Algal Research 78 (2024) 103406

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Microwave hydrolysis, as a sustainable approach in the processing of seaweed for protein and nanocellulose management

Anna Trubetskaya [a,b,*], Huy Quang Lê [c], Jenni Leppiniemi [d], Tetyana Koso [e,f], Teemu Välisalmi [c], Markus B. Linder [c], Italo Pisano [b], Jinze Dou [c], J.J. Leahy [b], Eero Kontturi [c,**]

[a] Department of Biosciences and Aquaculture, Nord University, Norway
[b] Department of Chemical Sciences, University of Limerick, Ireland
[c] Department of Bioproducts and Biosystems, Aalto University, Finland
[d] Department of Medicine, Tampere University, Finland
[e] Department of Functional Cellulose, VTT Technical Research Center of Finland, Finland
[f] Materials Chemistry Division, Department of Chemistry, University of Helsinki, Finland

ARTICLE INFO

Keywords:
Microwave hydrolysis
Cellulose nanocrystals
Protein
Seaweed
Alginate

ABSTRACT

The nature of marine biomass is very complex for a material scientist due to the large seasonal variation in the chemical composition that makes it difficult to prepare standardized products. A systematic investigation of the interaction of microwave irradiation with seaweed from Norway and Caribbean region was performed, covering a broad temperature range (130 → 170 °C) and without and with addition of γ-valerolactone (GVL) in ratios of 1:4 and 1:2. The temperatures above 150 °C and without addition of GVL led to the closure of mass balances up to 90 % that includes polysaccharides, "pseudo-lignin" fraction, fatty acids, and proteins. Fucoidan and mannose represented >50 % of all detected polysaccharides in *ascophyllum nodosum (AN)*, while *aegagropila linnaei (AL)* contained mostly glucose. The presence of arabinose and rhamnose in the upper surface of the cell wall hinders the glucose release during microwave treatment. The differences in the polysaccharide composition among both algae samples hindered the definition of a parameters set that can be used in microwave treatment of various seaweed species. A large fraction of protein (> 95 %) remained in the seaweed solid residue. Higher amount of protein was determined in *AL*, which was dominated by leucine and lysine. Another potential barrier to the application of seaweed in industry is the limited knowledge on the chemical composition of "pseudo-lignin" and extractives. The total amino acid analysis was identified as the most accurate to characterize the protein yield and composition. The results showed that microwave treatment of seaweed is indeed a viable method for producing bioactives in the temperature range 120–150 °C, and proteins and nanocellulose at temperatures above 170 °C without using GVL. The microwave temperature and seaweed type played a dominating role in the mass closure balances leading to >95 % identified compound.

1. Introduction

Environmental concerns and the need for sustainable alternatives to traditionally used non-renewable materials are rising globally as consumers, governments, and international organizations demand cleaner and more environmentally friendly products and processes [1]. Arable land area is used to a much greater degree globally than the seas [2]. Therefore, it is suggested that cultivated seaweed could be one of the solutions in meeting the consumption demands of a growing population and solving environmental issues like deforestation and freshwater shortage [3]. Currently, seaweed is harvested mainly for its extractives alginate and agar, which are used as thickening agents in the chemical and food industry [4]. Other uses include nanocellulose manufacturing, biofuel production, medicinal uses, and animal feed, although marginal compared to the production of agar and alginate [5]. Seaweed is also considered as a suitable source of protein that has promising bioactive and functional properties [6]. Proteins derived from plants are generally harder to digest than animal proteins, which is attributable to the high concentration of insoluble polysaccharides. Growing concern about consumption of foods of animal origin that contains high levels of

* Correspondence to: A. Trubetskaya, Department of Biosciences and Aquaculture, Nord University, Norway.
** Corresponding author.
E-mail addresses: anna.trubetskaya@nord.no (A. Trubetskaya), eero.kontturi@aalto.fi (E. Kontturi).

https://doi.org/10.1016/j.algal.2024.103406
Received 28 August 2023; Received in revised form 11 January 2024; Accepted 20 January 2024
Available online 22 January 2024

saturated fats and cholesterol add value to seaweed-based proteins in human and animal nutrition [7] [8].

Differences in carbohydrate, protein, and lipid contents between and within seaweed species were observed between seasons and thus, variation in nutrients supply can be a potential barrier to the application of seaweed in industry [9] [10]. Seaweed cell wall composition differences and variety of methods for the protein extraction may also cause challenges in identification of amino acids after hydrolysis and further process scale up [11]. Purified, acid and base treated algal cellulose has been used in a number of applications from biomedicals to energy storage [12], whereas the enzymatically hydrolysed proteins have been used in animal and fish feeding sectors [13] [14] [15] [7]. Hydrolysis aims to break a seaweed structure into various compounds, whereas amino acids are released through breakage of peptide bonds within a protein [16]. Proteins are inclined to chemical modifications, and various efforts have been established to minimize unwanted reactions in hydrolysis [17]. The operating conditions of the hydrolysis treatment must be optimized to prevent the fractionation process from formation of contaminants and undesired low value products via unwanted reactions [18]. Currently, the multi-stage acid pre-treated depolymerization and enzymatic processes for the seaweed fractionation to nanocellulose and protein have limitations, concurrently having a strong environmental impact due to the low recyclability of the acid. Another challenge in the biomass fractionation is the lack of a low energy intensive and sustainable approach with the overall reduced use of chemicals and raw material, but with great product selectivity within minimized operational time [19]. Recently, a one-stage, low-energy microwave process for the depolymerization of lignocellulose which provides high sugar yields with minimum energy inputs was reported [20] [21]. The main advantages of the microwave treatment over the conventional hydrolysis are short conversion time, uniformity, and selective heating over the entire material. It was shown that each of the components of macroalgae has its own temperature of activation and a certain time for transition to a water-soluble form convenient for isolation [22]. The previous research has demonstrated that low temperature (pressure) acid-free microwave hydrolysis of cellulose is an effective tool for a highly selective fractionation of polysaccharides and proteins from biomass matrix [23]. The optimal conditions for the conversion of citrus and herbaceous feedstocks can be achieved at temperatures below 180 °C [24]. Thus, a broad range of parameters (temperature/pressure, power/power density, sample to water ratio, solvent addition) must be optimized to achieve a high yield of nanocellulose by preventing the process from formation of contaminants and undesired low value non-cellulosic products.

The objective of this study is to implement the microwave-assisted hydrolysis using γ-valerolactone (GVL) as a sustainable solvent and reviewing existing microwave operating conditions by varying the operating temperature and using two types of seaweed such as the *aegagropila linnaei, AL* (marimo moss ball) and *ascophyllum nodosum, AN* (brown algae). AL and AN were selected because the cellulose isolation from red algae is already an established procedure, less so for green and brown algae [25]. The GVL has been extensively used in separation of lignin from biomass matrix including proteins and starch, and thus, it was selected as a green solvent for nanocellulose fractionation [26]. Another objective is to obtain unprecedented visual data on the composition of green and brown seaweed fractions after microwave treatment to close the process mass balances. We aim in this study to analyze the value-added products after microwave treatment using various methods to identify new commercialization routes.

2. Materials and methods

2.1. Chemicals and materials

Ascophyllum nodosum (AN) and *Aegagropila linnaei* (AL) were collected in winter 2021, then cut into smaller pieces with scissors, and dried at room temperature for > 4 days. Then, samples were comminuted using a 20-pass mesh Wiley Mini Mill 475-A mill (Thomas Scientific, US) with an operational speed of 1750 rpm. The GVL was supplied by Sigma Aldrich with ≥98 wt% purity. Pure water was obtained from Millipore Synergy® UV purification system (water resistivity of 18.2 MΩ cm).

2.2. Measurement uncertainty

The error bars represent standard deviation from the mean of the series of experiments at each condition in the microwave reactor. The yield of each fraction (See Fig. 1) in solid matrix and liquor for each condition was plotted as a representative average of at least three experiments. The absolute extended uncertainty of the product yields was determined by Gaussian error propagation procedure [27]. The average standard measurement error in the present study was ±5 wt%, within a 90 % confidence interval for the microwave hydrolysis experiments. The inaccuracy in determining the product yields was mainly caused by weighting and water filling errors in a quartz tube of the microwave reactor, the temperature and heating rate effect of the reactor's controller during hydrolysis. The protein yields obtained from the amino acids analysis showed the higher average standard measurement error of ±10 wt% due to the material loss in HCl hydrolysis and extraction.

2.3. Raw seaweed analysis

Proximate analysis was performed for both seaweed samples, following the ASTM standard E1755–01. Ultimate analysis results (CHNS) were acquired using a Thermo Flash Smart instrument (Thermo Fisher Scientific, UK), following the procedure in ASTM D5373–02. The oxygen content was calculated by the difference according to previous study [28]. The higher heating value (HHV) was determined by a bomb calorimeter (IKA C-200, Germany) following ASTM D2015–95. Ash compositional analysis was performed by inductively coupled plasma-optical emission spectrometry (ICP-OES) with prior microwave digestion according to ASTM D6349–13 [29]. The Cl content in the ash was determined by inductively coupled plasma-optical emission spectrometry/ion chromatography (ICP-OES/IC) at ASG Analytics (Neusäß, Germany).

