqueous extraction was significantly higher ($p<0.05$), i.e., most proteins are soluble in water (possibly protein–pigment complexes, since the extract had a red tinge). Most proteins are negatively charged under alkaline conditions due to the deprotonation of amine groups, resulting in increased protein-solvent interaction and protein solubility. Additionally, many water-insoluble polysaccharides are solubilized under basic conditions which could promote cell wall disruption and result in higher protein recovery yields [188]. A single alkaline extraction step should be studied to understand the influence of the preceding aqueous extraction. Protein denaturation due to the extremely basic pH is expected but necessary to achieve protein solubilization.

Comparing all 4 experiments, I and III ($p<0.05$), I and IV ($p<0.05$), and II and IV ($p<0.01$) are significantly different. Even though procedures I and II are not significantly different ($p>0.05$), an additional

alkaline extraction resulted in a 22% protein recovery yield increase, whereas an aqueous re-extraction (III) resulted in a 31% increase. Although significantly better than procedures I and II, procedure IV is a lot longer (34h) and results in a more diluted extract which can be more demanding in terms of downstream processing. It is important to mention that the extraction conditions used were not optimized for *Gelidium sesquipedale*. The influence of parameters like weight to volume ratio, stirring type and time, temperature, NaOH concentration and the presence of reducing agents (which dissociate proteins from polysaccharides, improving alkaline soluble protein yield) was not explored.

3.2.2. Ultrasound-Assisted Extractions

As mentioned in Materials and Methods, sequential aqueous and alkaline extractions were performed with or without ultrasound assistance (procedures V and VI). Aqueous extractions were performed at 4ºC for 16h, while alkaline extractions were carried out at RT for 1h. In experiment V, a sonication step was performed before the aqueous extraction, whereas in experiment VI a second sonication step was performed before the alkaline extraction. During aqueous extractions, the pH of the algal suspension reached values of around 7.4 ± 0.03, while during alkaline extractions, it reached values of 13 ± 0.07. Extraction parameters including volume, extraction time, protein mass extracted, concentration and recovery yield are presented in Table 3.3. The total protein extracted in grams of protein per 100 grams of alga (dry weight) is presented in Figure 3.2.

Comparing procedures V and VI with each other and with procedure I, no significant differences were observed ($p>0.05$), even though sonication was expected to induce protein release by promoting cell disruption. This means that either the sonication method was ineffective or that the grinding previously applied to the biomass reduced the alga to its minimal size, rendering this physical method useless. Similarly, an ultrasound-assisted procedure in dried and milled *P. Palmata* resulted in a protein extraction of 6.9 ± 0.1% dw, instead of 6.7 ± 0.2% dw when following sequential aqueous and alkaline extraction [75].

Table 3.3: Initial volume, volume collected, total protein mass extracted, protein concentration in the extracts, actual protein recovery yield (considering the volume collected), maximum protein recovery yield (considering the volume used) and duration time of procedures V (one ultrasound-assisted aqueous extraction and one alkaline extraction), and VI (one ultrasound-assisted aqueous extraction and one ultrasound-assisted alkaline extraction). Protein recovery yields are expressed in % of total protein (Protein extracted/Total protein · 100). Values are expressed as mean ± standard deviation, n = 3. Lowry method was performed in triplicate.

Procedure	***V_{used} (mL)***	***$V_{collected}$ (mL)***	***Total Mass (g)***	***Concentration (g/L)***	***Actual Protein Recovery Yield (%)***	***Maximum Protein Recovery Yield (%)***	***Time (h)***
V	300	249	$0.23 \pm 1 \cdot 10^{-2}$	$0.91 \pm 6 \cdot 10^{-5}$	16.6 ± 1.0	20.2 ± 0.9	17
VI	300	249	$0.22 \pm 1 \cdot 10^{-2}$	$0.90 \pm 4 \cdot 10^{-5}$	16.2 ± 0.9	19.6 ± 0.8	17

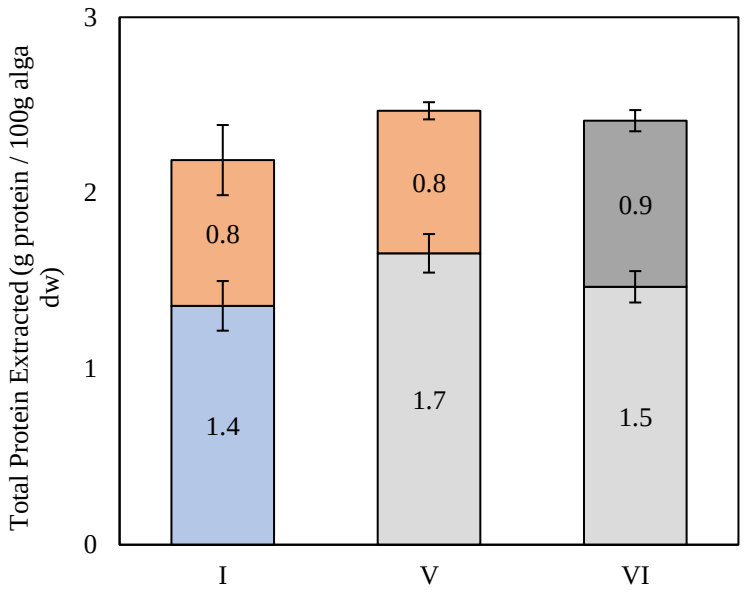

Figure 3.2: Total protein extracted in procedures V (one ultrasound-assisted aqueous extraction and one alkaline extraction), and VI (one one ultrasound-assisted aqueous extraction and one ultrasound-assisted alkaline extraction), in grams of protein per 100 grams of alga (dry weight), using the Lowry method (n=3). Values are expressed as mean ± standard deviation, n = 3.
(■- aqueous extraction; ■ - alkaline extraction; ■ - ultrasound-assisted aqueous extraction; ■ - ultrasound-assisted alkaline extraction)

3.2.3. Acid Extractions

As mentioned in Materials and Methods, acid extractions were performed in sequence with aqueous and alkaline extractions (procedures VII to IX). Procedure VII involves one aqueous extraction and one acid extraction, procedure VIII involves one aqueous extraction, one acid extraction and one acid re-extraction, and procedure IX one aqueous extraction, one acid extraction and one alkaline extraction.

Aqueous extractions were performed at 4ºC for 16h, while acidic and alkaline extractions were carried out at RT for 1h. During aqueous extractions, the pH of the algal suspension reached values of around 7.1 ± 0.04, while during acidic and alkaline extractions, it reached values of 1.6 ± 0.03 and 11.8 ± 0.09, respectively. Extraction parameters including volume, extraction time, protein mass extracted, concentration and recovery yield are presented in Table 3.4. The total protein extracted in grams of protein per 100 grams of alga (dry weight) is presented in Figure 3.3.

In procedure VII, sequential aqueous and acid extractions led to the extraction of 1.4 ± 0.02 g/100 g alga dw (9.3 ± 0.1% of protein recovery yield) and 0.4 ± 0.1 g/100 g alga dw (2.5 ± 0.5% of protein recovery yield). When comparing the water-soluble and the acid-soluble protein fractions, the protein extracted during the aqueous extraction was significantly higher ($p<0.01$). In procedure IX, when comparing the water-soluble, the acid-soluble and the alkaline-soluble protein fractions, the protein extracted during the aqueous extraction was significantly higher than the other fractions ($p<0.01$). The protein extracted under alkaline conditions was significantly higher than the one extracted under acidic ones ($p<0.01$).

When comparing experiment VII to experiment I (sequential aqueous and alkaline extractions), no significant difference was observed ($p>0.05$). However, the protein yield of one aqueous extraction followed by

two alkaline extractions (procedure II) was deemed significantly higher ($p<0.05$) than when it was followed by two acidic ones (procedure VIII).

Table 3.4: Initial volume, volume collected, total protein mass extracted, protein concentration in the extracts, actual protein recovery yield (considering the volume collected), maximum protein recovery yield (considering the volume used) and duration time of procedures VII (one aqueous extraction and one acid extraction), VIII (one aqueous extraction, one acid extraction and one acid re-extraction), and IX (one aqueous extraction, one acid extraction and one alkaline extraction). Protein recovery yields are expressed in % of total protein (Protein extracted/Total protein · 100). Values are expressed as mean ± standard deviation, n = 3. Lowry method was performed in triplicate.

