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# Extraction of proteins from six seaweeds of south-east coast of India: Proximate composition and effect of process variables on protein solubilisation

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## Abstract

Seaweed resources have received huge interest as a dietary source of proteins. In this study, we investigated the effects of process variables such as solvent-to-solid ratio, duration, temperature and pH on solubilisation of proteins from *Enteromorpha compressa* with the aim of developing a process of seaweed protein extraction. Protein solubilisation was also assessed for five additional seaweeds *viz,* the brown seaweed *Turbinaria conoides*, the red seaweeds *Kappaphycus alvarezii, Gracilaria edulis* and *Hypnea valentiae* and the green seaweed *Halimeda gracilis*, using the optimum conditions derived for *E. compressa*. Protein content in the dried seaweed samples ranged from 3.05-11.29%. Based on the extent of protein solubilisation, the optimal conditions were found to be a duration of 30 min, pH of 9, temperature of 90°C and dried seaweed-to-solvent ratio of 1:30. Under these conditions, 19.90% of proteins from *E. compressa* were solubilised. Under the same process conditions, protein solubilisation from *T. conoides, K. alverzii, H. gracilis, H. valentiae* and *G. edulis* was 27.39, 30.88, 45.57, 19.68 and 40.32%, respectively. The investigation condluded that protein extraction from seaweeds is highly challenging and needs further research for improved recovery.

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## Introduction

The increasing global population is leading to depletion of natural resources. Despite their abundance as a food source, seaweed (marine macro-algae) resources remain largely unexploited. India has a coastline of 8129 km, excluding its island territories with 0.2 million km$^2$ Exclusive Economic Zone (EEZ). India is home to 844 species of seaweeds, including 434 species of red seaweeds, 194 species of brown seaweeds and 216 species of green seaweeds. These are abundantly found along the coasts of Tamil Nadu and Gujarat as well as around Lakshadweep and Andaman and Nicobar Islands (Rao and Mantri, 2006). Seaweeds are rich in dietary fibres, minerals, polysaccharides and proteins and is used in various culinary delicacies such as salads, soups and traditional food products in different countries. Valorisation of seaweeds as source of ingredients has been explored for ages owing to its anti-microbial, anti-oxidant, anti-diabetic, anti-viral and cosmeceutical properties (Balti *et al*., 2011). However, the utilisation of seaweed as a source of protein has received attention only recently, creating ample opportunities for research and development in seaweed proteins.

Naturally, the protein content of macro-algae varies by species, locations and environmental conditions (Fleurence *et al*., 2012; Vilg *et al*., 2015). Generally, brown seaweeds contain lower protein levels (3-15% of dry weight), green algae have moderate (10-30% of dry weight) and red seaweeds have the highest protein content (up to 47% of dry weight) (Wong and Cheung, 2001; Burtin, 2003). However, extracting these proteins is challenging due to the polysaccharides bound to the cell wall, which results in poor extractability (Mabeau and Fleurence, 1993). Protein extractability is further complicated by the high viscosity of the polysaccharides in aqueous solution, the abundance of phenolic compounds and the ionic interactions between the cell wall components and proteins (Jordan and Vilter, 1991; Joubert and Fleurence, 2008). Meanwhile, several studies have validated methods of extraction of seaweed proteins (Vilg and Undeland, 2017). These studies generally employed ultrasonic treatment, mechanical grinding, osmotic shock, enzyme treatment and isoelectric precipitation to disrupt macro-algal cells, facilitating easier protein extraction (Wong and Cheung, 2001; Joubert and Fleurence, 2008). While isoelectric point precipitation of protein (pI) has primarily been used for isolation of protein, from soybean, wheat and fishery products (Vareltzis and Undeland, 2012), there are limited studies on using pH shift techniques for protein isolation from seaweeds such as *Sargassum* spp., *Enteromorpha* spp., *Palmaria palmata* and *Kappaphycus alvarezii* (Wong and Cheung, 2001; Kandasamy *et al*., 2012; Harnedy and FitzGerald, 2013; Kumar *et al*., 2014).

In many protein extracting processes, solubilisation is the first step followed by precipitation through pH adjustments, salts and solvents. The precipitation process has been attempted with modification such as extraction in the presence of mercaptoethanol followed by precipitation with ammonium sulphate (Wong and Cheung, 2001; Kandasamy *et al*., 2012; Harnedy and FitzGerald, 2013; Kumar *et al.*, 2014). A robust highly efficient process is required for separation of proteins from polysaccharides and phenolic compounds in seaweed for developing protein-rich seaweed-based food products, which remains a great challenge. Extensive studies on the process variables affecting solubilisation of seaweed proteins are necessary to maximise protein recovery. With this rationale, the study investigated the effect of process variables (time, temperature, pH and solvent ratio) on the solubilisation of proteins from *E. compressa*. Further, to evaluate the use of derived optimal conditions for protein solubilisation from other commercially important seaweeds, five more seaweeds *viz*, *Turbinaria conoides*, *K. alvarezii, Gracilaria edulis, Hypnea valentia* and *Halimeda gracilis* were studied.

