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High-Solids Processing of *Palmaria palmata* for Feed Applications: Effects of Alkaline Autoclaving and Sequential Enzymatic Treatment

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Abstract

Macroalgae are increasingly recognized as a valuable source of nutrients and bioactive compounds for animal nutrition, including for aquatic species. However, the complex structure of the macroalgal cell wall limits the accessibility of intracellular components, restricting their use in feeds. To overcome this limitation, macroalgal hydrolysis using various technological treatments has been tested, often employing a low solid-to-water ratio, which complicates downstream processing due to phase separation. In contrast, high-solids loading hydrolysis has the advantage of producing a single and consolidated fraction, simplifying subsequent processing and application. The present study assessed the effectiveness of high-solids loading water or alkaline (0.5 and 1N NaOH) autoclaving for 30 or 60 min, applied alone or followed by sequential enzymatic hydrolysis, using a xylanase-rich enzymatic complex aimed at promoting cell wall disruption and increasing the extractability of intracellular components in the red macroalga *Palmaria palmata* with minimal free water. The 1N NaOH treatment for 30 min decreased neutral and acid detergent fiber while increasing Folin–Ciocalteu total phenolic content (GAE) (expressed as gallic acid equivalent) and the water-soluble protein fraction and decreased crude protein, indicating enhanced extractability of these components. Microscopic examination showed relatively mild structural changes on the surface of *P. palmata* after high-solids loading alkaline (1N NaOH) autoclaving for 30 min. Following alkaline or water treatment, the enzymatic complex hydrolysis further increased the Folin–Ciocalteu total phenolic content (GAE), with minimal effects on NDF, ADF, or crude protein. Overall, these results showed that high-solids loading alkaline autoclaving, with or without subsequent enzymatic hydrolysis, effectively disrupts *P. palmata* cell walls and induces substantial modifications while simplifying processing by avoiding phase separation.

Keywords: animal nutrition; enzymatic hydrolysis; feedstuff sustainability; high-solids loading; macroalgae; solid-state hydrolysis

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1. Introduction

Macroalgae have emerged as an attractive and sustainable biomass source due to their rapid growth, high productivity, and capacity for CO_2 sequestration. These attributes and their potential health benefits have expanded their applications in feed and food supplements, hydrocolloids, nutraceuticals, pharmaceuticals, biofuels, and biomaterials [1,2].

Macroalgae have gained attention as potential feed additives due to their distinctive composition, which includes proteins, carbohydrates, minerals (such as sodium, potassium, calcium, and magnesium), vitamins (A, C, E, and the B-complex), phytochemicals, and polyunsaturated fatty acids (DHA and EPA) [3,4]. These characteristics, combined with their ability to grow in marine environments, make macroalgae a promising option for sustainable biomass production, particularly for use in animal feed formulation [5]. However, the structural complexity of the macroalgae polysaccharide matrix, which is resistant to intestinal enzymatic degradation, limits the accessibility of intracellular components and the efficient utilization of macroalgal nutrients. Macroalgae polysaccharide matrix, composed of diverse cyclic ether sugar molecules with multiple hydroxyl groups and additional functional groups such as amines and carboxylic acids, requires disruption into smaller structural units to improve extractability and processing efficiency [6]. Furthermore, the diversity of sugar monomers and complex polysaccharides complicates the characterization of macroalgae cell walls and the selection of optimal pretreatment methods, as approaches routinely applied to lignocellulosic terrestrial biomass cannot be directly transferred to macroalgae due to the presence of alginates, agar, and/or sulfated polysaccharides with distinct chemical reactivities [6,7].

In aquafeeds, macroalgae have been used as a dietary ingredient or a source of bioactive extracts. Bioactive compounds in macroalgae have potential functional benefits for fish, including antimicrobial and antioxidant effects, which can improve fish immune status and disease resistance [8]. However, the macroalgal inclusion rate in aquafeeds is limited mainly due to their relatively low protein content and reduced energy digestibility [9]. Despite these limitations, several macroalgal species have been evaluated for their nutritional and functional effects when incorporated into aquafeeds at varying inclusion levels, including those as low as 10–15%, depending on the feeding habits of the target species, ranging from carnivorous to herbivorous [10]. Overall, outcomes vary with both macroalgal species and fish species. In general, carnivorous fish appear less tolerant than herbivorous species. Still, low inclusion levels can yield promising effects, such as improved growth performance, feed efficiency, body composition, survival, and disease resistance, whereas higher inclusion levels often lead to the opposite results [10]. Moreover, the nutritional value of macroalgae is strongly dependent on their digestibility [10]. Processing strategies that modify macroalgal structure and improve nutrient bioavailability are therefore recommended to enhance their dietary inclusion in aquafeeds [11].

The edible red macroalgae *Palmaria palmata*, commonly found in the North Atlantic, has long been used as a food source and holds significant commercial value. It has also been recognized as a potential feed ingredient for livestock, with a protein content ranging from 80 to 350 g/kg [12], including a high proportion of essential amino acids [13]. To ensure its sustainable supply, cultivation methods have been developed for large-scale production [13], with commercial cultivation now established in Portugal under integrated multi-trophic aquaculture (IMTA) conditions. Regardless of the target application, processing macroalgal biomass requires pretreatment to reduce the complexity of the carbohydrate fraction, which remains challenging due to its unique polysaccharide composition. A key distinction between macroalgae and terrestrial plants lies in their polysaccharide profiles. While terrestrial plants predominantly contain starch, cellulose, hemicellulose, and lignin, macroalgae produce a range of unique polysaccharides, including alginate, carrageenan,

ulvan, mannitol, laminarin, and others [14]. This biochemical disparity poses challenges for conventional processing methods, particularly enzymatic hydrolysis, as the specific enzymes required to break down these marine polysaccharides are either unavailable or prohibitively expensive [2].