Procedure	***V_{used} (mL)***	***$V_{collected}$ (mL)***	***Total Mass (g)***	***Concentration (g/L)***	***Actual Protein Recovery Yield (%)***	***Maximum Protein Recovery Yield (%)***	***Time (h)***
VII	300	251	$0.16 \pm 9 \cdot 10^{-3}$	$0.65 \pm 4 \cdot 10^{-5}$	11.8 ± 0.7	14.9 ± 0.8	17
VIII	400	349	$0.18 \pm 1 \cdot 10^{-2}$	$0.52 \pm 4 \cdot 10^{-5}$	13.1 ± 0.9	16.2 ± 1.1	18
IX	400	329	$0.25 \pm 2 \cdot 10^{-2}$	$0.75 \pm 5 \cdot 10^{-5}$	17.9 ± 1.3	22.6 ± 0.8	18

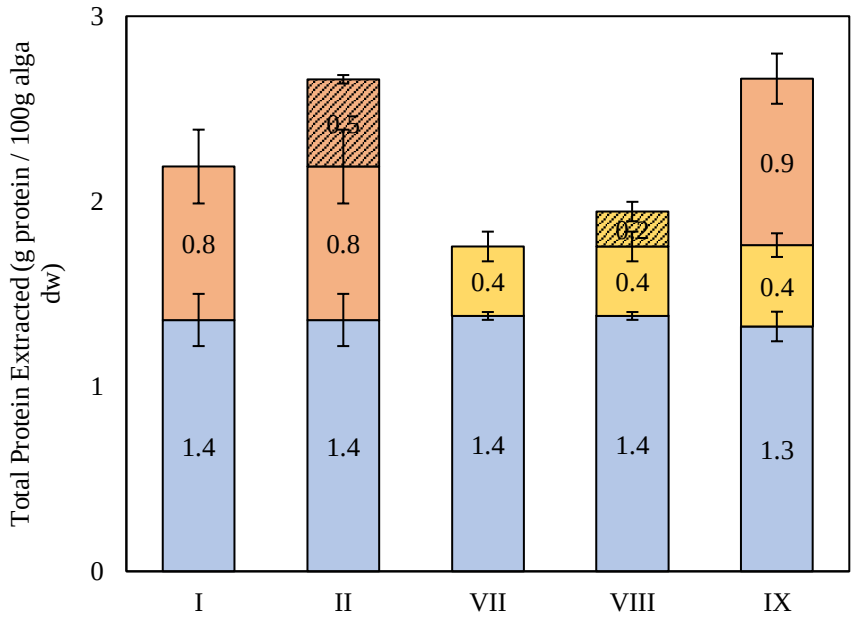

Figure 3.3: Total protein extracted in procedures I (one aqueous extraction and one alkaline extraction), II (one aqueous extraction, one alkaline extraction and one alkaline re-extraction), VII (one aqueous extraction and one acid extraction), VIII (one aqueous extraction, one acid extraction and one acid re-extraction), and IX (one aqueous extraction, one acid extraction and one alkaline extraction), in grams of protein per 100 grams of alga (dry weight), using the Lowry method (n=3). Values are expressed as mean ± standard deviation, n = 3.
(- aqueous extraction; - alkaline extraction; - alkaline re-extraction; - acid extraction; - acid re-extraction)

The use of acid prior to solubilization with an alkaline solution has been shown to promote the release of polysaccharides and proteins located in the cell wall matrix and is reportedly more effective for red and brown macroalgae [83]. This approach was tested in procedure IX to facilitate protein solubilization by subsequent alkaline extraction, however the alkaline extraction was not significantly different when compared to the one performed after an aqueous extraction (procedure I), which means protein solubilization was not facilitated like speculated. Acidic solutions have been used to obtain protein-rich pellets from *Ulva ohnoi* using HCl 0.05M at

85ºC (ulvan is extracted) [189]. Sequential alkaline and acid extraction of red and brown seaweeds, *Porphyra umbilicalis* and *S. latissima*, resulted in a protein recovery of 22.6 and 25.1%, respectively [83]. Although a very low pH is achieved, extractions with HCl concentrations as high as 0.4M have been reported. Acid extraction with HCl 0.4M at 4ºC followed by alkaline extraction with NaOH 0.4M yielded a protein recovery of 59.8% for *Ascophyllum nodosum* [140]. Once again, the influence of certain parameters should be investigated, namely acid concentration and temperature. In the case of *Gelidium sp.*, co-elution of polysaccharides and proteins is not very desirable since the agar fraction of the biomass would most likely be affected. As mentioned for the alkaline extractions, a single acid extraction should be performed to assess its potential without being preceded by an aqueous extraction step.

3.2.4. Hot Aqueous Extraction and Enzyme-Assisted Extractions

All enzyme-assisted extractions were performed at 50ºC, therefore a hot aqueous extraction at the same temperature was also performed, followed by an alkaline extraction (procedure X). During this experiment, the pH reached values of 6.3 ± 0.05 which were lower than the pH observed in previous aqueous extractions. During the alkaline extraction, the pH reached values of 12.6 ± 0.07. In procedures XI (one enzymatic-assisted aqueous extraction with Viscozyme and one alkaline extraction), XII (one enzymatic-assisted aqueous extraction with Celluclast and one alkaline extraction), XIII (one enzymatic-assisted aqueous extraction with Viscozyme and Celluclast and one alkaline extraction), XIV (one enzymatic-assisted aqueous extraction with Alcalase and one alkaline extraction), and XV (one enzymatic-assisted aqueous extraction with Celluclast, one enzymatic-assisted aqueous extraction with Alcalase, one alkaline extraction and one alkaline re-extraction), the initial pH of the first extraction method was set to values of 4.5 ± 0.02, while the initial pH in procedure XIV was set to 8 ± 0.03. In procedure XI, the pH reached values of 5.2 ± 0.2, whereas values of 5.4 ± 0.2, 4.8 ± 0.2 and 6.6 ± 0.05 were reached in procedures XII, XIII, and XIV, respectively. In procedure XV, the initial pH of the aqueous extraction solutions with Celluclast were set at 4.5 ± 0.01 and values of 5 ± 0.3 were obtained. The initial pH of the aqueous extraction solutions with Alcalase were set at 8 ± 0.01 and values of 5.6 ± 0.1 were reached. The first alkaline extractions registered pH values of 12.6 ± 0.2 in every experiment. In procedure XV, the alkaline re-extraction reached pH values of 13.1 ± 0.01. Other extraction parameters and the total protein extracted in grams of protein per 100 grams of alga (dry weight) are presented in Table 3.5 and Figure 3.4., respectively.

Table 3.5: Initial volume, volume collected, total protein mass extracted, protein concentration in the extracts, actual protein recovery yield (considering the volume collected), maximum protein recovery yield (considering the volume used) and duration time of procedures X (one hot aqueous extraction and one alkaline extraction), XI (one enzymatic-assisted aqueous extraction with Viscozyme and one alkaline extraction), XII (one enzymatic-assisted aqueous extraction with Celluclast and one alkaline extraction), XIII (one enzymatic-assisted aqueous extraction with Viscozyme and Celluclast and one alkaline extraction), XIV (one enzymatic-assisted aqueous extraction with Alcalase and one alkaline extraction), and XV (one enzymatic-assisted aqueous extraction with Celluclast, one enzymatic-assisted aqueous extraction with Alcalase, one alkaline extraction and one alkaline re-extraction). Protein recovery yields are expressed in % of total protein (Protein extracted/Total protein · 100). Values are expressed as mean ± standard deviation, n = 3 (except for XV with n = 2). Lowry method was performed in triplicate.

Procedure	***V_{used} (mL)***	***$V_{collected}$ (mL)***	***Total Mass (g)***	***Concentration (g/L)***	***Actual Protein Recovery Yield (%)***	***Maximum Protein Recovery Yield (%)***	***Time (h)***
X	300	233	$0.26 \pm 1 \cdot 10^{-2}$	$1.11 \pm 5 \cdot 10^{-5}$	18.9 ± 1.1	24.3 ± 0.9	17
XI	300	251	$0.26 \pm 4 \cdot 10^{-2}$	$1.05 \pm 1 \cdot 10^{-4}$	20.3 ± 2.2	24.3 ± 2.5	17
XII	300	247	$0.30 \pm 3 \cdot 10^{-2}$	$1.23 \pm 1 \cdot 10^{-4}$	22.2 ± 2.5	26.8 ± 2.6	17
XIII	300	239	$0.25 \pm 8 \cdot 10^{-3}$	$1.03 \pm 3 \cdot 10^{-5}$	17.9 ± 0.6	20.7 ± 1.0	17
XIV	300	245	$0.44 \pm 3 \cdot 10^{-2}$	$1.80 \pm 1 \cdot 10^{-4}$	32.2 ± 2.2	39.6 ± 2.5	17
XV	600	535	$0.62 \pm 4 \cdot 10^{-2}$	$1.17 \pm 6 \cdot 10^{-5}$	45.5 ± 2.8	49.0 ± 3.0	34

