COMPARISON OF PROTEIN EXTRACTION PROTOCOLS AND CHARACTERIZATION OF PROTEIN FROM RED SEAWEED

(*Gracilaria fisheri)*

Fariha Mahmud

Roll No. 0219/17

Registration No. 778

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A thesis submitted in the partial fulfillment of the requirements for the degree of Master of Science in Food Chemistry and Quality Assurance

Department of Applied Food Science and Nutrition
Faculty of Food Science and Technology
Chattogram Veterinary and Animal Sciences University
Chattogram-4225, Bangladesh

AUGUST 2022

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Fariha Mahmud

August, 2022

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This is to certify that we have examined the above Master's thesis and have found that is complete and satisfactory in all respects, and that all revisions required by the thesis examination committee have been made

……

(Dr. Md. Abdul Alim)
Professor
Department of Pathology and Parasitology
Supervisor

……

(Ms. Kazi Nazira Sharmin)
Associate Professor
Dept. of Applied Food Science and Nutrition
Co-Supervisor

……

(Ms. Kazi Nazira Sharmin)
Associate Professor
Dept. of Applied Food Science and Nutrition
Chairman of the Examination Committee

Department of Applied Food Science and Nutrition

Faculty of Food Science and Technology

Chattogram Veterinary and Animal Sciences University

Chattogram-4225, Bangladesh

August, 2022

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Title of the Thesis: Comparison of protein extraction protocols and characterization of protein from red seaweed (*Gracilaria fisheri*)

Name of the Student: Fariha Mahmud

Roll Number: 0219/17

Reg. Number: 778

Department: Applied Food Science and Nutrition

Faculty: Food Science and Technology

Name of the Supervisor: Dr. Md. Abdul Alim

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Professor
Department of Pathology and Parasitology
Supervisor

DEDICATATION

I Dedicated My Small Piece of Work to My Beloved Family Members and Respected Teachers.

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Abbreviations

AOAC	:	Association of Official Analytical Chemists
^0C	:	Degree Celsius
%	:	Percentage
DPPH	:	2, 2, diphenyl 1 picrylhydrazyl
EAA	:	Essential amino acid
g	:	Gravity
gm	:	Gram
hr	:	hour
HCl	:	Hydrochloric acid
H_2SO_4	:	Sulphuric acid
HPLC	:	High Performance Liquid Chromatography
MAE	:	Microwave assisted extraction
meq.	:	milliequivalent
mL	:	milliliter
min	:	minute
µL	:	microliter
NF-κB	:	Factor atomic kappa B
PFE	:	Pressurized fluid extraction
PLE	:	Pressurized liquid extraction
ppm	:	Parts per million
rpm	:	Revolutions per minute
SFE	:	Supercritical fluid extraction
TPTZ	:	Tripyridyltriazine
UAE	:	Ultrasound assisted extraction
UPE	:	Ultrahigh pressure extraction
w/v	:	Weight per Volume
WHO	:	World Health Organization

Abstract

Some species of the genus *Gracilaria* account for over 80% of the world's production of agar, making the genus a valuable marine bio-asset. Additionally, many species of *Gracilaria* are utilized as human food and medicinal purpose in various parts of the world. The purpose of this study was to examine the effects of osmotic shock, high shear force, alkaline, and polysaccharide treatments, as well as their many variants and combinations, on protein extraction from *Gracilaria fisheri*. It was required to compare the methods that provide the best protein extraction and purity in order to choose the protein with the highest percentage of purity. Protein profiles of *Gracilaria fisheri* were compared across four different extraction techniques. Protein was extracted using a variety of methods, including treatments with and without enzyme (Acetate buffer/NaOH buffer) and enzyme (cellulase/alcalase) at two distinct pH levels (4.5 and 7). Amino acid profiling also carried out in this study through acid oxidation, acid hydrolysis & alkali hydrolysis by utilizing the HPLC method. To extract the maximum amount of protein, researchers used a combination of acetate buffer, cellulase enzymes, sonication, and ammonium sulfate in Method 1. The study found that the efficiency of protein extraction ranged from 86 % for Method 1 to 10 % for Method 4. According to the results of this study, method 1 had the lowest separated yields of protein content (8.74%), while method 4 had the highest (25.11%). It was determined that Method 1 was the most effective for amino acid profiling in *G. fisheri*. Based on our findings, pure protein concentrates extracted from *Gracilaria* species possess the potential as sustainable alternative protein sources for human nutrition, and they will be useful in meeting our estimated global protein need in the future.

Keywords: Protein, *Gracilaria fisheri*, Seaweed, Acid oxidation, Acid hydrolysis, Alkali hydrolysis, Extraction.

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Chapter-1: Introduction

Food production must be increased and new food sources must be discovered as the global population continues to grow. Together, these factors and the decreasing availability of arable land and potable water make marine agriculture (also called Mariculture or Seagriculture) an attractive alternative option for human food production. The likelihood of a shortage of protein, a vital nutrient, in the future has been raised. Therefore, it is generally agreed that new protein production methods and sources will be necessary to meet future world protein demands. Minerals and trace elements are plentiful in seaweeds (Klnç et al., 2013).

The protein content of several macro algal species, especially red seaweeds, has been shown to be rather high; in some cases, it is even higher than that of more traditional sources of protein such soy, cereals, eggs, and fish (Kaliaperumal, 2003).Since macro algal proteins have both technological and biological applications, they represent a promising raw resource for creating new food items that are high in protein. The food industry stands to gain greatly from the abundance of essential minerals contained in edible algae (Plaza et al., 2008).

Seaweed is not only a staple meal in many Asian countries, but also a source of biochemicals for use in Western industries like the food, pharmaceutical, and beauty industries. There is a significant amount of seaweed in the food industry for stabilizing and thickening applications because of their high polysaccharide content. More and more people are turning to seaweeds for their high protein content. However, protein, peptide, and amino acid extraction and fractionation from macro algae have otherwise been conducted on a laboratory.

One of the largest genera of the red algae phylum Rhodophyta, *Gracilaria* has more than one hundred species distributed across the world's intertidal and subtidal zones. *Gracilaria*, a red marine macroalgae, is abundant in the tropical Atlantic and is well-known for its rapid growth rates (Melo et al., 2002). *Gracilaria* genus is important to humans because it provides a rich source of sulphated polysaccharides for use in the pharmaceutical and biotechnology sectors (Coura et al., 2012). Still, *G. fisheri* has seen only a little amount of widespread use. Traditionally it provides both dry products and perishables like fruits and vegetables (Benjama and Masniyom, 2011).

Following centrifugation, the protein-rich supernatant can be collected and the proteins are then purified utilizing different techniques such as- ultrafiltration, ammonium sulfate precipitation, or chromatographic (Galland-Irmouli et al., 1999). For the most part, the protein content of dried seaweed is extracted via enzymatic hydrolysis, water extraction, acid extraction, or alkaline extraction. Cellulases and alkalases are used in enzyme-based extraction to decimate the seaweed matrix and free the proteins that are contained within (Kadam et al., 2015).

Ultrahigh pressure extraction (UPE), pressurized fluid extraction (PFE), pressurized liquid extraction (PLE), microwave assisted extraction (MAE) (Chemat and Cravotto, 2012), supercritical fluid extraction (SFE) (Liang and Fan, 2013; Sereewatthanawut et al., 2008), and ultrasound assisted extraction (UAE) are only a few of the non-traditional methods of extraction that are now being studied and developed to boost extraction yield in a shorter amount of time. All these strategies improve mass transfer, which in turn boosts solvent-solute interaction and facilitates the reduction of extraction causes difficulties due to seaweed's complicated matrix.

High viscosity and ionic interactions due to the cell wall and interior polysaccharides may make it challenging to extract proteins from macro algae (Joubert and Fleurence, 2008). As a result of the cells being broken down and the addition of specific chemicals, protein extraction from macro algal biomass is now much simpler. Currently, the most popular procedures for eliminating macro algal cells include osmotic shock, mechanical grinding, ultrasonic treatment, and polysaccharide digestion (Sun et al., 2009; Joubert and Fleurence, 2008; Rouxel et al., 2001; Wong and Cheung, 2001a; Galland-Irmouli et al., 2000; Fleurence et al., 1995).

The utilization of polysaccharidases as a method of cell disruption for macro algal protein extraction has been demonstrated (Denis et al., 2009; Fleurence et al., 2001; Fleurence et al., 1995; Amano and Noda, 1992, 1990). However, the effectiveness of the enzymatic activity differs between algae species due to the structure of the cell wall, therefore careful consideration must be given while selecting enzymes.

One of the aforementioned methods for cell disruption is commonly used in water for protein extraction from macroalgae. In addition to the most typical alkaline extraction, macroalgae protein can also be extracted in low salt and high salt soluble forms. Macroalgal proteins are hydrophobic and therefore very difficult to dissolve in water.

However, alkaline solutions have long been known to be efficient (Wong and Cheung, 2001; Fleurence et al., 1995; Rice and Crowden, 1987).

This study set out to determine whether or not several treatments (osmotic shock, high shear force, alkaline, and polysaccharide) improved protein extraction from the red alga *Gracilaria fisheri*. Comparison of the methods that result in the greatest quality protein extraction and purity allowed us to choose the protein with the highest purity level.

Objectives:

1) To access a range of parameters associated with the extraction of protein from *Gracilaria fisheri.*
2) To compare the methods that gives better pure protein extraction.
3) To analyze the characteristics of highest percentage of extracted pure protein.

Chapter-2: Review of Literature

We have studied the literature on red seaweed, *Gracilaria* species, and their anticancer, anticoagulant, antibacterial, and anti-inflammatory actions, as well as their general biology, traditional applications, economic value, and health advantages. The review founding of the articles are presented in this chapter.

2.1 General Biology of *Gracilaria* species:

Among the red algae, the genus *Gracilaria* is located in the phylum Rhodophyta, the class Florideophyceae, the order Gracilariales, and the family Gracilariaceae. *Gracilaria* species can be found all over the world, from the tropics to the subarctic, but the Indo-Pacific and Western Atlantic are particularly rich in *Gracilaria* diversity.However, there are many species whose distribution is still little understood and is severely limited on a global scale (Guiry M.D. and Guiry G.M., 2018).

Figure 2.1: *Gracilaria* species (Guiry M.D. and Guiry G.M., 2018)

A common and easily recognizable group of benthic plants, *Gracilaria* species are often found between 0.5 and 10 meters deep in the intertidal and subtidal zones (Lyra et al., 2016). Members of this genus typically have at least some of their thallus covered by sand as they grow, whether they are linked to a solid (e.g. rocky) substratum or anchored in sand or sand and mud. Some species are also capable of living alone, forming dense mats on the surface of still water (Mello, 2001). While *Gracilaria* species can be found in a variety of maritime environments, they are most commonly discovered in protected places such as estuaries, bays, mangroves, reefs, and mudflats.

Polysiphonia-type life cycles have been identified in the *Gracilaria* class, where two asexual isomorphic epochs (the gametophyte and tetra sporophyte phases) are followed by a third asexual epoch (the carp sporophyte stage) that generates a female gametophyte (Kain and Destombe, 1995). The literature on *Gracilaria* species frequently describes alterations in morphology, life structures, physiology, development, proliferation, and synthetic piece in response to ecological (such as water temperature, saltiness, type of substrate, light power, and supplements) and biotic (such as epiphytism) factors, implying a remarkable phenotypic versatility. Attempting to identify a species only by its outward appearance is a difficult endeavor due to the large number of species and the considerable phenotypic variability they present (Gurgel and Fredericq, 2004). In recent years, molecular analyses based on DNA sequence data have become increasingly popular, allowing for more accurate predictions in areas such as scientific classification, biogeography, and phylogenetic relationship. In this analysis, we opted to include the species *Gracilaria* that has been recently proposed by Iha et al. (2018).

2.2 Traditional applications and economic significance:

The economic value of *Gracilaria* species is extremely high, and they are widely exploited by humans. China has a long history of misusing *Gracilaria* species for medicinal purposes. These vegetative expansions had dual use as both sustenance and folio material for architects fixated on lime in their wall compositions (Tseng, 1981). It's generally agreed that China was the pioneer in the spread of this eco-friendly development to the rest of the East.

Many people in Japan, Southeast Asia, Hawaii, and the Caribbean eat a type of *Gracilaria* called "ocean vegetable." Examples of dishes that benefit from the use of these algae as a garnish include the Hawaiian dish Poke, a fish salad (Jensen, 2004). To the Japanese, they are ogonori (or ogo), and they are typically eaten raw in a salad or as a garnish for sushi. Jamaicans call them "Irish moss," and they're used to make a popular drink by the same name. (Red macroalga *Chondrus crispus* Stackhouse is known as "Irish moss" in other parts of the world) (Gordon, 2017)

Gracilaria agar is a type of agar that has been approved for human consumption, thus it can be used in baked goods like pies, icings, and jam confections to add thickness,

stability, or gelling (Nussinovitch, 1997). Due to its animal origin, gelatin has been replaced with the plant-based thickener agar in Western diets. Common uses for agar in Asian cuisines include desserts like "Anmitsu" (China), "Ykan" (Japan), and "Halo-halo" (Philippines), as well as soups, snacks, and everyday foods like "Tokoroten" (Japan).

Numerous remedies involving *Gracilaria* species have their origins in traditional medicine. Some strains of *Gracilaria* have been shown to be effective in China for treating a variety of conditions, including those involving the digestive tract (such as bloating, gas, and diarrhea), the urinary tract, the thyroid gland, and the lungs (Fu et al., 2016). As an emollient and demulcent, *Gracilaria* species is used in Indian medicine to treat respiratory sickness, loose stools, and diarrhea (Khare, 2007; Watt, 2004; Nadkarni, 1996).

The most specific application of this genus is as a diuretic or for the treatment of intestinal constipation. In some countries, you can buy powdered *Gracilaria* or straight agar to help with gastrointestinal issues and curb your appetite.

Gracilaria species have been put to good use in a wide variety of products, including shampoos (such as the *Gracilaria* Cleanser by Thermalabs), cleansers (such as the hydrogel Cleanser by Ocean Laria), hydrating creams (such as the Day Cream by Thalasso), a facial cover (such as the Detox Facial Veil by Balinique), moisturizers (such as the *Gracilaria* Hydrogel by Ocean Laria), and even deodorants (e.g., normal antiperspirant by Bali secrets). *Gracilaria* is commonly used in the cosmetic industry because to the agar's thickening, settling, or gelling properties (Pereira, 2018)

2.3 Use of *Glacilaria* species in human health fields:

2.3.1 Anticancer activities:

According to the World Health Organization (2018), cancer is a major killer worldwide, responsible for the loss of life of almost 8 million people each year. Chemotherapy is a mainstay in the treatment of many diseases, but it's not without drawbacks. Drug resistance is also on the rise (Luqmani, 2005). Thus, the earnest and rational search for novel anticancer drugs isn't surprising. Most studies evaluating the efficacy of *Gracilaria* extracts used either a reduction in tetrazolium salts (67%) or a

change in resazurin color (10%) to determine whether or not the cells were undergoing metabolic activity.

