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Combining pressing and alkaline extraction to increase protein yield from *Ulva fenestrata* biomass

L. Juul[a,b,1], S. Steinhagen[f,2], A. Bruhn[b,d,3], S.K. Jensen[b,e,4], I. Undeland[g,5], T.K. Dalsgaard[a,b,c,*,6]

[a] Department of Food Science, Faculty of Technology, Aarhus University, Agro Food Park 48, 8200 Aarhus N, Denmark
[b] CBIO, Centre for Circular Bioeconomy, Aarhus University, Aarhus, Denmark
[c] CiFOOD, Centre for Innovative Food Research, Aarhus University, Aarhus, Denmark
[d] Department of Bioscience, Faculty of Technology, Aarhus University, Vejlsøvej 25, 8600 Silkeborg, Denmark
[e] Department of Animal Science, Faculty of Technology, Aarhus University, Blichers Allé 20, 8830 Tjele, Denmark
[f] Department of Marine Sciences, Tjärnö, University of Gothenburg, SE-452 96 Strömstad, Sweden
[g] Department of Biology and Biological Engineering, Food and Nutrition Science, Chalmers University of Technology, SE-41296 Gothenburg, Sweden

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ABSTRACT

Many seaweed species have a high production potential and attract interest as future protein sources. A high fiber and ash content, however, demand extraction of the protein to improve its digestibility and protein utilization in food or feed. This study explores three different approaches for protein extraction from *Ulva fenestrata* in order to maximize the protein extraction yield. Soluble protein was recovered either by mechanical pressing or by homogenization and osmotic shock of the biomass followed by alkaline extraction. The soluble protein was then concentrated by isoelectric precipitation. A combined procedure was carried out by pressing the biomass and following subjecting the residual pulp fraction to homogenization, osmotic shock and alkaline extraction. The three methods were ranked as follows with respect to protein extraction yield (as % of biomass protein); the combined method (23.9 ± 0.3%) > the alkaline extraction (6.8 ± 0.2%) > mechanical pressing (5.0 ± 0.2%). The significant increase when combining the methods was ascribed to a high precipitation yield after alkaline extraction of the pulp, hypothesized to be due to a reduced conductivity of the alkali-soluble protein fraction when derived from pulp rather than whole biomass.

1. Introduction

There is an increasing need for finding alternative and sustainable protein sources with reduced negative environmental impact in order to meet the increased demand for food and feed protein (Godfray et al., 2010). The green seaweed *Ulva* is explored as one of these potential protein sources, due to the high productivity, ability to provide ecosystem services and high proportion of essential amino acids

* Correspondence to: Department of Food Science, Aarhus University, Agro Food Park 48, DK-8200 Aarhus N, Denmark.
E-mail address: trine.dalsgaard@food.au.dk (T.K. Dalsgaard).
[1] https://orcid.org/0000–0002-2719–1447
[2] https://orcid.org/0000–0001-8410–9932
[3] https://orcid.org/0000–0002-7940–1338
[4] https://org/0000–0001-9852–4774
[5] https://orcid.org/0000–0002-9732–3644
[6] https://orcid.org/0000–0002-5635–4102

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(36–42% of total amino acids) (Fleurence, 1999, Wong and Cheung, 2001, Elizondo-González et al., 2018, Neveux et al., 2018, Magnusson et al., 2019). To utilize this potential protein source, extraction of the protein is needed in order to reduce the high content of fibers and phenolic compounds, as it hampers the protein digestibility (Horie et al., 1995, Wong and Cheung, 2001, Bikker et al., 2016, Trigo et al., 2021). Protein extraction from *Ulva* spp. has shown varying protein yields, with some of the most promising methods including alkaline extraction followed by isoelectric or ammonium sulfate-induced precipitations, recovering up to 36% of the initial protein in the biomass (Wong and Cheung, 2001, Harrysson et al., 2019). However, higher protein yields would be preferred.

This study aimed to compare three extraction methods to maximize protein extraction yield from *Ulva fenestrata* Postels & Ruprecht 1840. The methods comprised pressing and/or alkaline extraction followed by acid precipitation.

