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

Thesis to obtain the Master Science degree in Biological Engineering

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EXTENDED ABSTRACT

Info

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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.

1. INTRODUCTION

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. Products can have direct market applications, or they can be used as raw biomass for further manufacturing operations in a cascading approach. The concept of seaweed biorefinery can contribute to 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 ($t \cdot ha^{-1} \cdot year^{-1}$), 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.

Proteins of marine origin have been the focus of several studies due to their bioactive potential. The protein content of algae 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) [3][4]. 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) [5].

The protein content of macroalgae also 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 [3]. Protein content in macroalgae includes phycobiliproteins (water-soluble pigments, ~50% of total proteins in red algae), glycoproteins and lectins, and enzymes. Although the structure and biological properties of algal proteins are still relatively poorly documented, the amino acid composition is not. Most species contain all essential amino acids (EAA) that may represent about 50% of total amino acids [6].

The successful extraction of proteins highly depends on their accessibility, being the complex nature of algal cell walls its main challenge. Their cell wall is composed of a highly integrated network of biopolymers, mainly polysaccharides, which interact with water and metal cations, amongst other molecules [7]. It can be divided into three main domains: the fibrillar wall (most inert and resistant cell wall component, with cellulose being the most significant element), the amorphous matrix (with carboxylic and/or sulfated polysaccharides, like sulfated galactans), and the glycoprotein domain [8]. 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 [9]. The presence of polysaccharide-bound cell wall mucilage including anionic or neutral polysaccharides, and polyphenols reduces protein extractability and requires additional steps. Polysaccharides induce strong electrostatic interactions [10], whereas polyphenols may form reversible hydrogen bonds with proteins or oxidize. Oxidized phenolic compounds can react with amino acids and form insoluble complexes [11]. 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 [6].

Algal proteins are conventionally extracted by means of aqueous, acidic, and alkaline methods, followed by fractionation and enrichment techniques such as centrifugation, ultrafiltration, precipitation and/or chromatography [12]. Physical methods, such as osmotic shock, freeze/thawing or grinding, can enhance the extraction in some seaweeds.

Enzyme-Assisted Extraction (EAE) is often the preferable method to extract proteins/hydrolysates from seaweed [13]. Polysaccharidases can be applied as a cell disruption treatment prior to protein extraction to increase protein recovery yields. Other strategies include ultrasound-assisted, pulsed electric field, microwave-assisted, pressurized liquid, supercritical fluid, and switchable or smart solvents assisted extractions [6].

Single or combined purification methods that include chromatography, membrane technologies, and precipitation (e.g., isoelectric, salt) are used to isolate and concentrate seaweed proteins [6]. Purification of extracted proteins from poorly explored species represents a challenge because of their unknown physicochemical properties.

Gelidium sp., is a genus of thalloid red algae which are important agarophytes. Natural populations of *Gelidium* are exploited worldwide for the extraction of technical agars (e.g., bacteriological agar and agarose). *Gelidium* agar only represents about 1.6% of the world production [14], however its natural high gelling strength and low gelling temperatures make it attractive. Protein and carbohydrate contents of some *Gelidium* species are presented in Table 1 and Table 2.

Table 1: Protein content in some Gelidium species.

Species	*Protein Content*	*Quantitative Method*	*Ref.*
Gelidium amansii	18.5% dw	Semi-micro Kjeldahl method [15].	[15]
	10.5 ± 0.1% dw	Kjeldahl method (conversion factor of 6.25).	[16]
Gelidium pusillum	11.3 ± 1.0% dw	Kjeldahl method (conversion factor of 6.25).	[17]
Gelidium microdon	23.4 ± 0.7% dw	Kjeldahl method (conversion factor of 6.25).	[18]
Gelidium sp.	13.2 ± 1.1% dw	Kjeldahl method (conversion factor of 6.25).	[19]
Gelidium corneum	16.5 ± 0.2 % dw	Kjeldahl method (conversion factor not specified).	[20]
	9.6% dw		
	13.4% dw		

Table 2: Carbohydrate content in some Gelidium species.