In most instances, organic solvents yielded the most dynamic and thoroughly researched concentrations. Phytol was previously identified as a component responsible for the cancer-fighting actions of ethyl acetic acid derived extracts from *G. edulis* (Sakthivel et al., 2016; Sheeja et al., 2016). *Gracilaria* species were rarely subjected to a variety of tests, such as those designed to determine how resistant they are to translocation or cytotoxicity. Lao et al. (2017) provided the first comprehensive analysis of the effects of anti-migration policies.

2.3.2 Anticoagulant activities:

Blood clots can be avoided or reduced with the help of anticoagulants. Anticoagulants are widely used in experimental purpose and for the control of illness like thrombosis (Koenig-Oberhuber and Filipovic, 2016). The effectiveness of *Gracilaria* water concentrates or sulfated agarans (Subramanian, 1997) was tested against the activity of heparin (the gold standard) and other algae, however the results were disappointing.

2.3.3 Anti-inflammatory and Anti-nociceptive activities:

Anti-inflammatory drugs work to alleviate symptoms like pain, fever, and swelling. The majority of *Gracilaria* species anti-inflammatory exercise studies used aqueous extracts or sulfated agarans as the experimental material (Chaves et al., 2013). All things considered, the anti-inflammatory potential observed in these studies was fairly high. It's interesting to note that the findings using organic extracts were moderate at best (Shu et al., 2013; Lee et al., 2006). There was a decrease in edema and leukocyte migration due to the use of fluid concentrates and sulfated agarans, and the announcement of major combinations for inflammation was muted.

Tseng et al. (2014) provide evidence that fluid concentrates and sulfated agarans operate on mass cells, preventing the arrival of their substance by inhibiting various inflammatory mechanisms, such as restraint of the NF-kappa B (factor atomic kappa B) and MAPK (mitogen-enacted protein kinase) pathways (Coura et al., 2015). Equally intriguing is the potential role of the heme oxygenase-1 pathway in the

mitigating component of these concentrates and fractions (Coura et al., 2015; Vanderlei et al., 2011).

Pain-inducing stimuli are not felt as strongly by those with antinociceptive activity. The sulfated agarans of the genus *Gracilaria* were used to characterize this phenomenon (Makkar and Chakraborty, 2017; Coura et al., 2015; Chaves et al., 2013; Vieira et al., 2012). The discomfort felt during these tests resulted from inflammation. Sulfated agarans typically had analgesic benefits. However, Chaves et al. (2013) pointed out that the agarans' pain-relieving effect was not caused by their direct activity on pain receptors but rather by the attenuation of provoking effects.

2.3.4 Antimicrobial activities:

Antimicrobials are chemicals that stop the growth of bacteria, fungi, and other microorganisms (Sirois, 2016). The World Health Organization (WHO) states that due to the prevalence of adverse impacts and the rapid evolution of antibiotic-resistant microorganisms, the search for novel antimicrobial drugs to combat infectious diseases such pneumonia, tuberculosis, herpes, malaria, and AIDS (acquired immunodeficiency syndrome) is recommended. In this context, we shall only discuss microorganisms that have direct bearing on human health.

Among the most commonly tested microorganisms in *Gracilaria* species investigations were microscopic organisms, growths, protozoa, and diseases. Over eighty-nine percent of the bacteriological and infectious microorganism tests were carried out utilizing some sort of plate or well dispersion test. Problems with these tests stemmed from inaccurate portrayals of methods, and that was true even for anticancer treatments. Greater than 70% of the articles focused on screening for macroalgae. Nearly half of those who used bacterial microbes and 20% of those who used infectious microbes named *Gracilaria* species as one of the most effective algae against a single bacterium or parasite. The pathogenic bacterium *Staphylococcus aureus*, the source of some infections acquired in emergency rooms, was shown to be particularly susceptible to this concentration, making it one of the most effective antimicrobial weapons available. Herpes simplex virus types 1 and 2 (HSV 1 and HSV 2) were the most researched viruses (57.8% of all articles).

When tested against the various diseases, organic extracts proved ineffective, but aqueous extracts (or sulfated agaran) performed admirably. According to research by

Mazumder et al. (2002) a sulfated agaran component of *G. corticata* is more effective at inhibiting the replication of HSV1 and HSV2 than the positive control (Heparin). *Gracilaria's* antiprotozoal abilities have been studied in conjunction with a wide variety of other algae species.

2.3.5 Gastrointestinal diseases:

The term "gastrointestinal sickness" refers to conditions that manifest in the digestive tract and its supporting structures and appendages. *Glacilaria* species agarans, both in their raw and sulfated forms, have demonstrated protective effects against gastrointestinal injury, including the development of ulcers in rats induced by pylorus ligation (Senthil and Murugan, 2013), ethanol (Shu et al., 2013; Silva et al., 2011) or naproxen (Silva et al., 2012).

Studies on the antidiarrheal activity of sulfated agarans isolated from *G. caudata* (Costa et al., 2016) and *Gracilaria* intermedia J.Agardh (Leódido et al., 2017) were conducted. These polysaccharides' antidiarrheal effects included, among other things, an adversarial action on the secretory apparatus and a slowing of the gastrointestinal motility due to anticholinergic components.

2.3.6 Metabolic syndrome:

Components of metabolic illness that increase the complications of developing cardiovascular disease and type 2diabetes include hyperglycemia, hypocholesterolemia, hypertension, hypertriglyceridemia, and obesity (Cornier et al., 2008). *Gracilaria* species were used to investigate these potential dangers. Exercises to lower blood sugar levels or combat diabetes were typically performed in vitro, where the inhibitory effects of substances like alpha-glucosidase and amylase could be evaluated. The anti-diabetic action was often weak when compared to other macroalgae (red, brown, and green) or positive controls. However, (Xiancui et al., 2005) provided a notable exception when they described *G. textorii* as one of the most energetic algae. Researchers studied the effects of exercise on rats given a diet high in cholesterol and fat to determine if it may prevent hyper-lipidemia (high blood lipid levels).

Plasma levels of fatty oils, total cholesterol, and LDL (Low Density Lipoprotein) decreased with a lower caloric intake supplemented with algae (Lin et al., 2011)

Histopathological studies conducted by Chan et al. (2015) showed that *G. changii* enriched meals had no toxic effects on cadiac muscle or kidney and had protective benefits on the livers of hyperlipidemic rats. There was also an increase in the activities of cell reinforcement chemicals in these organs, which may be related to the decreased oxidative stress brought about by *G. changii* supplemented meals (Chan, et al., 2016).

2.3.7. Neurological disorders:

Diseases of the nervous system, often known as neurological disorders, include brain tumors, epilepsy, Parkinson's disease, stroke, and Alzheimer's disease (Ishwarya and Narendhirakannan, 2016). Sulfated agarans of *G. cornea* showed neuroprotective effects in rats that were being used for research on Parkinson's disease (Souza et al., 2017), as well as anxiolytic qualities (Monteiro et al., 2016). Several studies looked into the opposite of acetylcholinesterase's catalytic role. Neurological symptoms of Alzheimer's disease can be alleviated with the use of enzyme inhibitors. Some authors, including Natarajan et al. (2009), found encouraging results in their work. These authors demonstrated that methanolic concentrates of *G. gracilis* and *G. edulis* have promising therapeutic potential, with IC50 values that are lower than donepezil (reference standard). Suganthy et al. (2010) observed comparable results for *G. edulis* methanol concentrates. However, as compared to other algal species, *Gracilaria* strains demonstrated moderate (Bianco et al., 2015) and poor (Ghannadi, 2013) physical activity.

2.4 Use of *Glacilaria* species in other fields:

2.4.1 Agglutinating activities:

Compounds called agglutinins cause natural or cellular particles to clump together (Stegman, 2012). Antibodies and lectins are two well-known examples of such molecules. As a class, agglutinins have several practical applications outside of blood type, including antibacterial and cytotoxic properties (Stegman, 2012; Sharon and Lis, 2004). Here's something that's never happened before: over eighty percent of published publications on the subject of agglutinating characteristics of concentrates from species of *Gracilaria* evaluate the ability to agglutinate red platelets. Comparing the agglutinating activity of various groups of macroalgae revealed wide variation (red, brown, and green). Dinh et al. (2009) evaluated 44 different algae species and

found some encouraging results. Researchers discovered that the erythrocytes of rabbits, chickens, and sheep were agglutinated most effectively by the crude extract of *Gracilaria eucheumatoides* Harvey and *G. salicornia.*

2.4.2 Agriculture:

Increased production and tolerance to biotic and abiotic difficulties are two of the many benefits of employing macroalgae in plant crops (Battacharyya et al., 2015). In recent years, it has become clear that numerous members of the genus *Gracilaria* also have positive effects on plants that grow in soil. Mung bean plants benefited from *G. corticata* aqueous extract in terms of both leaf output and stem or root size (Chitra et al., 2013).Studies on *G. caudata* and *G. domingensis* extracts have led to the discovery of several biostimulants for lettuce, including sulfated agarans, palmitic acid, and phenylacetic acid (auxin) (Torres et al., 2018).

Gracilaria extracts have also been studied for their potential to combat several pathogenic fungi that are a problem in agricultural settings. When results were compared to those obtained with other varieties of macro algae (red, brown, and green) or with controls, they were disappointing. Jiménez et al. (2011) found that *G. chilensis* extracts in both water and alcohol were equally effective at inhibiting the development of root-rot fungus (*Phytophthora cinnamomi*).

2.4.3 Antioxidant activities:

Antioxidants are chemical molecules that prevent or slow the oxidation of organic matter or living organisms when they are exposed to oxidative stress. These chemicals have many potential applications, including disease prevention and use as food and cosmetic additives (Halliwell and Gutteridge, 2015).

Half of the papers looked at differences and similarities between different types of macroalgae (red, brown, and green). The *Gracilaria* species studied here showed weak to moderate cell reinforcement potential. Some examples of models include the ones by Zhang et al. (2007) that focus on Chinese algae and Zubia et al. (2007) that focus on Mexican algae. While research on sulfated agarans, such as that conducted by Seedevi et al. (2017) has yielded promising results, such research has been rarely concentrated. Results obtained by these writers were consistent with those found in established normative sources (ascorbic acid and butylated hydroxytoluene – BHT).

To give just one example, Murakami et al. (2005) revealed that the chloroform concentrate of *Gracilaria blodgettii* Harvey was among the best of a small group of land plants and algae at alleviating oxidative and nitrosative stress caused in leucocytes.

2.4.4 Aquaculture:

Aquaculture is the practice of raising marine organisms in captivity, such as fish, macroalgae, and crustaceans. A number of *Gracilaria* species have been noted as having showed potential in specific uses, most notably in the shrimp and fish farming industries. About 37% of all shrimp distributions in hydroponics come from in vivo experiments (Chen et al., 2012; Chen et al., 2015; Yeh et al., 2010).

Whiteleg shrimp (*Litopenaeus vannamei*) infected with WSSV survived longer when exposed to saltwater containing an aqueous extract of *G. tenuistipitata* (Lin et al., 2011). For this analogy, we used whiteleg shrimp infected with Vibrio alginolyticus. The liquid concentrate from *G. tenuistipitata* activates the prophenoloxidase framework, which increases the resistance of whiteleg shrimp, as reported by Chen et al. (2016)

Supplementation with *Gracilaria* species has been shown to improve the safety of reactions in European seabass (*Dicentrarchus labrax*) (Peixoto et al., 2016) and rainbow trout (*Oncorhynchus mykiss*) (Arajo et al., 2016), as well as reduce mortality in gilt-head bream (*Sparus aurata*) exposed to hypoxia (Magnoni et al., 2017).

The bioactivity properties and effects of dietary supplementation with *Gracilaria* species support their use as functional feed in aquaculture. *Gracilaria* species were shown to have moderate to high antibacterial activity, which was low to nonexistent in other macroalgae (red, brown, and green). One of the best results possibly was achieved by Bansemir et al. (2006) when they zeroed in on 26 different macroalgae. Results showed that the *G. cornea* dichloromethane extract was the most effective at inhibiting the growth of the fish pathogen *Pseudomonas anguilliseptica*.

2.5 Different extraction method of red seaweed protein with advantages and limitations:

There are a number of techniques used to extract the beneficial compounds from red seaweed; for example, microwave-assisted extraction (MAE), enzyme-assisted

extraction (EAE), ultrasound-assisted extraction (UAE), supercritical fluid extraction (SFE), pressurized solvent extraction (PSE), reaction extrusion, and photo-bleaching, each of which has its own set of benefits and drawbacks (Khalil et al., 2018).

Table 2.5: Advantages and limitations of different extraction methods of red seaweed protein (Khalil et al., 2018)

Extraction methods	**Advantages**	**Limitations**
Microwave-Assisted Extraction(MAE)	▪ Quick recovery time ▪ Both organic solvents and water are acceptable. ▪ Suitable for materials that quickly deteriorate in high temperatures ▪ Superior to the traditional Soxhlet procedure	▪ Solvents with low dissipation factors and high dielectric constants are required. ▪ High capital cost ▪ Expansion could occur, especially in a closed tank containing MAE.
Enzyme-Assisted Extraction(EAE)	▪ Permitted a sizable amount of bioactive chemical to be produced	▪ Extraction takes a long time (from hours to days) ▪ Insufficient moisture in the starting material greatly reduces the efficacy of enzymatic hydrolysis.
Ultrasound-Assisted Extraction(UAE)	▪ Rapid recovery time ▪ Lower usage of solvents ▪ Extraction yields are very high.	▪ Extraction efficiency is highly dependent on the plant matrix.

