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Food grade method of protein extraction of an under explored plant-based protein: Brief study on seaweed protein

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Abstract
This study investigates the effect of ultrasound assisted extraction of protein from three seaweeds *Kappaphycus alvarezii, Gracilaria salicornia, Sargassum wighti.* Initially proximate analysis of all three seaweeds were performed. Later, food grade method of protein extraction was adopted and the protein recovery of these seaweeds, enhancement in protein yield using ultrasound treatment and untreated sample was observed. The protein recovery found in *Sargassum wightii* was found to be higher than *Gracilaria salicornia*. Ultrasound treated sample in *Sargassum wightii* showed 159.3% recovery compared to 125.51% recovery in untreated sample. Whereas, the protein recovery in *Gracilaria salicornia* for treated sample showed 82.74% compared to 73.23% recovery in untreated sample. The study on protein recovery using ultra sound treatment at different stages of extraction was also observed. This study reports that chemical method of extraction assisted with ultrasound improves the yield, as cavitation effect helps in complete disintegration of biogolical matrix of seaweeds thereby increasing the release of protein molecules, as the tough seaweed matrix acts as a major hindrance for protein extraction from seaweeds.

Keywords: ultrasound assisted extraction, food grade extraction, seaweed, plant-based protein, protein recovery, *Kappaphycus alvarezii, Gracilaria salicornia, Sargassum wightii*

1. Introduction
With an increasing concern for health and nutritious food there increases huge demand for protein food. The current needs for novel plant sourced proteins are raising. Though animal sourced proteins are rich in essential amino acids and has higher digestibility value but the major drawbacks in consumption of animal proteins are the associated health risk, due to its resource intensive production and its impact on climate change (Budhathoki *et al*., 2019) [6]. The researchers and food industry are continuously working on better source of protein alternatives, and in development of processing methods to improve their functional and sensory attributes in order to ensure their application. The novel protein sources which have been exploited are pesudocereals (amaranth, quinoa and buckwheat), agricultural by- products such as oil cakes, tubers and roots, terrestrial and aquatic green biomass such as micro algae, leaves, seaweed and aquatic plant (Schweiggert-Weisz, Eisner, Bader-Mittermaier, & Osen, 2020) [15].

However, plant sourced proteins lack few of the essential amino acids, hence they are not considered as a complete or better protein when compared with animal sourced protein but seaweeds or macro-algae are considered to be a good sources of plant-based protein with all the essential amino acids and moreover, seaweeds are of low cost, easily available, requires less resources for the generation and the fast production rate is also relatively high (Fleurence, 1999) [7]. Besides, seaweed cultivation helps in combatting climate change as it sustains the marine ecosystem by carbon sequestration; thereby seaweed acts as a sustainable crop.

Furthermore, seaweeds or marine macro-algae are rich source of bioactive compounds such as polysaccharides, proteins, fatty acids, vitamins and minerals. The functional property of major bioactive compounds present in seaweed has vast application in major economic sectors such as agriculture, food industries, pharmaceutical and cosmetic industries. Additionally, seaweeds are rich in polysaccharides and minerals. And so, they are majorly used as bio-manures, plant stimulants in agricultural sector for improving crop yield.

Seaweed polysaccharides being the major proportion is widely used in food industry as food stabilisers and thickeners, they contain sulphated polysaccharides such as carrageenan, sodium alginate, agar are widely commercialised as it has good functional properties (Bixler & Porse, 2011) [4]. Recently, the application has been wide spread out towards as an encapsulate, for edible films and edible coating.

Generally, the protein content of seaweed varies from 3 to 47% the large variability is based on species, geographical location and climatic conditions. Normally, seaweeds are classified into three major groups namely red algae, green algae and brown algae based on the type of pigment present (Kadam, Tiwari, & O'Donnell, 2013) [10]. In addition, the protein content is higher in red algae followed by green and brown algae. The common methods adopted for protein extraction from seaweed powder are aqueous extraction, pH shift-based extraction, enzymatic hydrolysis. Extraction and isolation of protein is greatly influenced by the seaweed matrix as it acts as a physical barrier (Harrysson *et al*., 2018) [8]. This is because, Macro-algae have complex cell wall structure, and the proteins are tightly bound with phenolic and polysaccharide complexes affecting extraction efficiency.

(Okolie, Akanbi, Mason, Udenigwe, & Aryee, 2019) [14] elucidates that the effect of novel extraction techniques on improving the extraction efficiency of seaweed protein. As conventional methods are time consuming and requires more solvent and provide less yield, the novel extraction techniques such as ultrasound assisted extraction, pressurised fluid extraction, supercritical fluid extraction, microwave assisted extraction can also be adopted. These techniques can be used to enhance the extraction rate and yield by improving the mass transfer rate (interaction between the solvent and solute) thereby reducing the hindrance caused by the seaweed matrix.