# Materials and methods

## Collection of seaweeds

The seaweeds used in the present study were collected from Mandapam coastal region on the south-east coast of Tamil Nadu, India through a private farm engaged in such activities for over 15 years (R. K. Algae Project Centre, Mandapam, Tamil Nadu, India). The seaweeds received in semi-dried form comprised the brown seaweed *T. conoides*, red seaweeds *H. valentiae, G. edulis* and *K. alvarezii*) as well as green seaweeds *E. compressa* and *H. gracilis*. The seaweed samples received were washed and further dried at 50˚C using an electrical drier (Kraftwork drier-KSD 100, India) for 72 h. The dried samples (200 g each) were powdered in a household blender (Philips HL7756100) and packed in polythene pouches, sealed and stored in airtight containers at room temperature until further use.

## Effect of process variables on the extractability of seaweed proteins

### Solid-to-solvent ratio

In order to determine the amount of solvent *i.e.,* water to be used for the extraction of protein from seaweed samples. *E. compressa* was studied at three different solid mass-to-solvent volume ratio (1:6; 1:30; 1:60 w/v). In brief, 1 g of seaweed powder was weighed accurately in a 50 ml centrifuge tube (Tarsons 16K) and a predetermined volume of water was added. The seaweed-water mixture was allowed to hydrate at room temperature under magnetic stirring at 1200 rpm for 30 min. The seaweed-water mixture was centrifuged at 8000 rpm (Thermo Fisher Scientific, Heraeus Fresco 17, Germany) for 10 min at room temperature. The clear supernatant was filtered through a Whatman filter paper (Sigma-Aldrich, No. 1, 150 mm dia) and the filtrate was collected. The pellet was squeezed manually by placing between two layers of cheese cloth and the liquid obtained was filtered using a Whatman filter paper (Sigma-Aldrich, No. 1, 150 mm dia). Both the supernatants were combined and the total volume was recorded. From the supernatant, 100 µl of the extract was pipetted out using a micropipette and the protein was quantified using Lowry's method as described by Markwell *et al*. (1978). Dilution factor and volume of supernatant was taken into account to calculate the total protein extracted from the given mass of seaweed. The extractability of protein was expressed as mg of protein extracted per gram of seaweed sample.

### Hydrogen ion activity (pH)

Extractability of seaweed protein at different pH values was measured according to the method of Harrysson *et al*. (2018). Accurately one gram of seaweed (*E. compressa*) powder was weighed and mixed with distilled water at a ratio of 1:6, 1:30, 1:60 (w/v) and homogenised using a homogeniser (Velp Scientifica, OV5, Italy) at 1200 rpm for 2 min. The pH was adjusted to different values (1, 3, 5, 7, 9, 11, 13) using 1 M hydrochloric acid (HCl) or 1 M sodium hydroxide (NaOH). The slurry was incubated for 30 min under magnetic stirring and centrifuged at 8000 rpm for 10 min (Thermo Fisher Scientific, Heraeus Fresco 17, Germany). Then the supernatant was collected and protein content in the supernatant was determined by Lowry's method (1951) with some modifications as per Markwell *et al*. (1978).

### Temperature

The extractability of seaweed proteins was evaluated as influenced by temperature. Seaweed-water mixture (1:30) was prepared as explained in the previous section and incubated at different temperatures of 30 (room temperature), 50, 60, 70, 80, 90 and 100˚C for 30 min. Further steps to collect the supernatant was followed as explained previously and the total protein was quantified following the Lowry's method as modified by Markwell *et al.* (1978).

### Duration of extraction

To evaluate the effect of extraction time on the extractability of protein from powdered seaweed sample, accurately one gram of sample was weighed in a clean beaker and 30 ml of distilled water was added. The seaweed-water dispersion was hydrated for 5 min under magnetic stirring at 1500 rpm and then the pH was adjusted to 9 using 1M NaOH. The samples were incubated for different time periods of 30, 60, 90, 120 min under magnetic stirring at room temperature. The zero-time sample was prepared similarly with the omission of the incubation step. Intermittently the pH was checked and adjusted back to 9.0 using 1M NaOH. The protein extracted was collected by centrifuging the reaction mixture at 8000 rpm for 10 min at room temperature. The supernatant was again passed through a Whatman filter paper (Sigma-Aldrich, No.150). The pellet was collected and squeezed manually by placing between two layers of cheese cloth and filtered using Whatman filter paper (No.150). Both the extracts were combined and the volume was recorded and from this, an aliquot of 100 µl was used to quantify the protein content by Lowry's method as described in the previous section.