	▪ Inexpensive	▪ It is recommended to use solvents that have a low vapor pressure, surface tension, and viscosity. ▪ The quality of extracts could be diminished by over-sonication.
Supercritical Fluid Extraction(SFE)	▪ Extremely pure, residue-free extracts ▪ Non-solvent-based extracts ▪ Short extraction time ▪ Perfect for the isolation of heat-sensitive substances	▪ High pressure requires expensive equipment. ▪ It could be challenging to isolate polar substances. ▪ Cost and energy to process are very high.
Pressurized Solvent Extraction(PSE)	▪ Extraction efficiency is high, and fewer solvents are used. ▪ Minimal time required for extraction	▪ The high-pressure equipment required is expensive. ▪ Degradation of thermo labile compounds is a possible side effect of high-temperature extraction.
Reaction extrusion	▪ Lessen the use of time and solvents. ▪ Quality of extracted yields	▪ Not defined

	has increased.	
Photo-bleaching	▪ Because less bleaching agents are used, it is less harmful to humans and the environment. ▪ Better efficiency and quality in extraction.	▪ Not defined

2.6 Amount of protein extraction from different red seaweed species:

The amount of protein extraction after application of different extraction methods from different red seaweed species are given below-

Table 2.6.1: Amount of protein extraction from *Palmaria palmata*

Name of the species	**Extraction methods**	**Yield of extracted protein (%)**	**References**
Palmaria palmata	Classical sonication and salting out method	12.5 ± 2.3	O'Connor et al., 2020
	High-pressure processing (HPP)	14.9 ± 1.1	
	Laboratory autoclave processing	21.5 ± 1.4	

Table 2.6.2: Amount of protein extraction from *Chondrus crispus*

Name of the species	**Extraction methods**	**Yield of extracted protein (%)**	**References**
Chondrus crispus	Classical sonication and salting out method	35.2 ± 3.9	O'Connor et al., 2020
	High-pressure processing (HPP)	16.1 ± 0.5	
	Laboratory autoclave processing	21.9 ± 3.3	

Table 2.6.3: Amount of protein extraction from *Eucheuma cottonii*

Protein extraction from *Eucheuma cottonii* (Lim et al., 2015)			
Extraction methods	**Concentration (µg/mL)**	**Mass (µg)**	**Total protein yields (mg/g)**
Phenol/lysis buffer extraction	675.410±11.375	135.082±2.275	0.027±0.000
Chloroform extraction	448.330±22.793	89.666±4.559	0.018±0.001
Phenol/sodium dodecyl sulphate (SDS) buffer extraction	597.086±38.067	119.417±7.613	0.024±0.002

2.7 Amino acid profiling method of different red seaweed species:

Different amino acid profiling method of different seaweed species are given below-

Table 2.7: Amino acid profiling method of different red seaweed species

Name of the species	Amino acid profiling method	References
Palmaria palmata	Liquid chromatography using mass spectrometry	Naseri et al., 2020
Porphyra dioica, Porphyra umbilicalis,Gracilaria vermiculophylla, and Ulva rigida	High performance liquid chromatography.	Machado et al., 2020
Laurencia Filiformis, Laurencia intricate, Gracilaria domingensis. Gracilaria birdiae	Gas chromatography-mass spectrometry	Gressler et al., 2012
Gracilaria changii	High performance liquid chromatography.	Benjama and Masniyom, 2011

2.8 Amino acid profiles of different red seaweeds:

Gressler et al. (2010) conducted research on the amino acid profiles of several distinct species of red seaweed. These included *Kappaphycus alvarezii, Gracilaria domingensis, Gracilaria birdiae, Laurencia filiformis,* and *Laurencia intricate.*

Table 2.8: Amino acid profiles of different red seaweed species

Amino acid(%)	***Kappaphycus Alvarezii***	***Gracilaria domingensis***	***Gracilaria birdiae***	***Laurencia filiformis***	***Laurencia intricate***
Cysteine	Not defined	0.03	0.04	0.1	0.04
Isoleucine	0.12	0.4	0.4	0.5	0.3
L-Arginine	0.15	0.4	0.6	0.6	0.2
Leucine	0.22	0.7	0.7	0.8	0.5
L-Tyrosine	0.07	0.2	0.2	0.6	0.3
Lysine	0.14	0.4	0.6	1	0.5
Methionine	0.05	0.2	0.2	0.3	0.1
Phenylalanine	0.13	0.4	0.5	0.5	0.3
Threonine	0.15	0.4	0.5	0.6	0.4
Valine	0.16	0.4	0.5	0.5	0.3
Glycine	0.16	0.5	0.6	0.7	0.5
Histidine	0.02	0.1	0.2	0.2	0.1
L-Alanine	0.18	0.6	0.7	0.7	0.5
L-Aspartate	0.29	1	1.2	1.5	1
L-Glutamate	0.35	0.9	1	1.4	0.9
L-Proline	0.13	0.4	0.5	0.5	0.3
L-Serine	0.15	0.4	0.5	0.6	0.4
Tryptophan	Not defined	0.2	0.2	0.1	0.1

Chapter-3: Materials and Methods

3.1 Study period and Study area:

The research work was conducted for a period of 6 months from January 2022 to June 2022. Experimental procedure was carried out in the laboratory of the Department of Applied Food Science and Nutrition and Poultry Research and Training Center (PRTC) at Chattogram Veterinary and Animal Sciences University, Bangladesh.

3.2 Experimental Design:

At first, the red seaweed samples of *Gracilaria fisheri* were collected. The samples were already prepared for the experiment. Then the prepared freeze dried samples were used to determine the percentage of pure protein using different extraction protocols such as method 1, method 2, method 3 and method 4. The percentage of pure protein for each method was determined by the Kjeldahl method AOAC (2020). Finally, protein characterization was done according to Benjama and Masniyom (2011) with some slight modification applying the Water AccQ.Tag Amino acid analysis method using High Performance Liquid Chromatography (HPLC) (Waters Corporation e2695, Milford, MA, USA).

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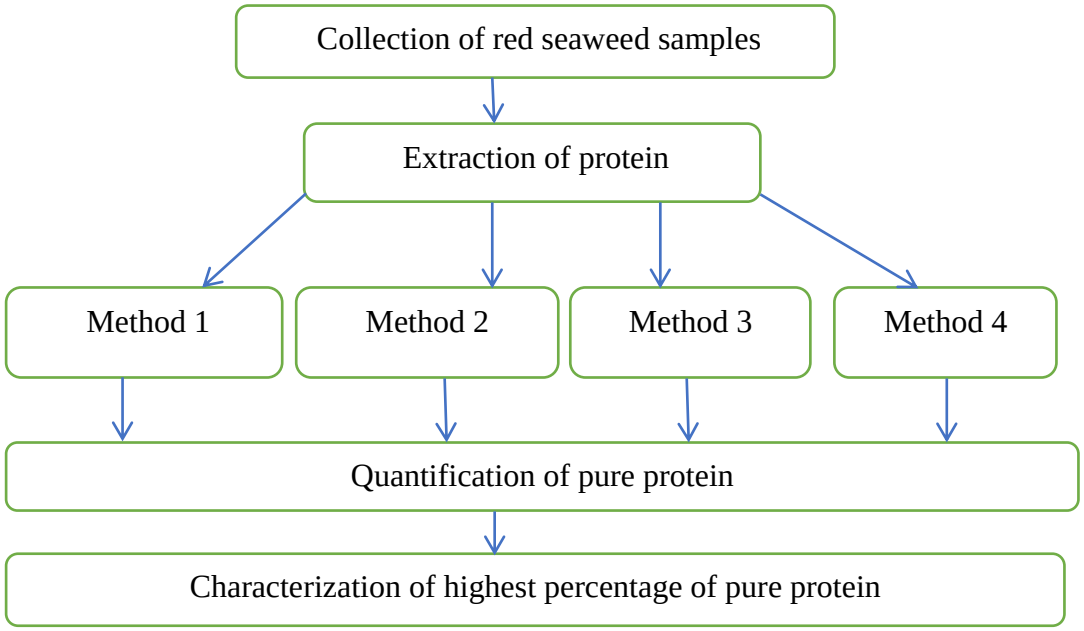


Figure 3.2: Flow diagram of Study Design

3.3 Collection of Samples and Preparation:

The red seaweed samples of *Gracilaria fisheri* were purchased from Thailand. We have also collected some samples from Cox´s bazar and Potenga seabeach but these were mixed with other seaweeds. As we need pure samples for extraction, so we decided to purchase pure sample from the reliable source (Hosni Ismael, seaweed supplier) from Thailand. We purchased from them 500gm freeze dried sample and 20gm raw sample of *G. fisheri* for our research purpose.

3.4 Apparatus, Machineries and Chemicals required for the extraction:

Table 3.4: List of apparatus, machineries and chemicals required for the extraction of protein from *Gracilaria fisheri*

Apparatus	Machineries	Chemicals
pH meter	Magnetic stirrer	Acetate buffer
Thermometer	Centrifuge machine	NaOH buffer
Centrifuge tube	Sonicator	Ammonium sulfate
Soxhlet apparatus	Chiller	HCL
Erlenmeyer flask	Freeze drier	Na_2CO_3
Dialysis tube		Ultrapure water
Plastic container		Deionized water
Blender / Grinder		
Water bath		

3.5 Protein extraction protocols of *Gracilaria fisheri* :

In the course of this research, a total of four different procedures for protein extraction were carried out in order to compare the protein profiles of *Gracilaria fisheri*. These are given below-

3.5.1 Method 1:

We used a modified version of the seaweed protein extraction method published by Galland-Irmouli et al. (1999). The procedure used a combination of sonication, ammonium sulfate extraction, acetate buffer, and cellulase enzyme.

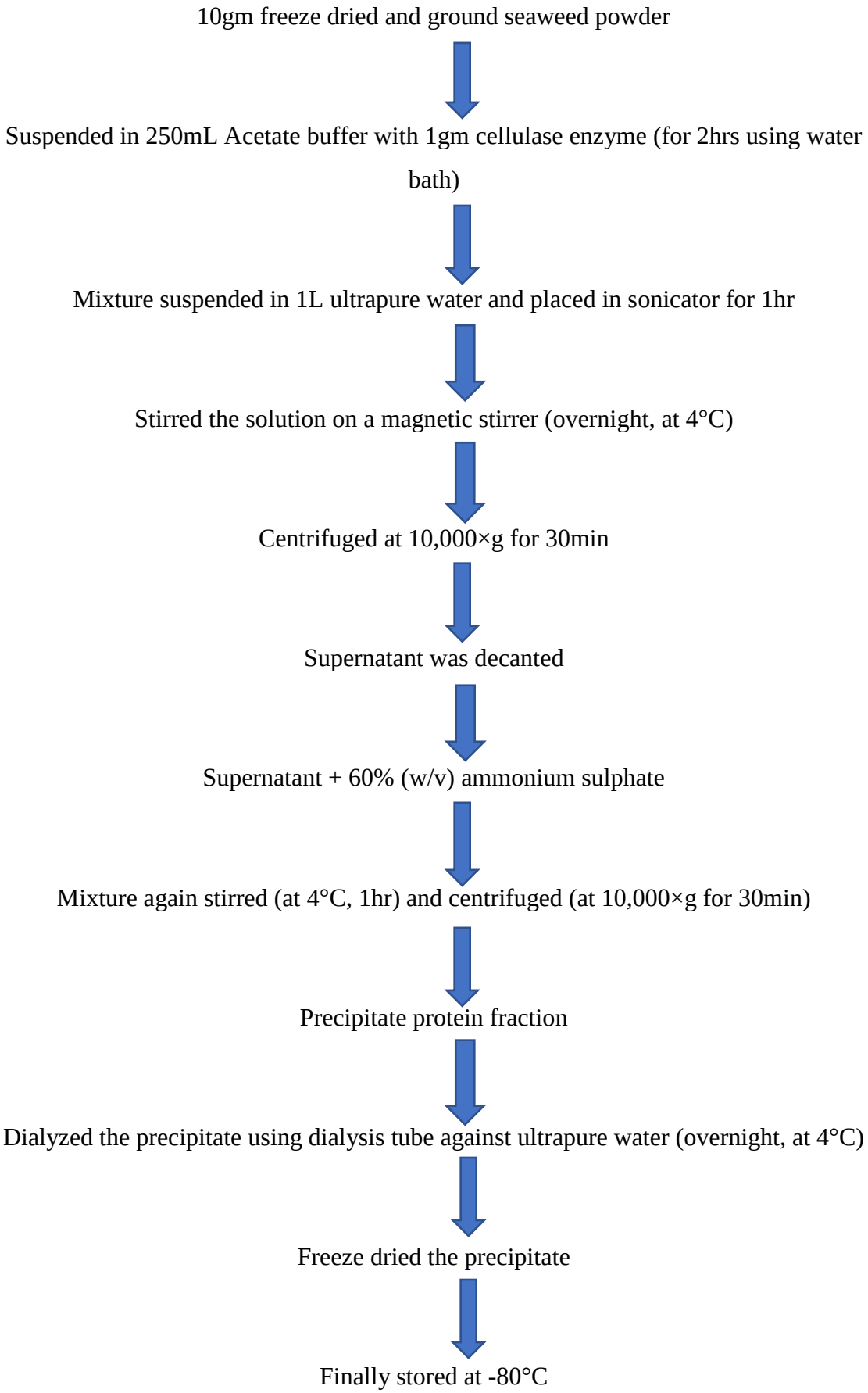


Figure 3.5.1: Flow chart of Method 1 protein extraction procedure

3.5.2 Method 2:

An enzymatic treatment was performed using a seaweed biomass/solution ratio of 1:20 w/w. The enzymatic extraction conditions such as pH and temperature were set in enzyme, pH, temperature accordingly 0.4% (cellulase), 4.5, 50 respectively and the pH was adjusted using either hydrochloric acid (HCl) or sodium carbonate (Na_2CO_3) described by Naseri et al. (2020).

500 mL of deionized H_2O was added to each of the 1 L Erlenmeyer flasks

↓

The enzymatic extraction conditions were constructed (such as 0.4% cellulase enzyme, pH 4.5, 50°C temperature, and pH adjustment with HCL or Na2CO3

↓

Placed the flasks in a water bath (at 50°C temperature and 80 rpm speed)

↓

Dried biomass was rehydrated for 1hr

↓

Add cellulase enzyme & enzymatic extraction was performed for 14hrs

↓

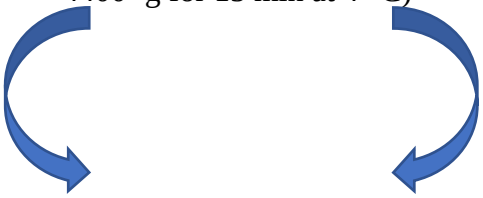
The pH of the total liquid fraction recovered from each Erlenmeyer flask during the aqueous/enzymatic extraction and alkaline extraction was adjusted from about 13 to 3 with HCl(5 M).