Species	*Carbohydrate Content*	*Quantitative Method*	*Ref.*
Gelidium amansii	75.2% dw (58.6% of agar)	HPLC after saccharification.	[15]
	71.4 ± 0.1% dw	Weight difference using crude protein, lipid, fiber, moisture, and ash content data.	[16]
Gelidium pusillum	40.6 ± 2.2% dw		[17]
Gelidium microdon	17.6 ± 0.3% dw	Phenol-sulphuric acid colorimetric method [18].	[18]
Gelidium sp.	53.7 ± 1.2% dw	Reductive hydrolysis [19]; gas chromatography.	[19]
Gelidium sesquipedale	Agar content of ~40% dw	Phenol-sulphuric acid colorimetric method [21].	[21]

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. The dried alga was milled to obtain a fine powder with an average granulometry of 0.25mm.

2.2. Chemicals and solutions preparation

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 [22] 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 [22].

2.3.2. Total Carbohydrate Content

Total carbohydrates were determined following the NREL's "Determination of Total Carbohydrates in Algal Biomass" analytical procedure [23], applied to 0.5 g samples. 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. 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 2.1 and Equation 2.2, 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 [24].

$$Cellulose + starch\ (\%) = \frac{c_{glucose} \cdot \frac{162}{180}}{c_{biomass} \cdot ODW} \cdot 100 \qquad \textit{Equation 2.1}$$

$$Agar\ (\%) = \frac{(c_{galactose} + 1.27 \cdot c_{galactose}) \cdot \frac{162}{180}}{c_{biomass} \cdot ODW} \cdot 100 \qquad \textit{Equation 2.2}$$

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 according to the Dumas method [174], using a nitrogen-to-protein conversion factor of 4.59 [25]. Samples of 0.1 g were used (n=3).

2.4. Protein Extraction

A list of the extraction procedures is presented in Table 4. Each extraction sequence started with 10 g of algal powder. The supernatants collected for protein assay were stored at -20°C.

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 4°C. 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 4°C. The supernatant was collected for protein assay. Hot aqueous extraction was performed identically but at 50°C 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 4°C. 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 4°C. 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 15°C 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. All procedures were carried out with a concentration of 0.2 % g_{enz}/g_{alga}. Extractions using Celluclast (Novozymes) and Viscozyme (Merck, Germany) were carried out at pH 4, whereas extractions with Alcalase (Merck, Germany) were carried out at pH 8. 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 50°C. 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 4°C. The supernatant was collected for protein assay.

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 40°C 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 extractions, 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% or 85%) was slowly added while stirring. Once the total mass of ammonium sulfate was added, the tubes were kept at 4°C 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 -20°C. 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 2.3, and the precipitation yield was determined using Equation 2.4. Precipitation with 85% ammonium sulfate was performed in triplicate.

$$Protein\ Mass_{pellet}(g) = [Protein]_{initial} \cdot V_{initial} - [Protein]_{supernatant} \cdot V_{supernatant} \quad \text{Equation 2.3}$$

$$Precipitation\ Yield\ (\%) = \frac{Protein\ Mass_{pellet}}{[Protein]_{initial} \cdot V_{initial}} \cdot 100 \quad \text{Equation 2.4}$$

The mass of ammonium sulfate necessary to attain the target saturations was calculated using Equation 2.5, 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.

$$Mass\ of\ (NH_4)_2SO_4\ (g) = \frac{G_{sat} \cdot (S_2 - S_1)}{1 - P \cdot S_2} \cdot V_{initial} \quad \text{Equation 2.5}$$

G_{sat} was determined considering the molarity of a saturated solution at 4°C (3.93 M [25]) and the MW of ammonium sulfate (132.14 g/mol). At 4°C the specific volume was estimated to be 0.53 mL/g [25].