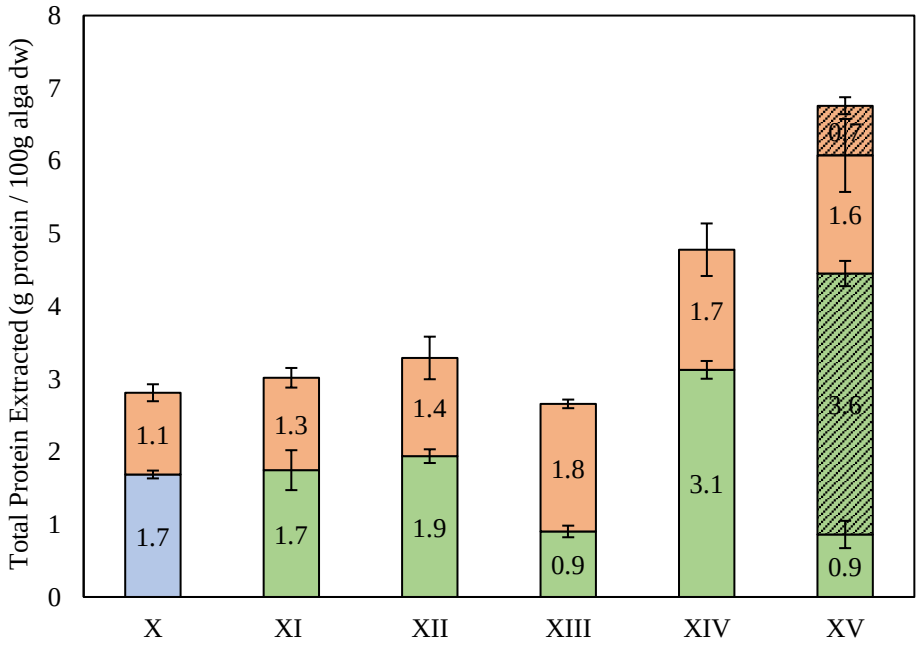

Figure 3.4: Total protein extracted in procedures X (one hot aqueous extraction and one alkaline extraction), XI (one enzymatic-assisted aqueous extraction with viscozyme and one alkaline extraction), XII (one enzymatic-assisted aqueous extraction with celluclast and one alkaline extraction), XIII (one enzymatic-assisted aqueous extraction with viscozyme and celluclast and one alkaline extraction), XIV (one enzymatic-assisted aqueous extraction with alcalase and one alkaline extraction), and XV (one enzymatic-assisted aqueous extraction with celluclast, one enzymatic-assisted aqueous extraction with alcalase, one alkaline extraction and one alkaline re-extraction), in grams of protein per 100 grams of alga (dry weight), using the Lowry method (n=3). Values are expressed as mean ± standard deviation, n = 3, except for XV with n=2.
(- aqueous extraction; - aqueous re-extraction; - alkaline extraction; - alkaline re-extraction; - enzyme-assisted extraction; - enzyme-assisted re-extraction)

In procedure X, sequential hot aqueous and alkaline extractions led to a protein extraction of 1.7 ± 0.02 g/100 g alga dw (11.4 ± 0.1% of protein recovery yield) and 1.1 ± 0.1 g/100 g alga dw (7.3 ± 0.8% of protein recovery yield), respectively, which was significantly higher when compared to procedure I ($p<0.05$). Although slightly more alkaline-soluble proteins were extracted, no significant difference was observed when compared to the alkaline extraction performed after aqueous extraction at 4ºC. 26.7% dw of extracted protein was reported for *Gelidium corneum* at T>100ºC [164], which suggests that temperature greatly affects the protein recovery yield, most likely due to the co-elution of polysaccharides.

XI, XII and XIII were not significantly different from X or from each other ($p>0.05$), and out of the three of them, only XII is significantly different from procedure I ($p<0.01$). Procedures XIV and XV reached protein values significantly higher than all the other procedures, reaching values of protein extraction of 4.8 ± 0.4 g/100 g alga dw and 6.8 ± 0.6 g/100 g dw, respectively, due to the use of Alcalase and the hydrolysis of peptide bonds.

A Celluclast treatment followed by an NAC-assisted alkaline extraction in *Eucheuma denticulatum* resulted in an increase of the extraction yield from 15.7% to 19.4%, which is comparable to the increase from 14.7% to 22.2% reported here. The use of Viscozyme fell short of expectations. For *Eucheuma denticulatum* a protein recovery yield of 48.5% using the same enzyme concentration was obtained. The combination of Celluclast and Viscozyme (procedure XIII) resulted in a lower recovery yield than when enzymes were used separately. Moreover, the Celluclast treatment in procedure XV resulted in lower protein recovery than in procedure XII when all experimental parameters were apparently maintained. In *Palmaria palmata*, the extraction yield could reach up to 90% when combining Celluclast and Alcalase at pH 8, even though 4.5 is widely reported as the optimum pH for Celluclast activity. Optimum enzymatic hydrolysis has also been reported to occur at a pH range of 5.8–6.0 [190].

Enzyme combinations, concentration, extraction time, temperature and pH should be optimized. Experiments performed at room temperature and neutral pH should be performed to assess any adverse effects on agar quality and to avoid possible gelling during the extraction process. Indeed, more viscous extracts were obtained during enzyme-assisted extractions, however the carbohydrate content of the extracts was not assessed.

To an extent, the results obtained are not comparable with the results described in the literature due to differences in alga species, the state of starting materials, extraction parameters and protein quantification methods used.

3.2.5. Summary

A summary of the protein recovery yields attained is presented in Figure 3.5. Extractions XIV and XV resulted in substantially higher yields due to the presence of Alcalase. However, they do not allow for the recovery of only intact proteins. Procedures IV and XII exhibited the highest protein recovery yields for the recovery of intact proteins (no significant differences observed). Procedure XII is faster and consumes less volume of solutions, although Celluclast is used as a consumable. Local conditions at the collection site such as light, salinity, nutrients, temperature, pollution, and water motion can considerably impact metabolite levels and bioactive composition. The biological status of the algae (e.g., life cycle, development stage and thallus structure) can also have an impact on their biochemical composition. Variability between batches is evident, but intra-batch variability could also be a factor. Therefore, the scaling up and/or and the repetition of extraction procedures is important.

It should also be noted that setting the pH of the algal suspensions was extremely difficult and time-consuming which might have negatively impacted the enzyme-assisted extractions results. The use of buffers

instead of deionized water could then be studied. It should also be noted that due to the seaweed's hygroscopic nature, some water is absorbed during extraction steps, leading to lower collected volumes when compared to the initial volume.

As mentioned, most extraction parameters must be optimized for the macroalga being used. It is also worth noting that the protein extraction yields were calculated considering a content of 14.8% dw of total protein in the biomass which was determined using a different protein quantification method than the one used in the extracts. If the quantification of total nitrogen and the use of a SNP of 4.59 does result in protein overestimation, then the actual protein extraction yields would be higher. Amino acid analysis and the sum of their concentrations would lead to more reliable results. This could be done once the screening and optimization of the extraction procedures are completed, since this quantification method uses expensive equipment and consumables. Studies focusing on protein quantification in *Gelidium* would be ideal.

The presence of proteins that are soluble in salt solutions or in 70% alcohol [63] was not investigated. The agar extraction from *Gelidium* includes a pre-treatment with a mild alkaline solution (e.g., Na_2CO_3) to remove pigments (phycoerythrin) and to macerate the seaweed. A protein extraction procedure that follows the same conditions should be performed to know how much protein is potentially lost in this step.

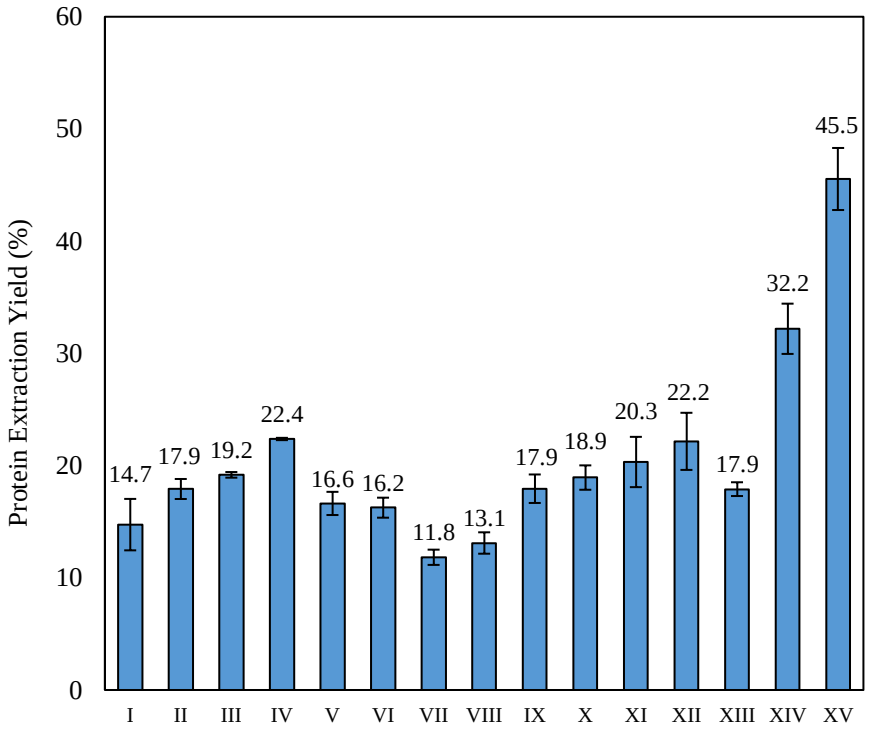

Figure 3.5: Actual protein recovery yield for all 15 extraction procedures (described in Table 2.1) expressed in % of total protein (Protein extracted/Total protein · 100). ***I****: Aqueous extraction and alkaline extraction (sequential):* ***II****: Aqueous extraction, alkaline extraction, and alkaline re-extraction (sequential);* ***III****: Aqueous extraction, aqueous re-extraction, and alkaline extraction (sequential);* ***IV****: Aqueous extraction, aqueous re-extraction, alkaline extraction, and alkaline re-extraction (sequential);* ***V****: Sonication-assisted aqueous extraction and alkaline extraction (sequential);* ***VI****: Sonication-assisted aqueous extraction and sonication-assisted alkaline extraction (sequential);* ***VII****: Aqueous extraction and acid extraction (sequential);* ***VIII****: Aqueous extraction, acid extraction, and acid re-extraction (sequential);* ***IX****: Aqueous extraction, acid extraction, and alkaline extraction (sequential);* ***X****: Hot aqueous extraction and alkaline extraction (sequential);* ***XI****: Enzyme-assisted aqueous extraction using Viscozyme and alkaline extraction (sequential);* ***XII****: Enzyme-assisted aqueous extraction using Celluclast and alkaline extraction (sequential);* ***XIII****: Enzyme-assisted aqueous extraction using Viscozyme and Celluclast and alkaline extraction (sequential);* ***XIV****: Enzyme-assisted aqueous extraction using Alcalase and alkaline extraction (sequential);* ***XV****: Enzyme-assisted aqueous extraction using Celluclast, enzyme-assisted aqueous extraction using Alcalase, and alkaline extraction (sequential). Values are expressed as mean ± standard deviation, n = 3 (except for XV with n = 2).*

