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Red Seaweed *Mastocarpus stellatus* as Sustainable Source of Protein and Bioactive Peptides

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Abstract

The current rapid growth of the global population calls for an increase in the food supply, particularly protein, as an essential macronutrient in the human diet. Animal and plant protein sources are somehow unreliable in terms of supply, yet they have enormous impacts on health and the environment. Seaweed, on the other hand, is a sustainable and 'green' source of protein – possessing high nutritional value and many nutraceutical properties, namely bioactive peptides.

In this work, the protein from the red macroalga *Mastocarpus stellatus* was characterized. The amino acid (AA) composition was ascertained by high-performance liquid chromatography (HPLC) – showing 16 AAs, 7 of which are essential, with the highest AA content recorded for Valine and Phenylalanine.

The algal protein extraction was optimized, studying the influence of the type of solvent, order and concentration of the solution, temperature, and time and volume used in the extracted protein yield. The optimal procedure consisted of a bead milling device and used HCl 0.4 M as solvent followed by extraction with NaCl 0.4 M for 1 h at 40 ^{o}C. This procedure retrieved 93% of the total protein present in *M. stellatus*. The size of the proteins extracted was analysed via size exclusion chromatography (SEC). Results unfolded two distinct portions, high molecular weight proteins (>669 kDa) and smaller macropeptides (<66.4 kDa).

Subsequently, the protein extracts were concentrated – and response surface methodology was employed to optimize enzymatic hydrolysis of the *M. stellatus* protein concentrate affected by Alcalase. The responses studied were degree of hydrolysis (DH), two antioxidant capacity assays, the oxygen radical absorbance capacity (ORAC) assay, and the 2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) scavenging assay and the inhibition of angiotensin-converting enzyme (IACE) activity. The optimal hydrolysis conditions were found to be 6.5 h of hydrolysis time and 2.6 %(v/v) of enzyme to substrate (E/S) ratio. The optimal responses were 4.2 ± 0.1 and 0.89 ± 0.07 µmol Trolox Equivalent (TE)/mg of hydrolysed protein (HP) for the ORAC and ABTS assays respectively, an IACE activity (IC_{50}) of 48.2 ± 0.6 µg/mL and a DH of 31.7 ± 0.8%.

Overall, *M. stellatus* could be considered a good source of protein, with highly bioactive peptides having possible applications in the food and pharmaceutical industries. Nevertheless, the relatively poor yield (ca. 29%) call for further optimization, appropriate scale-up, and preliminary economic analysis.

Keywords: *Mastocarpus stellatus*, Protein Extraction, Hydrolysis, Response Surface Optimization, Bioactive Peptides.

Abbreviations

AA	Amino acid
AAPH	2,2'-Azobis(2-amidinopropane) dihydrochloride
ABTS	2-Azinobis (3-ethyl-benzothiazoline-6-sulfonic acid)
$ABTS^{\bullet+}$	2-Azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) radical cation
Abz–Gly	*O*-amino benzoyl glycyl
ABz–Gly–Phe(NO2)–Pro	*O*-amino benzoyl glycyl-*p*-nitrophenilalanilproline
ACE	Angiotensin-converting enzyme
ANOVA	One-way analysis of variance
AQC	6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate
AUC	Area under curve
BCA	Bicinchoninic acid
BCAA	Branched-chain amino acid
BSA	Bovine serum albumin
BW	Body weight
CCD2	Central composite design of two factors
CD	Cell disruption
CO_2	Carbon dioxide
DH	Degree of hydrolysis
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DW	Dry weight
EAA	Essential amino acid
E/S	Enzyme to substrate ratio
EC_{50}	Half maximal effective concentration
EFSA	European food safety authority
FDA	Food and drug administration
FL	Fluorescein
FRAP	Ferric reducing antioxidant power
GRAS	Generally regarded as safe
HCl	Hydrochloric acid
HP	Hydrolysed protein
HPLC	High-performance liquid chromatography
IACE	Inhibition of angiotensin-converting enzyme activity
IC_{50}	Half maximal inhibitory concentration
LDL	Low-density lipoprotein
LOD	Limit of detection
LOQ	Limit of quantification
MW	Molecular weight
NaOH	Sodium hydroxide
ORAC	Oxygen radical absorption capacity
PBS	Phosphate-buffered saline
pI	Isoelectric point
RSM	Response surface methodology
SEC	Size exclusion chromatography
TE	Trolox equivalent
TEAC	Trolox equivalent antioxidant capacity
TMA	Trimethylamine
TMAO	Trimethylamine N-oxide
TNBS	2,4,6-Trinitrobenzenesulfonic acid
Trolox	(±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid
UAE	Ultrasound-assisted extraction
W/E	Water to ethanol ratio

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Objectives

This dissertation aimed at studying the possibility of using a red seaweed, *Mastocarpus stellatus*, as a sustainable source of high-quality protein and bioactive peptides, thus reducing the environmental and health impact related to the animal food industry and excessively intensive agriculture. In order to achieve that objective, the goals of this work were as follows:

Optimization of a 'food-grade' protein extraction method to recover the protein present in the red seaweed while minimizing solvent usage and extraction time;

Optimization of alcalase-mediated enzymatic hydrolysis in order to maximize the biological activities – inhibition of angiotensin converting enzyme activity and antioxidant activities – via response surface methodology, involving the combined effect of hydrolysis parameters, namely, reaction time and enzyme/substrate ratio.

1. Introduction

1.1. What is the Problem?

1.1.1. Food Shortage

By 2050 the world food supply needs to increase by 70% to feed the expected 9 billion people (Grahl et al., 2018). Global food production is now getting more complex than ever. The currently utilized agro-intensification techniques will soon become obsolete due to their significant environmental trade-offs. Protein is one of the most important nutrients that will be in low supply in the future. Considering the expected global protein requirements, alternative protein sources and production techniques are needed (Bleakley and Hayes, 2017).

1.1.2. Protein

Protein is one of the three essential macronutrients. It is found throughout our body, in muscle, bone, skin, hair, and many more organs. It is essential for many chemical reactions, making up enzymes and carrying oxygen as haemoglobin (Cheung, 2022). A systematic review of the health effects of daily protein intake by Pedersen and Cederholm (2014) reports that 0.83 g of good-quality protein per kg of body weight (BW) per day is the minimum dietary protein needed for all adult age groups. Intakes of up to 1.2-1.5 g protein/kg BW/day are entirely safe, and may render numerous benefits in the long term. It prevents muscle mass loss, and improves muscle strength and gain when coupled with adequate resistance training, besides maintaining bone mass (Perdersen and Cederholm, 2014). Not all proteins act the same; different protein qualities and sources have many implications on a person's health and the environment.

1.1.2.1. Animal Protein

Animal protein is the most common and preferred source of protein in a person's diet, with beef and chicken being excellent foods that provide high-quality protein (McFarlin et al., 2006). Animal proteins are considered complete proteins, containing all essential amino acids (EAA), whereas proteins derived from plant sources may be deficient in one or more amino acids (AA). Nevertheless, health, environmental and ethical issues have been related to animal protein consumption, and the underlying excessively intensive animal farming industry (Tharrey et al., 2018; Xu et al., 2020).

Health Problems

Differently from the other macronutrients, protein is mainly metabolized by fermentation by the intestinal flora. The resulting product compositions depend on the type of metabolites, their diversity, and gut microbiota. Many animal protein fermentation products are toxic if in high concentrations. In the long run, a high animal protein diet will imply an increased risk of contracting numerous diseases, including obesity, type 2 diabetes, intestinal disease, and many others (Cai et al., 2022).

Furthermore, red meat and dairy products have high amounts of dietary fat, including low-density lipoprotein (LDL) cholesterol, and saturated fatty acids that also raise the overall cholesterol levels in the blood. Both fats are associated with cardiovascular diseases, including stroke, atherosclerosis, and hypertensive and coronary heart disease (Campbell, 2017). In

addition, red meat and processed meat contain the AA L-carnitine, which can be metabolised into trimethylamine (TMA) and then trimethylamine N-oxide (TMAO). High TMAO levels, combined with other complications such as kidney disease, diabetes, and peripheral artery disease, double patients' mortality (Kanitsoraphan et al., 2018).

Environmental Problems

Industrial animal farming severely harms the environment. It heavily contributes to deforestation, biodiversity loss, and emergence and spread of infectious diseases. In addition, it contributes to climate change, and air and water pollution (Lavaine et al., 2020).

Cattle ranching has the highest impact upon forests globally, and is responsible for 65-70% of its total deforestation. Although many farmers use deforested land to raise livestock, well-known companies do it on a much larger scale, causing most damage (Bennett, 2017). Deforestation affects the climate directly, responsible for 25% of the world's total greenhouse gas production (**Figure 1**). In addition, forests hold almost half of the world's terrestrial carbon. Therefore, deforestation will generally release carbon dioxide into the atmosphere (Moutinho and Schwartzman, 1988).

Infectious diseases, biodiversity loss, and livestock expansion are also increasing globally, and exam of the patterns that link them is crucial for public health purposes. There is a direct relationship between the increasing number of cattle, the number of endangered species, and the number of human disease outbreaks. All of this endangers people's and animals' health and welfare (Morand, 2020).

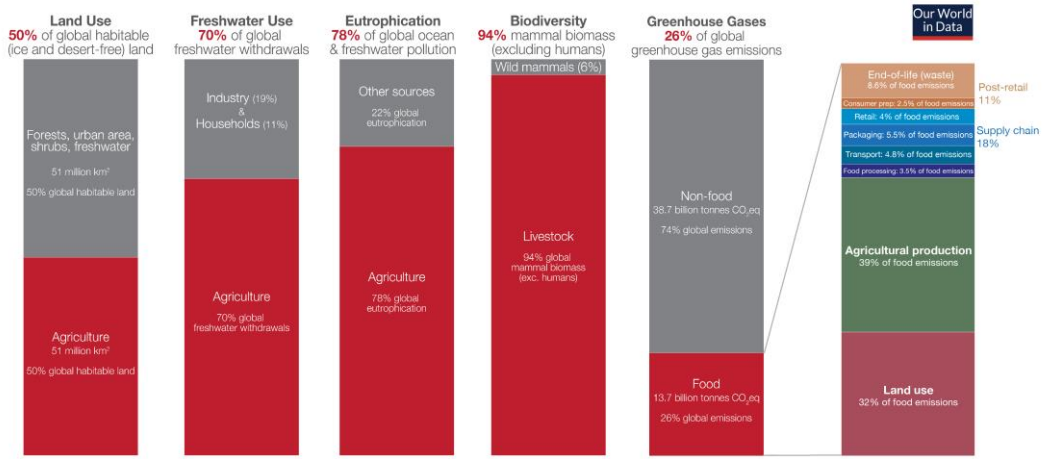

Figure 1. Global impact of food and agriculture upon the environment: land and freshwater use, eutrophication, biodiversity, and greenhouse gases (adapted from Ritchie and Roser, 2020).

Ethical Problems

Besides health and environmental concerns, ethical distress related to industrial food animal production is another factor to consider when replacing animal protein. The mass production of meat and other animal products results in poor animal well-being or even animal suffering (Hernandez et al., 2022). The ever-growing Veganism movement is a popular social movement that stands against this animal exploitation and abuse. It supports an alternative diet, mainly composed of plant-based foods, considered more ethical, environmentally sustainable, and healthier (Ulusoy, 2015; Tharrey et al., 2018; Arenas-Jal et al., 2020).

1.1.2.2. Plant protein

Many plant-based foods have a decent protein content, around 36% (Gorissen et al. 2018). These include grains, nuts, seeds, potatoes, legumes, soya, and many others (Tharrey et al., 2018). Soybeans and peas are considered adequate substitutes for animal proteins. Soybeans contain up to 40% of protein per dry weight (DW) and similar AA profiles except for the sulfur containing AAs, methionine and cysteine (Xu et al., 2020). Even though pea seeds contain only 20-25% protein, they have better digestibility, have AAs containing sulfur, and show even less allergenic responses than soya (Hadidi et al., 2022).

Overall, a diet based on plant protein should be superior to that based on animal protein. In contrast with animal protein, plant protein has no correlation with the increase in cardiovascular disease and type 2 diabetes mortality – and may even prevent diseases and improve gut microbiota (Cai et al., 2022; Wang et al., 2022). Plant proteins are also less allergenic, cheaper, and more widely available than their animal counterparts (Hadidi et al., 2022).

Essential Amino Acids Problem

Although mainly beneficial, as mentioned before, plant protein has its limitations; it is indeed mostly considered as incomplete protein. The human body can produce various AAs through AA synthesis, although nine of the twenty cannot be synthesized – meaning that they need to be ingested via our food. These thermed EAAs, viz. histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine (Cheung, 2022). Both animal and plant products contain all twenty AAs, but their quantity varies considerably depending on the source (Xu et al., 2020). It is believed that the effects of dietary restriction on host health depend more on specific AA restrictions than on total calorie intake (Wang et al., 2022).

The most popular protein concentrate on the market is whey protein, characterized by an extremely high-quality nutritional value – with all EAAs, easily digestible, and rapidly absorbed, thus making it an ideal building block for the growth and recovery of lean muscle mass (Arenas-Jal et al., 2020). Plant protein concentrates have been shown to be as effective as animal proteins. Nevertheless, this ends up being a sizable challenge, requiring adequate knowledge of nutrition to correctly select the plant protein sources to deliver all EAAs (Arenas-Jal et al., 2020).

Erosion and Nutrients of the Soil Problem

Additionally, the mass production of plant proteins is not necessarily better for the environment than animal protein (Grahl et al., 2018). Most agricultural regions experience greater erosion rates than soil formation, which over time, degrades the land (Pimentel et al., 1987). For example, in Brazil, coffee crops were first introduced as permanent crops that required little mechanization, having minimal impact on the soil condition. Unlike coffee, soybean planting is a seasonal and mechanized activity, requiring soil correction and substantial fertilization. The irrigation systems, coupled with heavy mechanization and harvests twice a year, further to water erosion impact the soil viability (Castro and Neto, 2009). The inadequate or non-existent management planning is a primary concern nowadays with regard to said areas. More data on the environmental impact of agriculture can be seen in **Figure 1**.

1.2. What is the Solution?

1.2.1. Blue economy

Animal protein is complete and abundant, but associated with health complications and has a bad reputation due to all the problems related to cattle ranching. Plant protein solves some of the health and animal cruelty issues related to the animal industry, but it is less nutritious and also deteriorates land due to excessive cultivation (Rawiwan et al., 2022). It is unbelievable that more than 95% of the world food is still produced on land instead of in the oceans, or other aquatic systems (FAO, 2021). Local seas have the potential to support regional economies and could be utilized more efficiently and sustainably.

Blue economy or "blue growth" encompasses a wide variety of activities that benefit the economy by sustainably using the sea. It includes marine trading and transportation, harvesting food and nutritional resources, obtaining renewable energy and raw material resources, aquaculture, biotechnology, and maritime tourism and security (Katila et al., 2019). A blue economy entails strictly sustainable economic growth, while preserving social cohesion and the environment; this means that the population's needs must be met without compromising the subsequent generations (Katila et al., 2019; Apitz, 2020). It is of the utmost importance that these strategies are not implemented carelessly, otherwise resulting in a "blue rush" – where the ocean resources would be excessively harvested and depleted (Apitz, 2020).

1.2.2. Algae

It has recently been recognized that seaweed, which is primarily consumed in Asia, is a nutrient-dense food that contains high-quality protein – thus potentially making it an excellent sustainable source of alternative protein to help combat the problems mentioned previously. Around 85% of the global seaweed industry is already used directly for human consumption (Milinovic et al., 2022) and by 2019, seaweed cultivation was worth 14.7 billion USD, with approximately 34.7 million tonnes of algae produced that year (Rawiwan et al., 2022).

Algae are thallophytes – plants without roots, stems, or leaves that produce oxygen through photosynthesis – with their primary photosynthetic pigment being chlorophyll a (Hoek et al., 1995). Most algae appear in water, either freshwater or saltwater; however, they can be found in many other environments, from snow in freezing mountains to desert soil and bare rocks (Hoek et al., 1995). Regarding morphology, degree of complexity, and size, algae hold a great diversity; however, two groups can be distinguished – microalgae, microscopic algae only visible through a microscope, and macroalgae, commonly known as seaweed, visible to the naked eye (Pereira, 2021).

Types of algae

The typical phytoplankton is made up of microalgae in surface waters. They are the main producers in most habitats, since they can produce organic matter from water, CO_2, and sunlight. (Hoek et al., 1995; Pereira, 2021). Macroalgae or seaweeds are usually found in the marine environment, and can reach up to 65 m in length. As primary producers, macroalgae are at the base of the marine food chain and support the diet of herbivores. Therefore, many macroalgae developed complex defence mechanisms, such as calcification, and production of secondary metabolites such as terpenes and polyphenols that are unpleasant to digest (Scheuer,

1987; Pereira, 2021). They can also adapt to harsh environments by assembling thicker cell walls, increasing their growth rate at low temperatures and low nutrient habitats, , and producing protective pigmentation that blocks high-energy ultraviolet and blue radiation (Sheath et al., 1996).

Algae can either be prokaryotic, mainly cyanobacteria, or eukaryotic, i.e. all other algae (**Figure 2**) (Hoek et al., 1995). Eukaryotic algae cells often have an outer cell wall composed of polysaccharides, and an inner plasma membrane surrounds the rest of the cell and controls the flow of substances in and out of the cell. Other important organelles include the flagella for locomotion; the nucleus, which contains the genetic material; and the chloroplasts that contain the chlorophyll and carry out photosynthesis (Hoek et al., 1995).

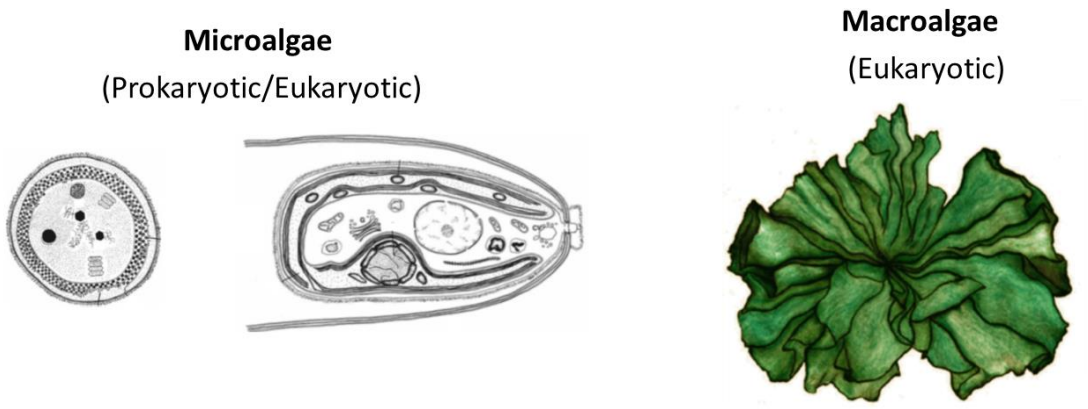

Figure 2. Type of algae, micro and macroalgae and type of cell, prokaryotic or eukaryotic (adapted from Hoek et al., 1995; Pereira, 2021).

The colour of algae depends on their different photosynthetic pigments. Algae can be divided into four main phyla, depending on their pigmentation (Pereira, 2021). Green macroalgae belong to the phylum Chlorophyta and have similar pigmentation to vascular plants with chlorophylls a, b, and some carotenoids. Red macroalgae, which represent more than 50% of the seaweed cultivation market (Rawiwan et al., 2022), are members of the phylum Rhodophyta, and their photosynthetic pigments include chlorophyll a, phycobilins, and carotenoids. The phylum Ochrophyta or Phaeophyta is home to brown macroalgae, with their predominant carotenoid, fucoxanthin.

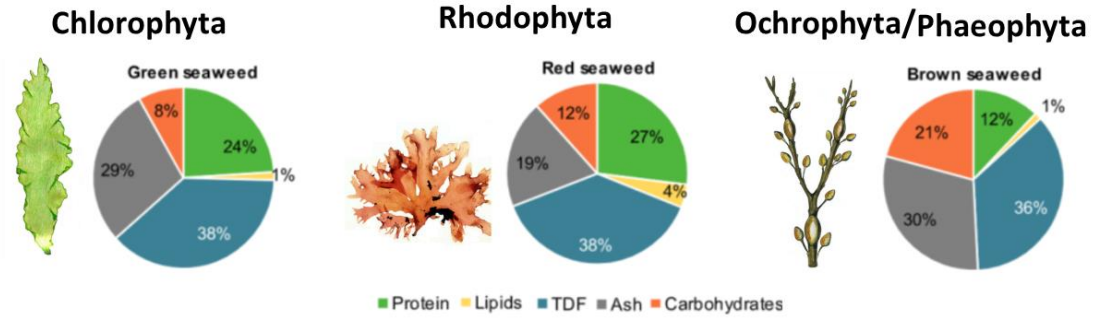

Figure 3. Macroalgae phyla and general nutrients composition (adapted from Tenorio et al., 2018; Pereira, 2021).

At last, the phylum Cyanobacteria encompasses blue-green algae. Green and red algae belong to the kingdom Plantae, and brown algae to the kingdom Chromista (Pereira, 2021). The same species of macroalgae can also have a highly variable composition, depending on the time of collection, habitat and external conditions, such as water temperature, light intensity, and water nutrient concentration (Pereira et al., 2019). The general composition of each macroalgae phylum can be seen in **Figure 3**.

Macroalgae Composition

As mentioned before, algae can be a valuable and nutritionally dense food with many benefits. Algal biomass presents lipids with higher degrees of unsaturation when compared with lipids from animal and plant sources (Foley et al., 2011). The carbohydrates concentration is high in macroalgae, 33-50 % of dry weight, and their composition varies from species to species. The human gastrointestinal tract cannot digest most of these; therefore, they are considered dietary fibre (Pereira, 2016). This fibre content is usually higher than in traditional vegetables and fruits, having both soluble and insoluble dietary fibre. In particular, soluble fibres slow the rate of absorption of polysaccharides, which helps lower blood sugar levels (Pereira, 2016).

Alhough lipid content tends to be low in macroalgae, most lipids are ω-3 and ω-6 polyunsaturated fatty acids. These fatty acids have high economic interest due to their effectiveness in reducing the risk of cardiovascular diseases, osteoporosis, and diabetes (Mišurcová et al., 2011). Due to the lack of sufficient ω-3 polyunsaturated fatty acids in the Western diet, seaweed could be used to fix this problem, by substituting the ω-3 from fish – often associated with undesirable smell and taste (Mišurcová et al., 2011).

Moreover, marine algae contain a very high concentration of vitamins and essential minerals in their chelated and colloidal forms, thus potentially enhancing their bioavailability in the body (Pereira, 2016). More importantly, some species of algae have relatively high amounts of protein, while having all the EAAs and even non-standard AAs like ornithine, an effective anti-fatigue supplement (Sugino et al., 2008).