Several methods have been employed to disrupt macroalgal cells, including osmotic shock [15], mechanical grinding [16], ultrasonic treatment [16,17], chemical hydrolysis [17], and biological treatments [18]. Recent studies suggest that combining multiple treatments, such as the combination of alkaline or acid hydrolysis and enzymatic treatments, ultrasonic and microwave treatments, and mechano-chemical or mechano-biological treatments, can enhance cell wall disruption [19]. Chemical treatments using alkali and acid reagents have been proven effective for breaking down macroalgal polysaccharides by dissolving polymers and improving the accessibility of organic compounds [19,20]. This approach increases surface area exposure through fiber swelling and reduction in crystallinity [19,21]. However, chemical treatments require severe processing conditions and may generate inhibitory products, such as furfural, organic acids, and phenolic compounds, that can restrict the downstream process [20]. Moreover, the large-scale use of chemicals on an industrial scale poses environmental concerns, particularly due to the difficulties in managing and disposing of the produced effluents [22].

In contrast, biological treatment may represent a more sustainable and energy-efficient alternative. This approach utilizes enzymes to hydrolyze polysaccharides into smaller molecules and is primarily used to modify the structure and composition of biomass, thereby enhancing the bioavailability of macroalgae [19,23]. However, these treatments face challenges due to the heterogeneous composition of macroalgal cell walls and structure, enzyme-to-substrate specificity, and cost-effectiveness [19]. To overcome these limitations and enhance the efficiency of biological treatments, they are often combined with chemical or mechanical pretreatments, which improve enzyme accessibility and overall process effectiveness [19].

For *P. palmata*, alkaline treatments using sodium hydroxide (NaOH) under a low solid-to-liquid ratio (1:15, w/v) have been shown to promote cell disruption and enhance protein extractability [18]. Combining alkaline extraction with enzymatic hydrolysis, particularly using xylanase and cellulase, at low biomass-to-solvent ratios (1:20–1:80, w/v), has further enhanced protein recovery and extractability [24,25]. Heat-assisted processing has also been reported to increase cell disruption and the amount of amino acids released [26,27]. However, these approaches rely on a low solid–liquid ratio, resulting in phase separation, which hinders the efficient recovery of both liquid and solid fractions [28–30].

In contrast, high-solids loading processing ($\geq$15% solids, w/w), with minimal free water or other chemicals, presents a valuable advantage in biomass valorization by eliminating the need for separate fractions. This approach yields a single, concentrated fraction, thereby reducing solvent consumption, lowering production costs, and enhancing environmental sustainability [14]. High-solids loading treatments may enhance cell wall disruption while avoiding phase separation, allowing for the direct incorporation of a single phase and thereby increasing their potential for incorporation into animal feed or other applications [31]. However, reducing the amount of free water may decrease the effectiveness of hydrolysis due to increased viscosity, poor mixing, and limited water availability, which are essential for the depolymerization processes [32]. This high-solids loading approach has recently been applied to the chlorophyte macroalga *Codium tomentosum*, yielding promising results, including a decrease in fiber content and an increase in soluble protein and phenolic-related compounds [33]. Given the distinct polysaccharide composition of Rhodophyta, it remains unclear whether similar outcomes can be achieved in red macroalgae, such as *P. palmata*.

Recognizing the potential of *P. palmata* as an alternative ingredient for aquafeeds, the present study aimed to evaluate the effects of high-solids loading autoclave treatments using water or alkaline solutions, applied alone or followed by sequential enzymatic hydrolysis, on cell wall disruption and compositional changes in *P. palmata*. The study focuses on the structural modification and extractability of biomass components under industry-relevant, low-free-water conditions, providing a basis for future studies that directly assess digestibility and nutritional performance in fish.

2. Materials and Methods

2.1. Macroalgae Treatments

P. palmata was provided by Algaplus (Aveiro, Portugal), cultivated under the IMTA system, and used as an unwashed dried powder (<0.250 mm). Based on previous results reported by Harnedy and FitzGerald (2013) [18] and Idowu et al. (2024) [24], the alkaline hydrolysis conditions (alkali type and concentration) and the carbohydrase preparation used in this study were selected. Hydrolysis with water was used as the control. *P. palmata* was mixed with water or alkaline solutions at a ratio of 25% algae to 75% liquid (*w*/*w*) and autoclaved. The treatments that yielded the highest hydrolysis efficiency were selected for subsequent enzymatic hydrolysis. Following the treatments, the samples were dried at 40 °C for 24 h, ground to a particle size of 1 mm, and stored in sealed plastic containers at room temperature until further analysis. All treatments were performed in triplicate.

2.1.1. High-Solids Loading Water and Alkaline Autoclave Hydrolysis

High-solids loading, water, and alkaline autoclave hydrolysis were performed in triplicate. For each replicate, 100 g of dry *P. palmata* was used. The total moisture content was adjusted to 75%, taking into account the residual moisture of the dried biomass, using either water or 0.5N or 1N NaOH (pH 11). Samples were then autoclaved at 121 °C for 30 or 60 min (treatments labeled as H_2O-30, H_2O-60, Alk(0.5N)30, Alk(1N)30, Alk(0.5N)60, and Alk(1N)60, respectively).

2.1.2. Enzymatic Hydrolysis

Natugrain® TS-Feed Enzyme from BASF (NAT), a highly purified enzymatic complex containing endo-1,4-β-xylanase (5600 TXU/g) and endo-1,4-β-glucanase (2500 TGU/g), was used for the enzymatic hydrolysis. The enzymatic treatment was performed following the general procedure described by Fernandes et al. [34].

P. palmata, previously hydrolyzed with water or 1N NaOH for 30 min, was cooled to 40 °C before enzymatic treatment. The biomass was then subjected to enzymatic hydrolysis with 0%, 0.2%, or 0.4% NAT (*w*/*w*, dry biomass basis), corresponding to 0, 1120, and 2240 TXU xylanase, and 0, 500, and 1000 TGU of glucanase per 100 g of dry biomass, respectively. The enzyme was selected based on previous evidence of xylanase-assisted hydrolysis of *P. palmata* [12].

Before enzyme addition, the pH of the biomass was adjusted to 3.5 using 5N HCl. The enzyme suspension was prepared in phosphate buffer (pH 5.5, 0.4 M) and added to the biomass to increase the moisture content to approximately 80%, considering an initial moisture content of ~75%, resulting in a final reaction pH of approximately 5.0. For the no-enzyme controls, the same volume of phosphate buffer (without enzyme) was added to both water- and alkaline-treated groups. All samples were incubated at 40 °C for 6h with manual stirring every 30 min to ensure homogeneity.