3.3. Extraction Scale-up

The scale up of two procedures was deliberated to assess the feasibility of the scale up itself, and to generate enough extract volume (i.e., supernatants) for future studies (e.g., precipitation). Extraction procedures XII and XV were chosen – because one likely originates intact proteins, while the other also originates peptides due to the protease activity. It was decided to implement an alkaline re-extraction in procedure XII as a way of improving the recovery yield – XII + AR. The scale up procedures were carried out using 50 g of algal power. The pH of the aqueous extractions with Celluclast was set at a pH of 4.6 ± 0.01 and the extracts obtained had pH values of 5.3 ± 0.05. After alkaline extraction, the pH of the XII + AR extracts reached values of 12. In procedure XV, the pH of the aqueous extraction with Alcalase was set at a pH of 8 while the extracts obtained had pH values of 5.7. After alkaline extraction, the pH of the XV extracts reached values of 12.2. Both alkaline re-extractions originated extracts with a pH of 12.6. Extracts from the same procedure were pooled.

The use of Celluclast resulted in underwhelming results – 0.4 ± 0.08 g/100g alga dw and 0.3 ± 0.06 g/100g alga dw in the scale up of XV and XII + AR, respectively – which were significantly different from the result obtained in the small scale XII (1.9 ± 0.09 g/100g alga dw), suggesting that the 0.9 ± 0.2 g/100g alga dw obtained in the small scale XV was a more reproducible result than expected. This procedure step should then be repeated or substituted for an extraction step with deionized water or another enzyme (e.g., Viscozyme), or simply skipped (procedure XIV + AR), if the use of Celluclast is rendered ineffective. Visually, agitation seemed to be a bottleneck, especially during the enzyme-assisted aqueous extraction using Celluclast. Magnetic agitation was maintained using a larger stir bar. The $\frac{Diameter_{stir\ bar}}{Diameter_{vessel}}$ and the $\frac{Width_{stir\ bar}}{Diameter_{vessel}}$ ratios were kept constant – 0.43 and 0.1, respectively, however, centrifugal bottles and Erlenmeyer flasks differ in geometry and enzyme-assisted extractions are rather sensitive to agitation changes. Such differences may then result in inadequate mass transfer and reduced yields. Alkaline extractions and re-extractions yielded the same results when compared to the smaller scale procedures ($p>0.05$), whereas the Alcalase extraction resulted in a slightly lower yield ($p>0.05$).

Table 3.6: Initial volume, volume collected, total protein mass extracted, protein concentration in the extracts, actual protein recovery yield (considering the volume collected), maximum protein recovery yield (considering the volume used) and duration time of the scale up of procedures XV (one enzymatic-assisted aqueous extraction with Celluclast, one enzymatic-assisted aqueous extraction with Alcalase, one alkaline extraction and one alkaline re-extraction) and XII + AR (one enzymatic-assisted aqueous extraction with Celluclast, one alkaline extraction and one alkaline re-extraction). Protein recovery yields are expressed in % of total protein (Protein extracted/Total protein · 100). Values are expressed as mean ± standard deviation, n = 1.

Procedure scale-up	***V_{used} (L)***	***$V_{collected}$ (L)***	***Total Mass (g)***	***Concentration (g/L)***	***Actual Protein Recovery Yield (%)***	***Maximum Protein Recovery Yield (%)***	***Time (h)***
XV	2	1.7	$2.6 \pm 2 \cdot 10^{-1}$	$0.97 \pm 9 \cdot 10^{-5}$	38.5 ± 3.5	40.3 ± 3.6	34
XII + AR	3	2.7	$1.1 \pm 4 \cdot 10^{-2}$	$0.64 \pm 3 \cdot 10^{-5}$	15.8 ± 0.6	17.1 ± 0.6	18

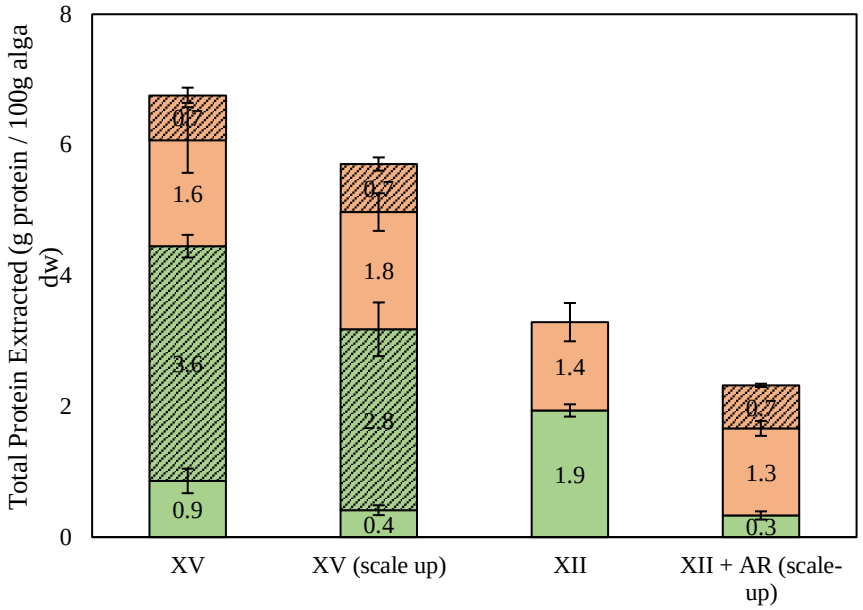

Figure 3.6: Total protein in grams of protein per 100 grams of alga (dry weight) extracted in extraction procedures XV (one enzymatic-assisted aqueous extraction with Celluclast, one enzymatic-assisted aqueous extraction with Alcalase, one alkaline extraction and one alkaline re-extraction) and XII (one enzymatic-assisted aqueous extraction with Celluclast and one alkaline extraction), and in the scale up of procedures XV and XII + AR (procedure XII with an alkaline re-extraction). Values are expressed as mean ± standard deviation, n=3 for small scale procedures, n = 1 for scale up procedures. Extraction procedures are described in Table 2.1.
(- enzyme-assisted extraction; - enzyme-assisted re-extraction; - alkaline extraction; - alkaline re-extraction).

Both procedures were performed in parallel but not in triplicate due to space limitations which lowers the confidence in the results obtained. It is worth mentioning that the scale up was performed without a proper optimization of the enzymatic process for this specific alga (as suggested in Hot Aqueous Extraction and Enzyme-Assisted Extractions).

3.4. Protein Precipitation

30 mL of the pooled extracts (i.e., supernatants) underwent precipitation with 70 to 85% ammonium sulfate saturation during 16h at 4ºC. Incubation time could be decreased as some authors suggest incubations of only 1h [83], however, overnight stirring was chosen so that maximum precipitation was ensured. After centrifugation, the supernatant's pH had values of 7.8 ± 0.08. Precipitation results are presented in Table 3.7 and Figure 3.7.

Ammonium sulfate is widely used in protein purification processes (e.g., precipitation, chromatographic gels saturation), however it is one of the interferences of the Lowry method, leading to protein overestimation [191]. To understand the extent of its interference, ammonium sulfate solutions were prepared and diluted with the same dilution factor as the supernatants collected for protein assay. When the Lowry method was performed, these solutions had similar absorbance values as distilled water, so it was assumed that, for a dilution factor of 4, no major interference occurs. However, if an overestimation of protein did occur, higher precipitation yields than the ones reported were obtained, since protein determination was carried out in the supernatant. Other reagents have been used to precipitate protein from macroalgae, such as trichloroacetic acid, urea, and organic solvents [18].

Table 3.7: Parameters of the ammonium sulfate precipitation applied to the pooled extract of the scale up of procedures XV (one enzymatic-assisted aqueous extraction with Celluclast, one enzymatic-assisted aqueous extraction with Alcalase, one alkaline extraction and one alkaline re-extraction) and XII + AR (one enzymatic-assisted aqueous extraction with Celluclast, one alkaline extraction and one alkaline re-extraction). Protein mass in the pellet was calculated indirectly using Equation 2.7. Protein mass in the supernatant was determined using the Lowry method (n=3). Values are expressed as mean ± standard deviation,and n=1 for each saturation.