### Determination of protein solubility of six different seaweeds by pH shifting method

The protein extractability of six different seaweed species was estimated by pH assisted solubilisation following the method of Vilg and Undeland (2017). One gram of powdered seaweed sample (dry weight) was mixed with 30 ml of distilled water (1:30) and adjusted the pH to 9 using 1M NaOH. The seaweed-water mixture was stirred continuously using a magnetic stirrer for 30 min under chilled conditions. The slurry was centrifuged at 8000 rpm for 10 min at room temperature and the supernatant was collected for determination of total protein by Lowry's method as described in the previous section.

## Analysis

### Proximate composition and non-protein nitrogenous fractions of seaweeds

The proximate composition of powdered seaweed samples including moisture (3.1.03), protein (3.5.10), ash (3.1.04) and lipid (3.5.07) were analysed according to the methods described in AOAC (2019). The moisture content was determined by drying in a hot air oven at 105°C for 12 h. Total nitrogen content was analysed using Kjeldahl technique and crude protein was calculated using a nitrogen conversion factor of 5.0 (Angell *et al*., 2016). Ash content was determined by incinerating the sample at 550°C for 6 h in a muffle furnace. Crude lipid (ether extractable) of seaweed samples was quantified gravimetrically by extracting the powdered samples using petroleum ether in a Soxhlet apparatus followed by oven-drying the extract at 80°C. The total carbohydrate and fibre were calculated based on the mass difference. The non-protein nitrogen fractions of whole seaweed were determined using 10% trichloroacetic acid (TCA) extract (AOAC, 2019). Analyses were carried out in triplicate for each seaweed. The values are represented in percentage.

### Protein determination by Lowry's method

For determination of protein concentration, in 1 ml sample, 5 ml of freshly made reagent (1 part 4% $CuSO_4 \cdot 5H_2O$ into 100 parts of a mixture of 2% $Na_2CO_3$, 0.4% NaOH, 0.16% Na-tartrate and 1% SDS) was added and incubated for 10 min at room temperature. After that, 0.5 ml freshly made phenol reagent (1-part Folin-Ciocalteu phenol reagent into 1 part Milli-Q water) was added and the samples were incubated for 30 min in darkness at room temperature. Absorbance was measured using a UV-VIS spectrophotometer at 750 nm. A standard curve made from bovine serum albumin (BSA) was used for quantification. The values were calculated as mg of protein per g of sample.

## Statistical analysis

Data were analysed with SPSS software (20.0). One-way ANOVA was used to compare the means and the differences were found by Duncan's multiple range test and differences were considered significant at $p<0.05$. All the analyses were carried out in triplicate.

## Results and discussion

Initially, six different species belonging to various categories of brown, red and green seaweeds including *T. conoides* (Phaeophyceae), *H. valentiae, G. edulis* and *K. alvarezii* (Rhodophyceae), *E. compressa* and *H. gracilis* (Chlorophyceae) were screened to quantify the protein content. Other major chemical constituents such as fat, ash and carbohydrate (including fibres) were also quantified. The results are presented in Table 1. Crude protein, ash, fat and carbohydrate contents of seaweeds studied were in the range of 3.05±0.38-11.29±0.08%, 22.50±3.53-65.50±4.94%, 0.96±0.03-2.93±0.11% and 18.33±0.33-69.87±0.46%, respectively. It is a well-established fact that the major chemical constituents of seaweed are influenced by various biological and environmental factors. The biotic factors include the type of species and biological cycle, whereas abiotic factors include growth stage and photosynthetic cycle (Mabeau and Fleurence, 1993). The environmental factor includes seasons, geographical location, water quality, temperature, salinity and nutrients availability (Mabeau and Fleurence, 1993).