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 cm2, 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 times were 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 2.6 is the mass balance equation. The protein mass in each retentate was calculated by applying Equation 2.6 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 2.6, Equation 2.7, and Equation 2.8 Equation 2.9, respectively.

$$(Protein\ Mass)_{load} = (Protein\ Mass)_{retentate} + (Cumulative\ Protein\ Mass)_{permeate} \quad \text{Equation 2.6}$$

$$Yield\ (\%) = \frac{(Protein\ Mass)_{retentate}}{(Protein\ Mass)_{load}} \cdot 100 \quad \text{Equation 2.7}$$

$$n_D = \frac{(Cumulative\ Volume)_{permeate}}{Volume_{load}} \quad \text{Equation 2.8}$$

$$\sigma = \frac{[Protein]_{permeate}}{[Protein]_{retentate}} \quad \text{Equation 2.9}$$

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 2.6, Equation 2.7, and Equation 2.8, respectively. The volumetric concentration factor (VCF) was calculated using Equation 2.10.

$$VCF = \frac{Volume_{retentate}}{Volume_{load}} \cdot 100 \quad \text{Equation 2.10}$$

2.7. Analytical Methods

2.7.1. Protein Quantification

The Lowry method was used to determine protein concentration in the extracts as described by Walker [26]. 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). Equation 2.11 and Equation 2.12 were used to calculate the protein extracted and the protein extraction yield, respectively.

$$[Protein](g/100g\ dw) = \frac{[Protein]_{supernatant}(g/L) \cdot V_{supernatant}(L) \cdot dilution\ factor}{ODW\ (g)} \cdot 100 \quad \text{Equation 2.11}$$

$$Protein\ Extraction\ Yield\ (\%) = \frac{[Protein]_{supernatant}(g/L) \cdot V_{supernatant}(L) \cdot dilution\ factor}{ODW\ (g) \cdot \frac{Total\ Protein\ (\%)}{100}} 100 \quad \text{Equation 2.12}$$

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 °C (column heater for large columns connected externally to the HPLC system, Croco-CIL 100-040-220 P, 40cm × 8cm × 8 cm, 30–99°C).

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.

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. The high content in total solids and, subsequently, the low moisture content is coherent with the drying treatment applied to the alga.

Table 3: 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, 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. 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*) over a range of different growth conditions [27], thus the actual protein content will vary. A study conducted on 34 algae strains revealed an average protein content of 18.8 ± 7% dw which is also consistent with the value obtained [27]. 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 [28]. 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 Introduction).

The total carbohydrate content (62.4 ± 3.5% dw) is also in agreement with some values reported for *Gelidium* in the literature (Table 2). 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 [29]. 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 [27]. Other values reported in Table 2 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 [23]. Quantification of total carbohydrates by phenol-sulfuric acid (method used in most cases reported in Table 2) often fails to match HPLC quantification of even simple mixtures of sugars [23]. Both procedures account for sugars regardless of their origin, so fractions of glycolipids and/or glycoproteins can be accounted for in both carbohydrate and lipid or protein 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 [30].

The ash content (19.6 ± 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* [18], *Gelidium pusillum* [17], and *Gelidium sp.* [19], respectively. 34 algae strains revealed an average of 22.9 ± 11% dw [27].

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* [31], Gelidium *latijohum* [31], *Gelidium microdon* [18], and Gelidium sp. [19], 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

Protein extraction results are presented in Table 5 and Figure 1.

3.2.1. Aqueous and Alkaline Extractions

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 [32]. A similar extraction procedure rendered 6.7 ± 0.2% dw of recovered protein in the red alga *Palmaria palmata* [33], which is higher but comparable to the 2.2 ± 0.2 g/100 g 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 [34]. 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

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 [33].

3.2.3. Acid Extractions

In procedure VII, the 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).

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 [35]. This approach was tested in procedure IX to facilitate protein solubilization, but the results obtained in the alkaline extraction performed after an acid extraction were not significantly different when compared 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 85°C (ulvan is extracted) [36]. 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 [35]. 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 4°C followed by alkaline extraction with NaOH 0.4M yielded a protein recovery of 59.8% for *Ascophyllum nodosum* [12]. Once again, the influence of certain parameters should be investigated, namely acid concentration and temperature.

3.2.4. Hot Aqueous Extraction and Enzyme-Assisted Extractions

In procedure X, the 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 4°C. 26.7% dw of extracted protein was reported for *Gelidium corneum* at T>100°C [20], 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 alga 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 [37].