Procedure (scale-up)	***Ammonium sulfate saturation (%)***	***V_{pool} (mL)***	***$(Protein\ mass)_{initial}$ (mg)***	***$V_{supernatant}$ (mL)***	***$(Protein\ mass)_{pellet}$ (mg)***
XV	70	30	45.5 ± 3.9	36	8.1 ± 0.6
	75			36.5	8.9 ± 0.7
	80			37.5	9.9 ± 1.0
	85			38	13.6 ± 1.1
XII + AR	70		32.2 ± 2	37	10 ± 0.6
	75			37	10.4 ± 1.1
	80			38	10.6 ± 0.3
	85			39.5	13 ± 0.8

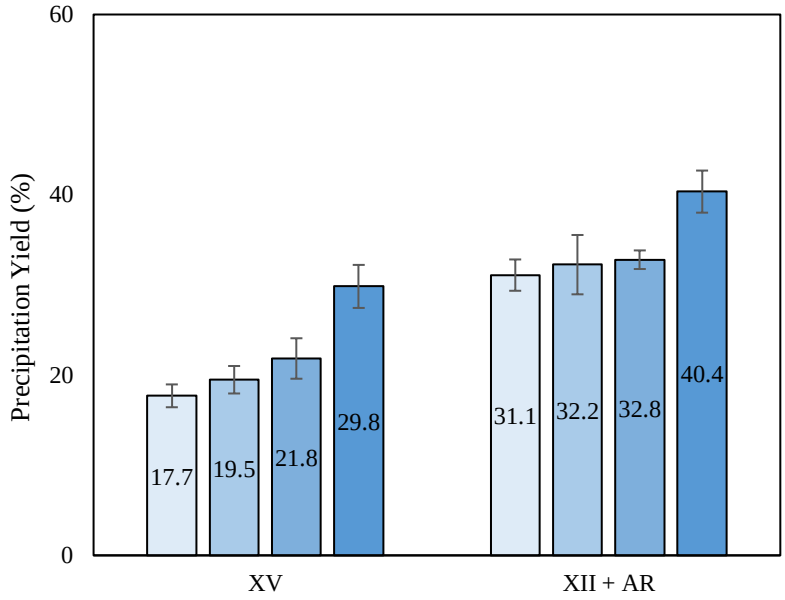

Figure 3.7: Graphic representation of the precipitation yields obtained for each ammonium sulfate saturation. Precipitation carried out for 16h at 4ºC. Protein was quantified in the respective supernatants using the Lowry method (n=3). Values are expressed as mean ± standard deviation, and n=1 for each saturation.
(□ - 70%; □ - 75%; □ - 80%; □ - 85%).

A saturation of 85% led to significantly higher results in both cases. When it comes to the XV extracts, significant differences were observed between the yields obtained with 70% and 85% ($p<0.01$), 75% and 85% ($p<0.01$), and 80% and 85% ($p<0.01$). The same results were observed for the XII + AR extracts, however the difference between the yields obtained with 80% and 85% had a p value inferior to 0.05. Precipitation with 85% of ammonium sulfate was performed again to obtain triplicates (Table 3.8). Lower precipitation yields were

obtained for the XV extract, which was expected since it suffered protease activity, hindering some protein-protein interactions during salting-out.

Table 3.8: Parameters of the ammonium sulfate (85%) precipitation applied to the pooled extract of the scale up of procedures XV (one enzymatic-assisted aqueous extraction with Celluclast, one enzymatic-assisted aqueous extraction with Alcalase, one alkaline extraction and one alkaline re-extraction) and XII + AR (one enzymatic-assisted aqueous extraction with Celluclast, one alkaline extraction and one alkaline re-extraction). Protein mass in the pellet was calculated indirectly using Equation 2.7. Values are expressed as mean ± standard deviation, n=3.

Procedure (scale-up)	***Ammonium sulfate saturation (%)***	***V_{pool} (mL)***	***$(Protein\ mass)_{initial}$ (mg)***	***$V_{supernatant}$ (mL)***	***$(Protein\ mass)_{pellet}$ (mg)***	***Precipitation Yield (%)***	***Average Precipitation Yield (%)***
XV	85	30	45.5 ± 3.9	38	13.6 ± 1.1	29.8 ± 2.4	24.6 ± 4.9
			47.6 ± 2.2	40	11.4 ± 1.4	24.0 ± 2.9	
				40	9.5 ± 1.1	20.0 ± 2.3	
XII + AR			32.2 ± 2.0	39.5	13.0 ± 0.8	40.4 ± 2.3	43.5 ± 3.2
			36.7 ± 2.3	40	15.9 ± 1.3	43.3 ± 3.4	
				39.5	17.2 ± 1.4	46.8 ± 3.9	

For the XII + AR extracts, 15.4 ± 2.2 mg of protein were obtained with the precipitation of 30 mL of extract. Considering that the precipitation of the total volume (1.7 L) leads to a pellet with 0.87 ± 0.1 g of protein, then the precipitation with 85% of ammonium sulfate results in 1.9 ± 0.3 g/100g dw, which corresponds to an overall protein recovery of 12.7 ± 1.8% (including the extraction yield). For the XV extracts, 11.5 ± 2.0 mg of protein were obtained with the precipitation of 30 mL of extract. Considering that the precipitation of the total volume (2.7 L) leads to a pellet with 1.0 ± 0.2 g of protein, then the precipitation with 85% of ammonium sulfate results in 2.2 ± 0.4 g/100g dw, which corresponds to an overall protein recovery of 15.1 ± 2.7%.

Kandasamy et al. reported the recovery of 5.7 to 6.5 % of total proteins in *E. tubulosa*, *E. compressa* and *E. linza* after sequential extraction with deionized water at 35ºC (overnight) and NaOH 1M (2h), and precipitation with 85% ammonium sulfate [192]. Kumar et al. reported a recovery of 7.8 % from *K. alvarezii* [120] and values of 7.8% to 48% were reported for *Sargassum* species [193]. The use of PEG-1450/potassium phosphate (pH 6.0) in combination with precipitation with ammonium sulfate was reported to efficiently extract R-PE from *Gelidium pusillum* (72% R-PE yield) [24].

Proteins in water obtain net positive or negative charges when adjusted to extremely acid or alkaline conditions, respectively. Alkaline solubilization followed by isoelectric protein precipitation has been applied mainly in the isolation of soy [194], whey [195], lentils [196], and microalgae [197] proteins. In *Saccharina*, the precipitation of solubilized proteins was possible below pH 4 and the highest precipitation yield of 34.5% was obtained at pH 2. This value is comparable to the precipitation yields of 24.6 – 43.5% obtained here. When combined alkaline extraction and acid precipitation was applied, 16% of the total protein was recovered [66]. When studying *U. lactuca, P. umbilicalis* and *S. latissima,* higher protein yields using the pH-shift method were obtained for the last two algae – 22.6 ± 7.3% and 25.1 ± 0.9%, respectively. Precipitation with 80% ammonium sulfate resulted in the greatest protein yield when applied to *U. lactuca* (19.6 ± 0.8%) [83].

Protein salting out concentrates the protein but does not purify it, since it is necessary to remove the salt from the protein sample meaning that further processing in the form of either dialysis, diafiltration or chromatography is required. Scaling up of the process also requires large quantities of salt.

3.5. Extract Diafiltration and Concentration

The pellets (one for each extraction procedure) that resulted from precipitation were re-dissolved in 15 mL of Tris HCl 20 mM pH 7 so they could undergo a desalting step by centrifugal ultrafiltration in diafiltration mode, with a MWCO of 3 kDa using the same buffer. Most desalting steps in the literature are, however, carried out by dialysis against deionized water at 4ºC.

A sample of the redissolved pellets was withdrawn for protein assay. Differences of 7.3 and 8 mg were observed for XV and XII + AR, respectively, when compared to the mass of protein determined in the pellet after precipitation (Table 3.9). This can be a result of analytical error and/or interferences. The distortion due to Tris was also corrected by simple blank correction, but a calibration curve with standards prepared in the same Tris buffer should have been done instead.

Table 3.9: Protein mass calculated after ammonium sulfate precipitation using Equation 2.7, and protein mass determined after re-dissolution in Tris HCl 20 mM pH 7, using the Lowry method (n=3). Values are expressed as mean ± standard deviation, n=3.

Procedure (scale-up)	***(Protein mass)$_{pellet}$ (mg)***	***(Protein mass)$_{re\text{-}dissolved}$ (mg)***
XV	13.6 ± 1.1	20.9 ± 0.3
XII + AR	17.2 ± 1.4	25.2 ± 0.7

To avoid protein precipitation during the diafiltration process, only 10 mL of the re-dissolved pellet were loaded into the centrifugal filter units and 5 mL of buffer were added. This resulted in an initial concentration of 1.39 ± 0.02 g/L and 1.68 ± 0.5 g/L for the XV and the XII + AR pellets, respectively. After diafiltration, final concentrations of 1.19 ± 0.02 g/L and 1.44 ± 0.03 g/L were determined by protein assay, respectively. Diafiltration was carried out until $n_{D,\,XV} = 4.28$ and $n_{D,\,XII+AR} = 4.16$. The protein mass decrease in the retentate (calculated using Equation 2.10), the subsequent protein mass increase in the cumulative permeate (determined using the Lowry method), and the protein retention yield are represented in Figure 3.8 and Figure 3.9. Rejection coefficients are represented in Figure 3.10.