Seaweeds are an excellent source of AAs. All three groups – green, red, and brown, have nearly half of their protein profile as EAAs, with overall similar amounts between phyla. Red seaweeds have recorded the highest protein content of up to 47% of dry weight (Pereira, 2016; Xu et al., 2020). Brown and green algae have lower protein contents, of 18 and 35%, respectively (Xu et al., 2020). The protein from algae can be separated into four distinct groups, depending on how well they dissolve in various solutions: albumins, which are soluble in water; prolamins, soluble in 70% alcohol; glutelins, soluble in diluted acids or bases; and globulins, which are soluble in salt solutions (Gordalina et al., 2021).

Other elements, such as metals, are naturally found in marine algae. Metallic elements are essential nutrients for algae and the levels of metals in the water directly correlate with the metals found in algae. Unfortunately, a toxicological effect in polluted waters with heavy metal concentrations above normal will distress the environmental quality, thus depleting oxygen and disrupting biochemical cycles. These problems influence mainly the foundation species, the primary producers, by reducing their nutrient uptake capacity and increasing their toxicity – as metals compete for cellular transportation (Costa et al., 2020).

Macroalgae Applications and Benefits

Seaweed cultivation on the sea is done throughout the world, with China and Japan being the countries with most consumption and commercial uses. Nevertheless, due to their therapeutic properties, macroalgae are used in many other countries for different reasons, namely food or animal feed, agriculture, and medicine. The increasing worldwide use of macroalgae has now entered the industrial scale (Gaspar et al., 2020). For example, in Norway in 2019, cultivation in just 3.8 square kilometres, produced approximately 111 tons of seaweed, valued at 0.4 million euros (Araújo et al., 2021). Some alga use date back hundreds of years, including food, animal feed, remedies, and fertiliser. Ancient records indicate that macroalgae have been used for human consumption since 500 B.C. in China and since 500 A.C. in Europe (Pereira, 2021). Macroalgae are the opposite to processed foods we are accustomed to today. They constitute natural food with a high nutritional value and low-fat content. Although we cannot break down the polysaccharides that make up macroalgae, thus ending up not absorbing them, they can still facilitate intestinal transit and lower the cholesterol level in the blood – while delivering a high content of minerals and vitamins (Gaspar et al., 2020). Algal extracts also contain beneficial compounds that can improve plant development, control diseases, and decrease insect damage (Pereira et al., 2019).

As industrial applications, algae have been used to produce calcium carbonate and formulate soft drinks and soaps. Some species of algae also store iodine that can be used as a dye (Pereira, 2016). Nowadays, the main compounds produced industrially from macroalgae are phycocolloids, mainly agar and carrageenans. Agar is an organic compound used as a gelling agent in medicine and food formulations, capable of geling at room temperature (Lira et al., 2016; Pereira, 2016). On the other hand, besides thickening, carrageenans can be used to interfere with the function of some viruses in the pharmaceutical field, serving as a preservative; or they can be used in cosmetics to wash hair due to their ability to bond with the keratin (Pereira, 2016). Alginic acid, derived from polysaccharides, is used in medical and cosmetic sectors due to its high stability regarding pH and salinity changes, healing, and neutralising ability in cases of heavy metal poisoning (Pereira, 2016). Other compounds of interest include fatty acids, for biofuel production, pigments, phenols, and polysaccharides – which can have various health benefits, such as anti-inflammatory and anti-thrombotic activity (Pereira, 2016). More recently, an increase in interest for seaweed has been encouraged by the awareness of its bioactive compounds, mainly protein-derived peptides, which may find use in the profitable food and nutraceutical industries. These applications could reduce metabolic risk factors, such as hyperglycemia, hypercholesterolemia, and hypertension (Xu et al., 2020).

Besides human consumption, seaweeds can be used as livestock feed – although their effects are inconclusive, with reports of increased, decreased, or no beneficial effects (Pereira et al., 2019). Macroalgae have also been used in agriculture as fertilisers for a long time; some are used to correct the pH of acidic soils and, at the same time, increase production yields due to the high contents of micronutrients (Pereira et al., 2019).

In that way, it is essential to explore this hidden potential, by incorporating algae into an ordinary person's diet. A more sustainable protein source is needed to meet the demands of food supplies. Protein is regarded as more sustainable if it can be obtained while using less land, energy, water, and chemicals, whilst producing higher yields. The main objective is to change

and increase our food options by adding algae to our diet so that they are more accessible, less expensive, sustainable for the environment, and socially acceptable (Grahl et al., 2018).

1.2.3. Portuguese coastline

Around 85% of the seaweed industry is directly used for human consumption. The Portuguese coast comprises over 800 kilometers of vast beaches and rocky shorelines, most of which are exposed to the sea movement and abundant in both microalgae and macroalgae (Lewis, 1964; Lira et al., 2016). The Northern region of Portugal has a similar flora to that of central Europe and the British coast. By contrast, in the southern half of the country, the algal species are considerably distinct, clearly influenced by the Mediterranean and the west coast of North Africa (Gaspar et al., 2019). On the northern coast, the checklist by Araújo et al. (2009) reports 346 distinct species of algae – 200 red, 70 brown, 50 green, and 26 Cyanobacteria.

Mastocarpus stellatus

Due to the dominance of Rodophitas in the market (Rawiwan et al., 2022), the abundance of red seaweed on the Portuguese coast (Araújo et al., 2009), and the fact that this phylum presents the highest amount of protein content (Pereira, 2016; Xu et al., 2020), the species *Mastocarpus stellatus* was selected for this study. *M. stellatus* is a red seaweed from the order Gigartinales, native to the coasts of the Northern Atlantic Ocean (Nguyen et al., 2016). As for its composition, by % of dry weight (DW), it has a moisture content of 7.5-8.9 %; an ash content of 20.8-25.0%; a lipid content of 0.4-0.8%; a total dietary fibre content of 31.7-48.6%, of which around 20% is carrageenan; and a total protein content of 21.1-25.4% (Marsham et al., 2007; Gómez-Ordóñez et al., 2010; Nguyen et al., 2016; Lab.Proj., 2022). *M. stellatus* is often used for its κ/ι-hybrid carrageenans due to their gelling properties and bioactivity (Hilliou et al., 2006; Blanco-Pascual et al., 2014).

1.3. How to Achieve the Solution?

A scalable, cost-effective, viable for the food industry and environmentally friendly method of extracting protein is an important goal in the algae industry. Some extraction processes might not be suitable for the food industry, for relying on dangerous solvents or wasting too much energy – thus leading to a non-economically viable process. It is necessary to understand if a method is sustainable and if not, what can be changed.

1.3.1. Environmental Impact

Even though algae represent a potentially environmentally sustainable source of protein (Cermeño et al., 2020), they still cannot compete with animal or plant protein (Bleakley and Hayes, 2017). An environmentally friendly extraction process should produce multiple products of interest, minimize waste, implement biorefinery shcemes, reduce the number of operations, reduce energy consumption, implement energy recovery, and, use environmentally safe solvents, mainly water (Gateau et al., 2021). The use of organic solvents such as chloroform and methanol, is common in multi-phasic extractions. These solvents are very harmful to the environment; they can and should be replaced by “greener” solvents, such as toluene and ethanol (Wahlström et al., 2017). Unfortunately, the environmental impact is not prioritized as much as algal protein extraction scalability and economic viability. This is problematic, since one

of the main reasons to use seaweed as a protein source is to prevent the environmental impact that other sources have on the planet (Lab. Proj., 2022).

1.3.2. Industrial Scale

The main limitation for algal protein commercialization resides on the scalability of the method. Only a few scalable methods are suitable for upscaling lab-scale methodologies and can be sustainable in industrial-scale operations (Bleakley and Hayes, 2017). For example, filtration is one of the most effective methods to scale up, with consistent and predictable results (Cermeño et al., 2020). In the last decade, researchers have progressively explored new methods and scale-up strategies to create viable large-scale methods to extract protein from algae (Lab. Proj., 2022). At a large scale, algae have the potential to produce 100-fold more protein than terrestrial plant crops (Bleakley and Hayes, 2021).

1.3.3. Economic Impact

Economic viability is a crucial factor in the sustainability of an industrial process. It would be pointless to implement an industrial process if it is not profitable. The main factor affecting a method economic viability is the use of expensive substances and/or high-energy consumption. For example, plain solvent extraction is easily scalable, but the amount of solvents used at a larger scale limits the economic viability of the process (Timira et al., 2021).

This economic burden can be mitigated by recycling and reusing the solvents (Chia et al., 2019). Since algae possess various compounds of interest in their composition, the extraction of multiple high-value co-products would benefit the overall economic viability of a sequence of extractions (Soto-Sierra et al., 2018). Another way to increase the economic value of a process comes with the selection of the algae species. Species with higher protein content or less resistant cell walls should allow higher extraction yields. The analysis and discussion of the economic impact and feasibility of the protein extraction methodology have witnessed a significant increase in recent years (Proj. Lab., 2022).

1.3.4. Food Industry

The first concern when it comes to using algae as a protein source is the safety of the species of algae to be processed. The algae used must be food-grade, and the level of heavy metals must not exceed the regulated limits – which require thorough analysis of these compounds to prevent contamination during processing (efsa.europa.eu; Li et al., 2021). The alga used has to earn the “Generally Regarded As Safe” (GRAS) status under the Food and Drug Administration (FDA) (Cermeño et al., 2020), or the European Food Safety Authority (EFSA) (Bleakley and Hayes, 2017) regulations. The process must also be validated, especially the solvents used in the extraction step. The pH range for most food products resides between 3 and 7, meaning that the pH has to be regulated when acid or alkaline extraction and/or hydrolysis are used (Grossmann et al., 2019a; Grossmann et al., 2019b).

Another concern is related to protein hydrolysates, which have been regarded as carrying significant health benefits. Although proven effective bioactive compounds, their long-term efficacy and safety are still to be determined, thus requiring further research and clinical trials (Barkia et al., 2018). The interest in protein extraction from algae as a food source has increased in the past few years. However, most studies only focus on compound extraction yield and

bioactivity, while disregarding their potential use in food formulation and not exploring any safety concerns or compliance with regulations (Lab. Proj., 2022).

1.3.5. Biorefinary concept

Biorefinery is a term that can be summed up as taking advantage of multiple algae components, thus reducing waste while increasing value. In the case of seaweed protein extraction, other compounds of interest could be extracted simultaneously (for example, other macronutrients, pigments, chlorophyll, or bioactive peptides). Biofuels are an excellent example, since protein extraction usually does not affect the hydrophobic portion of algae. Some algal species have high lipid contents that can undergo transesterification and produce biofuel. However, the protein is degraded during the biofuel conversion process, and the nitrogen is converted to ammonia, a toxic greenhouse gas (Garcia-Moscoso et al., 2013). Algae processing could become more sustainable if protein extraction could be implemented before biofuel production, using the organic solvents to extract the remaining hydrophobic fraction that was separated from the protein-rich fraction (Muñoz et al., 2015). Other products with a market value in algal biomass include chlorophylls and carotenoids. Chlorophyll can have multiple health benefits, such as anti-inflammatory properties, immune system stimulation, and blood and liver detoxification (Kulkarni and Nikolov, 2018). Carotenoids have been shown to prevent early atherosclerosis and cataract progression (Kulkarni and Nikolov, 2018). These compounds can be pre-extracted via simple solvent extraction, using ethanol before protein extraction. Carrageenan is another compound of interest, with 20% of its global production already coming from seaweed (Naseri et al., 2020).

1.4. Extraction methods

Seaweed harvested directly from its habitat has poor protein digestibility. Therefore, seaweed needs to be processed so that the protein will attain its maximal bioactivity. In that order, a series of techniques can be applied, including drying, cell disruption, extraction, and purification. Cell disruption and extraction methods can be divided into conventional, and more recent, novel methods (Bleakley and Hayes, 2017). Conventional protein extraction methods include milling/grinding (Postma et al., 2015), solvent extraction (Chotipan et al., 2016), and enzymatic cell disruption (Mæhre et al., 2016). Novel methods include ultrasound-assisted extraction (UAE) (Safi et al., 2014; Lupatini et al., 2016), pulse electric field extraction (Gateau et al., 2021; Azmi et al., 2020, high-pressure homogenization (Safi et al., 2014; Shene et al., 2015), microwave-assisted extraction (Chew et al., 2019), freeze-thawing (Chen et al., 2019; Sommer et al., 2021) and subcritical water extraction (Álvarez-Viñas et al., 2020).

1.4.1. Biomass drying

Drying affects the sustainability of the extraction process, since the drying step entails a high-energy consumption and, consequently, a high cost (Sierra et al., 2017). There are numerous drying methods used to dry seaweed, of which the main ones are sun-drying, oven-drying, and freeze-drying. Sun-drying is the best environment and economic wise, but is the least effective for protein extraction – and has longer durations, up to three times freeze-drying and six times oven-drying. Freeze-drying has the highest consumption and yield when extracting protein (Ansari et al., 2015; Ansari et al., 2018). Overall, drying methods lead to losses in productivity in any industrial process, thus lowering its sustainability and economic viability.

1.4.2. Milling/Grinding

Milling or grinding are physical cell disruption steps in the protein extraction process. Bead milling is commonly used for protein extraction by utilizing steel, glass, or ceramic beads that move at high speeds to disrupt the rigid cell wall of the seaweed (Soto-Sierra et al., 2018). It causes a very high disintegration degree, and protein release within a few minutes of operation, making it the ideal cell disruption method for the laboratory scale (Postma et al., 2015). Bead milling has such high disruption yields that it is usually used as a positive control for other cell disruption techniques (Gateau et al., 2021). Additionally, it can be up-scaled and used at the industrial scale (Soto-Sierra et al., 2018; Kazir et al., 2019). However, it requires a high energy output and is not the most efficient regarding volume usage, since the beads can occupy a considerable amount of the total grinding chamber volume (Soto-Sierra et al., 2018; Timira et al., 2021).

1.4.3. Solvent Extraction

Solvent extraction in the context of algae is a solid-phase extraction using any type of liquid solution, including water, which will separate the compound of interest from the biomass. Some solutions used include ionic liquids/salts (Wang and Zhang, 2012), alkaline and acid solutions (Chotipan et al., 2016), and organic solvents (Grossmann et al., 2018). Solvent extraction is easy to use, combines with most other extraction techniques, and has reasonable protein yields (Cermeño et al., 2020). The solubilized protein can be precipitated through pH change and salting, separating it from other soluble compounds. Multiple solvents can also be used simultaneously, for example, as a three-phasic extraction where an organic phase separates lipids, an aqueous phase separates sugars, and a third phase where the protein is precipitated through salting (Zeb et al., 2019). In contrast, some solvents, such as methanol and chloroform, are incredibly harmful to the environment, and are not compatible with the food industry (Wahlström et al., 2017). Another disadvantage is the operation time, which is usually long – and, depending on the solvent, can lead to partial protein degradation (Cermeño et al., 2020). A large amount of solvent is also needed to achieve high yields, having solvent:biomass ratios up to 40:1 (v/w) (Pagels et al., 2021), which is unfeasible at industrial scale.

1.4.4. Separation and Concentration

The two main separation methods in algal protein extraction are centrifugation and filtration. Centrifugation is done in almost every protocol used for protein extraction, for separating the liquid solvent phase from the solid phase, which can be either cell debris or precipitated protein, depending on the solubility of the protein in the liquid phase (Gifuni et al., 2020; Proj. Lab., 2022). Filtration is used to replace centrifugation (Eboibi et al., 2015) or after it as further purification (Garcia et al., 2018; Gifuni et al., 2020). It separates low molecular weight proteins from higher molecular weight proteins or other cell components (Bleakley and Hayes, 2017; Garcia et al., 2018). Filtration is ideal for separating compounds of interest, like chlorophyll from the sample, thus reducing both undesirable colour and taste, or separating the protein hydrolysates from non-hydrolysed proteins (Gifuni et al., 2020). In addition, it is widely used on an industrial scale, use no harmful chemicals and holds a low-energy consumption (Gifuni et al., 2020).

1.5. Protein Quantification

The assay selection to determine the protein concentration during extraction and purification steps is often tricky. The most common quantitative methods include Kjeldahl method, Ultraviolet (UV) Absorbance at 280 nm, and colorimetric assays such as the Bradford assay, Lowry assay and the Bicinchoninic Acid (BCA) assay.

The Kjeldahl method consists of digesting, neutralizing, and distilling the organic nitrogen from the protein in the sample, and quantifying it through titration using a nitrogen-to-protein conversion factor (Ballentine, 1957). This method is highly accurate and reproducible; however, it measures any nitrogen present in the sample, and different proteins require different correction factors (Goulding and O'Mahony, 2020).

A direct determination of the protein content of a sample can be done by measuring its absorbance at 280 nm. Most aromatic AAs can absorb ultraviolet light and the optical density is proportional to the concentration of those AAs (Simonian, 2000). This procedure is simple, sensitive, and fast, and the sample can be recovered; however, it has low specificity, being susceptible to interferences. Besides, since only aromatic AAs are quantified, an extinction coefficient needs to be experimentally determined for the sample studied, which requires AA sequencing (Olson and Markwell, 2007).

In Bradford assay, Coomassie's brilliant blue G-250 dye changes the protein conformation. It then binds to the hydrophobic AAs, forming a blue complex – and leading to a linear relationship between absorbance and protein concentration (Bradford, 1976). This method is quick, convenient, and sensitive; however, it has unpredictable effects, depending on the structure of the proteins, and has many interferents (Olson and Markwell, 2007).

In the Lowry assay, alkaline conditions reduce copper ions (Cu^{2+}) by the peptide bonds. Folin-Ciocalteu reagent reacts with the previously formed cuprous ions (Cu^{+}), and some AAs produce a blue complex that directly supports a correlation between absorbance and protein content in the sample (Lowry and Brough, 1951). Lowry assay is a sensitive and specific method that is not affected by turbidity. However, it is time-consuming and susceptible to interferences from some ions, carbohydrates, and other reducing agents (Olson and Markwell, 2007).

BCA method is another colourimetric assay. Also known as Smith assay, it is an improved version of the Lowry assay. As in the Lowry assay, Cu^{2+} (blue copper solution) bonds to the protein peptide bonds and gets reduced to Cu^{+} (green copper solution), all under alkaline conditions (Smith et al., 1985; Otieno, 2016). The Bicinchoninic Cu^{+} complex is primarily sensible to AAs such as tyrosine, tryptophan, and cysteine, by reacting with their side chains. Because of this, the assay should be performed at elevated temperatures, thus allowing for peptide bonds to reduce the copper ions and exposing AAs – which increases the sensitivity and reduces the variations between samples with different AA profiles (Olson and Markwell, 2017; Otieno, 2016). With the BCA assay, the determination of protein content is sensitive, precise, and rapid. BCA has the advantage of interacting less with contaminants and buffer components than the Folin-Ciocalteu reagent from the Lowry assay. As a disadvantage, the assay should be run at higher temperatures to maximize its sensitivity. It is vulnerable to interference from several compounds found in protein samples, mainly copper chelators and high concentration buffers (Olson and Markwell, 2007).

1.6. Bioactive Peptides Production

Hydrolysis consists on cleavage of the peptide bond of proteins, producing smaller peptides and even AAs. Hydrolysis can be brought about through heat treatment (Soto-Sierra et al., 2018) or acid and alkaline solvents (Wang et al., 2019) – althoug, the most common hydrolysis utilizes enzymes that hydrolyse protein by cutting the peptide bonds (Darvish et al., 2018; Hou et al., 2019). The most frequently used food-grade enzymes are alcalase and flavourzyme (Li et al., 2019). Due to their complex and unique metabolic pathways, macroalgae produce an extraordinary amount of bioactive organic compounds, including proteins, linear and cyclical peptides, and AAs (Sánchez and Vázquez, 2017).

The bioactive peptides exhibit an enhanced biological activity, including health benefits, such as hypolipidaemia, anti-oxidation, insulin regulation activity, antihypertensive properties (Medina et al., 2015; Li et al., 2019), and cytotoxic and antibacterial properties (Beaulieu et al., 2015; Darvish et al., 2017; Sánchez and Vázquez, 2017) – see **Figure 4**. They have a much higher economic importance, being valued four times over their corresponding protein concentrates (Soto-Sierra et al., 2018). However, it is impossible to predict how a peptide chemical structure will affect its function. Their activity depends on various factors including AA profile, especially their N- and C-terminal AA, length of the peptide, and hydrophobic, hydrophilic, and charged characteristics (Sánchez and Vázquez, 2017).

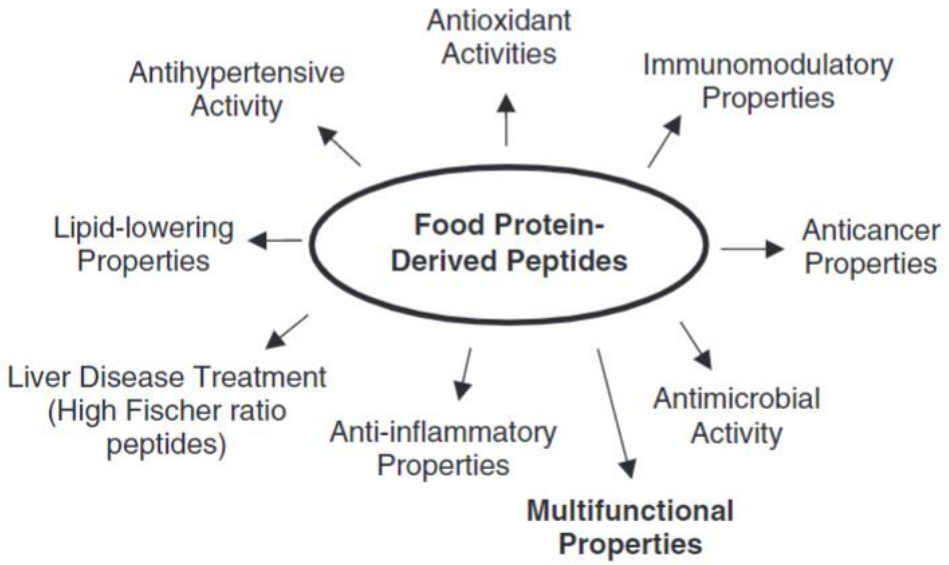

Figure 4. Bioactive properties of protein-derived peptides related to health and disease prevention (Udenigwe and Aluko, 2012).

1.6.1. Degree of Hydrolysis

The peptide amount and size obtained will vary depending on the conditions of the enzymatic hydrolysis, viz. pH, time of hydrolysis, type of enzyme, and enzyme to substrate ratio. These factors will determine the number of peptide bonds broken and, consequently, the size of said peptides. A higher amount of cuts will result in reduced average size peptides, and fewer broken bonds will result in longer peptides. This balance is defined as the degree of hydrolysis (DH), the proportion of the total peptide bonds cleaved during hydrolysis (Rutherfurd, 2010). Various techniques have been developed to determine the DH of protein hydrolysates.