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

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 proteins without an Alcalase step (no significant differences observed). Procedure XII is faster and consumes less volume of solutions, although Celluclast is used as a consumable

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. 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.

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 for the extracts. If the quantification of total nitrogen and the use of a Specific Seaweed-Nitrogen-To-Protein 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.

The presence of proteins that are soluble in salt solutions or in 70% alcohol [46] 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 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.

Table 4: List of protein extraction procedures.

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)

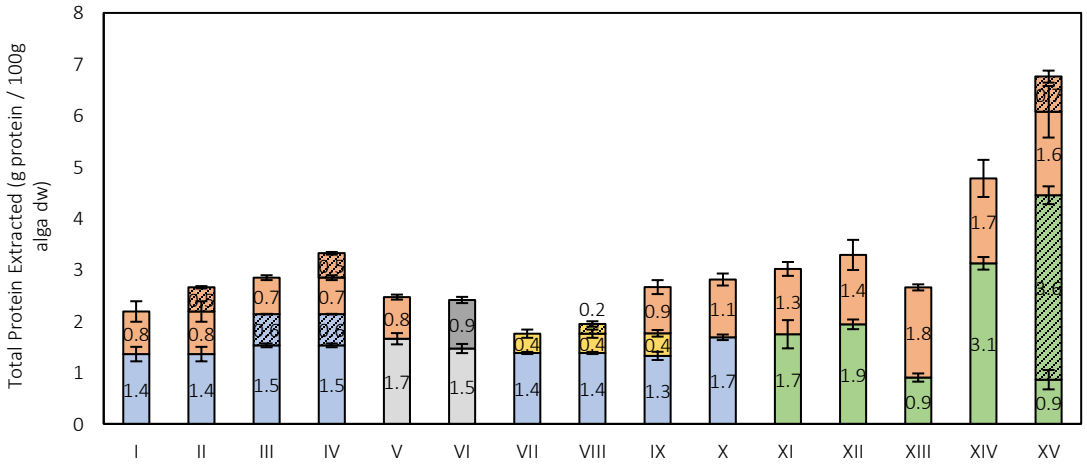

Figure 1: Total protein recoverd in *all 15 extraction procedures (described in Table 4) in grams of protein per 100 grams of alga (dry weight), using the Lowry method (n=3). Values are expressed as mean ± standard deviation, n = 3, except for XV with n=2.*

(- aqueous extraction; - aqueous re-extraction; - alkaline extraction; - alkaline re-extraction; - enzyme-assisted extraction; - enzyme-assisted re-extraction; - ultrasound-assisted aqueous extraction; - ultrasound-assisted alkaline extraction; - acid extraction; - acid re-extraction)

Table 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 I to XV. 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).

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
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
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
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

3.3. Extraction Scale-up

The scale up of procedures XII + AR (XII with an alkaline re-extraction) and XV was carried out using 50 g of algal power. Extracts from the same procedure were pooled. Results are presented in Figure 2 and Table 6.

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 ($p>0.05$). 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 and Enzyme-Assisted Extractions).

Table 6: Volume collected, total protein mass extracted, protein concentration in the extracts, actual protein recovery yield (considering the volume collected), and duration time of the scale up procedures XV and XII + AR.

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

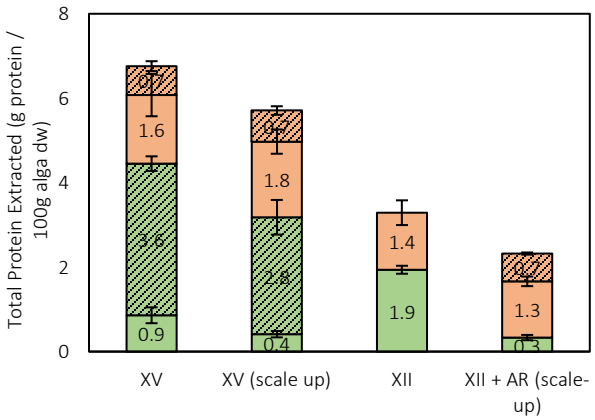

Figure 2: Total protein in grams of protein per 100 grams of alga (dry weight) extracted in extraction procedures XV and XII, and in the scale up of procedures XV and XII + AR. Values are expressed as mean ± standard deviation, n=3 for small scale procedures, n = 1 for scale up procedures.
(- enzyme-assisted extraction; - enzyme-assisted re-extraction;
- alkaline extraction; - alkaline re-extraction