2. Materials and methods

2.1. Chemicals

NaOH, HCl 37%, sodium dodecyl sulfate (SDS), Folin-Ciocalteu phenol (FC) reagent, bovine serum albumin (BSA), Na_2CO_3, and Na-K-tartrate were purchased from Sigma-Aldrich (Germany). $CuSO_4 \cdot 5H_2O$ was from Fluka (Switzerland) and ethylenediamine-tetra-acetic acid (EDTA) from Merck (Germany).

2.2. Raw material

The biomass, *Ulva fenestrata*, was provided by Tjärnö Marine Laboratory, harvested from a long-term indoor tank culture November 1st, 2019. The biomass was grown in cultivation tanks of 90 L volume under permanent aeration with a flow-through system with filtered seawater (5 µm filter and UV filter) (flow = 10–14 L h^{-1}), thus salinity and temperature fluctuated as a result of local weather (58°52'36.4" N, 11°6'42.84" E) and seasonal conditions. The biomass was grown with a light:dark cycle of 16:8 h at an irradiance of 140 µmol m^{-2} s^{-1} and light source INDY66 LED 60 W 4000 K 6000 lumen. No additional medium or chemicals were added to the water. Molecular identification details of the biomass can be found in Toth et al. (2020) (GenBank accession numbers: MN240309-MN240311).

2.3. Biomass pre-treatment

Upon harvest, the biomass was stored at –80 °C. To secure homogenous biomass batches, the frozen biomass was roughly chopped with a knife and mixed before being divided and allocated to the different extraction methods. Biomass for alkaline extraction was further grinded in a semi-thawed state with a Titracarne grinder C/E22N (Minerva Omega, Italy) with a 4.5 mm hole plate and stored at –80 °C. Prior to protein extraction, biomass was thawed in a bag under cold running water. No liquid run-off from the biomass was visible upon thawing.

2.4. Protein extraction techniques

Two different methods and a combination hereof was used for protein extraction (Fig. 1). The first method included mechanical pressing, resulting in two juices with soluble protein and a pulp residue. Juices were subjected to centrifugation and resulting soluble proteins of the supernatants were acid precipitated. The second extraction technique followed the so called pH-shift method suggested by Harrysson et al. (2019), using osmotic shock and alkaline extraction to solubilize protein from the biomass followed by removal of non-soluble matter and afterwards acid precipitation. The combined method was initiated by mechanical pressing followed by alkaline extraction of the pulp residue (pulp 2, Fig. 1) after pressing. The pressing and alkaline extracted fraction were separately subjected to centrifugation and acid precipitation. In all three methods, the acid precipitation was performed by adding 1 M HCl to