1.6.1.1. TNBS Method

The TNBS method uses the reagent 2,4,6-trinitrobenzenesulfonic acid (TNBS) to determine peptide content, as it reacts with N-terminal amino groups. The reaction products are trinitrophenyl-amino acid derivatives (**Figure 5**), which exhibit a stronger yellow colour than TNBS, with an absorbance peak at 340 nm (Rutherfurd, 2010). It is essential for the reaction to occur that pH is alkaline, to preserve the primary amino groups of the peptides. Unfortunately, since the reaction only occurs when a primary amine is present, AAs such as proline and lysine do not react with TNBS. Because lysine has two primary amino groups, it reacts more strongly with TNBS (Spellman et al., 2003). The reaction is quenched by decreasing pH with an acid (Rutherfurf, 2010). The DH can then be determined by correlating the number of N-terminal amino groups after hydrolysis with the total number of N-terminal amino groups possible. The amino-acid leucine is usually used for standard (Rutherfurd, 2010).

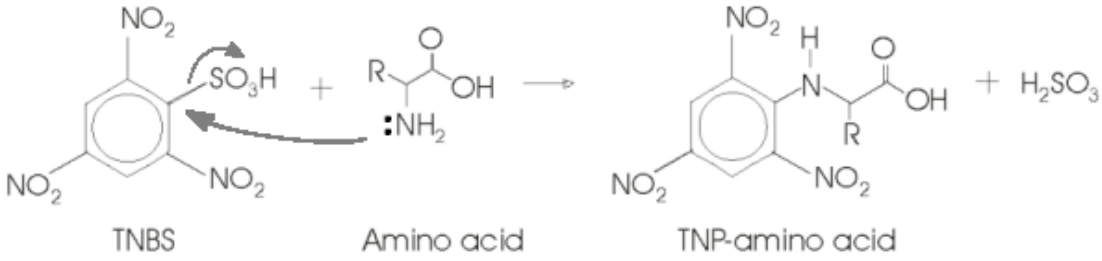

Figure 5. Nucleophilic substitution reaction between TNBS and an N-terminal amino group (adapted from Rutherfurd, 2010)

This method was first used for peptide content determination by Satake et al. (1960) and later optimized by Adler-Nissen (1979). The employment of borate buffer instead of bicarbonate buffer eliminated the inconvenient formation of carbon dioxide during the acidification step. The increase of reaction time from a few minutes to 1 h also allowed for completion of the reaction, which ensured a higher reproducibility of the method (Satake et al., 1960; Adler-Nissen, 1979).

1.7. Biological activities

1.7.1. Antioxidant

Reactive forms of oxygen (free radicals) are naturally produced in our body through various metabolic pathways and these damage the DNA, proteins and lipids of the membrane. Some causes for the appearance of these free radicals, include physiological stress, pollution, smoking and exposure to UV light. The oxidative damage provokes ageing, atherogenesis, ischemia and carcinogenesis (Guedes et al. 2011).

The ingestion of natural antioxidants frequently correlates with a decreased risk of these cardiovascular diseases and cancer. This effect is usually related to an increase in the dietary antioxidant intake of vitamins, pheolics, and carotenoids found in fruit and vegetable. (Thaipong et al., 2006). Recently, bioactive peptides from algal protein have gained more attention as natural antioxidants, possessing a tremendous radical scavenging activity (Cian et al., 2013). The measurement of antioxidant capacity of foods and compounds can be done via several different

assays, some of which including the oxygen radical absorption capacity (ORAC) and the 2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) (Thaipong et al., 2006). **Table 1** shows some advantages and disadvantages of both assays.

Table 1. Advantages and disadvantages of the ORAC and ABTS assays (Thaipong et al., 2006; Zulueta et al., 2009; Kedare and Singh, 2011)

Assay	Advantages	Disadvantages
ORAC	• Uses biologically relevant free radicals • Standardised assay • Evaluates time and degree of antioxidant reaction • Less affected by turbidity	• Most expensive • Vast quantity of results • High data variability
ABTS	• Inexpensive • Stable to pH changes • 2-5 h reaction (sample dependent) • Used in water and organic solvents	• Long preparation (12 h) • Free radical is not stable for a long time • Non-physiological radical

1.7.1.1. ORAC Method

The oxygen radical absorbance capacity (ORAC) method validated by Ou et al. (2001) consists of measuring the loss in fluorescence of fluorescein (FL) (3',6'-dihydroxyspiro[isobenzofuran-1[3H],9'[9H]-xanthen]-3-one) after the addition of AAPH (2,2'-Azobis(2-amidinopropane) dihydrochloride) – as shown in **Figure 6**.

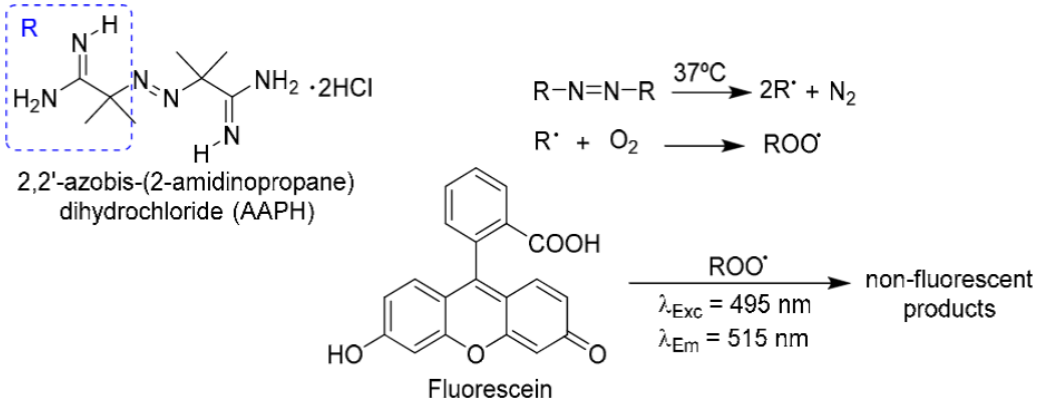

Figure 6. Simplified reaction scheme between AAPH and fluorescein in ORAC assay for detection of hydroxyl and peroxyl radicals (adapted from Santos et al., 2020)

The decomposition of AAPH by heat produces peroxyl radicals that then react with and oxidize FL. The reaction mechanism proceeds as a classic hydrogen atom transfer mechanism (Ou et al., 2001). Antioxidant agents can scavenge the free radicals, inhibiting or retarding their formation, interrupting the chain reaction, and slowing down the loss of fluorescence. Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), a water-soluble analogue of vitamin E, is commonly used as a standard for the ORAC assay and other antioxidant capacity assays. Trolox samples of different concentrations inhibit the fluorescence loss and are used to prepare a standard curve. The antioxidant capacity of the compound studied is then calculated using the calibration curve (Gómez-Ruiz et al., 2007). Dávalos et al. (2004) adapted the ORAC-FL method to manual handling, and used a conventional fluorescence 96-well plate reader to improve the efficiency considerably.

1.7.1.2. ABTS Method

The $ABTS^{\bullet+}$ scavenging assay, also known as Trolox equivalent antioxidant capacity (TEAC), is one of the most used spectrophotometric methods for determination of the antioxidant capacity of complex mixtures or single compounds – due to its simplicity, rapid and sensitive response, and reproducibility (Li et al., 2012; Sebastian et al., 2014). In this assay, the 2,2'-azino-bis(3-ethylbenzthiazoline-6- sulfonic acid) (ABTS) is oxidized by oxidants to form its radical cation $ABTS^{\bullet+}$, a deep blue/green chromophore with maximal absorption at wavelengths 414, 645,734 and 815 nm. The measurements are done at 734 nm to minimize possible interferences from other components in the sample and sample turbidity (Gülçin, 2012; Sebastian et al., 2014). In the presence of an antioxidant, the radical $ABTS^{\bullet+}$ is reduced back to ABTS by a hydrogen-donating antioxidant, thus decreasing absorbance (Ratnavathi and Komala 2016). This colour decrease depends on various factors, from antioxidant activity and concentration of the antioxidant used to the duration of the reaction (Guedes et al., 2012). The reaction time between antioxidants and radical $ABTS^{\bullet+}$ varies considerably with different compounds and matrices. Trolox, generally used as a standard, is a fast-reacting compound, while more complex mixtures exhibit different and longer reaction times. Hence, the reaction kinetics between the radical and the studied sample must be measured to determine the endpoint conditions at which the total antioxidant capacity is obtained (Magalhães et al., 2014).

The original $ABTS^{\bullet+}$ scavenging assay was developed by Miller et al. (1993), where metmyoglobin and H_2O_2 were used to form ferrylmyoglobin that would then oxidize ABTS to $ABTS^{\bullet+}$. The radical cation is generated by a chemical reaction using potassium persulfate ($K_2S_2O_8$), a potent oxidizing agent (Guedes et al., 2012; Gülçin, 2012; Ratnavathi and Komala 2016). The overall reaction in the ABTS/$K_2S_2O_8$ system is summarised in **Figure 7**.

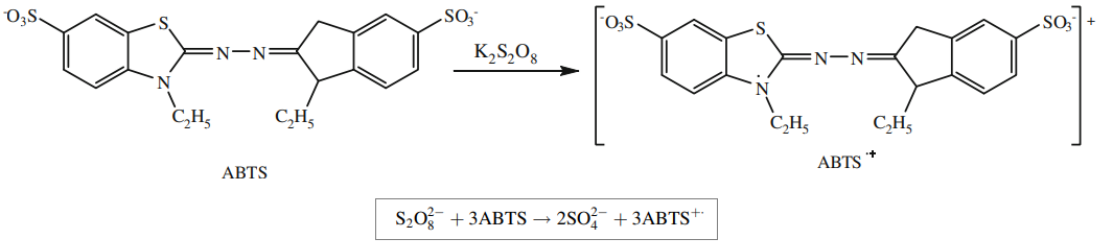

Figure 7. Overall oxidation reaction of ABTS with $K_2S_2O_8$ to from $ABTS^{\bullet+}$ (adapted from Gülçin (2012))

1.7.2. Antihypertension

Hypertension is considered a severe chronic medical condition that affects 15-20% of all adults worldwide, and increase the susceptibility to stroke and heart problems (He et al., 2013). Angiotensin-converting enzyme (ACE) is an enzyme that cleaves the decapeptide angiotensin I to octapeptide angiotensin-II, thus raising arterial pressure and increasing peripheral vascular resistance due to its vasoconstriction capabilities (Sentandreu and Toldrá, 2006; He et al., 2013). ACE also aids in degradation of the blood pressure-lowering nonapeptide bradykinin, and inactivation of the endogenous opioid peptide Met-enkephalin (Huang et al., 2013). Therefore, inhibiting ACE activity results in a decrease in formation of the vasoconstrictor angiotensin II, and an increase in concentration of the vasodilators bradykinin and Met-enkephalin, thus producing in a general antihypertensive effect as seen in **Figure 8** (Sentandreu and Toldrá, 2007; Huang et al., 2013). In addition to the direct affect, by inhibiting formation of angiotension II,

the levels of angiotension II-mediated aldosterone secretion from the adrenal cortex also decrease, thus decreasing water and sodium reabsorption and reducing cell volume, and consequently further lowering the blood pressure (Huang et al., 2013). Treatments for hypertension using synthetic drugs usually come with side effects (Vermeirssen et al., 2002). Therefore, it is important to find natural and safer alternative ACE inhibitors, such as seaweed protein, which have a unique composition due to their excellent adaptability to harsh environments (He et al., 2013). Bioactive peptides obtained via hydrolysis serve as a healthy and natural alternative, since they have been shown to inhibit ACE activity (He et al., 2013).

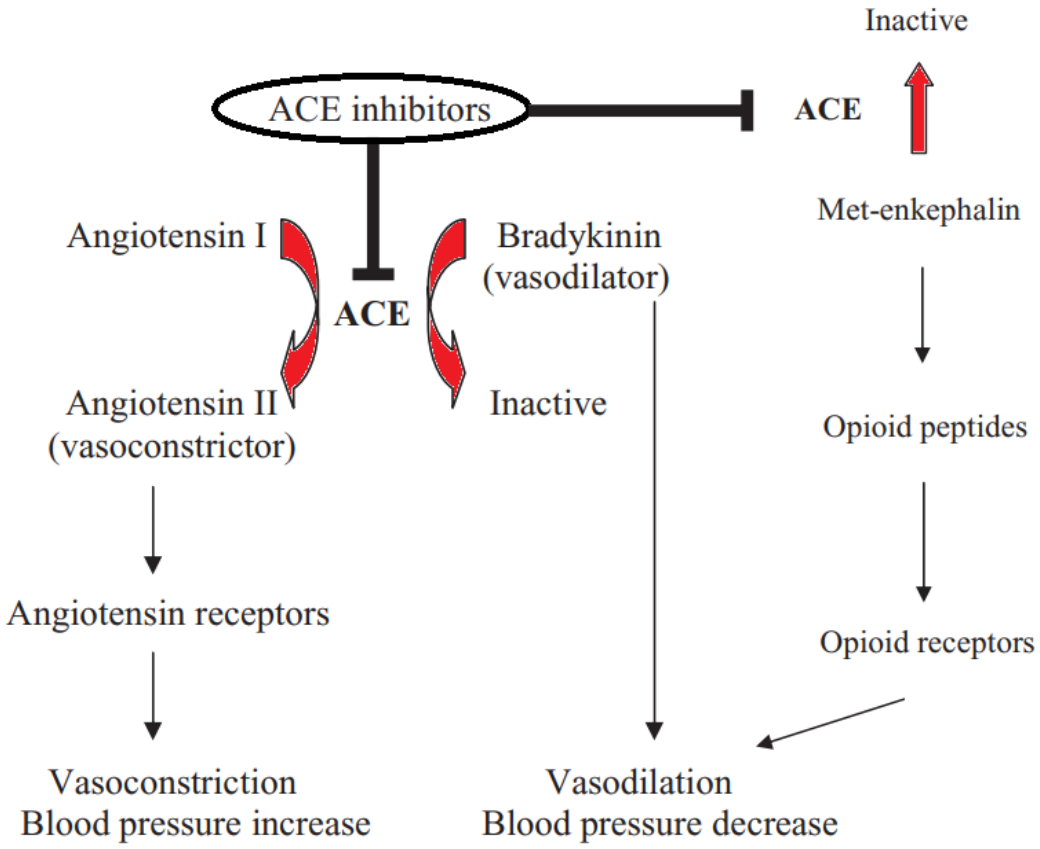

Figure 8. Mechanisms of antihypertensive effects in the presence of an ACE inhibitor (adapted from Huang et al., 2013)

1.7.2.1. Inhibition of Angiotensin-Converting Enzyme Activity

Carmel and Yarn (1978) first developed a measure of inhibition of ACE (IACE) activity. The method consisted of the use of the substrate *o*-amino benzoyl glycyl-*p*-nitrophenilalanilproline (ABz–Gly–Phe(NO2)–Pro), which the ACE would hydrolyse into *o*-amino benzoyl glycyl (Abz–Gly), a fluorescent compound. A positive control representing the entire activity of ACE could then be compared with the ACE activity when in the presence of an inhibitory substance. Later, Sentandreu and Toldrá (2006) optimized the method conditions and used a microplate fluorimeter to increase the speed of the assay (Mun'im et al., 2017).

2. Materials and Methods

2.1. Reagents

Hydrochloric acid, sodium hydroxide, potassium hydroxide, sodium phosphate monobasic, sodium sulfite, potassium phosphate dibasic, sodium phosphate monobasic monohydrate, sodium acetate anhydrous, potassium persulfate, glycerol, zinc chloride, sodium chloride, ethanol, acetic acid and tris-HCl were purchased from VWR (Leuven, Belgium). BCA Working agent (A and B) and Bovin Serum Albumin (BSA) from Thermo (Illinois, USA). Alcalase from *Bacillus licherniformis* (Lot:3851587), L-leucine and boric acid were obtained from Merck (Darmstadt, Germany) – TNBS, Fluorescein, Trolox, ABTS, ACE and all other AAs were purchased from Sigma-Aldrich (Steinheim, Germany). ABz–Gly–Phe(NO2)–Pro was purchased from Bachem (Bubendorf, Switerland) and AAPH from Cayman Chemicals (Michigan, USA).

2.2. Algae Sample Characterization and Preparation

Mastocarpus stellatus (a red seaweed) was chosen for this study due to its intrinsic high protein content. The species was harvested off the Portuguese coast in November 2021, and oven dried by CIIMAR. After dying, samples were portioned (roughly 200 g) and packed in plastic bags. Prior to extraction, samples were ground using a Knife Mill Grindomix GM 200 (Retsch, Germany) at 10,000 rpm for 20 seconds. An algal powder was obtained, ideal for cell disruption and solvent extraction afterwards. The nutritional values of the alga used were determined in a previous work (Lab. Proj., 2022), and are tabulated in **Table 2**.

Table 2. Nutritional content of *Mastocarpus stellatus* used for protein extraction in g/100 g of seaweed.

Water Content (%)	Ash Content (%)	Total Lipids (%)	Total Protein (%)	Total Dietary Fiber (%)*	Total Carbohydrates (%)
7.5 ± 0.1	21.8 ± 0.3	0.8 ± 0.1	21.5 ± 0.3	40.1 ± 0.4	48.4 ± 0.2

Values are expressed as mean ± standard error of the mean (SEM) (n=3);
*n=2; total carbohydrates include total dietary fiber.

2.2.1. Amino Acids Analysis

The AccQ-Tag method was used for derivatization and chromatographic determination. The AccQ-Tag Reagent Kit was purchased from Waters (Milford, Massachusetts, USA). The reagent kit consists of Waters AccQ-Fluor Borate Buffer, Waters AccQ-Fluor Reagent Powder, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), Waters AccQ-Fluor Reagent Diluent and Waters AccQ-Tag Eluent A (1:10).

About 20 mg of dried seaweed was weighed in 4 mL glass crimp vial. 3 mL of hydrochloric acid solution HCl (6 M) containing 0.5% (w/v) phenol was added, and sealed under vacuum by passing nitrogen gas in the vials. Acid hydrolysis was performed at 110 ◦C for 24 h, according to the reference hydrolysis method AOAC 982.30 (AOAC, 2000). 0.2 mL from the resulting hydrolysate was taken and neutralized with NaOH 6 M, and the final volume adjusted with borate buffer (0.1 M) to 1 mL. Undissolved particles were separated by ultrafiltration at 13,000 rpm for 1 min. Derivatization of AAs was performed with AccQ-Tag derivatization kit. Briefly, 10 µL of the sample was mixed with 10 µL of internal standard 50 µM (gamma-aminobutyric acid), and the mixture was added with 60 µL borate buffer and 20 µL derivatization agent. The resulting

derivatized AAs were then subjected to HPLC analysis using a Waters Alliance 2695 (Milford, MA, USA) equipped with an AccQ-Tag Amino Acids C18 Column (4 µm, 150 × 3.9 mm) (Waters, Ireland), a fluorescence detector (Waters, Milford, MA, USA) set at λEx = 250 nm and λEm = 395 nm (PMT = 100), a photodiode array (Waters, Milford, MA, USA) set to spectrum scanning within the range of λ = 210–600 nm, and a column heater (Waters, USA) set to 37.0 ˚C. After injection (5 µL sample), chromatographic separation was performed using a mixture of three eluents: a mobile phase A consisted of aqueous buffer, a mobile phase B consisted of milli-Q water, and a mobile phase C consisted of acetonitrile. Elution was performed at 1 mL/min, and chromatographic separation was accomplished as follows: 0 min (A, 100 %), 0.5 min (A, 99 %; B, 1%), 18 min (A, 97.5 %; B, 2.5%), 30.5 min (A, 86.4 %; B, 5 %; C, 8.6 %), 31.5 min (A, 82.4 %; B, 9 %; C, 8.6 %), 42 min (A, 74.4 %; B, 17 %; C, 8.6 %), 44.5 min (B, 60 %; C, 40 %) and 47.5 min (A, 100 %) for a total analysis time of 57.5 min.

AA calibration curves were constructed using the least squares linear regression model, plotting the peak area ratios of the different AAs and the corresponding internal standard versus the concentration of each analyte under study. Standard calibration curves were prepared using nine calibration points for AAs (ranging from 5 to 200 µM of each analyte).

Each test was performed in triplicate. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the calibration curve parameters – where the LOD was equal to the calculated intercept of the linear regression plus three times the Sy/x, and for LOQ ten times this value. Finally, the AA composition was reported as mg of AA/g algae DW.

2.3. Protein Extraction Optimization

The protein extraction optimization was done by studying various conditions and analysing the protein extracted. The optimum condition protocol would be maintained for the following condition studied. The conditions studied were type of solvent, order and concentration of extraction solution and temperature. A final optimization of time and solvent used in the protocol would also be implemented. The flowchart with all the conditions studied were presented in **Figure 9**.

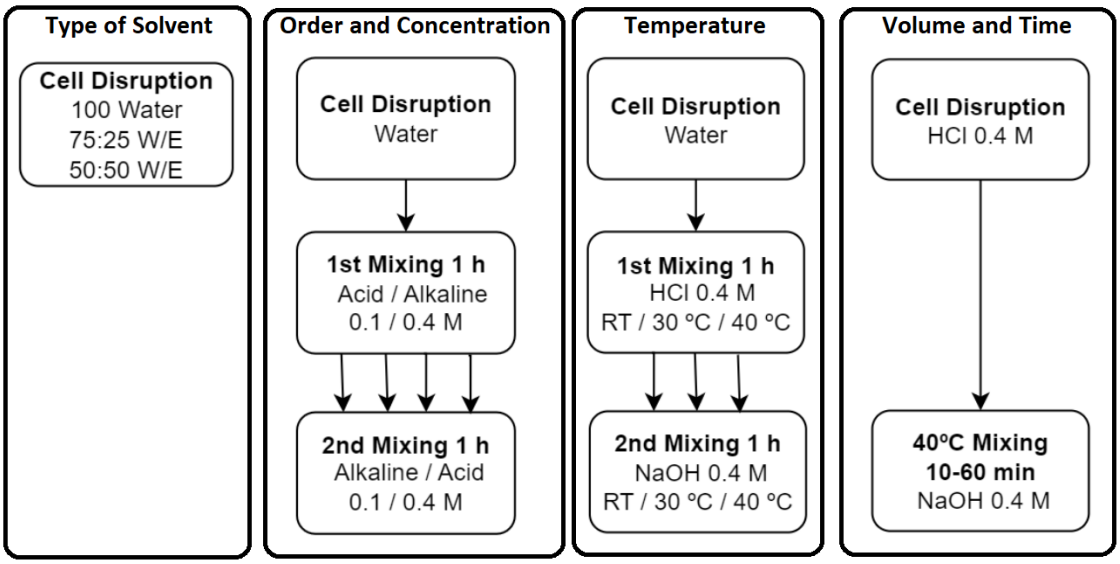

Figure 9. Flowcharts of the conditions studied in the protein extraction optimization.