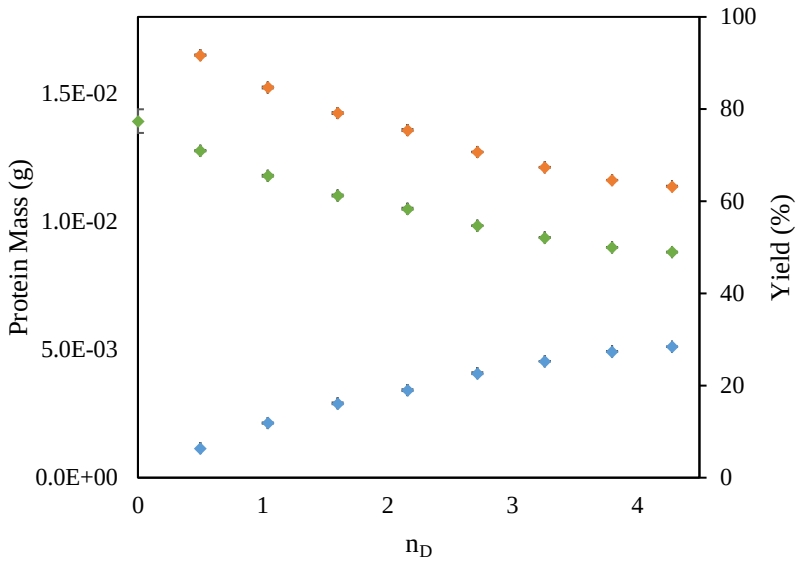

Figure 3.8: XV pellet diafiltration using an Amicon Ultra-15 centrifugal filter device (3 kDa MWCO); 3220 × g, swing-bucket rotor. Protein mass (g) in the retentate and in the permeate and protein retention yield (%) as a function of the number of diavolumes. Loading mass was 13.9 ± 0.2 mg. Average permeate flow rate of 6.1 ± 0.8 L/s. Protein mass in the permeate was determined using the Lowry method (n=3), while the protein mass in the retentate was calculated using Equation 2.10. Values are expressed as mean ± standard deviation, n=3. (♦ - permeate; ♦ - retentate; ♦ - yield).

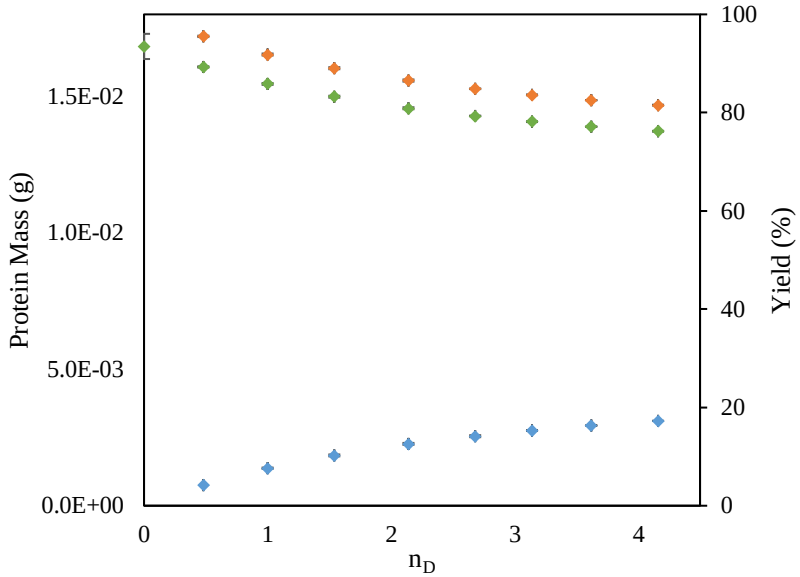

Figure 3.9: XII + AR pellet diafiltration using an Amicon Ultra-15 centrifugal filter device (3 kDa MWCO); 3220 × g, swing-bucket rotor. Protein mass (g) in the retentate and in the permeate and protein retention yield (%) as a function of the number of diavolumes. Loading mass was 16.8 ± 0.5 mg. Average permeate flow rate of 8.4 ± 1.5 L/s. Protein mass in the permeate was determined using the Lowry method (n=3), while the protein mass in the retentate was calculated using Equation 2.10. Values are expressed as mean ± standard deviation, n=3. (♦ - permeate; ♦ - retentate; ♦ - yield).

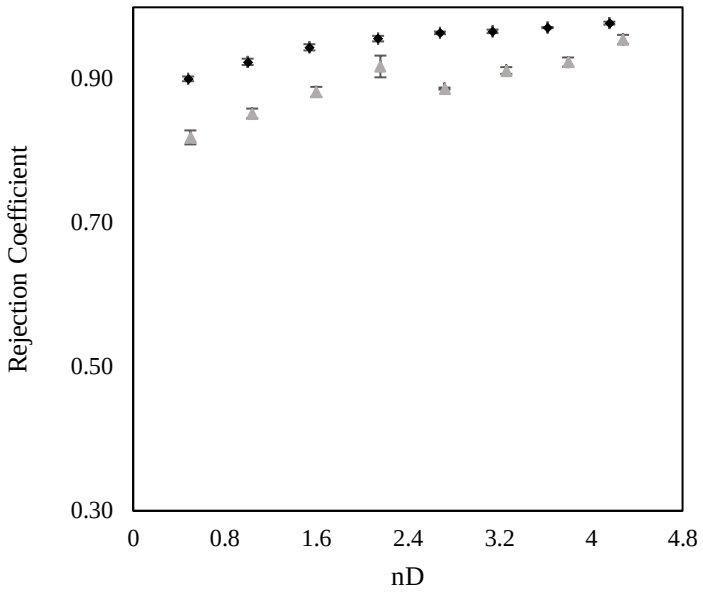

Figure 3.10: Rejection coefficients as a function of the number of diavolumes during the diafiltration of the XV (▲) and XII + AR (♦) pellets using an Amicon Ultra-15 centrifugal filter device (3 kDa MWCO); 3220 × g, swing-bucket rotor. Values are expressed as mean ± standard deviation, n=3.

Table 3.10: Diafiltration step using an Amicon Ultra-15 centrifugal filter device (3 kDa MWCO) - initial and final protein mass, initial and final retentate volume, cumulative protein mass in the permeate, protein retention yield and average rejection coefficient.

Procedure (scale-up)	***(Protein mass)*** $_{initial}$ ***(mg)***	***$V_{initial}$ (mL)***	***(Protein mass)*** $_{final}$ ***(mg)***	***V_{final} (mL)***	***(Protein mass)*** $_{cumulative\ permeate}$ ***(mg)***	***Retention Yield (%)***	***Average Rejection Coefficient (σ)***
XV	13.9 ± 0.2	10	10.7 ± 0.2	9	5.1 ± 0.03	76.7 ± 1.4	0.89 ± 0.01
XII + AR	16.8 ± 0.5		13.5 ± 0.3	9.4	3.1 ± 0.02	81.1 ± 1.8	0.95 ± 0.003

For XV, there was a generation of 1.9 mg of protein (13.4% of the initial protein mass). For XII + AR, there was a loss of 0.2 mg of protein (1.18% of the initial protein mass). The filter unit used for XV was re-used several times before this experiment, whereas the diafiltration of XII + AR was carried out using a new one. This and the fact that XII + AR likely has intact proteins in its composition could explain the higher yield and higher protein rejection coefficient. After each centrifugation, a certain volume of permeate was collected (~ 5 mL) and the same volume of buffer was added to the retentate. The successive volume measurements using graduated cylinders could lead to the propagation of errors. The ammonium sulfate concentration in the permeates also varies, which could interfere with the Lowry method differently.

A concentration step was performed with VCFs of 1.42 and 1.33 for XV and XII + AR, respectively (Table 3.11). The recovery yields and rejection coefficients were equal for both extraction protocols, suggesting that the protein content in each pre-purified extract had a similar nominal molecular weight. The concentration step was necessary for the bioaccessibility tests that will be performed in the future.

Table 3.11: Concentration step using an Amicon Ultra-15 centrifugal filter device (3 kDa MWCO) - initial and final protein concnetration, initial and final retentate volume, cumulative protein mass in the permeate, protein retention yield and average rejection coefficient.

Procedure (scale-up)	***[Protein] $_{initial}$ (g/L)***	***$V_{initial}$ (mL)***	***[Protein] $_{final}$ (g/L)***	***V_{final} (mL)***	***(Protein mass) $_{permeate}$ (mg)***	***Retention Yield (%)***	***Rejection Coefficient (σ)***
XV	1.26 ± 0.08	8.5	1.83 ± 0.1	6	0.29 ± 0.04	97.3 ± 0.3	0.93 ± 0.01
XII + AR	1.77 ± 0.09	8.8	2.17 ± 0.1	6.6	0.27 ± 0.01	97.4 ± 0.08	0.93 ± 0.002

Equation 2.10 was used to perform the mass balances of the processes using the data presented in Table 3.11. For XV, there was a generation of 0.54 mg of protein (5.01% of the initial protein mass). For XII + AR, there was a loss of 1.04 mg of protein (6.64% of the initial protein mass).

3.6. Overall Process

At the end of the concentration step, the 30 mL of combined XV extracts resulted in 10.97 ± 1.5 mg of protein (0.37 g/L). Assuming the 2.7 L of extract underwent the same purification strategies and produced the same results, a total of 0.99 g could be obtained, which corresponds to 2.14 g/100 g alga dw or an overall protein recovery yield of 14.4%. Following the same line of thought for the XII + AR extracts, the 30 mL result in 14.31 ± 0.7 mg of protein (0.48 g/L). Assuming the 1.7 L of extract underwent the same purification strategies, a total of 0.81 g could be obtained, which correspond to 1.76 g/100 g alga dw or an overall protein recovery yield of 11.8% (Figure 3.11).