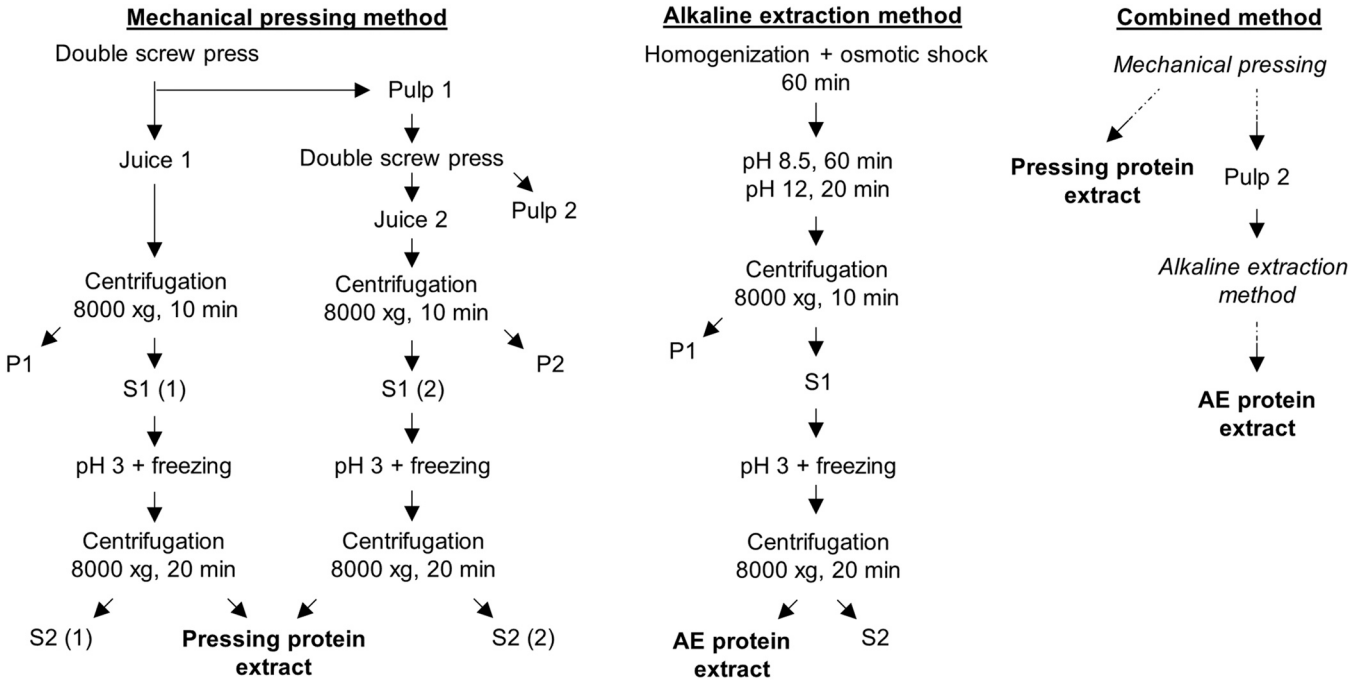

Fig. 1 – Flow chart of the protein extractions performed in this study. S: Supernatant, P: Pellet, AE: Alkaline extraction.