The cell disruption process utilized the commercial Precellys Evolution System (Bertin, France) (**Annex A1.1**), adapted from Pagels et al. (2021). For each condition studied, 250 mg of algal powder was weighed using an analytical scale (VWR, Belgium). To each flask, 8 ceramic beads were added, in addition to 5 mL of extracting solution. The extraction solutions varied depending on the condition being studied at the time. The homogenization was performed with 6 cycles of 8000 rpm for 30 seconds with a 45 second pause in between. Afterwards, 5 mL of the same extracting solution was added to each sample, and the same program of 6 cycles was run. Each sample was centrifuged at 5000 g for 10 min at 5 ^{o}C using a centrifuge 1580R (Gyrozen, Korea), to separate the extract from the seaweed pellet (**Annex A1.2**). After centrifugation, the supernatant was decanted (**Annex A1.2**) and stored at 4 ^{o}C until analysis.

2.3.1. Water/Ethanol Extraction

In this segment, adapted from Pereira et al. (2021), different ratios of water and ethanol were used as extracting solution for cell disruption. These extractions were performed in the Precellys Evolution System with 100% distilled water, 75/25, and 50/50 water to ethanol ratios, and then centrifuged as described earlier. The extractions were performed in triplicate. The protein content of the extracts was measured by the BCA protein assay method.

2.3.2. Acid and Base Extraction

In this segment, three sequential extractions were performed – one with water, one with acid, and one with alkaline solvents. The first extraction used 100% water with a final solvent ratio of 1:40 (w/v) (250 mg of dry algae in 10 mL), using Precellys Evolution System as described in **Section 2.3**. The supernatant was stored for further analysis; and the pellet was re-suspended and treated with HCl and NaOH at different concentrations (0.1 and 0.4 M), and following different orders of extraction between the second and third extraction steps, as done by Kadam et al. (2016). The mixture (pellet and HCl or NaOH) was stirred at 300 rpm for 1 h at room temperature (**Annex 1.3**), and then centrifuged at 5000 g for 10 min at 5 ^{o}C. The supernatants were collected for analysis, and the pellet was treated with NaOH or HCl (depending on the second step extraction solvent). The mixture was stirred at 300 rpm for 1 h at room temperature and then centrifuged at 5000 g for 10 min at 5 ^{o}C. The whole procedure was repeated under the different solvent conditions, concentration (the same for the same batch) and extraction order. All supernatants were collected for analysis, and the pellet was discarded. Those three sequential extractions were performed in triplicate. All three extracts (water, acid, and alkaline) for each of the 4 conditions (NaOH 0.1 M to HCl 0.1 M; NaOH 0.4 M to HCl 0.4 M; HCl 0.1 M to NaOH 0.1 M; HCl 0.4 M to NaOH 0.4 M) were analysed using the BCA protein assay method.

2.3.3. Temperature

After selecting the best extraction protocol (cell disruption with 100% water followed by HCl 0.4 M and NaOH 0.4 M) different extraction temperatures for the second and third extraction steps were studied. The following acid and alkaline extractions were performed at room temperature, 30 ^{o}C, and 40 ^{o}C, maintaining all other parameters constant between each condition. All three extracts (water, acid and alkaline) for each of the three temperatures (room temperature, 30 ^{o}C, and 40 ^{o}C) were done in triplicate, and analysed via the BCA protein assay method.

2.3.4. Extraction Time and Solvent Reduction

2.3.4.1. Cell disruption + Acid Extraction - Time and Solvent Reduction

After selecting the optimum temperature, the cell disruption and acid extraction steps were further studied in attempts to reduce the time necessary for extracting protein and/or reducing solvent usage. The conditions mentioned in the previous **Section 2.4.3** resulted in a total extraction time of 2 h 44 min (14 min for cell disruption (CD), 10 min for centrifugation, 1 h for acid extraction, 10 min for centrifugation, 1 h for alkaline extraction, and 10 min for centrifugation); for a total of 30 mL of extract solution (10 mL H_2O + 10 mL acid + 10 mL alkaline).

Two new optimization approaches were studied. First, the cell disruption and the 1 h acid extraction were combined into a single cell disruption protocol, thus replacing the initial solution of water with the acid solution of HCl 0.4 M; this reduced the solvent usage from two 10 mL solutions (water and acid) into a single 10 mL acid solution. Afterwards, the mixture would be stirred in the acid solution for 1 h as previously, thus reducing the extraction time to 2 h 34 min (minus 10 min for centrifuging and separating the water extract from the pellet). Alternately, the mixture would immediately be centrifuged for preparation to the alkaline extraction, thus reducing a considerable amount of time to 1 h 34 min (minus 1 h for acid extraction). Two extracts (acid and alkaline) obtained per each condition (acid cell disruption direct and 1 h) were obtained in triplicate, and analysed using the BCA protein assay method – and compared to the sum of both water and acid extracts of the previous 3-step extraction (water cell disruption, acid, and alkaline).

2.3.4.2. Alkaline Extraction - Time Reduction

As for the last optimization, different extraction times of the alkaline step were evaluated, by analysing the protein content in the alkaline solution at 10, 20, 30, 40, 50, and 60 minutes while maintaining the same conditions of stirring at 300 rpm at 40 ^{o}C – while utilizing the optimum cell disruption/acid extraction determined in the previous **Section 2.4.4.1**. Samples of 200 µL were collected in 1 mL eppendorf tubes at each minutes, and centrifuged using a Sorvall Legend Micro 17 microcentrifuge (Thermo, USA) operating at 4 g for 3 minutes. All samples were performed in triplicate, and the protein content was analysed using the BCA protein assay method.

2.3.5. Optimal Extraction Conditions

After optimizing the extraction conditions, the protein from *M. stellatus* was extracted under the following conditions: 250 mg of algal powder was weighted four times into four 15 mL flasks. To each flask, 8 ceramic beads were added in addition to 5 mL of HCl 0.4 M. The homogenization was done using 6 cycles of 8000 rpm for 30 seconds with a 45 second pause in between. Afterwards, 5 mL of HCl 0.4 M was added to each sample, and the same program was rerun. All 4 samples (1 g of alga in 40 mL of the acid solution, 1:40 (w/v) ratio) were then transferred to a 50 mL falcon tube (**Annex A1.4**). The 40 mL samples were centrifuged at 8000 g for 5 min at 5 ^{o}C, to separate the acid extract from the seaweed pellet. The supernatant was collected, and the pellet suspended in 40 mL of NaOH 0.4 M solution and stirred at 300 rpm for 1 h at 40 ^{o}C. The alkaline extract was then separated by centrifugation using the conditions stated previously. Optimal conditions were used again in order to produce enough extracts with

the desired protein content. The extracts (**Annex A1.5**) were stored at -75 ^{o}C until lyophilization or filtration, by using 50 mL 1K Omega Macrosep Advance Centrifugal Devices (Pall Corporation, USA) (**Annex A1.6**) to concentrate and promote sample desalting (**Annex A1.7**) – duly checked by conductivity measurement using a pHenomenal Multi-Parameter Meter, MU 6100 L (VWR, Belgium) – values shown in **Annex A1.8**. The protein content of the concentrates was analysed using the BCA protein assay method, and concentrates were stored at -75 ^{o}C until hydrolysis was in order.

2.4. Determination of Protein Content - BCA Protein Assay

The BCA assay was used to determine the protein content of the extracts. In a 96 well plate (**Annex A2.1**) 200 µL, 8 parts, of the BCA Working Reagent was mixed with 25 µL, 1 part, of the protein extract sample in each well. The BCA Working Reagent comprises 50 parts of Reagent A (BCA in alkaline buffer) and 1 part of Reagent B ($CuSO_4$). The reaction was performed at 37 ºC for 30 min (**Annex A2.1**), and absorbance was measured at 562 nm using a FLUOstar Omega microplate reader (bmglabtech, Germany). BSA was used as the protein standard curve between 200-1000 µg/mL (BCA Instructions; BCA Tech Bulletin)). Every condition studied led to biological triplicates that would be measured in three separate wells.

2.5. Statistical Analysis

All dependent variables were tested for distribution of residuals with Shapiro–Wilk's test. The different extracts were compared using a one-way analysis of variance (ANOVA) when the normal distribution of the residuals was confirmed. Welch correction was applied when homogeneity of variances was not verified. Whenever statistical significances were found, the multiple comparison Tukey´s HDS post-hoc test for homogeneous samples and Dunnett t3 post-hoc test for heterogeneous samples were applied for mean comparison. If normal distribution of the samples was not found, the protein contents of the different extracts were ascertained using a Kruskal–Wallis test, a non-parametric test that compares every variable pairwise. All statistical analyses were performed with a 5% significance level, using SPSS for Windows, version 28.0.1.1 (15) (IBM Corporation, New York, USA).

2.6. Protein Profile

The protein profile of the extracts was obtained by HPLC, performed with a Waters (Milford, MA, USA) Alliance HPLC system (model 2695), equipped with a photodiode array detector (model 2998). An XBridge Protein BEH SEC, 450 Å (3.5 µm 7.8 x 150 mm) (Waters) column was used for chromatographic separations. The protocol suggested by the producer was followed. 100 mM sodium phosphate, pH 6.8, was used as mobile phase at 0.86 mL/min under room temperature. Samples were preserved at 4 ºC. The calibration standard (BEH450 SEC Protein Standard Mix, Waters) was used to analyse sample compounds qualitatively. The injection volume for calibration standard and sample extracts was 20 µL; for each run and elution profile, detection was at 280 nm.

2.7. Enzymatic Hydrolysis

2.7.1. Response Surface Optimization

Protein concentrates were submitted to hydrolysis using the enzyme alcalase from *Bacillus licheniformis* (2.972 U/mL). Both acid and alkaline concentrates were mixed to a ratio of 56:44, so as to form a protein concentrate of 20 mg/mL with initial pH of 10.30.

The optimization of enzymatic hydrolysis was performed using Response Surface Methodology (RSM). As constant factors, the temperature of hydrolysis was kept at 55 °C using a Shaking Water Bath 18L (VWR, Belgium), and pH was adjusted to 7.50 ± 0.25 using HCl 5 M. The factors to study and optimize were time of hydrolysis (Factor A, Time(h)) and enzyme to substrate ratio (Factor B, E/S (%, v/v)) – as per their effects upon three biological activity assays, ORAC, ABTS, and IACE, as well as DH. A standard Central Composite design of two factors (CCD2) was used to construct the response surface, thus resulting in 13 experiment points with five levels. As shown in **Figure 10**, the circumscribed design uses four edge points (2 levels, -1 and 1), four axial points (2 levels, -α and α), and a total of five centre points (1 level, 0) to measure the process stability and variability (Bhattacharya, 2021). The alpha index (α) can be determined by the following **Equation (1),** and the actual values of the variables are listed in **Table 3**.

$$\alpha = (2^k)^{1/4} \qquad \textbf{Equation (1)}$$

where k is the number of factors, and α holds a value of ca. 1.414.

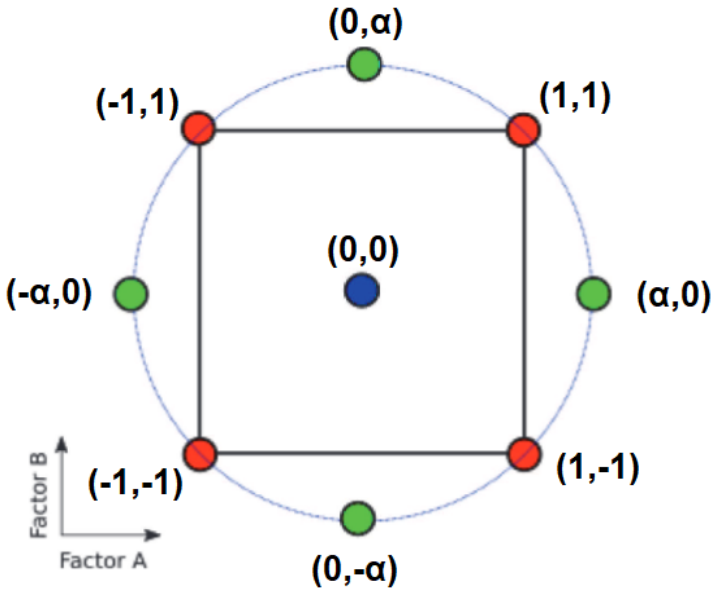

Figure 10. CCD2 model, circumscribed design and coded values of each point. (Adapted from Bhattacharya, 2021).

Table 3. Actual and coded levels of the variables used in the optimization of hydrolyses of the protein concentrates from *Mastocarpus stellatus,* using the response surface methodology.

Coded variable	**Factor level**				
	-α (-1.414)	-1	0	1	+α (1.414)
A: Time (h)	0.0	1.0	3.5	6.0	7.0
B: E/S (%, v/v)	0.1	0.5	1.5	2.5	2.9

The coded and actual values of the variable studied for each individual hydrolysis experiment can be seen in **Table 4**.

Table 4. Actual and coded levels of the independent variables of each individual hydrolysis run.

Run	Independent variables			
	A: Time		B: E/S	
	Coded	Actual (h)	Coded	Actual (%,v/v)
1	0	3.5	0	1.5
2	0	3.5	0	1.5
3	-1	1.0	1	2.5
4	0	3.5	0	1.5
5	1	6.0	-1	0.5
6	-1	1.0	-1	0.5
7	0	3.5	0	1.5
8	0	3.5	-1.414	0.1
9	1	6.0	1	2.5
10	0	3.5	1.414	2.9
11	-1.414	0.0	0	1.5
12	1.414	7.0	0	1.5
13	0	3.5	0	1.5

The results of each analytical method were assigned to their corresponding conditions, and served as experimental responses. The variables A (Time) and B (E/S) were optimized via a first-order model (**Equation (2)**) if linear, or a quadratic model otherwise (**Equation (3)**).

$$Y = \beta_0 + \beta_1 A + \beta_2 B \quad \textbf{Equation (2)}$$

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_{12} AB + \beta_{11} A^2 + \beta_{22} B^2 \quad \textbf{Equation (3)}$$

where Y is the dependent variable, A and B are the independent variables, β_0 is the intercept, β_1 and β_2 are the linear coefficients, β_{11} and β_{22} are the quadratic coefficients, and β_{12} is the two-factor interaction coefficient.

Experimental design results were analysed via response surface regression. Assessment of the goodness of fit was made via analysis of variance (ANOVA). Experimental design, data analysis, and response surface plottings were performed using the software Design-Expert v. 13.0.5.0.

After hydrolysis, the enzyme activity was discontinued by heating the sample at 100 °C for 10 min. Each sample was centrifuged, the supernatant recovered, and protein content analysed using the BCA Protein Assay method. The peptide-rich solution was then analysed using the bioactivity assays as per the next section. The optimal conditions were validated using the same protocol.

2.7.2. Degree of Hydrolysis (DH)

The DH was measured using the TNBS method, adapted from McKellar (1981). On semi-micro cuvettes (BRAND, Sigma-Aldrich), 50 µL of sample was mixed with 500 µL of borate buffer (1M) (pH 9.4) and 200 µL of TNBS solution (0.5 mM). The mixture was incubated in the dark at 25 °C for 30 min. After incubation, 200 µL of sodium sulfite (18 mM) in monobasic sodium phosphate (2 M) was added to each cuvette to quench the reaction (**Annex A2.2**). The

absorbance at 420 nm was measured using a Shimadzu UV-1800 UV/Visible Scanning Spectrophotometer (Cole-Parmer, USA). L-leucine was used as standard, and eight different concentrations between 0.4-2 mM were used for the calibration curve (**Annex A2.2**). All samples were tested as biological duplicates and chemical triplicates.

The DH of the samples was determined by correlating the number of N-terminal amino groups after hydrolysis with the total number of N-terminal amino groups possible (Rutherfurd, 2010). The total number of N-terminal amino groups was estimated by employing the TNBS method, and fully hydrolysing the protein concentrate in 5 M HCl at 100°C for 24 h (Soto-Sierra et al., 2021). The absorbance measured was converted to mg of N/mg of protein, and the %DH of each hydrolysate was calculated using **Equation (4)**:

$$\%DH = \frac{NS-N0}{NA-N0} \times 100 \qquad \textbf{Equation (4)}$$

where N0 and NS are the nitrogen content of the protein concentrates before and after hydrolysis, and NA is the nitrogen content of the protein concentrate after acid hydrolysis (maximum nitrogen content).

2.8. Bioactivity

2.8.1. ORAC Method

The ORAC method was adapted from Dávalos et al. (2004). A stock solution of FL sodium salt (1.20 mM) was prepared, and conserved at 4 °C. AAPH (46.6 mM) was prepared and FL diluted (0.117 µM) from the stock solution in 75 mM phosphate buffer (pH 7.4) on a daily basis. A stock solution of Trolox (1.05 mM) in 1 mL of methanol and phosphate buffer 75 mM pH 7.4. This solution was diluted in the same buffer and labelled as T0 solution (0.1 mM). The standard solutions were prepared from T0 solution with concentrations between 11 and 74 µM in phosphate buffer 75 mM pH 7.4 to produce the calibration curve. Each hydrolysate sample was diluted at ratios of 1:800, 1:1000, and 1:1200.

On a black polystyrene microplate (Nunc, Denmark), 20 µL of standard Trolox solutions was added in duplicate and in a "forward-then-reversed" order (11; 21; 32; 42; 53; 63; 74 µM), 20 µL of diluted hydrolysate sample was also added in triplicate (9 wells per sample, triplicates, 1 forward and 2 reverse, of each of the 3 dilutions). This approach was applied to guarantee some randomness and correct errors caused by signal drifting associated with the different positions of each well (Ou et al., 2001). In quadruplicate, a blank and a control were added with 20 and 80 µL of PBS solution, respectively. The microplate configuration used for the ORAC assay is shown in **Annex A2.3**. Afterwards, 120 µL of FL was added to every well, and the microplate incubated in the dark at 40 ºC for 10 min. After incubation, 60 µL of AAPH was added to every well, with the exception of the four control wells (the total volume in each well was 200 µL). The fluorescence was measured 104 times for 97 minutes (56 seconds/cycle), with 495 nm excitation and 515 nm emission filter, using a fluorimeter FluoSTAR OPTIMA (BMG Labtech, Offenburg, Germany). The software used was the Fluostar Control version 3.42 R5. The area under the curve (AUC) was calculated in Microsoft Excel via **Equation (5):**

$$AUC = 1 + \sum_{i=1}^{i=104} {}^{f_i}/_{f_0} \qquad \textbf{Equation (5)}$$

where f_0 is the initial fluorescence at 0 min, and f_i is the fluorescence reading at cycle *i*.

The net AUC was obtained by subtracting the mean AUC of the blanks from the corresponding microplates. The final ORAC values were calculated using the regression equation between Trolox standard solutions and net area under the FL decay curve (**Annex A2.3**). The results were expressed in µM Trolox equivalents per mg of hydrolysed protein.

2.8.2. ABTS Method

The ABTS method was adapted from Magalhães et al. (2014). A 200 mL stock solution of acetate buffer (0.05 M) at pH 4.6, a 5 mL stock solution of ABTS (7mM), and a 5 ml stock solution of potassium persulfate (2.45 mM) were prepared in distilled water, and stored at 4 ºC. A stock solution of Trolox 1.02 mM in 1 mL of methanol and acetate buffer 0.05 M pH 4.6. This solution was diluted in the same buffer and labelled T0 solution (0.1 mM). The standard solutions were obtained from T0 solution, with concentrations between 11 and 82 µM in acetate buffer 0.05 M and pH 4.6, to generate the calibration curve. Each hydrolysate sample was diluted to ratios 1:80, 1:100 or 1:120. A day before the analysis, an ABTS and potassium persulfate mixture was prepared using equal parts in volume (final ABTS concentration of 3.5 mM). The mixture was incubated in the dark, at room temperature, for 16h.

On the day of analysis, the solution of $ABTS^{\bullet+}$ (3.5 mM) was diluted multiple times in acetate buffer (pH 4.6), until obtaining a linear relationship between concentration and absorbance at 734 nm (**Annex A2.3**). The concentration of $ABTS^{\bullet+}$ that provides an absorbance value of 0.700 ± 0.020 was selected, and then used for the analyses (approximately 42.5 µM).

On a clear polystyrene microplate (Nunc, Denmark), 30 µL of standard Trolox solutions was added in duplicate (sorted as 10; 20; 31; 41; 51; 61; 72; 82 µM) for the calibration curve. For the samples and their blanks, 30 µL of diluted hydrolysate was added in triplicate (6 wells per sample, triplicates of each of the 2 dilutions) and in duplicates (4 wells per sample, duplicates of each of the 2 dilutions), respectively. In triplicate, a blank and control were filled with 30 µL of acetate buffer and water, respectively. Afterward, 170 µL of $ABTS^{\bullet+}$ (42.5 mM) was added to every well, with exception of the sample blanks, to which 170 µL of acetate buffer was added instead. A triplicate of a mixture of 30 µL of water and 170 µL of acetate buffer was also prepared for comparison with the sample blanks, to ascertain the effect of the diluted sample upon absorbance at 734 nm. The microplate configuration used for the $ABTS^{\bullet+}$ assay is shown in **Annex A2.3**.

Prior to analysis, the endpoint condition of the hydrolysates was obtained. The absorbance was measured 250 times for 417 minutes (100 seconds/cycle) at 734 nm, using a fluorimeter FluoSTAR OPTIMA (BMG Labtech, Offenburg, Germany). The software used was the Fluostar Control v. 3.42 R5. After determining the endpoint of 300 minutes (**Annex A2.3**), the absorbance values of each sample were interpolated in the Trolox calibration curve at the same reaction time, so as to obtain the results, expressed in µM Trolox equivalents per mg of hydrolysed protein.

2.8.3. IACE Activity

The IACE activity assay was adapted from Sentandreu and Toldrá (2006). A 25 mL solution of substrate ABz–Gly–Phe(NO2)–Pro (0,45 mM) was prepared in Tris-HCl buffer (150 mM) with NaCl (1125 mM), at pH 8.3. Afterwards, 0.75 mg of the Angiotensin-I Converting

Enzyme (peptidyl-dipeptidase A, EC 3.4.15.1) (Sigma Chemical, St. Louis, MO, USA) from rabbit lung was solubilized in a 5 mL water-glycerol 1:1 mixture. This enzyme solution was then diluted 1:10 in the Tris buffer (pH 8.3) with 1 µm of zinc chloride. Said solution was stored at 5 °C until analysis. Samples were diluted in six solutions of ratios of 1:1 to 1:32 or 1:4 to 1:128.

On a black polystyrene microplate (Nunc, Denmark), 160 µL of substrate was added to every well. The controls and sample blanks were filled with 40 µL of distilled water, in duplicate; and the blank was filled with 80 µL instead. For each sample, 40 µL of each of 6 diluted solution was added in triplicate for the samples, and in duplicate for sample blanks. Finally, 40 µL of ACE was added to the controls and samples. The microplate was immediately incubated at 37 ºC for 30 min. The fluorescence was measured with 350 nm excitation and 420 nm emission filter, using a fluorimeter FluoSTAR OPTIMA (BMG Labtech, Offenburg, Germany). The software used was the Fluostar Control v. 3.42 R5. The IACE activity was calculated using **Equation (6)**:

$$\%\, IACE = \frac{(Fc-Fb)-(Fs-Fsb)}{Fc-Fb} \times 100 \qquad \textbf{Equation (6)}$$

where Fc is the fluorescence emitted by Abz–Gly, the product of the reaction between substrate and ACE without inhibitor, Fb is the fluorescence emitted by ABz–Gly–Phe(NO2)–Pro, Fs is the fluorescence emitted by Abz–Gly in the presence of inhibitory sample, and Fsb is the fluorescence emitted by ABz–Gly–Phe(NO2)–Pro in the presence of inhibitory sample.