↓

Precipitate protein

↓

Immediately after protein precipitation, the resulting “soup” was centrifuged (at 4400×g for 15 min at 4 °C)



Supernatant (liquid) Pellet (solid)

Both were collected in a plastic container and stored at -80°C

Solid fraction was freeze dried

Finally milled before chemical analysis

Figure 3.5.2: Flow chart of Method 2 protein extraction procedure

3.5.3 Method 3:

The ratio of 1:20 w/w (seaweed biomass/solution) was utilized for the enzymatic treatment. Here, pH was adjusted using hydrochloric acid (HCl) or sodium carbonate (Na2CO3) and the enzymatic extraction conditions were set in enzyme, pH, temperature appropriately 0.4% (Alcalase), 7, 50 as indicated by Naseri et al. (2020).

500 mL of deionized H_2O was added to each of the 1 L Erlenmeyer flasks

Enzymetic extraction conditions were set (such as 0.4% Alcalase enzyme, pH 7, 50°C temperature respectively and pH was adjusted either HCL/ $Na_2CO_{3)}$

Placed the flasks in a water bath (at 50°C temperature and 80 rpm speed)

Dried biomass was rehydrated for 1hr

Add alcalase enzyme and enzymatic extraction was performed for 14hrs

Then pH of the total liquid fraction recovered from each Erlenmeyer flask during the aqueous/enzymatic extraction & alkaline extraction was lowered from approximately 13 to 3 using HCl (< 5 M).

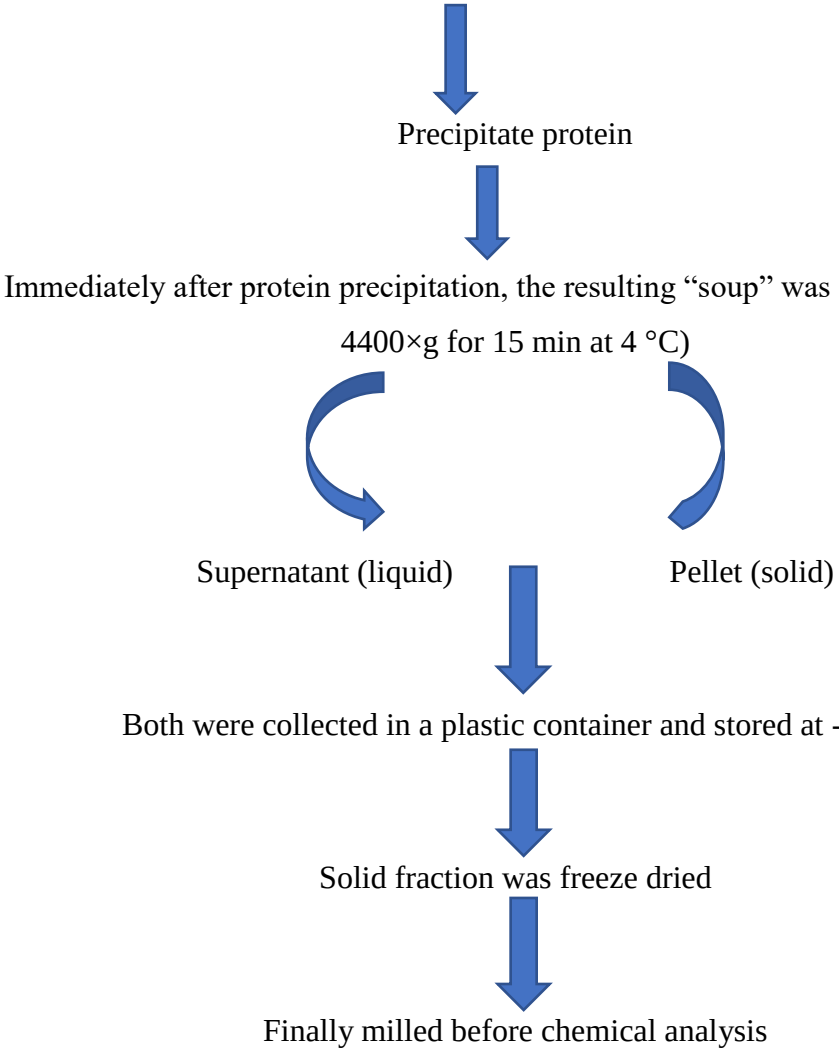
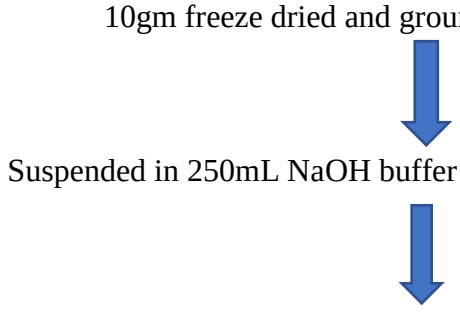


Figure 3.5.3: Flow chart of Method 3 protein extraction procedure

3.5.4 Method 4:

In this method protein extraction from seaweed was employed as described by Galland-Irmouli et al. (1999), with slight modification. The method used a combination of treatment using sodium hydroxide (NaOH) buffer, sonication and ammonium sulfate extraction without any enzymes.



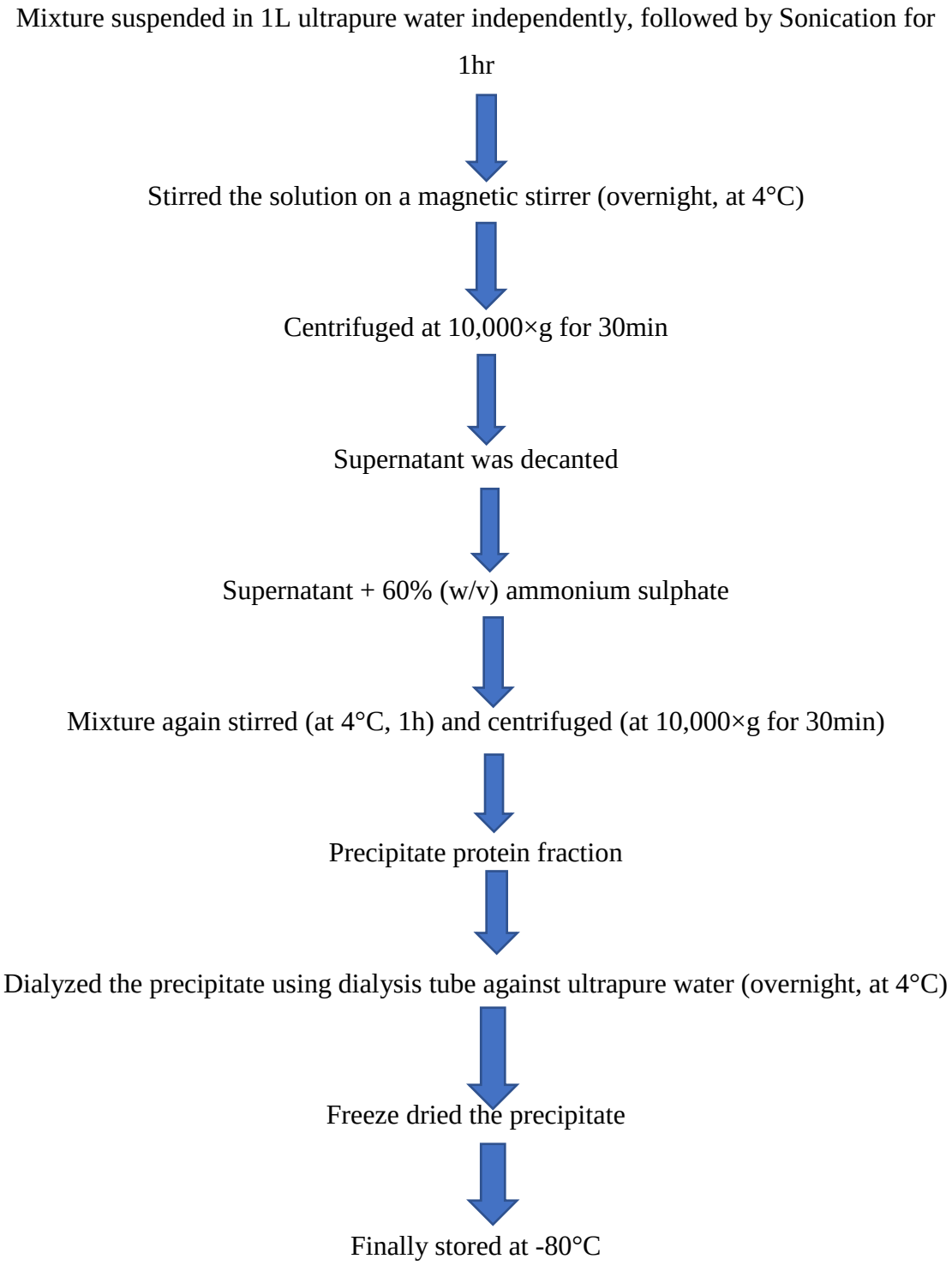


Figure 3.5.4: Flow chart of Method 4 protein extraction procedure

3.6 Determination of yield of the protein extract:

The amount of protein that can be produced from a single cell as well as the total number of cells that can secrete protein together defines the yield. Calculations were done to determine the amount of seaweed protein extract powder that could be derived from seaweed powder (in triplicate). The calculation was performed as followed by O'Connor et al. (2020):

$$\text{Seaweed protein extract powder (\%)} = \frac{\text{Weight of seaweed protein extract powder}}{\text{Weight of freeze dried seaweed powder}} \times 100$$

3.7 Determination of protein content of the protein extract:

Kjeldahl technique was used to determine protein content in accordance with AOAC guidelines (2000). The method was employed using a Kjeltech System (Foss Tecator Digestor DS6 2006).

Steps of kjeldahl method:

Digestion: Firstly, 1.0 gm of sample was weighed and transferred into a 250 mL digestive tube. Secondly, two tablets of Kjeltabs Cu 3.5 catalyst were added into the digestive tube containing the sample. Thirdly, 12 mL of concentrated H_2SO_4 was poured into digestive tube. The digestive tube was shaken gently to ensure the sample was mixed well with acid. Once the aspirator system was functioning well, the digestive tube was positioned on a rack for further inspection. The digestive tube was connected to exhaust system by placing on a preheated Digestive Heater Block D26 at 420^0C. Digestive process was conducted until the colour of sample solution change to green or light blue colour. The time consumption of the change in colour of sample commonly between 30 min and 60 min depend on the type of sample used.

Distillation: Next, the digested sample was cooled down for 2 min to 10 min, followed by the addition of 75 mL of distilled water into the cooled digestive tube. Afterwards, 50 mL of 40% NaOH was flowed into digestive tube automatically. Then, in a 250 mL conical flask, 25 mL of 4% boric acid and 5 drops of Green Bromocresol indicator were placed as a receiving solution. The distillation apparatus was then filled with the receiving solution (2100 Kjeltec Distillation Unit, FOSS, Sweden). After 4 minutes of distillation, a pale green solution was obtained.

Titration: Lastly, the distillation product was titrated with 0.1 N HCl until the colour of the solution turned into blue or grey colour and the volume of titration was recorded.

A blank was used during analysis. Blank solution contained two tablets of Kjeltabs Cu 3.5 catalyst and 12 mL of concentrated H_2SO_4 without any sample was used to undergo similar process which were digestion, distillation and titration.

The percentage of nitrogen and protein content in the sample was calculated as follows:

Calculation: The calculation of the percentage of protein in the sample using protein factor 5.

$$\% \text{ of Nitrogen} = \frac{(T-B)\times N\times 0.014}{\text{Weight of sample (mg)}} \times 100$$

Where, T = Volume of titration sample

B = Volume of titration for blank

N=Normality of acid (0.1N)

0.014= meq. of Nitrogen

% of Protein= % Nitrogen× 5

3.8 Determination of amino acid:

A small modification of Benjama and Masniyom (2011) was used to determine the amino acid composition of *G. fisheri*. This was accomplished by utilizing a High Performance Liquid Chromatography (HPLC) system (Waters Corporation e2695, Milford, MA, USA) equipped with a degasser, auto sampler, and fluorescence detector to carry out the Water AccQ.Tag Amino acid analysis method.

Each sample was analyzed twice, using the same amino acid analysis method. A volumetric flask holding 50 mL was labeled and filled with 2 gm of each sample. To each of the conical flasks, 20 mL of 6N HCL was added. The volumetric flasks' caps were then screwed on securely, making them airtight. The hydrolysis was carried out by placing each flask in an oven preheated to 105°C for a period of 24 hrs. After the hydrolysis was complete, the flasks were allowed to cool and 20 mL of water was added to each solution. After a thorough mixing, the solution was strained through

Whatman filter paper. The pH was adjusted to 7:10 with 0.1N NaOH after the filtrate was collected. After that, 2 mL of each solution was filtered using a 0.2 m syringe and stored in a sample vial. In order to replicate the results, 50 L of reagent powder and 50 L of reagent diluent were pipetted from one vial (reagent + sample) to another vial (reagent + diluent) and labeled accordingly. About 350 L of AccQ-Fluor Borate Buffer was pipetted into this vial and vortexed for ten minutes. About 150 L of the solution was pipetted into the HPLC inner tube for each vial (reagent + sample).

High Performance Liquid Chromatography System (Model: Waters e2695, Waters Corporation, USA) equipped with Fluorescence detector (FLR 2475) and C8 column (3.9 150 mm) was used to analyze amino acids. These are the instrumental parameters: Maintain a flow rate of 1 mL/min through a column kept at 37˚c, with an injection volume of 10 L and a detection wavelength of 250-395 nm in size using fluorescence detection. Aqueous Buffer, Acetonitrile, and Deionized Water were used as mobile phases in the procedure.

Chapter-4: Results

4.1 Results of Total protein (%) from raw sample by kjeldahl method:

Table 4.1 Percentage of Total protein from raw sample by kjeldahl method

Raw sample	Percentage of Total protein (%)
Before drying	13.23± 0.099
After drying	25.52±0.855

The term "Total protein" or "Crude protein" refers to the sum of the sample's nitrogen components, both protein nitrogen and all other forms of nitrogen. Table 4.1 shows the total protein content of raw seaweed sample before drying and after drying is 13.23%and 25.52% accordingly after application of Kjeldahl method.