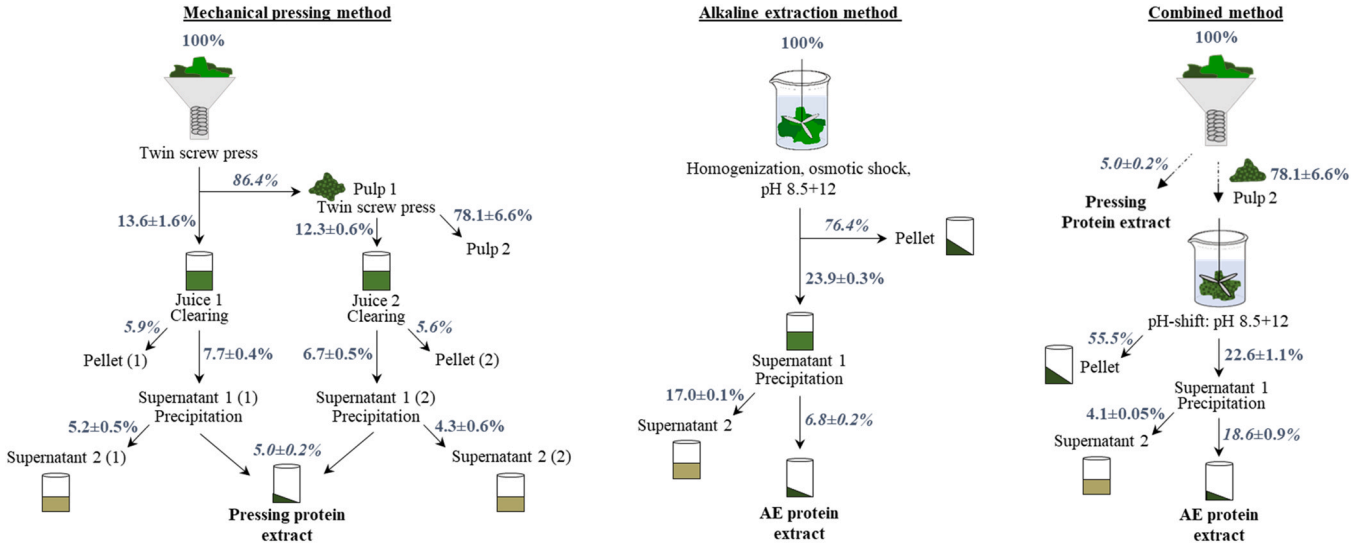

Fig. 2 – The protein mass balance of the three extractions. The protein mass balance is shown in the dark blue numbers on the flow chart, starting with 100% of the protein in the crude biomass. The numbers in *italics* are by indirect measurement (protein determined by the Lowry method from the liquid fractions). Measures are shown as mean ± SD, n = 2. The mass balance does not add up to exactly 100% due to deviations of measurements.

supernatant 1 (S1) to reach pH 3 whereupon the samples were frozen immediately at –20 °C overnight to increase precipitation. pH 3 was experimentally tested to be the highest yielding pH for protein precipitation (Juul et al. 2021). Acidified supernatants were centrifuged at 8000 x g, 20 min, 8 °C, after being thawed in a bag in cold water. Extractions were performed at 8 °C and in duplicates.

2.4.1. *Mechanical pressing*

The mechanical pressing was performed at the native pH of the seaweed; juice pH 4.6–4.8. Using a double screw press (Angel Juicer 8500 S, Domotech, Denmark), the biomass was separated into a juice (juice 1) and a pulp residue. The pulp was mixed with deionized water (w/w 1:1) for 10 min and pressed a second time in the double screw press, resulting in a second juice fraction (juice 2, Fig. 1) and a final pulp residue. Each juice was centrifuged at 8000 x g, 10 min, 8 °C. The supernatants were separately acid precipitated as described in Section 2.4.

2.4.2. *Alkaline extraction*

The alkaline extraction method was performed following the protocol by Harrysson et al. (2019). Biomass and de-ionized water in a ratio of 1:6 was homogenized on ice with an L5M mixer (Silverson, United States) at 8000 rpm for 2 min. The resulting homogenate was incubated 60 min at 8 °C with stirring in order to perform osmotic shock of the biomass. After the osmotic shock, pH was adjusted to pH 8.5 with 1 M NaOH, stirred and incubated at 8 °C for 60 min, followed by a pH adjustment to pH 12 with 1 M NaOH and finally incubated 20 min at 8 °C upon stirring. Pre-experiments showed that pH 12 gave the highest solubilization yield (Juul et al. 2021), however, it could be further improved by including the incubation at pH 8.5 (Harrysson et al. 2019). After incubation, the homogenate was centrifuged at 8000 x g, 10 min, 8 °C. Acid precipitation was performed on the resulting S1 (Fig. 1) as described in Section 2.4.

2.4.3. *Combined extraction method*

Mechanical pressing was performed as described in Section 2.4.1. Thereafter, the residual pulp after the second press (pulp 2, Fig. 1) was subjected to the alkaline extraction method as described in Section 2.4.2, i.e. the pulp was homogenized, and subsequently subjected to osmotic shock and alkaline protein solubilization. In this procedure, however, the pulp was mixed with de-ionized water in a ratio of 1:10 (w/w) instead of 1:6 to adjust for liquid loss during pressing and secure similar dry matter (DM)-to-water ratio as in 2.4.2. Isoelectric precipitation according to Section 2.4 was performed on both S1 from the pressing and from the pH-shift step.

2.4.4. *Calculations of protein yield*

Samples were taken from the biomass, homogenates, juices and supernatants (S) for protein analysis. The following calculations (Eqs. 1–3) were made to determine the protein yield. [protein] is the protein concentration of the given matrix. For the combined method, the final yield was calculated by adding the yield from pressing with the yield from pH-shift on the pulp. The final protein yield (the amount of protein in final extracts as percentage of protein in the initial biomass) equals the soluble protein yield, i.e. the amount of protein in S1 (Fig. 1) as percentage of protein in the initial biomass, multiplied with the protein precipitation yield, which is the amount of protein precipitated as percentage of protein in S1.