2.4. Microwave hydrolysis

Hydrolysis experiments were conducted in 30 mL vials heated in a microwave reactor (Anton Paar Monowave 300, Austria). For each trial, 1.5 g of AN algae (2.78 g, moist) and AL algae (1.287 g, dry) were used, with a liquid-to-wood ratio (1:2; 1:4) after 20 mL of pure water was added to all samples. The reaction mixture was rapidly heated (<2 min) to the maximum temperature (130, 150 or 170 °C), and held at such temperature for 120 min, and then firstly cooled down by compressed air to 70 °C and then to room temperature in a water bath. The solid residue and the spent liquor were separated by filtration with a Robu® glass crucible (porosity 4). The solid residue was washed with 200 mL water and then dried for 12 h under fume hood. The solid residue yield was determined gravimetrically. The spent liquor and washing water were collected and stored at −20 °C for subsequent analyses.

2.5. Lignocellulosic analysis

The carbohydrate and lignin contents in raw seaweed and samples after microwave treatment were analyzed in accordance with the 2-step hydrolysis method [30]. The pulps were firstly hydrolyzed in 72 % H_2SO_4, with an acid-to-material ratio of 10 mL g^{-1}, at 30 ± 3 °C, for 60 ± 5 min [31]. The hydrolyzed suspension was subjected to the second hydrolysis in 4 % H_2SO_4, with an acid-to-material ratio of 300 mL g^{-1} at 121 ± 1 °C, for 60 min and additional 60 min for heating and cooling of an autoclave (Systec DE23, Germany). The monosaccharides (i.e.,

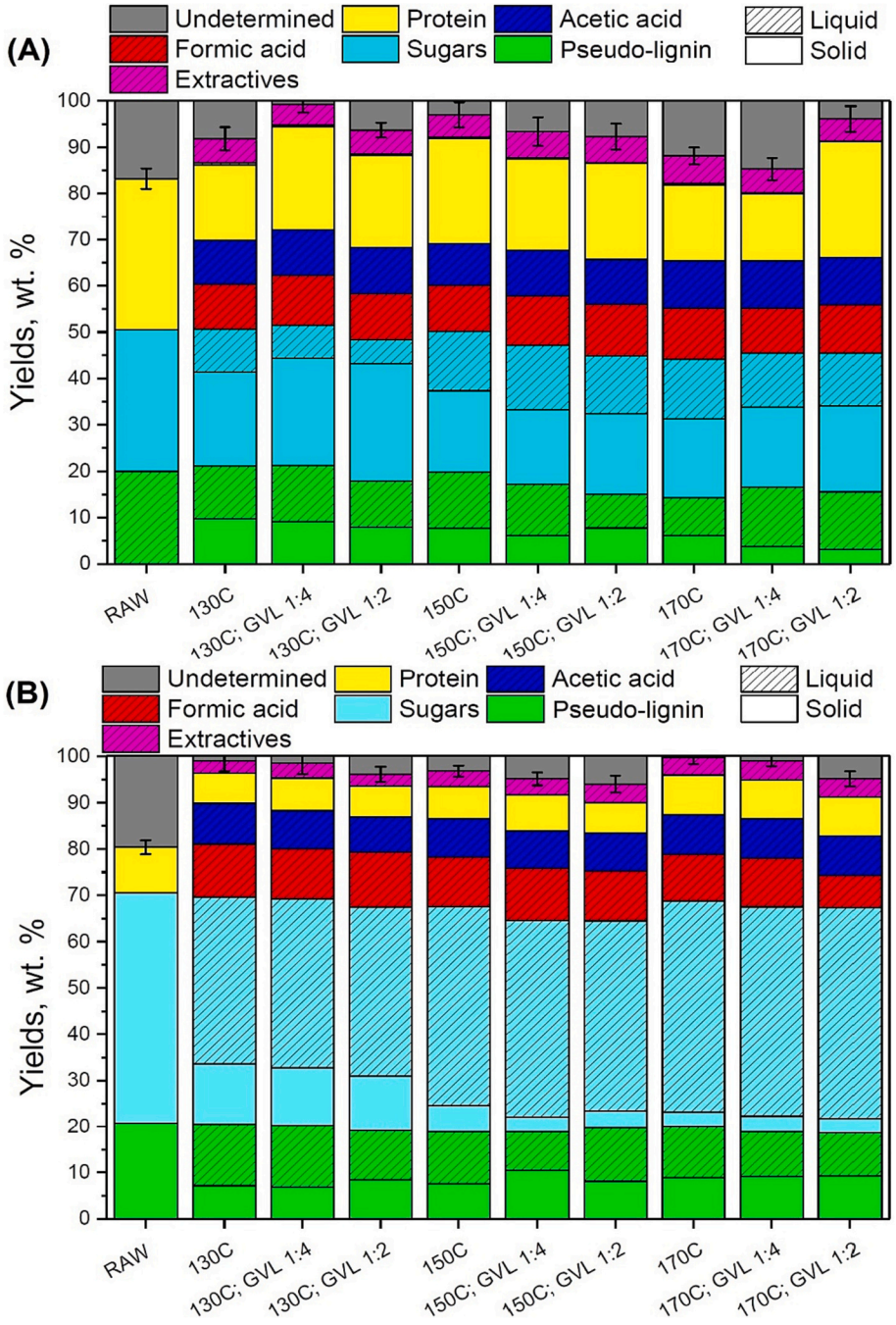

Fig. 1. Mass balance that was calculated per dry weight of the original seaweed of microwave treatment at temperatures of 130, 150, and 170 °C without GVL or with GVL (1:4; 1:2) (A) AL, and (B) AN. The protein content in the solid residue is measured using an elemental analysis, whereas protein in liquors was determined using amino acid analysis with HPLC.

glucose, xylose, mannose, arabinose, rhamnose, and galactose) were analyzed by high performance anion exchange chromatography (HPAEC-PAD, Dionex ICS-3000, Thermofisher, USA), equipped with a CarboPac PA20 column. Pure water with flow rate of 0.38 mL min^{-1} was used as the mobile phase at room temperature. From the amounts of neutral monosaccharides, the cellulose and hemicelluloses content in wood and solid residue samples was estimated with the Janson formula [32].

High-performance Liquid Chromatography (HPLC, Dionex Ultimate 3000, Thermofisher, USA) was used to determine dehydration products (HMF and furfural) and acids (acetic acid and butyric acid) using ultraviolet (UV) detector at 280 nm and 210 nm, respectively. The column used was Phenomenex Rezex ROA-Organic Acid H^+ (8 μm, 300 × 7.8 mm, Thermo Scientific, USA) and 0.0025 M H_2SO_4 was used as the eluent at a flow rate of 0.5 mL min^{-1} at 55 °C. The injection volume was 10 μL and the total run time was 60 min [33].

Acid insoluble (Klason) lignin was determined gravimetrically while acid soluble lignin (ASL) was determined by measuring the absorbance at the wavelength of 205 nm (Spectrophotometer Shimadzu UV-2550, Japan). An extinction coefficient of 110 L (g cm)$^{-1}$ was used for quantification of ASL in accordance with the previous research [34].

2.6. Protein analysis

The protein content of liquid liquor samples was determined using various methods (Bradford, elemental, and total amino acid analysis).

Coomassie (Bradford) protein assay kit (Thermo Scientific, USA) was prepared using microplate protocol on 96 well plates. A dilution series (0–250 μg mL^{-1}) of bovine serum albumin (BSA) was prepared (*See supplemental material, Table S-1*) using BSA stock (2 mg mL^{-1}) and MilliQ water for dilution. Then, 35 μL of BSA each BSA standard or each unknown sample was pipetted per well on 96 well plate. Three parallel samples (a, b and c) were studied. Then 200 μL of the Coomassie reagent was added per well and plate was mix on a plate shaker for 30 s at RT. Plate was incubated for 10 min at RT and then the absorbance at 595 nm was measured using Envision plate reader. Then an analytical curve was prepared by plotting the average blank corrected 595 nm measurement for each BSA standard vs. its concentration in μg/mL and the polynomial trendline was fitted that was used to determine the protein concentration (*See supplemental material Tables S-1, S-2, and S-3*).

In the second method, prior analysis, lipids were removed from microwave-treated liquor by extraction [35]. 5 mL of 1:1 volume mixture of chloroform and methanol were added to 1 mL of seaweed liquor sample in the presence of 10 μL of L-norleucine (Sigma-Aldrich, Finland) as an internal standard and then vortexed vigorously. The mixture was incubated in ice for 1 h and then centrifuged at 3600 rcf for 10 min. The upper layer was collected and placed in a glass dish to evaporate at 35 °C overnight on the heating block. The evaporated samples were weighed, mixed with 6 M HCl containing 0.1 v/v % phenol, flushed with nitrogen and hydrolysed at 110 °C for 24 h under nitrogen atmosphere. The HCl was evaporated under vacuum at 30 °C and the remaining solids were dissolved in sodium citrate buffer (pH 3.45). The mixture was filtered with 0.2 μm GHP filters to remove leftover particles. The total amino acid content was determined with S433 Amino Acid Analyzer (Sykam GmbH, Germany). Elution was done with a gradient of sodium citrate buffer A (pH 3.45) and B (pH 10.85). Eluted amino acids react with ninhydrin and the derivates were detected with 570 and 440 nm UV detectors. Internal standard norleucine was used to quantify the results.