Treatments were labeled as H_2O, H_2OEnz0.2%, H_2OEnz0.4%, Alk, AlkEnz0.2%, and AlkEnz0.4%, representing autoclaving with water; water autoclaving combined with 0.2%

or 0.4% NAT; alkaline autoclaving; and alkaline autoclaving combined with 0.2% or 0.4% NAT, respectively. Enzymatic hydrolysis treatments were performed in triplicate.

2.2. Chemical Analysis

The chemical composition of the samples was determined as follows. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) content were measured following the method of Van Soest et al. (1991) [35] using a Dosi-Fiber apparatus (J.P. Selecta, model 0361432). Crude protein (N × 6.25) was determined by the Kjeldahl method (Kjeltec system; digestor model 1015 and distillation models 1026; Tecator Systems, Höganäs, Sweden).

Soluble protein and Folin–Ciocalteu total phenolic content (GAE) were extracted using distilled water with a solid-to-liquid ratio of 1:5 (w/v) for 30 min at room temperature (≈25 °C), followed by centrifugation at 6500× g for 5 min. Soluble protein content was measured using the Bradford method [36].

Total phenolic compounds were determined using the Folin–Ciocalteu method, using gallic acid as the calibration standard, in accordance with Commission Regulation (EEC) No. 2676/90, and results are expressed as gallic acid equivalents (GAE). Because the Folin–Ciocalteu reagent reacts with a range of reducing substances in addition to phenolics, the values are interpreted as an estimate of total phenolic content (GAE) and overall reducing capacity rather than a definitive quantification of individual phenolic compounds.

2.3. Microscopic Analysis

The untreated and Alk(1N)30 *P. palmata* samples were attached to aluminum pin stubs using electrically conductive carbon adhesive tape (PELCO Tabs), and the excess tape was removed with compressed air. The samples were then placed on a Phenom Standard Sample Holder and examined in a desktop scanning electron microscope (Phenom ProX, Eindhoven, The Netherlands). The scanning electron microscopy was operated at 5 kV with a spot size of 3.3 for image capture. All the images were taken and processed using ProSuite software version 3.0.

2.4. Statistical Analysis

All data were checked for normality and homogeneity of variances and normalized when necessary, using a natural logarithmic (ln) transformation. Two-way ANOVA was conducted with solvent (water, NaOH 0.5N, and NaOH 1N) and autoclave duration (30 and 60 min) as fixed factors (Table 1), or solvent (water or NaOH 1N) and enzyme concentration (0%, 0.2%, 0.4%) as fixed factors (Table 2). Tukey's multiple range test was used to detect significant differences among means ($p < 0.05$). Non-orthogonal contrasts were used to compare the water and alkaline treatments with the untreated control. All statistical analyses were performed using the IBM SPSS Statistics software version 26 (IBM, NY, USA).

Table 1. Proximate composition of untreated and treated *Palmaria palmata* following high-solids loading autoclave treatment with water or alkaline solutions (0.5N or 1N NaOH) for 30 or 60 min (expressed on a dry matter basis).

Sample ID	Autoclave Time	Solvent	NDF (%)	ADF (%)	Crude Protein (%)	Soluble Protein (mg/g)	TPC (mg GAE/g)
Untreated	—	—	25.9	4.88	15.9	3.66	0.97
H_2O-30	30 min	H_2O	22.3	6.28	17.0	3.90	0.89
Alk(0.5N)30		0.5N NaOH	15.9	4.50	14.9	6.88	0.83
Alk(1N)30		1N NaOH	9.56	3.45	13.6	8.55	1.72

Table 1. *Cont.*

Sample ID	Autoclave Time	Solvent	NDF (%)	ADF (%)	Crude Protein (%)	Soluble Protein (mg/g)	TPC (mg GAE/g)
H_2O-60	60 min	H_2O	23.9	7.01	14.5	3.88	0.89
Alk(0.5N)60		0.5N NaOH	16.0	5.64	13.8	5.37	0.88
Alk(1N)60		1N NaOH	10.6	4.06	12.3	6.70	1.30
SEM			3.55	0.70	0.91	1.08	0.25

Non-orthogonal contrast (*p*-values)

	NDF	ADF	Crude protein	Soluble protein	TPC
Untreated vs. H_2O-30	0.000	0.000	0.015	0.622	0.839
Untreated vs. H_2O-60	0.001	0.000	0.008	0.622	0.761
Untreated vs. Alk(0.5N)30	0.000	0.079	0.048	0.000	0.685
Untreated vs. Alk(0.5N)60	0.000	0.006	0.000	0.005	0.839
Untreated vs. Alk(1N)30	0.000	0.000	0.000	0.000	0.043
Untreated vs. Alk(1N)60	0.000	0.004	0.000	0.000	0.318

Two-Way ANOVA	Variable Source			Solvent		
	Solvent	Time	Interaction	H_2O	Alk(0.5N)	Alk(1N)
NDF	***	*	ns	c	b	a
ADF	***	***	ns	c	b	a
Crude Protein	***	***	ns	c	b	a
Soluble Protein	***	**	ns	a	b	c
TPC	*	ns	ns	ab	a	b

Values are expressed as means (n = 3) with pooled standard error of the mean (SEM). NDF: Neutral detergent fiber; ADF: Acid detergent fiber; TPC: Folin–Ciocalteu total phenolic content (mg gallic acid equivalents/g). Two-way ANOVA: ns: non-significant ($p \geq 0.05$); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Different lowercase letters (a–c) indicate significant differences among treatments according to two-way ANOVA ($p < 0.05$); means sharing the same letter are not significantly different.

Table 2. Proximate composition of *Palmaria palmata* following high-solids loading autoclave pretreatments (121 °C, 30 min) with water or alkaline solution (NaOH, 1N), followed by sequential enzymatic hydrolysis using Natugrain® TS (dry matter basis).