Table1. Proximate composition of different groups of seaweeds

| Seaweed species | Parameters | | | | | |
|---|---|---|---|---|---|---|
| | Dry solids (%) | Protein (%) | Ash (%) | Lipid (%) | *Carbohydrate+ Fibre (%) | NPN (%) |
| *T. conoides* | $66.78 \pm 0.72^{a}$ | $5.80 \pm 0.40^{c}$ | $33.05 \pm 0.70^{b}$ | $1.00 \pm 0.01^{a}$ | $60.40 \pm 0.79^{d}$ | $0.08 \pm 0.01^{c}$ |
| *H. gracilis* | $94.19 \pm 0.42^{d}$ | $3.05 \pm 0.38^{a}$ | $32.83 \pm 0.23^{b}$ | $2.43 \pm 0.51^{b}$ | $61.83 \pm 0.25^{d}$ | $0.16 \pm 0.01^{e}$ |
| *E. compressa* | $88.94 \pm 0.54^{c}$ | $9.84 \pm 0.04^{d}$ | $65.50 \pm 4.94^{d}$ | $2.93 \pm 0.11^{c}$ | $18.33 \pm 0.33^{a}$ | $0.13 \pm 0.03^{d}$ |
| *K. alverzii* | $86.58 \pm 3.94^{c}$ | $3.96 \pm 0.80^{b}$ | $43.42 \pm 0.59^{c}$ | $2.50 \pm 0.36^{bc}$ | $50.00 \pm 0.42^{c}$ | $0.13 \pm 0.01^{b}$ |
| *H. valentiae* | $80.81 \pm 0.79^{b}$ | $11.29 \pm 0.08^{e}$ | $44.26 \pm 3.20^{c}$ | $1.00 \pm 0.00^{a}$ | $45.66 \pm 0.64^{b}$ | $0.14 \pm 0.01^{de}$ |
| *G. edulis* | $93.27 \pm 2.36^{d}$ | $4.66 \pm 0.40^{b}$ | $22.50 \pm 3.53^{a}$ | $0.96 \pm 0.03^{a}$ | $69.87 \pm 0.46^{e}$ | $0.02 \pm 0.01^{a}$ |

*-Total carbohydrate and fibre estimated by the mass difference
Results are presented on dry weight basis as Mean±SD (n=3). Different superscripts within the column indicates significant difference ($p<0.05$)

Among the six species, protein content was high in *H. valentiae* followed by *E. compressa*, *T. conoides, G. edulis, K. alverzii* and *H. gracilis*. It should be noted that in the present study the seaweed samples were collected from Mandapam coast (Gulf of Mannar region) during the month of December. The same *H. valentiae* species from the Persian coast of Iran has been reported to have protein content of 16.5±2.78% on dry matter basis (Ghadikolaei *et al*., 2012). In general, the protein content in seaweeds is relatively low compared to leafy vegetables of terrestrial origin. The values obtained in the present study for protein content were comparable with the values reported elsewhere for the seaweed species. The literature shows variation in nitrogen to protein conversion factor for seaweeds ranging from 3.59 to 6.25 (Hanisak, 1983). A conversion factor of 6.25 often results in reporting a higher protein content. In the present study, we used conversion factor 5 (Angell *et al*., 2016). Among the different groups of seaweeds studied in the present investigation, red seaweeds were found to have higher protein content than the green and brown seaweeds. An earlier study on brown seaweeds by Burtin (2003) concluded that the protein content in brown seaweeds is generally low. Phycobiliproteins, phycoerythrins, phycocyanins and allophycocyanins are the major pigmented proteins in red seaweeds. These pigments reflect red light and absorb blue light, allowing for greater water penetration, enabling red algae to photosynthesise efficiently, which may enhance the synthesis of pigmented proteins (Mabeau and Fleurence, 1993). Care should be taken when expressing the protein content of seaweeds calculated from nitrogen values to ensure accuracy.

In this study, the ash content was found to be more in *E. compressa i.e.,* 65.50% as compared to other species namely *H. valentiae*, *K. alvarezii*, *T. conoides*, *H. gracilis* and *G. edulis*. A different species under the same genus, *Enteromorpha intenstinalis* collected from Persian Gulf of Iran has recorded 22.4±1.46% ash on dry matter basis (Ghadikolaei *et al*., 2012). Ash content indicates the presence of micro and macro minerals with some trace elements (Mabeau and Fleurence, 1993). Seaweeds absorb minerals from seawater and accumulates in their thalli and therefore mineral content is higher in seaweeds compared to terrestrial vegetations. Ash content of seaweeds is influenced by the environmental and biological factors (Manivannan *et al*., 2009). In the present investigation, the acid insoluble ash was found to be more in *H. gracilis* showing around 42% of the total ash content, which indicated the presence of siliceous materials (data not shown). Generally, the lipid content in seaweeds vary from 1-5% on a dry weight basis (Mabeau and Fleurence, 1993). *E. compressa* contained high lipid content of 2.93% as compared to other species. The same species collected from Vedalai coastal waters, south-east coast of India showed comparatively less lipid content of 0.81% on dry weight basis (Manivannan *et al*., 2009). Similarly, the same seaweed species collected from the Tuticorin coast, India recorded higher lipid content as reported by Parthiban *et al*. (2013). The lipid content also varies from species to species and is influenced by environmental and biological factors (Benjama and Masniyom, 2012).