4.2 Results of Pure protein (%) from processed sample by different extraction methods:

Table 4.2 Percentage of Pure protein from processed sample by different extraction methods

Processed sample	Percentage of Pure protein (%)
Method 1	86±2.269
Method 2	28±0.764
Method 3	46±1.031
Method 4	10±0.049

"Pure protein" only accounts for the nitrogen found in proteins and excludes all other forms of nitrogen. The current research was mainly carried out to compare the four different protein extraction methods of red seaweed, *Gracilaria fisheri* based on their purity and highest percentage of extraction rate. The percentage of pure protein extract in processed seaweed samples of different extraction procedures ranged from 10% to 86%. Table 4.2 shows that, method 1 extraction protocol contained the highest

percentage of pure protein content about 86%, whereas method 4 extraction protocol found the lowest amount around 10% of pure protein.

4.3 Results of the yield of extracted pure protein (%):

Table 4.3 Percentage of the yield of extracted pure protein

Different extraction method	Yield of extracted pure protein (%)
Method 1	8.74±0.734
Method 2	19.21±2.167
Method 3	12.32±2.000
Method 4	25.11±2.774

A protein purification process has a yield if a significant amount of the original starting protein or enzyme remains in the final purified protein fraction. Activity rather than total protein is used to calculate yield in purification tables because total protein may contain unwanted by products. A high "% yield" indicates that a significant amount of the targeted enzyme was successfully extracted.

The yield of extracted protein ranged from 25.11% to 8.74% in different extraction procedures. Table 4.3 shows that, the lowest yield of protein extract (8.74%) was obtained when Method 1 extraction protocol was used, 19.21% and 12.32% yield were found accordingly followed by method 2 & method 3 whereas highest yield (25.11%) was experienced in Method 4 extraction process.

4.4 Results of amino acid analysis (ppm):

The following table shows the amino acid profile of the highest percentage of extracted protein obtained from method 1 extraction technique. The content of amino acids in *G. fisheri* was determined according to Benjama and Masniyom (2011) with some slight modification. The HPLC (Waters Corporation e2695, Milford, MA, USA) equipped with degasser, auto sampler, and fluorescence detector was used to analyse amino acids in *G. fisheri*. Amino acid profiling of protein helps to investigate the nutritional aspects of protein.

Table 4.4: Amino acid analysis (ppm) from highest percentage of extracted pure protein

No of amino acid	Mean	SD	Method of hydrolysis	Amino acid
1	0.8825	0.248194	alkaline hydrolysis	Tryptophan
2	2.252	0.079196	performic acid oxidation	Cysteine
3	3.6875	0.051619	performic acid oxidation	Methionine
4	9.39	1.398657	HCl hydrolysis	Aspartic acid
5	6.16535	0.46761	HCl hydrolysis	Serine
6	11.388	1.052175	HCl hydrolysis	Glutamic acid
7	7.1775	0.692258	HCl hydrolysis	Glycine
8	1.68	0.196576	HCl hydrolysis	Histidine
9	7.3695	1.132078	HCl hydrolysis	Arginine
10	7.6635	1.426234	HCl hydrolysis	Threonine
11	9.711	1.291177	HCl hydrolysis	Alanine
12	4.9515	0.509824	HCl hydrolysis	Proline
13	4.0775	0.509824	HCl hydrolysis	Tyrosine
14	7.6135	0.842164	HCl hydrolysis	Valine
15	6.106	0.729734	HCl hydrolysis	Lysine
16	4.326	0.280014	HCl hydrolysis	Isoleucine
17	7.8815	0.613062	HCl hydrolysis	Leucine
18	4.26	0.346482	HCl hydrolysis	Phenylalanie

Chapter-5: Discussions

5.1 Extracted yield & protein content of *Gracilaria fisheri*:

The total protein content of the raw sample of *Gracilaria fisheri* on a dry weight basis was found to be 25.52%, which is comparatively higher than the values that were previously reported for other red seaweed species such as Caulerpa veravelensis (7.77±0.59%), Caulerpa scalpelliformis (10.50±0.91%), Laminaria japonica (9.1%), Palmaria palmata (13.5%) (Suresh et al., 2014); *Gracilaria gracilis* (10.86%) (Rasyid et al., 2019) and *Gracilaria changii* (12.57%) (Sharmin et al., 2022).

During this study four different protein extraction protocols were studied for the comparison of the efficiency of pure protein extraction. Method1 was determined to have the highest rate of pure protein extraction. In essence, it was used in the same way that Galland-Irmouli et al. (1999) described it. Using a mixture of acetate buffer, cellulase enzyme, sonication, and ammonium sulfate extraction, the substance was treated. In this study, figure 4.4 shows that, Method 1 contained the highest efficiency (86%) of pure protein extraction, whereas, method 4 contained the lowest efficiency (10%) of extraction. In Method 4, NaOH were used as a buffer and no enzymes were used. Method 2 & Method 3 showed 28% & 46% efficiency of extraction accordingly.

Proteins in seaweed are often covalently bonded to polysaccharides and polyphenols, among other non-protein components, inside the seaweed cell. Macromolecular cell wall complexes and proteins cross-linked via disulphide linkages to polysaccharides have been identified in seaweed (Harnedy and FitzGerald, 2019; Deniaud et al., 2003; Amano et al., 1990). Alkaline solutions (NaOH) have been shown to successfully dissolve and facilitate the removal of extremely water-resistant proteins from seaweed and microalgae in a number of studies (Harnedy and FitzGerald, 2019; Lourenço et al., 2002; Amano et al., 1990). Extracting high-protein components from plant and muscle sources is another typical usage for food-grade NaOH. Soy protein isolates and surimi-based products both benefit from this process, as does the removal of fish muscle proteins (Harnedy and FitzGerald, 2019). However, as Naseri et al. (2020) shown, the protein extraction efficiency from *Palmaria palmate* was only around 45% when using the alkaline extraction outlined by Galland-Irmouli et al. (1999).

However our current research found a better result around 86% efficiency of extraction from method 1 extraction technique utilizing the combination of acetate buffer & cellulase enzyme rather than NaOH buffer alone.

In the current study, Table 4.5 revealed that the removed yield of protein content from freeze dried *Gracilaria fisheri* was viewed as 8.74%, 19.21%, 12.32%, and 25.11% for method 1, method 2, method 3 and method 4 respectively. Protein recovery rates for *Palmaria palmata* were 12.5%, 14.9%, and 21.5% after being subjected to traditional, high pressure processing, and autoclave pre-treatments, while rates for *Chondrus crispus* were 35.2%, 16.1%, and 21.5% (O'Connor et al., 2020).

Alkaline-soluble proteins were extracted from milled, oven-dried *Palmaria palmata* by Harnedy and FitzGerald using a variety of enzymes, including Celluclast 1.5 L, Shearzyme 500 L, Ultraflo, and Corolase, in addition to different amounts of sodium hydroxide (NaOH) and N-acetyl-L-cysteine (NAC). In a recent study, Umamizyme was used to increase the extraction yield from 24% to 80% (Bjarnadóttir et al., 2018). This is in contrast to the 24%, 40%, and 67% yields achieved using chemical extraction, physical procedure, and enzymatic hydrolysis (Harnedy and FitzGerald, 2019). Bjarnadóttir et al. (2018) used Xylanase and Umamizyme as proteases on frozen *Palmaria palmata*, achieving extraction yields of roughly 50% and 65% in the liquid fractions, respectively. Wet-milled samples (total 300gm) were included in a trial where 2 mg of pure xylanase and 200 mg of Umamizyme were added (Bjarnadóttir et al., 2018). The difference between their and our extraction efficiency could be due to the use of different enzymes (resulting in varied enzyme activity) or a greater enzyme concentration.

5.2 Amino acid profile of proteins:

Analyzing the amino acid profile of the processed *Gracilaria fisheri* sample, the researchers found that it was quite comparable to what had been described before. Method 1 protein extraction found the maximum protein purity, which was employed in this study for amino acid profiling of *Gracilaria fisheri* to investigate the nutritional aspects of protein. Since red seaweeds have a larger protein level than brown and green seaweeds, they also have greater amino acid content (Fleurence, 1999).

Table 4.6 shows, 18 amino acids obtained from control acid oxidation, acid hydrolysis and alkaline hydrolysis by using HPLC. Data reveal that amino acids were better preserved after HCl hydrolysis (Asparticacid, Serine, Glutamicacid, Glycine, Histidine, Arginine, Threonine, Alanine, Proline, Tyrosine, Valine, Lysine, Isoleucie, Leucine, Phenylalanine). Cysteine and Methionine were reported to be present in the sample obtained from performic acid oxidation, but alkaline hydrolysis only yielded tryptophan. It's easy for HCl to hydrolyse the amino acids tryptophan, cysteine, and methionine. These findings suggest that the proteins isolated from the processed *Gracilaria fisheri* sample are of a good quality, since this is determined by the amino acid content and digestibility of the protein (Černá, 2011).

It was found that glutamic acid (11.388%) was the most abundant essential amino acid in *G. fisheri*. Glutamic acid present in *G. fisheri* has a role in immunity, detoxification, digestive health, and even muscle development and repair (Tinsley, 2018).

According to the relevant study by Kadam et al. (2016), alkaline extraction is superior to acid extraction at preserving some amino acids (Phenylalanine, Serine, Glycine, and Valine), but threonine is destroyed only under alkaline extraction conditions. The samples extracted contained "essential" amino acids such as histidine, threonine, valine, and phenylalanine. Hydrolysis at high alkaline or acidic pH levels is known to totally destroy some amino acids (lvarez et al., 2013; Ravindran and Bryden, 2005; Fountoulakis and Lahm, 1998) leading to the production of new nitrogenous molecules.

In the previous study (Chan and Matanjun, 2017), nine essential amino acids including arginine, threonine, valine, methionine, lysine, isoleucine, leucine,

phenylalanine and seven non-essential amino acids including aspartic acid, glutamic acid, serine, glycine, histidine, alanine, and proline were detected in *G. changii* at different proportion, after acid hydrolysis of the protein samples tryptophan and cysteine were removed as per Benjama and Masniyom (2011), with a little adjustment using high-performance liquid chromatography (HPLC).

All of the essential amino acids (EAAs) were identified in *G. fisheri* using the same methodology as described by Benjama and Masniyom (2011) with the exception of asparagine and glutamine, which were destroyed during acid hydrolysis of the protein samples. This number was greater than that of the previous study (Norziah and Ching, 2000), but it is equivalent to other species of Gracilaria reported previously (Benjama and Masniyom, 2012; Gressler et al., 2010).

Chapter-6: Conclusions

The principal finding of the current research is that by utilizing the combination of enzymes with slight adjustment through an alkaline extraction strategy in method 1, it was possible to separate up to 86% of pure protein from *Gracilaria fisheri*. In this study method 1 was viewed as fruitful in light of the fact that it produced the highest percentage of pure protein than other species of red seaweed. The study revealed that the percentage of pure protein extracted using method 1 has both scientific and economic relevance. So, it was demonstrated to be a suitable technique for the reduction of processing time and better extraction of pure proteins. All extraction methods used in this study have a high potential for producing pure protein based on the extracted yield. Thus method 1 found to be optimum for amino acid profiling and it was carried out in this study through acid oxidation, acid hydrolysis and alkali hydrolysis by utilizing HPLC method. The outcomes of the amino acid profile helps for the investigation of nutritional aspects of protein. *Gracilaria fisheri* is widely recognized for its enormous value to human nutrition and its commercial potential in fields as diverse as food, cosmetics, and medicine. As such, it has potential as a backup plan to meet the growing need for protein around the world.

Chapter-7: Recommendations & Future Perspectives

However, more research is required and encouraged so that the liquid protein can be recovered more effectively. Ultrafiltration (UF) is one method that can be used to recover as much of the liquid as possible and it is something that has to be researched in the future. Furthermore, the peptides produced may have interesting physiological and nutritional features that require further research.

Protein in the solid fraction can be used as a substitute for total protein in the raw materials. Since this solid fraction contains protein, it can be put to use as a protein-rich by-product in a variety of contexts, such as a dietary supplement for poultry or fish. There should be more study done on the possibility of using seaweed protein as a supplement to meet the needs of a rapidly expanding population. The results of this investigation have revealed some huge gaps that require additional research.

More investigation is needed to identify new useful components in *Gracilaria fisheri* that can be incorporated into food products. Interesting discoveries emerge as we investigate the potential of seaweed protein in the development of new food products, the effects of different processing and packaging methods, and the interactions of seaweed protein with other components of the food matrix.

It appears that proteins, peptides, and amino acids extracted from seaweed will have significant use in the fields of nutrition, dietary supplementation, animal feed, and medical study. These elements have the potential to be refined into a variety of useful products, including nutraceuticals, treatments, cosmetics, and even animal feed. To sum up, scientists are entering a new era of human nutrition due to the discovery of seaweed protein, which has several potential health benefits.

References

Álvarez, C., Rendueles, M. and Díaz, M., 2012. Alkaline hydrolysis of porcine blood haemoglobin: applications for peptide and amino acid production. Animal production science, 53(2), pp.121-128.

Amano, H., & Noda, H. (1990). Proteins from protoplasts of red alga Porphyra yezoensis. Nippon Suisan Gakkaishi, 56, 1859e1864.

Amano, H., & Noda, H. (1992). Proteins of protoplasts from several seaweeds. Nippon Suisan Gakkaishi, 58, 291e299.

AOAC. (2000). Official methods of analysis of AOAC international (17th ed.). Gaithersburg: AOAC International.

Araújo, M., Rema, P., Sousa-Pinto, I., Cunha, L.M., Peixoto, M.J., Pires, M.A., Seixas, F., Brotas, V., Beltrán, C. and Valente, L.M., 2016. Dietary inclusion of IMTA-cultivated Gracilaria vermiculophylla in rainbow trout (Oncorhynchus mykiss) diets: effects on growth, intestinal morphology, tissue pigmentation, and immunological response. Journal of applied phycology, 28(1), pp.679-689.

Arizo, M.A., Beroncal, R.A., Chua, W.A., Lim, J.J., Rogando, K.C. and Maningas, M.B., 2016. Immune response of Macrobrachium rosenbergii immersed in aqueous extract of Gracilaria edulis challenged with white spot syndrome virus. Aquaculture, Aquarium, Conservation & Legislation, 9(2), pp.215-226.

Bansemir, A., Blume, M., Schröder, S. and Lindequist, U., 2006. Screening of cultivated seaweeds for antibacterial activity against fish pathogenic bacteria. Aquaculture, 252(1), pp.79-84.

Barba, F.J., Grimi, N. and Vorobiev, E., 2015. New approaches for the use of non-conventional cell disruption technologies to extract potential food additives and nutraceuticals from microalgae. Food Engineering Reviews, 7(1), pp.45-62.

Barbarino, E. and Lourenço, S.O., 2005. An evaluation of methods for extraction and quantification of protein from marine macro-and microalgae. Journal of Applied Phycology, 17(5), pp.447-460.