Sample ID	Pre-Treatment	PBS ♦	Enzyme ♦♦	NDF (%)	ADF (%)	Crude Protein (%)	Soluble Protein (mg/g)	TPC (mg GAE/g)
H_2O	H_2O	Yes	-	25.1	6.94	15.2	3.63	0.79
H_2OEnz0.2%		Yes	0.2%	23.6	8.47	14.1	3.93	1.29
H_2OEnz0.4%		Yes	0.4%	23.8	7.39	13.2	3.90	3.76
Alk	1N NaOH	Yes	-	12.8	6.19	12.1	11.1	1.93
AlkEnz0.2%		Yes	0.2%	9.8	5.03	11.2	9.40	1.53
AlkEnz0.4%		yes	0.4%	10.7	4.90	11.8	9.69	4.26
SEM				4.15	1.08	0.93	1.91	0.81

Two-way ANOVA	Variable Source			Enzyme		
	Pre-treatment	Enzyme	Interaction	0	0.2%	0.4%
NDF	***	ns	ns	-	-	-
ADF	*	ns	ns	-	-	-
Crude Protein	***	ns	ns	-	-	-
Soluble Protein	***	ns	ns	-	-	-

Table 2. *Cont.*

Sample ID	Pre-Treatment	PBS ◆	Enzyme ◆◆	NDF (%)	ADF (%)	Crude Protein (%)	Soluble Protein (mg/g)	TPC (mg GAE/g)
TPC		ns		***	ns	a	a	b

Values are expressed as means (n = 3) with pooled standard error of the mean (SEM). ◆ Phosphate buffer included in all treatments at 1 mL/1 g (dry weight). ◆◆ Natugrain® TS-Feed Enzyme, BASF. NDF: Neutral detergent fiber; ADF: Acid detergent fiber; TPC: Folin–Ciocalteu total phenolic content (mg gallic acid equivalents/g). Two-way ANOVA: ns: non-significant ($p \geq 0.05$); * $p < 0.05$; *** $p < 0.001$. Different lowercase letters (a,b) indicate significant differences among treatments according to two-way ANOVA ($p < 0.05$); means sharing the same letter are not significantly different.

Heatmaps with hierarchical clustering were generated using the ClustVis online tool. Data were scaled by unit variance (row-wise Z-score normalization) before clustering. Hierarchical clustering was performed using Euclidean distance as the similarity metric and the average linkage method as the clustering approach. The color scale represents standardized values, with blue indicating lower and red indicating higher relative values [37].

3. Results

The autoclaving treatments significantly affected the proximate composition of *P. palmata* (Table 1). Compared to the untreated *P. palmata*, the NDF content decreased, while ADF and crude protein increased, following water treatments for 30 min; however, soluble protein and Folin–Ciocalteu total phenolic content (GAE) remained unaffected. With alkaline treatments, NDF was also reduced, as was ADF, except after Alk(0.5N)30 treatment.

Irrespective of the treatment, treatment time significantly increased NDF and ADF and decreased crude and soluble protein content. NDF, ADF, and crude protein were lower in the alkali treatments than in the water treatment and were consistently lower in the 1 N NaOH treatment than in the 0.5 N NaOH treatment. In contrast, soluble protein showed the opposite response.

The crude protein content was lower in all treatments than in the untreated *P. palmata*, except for the H_2O-30 treatment. Regardless of the treatment time, alkaline treatments yielded a lower crude protein content than water treatments. Additionally, increasing the autoclave time or NaOH concentration led to a higher reduction in protein content, regardless of the treatment.

Soluble protein content increased after alkaline treatments (0.5N and 1N) compared to untreated *P. palmata*, but not after water treatments. When comparing the alkaline treatments, increasing the normality of NaOH resulted in a higher soluble protein content, whereas increasing the autoclave time reduced soluble protein levels.

The Folin–Ciocalteu total phenolic content (GAE) increased after Alk(1N)30 treatment compared to the untreated *P. palmata*. When comparing water and alkaline treatments, the Folin–Ciocalteu total phenolic content (GAE) was higher after the Alk(1N) treatment than after the Alk(0.5N) treatment.

The heatmap analysis of the effects of water and alkaline treatments on the proximate composition of *P. palmata* revealed two major sample clusters (Figure 1). The first cluster was divided into two subclusters. The first subcluster grouped the untreated *P. palmata* and the H_2O-30 treatment, showing minimal changes in proximate composition. The second subcluster included the H_2O-60 treatment and the Alk(0.5N)60 treatment, which showed a slight increase in soluble protein and Folin–Ciocalteu total phenolic content (GAE) and a reduction in ADF and NDF contents. The second major cluster comprised samples subjected to alkaline treatments, which exhibited more pronounced compositional changes. This cluster was also divided into two subclusters: the first comprised the Alk(0.5N)30 treatment, which showed intermediate changes, and the second grouped the most intensive

alkaline treatments (Alk(1N)30 and Alk(1N)60), which led to the most significant alterations, including a marked increase in soluble protein and Folin–Ciocalteu total phenolic content (GAE) and a substantial decrease in ADF and NDF levels.