In this study the food grade-protein extraction was performed for three different seaweeds namely *Kappaphycus alvarezii*, *Gracilaria salicornia*, *Sargassum wightii* was performed. Since, the studies related to the present work are relatively less and further, protein extraction on these seaweeds are not well explored, we have focused on the better explication and so better knowledge is obtained regarding the importance of plant-based protein over animal-based proteins. The objective of the study is to analyse the nutrient profile of the above mentioned samples and to extract protein using ultrasound assisted extraction and to investigate the extraction yield of these seaweeds (Harrysson *et al*., 2018) [8].

2. Materials and Methods

2.1 Sample preparation

Seaweeds such as *Kappaphycus alvarezii, Gracilaria salicornia* and *Sargassum wightii* were collected from Prasmo agri limited, kumbakonam, Tamil Nadu, India. These seaweeds were harvested from coastal region of Tamil Nadu. The sun dried sample was washed thoroughly to remove salts and other impurities. These cleansed samples were tray dried at 40ºC for 48 hours. Dried seaweed was milled using attrition mill and hammer mill and sieved to obtain uniform size (0.5mm) powder. The ground sample was stored in airtight aluminium pouches and stored at room temperature until further use.

2.2 Proximate analysis

Determination of moisture, fat, protein, ash and carbohydrate contents of seaweeds was conducted according to the standard methods (AOAC, 2016). The value of energy could be calculated by summation of the calories obtained from carbohydrates, proteins and lipids, using the conversion factors 4, 4 and 9 kcal/g respectively; provided by the proximate values (Theagarajan, Malur Narayanaswamy, Dutta, Moses, & Chinnaswamy, 2019) [17].

2.3. Extraction of protein

Protein extraction from seaweed was carried out using a modified method of Kadam, Álvarez, Tiwari, and O'Donnell (2017) [9]. Dried seaweed powder was dissolved in distilled water in 1:20 ratio, was incubated overnight at 4 ºC. The hydrated solution was centrifuged at 9000 rpm for 20 min at 4 ºC. The supernatant was collected and the pellet was dissolved in acid (HCl) solution at a concentration of 0.4M with solid to solvent ratio of 1:15 and was stirred for 1 hour at room temperature followed by centrifugation at 9000 rpm for 20 min at 4 ºC. Similarly, the pellets obtained after centrifugation were dissolved in alkali (NaOH) solution at 0.4M concentration and was kept for continuous stirring for 1 hour followed by centrifugation. The resultant pellet and supernatant was collected and estimated for its protein content, this procedure was considered as the control treatment, and however, ultra sound treatments were performed by the following method. Similar procedure was followed same as the control treatment, but instead of stirring for one hour during acid and alkali hydrolysis, application of ultrasound treatment was introduced for 5 min with 30s pulsation and at 50% amplitude, these conditions were optimised based on the available literature and initial trials. Probe type ultrasound system (Sonic CV334, 220 V, 50/60 Hz) with the system specification: 20 KHz frequency, 750 W power output and the diameter of probe tip 19mm was employed through the study (Sengar, Rawson, Muthiah, & Kalakandan, 2020) [16].

Five variations in the protocols were followed for the better protein extraction. Therefore, protein extraction without ultrasound treatment as control and application of sonic energy at different stages of extraction process as treated samples. For example, protein extraction without ultrasound treatment; sonication as pre-treatment before soaking phase; sonication during acid hydrolysis phase; sonication during alkali hydrolysis; and sonicaion at both the acid and alkali hydrolysis phase was performed.

2.4 Statistical Analysis

All experiments were performed three times and the data was represented in terms of mean ±SD. Significant differences among different species were obtained by Tukey test with a significant level of (p<0.05) and the one way analysis of variance was conducted using SPSS statistical software 2.0.

3. Results and Discussion

3.1 Proximate analysis

Proximate composition of three different seaweeds *Kappaphycus alvarezi*, *Gracilaria salicornia* and *Sargassum wightii* was done. Table 1 shows the results of proximate analysis, from the results we understand that there was no significant difference among the moisture content of all the three seaweed samples. Whereas, the values of ash, fibre, protein, carbohydrate and energy content exhibited significant difference. Further, there was no significant difference observed in the fat content of red algae (*Kappaphycus alvarezii and Gracilaria salicornia*) rather, brown algae (*Sargassum wightii*) showed significant different from them. Generally, the chemical composition of seaweeds differ among species, based on geographical location, season and

method of cultivation (Fleurence, 1999; Makwana, 2011) [7]. Protein content of seaweed usually varies based on the above mentioned factors.