In the third method, the nitrogen content of microwave-hydrolysed liquor samples was measured with an elemental analyzer (see Section 2.3). Prior to the elemental analysis, the microwave-treated liquor was extracted using chloroform-methanol procedure (see above). This value was converted to the protein content using a literature conversion factor of 4.78 [36].

2.7. Gas chromatography – mass spectroscopy (GC-MS)

The seaweed liquor samples were extracted with chloroform-methanol mixture as discussed above (see Section 2.6). In addition, dichloromethane (DCM) - hexene extraction was performed to evaluate the impact of extraction on the extractives yields. Prior to GCMS analysis, 1 ml of seaweed extracted samples were solubilized in 500 μL pyridine with tetracosane as the internal standard (0.1 mg mL^{-1}) [37]. *N*,*O*-Bis(trimethylsilyl) trifluoroacetamide (300 μL) was then added into the vial with the mixture that was wrapped with the alumina folie and kept at room temperature for 12 h. Samples were filtered using 0.2 μm GHP filter and analyzed using Shimadzu GCMS-QP 2010SE with EI ionization, the exact column information and temperature program are described previously [38].

3. Results and discussion

3.1. Raw feedstock characterization

The ultimate, proximate and ash compositional analyses of AL and AN algae samples were carried out and the results are shown in Table 1.

Seaweeds are known to have a higher ash content than terrestrial plants due to their excellent absorption and accumulation properties [39]. The macro minerals (K, Na, Ca, Mg, P, S, Cl) content was significantly higher in AN (482,000 mg kg^{-1}) than in AL (258,000 mg kg^{-1}). This is mostly due to the higher Cl content in AN (230,000 mg kg^{-1}) than in AL (172,000 mg kg^{-1}). During the analysis chloride was detected as a part of NaCl that usually comprises a huge portion of seaweeds dry weight [40]. For both seaweed species, the sulphur content (4–9 wt%) is considerably higher than common land-based biomasses such as wood (< 0.2 wt%) or wheat straw (< 2 wt%) [41]. The sulphated polysaccharides, e.g., ulvan, fucoidan, form typically a part of seaweed cell walls, which represents chemically distinguishing characteristics to land-based biomasses [42]. The high sulphur content resulted in low gross calorific values (2.1–10.2 MJ kg^{-1}), which was additionally affected by the low carbon content and relatively high ash content in seaweeds. The nitrogen content in both seaweed samples (2–7 wt%) was higher than in terrestrial biomass (0.02–1.5 wt%) due to the high protein presence [43]. The AL samples showed a higher nitrogen content than AN due to nitrogen consumption from fertilizers, fish or plant decaying material while the AN grows on minerogenic substrate [44] [45]. The Ca, Mg and P contents in AN were higher than in previously examined samples from Norway and Denmark [39]. The trace mineral content (I, Cr, Co, Cu, Fe, Mn, Se, Zn) was higher in AL (30,600 mg kg^{-1}) than in AN (5500 mg kg^{-1}). The AL contained higher concentrations of Fe compared to previous investigations [46]. Algae are natural accumulators of minerals in their natural environment, but they also have the capacity to accumulate heavy metals. Heavy metals (As, Cd, Hg, Pb) were detected in both samples. The maximum levels of toxic metals (on dry weight) stipulated by EU countries including special requirements used in France and Spain for edible seaweeds are Pb < 5 mg kg^{-1}, As <5 mg kg^{-1}, Cd < 0.5 mg kg^{-1}, and Hg < 0.1 mg kg^{-1}. In the present study, the concentrations of Pb (14–36 mg kg^{-1}) and Hg (4–5 mg kg^{-1}) were

Table 1
Proximate, ultimate and ash compositional analysis of AL and AN algae samples.

Properties	*Aegagropila linnaei*	*Ascophyllum nodosum*
Proximate analysis		
Moisture, wt% (as received)	4.3	11.9
Ash, wt% (as received)	19.4	15
Volatile matter, wt% (on dry basis)	66.9	73
Fixed carbon, wt% (on dry basis)	13.7	12
Gross calorific value, MJ kg^{-1}	2.1	10.2
Ultimate analysis, % (on dry basis)		
Carbon	36.4	45
Hydrogen	5.7	6.3
Oxygen	27.1	15.4
Nitrogen	6.8	2.1
Sulphur	4.1	8.6
Ash compositional analysis, mg kg^{-1} (on dry basis)		
Cl	172,000	230,000
I	900	1000
Al	13,400	2100
Au	10	10
Ca	7600	76,300
Co	11	6
Cr	35	11
Cu	74	21
Fe	29,000	3200
K	20,460	30,100
Mg	10,000	36,000
Mn	500	1000
Na	2400	20,500
Ni	20	25
Pb	36	14
Si	630	1000
Zn	83	200
As	4	10
Cd	0.2	0.4
Hg	4	5
Mo	5	2
P	3700	2400
Se	3	7
Ti	1500	300

significantly above the maximum concentrations allowed for human consumption in EU countries [47–49]. The high concentrations of N, S, heavy metals (As, Hg, Pb), and low gross calorific values limit the use of both AL and AN samples in the food and energy sectors [50,51]. Therefore, the biorefinery concept of fractionation and purification can support a seaweed customization towards edible food, hygiene and cosmetics items increasing the overall value of the product.

3.2. Mass balance

Fractionation trials with seaweed were first conducted to determine the optimal temperature and GVL/H_2O ratio for nanocellulose production. The behaviour of the main components of seaweed, i.e., polysaccharides, lignin, and protein, along with varying temperature from 130 to 170 °C and GVL/H_2O ratio from 1:4 to 1:2 is shown in Fig. 1. The undetermined fraction represents such compounds as fatty acids, extractives, etc. The protein fraction is shown in a mass balance using results only from elemental analysis, whereas the more detailed discussion on various methods used in protein quantification is given in the next section. The mass balance indicates that the type of seaweed has a significant impact on the fractionation.

The microwave treatment enabled only a small fraction of polysaccharides to be released from the AL's matrix, whereas >70 % of original polysaccharides were found in the AN liquor. Delignification process led to the release of >40 % "pseudo-lignin" compounds in both seaweed samples. The differences in lignin yields determined in spent liquors is due to the compositional differences of "pseudo-lignin" material and variations in "pseudo-lignin" compounds' bonding to other fractions in seaweed, e.g., polysaccharides, metals, etc.

Although genuine lignin has not been reported in seaweed, AN were confirmed to include a series of "pseudo-lignin" compounds such as phloroglucinol-derived polyphenols [43]. *Aegagropila linnaei* species are aggregations of the filamentous green macroalgae, and thus, "pseudo-lignin" polyphenols using phenylpropanoid formation pathway are present in high concentrations [52] [53]. The "pseudo-lignin" complexes in green macroalgae can be significantly affected by high temperatures, radiation, nutrient content, and the presence of heavy metals [54]. The increase in microwave treatment temperature had a strong impact on fractionation of polysaccharides and "pseudo-lignin". Thus, Fig. 1(A) illustrates that the increase in microwave temperature from 130 to 170 °C caused stronger delignification in AL algae leading to release of >85 wt% pseudo-lignin found in the raw feedstock into the liquor phase.

Fig. 1**(B)** illustrates that the release of polysaccharides did not change significantly with the temperature variation. In opposite, the low microwave treatment temperatures enhanced delignification process in AN, whereas the polysaccharide fraction in the spent liquor increased from 70 to 95 % with the higher temperatures. Similar amounts of acetic and formic acids were determined in both seaweed liquors. These organic acids are degradation products of the carbohydrates. Acetic acid originates from the cleavage of acetyl groups in glucuronoxylan, whereas formic acid is formed simultaneously from the degradation of hexoses or pentoses, following similar pathways of terrestrial plants [55]. The total amount of organic acids ranged between 10 and 18 wt%, with nearly equal percentages of formic and acetic acids over the entire temperature range. The impact of microwave treatment temperature was pronounced at 170 °C for AN leading to slight decrease in formation of formic acid. At low temperatures (120–150 °C), seaweed samples are mostly converted predominantly to small molecular weight components, whereas higher temperatures can cause the reaction of seaweed to both large and small molecular weight compounds. In opposite, no significant changes were observed during microwave treatment of AL over the entire temperature range. Both results confirm the previous results on correlation between formic acid formation and the feedstock type [56]. The addition of GVL at 170 °C microwave treatment decreased the formation of formic acid leading to increase in glucan. Formic acid is a well-known non-gaseous hydrogen donor and further be used as a reactant for the high glucose recovery. The precise definition of the reaction mechanism for glucose into formic acid at high hydrolysis temperature is still under debate [57]. However, the present results clearly indicate that the decrease in formic acid formation was observed only at high concentration of GVL/H_2O (1:2).