Carbohydrate and fibre are the major components of seaweeds. Carbohydrate contributes 50-60% on dry weight basis (Ghadikolaei *et al*., 2012) whereas the fibre content of seaweeds is higher than fruits and vegetables (Dawczynski *et al*., 2007). The estimated levels of carbohydrate and fibre were found to be more in *G. edulis* (69.87±0.46%) on dry weight basis followed by others. The seaweeds *G. corticata* from the same genus, collected from Mandapam coastal region has shown higher carbohydrate content (Narasiman and Murugaiyan, 2012). The high content of fibre in red algae might be due to higher phycocolloid content in their cell walls (Parthiban *et al*., 2013).

Green seaweed *H. gracilis* contained comparatively high non-protein nitrogen (NPN) (0.16%) followed by *H. valentiae, K. alverzii*, *E. compressa*, *T. conoides* and *G. edulis*. Dawczynski *et al.* (2007) studied some red, green and brown algae and reported that red algae species contributed higher NPN fraction than brown algae. The NPN-fraction of algae mainly consists of chlorophyll, phycoerythrins, nitrite, nitrate, nucleic acids, ammonium compounds and free amino acids (Lourenco *et al*., 1998).

## Effect of process variables on solubilisation of seaweed (*E. compressa*) protein

### Solid-to-solvent ratio

The ratio between seaweed and the amount of solvent can affect the extraction yield. To find out the protein extractability in water, seaweed samples (*E. compressa*) were dispersed in water at three different ratios (1:6, 1:30, 1:60) (dry weight basis). In this investigation, the extracted protein value was more in 1:60 (16.05±2.41 mg $g^{-1}$) whereas the value was less in 1:6 (6.23±0.66 mg $g^{-1}$) (Fig. 1). There was a significant ($p<0.05$) relationship between the extraction yield of protein and the relative amount of water added. It was found that, when the water ratio was increased, the extraction yields also gradually increased as the availability of more solvent facilitated solvation of protein to a larger extent (Vareltzis and Undeland, 2012). It could be expected that seaweed contains salt and other minerals like sodium, calcium and magnesium. Protein extraction in the aqueous medium increased in the presence of chloride ions through electrostatic repulsion after binding to the positively charged protein groups. The degree of protein solubility in an aqueous medium is governed by the electrostatic and hydrophobic interactions between the protein molecules. Protein solubility is also affected by other factors like mechanical grinding, homogenisation and speed of centrifugation. Another factor which influences the protein concentration is the volume of supernatant recovered, since volume of supernatant is used to calculate the total amount of protein extracted from the given mass of seaweed. Vilg and Undeland (2017) stated that by increasing the volume of water upto 1:60, the yield of protein also increased by 58% from the seaweed (*Saccharina latissima*). In our study, increasing the solid to the solvent ratio from 1:6 to 1:30 (fivefold), the protein solubilisation increased by 54.25%. Further increase of solid-to-solvent ratio to 1:60 (tenfold) increases the protein solubilisation by 157% (2.57-fold) compared to the extractability obtained at 1:6.

## Effect of pH on the extractability of seaweed proteins

pH is the most determining factor of protein recovery as solubility is mainly governed through interaction between the protein molecules and the medium (water). The interaction is controlled by the charged functional groups in the side chain of amino acid residues. The degree of protein solubility in aqueous medium depends on the electrostatic and hydrophobic interaction between the protein molecules. However, if the electrostatic repulsion between the two

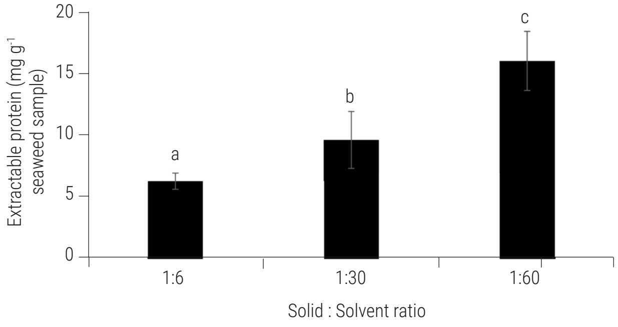


Fig. 1. Solid to solvent ratio (seaweed: water) on extractability of seaweed protein. Results are presented as Mean±SD (n=3). Different alphabets on error bars indicate significant difference ($p<0.05$)