The inverse of IACE activity was plotted versus the inverse of concentration (**Annex A2.4**), thus a linear relationship and obtaining the IC_{50} (µg protein/mL) of each hydrolysate by interpolation (Sentandreu and Toldrá, 2007).

3. Results and Discussion

3.1. Amino Acid Composition

The following 16 AAs were detected and quantified in *M. stellatus*: L-Aspartic acid, L-Alanine, L-Arginine, L-Glutamic acid, L-Glutamine, L-Glycine, L-Proline, L-Serine, L-Tyrosine, L-Isoleucine, L-Leucine, Lysine, Methionine, Phenylalanine, L-Threonine, Valine. The algal protein AA composition can be seen in **Table 5**.

M. stellatus AA composition had the highest content of Val and Phe (8.72 ± 5.50 and 13.35 ± 7.12 mg AA/g algae DW), two of the seven EAAs quantified. The AAs Asp, Ala, Arg, Glu, Gly, Gln and Ile were also found in concentrations between 2.89-7.24 mg AA/g algae DW. The remaining AAs were found at the lowest concentrations, less than 2 mg AA/g algae DW. From the total protein present in *M. stellatus,* note that a mere 28% (65 mg AA/232 mg protein in a g of alga DW) was quantified by the AccQ-Tag method.

Table 5. Amino acids present in *Mastocarpus stellatus,* and corresponding concentrations in mg of AA/g of alga DW.

Amino acid	3 letter-code	Concentration (mg AA/alga DW)[1]
Aspartic acid	Asp	4.19 ± 1.20
Alanine	Ala	5.24 ± 1.13
Arginine	Arg	7.24 ± 1.08
Glutamic acid	Glu	3.52 ± 0.84
Glutamine	Gln	5.25 ± 0.10
Glycine	Gly	4.39 ± 1.63
Proline	Pro	1.95 ± 0.10
Serine	Ser	1.17 ± 0.41
Tyrosine	Tyr	1.06 ± 0.33
Isoleucine*	Ile	2.89 ± 0.01
Leucine*	Leu	1.82 ± 0.68
Lysine*	Lys	1.71 ± 0.69
Methionine*	Met	0.12 ± 0.05
Phenylalanine*	Phe	13.35 ± 4.96
Threonine*	Thr	1.97 ± 0.90
Valine*	Val	8.72 ± 3.16

[1] Values are expressed as mean ± standard error; Obtained by the AccQ-Tag method (mg AA/g alga DW)
*Essential Amino Acids

Considering the total AAs detected (**Figure 11**), Val and Phe constitute 21% and 13% respectively, extraordinary high amounts of a single EAA compared to the standard 2-8% found in animal and plant protein sources (Gorissen et al. 2018). These EAAs have various benefits; val is a branched-chain amino acid (BCAA) that stimulates muscle growth, muscle regeneration and energy production. It also helps lowering high blood sugar levels and increases growth hormone production (pubchem.nlm). Phe plays a key role in the function and structure of proteins and enzymes, being an essential factor for the production of other AAs. It may also be used as an anti-depressant and to ease schizophrenia symptoms (pubchem.nlm). Additionally, the EAA content of *M. stellatus* is higher than other more common protein sources, having 47% compared to 26% in plant-based and 37% in animal-based proteins (Gorissen et al. 2018).

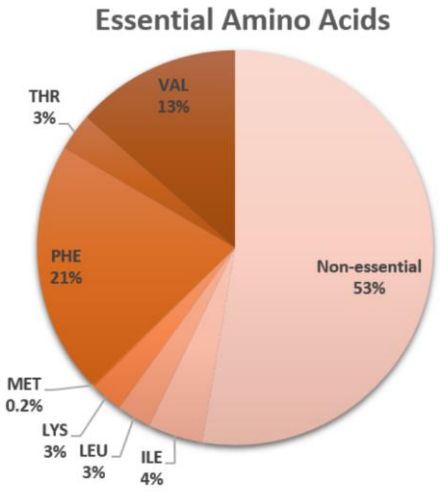

Figure 11. EAAs composition from the detected AAs from *M. Stellatus.*

Blanco-Pascual et al. (2014) also analysed the AAs in *M. stellatus,* using an AA analyser – and reported a total of 18 AAs, 9 of which were essential. Ser, Gly, Ala, Asp and Glu had the highest contents reported. Other articles that analysed green, red, and brown macroalgae species also reported 16-17 AAs, with at least 8 EAAs among them (Kadam et al., 2017; Kazir et al., 2019). Although only 7 of the 9 EAAs were quantified, the literature confirms the presence of all EAAs. M. *stellatus*, is a source of high-quality protein, proving that it can be used as a better substitute for animal protein than plant-based protein.

3.2. Protein Extraction Optimization

3.2.1. Water/Ethanol Extraction

The effects of different ratios of water and ethanol during the cell disruption step on the protein contents (g of protein/ 100 g alga) are shown in **Table 6**. Plain water extracted the most protein, 2.8%, compared to the solvent ratio of 75:25 water/ethanol (W/E), 1.5%, and 50:50 W/E, 1.2%. Water alone showed to be that at most effective protein extraction solution in the cell disruption phase, extracting approximately 86% more protein when compared to 75:25 water/ethanol and 133% more than 50:50 water/ethanol. However, the amount of protein extracted with water is only about 13% of the total crude protein present in *M. stellatus*, which has approximately 21.5% total protein per g of alga (Lab. Proj. 2022).

Table 6. Experimental design of water/ethanol ratio solutions and results – protein concentration in the solution (mg/mL), total protein extracted (mg), and yield (g of protein/100 g per g of alga).

Extraction Solution (W/E)	Protein concentration (mg/mL)[1]	Total Protein (mg) [2]	Yield (g protein/100 g alga) [3]
100/0	0.7 ± 0.1	7.2 ± 0.8	2.8 ± 0.3[a]
75/25	0.4 ± 0.1	3.7 ± 0.2	1.5 ± 0.1[b]
50/50	0.3 ± 0.2	3.2 ± 2.1	1.2 ± 0.1[c]

Values are expressed as mean ± standard error of the mean (SEM) (n=3). The final yields marked by the same letters were not significantly different ($p > 0.05$), so different letters indicate significant differences ($p < 0.05$). Analysis of variance was used to estimate the effect of extracting solution on the final yield: Tukey's HDS Post Hoc Test: *$p < 0.05$.

[1]obtained by BCA (bicinchoninic acid) protein assay method

[2]10 mL extract solution

[3]initial dry algae mass of 250 mg

The cell disruption method utilized in this study provides sufficient shear force that exerts high stress upon the cell wall – thus – weakening and disrupting its structure, and maximizing protein accessibility in a highly efficient manner (Geada et al., 2021). A large portion of anionic polysaccharides on the cell wall is bound to proteins that interfere with their release, thus making this cell wall breakdown essential to the protein extraction process (Bleakley and Hayes, 2017). Oven drying of biomass before extraction can decompose phenolic compounds that aid in disruption of the polysaccharides, and further increase protein extractability (Gordalina et al., 2021). Bead milling can also be used on an industrial scale. However, operating parameters must be optimized for the specific cell type aiming at increased disruption rates, and heating and power consumption problems may eventually affect sustainability of the industrial process (Geada et al., 2021).

Water and ethanol are two of the most used solvents to extract protein from seaweed. Studies utilizing each solvent separately have conclude that water extracts higher protein content than ethanol (Phong et al., 2016, and Janani et al., 2019). This study focused only on lower ethanol contents, because higher protein content extracts are commonly obtained when the water/ethanol ratio is above 50%. For example, Zhang et al. (2017) studied various water/ethanol mixtures with different cell disruption techniques upon microalga extraction, obtaining maximum protein extraction with only 11% ethanol content. Pereira et al. (2021) studied four water/ethanol ratios with red macroalgae, and reported a 20% increase in protein content when using 75:25 water/ethanol, and a 30% increase when using 50:50 water/ethanol when compared to 100% water. Such trend was not confirmed in this study, where water had a more significant yield compared to the mixtures. This could be due to the use of different algae with distinct protein profiles. Albumins are soluble in water, whilst other protein types, such as prolamins, are soluble in 70% alcohol in water (Gordalina et al., 2021). Hence, *M. stellatus* should have a higher content of albumins than prolamins, and a higher content of proteins bound to the anionic polysaccharides more soluble in water and acid solutions (Cermeño et al., 2020). Nevertheless, the amount of protein content extracted is not even one fourth of the total content of protein of *M. stellatus*, since only the water-soluble proteins were recovered – thus calling for implementation of other liquid extraction systems (acid and alkaline solutions) that generally lead to higher yields (Cermeño et al., 2020).

Additionally, pure ethanol is an effective solvent for pigment extraction, for extracting both carotenoids and chlorophylls from macroalgae (Kulkarni and Nikolov, 2018). The use of ethanol for pigment removal could be beneficial, as it leads to a high-value product – while not affecting protein extractability that much (Kulkarni and Nikolov, 2018). Although not studied, this increased extraction of pigments can be seen in the colouration of the different extracts, see **Annex A3.1**. Higher ethanol concentrations resulted in a more greenish extract, as reported in the literature (Pereira et al., 2021).

3.2.2. Acid and Alkaline Extraction

In this segment, different acid and alkaline solutions at different concentrations of HCl and NaOH were studied for their ability to extract further protein after the cell disruption step. The protein contents (g of protein/100 g alga) are shown in **Table 7**. The total protein recovery yield increased when higher concentrations were used. The increase from 0.1 M to 0.4 M on both acid and alkaline solutions showed an increase from 7.3% to 8.5% when the alkaline extraction was performed first (NaOH → HCl) and from 7.5% to 11.1% when the acid extraction was performed first (HCl → NaOH). The order of extraction did not unfold any significant difference when the concentrations of the solutions was 0.1 M, having total yields of 7.3 (NaOH → HCl) and 7.5 (HCl → NaOH). When the concentration used was 0.4 M, the order of the solutions varied significantly, from 8.5% when acid extraction was done first (HCl → NaOH) to 11.1% when alkaline extraction was done first (NaOH → HCl). The acid extraction at 0.4 M HCl, followed by the alkaline extraction at 0.4 M NaOH was the most effective for extracting protein among all conditions studied – extracting approximately 51.6% of the total protein in *M. stellatus*. The pH of the initial solutions (HCl_i and $NaOH_i$) and extracts (first and second) are found in **Annex A3.2**.

Table 7. Experimental design of sequential water cell disruption (CD) and acid/alkaline extractions and results – protein concentration in solution (mg/mL), total protein extracted (mg) in each step, yield (g of protein/100 g alga) of said step, and total yield (g of protein/100 g alga).

Extraction Steps	Protein concentration (mg/mL)[1]	Total Protein (mg) [2]	Step Yield (g protein/ 100 g alga) [3]	Total Yield (g protein/ 100 g alga) [3]
H_2O CD	0.7 ± 0.1	7.2 ± 0.8	2.8 ± 0.3^{C}	-
H_2O → 0.1 M NaOH	1.0 ± 0.1	9.9 ± 0.4	3.9 ± 0.2^{B}	6.7 ± 0.2^{d}
H_2O → 0.4 M NaOH	1.3 ± 0.1	12.5 ± 0.3	5.0 ± 0.1^{A}	7.8 ± 0.1^{c}
H_2O → 0.1 M HCl	0.1 ± 0.1	1.3 ± 0.1	0.5 ± 0.1^{D}	3.3 ± 0.1^{e}
H_2O → 0.4 M HCl	0.1 ± 0.1	1.0 ± 0.1	0.4 ± 0.1^{D}	3.2 ± 0.1^{e}
H_2O → 0.1 M NaOH → 0.1 M HCl	0.1 ± 0.1	1.3 ± 0.1	0.5 ± 0.1	7.3 ± 0.2^{c}
H_2O → 0.4 M NaOH → 0.4 M HCl	0.2 ± 0.1	1.6 ± 0.1	0.6 ± 0.1	8.5 ± 0.1^{b}
H_2O → 0.1 M HCl → 0.1 M NaOH	1.1 ± 0.1	10.6 ± 0.6	4.1 ± 0.2	7.5 ± 0.3^{c}
H_2O → 0.4 M HCl → 0.4 M NaOH	1.9 ± 0.2	19.5 ± 1.9	7.8 ± 0.8	11.1 ± 0.2^{a}

Values are expressed as mean ± standard error of the mean (SEM) (n=3). The final yields marked by the same lowercase letters were not significantly different ($p > 0.05$) and thus, different lowercase letters indicate significant differences ($p < 0.05$). The yields of the CD and first extractions steps were also marked by capital letters ($p < 0.05$). Analysis of variance was used to estimate the effect of extracting conditions on the final yield: Tukey's HDS Post Hoc Test: *$p < 0.05$.

[1]obtained by BCA (bicinchoninic acid) protein assay method

[2]10 mL extract solution

[3]initial dry algae mass of 250 mg

After further analysing **Table 7** – and although the extraction yields of the water cell disruption (2.8%) were significantly greater than those of the first acid extraction (0.4-0.5%), the influence of the acid extraction on the total yield is significant when compared with the treatment with alkaline solution alone. Implementing a previous acid extraction increased the total yield to 7.5% for 0.1 M and 11.1% for 0.4 M, from 6.7% and 7.8% respectively. This justifies implementation of the acid extraction step, since it benefits the overall extraction; even though it did not contribute much to the increase of total yield as a single step, it enhanced the next alkaline extraction.

Conventional algal protein extraction is usually based on a physical or enzymatic process to disrupt the cell wall, often aided by one or multiple solvent extractions (Bleakley and Hayes, 2017). The study by Kadam et al. (2016) reported on the extraction of protein from *Ascophyllum nodosum*, a brown seaweed, with the same HCl and NaOH solutions. They presented similar results, where the acid extractions extracted less protein (8.0-16.9%, increasing with the increase of acid concentration) compared to the alkaline extraction (49.6-56.4%, only increasing significantly with 0.4 M). Compared to this study, the percentage of protein extracted varies considerably, extracting only 14.9-15.3% with acid extraction and 31.2-36.3% with alkaline extraction. Nonetheless, the sequential extraction using acid and alkaline was the most efficient

method for extracting protein in both studies, with 59.8% in the study by Kadam et al. (2016) compared to the 51.6% observed in this study. The increase in yield when acid is used before alkaline extraction is thought to degrade the polysaccharides present in the cell wall, thus leading to release of proteins and promoting their solubility. This solubilisation is better perceived under alkaline conditions, once the water-insoluble hydrophobic proteins are present in seaweed (Kadam et al., 2016).

The variation between the study by Kadam et al. (2016) and this study is expected, mainly due to the comparison between two different species of seaweed, from different phyla; although the same solvent extraction was implemented, different cell disruption procedures were elected (Field et al., 2016; Cermeño et al., 2020). Different species of algae have distinct chemical compositions, including protein content and cell wall morphology, so they react differently to the same extraction procedure (Field et al., 2016). The disruption through soaking instead of a homogenizer also affects the outcome of the water extraction, thus affecting the total yield of extraction (Safi et al., 2014).

3.2.3. Temperature

In the present section, the mixing phases of both acid and alkaline extractions were performed at different temperatures, thus affecting the protein contents (g of protein/100 g alga), shown in **Table 8**. The increase in temperature produced an increase in total protein extracted. The increase from room temperature to 30 °C resulted in an increase of protein extracted from 11.1% to 12.5%; when increased to 40 °C, the total yield improved further to 14.2%. The acid and alkaline extraction at 40 °C was the most effective for extracting protein from each temperatures studied, extracting approximately 66.0% of the total protein in *M. stellatus*.

One of the main factors affecting protein viability is the extraction temperature (Cermeño et al., 2020). It may affect it positively, by increasing protein solubility in the solvent through an improvement in diffusion rate/mass transfer of the molecule (Xi, 2017). Many studies show that higher temperatures lead to a more significant protein content recovery in solvent extractions from algae (Slocombe et al., 2013; Chu et al.. 2015; Chotipan et al., 2016; and Amorim et al., 2020). Also, moderate heat can increase protein digestibility by partially denaturing the tertiary structure and revealing accession sites crucial to the gastrointestinal tract (GI) enzymes (O'Connor et al., 2020).

However, one of the reasons for not exploring higher temperatures of extraction in this study was the concern with stability of the proteins and thermolabile compounds. Higher temperatures denature the protein, thus decreasing solubility; by losing its structure, potential bioactivity is comprimised, and precipitation due to coagulation may occur, so a considerable amount is lost in the extraction process (Zocher et al., 2019; Amalia et al., 2021). More extreme temperatures also negatively affect the bioactive protein capacities, mainly through degradation of enzymes and racemization of AAs (Cermeño et al., 2020; Amalia et al., 2021). Racemization of AAs due to extreme heat influences negatively the accessibility and digestibility of proteins (O'Connor et al., 2020). L-amino acids become D-amino acids, which are less digestible and decrease the overall nutritional quality of the protein (Friedman, 1999). Finally, another concern would be the scalability of the extraction method. The higher the temperature, the more

dangerous and more expensive the process becomes – thus making it unfeasible and unsustainable at larger scales (Bleakley and Hayes, 2017).

Table 8. Experimental design of sequential water cell disruption (CD) and acid/alkaline extractions at different temperatures and results – protein concentration in solution (mg/mL), total protein extracted (mg) in each step, yield (g of protein/100 g alga) of the step, and total yield (g of protein/100 g alga).

Extraction Temperatures and Steps	Protein concentration (mg/mL) [1]	Total Protein (mg) [2]	Step Yield (g protein/ 100 alga) [3]	Total Yield (g protein/ 100 g alga) [3]
H_2O CD	0.7 ± 0.1	7.2 ± 0.8	2.8 ± 0.3	-
RT: H_2O → 0.4 M HCl	0.1 ± 0.1	1.0 ± 0.1	0.4 ± 0.1	3.2 ± 0.1
30 °C: H_2O → 0.4 M HCl	0.1 ± 0.1	1.4 ± 0.1	0.6 ± 0.1	3.4 ± 0.1
40 °C: H_2O → 0.4 M HCl	0.2 ± 0.1	2.1 ± 0.5	0.8 ± 0.2	3.6 ± 0.2
RT: H_2O → 0.4 M HCl → 0.4 M NaOH	1.9 ± 0.2	19.5 ± 1.9	7.8 ± 0.8	11.1 ± 0.2[c]
30 °C: H_2O → 0.4 M HCl → 0.4 M NaOH	2.3 ± 0.3	22.8 ± 0.1	9.1 ± 1.0	12.5 ± 0.3[b]
40 °C: H_2O → 0.4 M HCl → 0.4 M NaOH	2.6 ± 0.2	26.5 ± 2.4	10.6 ± 0.3	14.2 ± 0.4[a]

Values are expressed as mean ± standard error of the mean (SEM) (n=4); The final yields marked by the same letters were not significantly different ($p > 0.05$), so different letters indicate significant differences ($p < 0.05$); Analysis of variance was used to estimate the effect of extracting conditions on the final yield: Tukey's HDS Post Hoc Test: *$p < 0.05$.
[1]obtained by BCA (bicinchoninic acid) protein assay method
[2]10 mL extract solution
[3]initial dry algae mass of 250 mg

3.2.4. Extraction time and solvent reduction

3.2.4.1. Cell Disruption + Acid Extraction - Time and Solvent Reduction

After selecting the optimum temperature, combining both cell disruption and acid extraction steps led to a significant increase of total protein yield in both cases. The protein contents (g of protein/100 g algae) of the previous optimum condition and the two new conditions are shown in **Table 9**. The previous condition originated a total of 20 mL of extract, but extracting only 3.6%. The new conditions reduced the total extract solution to 10 mL, but increased the yields to 7.2 ± 0.1% for the direct method and 6.9 ± 0.6% for the 1 h acid-only extraction – an increase of 100% and 92% compared with the previous 3-step extraction. Although no significant difference was found between the direct and 1 h acid-only extractions since, the direct method takes only 1 h 34 min compared to the 2 h 34 min – a 64% increase in efficiency. The direct method was the most effective in extracting protein, extracting approximately 32.1% of the total protein in *M. stellatus* with the acid extraction alone.

Table 9. Experimental design of cell disruption (CD) and acid extraction and results – protein concentration in solution (mg/mL), total protein extracted (mg) for each condition, and total yield (g of protein/100 g alga) of the extraction.

Cell Disruption/ Acid Extraction Step	Protein concentration (mg/mL) [1]	Total Protein (mg) [2]	Total Yield (g protein/ 100 g alga) [3]
H_2O CD + 40 °C: 0.4 M HCl 1 h	-	9.3 ± 0.5[*]	3.6 ± 0.2[b]
0.4 M HCl CD	1.8 ± 0.1	17.9 ± 1.2	7.2 ± 0.1[a]
0.4 M HCl CD → 40 °C, 1 h	1.8 ± 0.4	17.5 ± 4.1	6.9 ± 0.6[a]

Values are expressed as mean ± standard error of the mean (SEM) (n=3); The final yields marked by the same letters were not significantly different ($p > 0.05$), so different letters indicate significant differences ($p < 0.05$); Analysis of variance was used to estimate the effect of extracting conditions on the final yield: Independent-Samples Kruskal-Wallis Test: *$p < 0.05$.

[1]obtained by BCA (bicinchoninic acid) protein assay method

[2]10 mL extract solution

[3]initial dry algae mass of 250 mg

[*]10 mL H_2O + 10 mL HCl extract solutions

The main disadvantages of solvent extraction are the use of vast amounts of solvent and the fact that it is time-consuming (Bleakley and Hayes, 2017). Minimizing these problems without compromising the protein extraction yield increases efficiency, productivity, and sustainability. Other studies that combine cell disruption techniques with acid and/or alkaline treatments have also reported increases in efficiency. Increases of over 500% in protein extraction, as well as reduction of process times to one-sixth of the original process (Kadam et al., 2017) and increases of 200% of disrupted biomass (Mendes-Pinto et al., 2001) indicate that this combination is an effective way to improve the sustainability of the process.

During cell disruption, the collision between beads causes a permanent production of thermal energy that increases the mixture temperature (around 55 °C). Possibly due to this increase in temperature, both direct and 1 h acid cell disruptions were characterized by similar reaction yields. As seen before, higher temperatures increase the diffusion speed of compounds, thus releasing protein quickly and reaching a maximum concentration within a short time (Xi, 2017; Garcia et al., 2018).

The decision to remove the water extraction portion over the acid portion was taken based on the previous conclusion in the **Section 3.2.2**. It was shown that the acid extraction enhances the alkaline extraction afterwards, although the acid step alone did not contribute much to the total yield.