Fig. 2 illustrates differences in polysaccharides composition among seaweed samples which were analyzed by HPAEC method. The AN sample additionally contained fucose and mannitol derived from mannose leading in total to higher concentration of polysaccharides (~ 50 wt%) than in AL (~ 30 wt%). The amounts of mannose and xylose were also higher in AN, supporting previous findings [58]. Glucose, arabinose, and galactose were the main polysaccharides determined in AL samples. The microwave treatment temperature had a dominant effect on the release of glucose, xylose, galactose, fucose, mannan and mannitol derived from mannose. A significantly higher fraction of polysaccharides in AN was transferred into the liquor with the increasing microwave treatment temperature than in the case of AL. The results on quantification of fucose and mannose derived from mannitol correspond to the data from 2D HSQC NMR analysis (*See supplemental material Fig. S-10*).

The further adjustment of parameters, e.g., increase in ^{1}H NMR analysis temperature, could provide a better resolution of peaks allocated in the range from 1 to 5 ppm, as Grasdalen suggested [59]. We stress that both algae contain polysaccharides whose monosugars after total hydrolysis cannot be resolved with the HPAEC column, namely

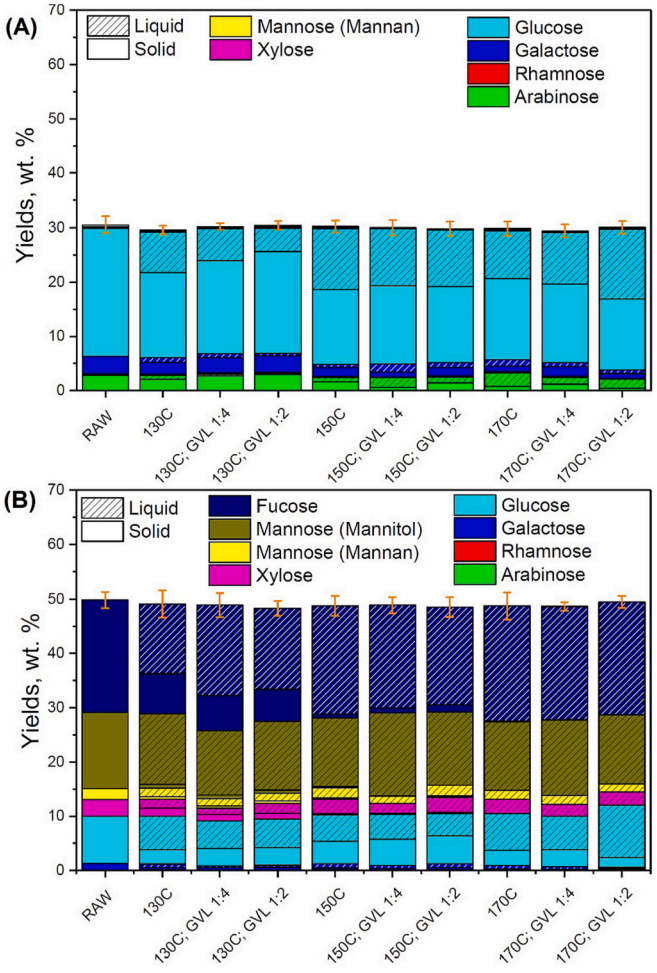

Fig. 2. Sugar mass balances of microwave treatment at temperatures of 130, 150, and 170 °C without GVL or with GVL (1:4; 1:2) (A) AL, and (B) AN which were calculated per dry weight of the original seaweed.

glucuronic and mannuronic acid (from alginate) and fucose (from fucoidan). Finer resolution of the carbohydrate composition will be subject to further studies. Also, the impact of the microwave treatment temperature was dominating over the additional GVL at various concentrations. This can be related first to the differences in the seaweed structure and composition. AL obtained a similar structure-composition to terrestrial plants, while AN shared several characteristics with the yellow-green seaweed. The undetermined fraction for both seaweed samples can represent non-measured extractives and mineral content. The AN contained very large grains of sand in the gas bladders which were separated from solid residue and liquor fractions and were not taken in a mass balance. The protein concentrations varied in both seaweed samples. However, no strong dependence was observed between protein concentration, temperature, and ratio of GVL. The microwave treatment did not cause strong release of protein into seaweed spent liquor based on the total amino acid results. The quantities of protein in a solid residue varied in AL from 15 to 26 wt% and in AN from 5 to 11 wt%.

The AFM analysis was performed (*See supplemental material Fig. S-11*). Based on the images, it appears possible to isolate nano- or microcellulose-like structures without additional chemicals or solvents [60]. The issue, however, requires a devoted study and rigorous optimization and is hence outside the scope of this contribution.

3.3. Characterization of seaweed proteins

Fig. 3 shows the large differences in protein yields which were determined in seaweed solid residue and liquor samples. Elemental nitrogen measurements for protein calculations provided almost 10 times higher values for seaweed liquors and 3 times higher protein contents for solid residue than it was determined with the total amino acid analysis. The recalculation procedure of elemental nitrogen adopted a conversion factor from literature that was estimated as an average between 12 strains of algae [36]. This strain of AN was a part of the approximation. However, a strain of AL was excluded from the research on the conversion factor average leading to the protein yields, which significantly differ from the actual protein content [61]. The total amino acid content using chromatographic identification of individual amino acids after extraction and HCl hydrolysis showed notable variation from 0.09 to 0.6 wt% in AL liquor and from 0.09 to 0.3 wt% in AN. The clear trends of the total amino acid yields and operating condition such as microwave treatment temperature and addition of GVL could not be established.

However, the comparison of protein yields in liquor samples and solid residues allows to conclude that the release of protein into liquor phase was very low and thus, the development of additional procedure for the separation of cellulose from protein is not required. The advantage of the total amino acid analysis is the possibility to obtain the compositional characteristic of different amino acids. Cystine was found limiting in raw AN, whereas the composition of both seaweed samples was similar qualitatively, as shown in Fig. 4. Glutamic acid (GLU), alanine (ALA), aspartic acid (ASP), arginine (ARG), leucine (LEU), lysine (LYS), and valine (VAL) make up >50 % of the total amino acid content in AL liquor. GLU and ASP are the main constituents of the AN liquor.

The qualitative composition of total amino acid content in seaweed liquor samples only slightly changed under various operational conditions of microwave treatment. LEU and LYS contents decreased with the increase in microwave treatment temperature in AL liquor. The intermediate contents of LYS and ARG were measured in raw AN which later decreased in the temperature range from 130 to 170 °C. The protein composition of both solid residue samples remained qualitatively similar to the raw seaweed. However, the AN solid residue showed decreased content of ASP and GLU, whereas AL solid residue contained less LYS, ARG, and PRO at 170 °C microwave treatment. The total amino acid procedure did not allow the quantification of tryptophan due to its loss during the HCl hydrolysis. However, taking the lack of tryptophan into consideration, the sum of the reported amino acids in liquor and solid residues is approximated to be ~10 wt% that seems to be similar to the actual protein content of seaweed.

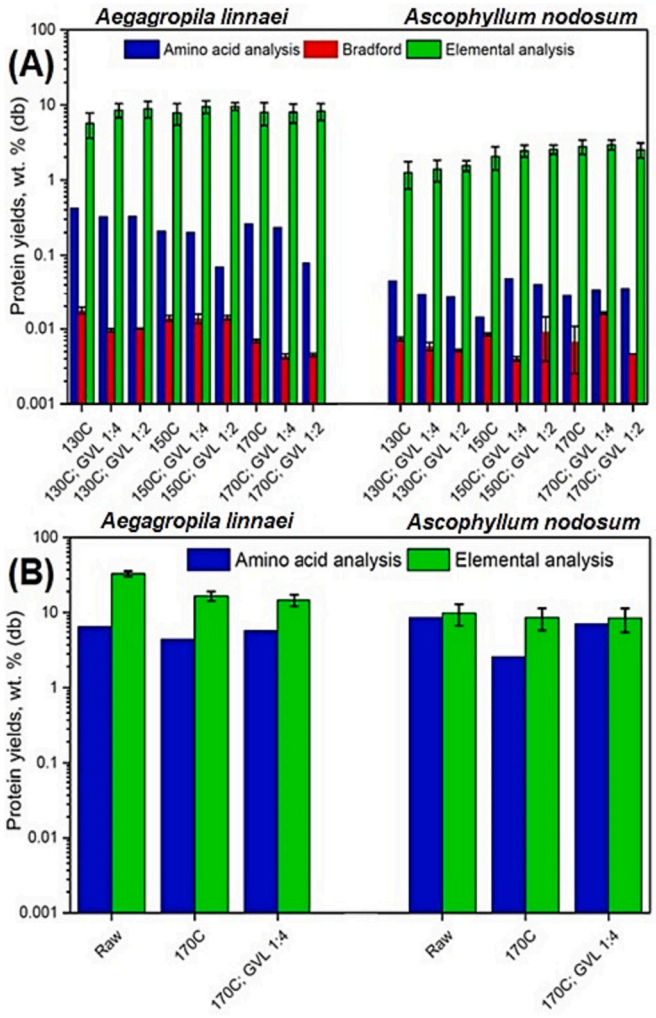

Fig. 3. Protein yields which were calculated per dry weight of the original seaweed of microwave treatment at temperatures of 130, 150, and 170 °C without GVL or with GVL (1:4; 1:2) detected in (A) liquid liquor samples of AL and AN using total amino acid analysis (AAA), Bradford, and elemental methods (EA) and (B) raw seaweed and solid residue from microwave treatment at 170 °C without GVL and with GVL 1:4 using AAA and EA methods. The Y-axis is shown using logarithmic scale.