Battacharyya, D., Babgohari, M.Z., Rathor, P. and Prithiviraj, B., 2015. Seaweed extracts as biostimulants in horticulture. Scientia Horticulturae, 196, pp.39-48.

Bean, S.R., Ioerger, B.P., Park, S.H. and Singh, H.A.R.M.I.T., 2006. Interaction between sorghum protein extraction and precipitation conditions on yield, purity, and composition of purified protein fractions. Cereal chemistry, 83(1), pp.99-107.

Benjama, O. and Masniyom, P., 2011. Nutritional composition and physicochemical properties of two green seaweeds (Ulva pertusa and U. intestinalis) from the Pattani Bay in Southern Thailand. Sonklanakarin Journal of Science and Technology, 33(5), p.575.

Benjama, O. and Masniyom, P., 2012. Biochemical composition and physicochemical properties of two red seaweeds (Gracilaria fisheri and G. tenuistipitata) from the Pattani Bay in Southern Thailand. Sonklanakarin Journal of Science and Technology, 34(2), p.223.

Bianco, É.M., Krug, J.L., Zimath, P.L., Kroger, A., Paganelli, C.J., Boeder, A.M., Santos, L.D., Tenfen, A., Ribeiro, S.M., Kuroshima, K.N. and Alberton, M.D., 2015. Antimicrobial (including antimollicutes), antioxidant and anticholinesterase activities of Brazilian and Spanish marine organisms–evaluation of extracts and pure compounds. Revista Brasileira de Farmacognosia, 25, pp.668-676.

Bjarnadóttir, M., Aðalbjörnsson, B.V., Nilsson, A., Slizyte, R., Roleda, M.Y., Hreggviðsson, G.Ó., Friðjónsson, Ó.H. and Jónsdóttir, R., 2018. Palmaria palmata as an alternative protein source: enzymatic protein extraction, amino acid composition, and nitrogen-to-protein conversion factor. Journal of Applied Phycology, 30(3), pp.2061-2070.

Bleakley, S. and Hayes, M., 2017. Algal proteins: extraction, application, and challenges concerning production. Foods, 6(5), p.33.

Buschmann, A.H., Troell, M., Kautsky, N. and Kautsky, L., 1996. Integrated tank cultivation of salmonids and Gracilaria chilensis (Gracilariales, Rhodophyta). Hydrobiologia, 326(1), pp.75-82.

Černá, M., 2011. Seaweed proteins and amino acids as nutraceuticals. Advances in food and nutrition research, 64, pp.297-312.

Chan, P.T. and Matanjun, P., 2017. Chemical composition and physicochemical properties of tropical red seaweed, Gracilaria changii. Food Chemistry, 221, pp.302-310.

Chan, P.T., Matanjun, P., Yasir, S.M. and Tan, T.S., 2014. Antioxidant and hypolipidaemic properties of red seaweed, Gracilaria changii. Journal of Applied Phycology, 26(2), pp.987-997.

Chan, P.T., Matanjun, P., Yasir, S.M. and Tan, T.S., 2015. Histopathological studies on liver, kidney and heart of normal and dietary induced hyperlipidaemic rats fed with tropical red seaweed Gracilaria changii. Journal of Functional Foods, 17, pp.202-213.

Chan, P.T., Matanjun, P., Yasir, S.M. and Tan, T.S., 2016. Oxidative stress biomarkers in organs of hyperlipidaemic and normal rats fed tropical red seaweed, Gracilaria changii. Journal of applied phycology, 28(2), pp.1371-1378.

Chaves, L.D.S., Nicolau, L.A.D., Silva, R.O., Barros, F.C.N., Freitas, A.L.P., Aragão, K.S., Ribeiro, R.D.A., Souza, M.H.L.P., Barbosa, A.L.D.R. and Medeiros, J.V.R., 2013. Antiinflammatory and antinociceptive effects in mice of a sulfated polysaccharide fraction extracted from the marine red algae Gracilaria caudata. Immunopharmacology and Immunotoxicology, 35(1), pp.93-100.

Chemat, F. and Cravotto, G. eds., 2012. Microwave-assisted extraction for bioactive compounds: theory and practice (Vol. 4). Springer Science & Business Media.

Chemodanov, A., Jinjikhashvily, G., Habiby, O., Liberzon, A., Israel, A., Yakhini, Z. and Golberg, A., 2017. Net primary productivity, biofuel production and CO2 emissions reduction potential of Ulva sp.(Chlorophyta) biomass in a coastal area of the Eastern Mediterranean. Energy Conversion and Management, 148, pp.1497-1507.

Chen, Y.Y., Chen, J.C., Kuo, Y.H., Lin, Y.C., Chang, Y.H., Gong, H.Y. and Huang, C.L., 2016. Lipopolysaccharide and β-1, 3-glucan-binding protein (LGBP) bind to seaweed polysaccharides and activate the prophenoloxidase system in white shrimp Litopenaeus vannamei. Developmental & Comparative Immunology, 55, pp.144-151.

Chen, Y.Y., Chen, J.C., Lin, Y.C., Yeh, S.T. and Huang, C.L., 2015. White shrimp Litopenaeus vannamei that have received Gracilaria tenuistipitata extract show early recovery of immune parameters after ammonia stressing. Marine drugs, 13(6), pp.3606-3624.

Chen, Y.Y., Sim, S.S., Chiew, S.L., Yeh, S.T., Liou, C.H. and Chen, J.C., 2012. Dietary administration of a Gracilaria tenuistipitata extract produces protective immunity of white shrimp Litopenaeus vannamei in response to ammonia stress. Aquaculture, 370, pp.26-31.

Chitra, G. and Sreeja, P.S., 2013. A comparative study on the effect of seaweed liquid fertilizers on the growth and yield of Vigna radiata (L.). Nature Environment and Pollution Technology, 12(2), p.359.

Chittapalo, T. and Noomhorm, A., 2009. Ultrasonic assisted alkali extraction of protein from defatted rice bran and properties of the protein concentrates. International Journal of Food Science & Technology, 44(9), pp.1843-1849.

Conde, E., Balboa, E.M., Parada, M. and Falqué, E., 2013. Algal proteins, peptides and amino acids. In Functional ingredients from algae for foods and nutraceuticals (pp. 135-180). Woodhead Publishing.

Cornier, M.A., Dabelea, D., Hernandez, T.L., Lindstrom, R.C., Steig, A.J., Stob, N.R., Van Pelt, R.E., Wang, H. and Eckel, R.H., 2008. The metabolic syndrome. Endocrine reviews, 29(7), pp.777-822.

Costa, D.S., Araújo, T.S., Sousa, N.A., Souza, L.K., Pacífico, D.M., Sousa, F.B.M., Nicolau, L.A., Chaves, L.S., Barros, F.C.N., Freitas, A.L.P. and Medeiros, J.V.R., 2016. Sulphated polysaccharide isolated from the seaweed Gracilaria caudata exerts an antidiarrhoeal effect in rodents. Basic & clinical pharmacology & toxicology, 118(6), pp.440-448.

Coura, C.O., de Araújo, I.W., Vanderlei, E.S., Rodrigues, J.A., Quinderé, A.L., Fontes, B.P., de Queiroz, I.N., de Menezes, D.B., Bezerra, M.M., e Silva, A.A. and Chaves, H.V., 2012. Antinociceptive and anti-inflammatory activities of sulphated polysaccharides from the red seaweed Gracilaria cornea. Basic & clinical pharmacology & toxicology, 110(4), pp.335-341.

Coura, C.O., Souza, R.B., Rodrigues, J.A.G., Vanderlei, E.D.S.O., de Araújo, I.W.F., Ribeiro, N.A., Frota, A.F., Ribeiro, K.A., Chaves, H.V., Pereira, K.M.A. and da Cunha, R.M.S., 2015. Mechanisms involved in the anti-inflammatory action of a polysulfated fraction from Gracilaria cornea in rats. PloS one, 10(3), p.e0119319.

Da Costa, E., Melo, T., Moreira, A.S., Bernardo, C., Helguero, L., Ferreira, I., Cruz, M.T., Rego, A.M., Domingues, P., Calado, R. and Abreu, M.H., 2017. Valorization of lipids from Gracilaria sp. through lipidomics and decoding of antiproliferative and anti-inflammatory activity. Marine Drugs, 15(3), p.62.

Deniaud, E., Quemener, B., Fleurence, J. and Lahaye, M., 2003. Structural studies of the mix-linked β-(1→ 3)/β-(1→ 4)-D-xylans from the cell wall of Palmaria palmata (Rhodophyta). International journal of biological macromolecules, 33(1-3), pp.9-18.

Denis, C., Le Jeune, H., Gaudin, P. and Fleurence, J., 2009. An evaluation of methods for quantifying the enzymatic degradation of red seaweed Grateloupia turuturu. Journal of applied phycology, 21(1), pp.153-159.

Destombe, C., 1995. A review of the life history, reproduction and phenology of Gracilaria. Journal of applied phycology, 7(3), pp.269-281.

Díaz-Rosales, P., Felices, C., Abdala, R., Figueroa, F.L., Gómez Pinchetti, J.L., Moriñigo, M.A. and Balebona, M.C., 2007. In vitro effect of the red alga Hydropuntia cornea (J. Agardh) on the respiratory burst activity of sole (Solea senegalensis, Kaup 1858) phagocytes. Aquaculture Research, 38(13), pp.1411-1418.

Dillehay, T.D., Ramírez, C., Pino, M., Collins, M.B., Rossen, J. and Pino-Navarro, J.D., 2008. Monte Verde: seaweed, food, medicine, and the peopling of South America. science, 320(5877), pp.784-786.

Dinh, H.L., Hori, K. and Quang, N.H., 2009. Screening and preliminary characterization of hemagglutinins in Vietnamese marine algae. Journal of applied phycology, 21(1), pp.89-97.

Dong, X.Y., Guo, L.L., Wei, F., Li, J.F., Jiang, M.L., Li, G.M., Zhao, Y.D. and Chen, H., 2011. Some characteristics and functional properties of rapeseed protein prepared by ultrasonication, ultrafiltration and isoelectric precipitation. Journal of the Science of Food and Agriculture, 91(8), pp.1488-1498.

Fan, D., Hodges, D.M., Zhang, J., Kirby, C.W., Ji, X., Locke, S.J., Critchley, A.T. and Prithiviraj, B., 2011. Commercial extract of the brown seaweed Ascophyllum nodosum enhances phenolic antioxidant content of spinach (Spinacia oleracea L.) which protects Caenorhabditis elegans against oxidative and thermal stress. Food Chemistry, 124(1), pp.195-202.

FAO, Fishery and Aquaculture Statistics - 2016, FAO Fisheries and Aquaculture Departament,2018,http://www.fao.org/fishery/static/Yearbook/YB2016_USBcard/root/aquaculture/yearbook_aquaculture.pf.

Farvin, K.S. and Jacobsen, C., 2013. Phenolic compounds and antioxidant activities of selected species of seaweeds from Danish coast. Food chemistry, 138(2-3), pp.1670-1681.

Fleurence, J. (2004). Seaweed proteins. In R. Y. Yada (Ed.), Proteins in food processing (pp. 197e213). Cambridge: Woodhead Publishing Limited.

Fleurence, J., 1999. Seaweed proteins: biochemical, nutritional aspects and potential uses. Trends in food science & technology, 10(1), pp.25-28.

Fleurence, J., Morançais, M. and Dumay, J., 2018. Seaweed proteins. In Proteins in food processing (pp. 245-262). Woodhead Publishing.

Fleurence, J., Morançais, M., Dumay, J., Decottignies, P., Turpin, V., Munier, M., Garcia-Bueno, N. and Jaouen, P., 2012. What are the prospects for using seaweed in human nutrition and for marine animals raised through aquaculture?. Trends in food science & technology, 27(1), pp.57-61.

Fountoulakis, M. and Lahm, H.W., 1998. Hydrolysis and amino acid composition analysis of proteins. Journal of chromatography A, 826(2), pp.109-134.

Friedlander, M., 2008. Israeli R & D activities in seaweed cultivation. Israel Journal of Plant Sciences, 56(1-2), pp.15-28.

Fu, X.M., Zhang, M.Q., Shao, C.L., Li, G.Q., Bai, H., Dai, G.L., Chen, Q.W., Kong, W., Fu, X.J. and Wang, C.Y., 2016. Chinese marine materia medica resources: status and potential. Marine Drugs, 14(3), p.46.

Galland-Irmouli, A.V., Fleurence, J., Lamghari, R., Luçon, M., Rouxel, C., Barbaroux, O., Bronowicki, J.P., Villaume, C. and Guéant, J.L., 1999. Nutritional value of proteins from edible seaweed Palmaria palmata (Dulse). The Journal of Nutritional Biochemistry, 10(6), pp.353-359.

Galland-Irmouli, A.V., Pons, L., Lucon, M., Villaume, C., Mrabet, N.T., Guéant, J.L. and Fleurence, J., 2000. One-step purification of R-phycoerythrin from the red macroalga Palmaria palmata using preparative polyacrylamide gel electrophoresis. Journal of Chromatography B: Biomedical Sciences and Applications, 739(1), pp.117-123.

Ghannadi, A., Plubrukarn, A., Zandi, K., Sartavi, K. and Yegdaneh, A., 2013. Screening for antimalarial and acetylcholinesterase inhibitory activities of some Iranian seaweeds. Research in pharmaceutical sciences, 8(2), p.113.

Gordon, A., 2017. Case study: Improving the quality and viability of a traditional beverage—Irish Moss. In Food Safety and Quality Systems in Developing Countries (pp. 47-79). Academic Press.

Gressler, V., Fujii, M.T., Colepicolo, P. and Pinto, E., 2012. Characterization of volatile composition of Laurencia dendroidea by gas chromatography-mass spectrometry. Revista Brasileira de Farmacognosia, 22, pp.805-813.

Gressler, V., Yokoya, N.S., Fujii, M.T., Colepicolo, P., Mancini Filho, J., Torres, R.P. and Pinto, E., 2010. Lipid, fatty acid, protein, amino acid and ash contents in four Brazilian red algae species. Food chemistry, 120(2), pp.585-590.

Guiry, M.D. (2013) Ascophyllum nodosum (Linnaeus) Le Jolis http://www.algaebase.org/search/species/detail/?species_id=5. Accessed 20th May 2013.

Guiry, M.D. and Guiry, G.M., 2018. World-wide electronic publication, National University of Ireland, Galway. AlgaeBase.