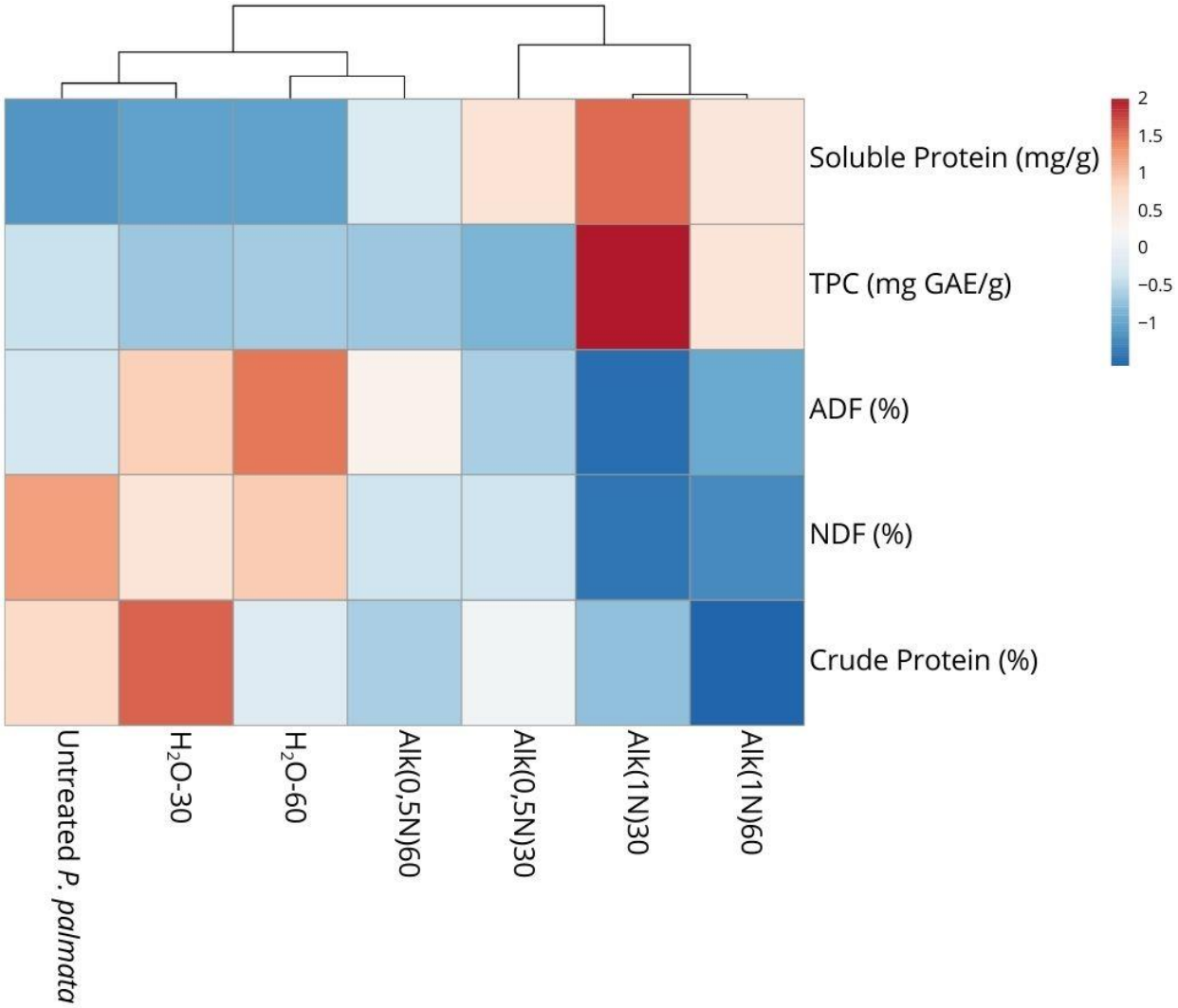


Figure 1. Heatmap of proximal composition (dry matter basis) of *Palmaria palmata*, either untreated or subjected to high-solids autoclave treatments with water, 0.5N NaOH, or 1N NaOH for 30 or 60 min (Alk(0.5N)30, Alk(1N)30, Alk(0.5N)60, Alk(1N)60). NDF: Neutral detergent fiber; ADF: Acid detergent fiber; TPC: Folin–Ciocalteu total phenolic content (mg gallic acid equivalents/g). Hierarchical clustering of samples is shown above the heatmap.

The high-solids loading water and alkaline (1N) autoclaving (30 min) treatments were selected for sequential hydrolysis with NAT, and the proximate composition of *P. palmata* submitted to these treatments is presented in Table 2. Enzymatic hydrolysis did not promote further changes in the measured parameters, except for an increase in Folin–Ciocalteu total phenolic content (GAE) with the 0.4 NAT treatments.

The heatmap analysis divided the treatments into two clusters. The first cluster is divided into two subclusters (Figure 2). The first subcluster grouped untreated *P. palmata* and the water treatment, indicating that autoclaving with water had a minimal impact on the composition of P. palmata. The second subcluster grouped water treatments followed by enzymatic hydrolysis (H_2OEnz0.2% and H_2OEnz0.4%). H_2OEnz0.2% showed a slight increase in ADF and reduced crude protein, while H_2OEnz0.4% exhibited a more pronounced decrease in crude protein and increased Folin–Ciocalteu total phenolic content

(GAE). The second cluster grouped the alkaline-treated treatments (Alk, AlkEnz0.2%, and AlkEnz0.4%) and is further divided into two subclusters. The first subcluster includes Alk and AlkEnz0.2%, which exhibited a substantial reduction in NDF and crude protein, accompanied by an increase in soluble protein. The second subcluster consists of AlkEnz0.4%, which differs from the other alkaline treatments due to its significantly higher Folin–Ciocalteu total phenolic content (GAE).