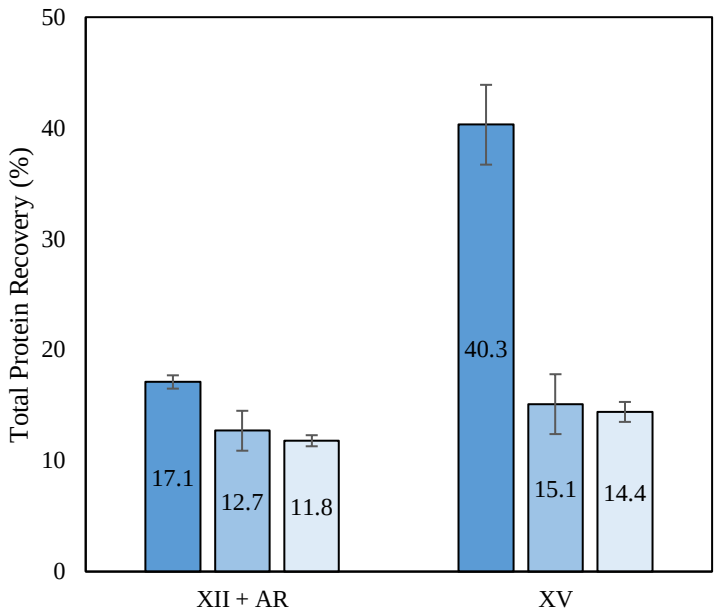

Figure 3.11: Cumulative total protein recovery (Protein extracted/Total protein · 100) after each process step (extraction, precipitation with 85% ammonium sulfate, and centrifugal centrifugation in diafiltration and concentration mode), for extraction procedures XV (one enzymatic-assisted aqueous extraction with Celluclast, one enzymatic-assisted aqueous extraction with Alcalase, one alkaline extraction and one alkaline re-extraction) and XII + AR (one enzymatic-assisted aqueous extraction with Celluclast, one alkaline extraction and one alkaline re-extraction).
(■ - protein extraction; ■ - protein extraction + ammonium sulfate precipitation; □ - protein extraction + ammonium sulfate precipitation + diafiltration and concentration).

3.7. Carbohydrate Concentration

As mentioned before, the co-elution of carbohydrates during the extraction procedures can happen. Although protein extractability is favored and the extracts produced might have interesting biological activity, the preservation of the agar fraction is crucial when it comes to the processing of *Gelidium* species. To quantify the loss of carbohydrates during the protein extraction procedures, the solid residues obtained after the scale up extractions were oven dried and underwent carbohydrate quantification. The results in Figure 3.12 are expressed in grams of carbohydrate per 100 grams of initial algal biomass (dry weight). The conversion from grams of carbohydrate per 100 grams of residue (dry weight) to grams of carbohydrate per 100 grams of initial algal biomass (dry weight) was carried out using the weight ratio between the two (0.64 g residue dw/g of initial algal biomass dw and 0.59 g residue dw/g of initial algal biomass dw for extractions XII + AR and XV, respectively). However, the ratio used does not fully translate the real weight ratio since some biomass was lost during transfers (for example, from and to centrifugal bottles and to the glass dishes where they were dried). That being said, the percentage of carbohydrates obtained in the residue was underestimated.

The residue after extraction XV (Residue XV) had a concentration of cellulose and agar of 9.7 ± 0.9 g/100 g dw and 63.6 ± 3 g/100 g dw, respectively, which correspond to concentrations of 5.8 ± 0.5 g/100 g initial algal biomass dw and 37.7 ± 1.8 g/100 g initial algal biomass dw. The residue after extraction XII + AR (Residue XII + AR) had a concentration of cellulose and agar of 11.3 ± 1.8 g/100 g dw and 58.9 ± 2.7 g/100 g dw, respectively, which correspond to concentrations of 7.3 ± 1.2 g/100 g initial algal biomass dw and 37.9 ± 1.7 g/100g initial algal biomass dw.

Regarding the cellulose fraction, only Residue XV showed a significant difference when compared to the initial content in the crude biomass ($p<0.05$). No significant difference was observed between cellulose content in the two types of residues. The agar content differed significantly between the two residues and the initial agar content ($p<0.01$), however no difference was reported between each other. The same correlation was observed when comparing the total carbohydrate fraction. One can conclude that the extraction step using Alcalase does not seem to influence carbohydrate extraction, although it is carried out at 50ºC. The use of a pH = 8 could explain this since agar extraction is usually performed at pH values near 6 [168]. Note that only the solid residues obtained after the scale up procedures were analyzed, due to the limitations of the method when applied to liquid samples. Extractions performed at smaller scales, which show higher protein recovery yields, must be analyzed to understand the extent of the correlation between carbohydrate loss and protein extractability here. Even with a decrease of ~30% of total carbohydrate content, the concentration of agar found in the residues still matches values reported for unprocessed *Gelidium sesquipedale* (e.g., 40% [168]). As mentioned in Biomass Characterization, a colorimetric assay for determination of total carbohydrates should be performed to confirm the results obtained.

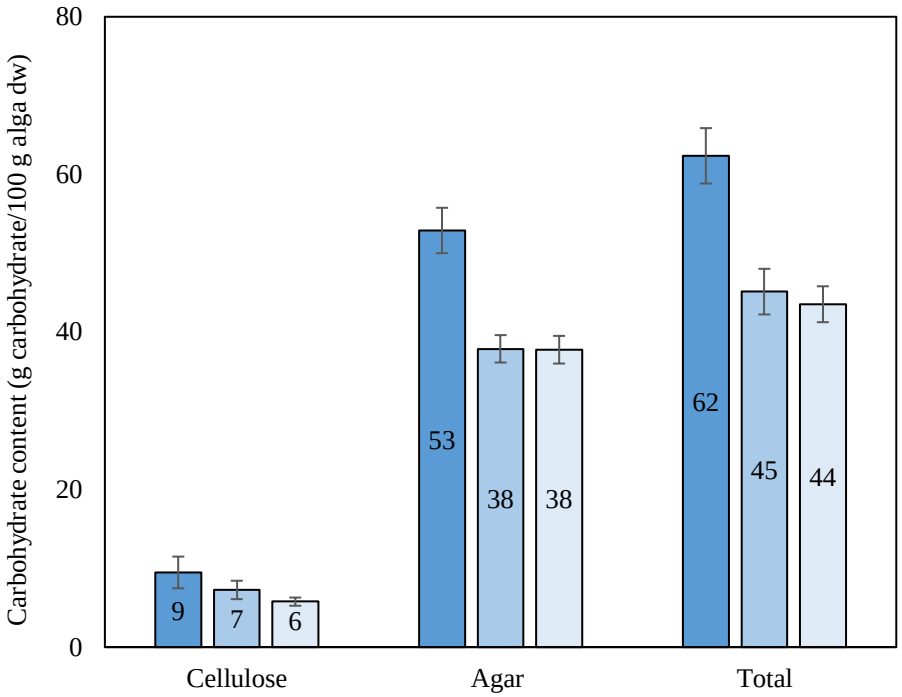

Figure 3.12: Cellulose, agar and total carbohydrate content, in grams of carbohydrate per 100 grams of initial algal biomass (dry weight), in the crude algal biomass, and in the solid residues that resulted from the scale up of protein extraction procedures XV (one enzymatic-assisted aqueous extraction with Celluclast, one enzymatic-assisted aqueous extraction with Alcalase, one alkaline extraction and one alkaline re-extraction) and XII + AR (one enzymatic-assisted aqueous extraction with Celluclast, one alkaline extraction and alkaline re-extraction). Values are expressed as mean ± standard deviation, n=3. Quantification carried out by saccharification followed by HPLC.
(■ - crude algal biomass; ■ - algal residues after protein extraction following procedure XII + AR; □ - algal residues after protein extraction following procedure XV).

4. Conclusions and Future Prospects

The present work successfully demonstrated that it is possible to extract protein from *Gelidium sesquipedale*, by applying multi-extraction steps. The conventional procedure of sequential aqueous extraction (deionized water, 16h, 4ºC) and alkaline extraction (0.1M NaOH, 1h, RT) yielded a protein recovery of 14.7 ± 2.3% which was lower but comparable to the results obtained for other macroalgae, like *Palmaria Palmata, Ulva rigida* and *Ulva rotundata* [75] [82]. Performing aqueous or alkaline re-extractions (or both) led to a slight increase in protein recovery, however the simultaneous increase in extraction time and volume makes them less attractive. The implementation of sonication step(s) before aqueous or alkaline extraction (or both) did not result in any significant increase in protein recovery, meaning the conditions applied were either too weak thus more aggressive ones should be applied, or that the drying and grinding of the seaweed already resulted in enough mechanical cell wall damage to the cells. Acid extractions after aqueous extraction and the use of acid prior to alkaline extraction did not significantly impact the protein recovery yield. An aqueous extraction with Celluclast (0.2% g_{enzyme}/g_{alga}, pH 4.5, 50ºC) followed by an alkaline extraction yielded a protein recovery of 22.2 ± 2.5%, whereas an aqueous extraction with Alcalase (0.2% g_{enzyme}/g_{alga}, pH 8, 50ºC) followed by an alkaline extraction resulted in a 32.2 ± 2.2% yield. By implementing both enzyme-assisted extractions sequentially and following them by two sequential alkaline extractions, a protein recovery of 45.5 ± 2.8% was achieved. Celluclast did not show reproducible results, suggesting that optimization is necessary and that other enzymes could be studied. Future work includes the optimization of extraction parameters for *Gelidium sesquipedale*, namely extraction time, temperature, concentration of reagents and/or enzymes, and speed/type of agitation. Implementing higher enzyme concentrations could improve extraction yields but that could compromise the process's feasibility when considering higher process scales. Procedures with Alcalase produced better results but its protease activity leads to the production of peptides, meaning it cannot be applied when the recovery of intact proteins is intended.