Gurgel, C.F.D. and Fredericq, S., 2004. SYSTEMATICS OF THE GRACILARIACEAE (GRACILARIALES, RHODOPHYTA): A CRITICAL ASSESSMENT BASED ON RBCL SEQUENCE ANALYSES 1. Journal of phycology, 40(1), pp.138-159.

Gurgel, C.F.D., Fredericq, S. and Norris, J.N., 2004. Gracilaria apiculata and G. flabelliformis (Gracilariaceae, Rhodophyta): restoring old names for common tropical western Atlantic species, including the recognition of three new subspecies, and a replacement name for G. lacinulata. Cryptogamie. Algologie.

Halliwell, B. and Gutteridge, J.M., 2015. Free radicals in biology and medicine. Oxford university press, USA.

Harnedy, P.A. and FitzGerald, R.J., 2011. Bioactive proteins, peptides, and amino acids from macroalgae 1. Journal of Phycology, 47(2), pp.218-232.

Harnedy, P.A. and FitzGerald, R.J., 2013. Extraction of protein from the macroalga Palmaria palmata. LWT-Food Science and Technology, 51(1), pp.375-382.

Hill, R.L., 1965. Hydrolysis of proteins. Advances in protein chemistry, 20, pp.37-107.

Iha, C., Grassa, C.J., Lyra, G.D.M., Davis, C.C., Verbruggen, H. and Oliveira, M.C., 2018. Organellar genomics: a useful tool to study evolutionary relationships

and molecular evolution in Gracilariaceae (Rhodophyta). Journal of phycology, 54(6), pp.775-787.

Ishwarya, M. and Narendhirakannan, R.T., 2016. The advances in neurobiology. The Benefits of Natural Products for Neurodegenerative Diseases, pp.293-306.

Jambrak, A.R., Mason, T.J., Lelas, V., Paniwnyk, L. and Herceg, Z., 2014. Effect of ultrasound treatment on particle size and molecular weight of whey proteins. Journal of Food engineering, 121, pp.15-23.

Jensen, M.N., 2004. Cultivating edible seaweed in Hawaii: new technique helps local farmers.

Jiménez, E., Dorta, F., Medina, C., Ramírez, A., Ramírez, I. and Peña-Cortés, H., 2011. Anti-phytopathogenic activities of macro-algae extracts. Marine drugs, 9(5), pp.739-756.

Joubert, Y. and Fleurence, J., 2008. Simultaneous extraction of proteins and DNA by an enzymatic treatment of the cell wall of Palmaria palmata (Rhodophyta). Journal of Applied phycology, 20(1), pp.55-61.

Júnior, F.D.C.V., Sales, A.B., Barros, F.C.N., de Sousa Chaves, L., Freitas, A.L.P., Vale, M.L., de Albuquerque Ribeiro, R., Souza, M.H.L.P., Medeiros, J.V.R. and dos Reis Barbosa, A.L., 2012. Involvement of the NO/cGMP/PKG/KATP pathway and endogenous opioids in the antinociceptive effect of a sulphated-polysaccharide fraction extracted from the red algae, Gracilaria caudata. Biomedicine & Preventive Nutrition, 2(4), pp.303-309.

Kadam, S.U. and Prabhasankar, P., 2010. Marine foods as functional ingredients in bakery and pasta products. Food Research International, 43(8), pp.1975-1980.

Kadam, S.U., Álvarez, C., Tiwari, B.K. and O'Donnell, C.P., 2015. Extraction of biomolecules from seaweeds. In Seaweed sustainability (pp. 243-269). Academic Press.

Kadam, S.U., O'Donnell, C.P., Rai, D.K., Hossain, M.B., Burgess, C.M., Walsh, D. and Tiwari, B.K., 2015. Laminarin from Irish brown seaweeds Ascophyllum

nodosum and Laminaria hyperborea: Ultrasound assisted extraction, characterization and bioactivity. Marine drugs, 13(7), pp.4270-4280.

Kadam, S.U., Tiwari, B.K. and O'Donnell, C.P., 2013. Application of novel extraction technologies for bioactives from marine algae. Journal of agricultural and food chemistry, 61(20), pp.4667-4675.

Kadam, S.U., Tiwari, B.K., Smyth, T.J. and O'Donnell, C.P., 2015. Optimization of ultrasound assisted extraction of bioactive components from brown seaweed Ascophyllum nodosum using response surface methodology. Ultrasonics Sonochemistry, 23, pp.308-316.

Kain, J.M. and Destombe, C., 1995. A review of the life history, reproduction and phenology of Gracilaria. Oceanographic Literature Review, 3(43), p.297.

Kaliaperumal, N., 2003. Products from seaweeds. SDMRI Research Publication, 3, pp.33-42.

Khalil, A., Lai, T.K., Tye, Y.Y., Rizal, S., Chong, E.W.N., Yap, S.W., Hamzah, A.A., Fazita, M.R. and Paridah, M.T., 2018. A review of extractions of seaweed hydrocolloids: Properties and applications, Express Polym. Lettres, 12, pp.296-317.

Khare, C.P., 2007. Indian medicinal plants: an illustrated dictionary. Springer Science & Business Media.

Kılınç, B., Cirik, S., Turan, G., Tekogul, H. and Koru, E., 2013. Seaweeds for food and industrial applications. In Food industry. IntechOpen.

Koenig-Oberhuber, V. and Filipovic, M., 2016. New antiplatelet drugs and new oral anticoagulants. BJA: British Journal of Anaesthesia, 117(suppl_2), pp.ii74-ii84.

Kumar, K.S., Ganesan, K., Selvaraj, K. and Rao, P.S., 2014. Studies on the functional properties of protein concentrate of Kappaphycus alvarezii (Doty) Doty–An edible seaweed. Food chemistry, 153, pp.353-360.

Lee, W.K., Lim, Y.Y., Leow, A.T.C., Namasivayam, P., Abdullah, J.O. and Ho, C.L., 2017. Factors affecting yield and gelling properties of agar. Journal of Applied Phycology, 29(3), pp.1527-1540.

Leódido, A.C.M., Costa, L.E., Araújo, T.S., Costa, D.S., Sousa, N.A., Souza, L.K., Sousa, F.B., Manoel Filho, D.S., Vasconcelos, D.F., Silva, F.R. and Nogueira, K.M., 2017. Anti-diarrhoeal therapeutic potential and safety assessment of sulphated polysaccharide fraction from Gracilaria intermedia seaweed in mice. International journal of biological macromolecules, 97, pp.34-45.

Liang, X. and Fan, Q., 2013. Application of sub-critical water extraction in pharmaceutical industry. Journal of Materials Science and Chemical Engineering, 1(05), p.1.

Lim, H.T. and Teo, S.S., 2015. Comparison of Protein Extraction Protocols for Proteomic Analysis of Red Algae, Eucheuma cottonii. Pertanika Journal of Tropical Agricultural Science, 38(2).

Lin, Y.C., Yeh, S.T., Li, C.C., Chen, L.L., Cheng, A.C. and Chen, J.C., 2011. An immersion of Gracilaria tenuistipitata extract improves the immunity and survival of white shrimp Litopenaeus vannamei challenged with white spot syndrome virus. Fish & Shellfish Immunology, 31(6), pp.1239-1246.

Lin, Y.H., Tsai, J.S., Hung, L.B. and Pan, B.S., 2011. Plasma lipid regulatory effect of compounded freshwater clam hydrolysate and Gracilaria insoluble dietary fibre. Food chemistry, 125(2), pp.397-401.

Lourenço, S.O., Barbarino, E., De-Paula, J.C., Pereira, L.O.D.S. and Marquez, U.M.L., 2002. Amino acid composition, protein content and calculation of nitrogen-to-protein conversion factors for 19 tropical seaweeds. Phycological Research, 50(3), pp.233-241.

Lozano, I., Wacyk, J.M., Carrasco, J., Martín, C.S. and Marcelo, A., 2016. Red macroalgae Pyropia columbina and Gracilaria chilensis: sustainable feed additive in the Salmo salar diet and the evaluation of potential antiviral activity against infectious salmon anemia virus. Journal of applied phycology, 28(2), pp.1343-1351.

Luqmani, Y.A., 2005. Mechanisms of drug resistance in cancer chemotherapy. Medical principles and practice, 14(Suppl. 1), pp.35-48.

Lyra, G.D.M., Gurgel, C.F.D., Costa, E.D.S., de Jesus, P.B., Oliveira, M.C., Oliveira, E.C., Davis, C.C. and Nunes, J.M.D.C., 2016. Delimitating cryptic species in the Gracilaria domingensis complex (Gracilariaceae, Rhodophyta) using molecular and morphological data. Journal of phycology, 52(6), pp.997-1017.

Machado, M., Machado, S., Pimentel, F.B., Freitas, V., Alves, R.C. and Oliveira, M.B.P., 2020. Amino acid profile and protein quality assessment of macroalgae produced in an integrated multi-trophic aquaculture system. Foods, 9(10), p.1382.

Magnoni, L.J., Martos-Sitcha, J.A., Queiroz, A., Calduch-Giner, J.A., Gonçalves, J.F.M., Rocha, C.M., Abreu, H.T., Schrama, J.W., Ozorio, R.O. and Pérez-Sánchez, J., 2017. Dietary supplementation of heat-treated Gracilaria and Ulva seaweeds enhanced acute hypoxia tolerance in gilthead sea bream (Sparus aurata). Biology open, 6(6), pp.897-908.

Makkar, F. and Chakraborty, K., 2017. Antidiabetic and anti-inflammatory potential of sulphated polygalactans from red seaweeds Kappaphycus alvarezii and Gracilaria opuntia. International Journal of Food Properties, 20(6), pp.1326-1337.

Mazumder, S., Ghosal, P.K., Pujol, C.A., Carlucci, M.J., Damonte, E.B. and Ray, B., 2002. Isolation, chemical investigation and antiviral activity of polysaccharides from Gracilaria corticata (Gracilariaceae, Rhodophyta). International journal of biological macromolecules, 31(1-3), pp.87-95.

Mello, J., Gracilaria and Gracilariopsis, in: W.S. Leet, C.M. Dewees, R. Klingbeil, E.J. Larson (Eds.), California's Living Marine Resources: A Status Report, University of California, 2001, pp. 491–492.

Monteiro, V.S., Teles, F.B., Coura, C.O., Souza, R.B., Lima, C.N.D.C., Costa, D.V.D.S., Honório Junior, E.R., Escudeiro, S.D.S., Chaves, E.M.C.,

Vasconcelos, S.M.M. and Benevídes, N.M.B., 2016. Involvement of the GABAergic system in the anxiolytic effect of sulfated polysaccharides from the red seaweed Gracilaria cornea. Journal of Applied Phycology, 28(3), pp.1997-2004.

Murakami, A., Ishida, H., Kobo, K., Furukawa, I., Ikeda, Y., Yonaha, M., Aniya, Y. and Ohigashi, H., 2005. Suppressive effects of Okinawan food items on free radical generation from stimulated leukocytes and identification of some active constituents: implications for the prevention of inflammation-associated carcinogenesis. Asian Pacific Journal of Cancer Prevention, 6(4), p.437.

Nadkarni, K.M., 1996. [Indian materia medica]; Dr. KM Nadkarni's Indian materia medica: with Ayurvedic, Unani-Tibbi, Siddha, allopathic, homeopathic, naturopathic & home remedies, appendices & indexes. 1 (Vol. 1). Popular Prakashan.

Naseri, A., Marinho, G.S., Holdt, S.L., Bartela, J.M. and Jacobsen, C., 2020. Enzyme-assisted extraction and characterization of protein from red seaweed Palmaria palmata. Algal Research, 47, p.101849.

Natarajan, S., Shanmugiahthevar, K.P. and Kasi, P.D., 2009. Cholinesterase inhibitors from Sargassum and Gracilaria gracilis: seaweeds inhabiting South Indian coastal areas (Hare Island, Gulf of Mannar). Natural product research, 23(4), pp.355-369.

Norziah, M.H. and Ching, C.Y., 2000. Nutritional composition of edible seaweed Gracilaria changgi. Food chemistry, 68(1), pp.69-76.

Nunes, N., Ferraz, S., Valente, S., Barreto, M.C. and Pinheiro de Carvalho, M.A.A., 2017. Biochemical composition, nutritional value, and antioxidant properties of seven seaweed species from the Madeira Archipelago. Journal of Applied Phycology, 29(5), pp.2427-2437.

Nussinovitch, A., 1997. Gum technology in the food and other industries. Blackie Academic and Professional. London, UK.

O'Connor, J., Meaney, S., Williams, G.A. and Hayes, M., 2020. Extraction of protein from four different seaweeds using three different physical pre-treatment strategies. Molecules, 25(8), p.2005.

Parniakov, O., Apicella, E., Koubaa, M., Barba, F.J., Grimi, N., Lebovka, N., Pataro, G., Ferrari, G. and Vorobiev, E., 2015. Ultrasound-assisted green solvent extraction of high-added value compounds from microalgae Nannochloropsis spp. Bioresource Technology, 198, pp.262-267.

Parniakov, O., Barba, F.J., Grimi, N., Marchal, L., Jubeau, S., Lebovka, N. and Vorobiev, E., 2015. Pulsed electric field and pH assisted selective extraction of intracellular components from microalgae Nannochloropsis. Algal Research, 8, pp.128-134.

Patra, S. and Muthuraman, M.S., 2013. Gracilaria edulis extract induces apoptosis and inhibits tumor in Ehrlich Ascites tumor cells in vivo. BMC complementary and alternative medicine, 13(1), pp.1-12.

Peixoto, M.J., Salas-Leitón, E., Pereira, L.F., Queiroz, A., Magalhães, F., Pereira, R., Abreu, H., Reis, P.A., Gonçalves, J.F.M. and de Almeida Ozório, R.O., 2016. Role of dietary seaweed supplementation on growth performance, digestive capacity and immune and stress responsiveness in European seabass (Dicentrarchus labrax). Aquaculture Reports, 3, pp.189-197.

Peixoto, M.J., Svendsen, J.C., Malte, H., Pereira, L.F., Carvalho, P., Pereira, R., Gonçalves, J.F. and Ozório, R.O., 2016. Diets supplemented with seaweed affect metabolic rate, innate immune, and antioxidant responses, but not individual growth rate in European seabass (Dicentrarchus labrax). Journal of Applied Phycology, 28(3), pp.2061-2071.

Pereira, L., 2018. Seaweeds as source of bioactive substances and skin care therapy—cosmeceuticals, algotheraphy, and thalassotherapy. Cosmetics, 5(4), p.68.