molecules is higher than the hydrophobic interaction, the solubility gradually increases (Zayas, 1997). To determine the solubility of seaweed proteins in an aqueous medium at different pH, the green seaweed (*E. compressa*) was homogenised in water as solvent at a ratio of 1:6; 1:30; 1:60 (w/v) and the pH was adjusted between 1 and 13 using 1M HCl/1M NaOH. At solid-to-solvent ratio of 1:6, the protein solubility was higher at pH 9 with the extractability value of 12.40±0.43 mg $g^{-1}$ of sample. The lowest protein solubilisation was at the pH 7 (3.06±0.12 mg $g^{-1}$). At the solid-to-solvent ratio of 1:30, the solubilisation of seaweed protein was the highest at pH 9 (40.87±1.39 mg $g^{-1}$). At solid-to-solvent ratio of 1:60, the protein solubilisation was high (43.07±2.20) at pH 9 and the lowest extraction was at pH 3 (20.82±0.39 mg $g^{-1}$). Results clearly indicated that alkaline pH specifically had a profound effect on protein extraction. Further increase in pH to 11 and 13 reduced the protein solvation. Another salient finding is that the solvent availability (volume) is the major limiting factor even at the same pH. Sufficient quantity of solvent should be made available in seaweed protein extraction process. In the presence of abundant solvent, acidic conditions also facilitate the solvation of seaweed proteins. However, this effect is not observed when the solvent quantity is limited. Overall, a pH of 9 is more conducive for the interaction of seaweed proteins, whether at a solid-to-solvent ratio of 1:30 or 1:60 (Fig. 2). The protein extractability values obtained in the present study is comparatively less than the values already reported (Harrysson *et al.,* 2008; Kandasamy *et al.,* 2012). Solubilisation of seaweed proteins depends on different factors such as original protein value of the particular seaweed, geographical location, efficiency of homogenisation/filtration. Solubilisation capacity of proteins in the seaweeds may also depend on the characteristics of the seaweed matrix such as the amount of anionic or neutral polysaccharides, protein folding and interaction with protein binding compounds such as phenolic compounds (Jordan and Vilter, 1991). The protein solubility at different pH varies from species to species. Harrysson *et al*. (2018) reported that pH 12 is the most appropriate pH for protein solubilisation in *Ulva lactuca* (62.1±5.1 mg $g^{-1}$) and *Porphyra umbilicalis* (54.2±4.9 mg $g^{-1}$). According to Kandasamy *et al.* (2012), the use of 2-mercaptoethanol, increasing the pH up to 12 and precipitation with ammonium sulphate, increased the protein concentration of *E. compressa* from 17.48±0.41 (raw seaweed) to 60.35±2.01% (protein concentrate/extract). Similar increases were observed for *Enteromorpha tubulosa* (from 19.09±0.91 to 53.83±0.70%) and in *E. linza* (from 12.5±1.26 to 33.36±1.04%) on a dry weight basis, with protein recovered up to 6.48, 6.16 and 5.71% respectively. These results underscores that the enhanced solubilisation of seaweed proteins through pH assisted process depends significantly on the solvent quantity and pH level rather than being solely limited by pH alone.

## Effect of time on the extractability of protein from seaweed

Duration of extraction is the most crucial factor for protein recovery. For knowing the actual extraction time at pH 9, the seaweed samples were incubated at different time periods (0, 30, 60, 90, 120 min) under room temperature and the total protein values were calculated. Significant differences ($p<0.05$) in protein solubility were observed across different extraction times, ranging from 0 min to 120 min. Initially, at 0 min, the protein solubility was 11.51±0.63 mg $g^{-1}$. The solubility peaked at 30 min with 16.25±1.37 mg $g^{-1}$, followed by 10.90 mg $g^{-1}$ at 60 min, 14.42 mg $g^{-1}$ at 90 min and 15.62 mg $g^{-1}$ at 120 min (Fig. 3). The observed reduction in the yield at 60 min could be due to the interaction of the extracted protein with other biomolecules including polyphenols and formation of insoluble aggregates. Venkataraman and Shivashankar (1979) found a major increase in the yield of protein extracted, when the extraction time increased from 45 to 60 min in the microalga, *Scendesmus acutus*. Harnedy and Fitzgerlad (2013) reported that the protein solubility was increased with increasing the agitation time from 0.5 to 3 h in *Palmaria palmata* in alkaline medium. Vilg and Undeland (2017) reported that the total protein yield was more in alkaline medium for the incubation period of 1-2 h. The degree of agitation also affects protein solubility. For further studies, extraction time of 30 min was selected and the solution was kept under magnetic stirring at 1200 rpm. Several other factors like types of species, particle size, processing condition and the molecular interaction also affect the protein solubility.