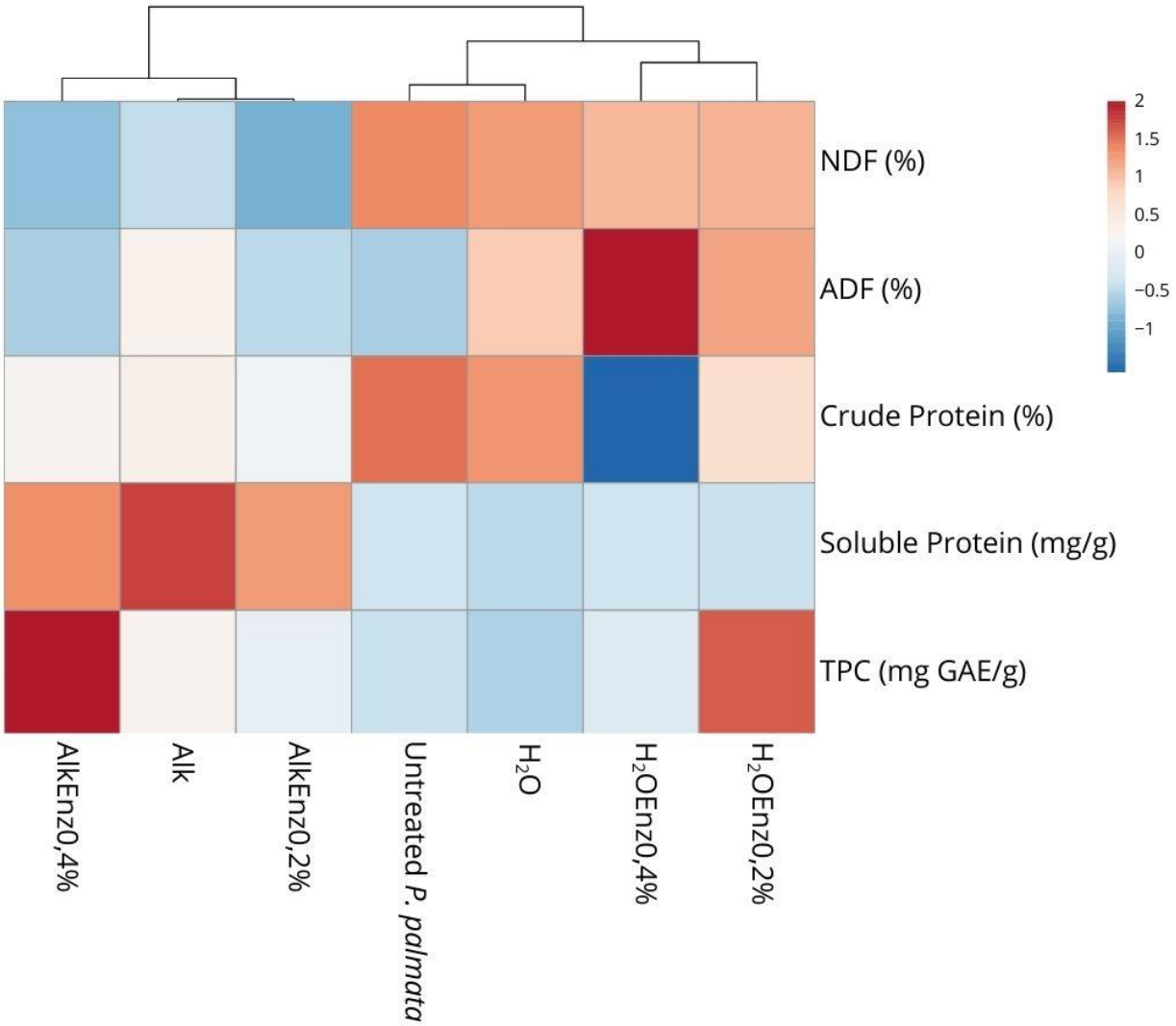


Figure 2. Heatmap of proximal composition (dry matter basis) of untreated and pretreated *Palmaria palmata* (water or 1 N NaOH autoclave, 121 °C, 30 min), followed by sequential enzymatic hydrolysis with Natugrain® TS (treatments: Untreated, H_2O, H_2OEnz0.2%, H_2OEnz0.4%, Alk, AlkEnz0.2%, AlkEnz0.4%). NDF: Neutral detergent fiber; ADF: Acid detergent fiber; TPC: Folin–Ciocalteu total phenolic content (mg gallic acid equivalents/g). Hierarchical clustering is shown above the heatmap.

The surface structure of untreated *P. palmata* and following Alk(1N)30 treatment is presented in Figure 3. The untreated *P. palmata* exhibited a thoroughly coated surface with visible salts and displayed a highly uneven structure (Figure 3a). Following Alk(1N)30 treatment, the *P. palmata* surface showed several cracks, albeit with a relatively mild presence (Figure 3b).

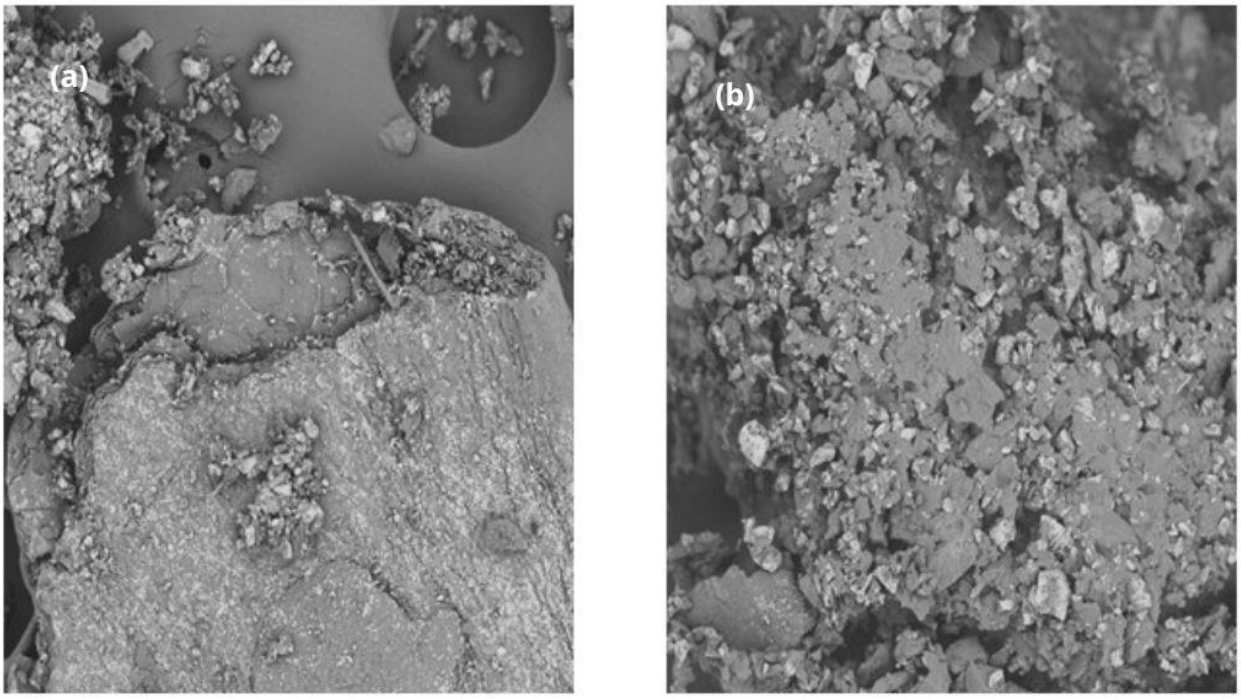


Figure 3. Scanning Electron Microscopy images (100 μm) of *Palmaria palmata*: (**a**) Untreated; (**b**) High-solids loading alkaline autoclaving treatment with 1N NaOH for 30 min (Alk(1N)30).