The scaling up of two procedures (aqueous extraction with Celluclast followed by two alkaline extractions; and aqueous extraction with Celluclast followed by aqueous extraction with Alcalase and two alkaline extractions) led to recovery yields of 15.8 ± 0.6% and 38.5 ± 3.5%, respectively. These are expected to be higher once process optimization is achieved. Scaling up resulted in extract volumes of 1.7 L and 2.7 L, respectively. The solid residues showed that ~30% of the carbohydrate fraction was lost during extraction, although agar levels were still high (~40% dw). The question is if or how detrimental the protein extraction procedures are to the extraction and quality of the remaining agar fraction.

After extraction, precipitation with 85% ammonium sulfate saturation resulted in a higher precipitation yield when compared to salt concentrations of 70, 75 and 80%. Using an 85% ammonium sulfate after extraction resulted in a total protein recovery of 12.7 ± 1.8% and 15.1 ± 2.7% for the two scaled up procedures (aqueous extraction with Celluclast + two alkaline extractions; and aqueous extraction with Celluclast followed by aqueous extraction with Alcalase and two alkaline extractions), respectively. These values were comparable to precipitation yields reported for different types of algae in the literature. Future work should include assessing precipitation using pH-shift methods since no desalting step would be necessary.

Desalting by centrifugal ultrafiltration in diafiltration mode (3 kDa MWCO) followed by a concentration step resulted in an overall protein recovery of 11.8% and 14.4%, respectively. Unprocessed protein extracts (30

mL) with initial concentrations of $0.64 \pm 3 \cdot 10^{-5}$ g/L and $0.97 \pm 9 \cdot 10^{-5}$ g/L resulted in a product of ~6 mL with concentrations 2.17 ± 0.1 and 1.83 ± 0.1 g/L, respectively.

Assuming an average productivity for seaweed of 25 $t \cdot ha^{-1}$ [2] and a moisture content of 82% in fresh harvested *Gelidium*, 4.5 $t\ dw \cdot ha^{-1}$ are harvested which corresponds to 668 kg of protein $\cdot\ ha^{-1}$ that could be produced yearly. To produce enough protein to compete with protein-rich foods like soy (400 kg of edible protein per ha [198]), an extraction procedure of ~60% would be needed. This is, however, a very rough estimate. The protein content in harvested algae is not constant throughout the year and over different locations and water depths. Although the recovery yields obtained can still be improved, *Gelidium sesquipedale* could be considered a possible future protein resource and its recovery could contribute towards a "no waste" agar extraction industrial process. Following this line of thought, an evaluation of the protein loss that might happen during the alkaline pre-treatment prior to agar extraction is of great interest.

Amino acid profiling, bioaccessibility and biological activity tests of both the purified and the unpurified extracts must be performed. From a biorefinery perspective, efforts should be made not only to improve protein extraction yields but also to decrease protein loss during purification steps.

To conclude, and although not all the objectives were met, the outcome of the present work presents important insights towards the valorization of *Gelidium sesquipedale*, a still poorly explored macroalga when considering protein extraction.

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Appendix A – Calibration Curves

A.1. Protein Quantification

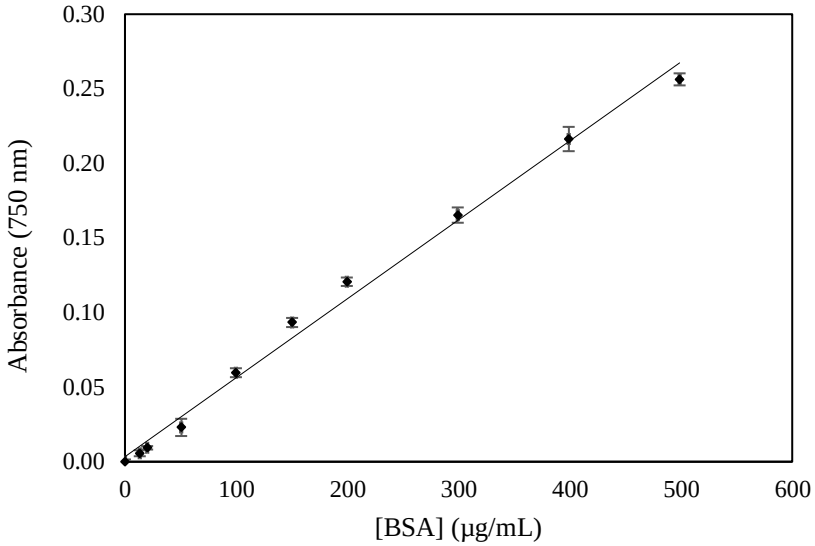

Figure A.1: Calibration curve for protein quantification using Lowry's method prepared with BSA for a working range of 0–499 µg/mL. Standards were analysed in triplicate.
$Abs_{750\,nm} = 5.29 \cdot 10^{-4} \cdot [Protein]\ (\mu g/mL) + 3.38 \cdot 10^{-3}$*, with a correlation factor of* 0.994

A.2. Monosaccharides Quantification

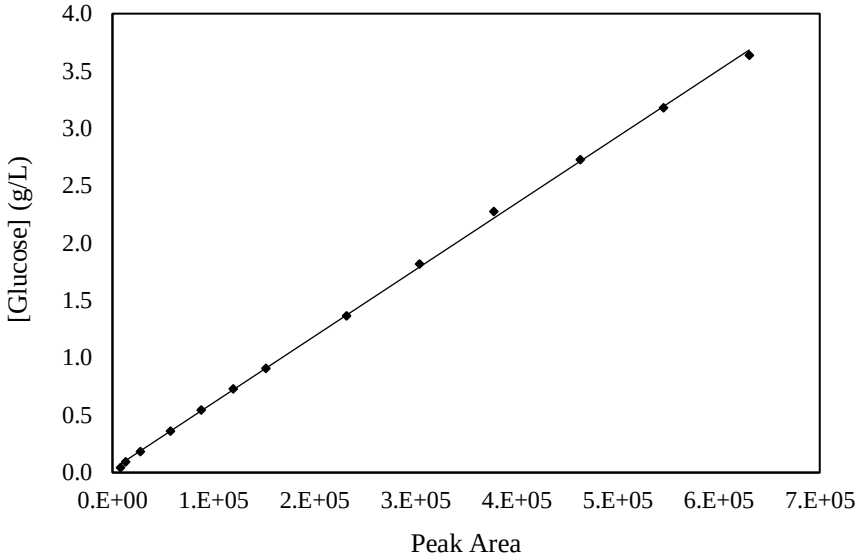

Figure A.2: Calibration curve for glucose quantification using High-Performance Liquid Chromatography prepared for a working range of 0–3.64 g/L.
$[Glucose]\ (g/L) = 5.80 \cdot 10^{-6} \cdot Peak\ Area + 2.88 \cdot 10^{-2}$*, with a correlation factor of* 0.999.

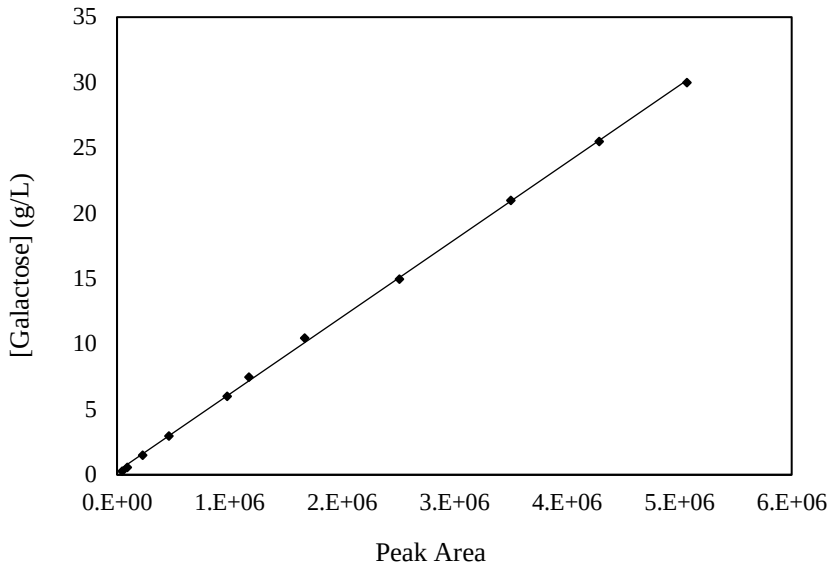

Figure A.3: Calibration curve for galactose quantification using High-Performance Liquid Chromatography prepared for a working range of 0–30 g/L.
$[Galactose]\ (g/L) = 5.90 \cdot 10^{-6} \cdot Peak\ Area + 2.56 \cdot 10^{-1}$*, with a correlation factor of* 0.999.