3.2.4.2. Alkaline Extraction - Time Reduction

The effects of different times of alkaline extraction step upon the protein contents (g of protein/100 g alga) are shown in **Table 10**. The alkaline extraction step yield were 8.2%, 8.6%, 9.7%, 10.5%, and 11.0% for 10, 20, 30, 40 and 50, and 60 min respectively. The time of extraction showed an increase in protein extracted, except for 40 and 50 min, with no significant difference found. The longest extraction was the most effective at extracting protein, approximately 51% of the protein present in *M. stellatus* on the alkaline extraction alone, and extracting approximately 83.3% (32.1% in acid CD and 51.2% in alkaline extraction) of the total protein present in *M. stellatus*.

Table 10. Experimental design of the alkaline extraction at 40 °C with different durations and results – protein concentration in solution (mg/mL), total protein extracted (mg) for each time, and yield (g of protein/100 g alga) of the extraction.

Alkaline Extraction Time (0.4 M NaOH 40 °C)	Protein concentration (mg/mL) [1]	Total Protein (mg) [2]	Step Yield (g protein/ 100 g alga) [3]
10 min	2.1 ± 0.1	21.1 ± 0.9	8.2 ± 0.1^{e}
20 min	2.2 ± 0.1	22.2 ± 0.7	8.6 ± 0.1^{d}
30 min	2.5 ± 0.1	24.9 ± 0.7	9.7 ± 0.1^{c}
40 min	2.7 ± 0.2	26.9 ± 1.7	10.5 ± 0.5^{b}
50 min	2.7 ± 0.2	27.1 ± 2.0	10.5 ± 0.7^{b}
60 min	2.8 ± 0.2	28.2 ± 2.1	11.0 ± 0.7^{a}

Values are expressed as mean ± standard error of the mean (SEM) (n=4); The final yields marked by the same letters were not significantly different ($p > 0.05$), so different letters indicate significant differences ($p < 0.05$); Analysis of variance was used to estimate the effect of extracting conditions on the final yield: Tukey's HDS Post Hoc Test: *$p < 0.05$.

[1]obtained by BCA (bicinchoninic acid) protein assay method

[2]10 mL extract solution

[3]initial dry algae mass of 250 mg

The main objective here encompassing protein extraction was to obtain the most protein and as efficiently as possible. Since there is a significant increase in yield between 60 min and the remaining extraction times, this was chosen as the alkaline extraction time of the final process. Obviously that, the greater the time of extraction, the more protein content can be solubilized, thus leading to greater yields. However, various studies have shown that this benefit is limited, specifically when it is combined with a higher temperature (Amorim et al., 2020; Bai et al., 2021). Lower yields of protein are obtained under higher temperatures and extraction times. The results pertaining to denaturation and protein degradation possibly favour Maillard reactions between AAs and carbohydrates (Amorim et al., 2020). This was the main reason for not increasing the extraction time even more, as it would eventually lead to less protein extracted. Moreover, this segment focused on optimizing the time of the overall process, so any increase in extraction time would lower the productivity of the overall process.

3.2.5. Optimal Extraction

The implementation of the optimum conditions determined earlier as well as a small alteration to the scale of the extraction – where the ratio of solid to liquid was maintained but the total solid and liquid were quadrupled, resulted in a slight change in step yields. The effects of the scale of extraction on protein contents (g of protein/100 g algae) are shown in **Table 11.** A significant increase ($p < 0.05$) in total protein extracted was observed compared to the previous protocol, increasing from 17.9 to 20.1 g of protein/ 100 g of alga, approximately 93.5% of the total protein present in *M. stellatus*. This increase in yield was due to the excessive use of the Precellys equipment, increasing the temperature of the flasks due to the successive homogenizations. This increase in temperature (above 55 °C) resulted in an increase in protein solubility in the acid extraction phase, extracting 10.8 instead of 7.2%.

During ultrafiltration, the 1 kDa cut-off is ideal for removing the HCl and NaOH present in the concentrates while not allowing protein to permeate through the filter. The yield of the

filtration step for the acid and alkaline concentrates was 55% and 72%, respectively. The protein loss is due to small peptides (<1 kDa) (Wu et al., 2005) and AAs (average molecular weight (MW) of 118.9 Da) (Piques et al., 2009) permeating through the membrane. The acid concentrate experienced a greater loss in protein, probably because of retention of the protein in the gel-like substance composed of polysaccharides when decanting and because of the more significant number of peptides and AAs due to partial hydrolysis from the acid (Soto-Sierra et al., 2021). Nevertheless, the acid and alkaline extracts were concentrated 6.56 and 10.1-fold.

Table 11. Optimal extraction conditions and results – protein concentration in solution (mg/mL), total protein extracted (mg) in each step, yield (g of protein/100 g alga) of the step, and total yield (g of protein/100 g algae).

Extraction Steps	Protein concentration (mg/mL)[1]	Total Protein (mg) [2]	Step Yield (g protein/ 100 g alga) [3]	Total Yield (g protein/ 100 g alga) [3]
0.4 M HCl CD	2.7 ± 0.3	108 ± 11	10.8 ± 1.1	-
0.4 M HCl → 40 °C, 0.4 M NaOH, 1h	2.3 ± 0.1	92 ± 6	9.2 ± 0.6	20.1 ± 0.6

Values are expressed as mean ± standard error of the mean (SEM) (n=4).
[1]obtained by BCA (bicinchoninic acid) protein assay method
[2]40 mL extract solution
[3]initial dry algae mass of 1000 mg

3.3. Peptides Size

The molecular weight (MW) distribution of M. *stellat*us protein extracts and concentrates before and after hydrolysis was analysed by size exclusion chromatography. The calibration standard (BEH450 SEC Protein Standard Mix, Waters) originated a chromatogram with 5 distinct analyte peaks (**Figure 12a**). **Table 12** displays each analyte, with the corresponding MW and retention time.

Table 12. BEH450 SEC Test calibration standard mix – analytes and corresponding molecular weight and retention time.

Analyte	MW (Da)[1]	Retention time (min)
Thyroglobulin	669000	5.3
Immunoglobulin G	150000	6.2
Bovine serum albumin	66400	6.5
Myoglobin	17000	7.0
Uracil	112	7.4

[1]Values from Waters care and use manual.

By analysing chromatogram **b)**, two peaks can be distinguished. When comparing the retention time with the analyte standard mix, a protein complex with MW greater than 669 kDa, and a smaller protein that ranges between 17-66.4 kDa can be identified. All solvent extractions contained the smaller proteins, whilst only the acid and alkaline extraction contained the bigger protein complexes; with the alkaline extract, a much more significant fraction was detected. Kadam et al. (2017) reported this same behaviour between the acid and alkaline extracts, where high MW proteins of over 1000 kDa were identified in the alkaline extract. This seems to happen only when there is a previous extraction with acid (Kadam et al., 2017), probably due to

solubilisation of a different faction of proteins that were not accessible before acid treatment. The proteins in question could be glycoproteins previously attached to the cell wall, released by the acid treatment due to the anionic nature of the algal cell walls (Domozych et al., 2012; Cermeño et al., 2020).

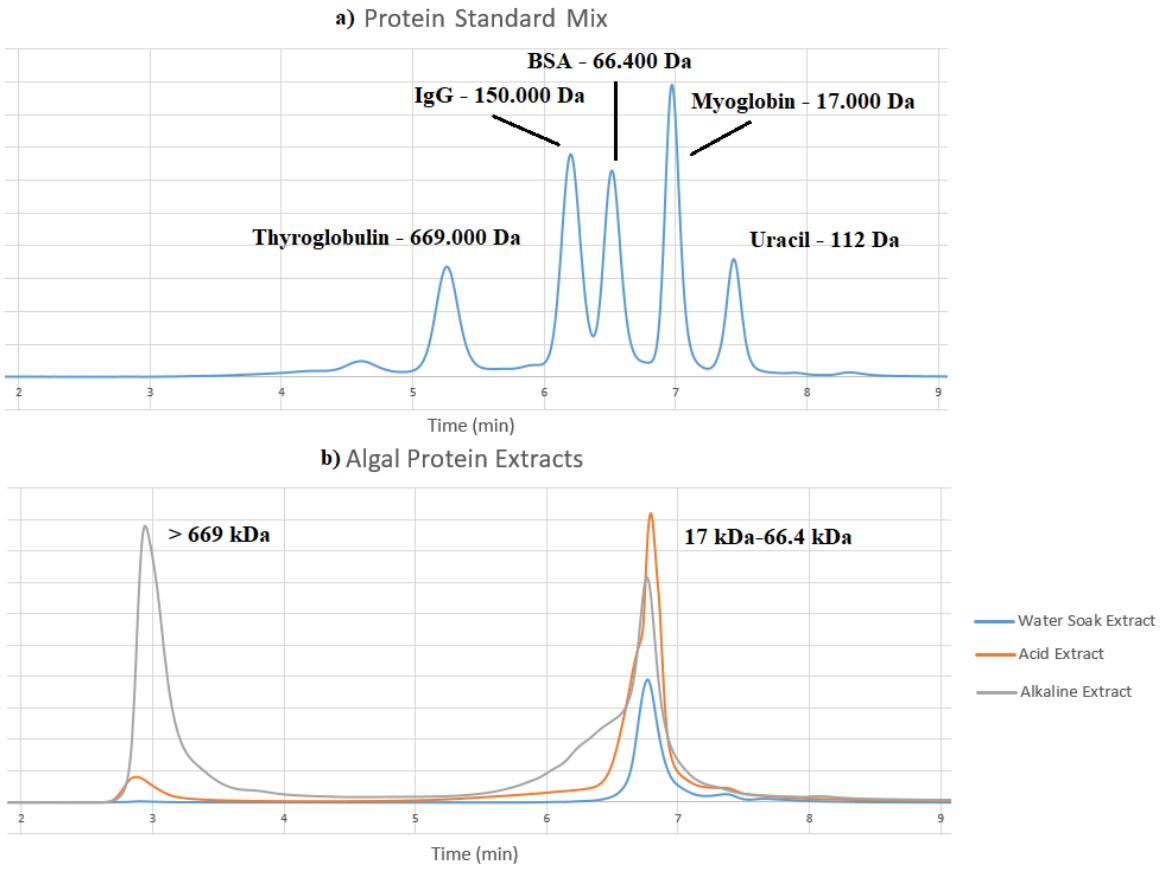

Figure 12. Protein separation on the XBridge Protein BEH SEC, 450 Å, 3.5 µm, 7.8 x 150 mm Column; a) Protein standard mix; b) Algal protein extracts; c) Concentrate mix before and after hydrolysis

Although use of acid and alkaline solutions for extraction could lead to hydrolysis, thus justifying the low MW proteins (Kadam et al., 2017), the water soak extraction served as a control – and confirmed that the smaller sizes of the extracted protein from *M. stellatus* is not get influenced by acid or alkaline hydrolysis. After all, the solutions had only 0.4 M, a small amount compared to the usual 5-6 M used for hydrolysis (Soto-Sierra et al., 2021).

3.4. Enzymatic Hydrolysis – Response Surface Optimization

3.4.1. Model Fitting

The response surface methodology (RSM) statistical technique was used to optimise the enzymatic hydrolysis of concentrated extracts from *M. stellatus* using alcalase. The effect of hydrolysis time and E/S ratio upon the bioactivity of the hydrolysed protein concentrates was achieved using CCD2. The 13 independent runs conditions and response variables results are presented in **Table 13**.

Table 13. Central composite design with natural hydrolysis conditions (Time and E/S) and experimentally obtained values of all investigated responses, antioxidant capacity (ORAC and ABTS (µmol Trolox Equivalent/mg Hydrolysed Protein)), ACE inhibitory activity (IACE (IC_{50}, µg Hidrolysed Protein/mL)), and degree of hydrolysis (DH (%)).

Run	Independent variables		Responses			
	Time (h)	E/S (%,v/v)	ORAC[a]	ABTS[b]	IACE[c]	DH[d]
1	3.5	1.5	3.9 ± 0.1	0.68 ± 0.03	57.9 ± 1.7	24.3 ± 2.3
2	3.5	1.5	3.9 ± 0.2	0.60 ± 0.01	76.1 ± 1.1	25.5 ± 0.1
3	1.0	2.5	4.0 ± 0.1	0.64 ± 0.04	65.8 ± 0.8	20.7 ± 1.8
4	3.5	1.5	3.8 ± 0.2	0.66 ± 0.02	59.8 ± 2.1	24.8 ± 0.4
5	6.0	0.5	3.1 ± 0.1	0.82 ± 0.01	70.1 ± 0.8	4.7 ± 0.4
6	1.0	0.5	3.4 ± 0.2	0.50 ± 0.02	63.0 ± 0.7	10.1 ± 0.3
7	3.5	1.5	4.2 ± 0.1	0.72 ± 0.09	55.6 ± 2.2	24.1 ± 0.6
8	3.5	0.1	2.5 ± 0.2	0.67 ± 0.03	71.6 ± 2.1	14.9 ± 0.3
9	6.0	2.5	4.1 ± 0.1	0.84 ± 0.01	54.0 ± 1.4	30.5 ± 3.2
10	3.5	2.9	4.1 ± 0.3	0.75 ± 0.01	43.8 ± 2.8	26.8 ± 0.6
11	0.0	1.5	2.0 ± 0.1	0.34 ± 0.02	92.6 ± 4.0	0.0 ± 0.0
12	7.0	1.5	4.1 ± 0.1	0.97 ± 0.02	57.0 ± 1.9	29.3 ± 2.3
13	3.5	1.5	3.8 ± 0.3	0.79 ± 0.02	65.6 ± 0.3	23.1 ± 0.1

Response values are expressed as mean ± standard error of the mean (SEM) (n>=3)
[a]Obtained by the ORAC assay (µmol TE/mg HP)
[b]Obtained by the ABTS assay (µmol TE/mg HP)
[c]Obtained according to Sentandreu and Toldrá (2007) (IC_{50}, µg HP/mL)
[d]Obtained by the TNBS assay (%)

The coefficients for each linear and quadratic term are shown in **Table 14**. Analysing the coefficients of determination (R^2), the degree of fit was 0.933, 0.910, 0.954, and 0.996 for ORAC, ABTS, IACE, and DH respectively – thus demonstrating a good fit of the models to the experimental data, since every R^2 is greater than 0.900. The lack of fit also indicates that the models are appropriate to describe the experimental responses. The p-values were greater than 0.100, i.e. the lack of fit was non-significant – meaning that the model fits well. Additionally, the adjusted R^2 values were all greater than 0.850, thus indicating that the model degree of fit after removing the non-significant ($p > 0.10$) coefficients was still high. The predicted R^2 values indicate how well a model predicts the responses of new observations. It should be in reasonable agreement with the adjusted R^2, i.e. the difference between the two values should be less than 0.2. This agreement was observed on every response with the exception of IACE, which exhibits

a difference of 0.386. This may compromise the model ability to predict responses, meaning that the model may not represent the actual response.

Table 14. Fitted model and coefficients for each term, hydrolysis time and E/S ratio (linear and quadratic) and statistics – R^2, Adjusted R^2, Predicted R^2, and lack of fit.

Factors	Actual Coefficient Estimate			
	ORAC (µmol TE/mg HP)	ABTS (µmol TE/mg HP)	IACE (µg HP/mL)	DH (%)
Constant (β_0)	2.205***	0.3949***	92.96***	0.2794***
Time (β_1)	0.06768	0.06557***	-9.281**	4.816***
E/S (β_2)	1.627***	0.04318**	-10.27***	11.12***
Time x E/S (β_{12})	-0.008259	-	-0.3010	-0.4303**
Time^2 (β_{11})	-0.004401	-	1.009**	-0.2606***
E/S^2 (β_{22})	-0.3642***	-	0.9100	-1.758***
Statistics				
R^{2a}	0.933	0.910	0.954	0.996
Adjusted R^{2b}	0.885	0.890	0.896	0,992
Predicted R^{2c}	0.693	0.817	0.510	0.979
Lack of Fit[d]	0.156	0.425	0.123	0.726

Regression coefficient significantly different from zero: *p < 0.10, **p < 0.05, ***p < 0.01.

[a] coefficient of determination, should be maximized

[b] adjusted coefficient of determination, should be maximized

[c] predicted coefficient of determination, should be maximized

[d] p-value, should be > 0.100, not significant

By inspection of **Table 14**, it became clear that different responses are affected differently by the independent variables. The regression model for the ORAC assay shows that mainly E/S ratio affects the ORAC antioxidative system of the hydrolysed peptides. Both linear and quadratic terms related to E/S variable were significant ($p < 0.01$). In contrast, the remainder of terms have p-values greater than 0.10, meaning they were not significant – so their exclusion from the model would probably not be noticeable. If there were many insignificant model terms, the model reduction could improve the model fit, a parameter measured by the adjusted R^2 as previously stated. The other antioxidant model behaves quite differently, being a first-order model – since the quadratic coefficients of the ABTS model were not significant ($p > 0.1$). Both p-values of Time and E/S were significant (< 0.05), with Time being the most significant term ($p < 0.01$). This means that both independent variables significantly affect the ATBS response, with Time having a more significant influence when compared with E/S. This effect can also be preceived in the 3D fitted models presented in **Figure 13** as 3D surfaces. The same coded value increase in the time factor results in a more significant increase in the ABTS response than the E/S factor. The IACE and DH response models displayed a more balanced quadratic equation where both time and E/S significantly affect the results. The IACE model has both the two-factor interaction term and the E/S quadratic term as non-significant ($p > 0.05$); while the DH regression model coefficients were all significant ($p < 0.05$).

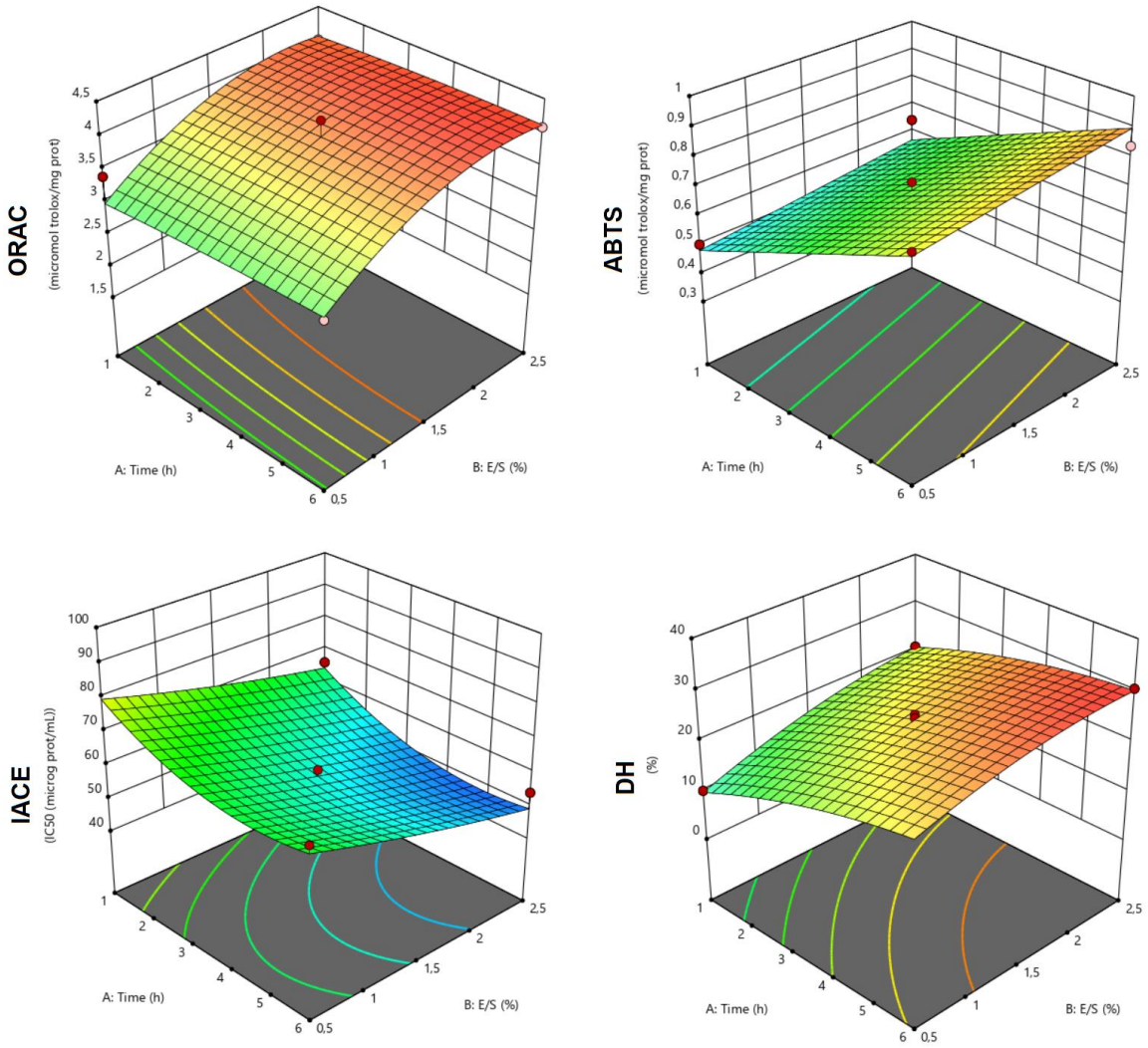

Figure 13. Model graphs (3D surfaces) of each response, ORAC, ABTS, IACE, and DH as a function of Time and E/S.

3.4.2. Optimal Conditions

After obtaining the models for each response (**Figure 13**), the optimal hydrolysis time and E/S ratio that would produce the most bioactive peptide concentrate were determined. In order to obtain the most desirable responses, a goal and an importance rank were assigned to each response. Both antioxidant activity models, ORAC and ABTS, were maximized – since a greater mass of Trolox equivalent per mass of hydrolysed protein is desirable to limit oxidation. For the ACE-inhibitory activity, the opposite was done, thus minimizing the IC_{50} – since the bioactivity of the peptides is greater if the doses to inhibit 50% of the ACE activity are smaller. The same level of importance was given to ORAC, ABTS, and IACE; while no importance was given to the response of DH since it is a non-bioactivity assay, which measure only the quantity of peptides in the hydrolysed sample and disregard the activity of these peptides. In addition (and as seen previously), the different responses are affected differently by the variation of hydrolysis time and E/S ratio. DH is highly dependent on both time and E/S, while ORAC is mainly dependent on E/S and ABTS on time, respectively. All in all, a greater number of peptides does not translate to a greater bioactivity of the hydrolysate – meaning that it should not be

considered in the determination of the most desirable conditions (Sánchez and Vázquez, 2017). A desirability model was obtained with both goal and importance set for each relevant response.

Typically, the optimum response is estimated by interpolation, having the process space limited to low and high coded limits (-1 and +1) (Broniatowski and Celant, 2014). Anywhere outside the design range is considered an extrapolation, making the polynomial function less representative the further away the optimal point is from the design points. Therefore, the optimum conditions were located by interpolation at the upper right edge point (1,1) – and translate to hydrolysis time of 6 h and E/S ratio of 2.5%. The experimental response values were already shown in **Table 13**, and this solution desirability can be seen in **Table 15**. Alternatively, if extrapolated without any restriction, the desirable conditions of time and E/S fall outside the experimental design, thus unfolding an increase of desirability from 0.911 to 0.928 – as shown in **Table 15**. The design space-coded limits were increased from (-1,1) to (-α,α), and the maximum desirability conditions and responses can be seen in **Figure 14**.