Comparing the sum of the amino acids with the calculation using the elemental nitrogen, the total amino acid amount, resulting from chromatographic identification, seems to be better for quantification, with regards to accuracy and precision of the measurement, of the total protein content. The protein yields using Bradford method were significantly less than using other methods varying from 0.005 to 0.02 wt%. The Bradford method is based on the measurement of a colour change caused by oxidation of aromatic amino acid residues by the Bio-Rad Bradford dye reagent (*See supplemental material Fig. S-12*). The over- and underestimation of the total amino acid content can occur when the average sample protein profile is different from the Bradford assay since it relies on dye binding specifically to lysine and arginine residues rather than reacting more generally with peptide bonds [62]. The compositional analysis using chromatographic identification indicated that the sum of lysine and arginine in AL liquor was <10 wt% and in AN <3.5 wt %. Additionally, the AN liquor samples were not homogeneous with respect to particle size in a suspension that could lead to high analysis uncertainty.

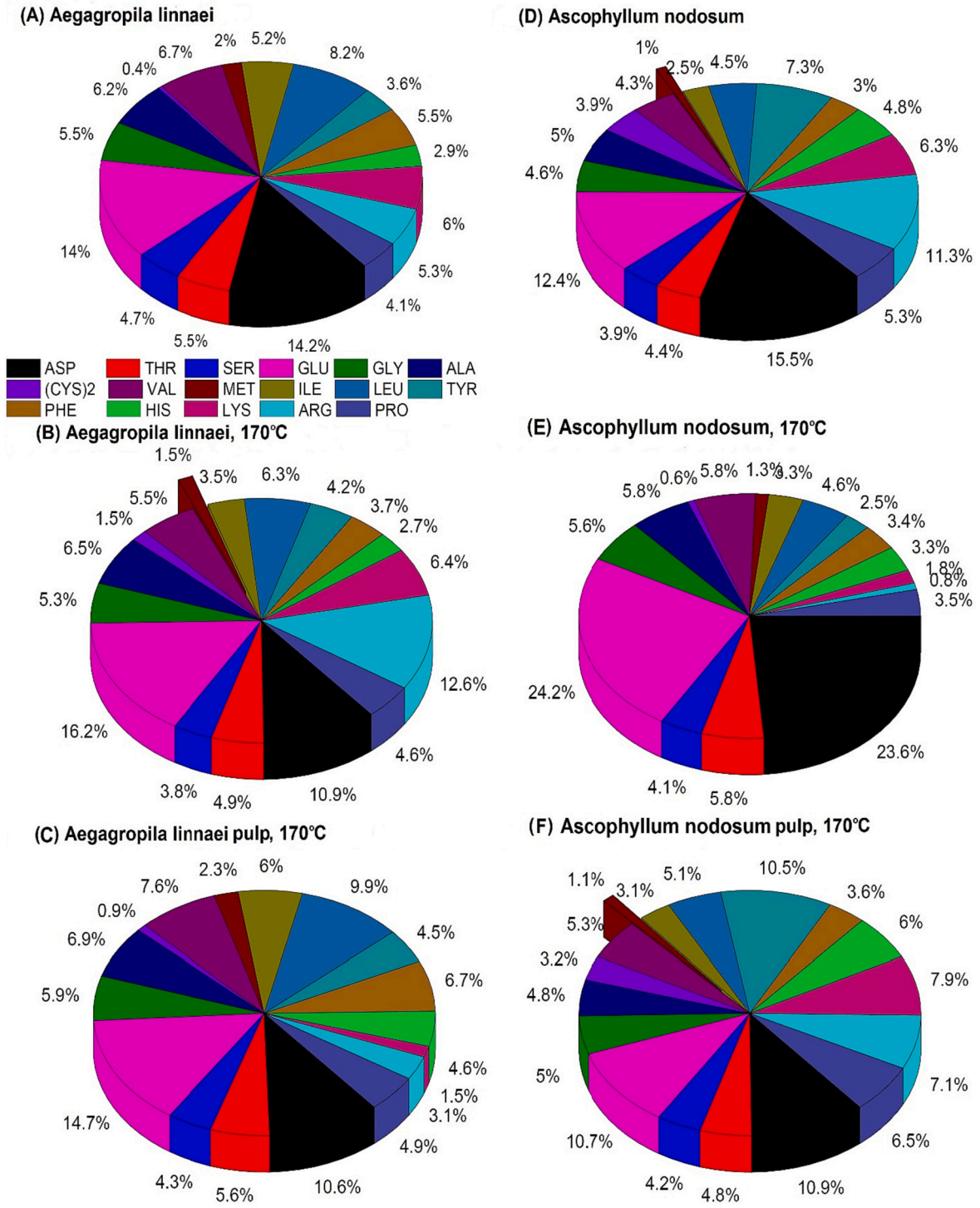

Fig. 4. Protein composition using the total amino acid method (A) raw AL; (B) AL liquor, 170C; (C) AL pulp, 170C; (D) raw AN; (E) AN liquor, 170C, and (F) AN pulp, 170C. The abbreviations mean: Aspartic acid (ASP), Threonine (THR), Sericin (SER), Glutamic acid (GLU), Glycine (GLY), Alanine (ALA), Cystine (CYS)2, Valine (VAL), Methionine (MET), Isoleucine (ILE), Leucine (LEU), Tyrosine (TYR), Phenylalanine (PHE), Histidine (HIS), Lysine (LYS), Arginine (ARG), Proline (PRO).

3.4. GC–MS analysis of extractives

Fig. 5**(A)** illustrates the relationship between the extraction type and yield of extractives in AL and AN liquors. Both extractions using chloroform-methanol and DCM-hexene give similar extractives yields in spent liquors from microwave treatment at selected operational condition. The yields slightly vary in the range from 4.4 to 5.8 wt% for AL samples and from 2.4 to 3.5 wt% for AN liquors, respectively. Saturated fatty acids and their derivatives were identified to 90 % of the totally measured compounds. These compounds were 2-methoxy-butanoic acid (characteristic trimethylsilyl (TMS) derivatives ion *m/z* 262), palmitic acid (*m/z* 313), stearic acid (*m/z* 341), glycerol monostearate (*m/z* 399), 1-monopalmitin (*m/z* 371), and dodecanal (*m/z* 385) at retention times of 8.7, 30.5, 34.8, 41.9, 45.4, and 52.4 min, as shown in Fig. 5(B). The identity of the aforementioned compounds was confirmed by comparison with the retention times and mass spectra of the authentic compounds. The further recalculation of yields in wt% was performed using the external standard (tetracosane at 37.9 min, *m/z* 338). The