## Effect of temperature on protein leaching

Temperature can be an important factor for protein solubilisation. Generally, higher temperatures decrease the solubility of proteins mainly due to denaturation. As the temperature increases,

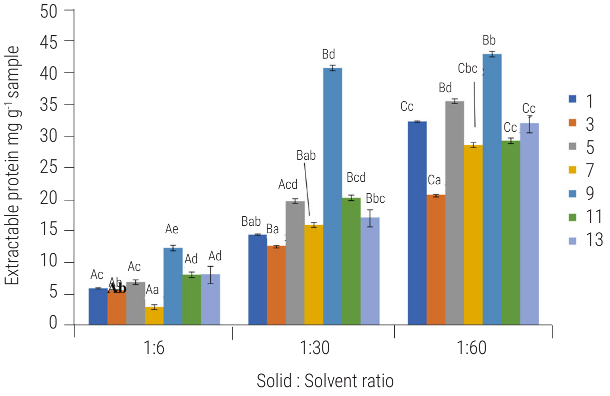


Fig. 2. Effect of pH on extractability of seaweed proteins (*E. compressa*) at different solid to solvent ratio. Different uppercase alphabets on the error bars indicate significant difference between solid to solvent ratio within pH and different lowercase alphabets on the error bars indicate significant difference between pH within solid to solvent ratio ($p < 0.05$)

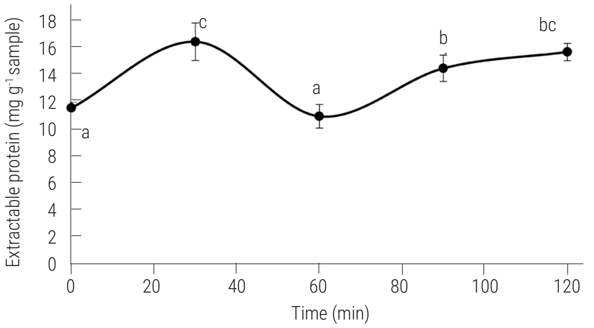


Fig. 3. Effect of time on extractability of seaweed proteins (*E. compressa*). Results are presented as Mean±SD (n=3). Different alphabets on error bars indicate significant difference ($p<0.05$)

the solubility of protein is less than that of the native protein and the conformational structure of protein also changes (Zayas, 1997). In this experiment, for assessing the protein solubility as influenced by temperature [30 (room temperature) 50, 60, 70, 80, 90 and 100°C] the seaweed water mixture (1:30) was kept for incubation for 30 min. The protein extractability was in the range of 11.58±0.20 to 18.22 mg g$^{-1}$ of raw materials (Fig. 4). At room temperature, the protein solubility was 11.58±0.20 mg g$^{-1}$ of raw materials which was found to be less. There was no significant effect ($p<0.05$) of temperature on protein solubility. The solubility was slightly higher at 90°C (20.64±1.96 mg g$^{-1}$ of raw materials). Further rise in the temperature to 100°C, reduced the protein extractability (18.22±2.19 mg g$^{-1}$ of raw materials). Vilg and Undeland (2017) studied different temperatures like 4, 20 and 50°C for protein extraction in *Saccharina latissima* and found the protein extractability was slightly higher at 50°C than at 20°C and 4°C. It could also be expected that the protein extraction from seaweed at different temperatures is influenced by the nature of seaweed (type of species) and the physical characteristics of raw materials (particle size and surface area). Based on the results obtained, the temperature of 90°C, time duration of 30 min, solid-to-solvent ratio of 1:30 and pH of 9 were selected for studying the protein solubilisation in other seaweed species.