Soluble protein yield

$$= 100 \times \frac{\text{S1}\quad[\text{protein}] \times \text{mass(S1)}}{(\text{biomass}[\text{protein}] \times \text{mass(biomass)})} \tag{1}$$

Protein precipitation yield

$$= 100 \times \left(1 - \frac{\text{S2}\quad[\text{protein}] \times \text{mass(S2)}}{\text{S1}\quad[\text{protein}] \times \text{mass(S1)}}\right) \tag{2}$$

Final protein yield

$$= \text{soluble protein yield} \times \text{protein precipitation yield} \tag{3}$$

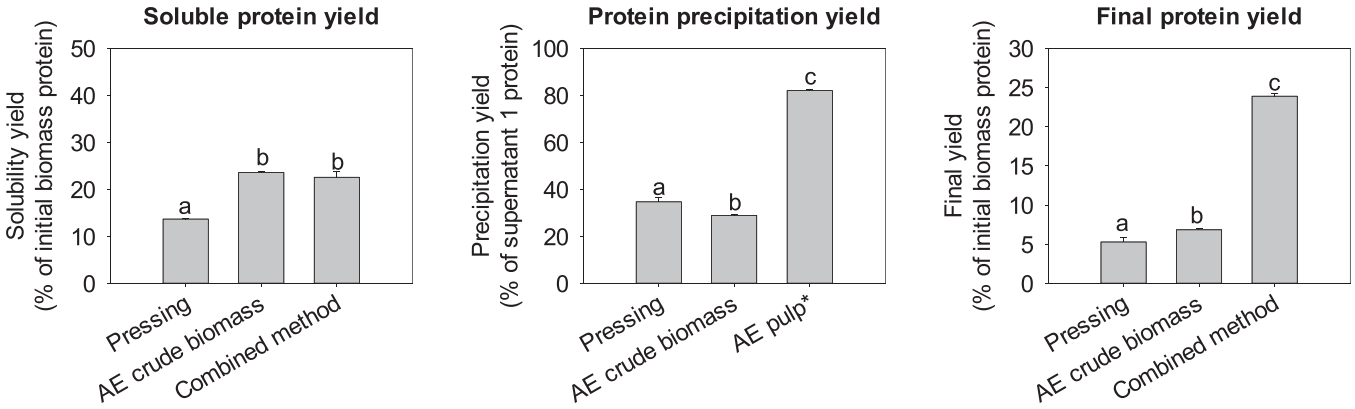

Fig. 3 – Protein yields as defined in Eqs. 1–3 in Section 2.4.4, showing the soluble protein yield (amount of protein in S1 as percentage of initial biomass protein), the protein precipitation yield (amount of protein precipitated into final extract as percentage of protein in S1), and the final protein yield (amount of protein in final extracts as % of initial biomass protein). *Results of the protein precipitation yield of the combined method is only shown for the alkaline extraction part of the combined method. For the first part of the combined method, the protein precipitation yield is the same as for the pressing method. Notations on bars indicate significance of difference between extraction methods ($p < 0.05$). Data are presented as mean ± standard deviation, n = 2.

2.5. *Dry matter content*

A known amount of fresh biomass was dried in an oven at 105 °C for 24 h. The dry matter (DM) was calculated as percentage of the initial fresh weight of the biomass.

2.6. *Protein content*

The protein content of the raw biomass and the pulp (mechanical pressing) was determined by combustion using a LECO Trumac nitrogen analyzer (TruMac N, Leco Corporation) with EDTA as standard, using a nitrogen-to-protein conversion factor of 5 (Angell et al. 2016). The protein content of the liquid samples was determined by a modified Lowry method (Markwell et al. 1978), diluting samples 20–100 times in 0.1 M NaOH. One mL of the diluted samples was mixed with 3 mL of freshly made Lowry reagent and incubated 30 min at room temperature (RT). Lowry reagent consisted of 100 parts mixed 2.0% Na_2CO_3, 0.40% NaOH, 0.16% Na-K-tartrate, and 1% SDS mixed with 1 part 4% $CuSO_4 \cdot 5H_2O$. After the first incubation, 300 µL freshly made phenol reagent (1 part 2 N FC reagent, 1 part distilled water) was added to the sample and incubated at RT in darkness for 45 min. Absorbance was measured at 750 nm with a Cary 60 UV-Vis spectrophotometer (Agilent technologies). A standard curve with bovine serum albumin (BSA) was used for quantification. All protein determinations were carried out in triplicates.