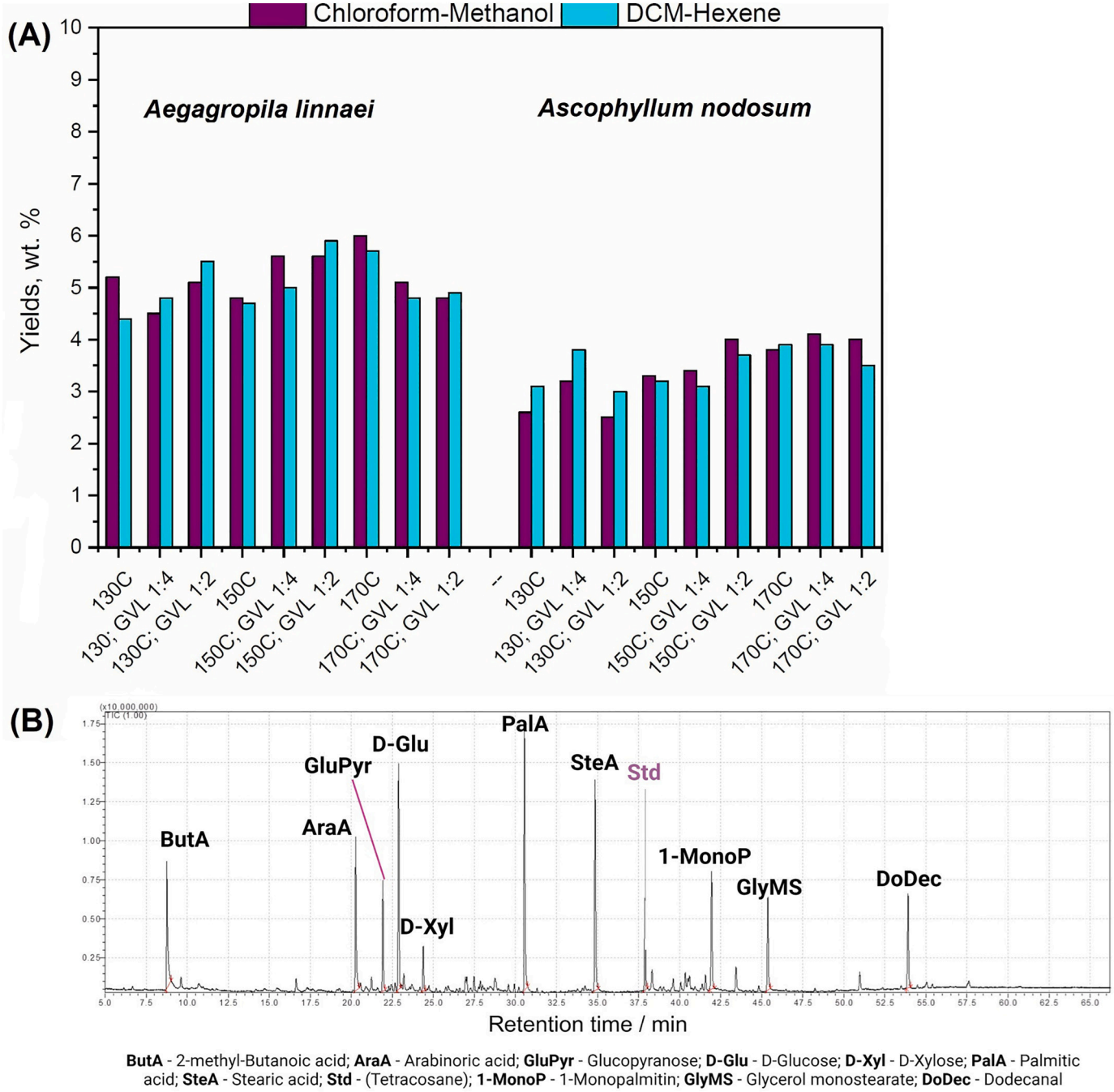

Fig. 5. GCMS analysis (A) Comparison of DCM-Hexene and Chloroform-Methanol on extractives in liquor samples of AL and AN from microwave treatment at temperatures of 130, 150, and 170 °C without GVL or with GVL (1:4; 1:2) and (B) A representative chromatogram with detected compounds using an example of AN liquor from 170 °C with GVL (1:4). The extractives yields were calculated per dry weight of the original seaweed.

unquantified compounds could be unusual lipids [63], flavonoids, saponins, phenolic compounds, tannins, carotenoids, etc. [64]. The microwave treatment temperature and GVL ratios did not have strong impact on extractives yields and composition.

The presence of further compounds, e.g., levoglucosan (*m/z* 204), phenol (*m/z* 151), L-fucose (*m/z* 361), D-mannose (*m/z* 204), D-glucose (*m/z* 204), D-xylose (m/z 204), arabinonic acid (*m/z* 232), D-galactopyranose (*m/z* 204) at high temperatures was observed in AN samples, whereas the composition of AL liquors remained homogeneous under various operating conditions. This indicates that the impact of algae type on the yields and composition of extractives was stronger than other factors. Moreover, the presence of D-glucose, D-mannose and glycerol compounds in AN indicate the potential formation of uronic acids (β-1,4-d-mannuronic acid and α-L-guluronic acid) and further alginate via phosphate pathways [65].

The presence of L-fucose and alginate pre-cursors using GC–MS is a comparable result to alginate and fucoidan's pattern detected in 2D HSQC NMR analysis (*See supplemental material from Figs. S-1 to S-9*).

4. Discussion

This work has shown that seaweed is a significant source of polysaccharides, protein, and extractives, including alginate or fatty acids. Both seaweed type and microwave temperature are critical for the

isolation of polysaccharides. The temperatures in the range from 150 to 170 °C lead to >90 % release of polysaccharides into the liquor from AN matrix. In opposite, the AL released <50 % of the total polysaccharide content in the same temperature range. This is because the structure of AL is more similar to terrestrial plants whose more elaborate, hierarchical construct efficiently resists hydrolytic conditions. Cell walls store carbohydrates in the form of starch which is difficult to separate from the AL algae matrix [66]. This study also indicated that both seaweeds are significant sources of polysaccharides with the dominating arabinose and glucose in AL and fucose and mannose in AN. The AL samples that showed lower release of polysaccharides into aqueous phase will require a prior extraction of arabinose, galactose, and xylose to make the cell wall more flexible [67]. Fucose and mannose derived from mannitol can be separated from the AN using a priori extraction. The high yields of fucose and mannose derived from mannitol after microwave treatment would enable the application in biomedical and food sectors as a separated product stream, but it is not a mandatory requirement. Limited studies have been done on nanocellulose manufacturing or even the isolation of the cellulose fraction from seaweed. These results have clearly indicated that seaweed from Nordic and Caribbean regions has potential for the development and industrial future growth in nanocellulose and manufacturing of bioactive products.

This work showed that only a combined use of measurement techniques for the product characterization, e.g., protein, extractives will make it possible to close the mass balance up to >90 wt%. The value-added by-products detected in AN liquor are alginate and fucoidan, whereas AL liquor is rich in protein and fatty acids. The observed fatty acids and other extractives are direct products of combined microwave extraction with the hydrolysis process and not a result of hydrolysis decomposition. The alginate, fucoidan, and fatty acids in seaweed were characterized using NMR and GCMS techniques. However, the undetermined fraction in both seaweed samples could be formed by bioactive compounds, e.g., carotenoids, flavonoids, and unusual lipids. The seaweed species could also contain sulphonated polysaccharides which were not possible to analyze with the HPAEC and HPLC methods due to unequivocal structure of intact seaweed fucoidan and ulvan [68].

Compared with the previous studies [69,70], the present results clearly showed that a procedure for the polysaccharide separation from proteins is not required. The micron-sized cellulosic structures were observed during AFM analysis of the fraction composed of cellulose and proteins. Furthermore, only a small protein fraction (< 2 %) was released into the aqueous phase during microwave hydrolysis. In addition, this study demonstrates that the yields and composition of proteins vary with the seaweed type by depending less on other factors. In addition, seaweeds are known to have “pseudo-lignin” structures in their composition of the polyphenolic origin. The polyphenolic compounds are easier to separate in the AL samples, whereas the yields of “pseudo-lignin” species mostly remained unchanged in the AN liquor. The literature reports a similar composition of *ascophyllum nodosum* with the Japanese beechwood, whereas structures similar to P, G and S type lignin were found in green algae due to the cell wall evolution [71]. More research will be required to characterize the chemical composition of “pseudo-lignin” compounds in seaweed. This will guide with the choice of a potential green solvent to fractionate selectively polysaccharides of the highest purity.

Low temperatures for nanocellulose manufacturing have been suggested due to the lower footprint and lower energy consumption [24]. Despite all advantages of low microwave treatment temperatures, the present results demonstrate that the higher temperatures (> 150 °C) can lead to a more heterogenous composition of extractive products. As a disadvantage, the yields of some value-added compounds, i.e., formic acid used in biofuel and bioplastic manufacturing can decrease with an increase in microwave temperature. A balance must be found between the energy consumption, cost, reactor design, and environmental issues to obtain products of the highest purity for specific industrial use.

From the technical point of view, it is viable to use the two-step microwave treatment in one reactor. This work confirmed that a clear advantage of the two-step microwave treatment is a fine-tuning of products using a limited number of operating parameters in the sustainable manufacturing environment. While the high environmental and economic costs of nanocellulose production remain unsolved for the time being [72], the results of this study are promising with respect to reduction of process chemicals used and solvent free process operation. The high content of minerals in seaweed and a variety of seaweed species requiring the modification of extraction conditions hinder the immediate scale-up of microwave hydrolysis. However, the design of the high-pressure microwave reactor (up to 25–30 bar) could enable precise frequency and microwave power control to avoid significant misreadings of the actual values during the experiments. This will lead to less variations in product composition by tuning the liquor structure to several value-added products. In addition, it will make the overall product composition more homogeneous and less dependent on a seaweed type.

5. Conclusion

The microwave treatment of seaweed was investigated through a systematic experimental study. The effect of three variables, seaweed type (*Ascophyllum nodosum* and *Aegagropila linnaei*), microwave temperature (130–170 °C), and γ-valerolactone solvent addition (1:4; 1:2 ratios) on the product yield and composition was studied. The microwave temperature and seaweed type played a dominating role in the mass closure balances leading to >90 % identified compounds in the temperature range from 150 to 170 °C. Both seaweed cultures are significant sources of polysaccharides with the dominating arabinose and glucose in *Aegagropila linnaei* and fucose and mannose in *Ascophyllum nodosum*. The released extractives in the aqueous phase during *Ascophyllum nodosum* microwave treatment are dominated by alginate and fucoidan, whereas fatty acids were determined in both seaweed samples. The formic and acetic acids over the entire microwave temperature range are value-added products for the energy and chemical sectors. For the first time, the accurate characterization of protein composition using the total amino acid method showed that >95 % of protein remains in a seaweed solid residue during microwave treatment. The comparison of three protein characterization methods identified that the total amino acid analysis provides both quantitative and qualitative description of protein with the higher accuracy than other methods. This work clearly presents convincing arguments that the two-step microwave treatment of seaweed through the fine-tuning of products using a limited number of operating parameters is a promising sustainable process for both materials and energy sectors.