3.4. Protein Precipitation

Ammonium sulfate is widely used in protein purification processes, however it is one of the interferences of the Lowry method, leading to protein overestimation [38]. This distortion was eliminated by blank correction. However, if an overestimation of protein did occur, higher precipitation yields than the ones reported were obtained.

Precipitation with a saturation of 85% led to significantly higher results in both extracts (data not shown). For this saturation, average precipitation yields of 24.6 ± 4.9% and 43.5 ± 3.2% were obtained for XV and XII + AR, respectively. The lower precipitation yield obtained for the XV extract was expected since it suffered protease activity, hindering some protein-protein interactions during salting-out.

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 precipitation with 85% of ammonium sulfate resulted in 1.9 ± 0.3 g/100 g alga 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 precipitation with 85% of ammonium sulfate resulted in 2.2 ± 0.4 g/100 g alga 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 35°C (overnight) and NaOH 1M (2h), and precipitation with 85% ammonium sulfate [39]. Kumar et al. reported a recovery of 7.8% from *K. alvarezii* [40] and values of 7.8% to 48% were reported for *Sargassum* species [41]. Alkaline solubilization followed by isoelectric protein precipitation is an alternative method. 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.

3.5. 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 using centrifugal ultrafiltration in diafiltration mode, with a MWCO of 3 kDa using the same buffer. The distortion due to Tris was corrected by simple blank correction, however a calibration curve with standards prepared in the same Tris buffer should have been done instead.

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. An n_D of 4.28 and 4.16 was obtained for XV and XII + AR, respectively. The protein mass decrease in the retentate (calculated using Equation 2.6), the subsequent protein mass increase in the cumulative permeate (determined using the Lowry method), and the protein retention yield are represented in Figure 3.

For XV, there was a generation of 1.9 mg of protein (13.4% of the initial protein mass), and for XII + AR, there was a loss of 0.2 mg of protein (1.18% of the initial protein mass). The successive volume measurements using graduated cylinders could lead to the propagation of errors. The ammonium sulfate concentration variations in the permeates could also interfere with the Lowry method differently. Average yields of 76.7 ± 1.4% and 81.1 ± 1.8%, and rejection coefficients of 0.89 and 0.95 were obtained for XV and XII + AR, respectively.

A concentration step was performed with VCFs of 1.42 and 1.33 for XV and XII + AR, respectively. For XV, there was a generation of 0.54 mg of protein (5.01% of the initial protein mass), and for XII + AR, there was a loss of 1.04 mg of protein (6.64% of the initial protein mass). The recovery yields and rejection coefficients were equal for both extraction protocols (97% and 0.93), 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.