2.7. *Conductivity measurements*

Conductivity was measured at 8 °C on liquid fractions with a conductivity-meter (CDM210, MeterLab). NaCl was used to make a standard curve to be able to convert conductivity to NaCl equivalents.

2.8. *Statistics*

The software R, version 4.0.3 (R Core Team, 2020) was used for statistical analysis. Generalized linear models (McCullagh and Nelder, 1989) with an identity link function were used to study the soluble protein yield of the different approaches, using the Gaussian distribution for modelling. For the precipitation yield and final protein yield, a gamma distribution with a logarithmic link function was used for modelling, as this data was not normally distributed. Model adequacy was tested by residual analysis and post hoc analyses were made with the R package postHoc (Labouriau, 2020). Multiple testing was adjusted for by the method of controlling the false discovery rate (Benjamini and Hochberg, 1995). $p = 0.05$ was used as significance level.

3. Results and discussion

The DM content of the *Ulva fenestrata* biomass was 15.8 ± 0.5% and the protein content was 18.0 ± 0.7% of DM, corresponding to 2.8% of the fresh weight (FW). By mechanical pressing, 25.9 ± 1.1% of the protein from the crude biomass was pressed into the juices, leaving 78.1 ± 6.6% of the crude biomass protein in the residue pulp (pulp 2) (Fig. 2). After centrifugation of the juice to get rid of insoluble fibers, the mechanical pressing resulted in a soluble protein yield of 13.7 ± 0.2%, which was significantly lower than that obtained for the alkaline extraction; 23.9 ± 0.3% (Fig. 3). Due to the high protein content left in the pulp after mechanical pressing, further protein extraction was performed from the pulp using the alkaline extraction (combined method). This resulted in a soluble protein yield of 22.6 ± 1.1% of the initial biomass protein. This value is comparable with the protein solubilization yield from alkaline extraction of the crude biomass, even though, for the combined method, ¼ of the biomass protein was already pressed out prior to the alkaline processing. Performing the pressing before alkaline extraction might make the cells more receptive for alkaline protein extraction, possibly breaking cell walls during the double screw pressing. Further, freezing of the pulp before the pH-shift extraction, which was done for practical matters, might also have induced cell bursting. However, when the crude biomass was subjected to the alkaline extraction, the biomass was grinded, homogenized and underwent osmotic shock, which most likely also induced cell rupture.

Regarding the high pH used for the alkaline extraction, it can induce aminoacyl cross-links such as lysinoalanine and lanthionine, which is elaborated on and explored for the same biomass in the paper by Juul et al. (2021). Even though the extreme alkaline pH did induce cross-linking, it did not seem to affect the protein digestibility tested in vitro and further the solubility of the dried protein extract obtained from alkaline extraction method showed better than for the protein extract obtained by the pressing method (Juul et al., 2021).