It was reported that mostly, *Gracilaria* species contains around 7-13% of protein (Briggs & Funge-Smith, 1993) [5]. The protein content of *Gracilaria salicornia* was observed to be higher than other Gracilaria species (Norziah & Ching, 2000) [13]. Munandar *et al*. (2019) [12] reports similar range of protein and ash content of *Kappapphycus* species. The crude protein content of *Sargassum wightii* was found to be 8.3% which was similar to the protein content present in other *Sargassum* species (Wong & Cheung, 2001) [18].

Generally, seaweeds are rich in polysaccharides and minerals but fat content was found to be less. The lipid content of seaweeds are usually low, containing around 1- 3% dry weight (Azad, Alauddin, Islam, Hoque, & Chowdhury, 2007) [2]. Similarly, the lipid content of the seaweeds chosen was found to be low. In this study *Sargassum* species showed higher ash content and lower fiber content as compared to other species like *Sargassum vulgare* (Arguelles, Monsalud, & Sapin, 2019) [1].

Table 1: Proximate compostion of *Kappaphycus alvarezii, Gracilaria salicornia, Sargassum wightii*

Proximate content	*Kappaphycus alvarezii*	*Gracilaria salicornia*	*Sargassum wightii*
Moisture (%)	$6.9^{a} \pm 0.360$	$6.13^{a} \pm 0.808$	$6.27^{a} \pm 0.251$
Ash (%)	$17.36^{a} \pm 0.334$	$25.08^{b} \pm 0.190$	$35.33^{c} \pm 0.530$
Protein (%)	$3.77^{a} \pm 0.493$	$13.29^{c} \pm 1.12$	$8.33^{b} \pm 0.253$
Fat (%)	$1.13^{a} \pm 0.550$	$0.66^{a} \pm 0.285$	$3.17^{b} \pm 0.763$
Fiber (%)	$6.79^{a} \pm 0.321$	$14.77^{c} \pm 0.208$	$10.36^{b} \pm 0.480$
Carbohydrates (%)	$64.04^{c} \pm 0.65$	$40.07^{b} \pm 0.127$	$36.54^{a} \pm 0.78$
Energy (Kcal)	$281.41^{c} \pm 0.54$	$219.38^{b} \pm 0.61$	$208.01^{a} \pm 0.54$

Different letters in column represent significant difference at $P \leq 0.05$.

3.2 Protein content and yield of extraction

Among all the three seaweeds, *Sargassum wightii* showed better extraction yield than *Gracilaria salicornia* and *Kappaphycus alvarezii*. This extraction method was most suitable for brown algae and so the extraction yield of brown algae showed higher. *Kappaphycus alvarezii* showed very low crude protein content so the extraction study was observed only for *Gracilaria salicornia* and *Sargassum wightii*.

The percentage of protein recovery for *Gracilaria salicornia* ranged from 64.1 to 82.74% and for *Sargassum wightii* it the values ranged from 125.51 to 159.3% reporting that *Sargassum wightii* showed better recovery. Similar results were obtained for brown algae *Ascophyllum nodosum* using novel method of extraction (Kadam *et al*., 2017) [9]. Studies show that similar yield were obtained for other species among red and brown algae using classical method of extraction i.e sonication along with ammonium sulphate precipitation (O'Connor, Meaney, Williams, & Hayes, 2020). Similar results were obtained on protein yield from *Ulva* sp. and *Gracilari*a sp. (Kazir *et al*., 2019).

Sequential extraction of protein using both acid and alkali treatment was found to be the most efficient method for extraction of protein from seaweed, Kadam *et al*. (2017) [9] reports similar study on protein extraction from *Ascophyllum nodosum*. In this extraction method, the sample was first extracted by acid condition followed by alkali condition which helps in removing the polysaccharides bound to the protein; generally, extraction of protein from seaweed is a challenging process as the protein molecules are entrapped with other non-protein complexes such as polysaccharides and polyphenols.

During acid treatment the cell wall matrix gets eroded but complete cell disintegration was induced by ultrasound treatment as it induces cavitation which disrupts the biological matrix and ensures complete release of protein molecules. Thereafter, the proteins are easily solubilised in the alkali solution (Barbarino & Lourenço, 2005) [3], Ultrasound treatment was used during alkali hydrolysis as it ensures homogenisation which increases the mass transfer rate of solvent and solute thereby increasing the extraction yield.

3.3 Effect of ultrasound treatment at different stages of extraction

The effect of ultrasound treatment at three different stages of extraction for *Sargassum wightii* was observed and represented in the figure 1. Firstly, the ultrasound treatment was conducted prior to soaking, ultrasound as pre-treatment, then extraction was carried out. Then the ultrasound treatment was applied during acid hydrolysis, as it improves cell disintegration, requires less solvent and time for extraction. Similarly, the treatment effect was observed during alkali hydrolysis. Ultra sonication as pre-treatment showed higher protein recovery when compared to its effect during acid hydrolysis or alkali hydrolysis, as it ensures complete hydration.