4. Discussion

The increasing demand for low-carbon-footprint ingredients for livestock feed has increased the interest in macroalgae as a sustainable feed ingredient. Macroalgae are rich in several bioactive compounds, making them a promising ingredient for producing new diets aligned with the total nutrition concept, which integrates animal growth requirements, disease resistance, overall health maintenance, and a reduced environmental footprint. However, the large-scale incorporation of macroalgae into animal feed remains challenging, particularly due to their high content of indigestible complex carbohydrates [38–42]. In non-ruminant animals, high dietary levels of these complex carbohydrates have been associated with reduced feed digestibility [43]. Therefore, processing strategies to hydrolyze structural macromolecules and alter macroalgal cell wall structure are considered essential for improving their suitability as feed ingredients, particularly by enhancing the accessibility and extractability of intracellular components [6]. *P. palmata* has a complex cell wall composed of cellulose, glucomannan, and hemicellulose compounds, including sulfated glucan (carrageenan and agar) and xylogalactan [44]. Various treatments have been applied to macroalgae to extract proteins and bioactive compounds and to produce agar and biofuels [45,46]. However, many of these treatments involve high energy and solvent consumption, raising concerns about their environmental impact and cost-effectiveness.

Hydrothermal treatment is commonly used for extracting sulfated galactans (agar and carrageenans) from the cell walls of red macroalgae due to its simplicity and the high water solubility of these compounds at high temperatures [47]. In the present study, high-solids loading autoclaving with water alone resulted in limited structural disruption of *P. palmata*, as evidenced by minor microscopic structure and solubilization of water-soluble fiber. Compared with untreated *P. palmata*, water autoclaving resulted in a 14% decrease in NDF and a 6.9% increase in crude protein content, while ADF content increased, indicating limited solubilization of the acid detergent fiber fraction. This apparent increase in ADF may be attributed to carrageenan aggregation into a gel-like structure during hot-water treatment, which can restrict water penetration and hinder hydrolysis during detergent

fiber analysis [14,48]. Moreover, regardless of the solvent used, longer autoclaving times resulted in higher apparent NDF and ADF values, indicating a time-dependent effect. These results may be related to increased viscosity and gel network development of sulfated galactans under high-solids (limited free-water) conditions, which can reduce extractability cooling [49]. In macroalgae, viscosity has been reported to increase with longer hot-treatment time [49]. For *P. palmata,* higher viscosities have also been reported at 50 °C and 90 °C compared with 70 °C, and after 60 min compared with 30 min of extraction [27].

The relatively low efficiency of the hydrothermal treatment of *P. palmata* was likely due to the limited availability of free water. When higher water-to-solid ratios were used (1:50), higher solubilization was achieved, although protein recovery was always relatively low. Indeed, water extracts of *P. palmata*, prepared at a ratio of 1:50 (g/mL) and subjected to temperatures of 50, 70, and 90 °C for 15, 30, or 60 min, showed a low protein content but a high carbohydrate content [27]. Similarly, the hydrolysis of *P. palmata* using a solid-to-liquid ratio of 1:50 and autoclaving at 124 °C for two 15 min cycles demonstrated a moderate yield of protein and a high carbohydrate content [45]. In contrast, boiling dried *P. palmata* for 15–30 min significantly increased protein bioaccessibility without affecting the amino acid profile, resulting in higher levels of available essential amino acids [25]. Furthermore, hydrothermal pretreatment of *Porphyra umbilicalis* by autoclaving using a solid-to-liquid ratio of 1:10 (*w*/*v*) in reverse osmosis water effectively hydrolyzed the biomass, leading to the release of 3.6–4.6 g/L of total sugars in the hydrolysate [50].

Although water hydrolysis is considered a more eco-friendly and cost-effective process, alkaline hydrolysis under low-solid loading conditions has proven to be more efficient in breaking down structural polysaccharides, promoting fiber degradation, and releasing bioactive compounds [50,51]. In particular, alkaline treatments are more effective for carrageenan hydrolysis, resulting in higher carrageenan yields and selectively removing components with lower gel strength compared to water-based methods [52]. In the present study, alkaline treatments under high-solids loading conditions significantly reduced the NDF and ADF contents, indicating an enhanced disruption of structural polysaccharides despite the presence of minimal free water. Scanning electron microscopy analysis revealed structural changes consistent with the reductions in cell wall disintegration. Similar morphological changes have been reported in other macroalgal species. For example, scanning electron microscopy images of alkaline-pretreated *Laminaria digitata* (Phaeophyceae), *Ulva linza* (Chlorophyta), or *Porphyra umbilicalis* (Rhodophyta) (1.25 N NaOH; 1:10 solid/liquid) also confirmed the disruption of the morphology of all these macroalgae, converting them into an amorphous biomass [27]. Additionally, scanning electron microscopy images of cellulose extracts from *Ulva lactuca* (Chlorophyta) revealed larger cellulose aggregations in the untreated samples, whereas the alkali-treated samples showed smoother surfaces with smaller nanocellulose particles [53].

Scanning electron microscopy of untreated *P. palmata* revealed a surface with visible salt deposits, consistent with the use of unwashed biomass. Macroalgae typically contain high mineral levels, including sodium, potassium, and chloride, and this inorganic fraction can constrain feed formulation, potentially affecting the performance and health of terrestrial animals [54]. In aquafeeds, the inclusion of macroalgae has been widely studied, and the replacement of fishmeal with up to 15% is generally well accepted by fish, although this may be higher in some species [10]. Therefore, quantification of ash and mineral content is an important next step when evaluating high-solids alkaline processing for aquafeed applications.

The disruption of structural polysaccharides is crucial for releasing cell walls and intracellular components, mainly proteins and bioactive compounds. In the present study, a significant increase in soluble protein and a decrease in crude protein content of *P. palmata*

were observed after alkaline treatments, which were more effective with a stronger NaOH concentration (1N) and longer treatment times, reflecting enhanced protein extractability rather than an increase in total protein content. Similarly, increasing NaOH concentration for milled oven-dried *P. palmata* or *Saccharina latissima* (Phaeophyceae) also increased protein yield [18]. Also, for *S. latissima*, it was observed that protein yield and protein solubility were higher when extracted at alkaline (NaOH, 1N) compared to the control method at neutral pH [55]. Nevertheless, excessive exposure to alkalis can negatively affect the protein structure and functionality [56].