The protein precipitation yield was 34.7 ± 1.8% for the pressing extraction and 29.0 ± 0.5% for the alkaline extraction of the crude biomass. The final protein yield was 5.0 ± 0.2% and 6.8 ± 0.2% for the pressing extraction and the alkaline extraction of the crude biomass, respectively. For the combined method, 82.0 ± 0.2% of the soluble protein from the pulp was precipitated, resulting in a significantly ($p < 0.001$) higher final protein yield of 23.9 ± 0.3% (yield from pressing followed by acid precipitation (5.0%) plus yield from alkaline processing of pulp followed by acid precipitation (18.6%)) compared to the single methods (Fig. 3). Thus, the high precipitation yield during the alkaline processing of the pulp was crucial for the large increase in final protein yield of the combined method. Usually, relatively low isoelectric precipitation yields (< 50%) are observed for seaweed protein (Abdollahi et al., 2019, Harrysson et al., 2019, Juul et al., 2020, Naseri et al., 2020), as was also observed for the single methods in this study. This could be due to the relatively high salt content of the seaweed, which might increase the solubility of the proteins even at low pH due to their strong interactions with e.g. Cl^- ions (Schein, 1990, Maurer et al., 2011). This "salting-in" phenomena would further explain the lower precipitation yield of the single methods compared to the combined method. The S1 from the first press had a conductivity of 37.75 ± 0.07 mS cm^{-1} and S1 from alkaline extraction of crude biomass had a conductivity of 6.62 ± 0.31 mS cm^{-1}, corresponding to ~0.67 M and ~0.11 M NaCl equivalents, respectively. The conductivity of S1 from alkaline extraction of pulp was significantly lower, 0.38 ± 0.05 mS cm^{-1} (i.e. ~0.002 M NaCl equivalents). The low conductivity of S1 from alkaline extraction of pulp in the combined method was due to 50–60% of the salt being pressed into the juices, resulting in a pulp residue with reduced salt content, avoiding the "salting in"-effect in the combined method's alkaline extraction. Hence, a reduction in ionic strength seemed to enhance the *Ulva* protein precipitation yield. This was further supported by a preliminary trial (single replicate) in which S1 from the alkaline extraction was subjected to dialysis for 2.5 h and 18 h. When the conductivity was reduced to 0.031 M and 0.005 M NaCl equivalents (initial conductivity being 0.140 M NaCl equivalents), the protein precipitation was increased from ~14% to ~42% and ~64%, respectively.

In literature, some of the highest protein extraction yields from *Ulva* sp. were found by performing alkaline extraction followed by isoelectric precipitation. E.g. Harrysson et al. (2019) obtained a protein yield of 29%, using a pH-shift protocol identical to the alkaline process and acid precipitation used in this study where only a final protein yield of 6.8% was reached. The study by Harrysson et al. (2019) was performed on the same species (Toth et al., 2020) and cultivated in the same way, with the only difference being that the biomass was dried and grinded prior to extraction. The drying per se is however not expected to be a main reason for the large difference in yield. Wijers et al. (2020) showed that drying of *Ulva lactuca* biomass prior to protein extraction rather decreased the protein yield, as compared to extracting from fresh or frozen biomass. It is possible though that the larger particle size reached in our study when grinding wet *Ulva* biomass (here with a 4.5 mm hole plate) compared to when grinding dried *Ulva* biomass (Harrysson et al., 2019), reduced its interaction with water and thereby the protein solubilization. The biomass protein content also differed between the two studies; here 18.0% vs 12.8% in Harrysson et al. (2019) which could be due seasonal variation and/or a different maturation stage of the biomass. Regarding the latter, it has for instance been observed that the expression of the photosynthetic protein Rubisco differed between sporophytes and gametophytes in certain red and brown seaweed species. It can further not be excluded that the fact that biomass protein was measured based on N-analyses in the current study, while Harrysson et al. (2019) measured it by the Lowry-principle, detecting peptide bonds of solubilized proteins, affected protein data and thereby yield calculations. Incomplete protein solubilization of biomass during the Lowry-assay may have resulted in underestimations of total proteins, translating into higher calculated protein yields, whereas a too high protein-to-conversion factor in this study might influence the yield negatively.

4. Conclusion

The study showed that protein yield can be increased by performing consecutive extractions, combining extraction approaches. The final protein yield of the combined method (23.9 ± 0.3%) was higher compared to when adding the final yields of the single methods together; i.e., 11.8% (5.0% + 6.8%). Hence, combining the methods had an extra effect as opposed to just being a "double-extraction", which was hypothesized to be due to the significantly higher precipitation yield during the alkaline processing of pulp, as a result of highly reduced conductivity of the S1 fraction. This gives an indication that future seaweed biorefinery systems with proteins as one of the output products could benefit from salt extraction prior to protein extraction. This could possibly increase the yield if proteins are to be concentrated by isoelectric precipitation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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