Table 15. Natural hydrolysis optimum conditions (Time and E/S) and predicted values of all investigated responses, antioxidant capacity (ORAC and ABTS), ACE inhibitory activity (IACE), and degree of hydrolysis (DH).

Optimum conditions	**Independent variables**		**Predicted Responses**				
	Time (h)	E/S (%,v/v)	ORAC[a]	ABTS[b]	IACE[c]	DH[d]	Desirability
Interp.[1]	6.0	2.5	4.1 ± 0.2	0.90 ± 0.03	49.1 ± 3.3	30.2 ± 0.6	0.911
Extrap.[2]	6.9	2.8	4.0 ± 0.3	0.97 ± 0.04	49.6 ± 5.3	30.1 ± 0.9	0.928
Extrap.(Conf.)[3]	6.5	2.6	4.0 ± 0.2	0.93 ± 0.03	49.6 ± 4.0	30.3 ± 0.7	0.921

Predicted response values are expressed as mean ± standard error with exception of Desirability (valued between 0 and 1)

[1]Interpolation (-1;1)

[2]Extrapolation without any limitation (-α;α)

[3]Restricted extrapolation using the confidence band

[a] in µmol TE/mg HP

[b] in µmol TE/mg HP

[c] in IC50, µg HP/mL

[d] in %

It can be seen that the higher the distance of a point from its design space, the worse the confidence when interpreting the results. The predicted responses given by the polynomial functions may indeed be no longer representative of the valid responses. Via Design-Expert v13, the standard error of each response was calculated (**Table 15**); consequently, the extrapolation can be limited with a confidence band around the predicted responses – which cannot be wider than the most distant, highest deviation from the mean run (Extrapolating a Mixture Design, 2020). The model graph of desirability with the restrictive band for extrapolation can be seen in **Figure 15**; it indicates the maximum desirability within the design space. The optimum conditions of hydrolysis time and E/S ratio were 6.5 h and 2.6% (v/v), respectively (**Table 15**).

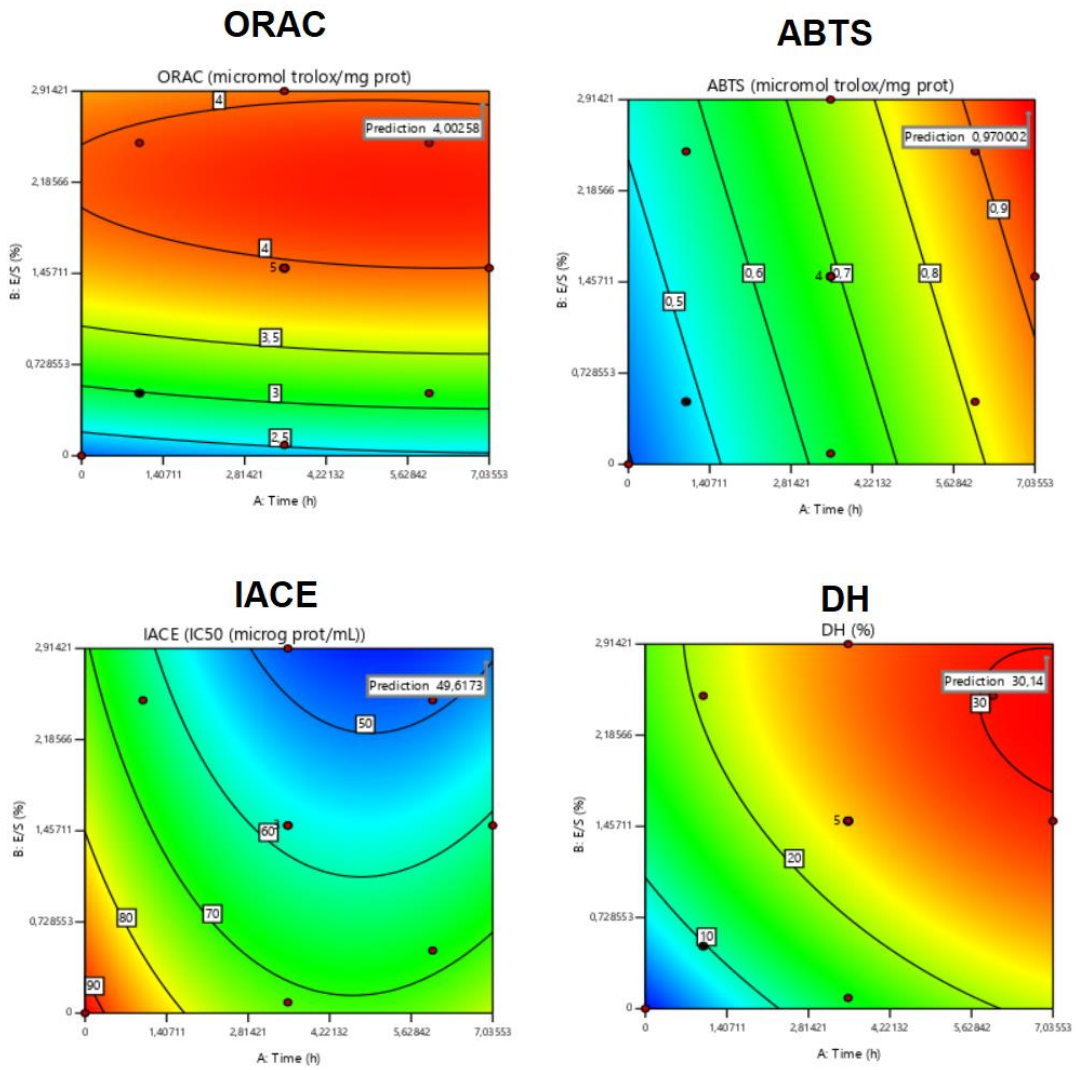

Figure 14. Model graphs (contours) of each response, ORAC, ABTS, IACE, and DH as a function of Time and E/S and predicted values for optimum conditions (blue – lower value; red – higher values).

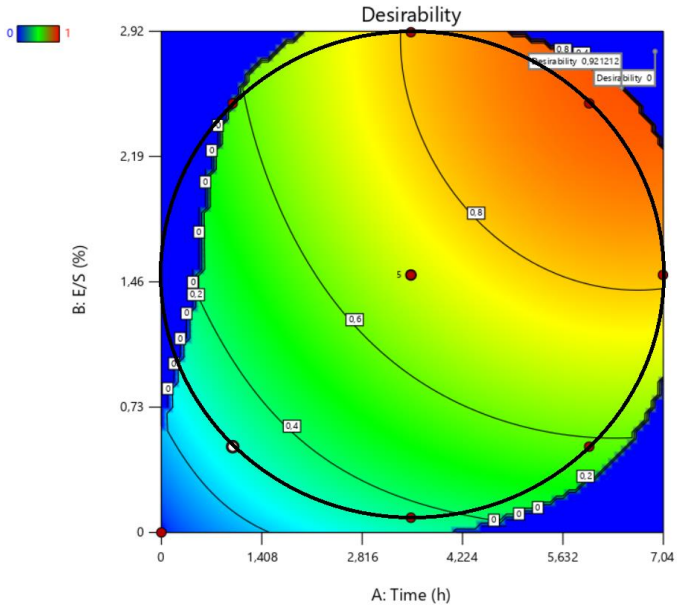

Figure 15. Model graph (contour) of Desirability as a function of time and E/S and desirability of the optimum conditions while extrapolating with a confidence band.

3.4.3. Verification of the Models

The optimum independent variables were checked by using the restricted extrapolation, with a maximal Desirability of 0.92. The previous optimal predicted values and the experimental values are shown in **Table 16**.

Table 16. Optimal predicted and experimental values of all investigated responses, antioxidant capacity (ORAC and ABTS), ACE inhibitory activity (IACE), and degree of hydrolysis (DH).

Results	Responses			
	ORAC (µmol TE/mg HP)	ABTS (µmol TE/mg HP)	IACE (IC50, µg HP/mL)	DH (%)
Predicted	4.0 ± 0.2	0.93 ± 0.03	49.6 ± 4.0	30.3 ± 0.7
Experimental	4.2 ± 0.1	0.89 ± 0.07	48.2 ± 0.6	31.7± 0.8

Response values are expressed as mean ± standard error

The bioactivity of the optimum hydrolysed protein was then compared with various other protein hydrolysates and extracts (**Table 17**). The ORAC result was expressed as EC_{50} (mg TE/mL) and the ABTS in IC_{50} (µg HP/mL), in agreement with common compared literature on the subject (Cian et al., 2013; Medina et al., 2015; Montone et al., 2018).

Table 17. Experimental values in EC_{50} and IC_{50} of all investigated responses of hydrolysed protein from *M. stellatus*, antioxidant capacity (ORAC and ABTS), ACE inhibitory activity (IACE), compared to values from the literature – values, origin of the extract/hydrolysate/peptide and corresponding reference.

Responses	*M. stellatus*	Literature	Origin	References
ORAC (EC_{50} (µg TE/mL))	12	26	Microalga *N. gaditana*	Medina et al. (2015)
ABTS (IC_{50} (µg HP/mL))	155	4.41	*Microalga M. neglectum**	Montone et al. (2018)
		600	Red seaweed *P. columbina*	Cian et al. (2015)
		1010	Red seaweed *P. columbina*	Cian et al. (2013)
IACE (IC_{50} (µg HP/mL))	48.2	91.6	Red seaweed *M. stellatus*	Blanco-Pascual et al. (2014)
		210	Microalga *C. pyrenoidosa*	Li et al. (2021)
		100	Microalga *A. platensis*	Li et al. (2021)
		1.06	Microalga *T. obliquus**	Montone et al. (2018)

*isolated peptide mixture

3.4.3.1. Antioxidant Capacity

As mentioned previously, the principle of antioxidant capacity methods is retardation and/or inhibition of an oxidation reaction. The antioxidant scavenges the free radical, and thus prevent oxidation of fluorescein in the ORAC assay, and revert the oxidation reaction that forms $ABTS^{\bullet+}$ radical in the ABTS assay. With this, both ORAC and ABTS values can be expressed as EC_{50} (half-maximal effective concentration) and IC_{50} (half-maximal inhibitory concentration), respectively – EC_{50} corresponding to the concentration required to quench 50% of the initial radical.

The ORAC assay EC_{50} obtained for the *M. stellatus* hydrolysate was 12 μg TE/mL, showing a greater antioxidant activity than a *N. gaditana* protein hydrolysates reported by Medina et al. (2015) (26 mg TE/mL). Medina et al. (2015) also reported an improvement in the antioxidant capacity of almost 28 times from the pure protein compared to the protein hydrolysates. For *M. stellatus*, an increase of 2.1 times is apparent after hydrolysis. The lower increase in antioxidant capacity was probably due to the already high number of peptides in the protein concentrate before hydrolysis. As seen in **Section 3.3**, the crude protein of *M. stellatus* possessed mostly smaller peptides, which should exert antioxidant activity even before hydrolysis.

Montone et al. (2018) studied the antioxidant capacity of specific peptide sequences, of which the protein from microalga *M. neglectum* originated the peptide SDWDRF. The peptide had an IC_{50} of only 4.41 μg/mL, almost 40 times more active than the peptides obtained from *M. stellatus*, with an IC_{50} of 155 μg/mL. Since the peptide solution contains only a single and bioactive peptide, it is expected that the mixture of unknown peptides obtained in this work holds a lower antioxidant capacity. The peptides will have varying bioactivities; since bioactivity is calculated depending on the total mass of protein in the sample, the overall activity will be lower.

Comparing the antioxidating capacity from the ABTS assay of *M. stellatus* hydrolysate to that of another red seaweed, *P. columbina*, the former IC_{50} was 155 μg HP/mL – compared to 1010 (Cian et al., 2013) and 600 μg HP/mL (Cian et al., 2015). These results showed that the *M. stellatus* hydrolysate can scavenge 50% of the $ABTS^{\bullet+}$ at much lower concentrations compared to other hydrolysates, indicating a high antioxidant ability. Nevertheless, in this work the antioxidant capacity increased 2.6-fold when the protein was hydrolysed, while Cian et al. 2015 reported an increase of 3.7-fold, which shows that the antioxidant properties of *M. stellatus*' peptides should be superior to that of *P. columbina*.

3.4.3.2. IACE Activity

The in vitro IACE activity exerted by *M. stellatus* was measured as IC_{50} and displayed a high inhibition capacity of 48.2 μg/mL compared to other algal protein extracts. Blanco-Pascual et al. (2014), who also extracted and hydrolysed *M. stellatus* protein, reported a minimum IC_{50} of 91.62 μg/mL – almost twice the concentration needed to inhibit 50% of ACE activity compared to the concentration reported in this work. This difference was influenced mainly by the different enzymatic hydrolysis employed in those studies. In the study by Blanco-Pascual et al. (2014), the hydrolysis was done directly on the algal biomass, using the same enzyme, alcalase, at 50 °C, pH 8, for 3 h and 0.132 AU/g of seaweed; conversely, the optimal conditions in this

work were 55 °C, pH 8, for 6.5 h and 3.860 AU/g of algal protein. As proven earlier in **Section 3.4.1**, higher values of time and E/S greatly influence the IACE activity response, by reducing IC_{50}.

The study by Li et al. (2021) reported values of IACE activity for two microalgal protein hydrolysates, having better IC_{50} when hydrolysed with pepsin than trypsin. The values reported in **Table 17** refer to the peptic hydrolysates of each species, having an IC_{50} of 210 and 100 µg/mL for *C. pyrenoidosa* and *A. platensis,* respectively. Both values were higher than those reported on IACE activity for *M. stellatus*. The greater inhibition of *M. stellatus* protein hydrolysate could be due to the use of alcalase instead of pepsin or trypsin, expected to produce different peptides with different biological activities (Li et al., 2021).

Lastly, Montone et al. (2018) also studied the IACE activity of specific peptide sequences, of which the protein from *Tetradesmus obliquus* originated the peptide WYGPDRPKFL. The peptide had an IC_{50} of only 1.06 µg/mL, almost 50 times more active than the peptides obtained from *M. stellatus* in this study. As per the comparison of antioxidant capacity, the same principle can justify the much lower concentration needed from the pure peptide to inhibit 50% of the ACE activity compared to the unknown peptides present in the hydrolysate obtained in this study. Additionally, the peptide WYGPDRPKFL has tryptophan as the N-terminal AA, and peptides with aromatic N-terminal AAs have been shown to exert more robust IACE activities (Sánchez and Vázquez, 2017).

Although the results obtained in this study showed higher antioxidant capacities and IACE activities, it is important to recognize that the hydrolysates obtained were not composed of only protein. Consequently, bioactivity is expressed not only arising from the peptides but also from any other compounds present in the mixture that might have a biological activity. The hydrolysates obtained in this study did not only have protein, but also other compounds that were solubilised during extraction – including polysaccharides, simple sugars, and pigments. Some of these compounds, namely carrageenan (Blanco-Pascual et al., 2014) and carotenoids (Pérez-Gálvez et al., 2020), are common among red algae, and have shown decent biological activities due to their complex structure.

3.5. Process Mass Balance

The overall process of extracting, concentrating, and hydrolysing the protein of *M. stellatus* was intended for a potential implementation in the food industry. The percentage of algal protein is generally higher than in other traditional protein sources. However, to compete with foods such as meat, eggs, fish, soy, or peas, algal protein needs to be processed to become more digestible and increase its bioactivity – thus increasing its appeal and consequently its value (Bleakley and Hayes, 2017). **Figure 16** shows the protein mass balance throughout the process implemented in this work.

In the extraction process, 93% of the total protein in *M. stellatus* was recovered, after optimising the protein extraction process. Based on optimization, the protein recovery yield went from 51.6% to 93.5%; whilst use of 2/3 of solvent reduced the extraction time to 57% of that of the original extraction. Other studies on algal protein extraction claimed a maximum protein recovery yield of 36.1%, namely Fleurence et al. (1995), and 25.1%, by Harrysson et al. (2018); with other yields as low as 9.4% (Fleurence et al., 1995).

The loss in protein during the concentration step leads to over 37% of protein loss in a single step, thus resulting in a total yield of only 58%. This step strongly affects sustainability of the algal protein processing; it reduces the process productivity, and removes mainly peptides that can pass through the filter. These small peptides with low MW and reduced number of AAs potentially possess high bioactivity (Gordalina et al., 2021), so they should preferably be retrieved.

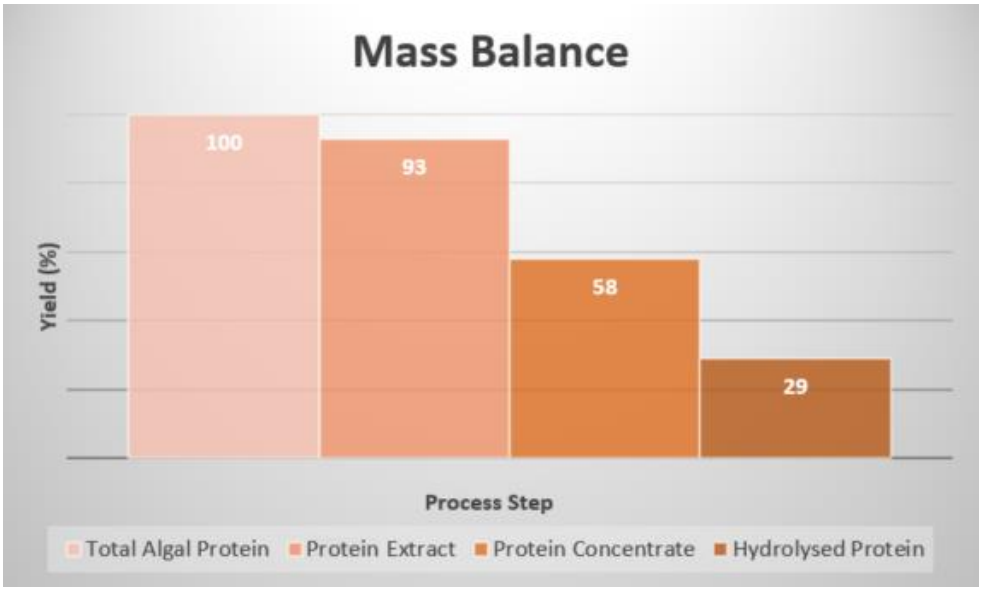

Figure 16. Protein mass balance in each step of the processing of *Mastocarpus stellatus* protein.

An alternative method of concentrating the protein extract is precipitation triggered by a pH change. Sánchez-Zurano et al. (2020) used RSM to optimize precipitation, where the optimal conditions for precipitation resulted in a 75.2% recovery of protein – almost 10% higher than that attained in this study, 66.7%.

Before hydrolysis, the two concentrates were combined and their pH corrected to 7.5. The initial concentration of the protein concentrate mix was theoretically 20 mg/mL, yet a precipitate was immediately formed (**Annex A3.3**). After hydrolysis and centrifuging of the mixture, the protein content of the hydrolysates was approximately 10 mg/mL. This step resulted in a loss of 50% of the protein, along with an overall process yield of only 29%.

4. Conclusion

It is increasingly urgent to find sustainable sources of protein for Humankind as a whole. Macroalgae are strong candidates for a realistic solution to the problems related to animal and plant protein. Their cultivation requires less land, energy and water, thus providing a highly sustainable solution relative to other sources. Seaweed possess high nutritional values, and entail various beneficial nutrients. As shown in this work, their AA profile includes most of the EAAs.

The main objective of this work was to study the biological activities present in a *M. stellatus* protein hydrolysate, with the ultimate goal of potential future implementation in the food industry.

The results generated show that combination of bead milling homogenizer with acid extraction, followed by alkaline extraction, supports a remarkable recovery of 93% of the protein present in *M. stellatus*.

The concentration of the algal protein extract obtained permits desalting and concentration of protein for at least six-fold. Nevertheless, a considerable amount of protein is still lost during this process.

The optimization of hydrolysis by RSM indicated that higher hydrolysis times and E/S ratios lead to a better degree of hydrolysis, antioxidant capacity (ORAC and ABTS), and IACE activity. Optimal conditions produced a hydrolysate with high bioactivities, up to twice those of other red algae and microalgae.

In conclusion, the protein from *M. stellatus* appears to carry a potential for practical application; it can be sustainably extracted and hydrolysed, thus forming bioactive peptides that rival and even surpass the physiological function of other novel foods. By further optimizing the methodologies used, an industrial-scale operation might result – along with an appropriate contribution for the future of the food industry. Nevertheless, complementary work is still in need to validate feasibility of large-scale operation.

5. Future Projects

In the research effort behind this dissertation, bioactive extracts were obtained from *M. stellatus* protein. We showed how protein from said red alga could be almost entirely extracted, and how it could be hydrolysed to release important bioactive peptides. However, there still is a considerable amount of work ahead to implement these methodologies on an industrial scale, aimed at mass production and food formulation.

First, more extensive optimization of the overall process needs to be studied. Although most protein was extracted from the red alga, a ratio of 1:40 between seaweed and solvent is highly problematic – for resulting in excessive amounts of solvent if considered at a larger scale. Furthermore, the acid extraction is thought to bring about a high amount of polysaccharides, undesirable since the protein is the target compound. The implementation of a separation step to separate polysaccharides from protein after acid extraction would be in order, possibly via pI precipitation. Novel protein extraction methods should also be tested, as they might have similar yields within much shorter timeframes and thus solve some of those problems. Scalability and economic feasibility should be thoroughly explored in order to validate this technology at industrial scale, from alga cultivation/harvest down to food formulation.

Other processing factors besides hydrolysis time and E/S ratio should be studied, namely pH and temperature, and using different enzymes. Other bioactive responses should also be studied, such as the other antioxidant methods or other biological activities such as anti-inflammatory ones.

For a better rationalization of the effects of the different types of peptides on the bioactivity mechanisms, it is essential to isolate and identify the peptides present in the hydrolysates – and eventually evolve assays from *in vitro* to *in vivo* level.

Since the main objective is implementing protein and peptides in food formulations, rheological and sensory tests are to be performed. In addition, simulating gastrointestinal digestion should be performed to check the stability of peptides and unfold putative losses in bioactivity; as well as implementing techniques to circumvent these problems, namely, microencapsulation. Following these studies, the biological benefits related to the hydrolysed peptides still need to be validated through clinical trials.

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Annexes

A1. Extraction Optimization

A1.1. Cell Disruption Equipment

Figure A1. a) Precellys Evolution System; b) 15 mL flasks containing the algal sample, solvent, and ceramic beads.