Piao, M.J., Kim, K.C., Zheng, J., Yao, C.W., Cha, J.W., Kang, H.K., Yoo, E.S., Koh, Y.S., Ko, M.H., Lee, N.H. and Hyuna, J.W., 2014. Gracilaria bursa-pastoris (Gmelin) Silva Extract Attenuates Ultraviolet B Radiation− Induced Oxidative Stress in Human Keratinocytes. Journal of Environmental Pathology, Toxicology and Oncology, 33(1).

Pimentel, D. and Pimentel, M., 2003. Sustainability of meat-based and plant-based diets and the environment. The American journal of clinical nutrition, 78(3), pp.660S-663S.

Porse, H. and Rudolph, B., 2017. The seaweed hydrocolloid industry: 2016 updates, requirements, and outlook. Journal of applied phycology, 29(5), pp.2187-2200.

Rasyid, A., Ardiansyah, A. and Pangestuti, R., 2019. Nutrient composition of dried seaweed Gracilaria gracilis. Indones. J. Mar. Sci, 24(1), pp.1-6.

Ravindran, G. and Bryden, W.L., 2005. Tryptophan determination in proteins and feedstuffs by ion exchange chromatography. Food Chemistry, 89(2), pp.309-314.

Rice, E.L. and Crowden, R.K., 1987. An improved method for the extraction and electrophoresis of proteins and active enzymes from fucalean macroalgae (Phaeophyta). Phycologia, 26(2), pp.235-246.

Rico, R.M., Tejedor-Junco, M.T., Tapia-Paniagua, S.T., Alarcón, F.J., Mancera, J.M., López-Figueroa, F., Balebona, M.C., Abdala-Díaz, R.T. and Moriñigo, M.A., 2016. Influence of the dietary inclusion of Gracilaria cornea and Ulva rigida on the biodiversity of the intestinal microbiota of Sparus aurata juveniles. Aquaculture international, 24(4), pp.965-984.

Rouxel, C., Daniel, A., Jérôme, M., Etienne, M. and Fleurence, J., 2001. Species identification by SDS-PAGE of red algae used as seafood or a food ingredient. Food Chemistry, 74(3), pp.349-353.

Sakthivel, R., Muniasamy, S., Archunan, G. and Devi, K.P., 2016. Gracilaria edulis exhibit antiproliferative activity against human lung adenocarcinoma cell line A549 without causing adverse toxic effect in vitro and in vivo. Food & function, 7(2), pp.1155-1165.

Santelices, B., 2014. Cultured aquatic species information programme. Gracilaria spp. FAO Fisheries and Aquaculture Department.

Seedevi, P., Moovendhan, M., Viramani, S. and Shanmugam, A., 2017. Bioactive potential and structural chracterization of sulfated polysaccharide from seaweed (Gracilaria corticata). Carbohydrate polymers, 155, pp.516-524.

Senthil, K.A. and Murugan, A., 2013. Antiulcer, wound healing and hepatoprotective activities of the seaweeds Gracilaria crassa, Turbinaria ornata and Laurencia papillosa from the southeast coast of India. Brazilian Journal of Pharmaceutical Sciences, 49, pp.669-678.

Sereewatthanawut, I., Prapintip, S., Watchiraruji, K., Goto, M., Sasaki, M. and Shotipruk, A., 2008. Extraction of protein and amino acids from deoiled rice bran by subcritical water hydrolysis. Bioresource technology, 99(3), pp.555-561.

Shahidi, F., 2007. Marine Algae and Polysaccharides with Therapeutic Applications. In Marine Nutraceuticals and Functional Foods (pp. 357-378). CRC Press.

Sharmin, K.N., Amiza, M.A., Ahmad, F., Razali, S.A. and Hashim, F., 2022. In silico analysis of Gracilaria changii proteins for potential bioactive peptides. In IOP Conference Series: Earth and Environmental Science (Vol. 967, No. 1, p. 012017). IOP Publishing.

Sharon, N. and Lis, H., 2004. History of lectins: from hemagglutinins to biological recognition molecules. Glycobiology, 14(11), pp.53R-62R.

Sheeja, L., Lakshmi, D., Bharadwaj, S. and Parveen, K.S., 2016. Anticancer activity of phytol purified from Gracilaria edulis against human breast cancer cell line (MCF-7). Int. J. Curr. Sci, 19(4), pp.36-46.

Shu, M.H., Appleton, D., Zandi, K. and AbuBakar, S., 2013. Anti-inflammatory, gastroprotective and anti-ulcerogenic effects of red algae Gracilaria changii (Gracilariales, Rhodophyta) extract. BMC complementary and alternative medicine, 13(1), pp.1-13.

Silva, R.O., dos Santos, G.M.P., Nicolau, L.A.D., Lucetti, L.T., Santana, A.P.M., de Souza Chaves, L., Barros, F.C.N., Freitas, A.L.P., Souza, M.H.L.P. and Medeiros, J.V.R., 2011. Sulfated-Polysaccharide Fraction from Red Algae G racilaria caudata Protects Mice Gut Against Ethanol-Induced Damage. Marine drugs, 9(11), pp.2188-2200.

Silva, R.O., Santana, A.P.M., Carvalho, N.S., Bezerra, T.S., Oliveira, C.B., Damasceno, S.R., Chaves, L.S., Freitas, A.L.P., Soares, P.M., Souza, M.H. and Barbosa, A.L.R., 2012. A sulfated-polysaccharide fraction from seaweed Gracilaria birdiae prevents naproxen-induced gastrointestinal damage in rats. Marine drugs, 10(12), pp.2618-2633.

Sirois, M., Pharmacology and pharmacy, 2016, Elsevier's Veterinary Assisting Textbook, pp. 104–130.

Smith, A.H., Nichols, K. and McLachlan, J., 1984. Cultivation of seamoss (Gracilaria) in St. Lucia, West Indies. In Eleventh International Seaweed Symposium (pp. 249-251). Springer, Dordrecht.

Souza, B.W., Cerqueira, M.A., Bourbon, A.I., Pinheiro, A.C., Martins, J.T., Teixeira, J.A., Coimbra, M.A. and Vicente, A.A., 2012. Chemical characterization and antioxidant activity of sulfated polysaccharide from the red seaweed Gracilaria birdiae. Food Hydrocolloids, 27(2), pp.287-292.

Souza, R.B., Frota, A.F., Sousa, R.S., Cezario, N.A., Santos, T.B., Souza, L.M.F., Coura, C.O., Monteiro, V.S., Cristino Filho, G., Vasconcelos, S.M.M. and da Cunha, R.M.S., 2017. Neuroprotective effects of sulphated agaran from marine alga Gracilaria cornea in rat 6-hydroxydopamine Parkinson's disease model: behavioural, neurochemical and transcriptional alterations. Basic & clinical pharmacology & toxicology, 120(2), pp.159-170.

Stegman, J.K., 2012, Stedman’s medical dictionary for the health professions and nursing, 7th ed., Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia.

Subramanian, A., Kannan, L., Pugalendhi, T., Muruganantham, A., Heparin from seaweeds, Curr. Sci. 73 (1997) 566.

Suganthy, N., Pandian, S.K. and Devi, K.P., 2010. Neuroprotective effect of seaweeds inhabiting South Indian coastal area (Hare Island, Gulf of Mannar Marine Biosphere Reserve): Cholinesterase inhibitory effect of Hypnea valentiae and Ulva reticulata. Neuroscience letters, 468(3), pp.216-219.

Sun, L., Wang, S., Gong, X., Zhao, M., Fu, X. and Wang, L., 2009. Isolation, purification and characteristics of R-phycoerythrin from a marine macroalga Heterosiphonia japonica. Protein Expression and Purification, 64(2), pp.146-154.

Tinsley, G., 2018. Glutamine: Benefits, Uses and Side Effects.

Torres, P., Novaes, P., Ferreira, L.G., Santos, J.P., Mazepa, E., Duarte, M.E.R., Noseda, M.D., Chow, F. and dos Santos, D.Y., 2018. Effects of extracts and isolated molecules of two species of Gracilaria (Gracilariales, Rhodophyta) on early growth of lettuce. Algal research, 32, pp.142-149.

Tseng, C.K., Lin, C.K., Chang, H.W., Wu, Y.H., Yen, F.L., Chang, F.R., Chen, W.C., Yeh, C.C. and Lee, J.C., 2014. Aqueous extract of Gracilaria tenuistipitata suppresses LPS-induced NF-κB and MAPK activation in RAW 264.7 and rat peritoneal macrophages and exerts hepatoprotective effects on carbon tetrachloride-treated rat. PloS one, 9(1), p.e86557.

Van Krimpen, M.M., Bikker, P., Van der Meer, I.M., Van der Peet-Schwering, C.M.C. and Vereijken, J.M., 2013. Cultivation, processing and nutritional aspects for pigs and poultry of European protein sources as alternatives for imported soybean products (No. 662). Wageningen UR Livestock Research.

Venkataraman, L.V., 1979. Studies on the extractability of proteins from the alga Scenedesmus acutus.

Vizcaíno, A.J., Mendes, S.I., Varela, J.L., Ruiz-Jarabo, I., Rico, R., Figueroa, F.L., Abdala, R., Moriñigo, M.Á., Mancera, J.M. and Alarcón, F.J., 2016. Growth,

tissue metabolites and digestive functionality in Sparus aurata juveniles fed different levels of macroalgae, Gracilaria cornea and Ulva rigida. Aquaculture research, 47(10), pp.3224-3238.

Wallace, J.S., 2000. Increasing agricultural water use efficiency to meet future food production. Agriculture, ecosystems & environment, 82(1-3), pp.105-119.

Wang, T., Jónsdóttir, R., Kristinsson, H.G., Hreggvidsson, G.O., Jónsson, J.Ó., Thorkelsson, G. and Ólafsdóttir, G., 2010. Enzyme-enhanced extraction of antioxidant ingredients from red algae Palmaria palmata. LWT-Food Science and Technology, 43(9), pp.1387-1393.

Watt, G., 2014. A dictionary of the economic products of India (Vol. 6). Cambridge University Press.

Wijesinghe, W.A.J.P. and Jeon, Y.J., 2012. Enzyme-assistant extraction (EAE) of bioactive components: a useful approach for recovery of industrially important metabolites from seaweeds: a review. Fitoterapia, 83(1), pp.6-12.

Wong, K. and Chikeung Cheung, P., 2001. Influence of drying treatment on three Sargassum species 2. Protein extractability, in vitro protein digestibility and amino acid profile of protein concentrates. Journal of applied phycology, 13(1), pp.51-58.

World Health Organization, Antimicrobial Resistance, (2018).

Xiancui, L., Rongli, N., Xiao, F., Lijun, H. and Lixin, Z., 2005. Macroalage as a source of alpha-glucosidase inhibitors. Chinese Journal of Oceanology and Limnology, 23(3), pp.354-356.

Yeh, S.T., Li, C.C., Tsui, W.C., Lin, Y.C. and Chen, J.C., 2010. The protective immunity of white shrimp Litopenaeus vannamei that had been immersed in the hot-water extract of Gracilaria tenuistipitata and subjected to combined stresses of Vibrio alginolyticus injection and temperature change. Fish & Shellfish Immunology, 29(2), pp.271-278.

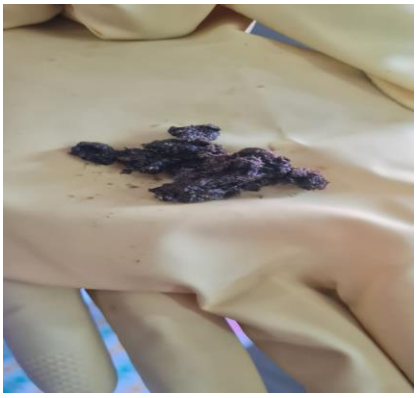
Zhang, J., Ge, C., Fang, J. and Tang, Q., 2018. Multi-Trophic Mariculture Practices in Coastal Waters. Aquaculture in China: success stories and modern trends, pp.541-554.

Zhang, W.W., Duan, X.J., Huang, H.L., Zhang, Y. and Wang, B.G., 2007. Evaluation of 28 marine algae from the Qingdao coast for antioxidative capacity and determination of antioxidant efficiency and total phenolic content of fractions and subfractions derived from Symphyocladia latiuscula (Rhodomelaceae). Journal of Applied Phycology, 19(2), pp.97-108.

Zhu, J. and Fu, Q., 2012. Optimization of ultrasound-assisted extraction process of perilla seed meal proteins. Food Science and Biotechnology, 21(6), pp.1701-1706.

Zubia, M., Robledo, D. and Freile-Pelegrin, Y., 2007. Antioxidant activities in tropical marine macroalgae from the Yucatan Peninsula, Mexico. Journal of applied phycology, 19(5), pp.449-458.

Appendix A: Photo Gallery



Raw sample

Freeze dried sample

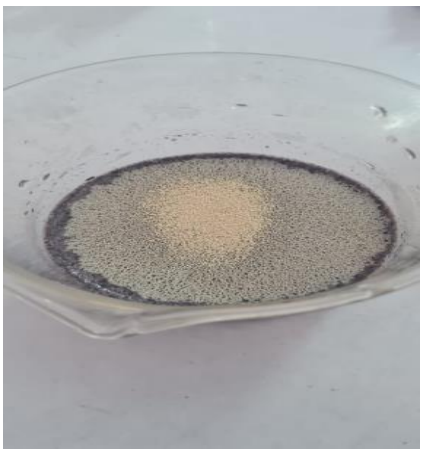
Weighing of processed sample

Preparation of buffer

Weighing of enzyme

Adding enzyme into buffer solution

Addition of seaweed powder



Mixture of seaweed powder, buffer and enzyme

Magnetic stirring

Water bath

Sonication

Sample after sonication

Centrifugation of the sample

Measurement of ammonium sulphate

Supernatant after Centrifugation

Dialysis tube

Dialysis

Sample after protein test

Brief Biography

Fariha Mahmud passed the Secondary School Certificate Examination in 2011 from Dr. Khastagir Govt. Girl´s High School, Chattogram, and then Higher Secondary Certificate Examination in 2013 from Govt. City College, Chattogram. She completed her B.Sc. (Hon's) in Food Science and Technology from the Faculty of Food Science and Technology at Chattogram Veterinary and Animal Sciences University, Chattogram, Bangladesh. Now, she is a candidate for the degree of Master of Science in Applied Human Nutrition and Dietetics under the Department of Applied Food Science and Nutrition, Chattogram Veterinary and Animal Sciences University (CVASU). She has an immense interest to work in improving health status of people through proper guidance and suggestions and to create awareness among people about food safety and nutrition.