CRediT authorship contribution statement

Anna Trubetskaya: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Investigation, Formal analysis, Conceptualization. **Huy Quang Lê:** Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Jenni Leppiniemi:** Writing – original draft, Visualization, Software, Formal analysis, Data curation. **Tetyana Koso:** Writing – original draft, Visualization, Formal analysis, Conceptualization. **Teemu Välisalmi:** Writing – original draft, Visualization, Validation, Resources, Formal analysis. **Markus B. Linder:** Writing – review & editing, Supervision, Resources, Formal analysis. **Italo Pisano:** Writing – review & editing, Formal analysis, Data curation. **Jinze Dou:** Visualization, Software, Formal analysis, Data curation, Conceptualization. **J.J. Leahy:** Writing – review & editing, Methodology, Investigation. **Eero Kontturi:** Writing – original draft, Resources, Methodology, Investigation, Conceptualization.

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.

Data availability

No data was used for the research described in the article.

Acknowledgments

Dr. Trubetskaya acknowledges Nansenfondet Oslo Norway for the financial support (project grant 1051).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2024.103406.

References

[1] K. Shak, Y. Pang, S. Mah, Nanocellulose: recent advances and its prospects in environmental remediation, Beilstein J. Nanotechnol. 9 (2018) 2479–2498.
[2] A. Bruhn, C. Duarte, D. Krause-Jensen, A seaweed aquaculture imperative to meet global sustainability targets, Nat. Sustain. 5 (2022) 185–193.
[3] C. Duarte, S. Agusti, E. Barbier, G. Britten, J. Castilla, J. Gattuso, R. Fulweiler, T. Hughes, N. Knowlton, C. Lovelock, H. Lotze, M. Predragovic, E. Poloczanska, C. Roberts, B. Worm, Rebuilding marine life, Nature 580 (2020) 39–51.
[4] E. Becker, Micro-algae as a source of protein, Biotechnol. Adv. 25 (2) (2007) 207–210.
[5] M. el Achaby, Z. Kassab, A. Aboulkas, C. Gaillard, A. Barakat, Reuse of red algae waste for the production of cellulose nanocrystals and its application in polymer nanocomposites, Int. J. Biol. Macromol. 106 (2018) 681–691.
[6] E. Ibañez, M. Herrero, J. Mendiola, M. Castro-Puyana, Extraction and characterization of bioactive compounds with health benefits from marine resources: macro and micro algae, cyanobacteria, and invertebrates, in: M. Hayes (Ed.), Marine Bioactive Compounds, Springer, Boston, MA, 2012, pp. 1–44.
[7] S. Bleakley, M. Hayes, Algal proteins: extraction, application, and challenges concerning production, Foods 6 (5) (2017) 1–33.
[8] A. Bocanegra, S. Bastida, J. Benedi, S. Rodenas, F. Sanchez-Muniz, Characteristics and nutritional and cardiovascular-health properties of seaweeds, J. Med. Food 12 (2) (2009) 236–258.
[9] H. Khairy, S. El-Shafay, Seasonal variations in the biochemical composition of some common seaweed species from the coast of Abu Qir Bay, Alexandria, Egypt, Oceanologia 55 (2) (2013) 435–452.
[10] M. Wang, C. Hu, J. Cannizzaro, D. English, X. Han, D. Naar, B. Lapointe, R. Brewton, F. Hernandez, Remote sensing of sargassum biomass, nutrients, and pigments, Geophys. Res. Lett. 45 (22) (2018) 359–367.
[11] S. Lourenco, E. Barbarino, J. Campos De-Paula, L. Otavio da S Pereira, U. Lanfer Marquez, Amino acid composition, protein content and calculation of nitrogen-to-protein conversion factors for 19 tropical seaweeds, Phycol. Res. 50 (2002) 233–241.
[12] M. Strømme, S. Zhou, L. Nyholm, Z. Wang, Cladophora cellulose: unique biopolymer nanofibrils for emerging energy, environmental, and life science applications, Acc. Chem. Res. 52 (8) (2019) 2232–2243.
[13] H. Doh, M. Lee, W. Whiteside, Physicochemical characteristics of cellulose nanocrystals isolated from seaweed biomass, Food Hydrocoll. 102 (2020) 105542.
[14] Z. Liu, X. Li, H. Deng, Extraction, isolation and characterization of nanocrystalline cellulose from industrial kelp (Laminaria japanica) waste, Carbohydr. Polym. 173 (2017) 353–359.
[15] S. Singh, K. Gaikwad, Y. Lee, S. Park, Microwave-assisted step reduced extraction of seaweed (Gelidiella aceroso) cellulose nanocrystals, Int. J. Biol. Macromol. 99 (2017) 506–510.
[16] M. Plaza, S. Santoyo, L. Jaime, G. Garcia-Blairsy Reina, M. Herrero, F. Senorans, E. Ibanez, Screening for bioactive compounds from algae, J. Pharm. Biomed. Anal. 51 (2) (2010) 450–455.
[17] J. Echave, M. Fraga-Corral, P. Garcia-Perez, J. Popovic-Djordjevic, E. Avdovic, M. Radulovic, J. Xiao, M. Prieto, J. Simal-Gandara, Seaweed protein hydrolysates and bioactive peptides: extraction, purification, and applications, Mar. Drugs 19 (500) (2021) 1–22.
[18] N. Bourgougnon, Chapter 9 - current knowledge and challenges in extraction, characterization and bioactivity of seaweed protein and seaweed-derived proteins, in: Seaweed Around the World: State of Art and Perspectives, Academic Press, 2020, pp. 289–326.
[19] D. Klemm, F. Kramer, S. Moritz, T. Lindström, M. Anke, D. Gray, A. Dorris, Nanocelluloses: a new family of nature-based materials, Angew. Chem. 50 (24) (2011) 79–86.
[20] Y. Zhou, L. Li, R. Zhang, C. Hu, Fractional conversion of microalgae from water blooms, Farraday Discussions 202 (2017) 197–212.
[21] M. De Bruyn, V. Budarin, G. Sturm, G. Stefanidis, M. Radoiu, A. Stankiewicz, D. Macquarrie, Subtle microwave-induced overheating effects in an industrial demethylation reaction and their direct use in the development of an innovative microwave reactor, J. Am. Chem. Soc. 139 (15) (2017) 5431–5436.
[22] S. Splinter, J. Pare, S. Kadali, Method for direct extraction and concentration of naturally-derived active compounds, US Patent 20130338234A1, 2012.
[23] A. Matharu, E. de Melo, J. Remon, S. Wang, A. Abdulina, E. Kontturi, Processing of citrus nanostructured cellulose: a rigorous design-of-experiment study of the hydrothermal microwave-assisted selective scissoring process, ChemSusChem 11 (8) (2018) 1344–1353.
[24] V. Budarin, Y. Zhao, M. Gronnow, P. Shuttleworth, S. Breeden, D. Macquarrie, J. Clark, Microwave-mediated pyrolysis of macro-algae, Green Chem. 9 (13) (2011) 2330–2333.
[25] E. Zanchetta, E. Damergi, B. Patel, T. Borgmeyer, H. Pick, A. Pulgarin, C. Ludwig, Algal cellulose, production and potential use in plastics: challenges and opportunities, Algal Res. 56 (2021) 102288.
[26] S. Tabasso, G. Grillo, D. Carnaroglio, E. Gaudino, G. Cravot, Microwave-assisted γ-valerolactone production for biomass lignin extraction: a cascade protocol, Molecules 21 (4) (2016) 1–9.
[27] A. Trubetskaya, P. Jensen, A. Jensen, M. Steibel, H. Spliethoff, P. Glarborg, Influence of fast pyrolysis conditions on yield and structural transformation of biomass chars, Fuel Process. Technol. 140 (2015) 205–214.
[28] N. Prochukhan, S. O'Brian, A. Davo-Quinonero, A. Trubetskaya, E. Cotter, A. Selkirk, R. Senthamaraikannan, M. Ruether, D. McCloskey, Room temperature fabrication of macroporous lignin membranes for the scalable production of black silicon, Biomacromolecules 23 (2022) 2512–2521.
[29] A. Trubetskaya, C. Lin, J. Ovadnevaite, D. Ceburnis, C. O'Dowd, J. Leahy, R. Monaghan, R. Johnson, P. Layden, W. Smith, Study of emissions from domestic solid-fuel stove combustion in Ireland, Energy Fuel 35 (6) (2021) 4966–4978.
[30] J. Dou, H. Kim, Y. Li, D. Padmakshan, F. Yue, Structural characterization of lignins from willow bark and wood, J. Agric. Food Chem. 66 (2018) 7294–7300.
[31] A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, Determination of structural carbohydrates and lignin in biomass, NREL Report Contract No. DE-AC36-08GO28308 1–15, 2012.
[32] J. Janson, Calculation of polysaccharide composition of wood and pulp, Peperi ja PUU 52 (5) (1970) 323–329.
[33] B. Pratto, V. Chandgude, R. Júnior, A. Cruz, Biobutanol production from sugarcane straw: defining optimal biomass loading for improved ABE fermentation, Ind. Crop. Prod. 148 (2020) 112265.
[34] M. Nguyen, P. Westerhoff, L. Baker, Q. Hu, Characteristics and reactivity of algae-produced dissolved organic carbon, J. Environ. Eng. 131 (11) (2005) 1574–1582.
[35] M. Schreiner, Optimization of solvent extraction and direct transmethylation methods for the analysis of egg york lipids, Int. J. Food Prop. 9 (2006) 573–581.
[36] S. Lourenco, E. Barnarino, P. Lavin, U. Lanfer Marquez, E. Aidar, Distribution of intracellular nitrogen in marine microalgae: calculation of new nirogen-to-protein conversion factors, Eur. J. Phycol. 39 (1) (2004) 17–32.
[37] J. Dou, D. Evtuguin, T. Vuorinen, Structural elucidation of Suberin from the bark of cultivated willow (Salix sp.), J. Agric. Food Chem. 69 (2021) 10848–10855.
[38] D. Branco, J. Campos, L. Cabrita, D. Evtuguin, Structural features of macromolecular components of cork from Quercus suber L, Holzforschung 74 (6) (2020) 625–633.
[39] A. Circuncisao, M. Catarino, S. Cardoso, A. Silva, Minerals from macroalgae origin: health benefits and risks for consumers, Mar. Drugs 16 (11) (2018) 1–30.
[40] C. Nufus, A. Abdullah, A. Nurjanah, Characteristics of green seaweed salt as alternative salt for hypertensive patients, IOP Conference Series: Earth and Environmental Science. The 3rd EIW (2019) 012050.
[41] A. Trubetskaya, Reactivity effects of inorganic content in biomass gasification: a review, Processes 15 (9) (2022) 1–36.
[42] J. McLachlan, Macroalgae (seaweeds): industrial resources and their utilization, Plant Soil 89 (1985) 137–157.
[43] J. Olsson, G. Toth, E. Albers, Biochemical composition of red, green and brown seaweeds on the Swedish west coast, J. Appl. Phycol. 32 (2020) 3305–3317.
[44] A. Beck, C. Mayr, Nitrogen and carbon isotope variability in the green-algal lichen Xanthoria parietina and their implications on mycobiont–photobiont interactions, Ecol. Evol. 2 (12) (2012) 3132–3144.
[45] K. Rousk, D. Jones, T. deLuca, Moss-cyanobacteria associations as biogenic sources of nitrogen in boreal forest ecosystems, Front. Microbiol. 4 (150) (2012) 1–10.
[46] I. Michalak, B. Messyasz, Concise review of Cladophora spp.: macroalgae of commercial, J. Appl. Phycol. 33 (2021) 133–166.
[47] E. Committee, Commission Regulation (EC) no 629/2008 of 2 July 2008 Amending Regulation (EC) no 1881/2006 Setting Maximum Levels for Certain Contaminants in Foodstuffs, EU Commission, Brussel, 2008.
[48] European Committee, Commission Regulation (EU) no 1275/2013 of 6 December 2013 Amending Annex I to Directive 2002/32/EC of the European Parliament and of the Council as Regards Maximum Levels for As, Cd, Pb, Nitrites, Volatile Mustard Oil and Harmful Botanical Impurities, EU Commission, Brussel, 2013.
[49] European Committee, Edible Seaweed-French & European Regulation [Online]. Available: http://www.cybercolloids.net/sites/default/files/seaweed%20and%20regulation2014.pdf, 2018 [Accessed 26 december 2022].
[50] M. Roleda, H. Marfaing, N. Desnica, R. Jonsdottir, J. Skjermo, C. Rebours, U. Nitschke, Variations in polyphenol and heavy metal contents of wild-harvested and cultivated seaweed bulk biomass: health risk assessment and implication for food applications, Food Control 95 (2019) 121–134.