A1.2. Separation of Pellet and Extract

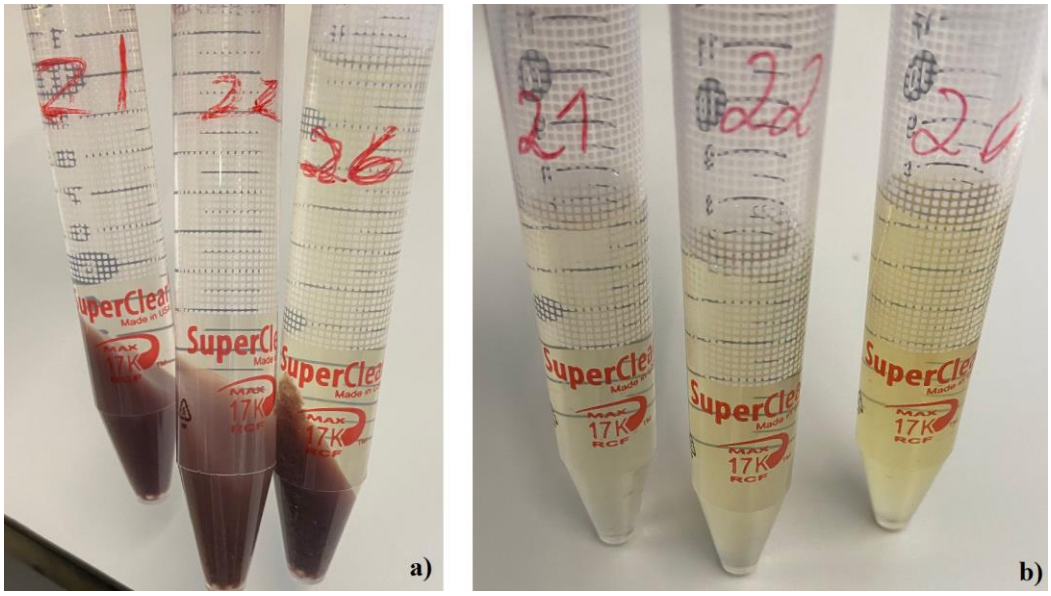

Figure A2. a)15 mL falcon tubes containing solvent and algal pellet after centrifugation; b) 15 mL falcon tubes containing the decanted algal extract.

A1.3. Mixing Phase

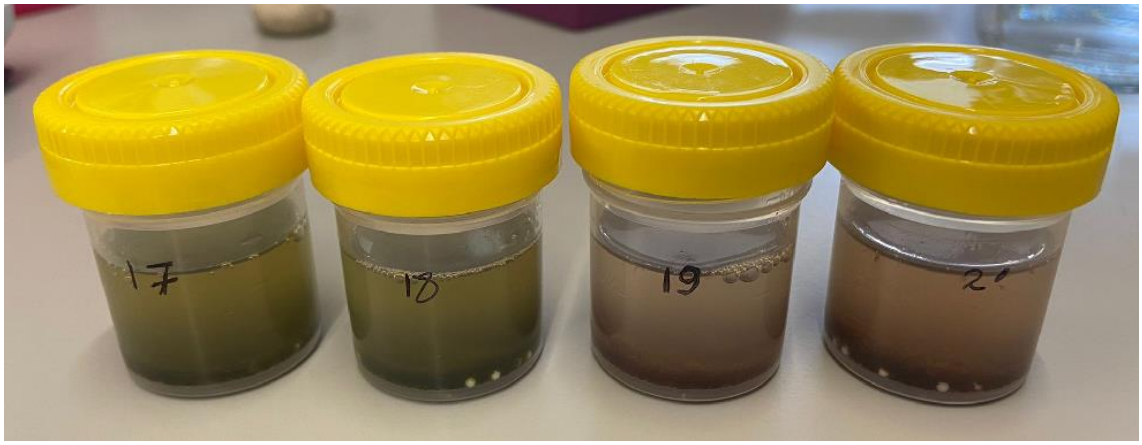

Figure A3. Mixing containers for alkaline extraction (left) and for acid extraction (right).

A1.4. Scaled Equipment

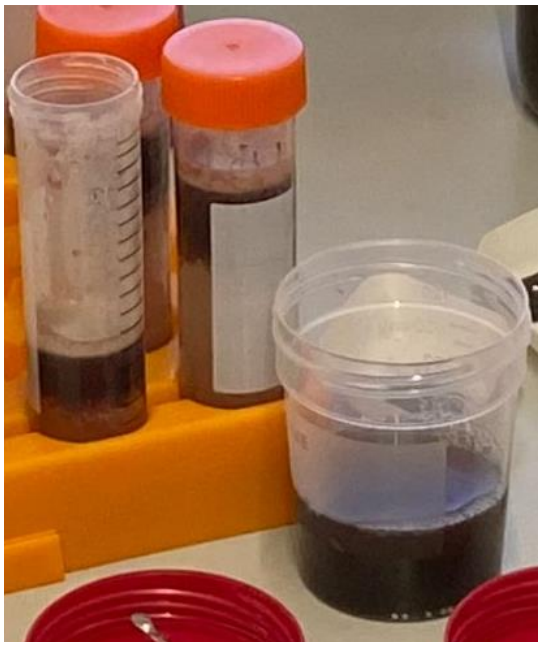

Figure A4. 50 mL falcon tube for centrifuging the acid extract (left) and goblet for the alkaline mixing phase (right).

A1.5. Final Algal Protein Extracts

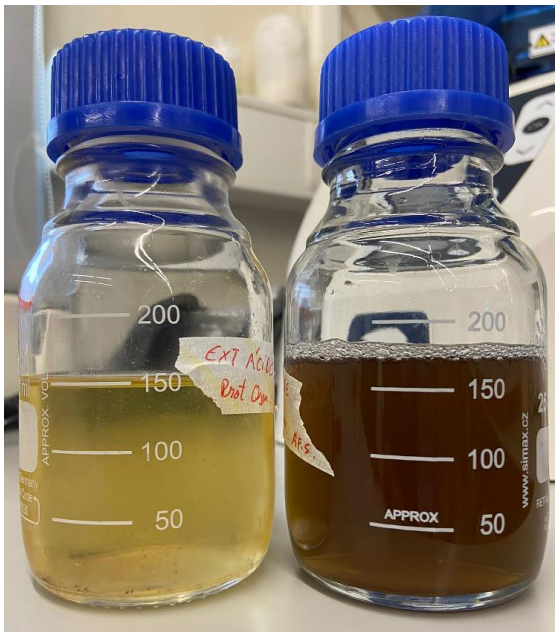

Figure A5. Final algal protein extracts, acid (left), alkaline (right).

A1.6. Ultra Filtration Devices

Figure A4. 1K Omega Macrosep Advance Centrifugal Devices (Pall Corporation), used to concentrate protein and dilute salts.

A1.7. Algal Protein Concentrates

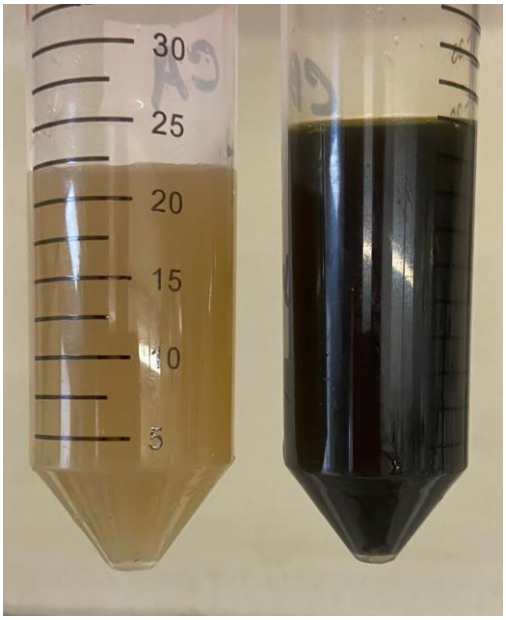

Figure A5. Algal protein concentrate, acid (left), alkaline (right).

A1.8. Extracts and Concentrates Characteristics

Table A1. Final acid and alkaline extracts and respective concentrates – protein concentration in the solution (mg/mL), pH and conductivity (mS/cm).

Extracts	Protein concentration (mg/mL)[1]	pH	Conductivity (mS/cm)
Acid Extract	2.7	0.70	125.9
Alkaline Extract	2.3	11.81	63.1
Acid Concentrate	17.7	1.85*	16.99
Alkaline Concentrate	23.2	11.55	25.5

A2. Protein Quantification and Bioactive Assays

A2.1. BCA Protein Assay Reaction

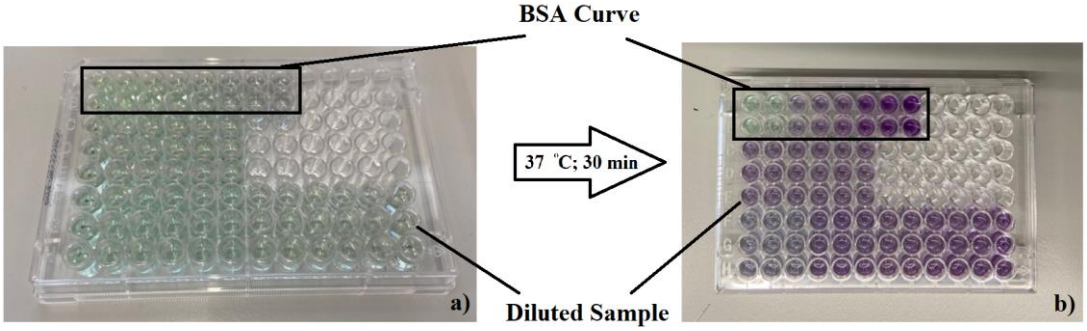

Figure A6. a) BCA Working agent and protein samples before incubation; b) BCA Working agent reacting with the protein samples after incubation (green- lower concentration; purple – higher concentration)

A2.2. TNBS Method

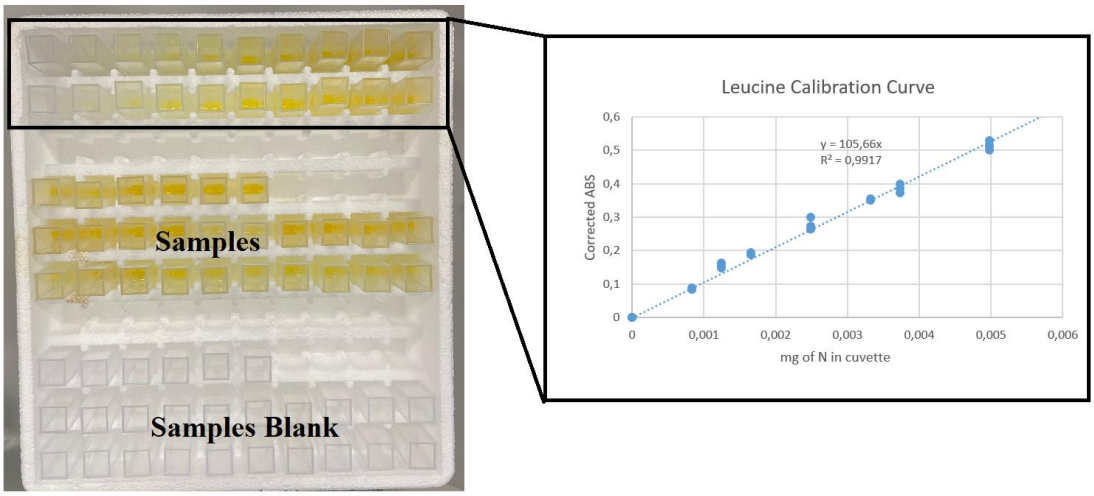

Figure A7. Leucine calibration curve (and function), samples, and blanks in semi-micro at reaction end-point.

A2.3. ORAC Method

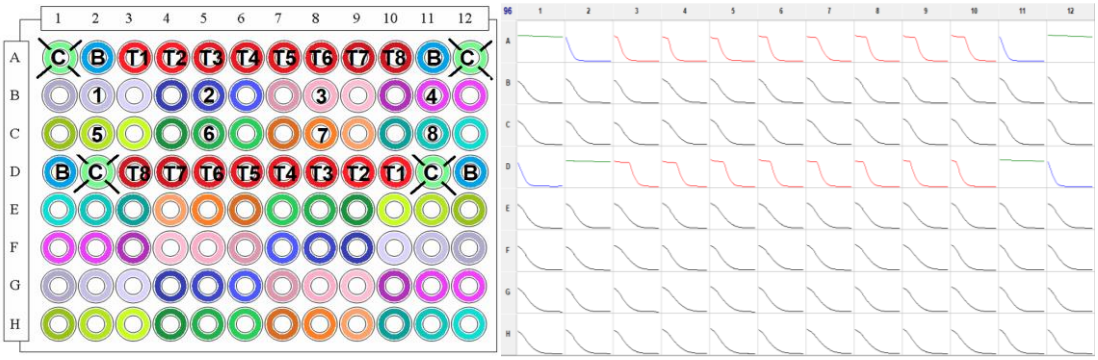

Figure A8. Microplate configuration for ORAC assay; C-Control; B- Blank; T1-8- Trolox standard curve (0.11-0.84 mM); Each number indicates each sample; Darker colors indicate less diluted samples (1:800), lighter colors more diluted samples (1:1200), middle tone (1:1000) (left); Fluorescence vs time curves of each well (right)

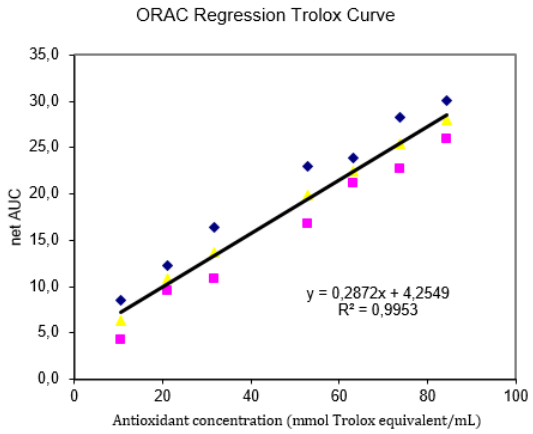

Figure A9. Regression curve and equation net AUC vs antioxidant concentration (mmol Trolox equivalent/mL) calculated using the Trolox standard solutions.

A2.3. ABTS Method

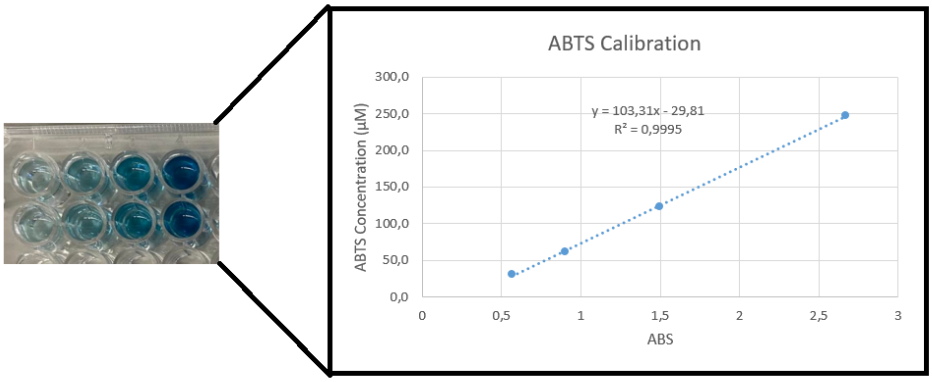

Figure A10. Linear relationship for determining the ideal concentration of ABTS (0.700 ABS).

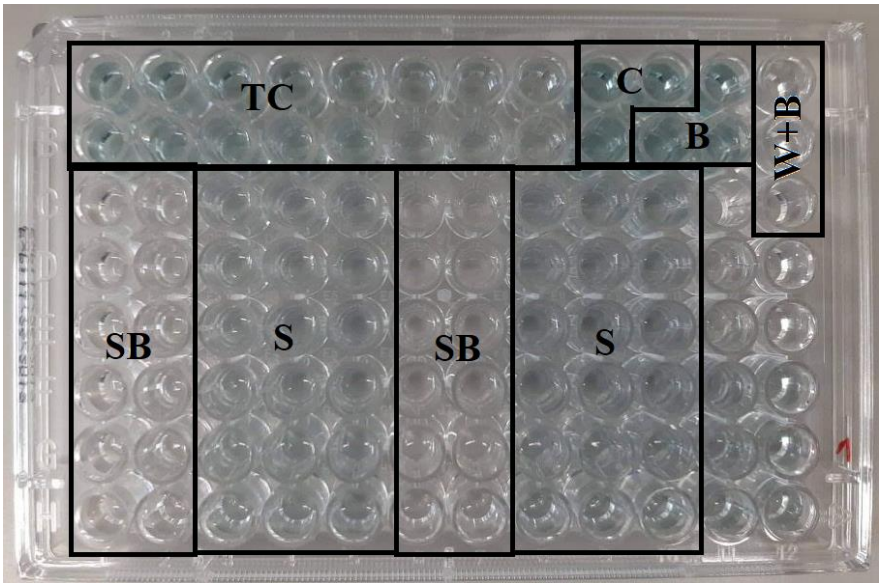

Figure A11. Microplate configuration for ABTS assay; TC- Trolox standard curve (0.11-0.84 mM); C- Control; B- Blank; W+B- Water + buffer; SB- Sample Blank; S- Samples.

Table A2. Values of the concentration of Trolox equivalent in a well (hydrolysate nº 7) dependency on time.

Time (min)	Concentration (mmol TE/mL)
10	0.034
30	0.048
60	0.059
150	0.076
300	0.088
350	0.087

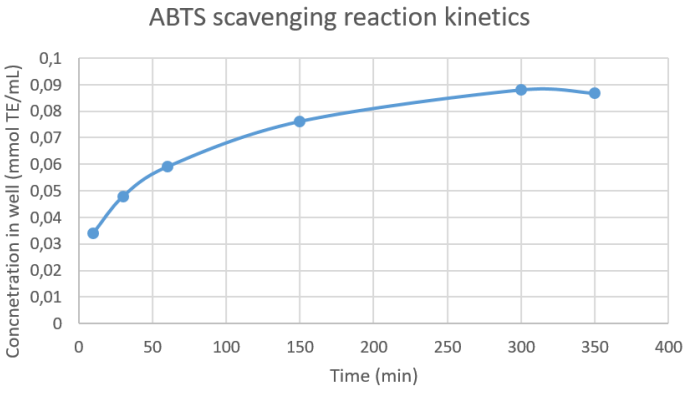

Figure A12. Graph of the concentration of Trolox equivalent in a well (hydrolysate nº 7) dependency on time.

A2.4. IACE Activity Assay

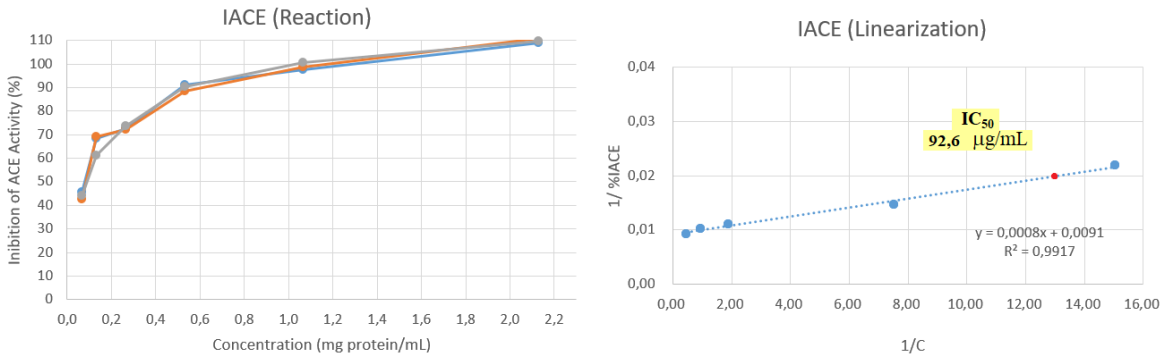

Figure A13. % of inhibition of ACE activity vs protein concentration, actual inhibition (left) and linearization (right) (hydrolysate nº 11).

A3. Extraction results

A3.1. Ethanol influence on pigment extraction

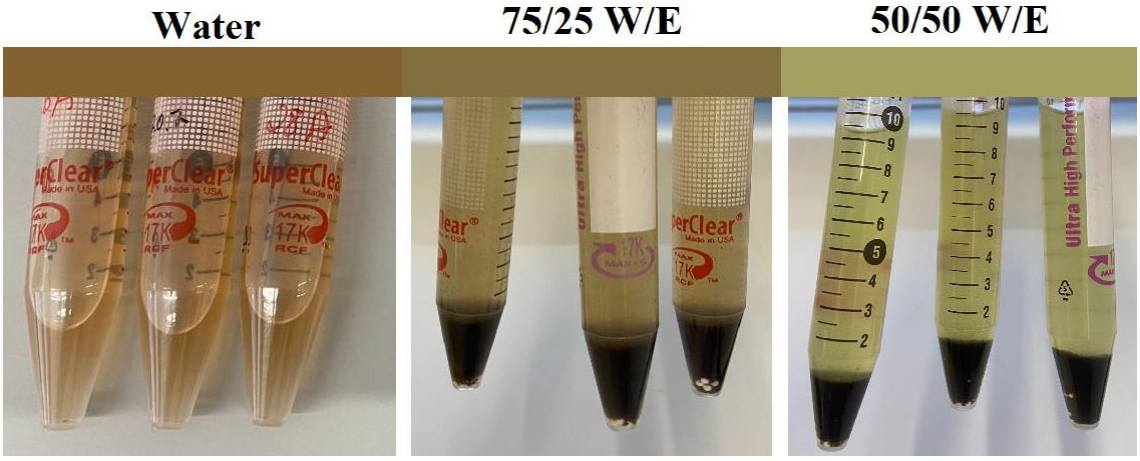

Figure A14. *Mastocarpus stellatus* extract color dependence on the W/E ratio.

A3.2. pH of Extraction solutions and Extracts

Table A3. pH of the initial solutions, HCl_i and $NaOH_i$ (left), and extracts obtained with the Acid and Base Extraction (right).

Solution	pH
0.1 M $NaOH_i$	11.70
0.4 M $NaOH_i$	12.05
0.1 M HCl_i	1.01
0.4 M HCl_i	0.55

Extract	pH
1st - 0.1 M NaOH	11.73
1st - 0.4 M NaOH	11.92
1st - 0.1 M HCl	1.64
1st - 0.4 M HCl	0.70
2nd - 0.1 M HCl	1.73
2nd - 0.4 M HCl	1.20
2nd - 0.1 M NaOH	11.72
2nd - 0.4 M NaOH	11.81

A3.3. Precipitated Protein

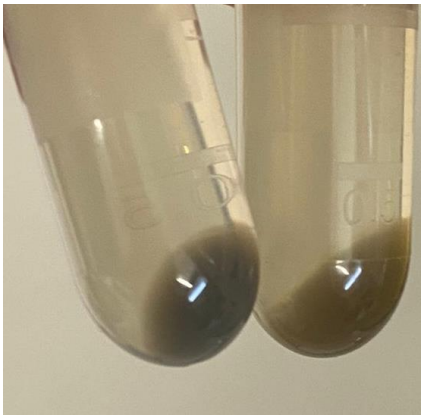

Figure A15. Precipitated protein from the mixture between the acid and alkaline concentrates.