Similarly, the figure 2 represents the protein recovery of *Gracilaria salicornia* at different conditions, it was observed that ultra sonication performed at both acid and alkali hydrolysis showed higher recovery.

The protein extracted without ultrasound treatment, where one hour of stirring was conducted at acid hydrolysis and alkali hydrolysis showed similar results to the samples which were treated with ultra sonication for 5 minutes instead of stirring. This shows that ultrasound assisted extraction enhances the yield, with less treatment time and requires minimum solvent quantity. This is a food grade method of extraction.

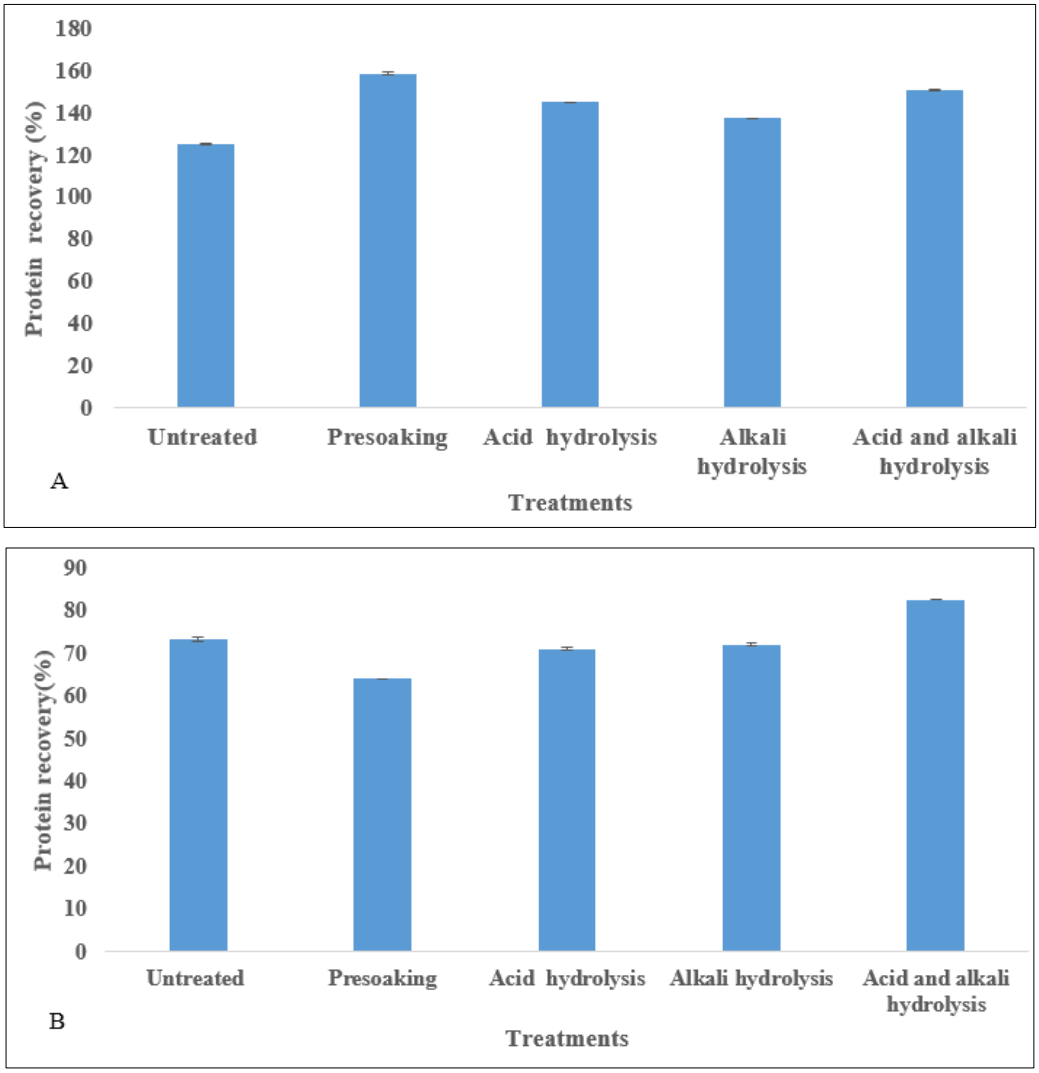

Fig 1: Protein recovery of (A) *Sargassum wightii* and (B) *Gracilaria salicornia*

4. Conclusion

This study states that different seaweed species respond differently to the extraction method. The focus of work was to develop a food grade method of protein extraction using novel extraction technique. Usually, conventional method of extraction was performed using 2-mercaptoethanol or ammonium sulphate precipitation which is highly unsafe for consumption. Moreover, ultrasound assisted pH shift technique enhanced the protein yield and observed to be the best method for protein extraction from seaweed as it is an safe and eco-friendly approach.

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