[51] R. Lawton, R. de Nys, N. Paul, Selecting reliable and robust freshwater macroalgae for biomass applications, PLoS One 8 (5) (2013) e64168.
[52] D. Cano-Raminez, T. Saskia de Fraine, O. Griffiths, A. Dodd, Photosynthesis and circadian rhythms regulate the buoyancy of marimo lake balls, Current Biology Manazine 28 (2018) 869–870.
[53] H. Renault, D. Werck-Reichart, J. Weng, Harnessing lignin evolution for biotechnological applications, Curr. Opin. Biotechnol. 56 (2021) 105–111.
[54] L. Alzate-Gaviria, J. Dominguez-Maldonado, R. Chable-Villacis, E. Olguin-Maciel, R. Leal-Bautista, G. Canche-Escamilla, A. Caballero-Vazquez, C. Hernandez-Zepeda, F. Barredo-Pool, R. Tapia-Tussell, Presence of polyphenols complex aromatic "lignin" in Sagassum spp. from Mexican Caribbean, J. Mar. Sci. Eng. 9 (6) (2021) 1–10.
[55] H. Le, M. Borrega, H. Sixta, Y. Ma, Wood biorefinery based on gamma-valerolactone/water fractionation, Green Chem. 18 (2016) 5466–5476.
[56] K. Swiatek, S. Gaag, A. Klier, A. Kruse, J. Sauer, D. Steinbach, Acid hydrolysis of lignocellulosic biomass: sugars and furfurals formation, Catalysts 10 (437) (2020) 1–18.
[57] N. Bocanegra, S. Suzrez Vazquez, L. Sandoval Rangel, M. Garza Navarro, J. Rivera de la Rosa, C. Lucio Ortiz, G. Flores-Escamilla, I. Santos-Lopez, E. Carrillo-Pedraza, M. Bravo Sanchez, D. De Haro Del Rio, Catalytic conversion of GVL to biofuels using cu and Pt catalysts over microwave-synthesized FAU zeolite, Catal. Today 392-393 (2022) 105–115.
[58] S. Maneein, J. Milledge, B. Nielsen, P. Harvey, A review of seaweed pre-treatment methods for enhanced biofuel production by anaerobic digestion or fermentation, Fermentation 4 (100) (2018) 1–31.
[59] H. Grasdalen, High-field, ^{1}H NMR spectroscopy of alginate; sequential structure and linkage conformations, Carbohydr. Res. 118 (1983) 255–260.
[60] J. Guo, K. Uddin, K. Mihhels, W. Fang, P. Laaksonen, J. Zhu, O. Rojas, Contribution of residual proteins to the thermomechanical performance of cellulosic Nanofibrils isolated from green macroalgae, ACS Sustain. Chem. Eng. 5 (8) (2017) 6978–6985.
[61] L. Laurens, T. Dempster, H. Jones, E. Wolfrum, S. Van Wychen, J. McAllister, M. Rencenberger, K. Parchert, L. Gloe, Algal biomass constituent analysis: method uncertainties and investigation of the underlying measuring chemistries, Anal. Chem. 84 (2012) 1879–1887.
[62] C. Niemi, A. Mols Mortensen, R. Rautenberger, S. Matsson, A. Gorzsas, F. Gentili, Rapid and accurate determination of protein content in North Atlantic seaweed by NIR and FTIR spectroscopies, Food Chem. 404 (2023) 134700.
[63] H. Nygren, T. Seppänen-Laakso, H. Rischer, Liquid chromatography-mass spectrometry (LC-MS)-based analysis of molecular lipids in algae samples, Methods Mol. Biol. 1980 (2017) 215–222.
[64] C. Ragunath, Y. Kumar, I. Kanivalan, S. Radhakrishnan, Phytochemical screening and GC-MS analysis of bioactive constituents in the Methanolic extract of Caulerpa racemosa (Forssk.) J. Agardh and Padina boergesenii Allender & Kraft, Current Appl Sci Tech 20 (3) (2020) 380–393.
[65] R. Ashby, The Production and Characterization of Alginate Produced by *Pseudomonas syringae*, Lousiana State University, LA, USA, 1994. PhD thesis.
[66] Nisshin Seifun group Inc, Product obtained using moss and process for producing the same, Japan Patent JP5732274B2, 2011.
[67] M. Øverland, L. Mydland, A. Skrede, Marine macroalgae as a source of protein and bioactive compounds in feed for monogastric animals, Science of Food and Agriculture 99 (1) (2019) 13–24.
[68] H. Gaspar, A. Martins, C. Alves, J. Silva, S. Pinteus, R. Pedrosa, H. Gaspar, Sulfated polysaccharides from macroalgae - a simple roadmap for chemical characterization, Polymers 15 (399) (2023) 1–20.
[69] V. Budarin, Y. Zhao, M. Gronnow, M. De Bruyn, J. Fan, P. Shuttleworth, S. Breeden, D. Macquarrie, J. Clark, Direct microwave-assisted hydrothermal depolymerization of cellulose, J. Am. Chem. Soc. 135 (32) (2013) 11728–11731.
[70] Z. Du, Y. Li, X. Wang, Q. Chen, Y. Wan, Q. Chen, C. Wang, X. Lin, Y. Liu, Microwave-assisted pyrolysis of microalgae for biofuel production, Bioresour. Technol. 102 (2011) 4890–4896.
[71] H. Rabemanolontsoa, S. Saka, Comparative study on chemical composition of various biomass species, RSC Adv. 3 (2013) 3946–3956.
[72] C. Abbati de Assis, C. Houtman, R. Phillips, E. Bilek, O. Roj, M. Soledad Peresin, H. Jameel, R. Gonza, Conversion economics of Forest biomaterials: risk and financial analysis of CNC manufacturing, Biofuels Bioprod. Biorefin. 11 (4) (2017) 682–700.