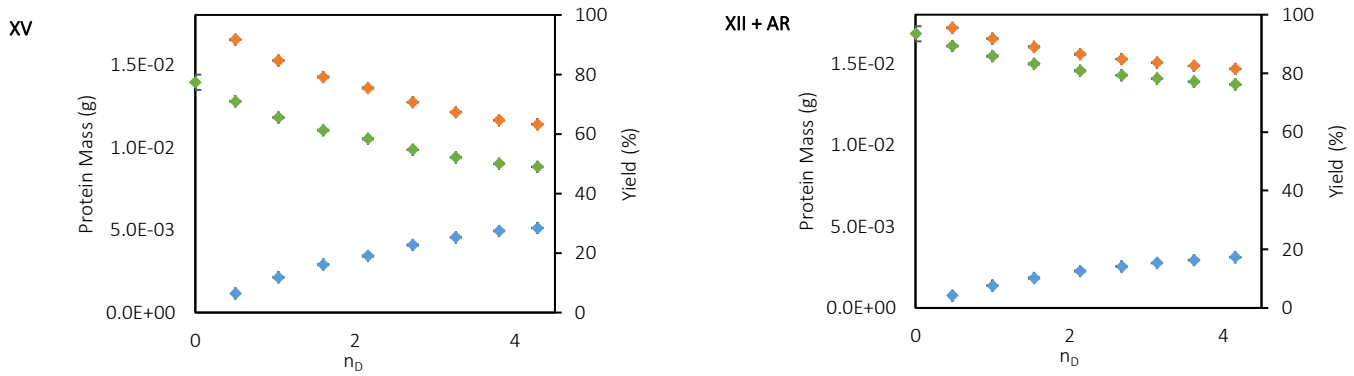

Figure 3: XV and XII + AR pellet diafiltration using an Amicon Ultra-15 centrifugal filter device (3 kDa MWCO), 3220 × g, swing-bucket rotor. Protein mass (g) in the retentate and in the permeate, and protein retention yield (%) as a function of the number of diavolumes. Loading mass was 13.9 ± 0.2 mg and 16.8 ± 0.5 mg, respectively. Average permeate flow rates of 6.1 ± 0.8 L/s and 8.4 ± 1.5 L/s, respectively. Protein mass in the permeate was determined using the Lowry method (n=3), while the protein mass in the retentate was calculated using Equation 2.6. Values are expressed as mean ± standard deviation, n=3. (- permeate; - retentate; - yield).

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 resulted 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 dw or an overall protein recovery yield of 11.8% (Figure 4).

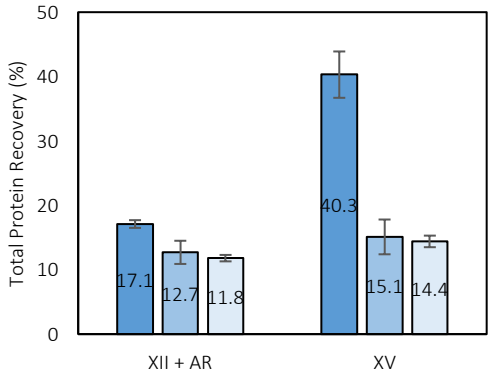

Figure 4 Cumulative total protein recovery (Protein extracted/Total protein · 100) after each process step (extraction, precipitation with 85% ammonium sulfate, and centrifugal centrifugation in diafiltration and concentration mode), for extraction procedures XV and XII + AR.
(- protein extraction; - protein extraction + ammonium sulfate precipitation; - protein extraction + ammonium sulfate precipitation + diafiltration and concentration).

3.7. Carbohydrate Concentration

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. A 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 one 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/100g dw and 63.6 ± 3 g/100g dw, respectively, which correspond to concentrations of 5.8 ± 0.5 g/100g initial algal biomass dw and 37.7 ± 1.8 g/100g 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/100g dw and 58.9 ± 2.7 g/100g dw, respectively, which correspond to concentrations of 7.3 ± 1.2 g/100g 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. 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. Even with a decrease of about 30% of total carbohydrate content, the concentration of agar found in the residues still matches values reported for unprocessed *Gelidium sesquipedale* (e.g., 40% [21]).

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, 4°C) and alkaline extraction (0.1M NaOH, 1h, RT) yielded a protein recovery of 14.7 ± 2.3%. 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. 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 followed by an alkaline extraction yielded a protein recovery of 22.2 ± 2.5%, whereas an aqueous extraction with Alcalase 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 feasibility when considering higher process scales.

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 resulted in a higher precipitation yield when compared to salt concentrations of 70, 75 and 80%. Using 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 ± 3 $\cdot 10^{-5}$ g/L and 0.97 ± 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 $t \cdot ha^{-1} \cdot year^{-1}$ [2] and a moisture content of 82% in fresh harvested *Gelidium*, 4.5 t dw $\cdot ha^{-1} \cdot year^{-1}$ are harvested which corresponds to 668 kg of protein $\cdot ha^{-1} \cdot year^{-1}$ that could be produced yearly. To produce enough protein to compete with protein-rich foods like soy (400 kg of edible protein per ha [42]), 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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