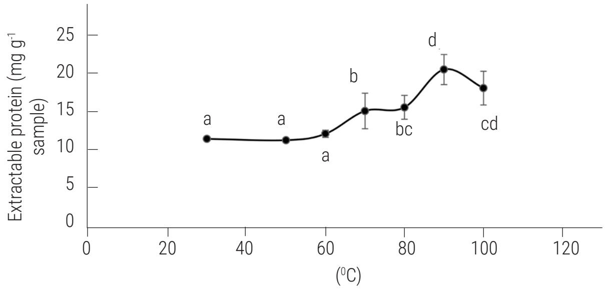
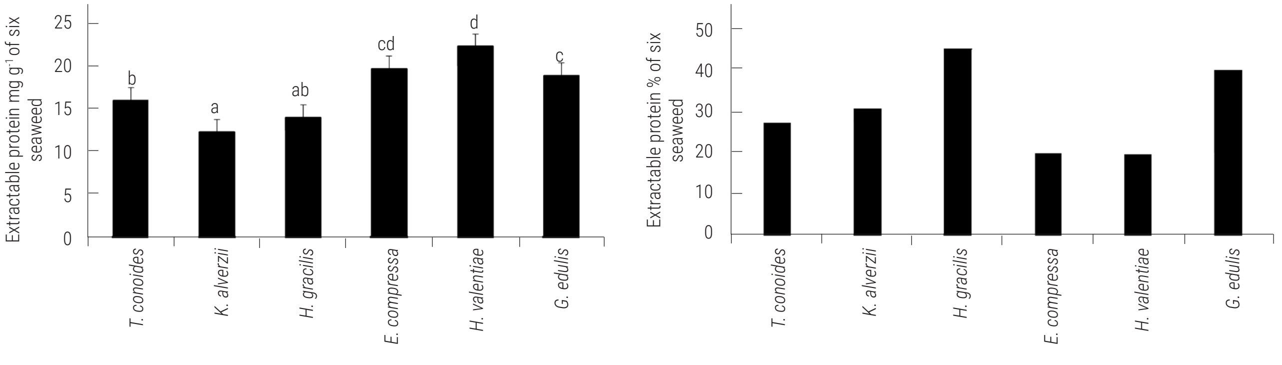


Fig. 4. Effect of temperature on extractability of seaweed proteins (*E. compressa*). Results are presented as Mean±SD (n=3). Different alphabets on error bars indicate significant difference ($p<0.05$)

## Determination of protein solubility of six different seaweeds by pH shifting method

The protein solubility was found to be more at the solid-to-solvent ratio of 1:30 at pH 9 for the seaweed *E. compressa*. This was considered as optimum. The same extraction conditions were applied on other brown, red and green seaweeds to find out the protein solubility. Among the seaweeds, the highest protein solubility was exhibited by the red seaweed *H. valentiae* (22.23±1.60 mg g$^{-1}$ *i.e.,* 19.68%) on dry weight basis followed by *G. edulis* (18.79±0.64 mg g$^{-1}$ *i.e.*, 40.32%) and *K. alvarezii* (12.23±1.99 mg g$^{-1}$ *i.e.,* 30.88%) (Fig. 5a and b). The green seaweeds showed protein solubility varying from 13.90± 0.64 mg g$^{-1}$ *i.e.*, 45.57% (*H. gracilis*) and 19.59±2.49 mg g$^{-1}$ *i.e.*, 19.90% (*E. compressa*) whereas the brown seaweed *T. conoides* showed protein solubility of 15.89±1.03 mg g$^{-1}$ (27.39%). Significant differences ($p<0.05$) in protein solubility were observed between different species. The protein extractability depends upon the type of species, method of handling (agitation, filtering), method of extraction (pH shifting method, solvent extraction), method of protein assay (Kjeldhal, Lowry, Bradford), process variables (time, temperature, pH, solid-to-solvent ratio) and presence of other chemical constituents like nitrogenous and phenolic compounds. The abundance and nature of phenol compounds vary largely in seaweeds which has been found to affect the protein solubility (Sabeena and Jacobsen, 2013). The cell wall composition and structural organisation of chemical constituents, differ from species to species, which also greatly



(a) (b)

Fig. 5. Protein solubility (a) in mg g$^{-1}$ and (b) in % of different seaweed species by pH shifting method. Results are presented as Mean±SD (n=3). Different alphabets on error bars indicate significant difference ($p<0.05$)

influence the extractability of protein from seaweeds (Joubert and Fleurence, 2008). Our results indicate that, a combination of pH and temperature treatment results in extraction of 20-45% of seaweed proteins.

The proximate compositional analysis of six seaweed species (*T. conoides*, *K. alvarezii, G. edulis, H. Valentiae*, *H. gracilis* and *E. compressa*) from the south-east coast of India clearly indicates that they can serve as potential sources of dietary ingredients with high nutritional value. In order to develop extraction process for seaweed proteins, we have studied the process variables influencing the solubilisation of protein from *E. compressa* such as solvent-to-solid ratio, duration, temperature as well as pH and applied the optimum conditions on five other seaweeds of commercial interest. The protein solubilisations varied in the range of 19.68-45.57% (protein to protein) for different species. Study indicated that further improvement is necessary for maximising the protein solubility as the optimum conditions employed resulted in incomplete solubilisation of proteins. Extraction of seaweed proteins is highly challenging and demands deep knowledge of process variables and processing aids.

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