Polyphenols are secondary metabolites with antioxidant, antimicrobial, and anti-inflammatory properties, and they have attracted interest in animal nutrition as functional feed components due to their potential effects on oxidative status, gut function, and performance [57]. Phenolic composition varies considerably depending on macroalgae species, chemical nature, storage conditions, and the presence of interfering substances [58]. The macroalgal cell wall matrix can limit the extractability and accessibility of these compounds. Therefore, different mechanical, physical, chemical, thermal, and enzymatic treatments have been reported to facilitate cell wall disruption, thereby improving the extraction and accessibility of total phenolic compounds [59]. In the present study, alkaline hydrolysis (1N NaOH) promoted the highest release of total phenolic content, as estimated by the Folin–Ciocalteu assay and expressed as gallic acid equivalents (GAE), in *P. palmata*. Because the Folin–Ciocalteu assay is not specific to phenolic compounds and responds to a range of reducing substances, the observed increases in total phenolic content are interpreted as changes in extractable phenolic-related compounds and overall reducing capacity rather than definitive evidence of increased individual polyphenols [60]. Similar trends have been reported for *Kappaphycus alvarezii* and *Eucheuma denticulatum* (Rhodophyta), where alkaline hydrolysis increased the release of total phenolic compounds [61]. Previously, aqueous extraction of *P. palmata* under different pH conditions (3, 6, and 9) showed that phenolic compounds were predominantly present in the liquid fraction, with alkaline extraction yielding the highest release of total phenolic compounds [62]. Although antioxidant activity was not directly measured in the present study, increases in phenolic compounds and reducing capacity have been associated with antioxidant potential in macroalgal ingredients, which may contribute to oxidative stress resilience and immune support in terrestrial animals [57] and fish [63].

Combining alkaline and enzymatic treatments is expected to promote macroalgal cell wall hydrolysis more efficiently. Specific enzymes targeting macroalgal polysaccharides are not yet available at a commercial stage; therefore, nonspecific enzymes, such as cellulases, hemicellulases, β-glucanases, and xylanases, which target the complex polysaccharide structures of macroalgae, have been used individually or as part of a multi-enzyme cocktail [64]. Previously, it was observed in *P. palmata* that protein extraction yield was higher with enzyme-assisted alkaline extraction [18,65,66]. For instance, studies employing Shearzyme® (a xylanase-based complex) in low-solid loading hydrolysis achieved a high protein extraction [67,68]. Similarly, enzymatic saccharification of *P. palmata* using Viscozyme® L, an enzyme complex including arabinase, cellulase, β-glucanase, hemicellulase, and xylanase, at low-solid loading hydrolysis also promoted the amount of extractable protein and total fatty acids [69]. Additionally, hydrolysis of *P. palmata* with an endo- and exo-peptidase complex (Umamizyme; 100 mg enzyme) at a low-solid loading also increased the total phenolic extract [70].

In the present work, water- or alkali-pretreated *P. palmata* was enzymatically hydrolyzed using a high-solids loading hydrolysis ratio and an enzymatic complex that included endo-1,4-β-xylanase and endo-1,4-β-glucanase, selected based on the carbohydrate profile of *P. palmata*, which is composed of xylose (57%; including mixed-linked

xylans, xylogalactans, and acid xylomannans) and galactose (22%) [71]. Contrary to what was observed in the low-solids hydrolysis, the enzymatic treatment in this study did not significantly affect the contents of NDF, ADF, crude protein, or soluble protein. However, Folin–Ciocalteu total phenolic content (GAE) release increased at the highest enzyme concentration. Although a xylanase-rich enzymatic complex was used, the degree of hydrolysis of *P. palmata* was lower than in previous studies employing xylanase-based enzymatic treatments. For example, alkaline pretreated *P. palmata* hydrolyzed with four enzymes (Celluclast® 1.5 L, Shearzyme® 500 L, Alcalase® 2.4 L FG, and Viscozyme® L) at 0.2% and 0.4% (*w*/*w*) using a 1:20 solid-to-liquid ratio showed that Shearzyme® (a xylanase-based complex) achieved the highest protein extraction [65]. In another study, the combination of cellulase (Celluclast®) and xylanase (Shearzyme®) at 24 U (24 U of cellulose and 24 U of xylanase) and a 1:6 solid-to-liquid ratio resulted in higher *P. palmata* protein extraction than the control (0 U) or lower enzyme concentration (12 U) [67]. Similarly, enzymatic saccharification of *P. palmata* using Viscozyme® L at a 5:100 solid-to-liquid ratio resulted in twice the amount of extractable protein and total fatty acids [68]. Additionally, hydrolysis of *P. palmata* with an endo- and exo-peptidase complex (Umamizyme; 100 mg enzyme) at a 1:25 solid-to-liquid ratio increased total phenolics from approximately 2.5 to 15.5 g GAE/kg extract [69]. Moreover, enzymatic pretreatment with xylanase and cellulase (from *Trichoderma longibrachiatum*), applied at 10, 50, or 100 U, further enhanced amino acid release, leading to a three-fold increase in extractable amino acids, and its combination with alkaline extraction resulted in higher protein recovery compared to conventional alkaline extraction [25]. The lower hydrolysis efficiency observed in the present study may be attributed to the high-solids loading in enzymatic hydrolysis. Indeed, enzymatic efficiency is influenced by substrate concentration and structure and composition of the macroalgal cell wall, enzyme–substrate interactions, and pretreatment conditions [71]. A limitation in macroalgal biomass processing is the use of commercial enzymes, which were initially designed for terrestrial biomass, that significantly differ in composition from macroalgae in their carbohydrate content [49]. Since macroalgae-specific enzymes are not yet commercially available, it is crucial to identify the specific enzyme activities present in these commercial mixtures and tailor their application to the unique carbohydrate composition of macroalgae [72]. Further studies are therefore required to optimize hydrolysis parameters, such as enzyme concentration and substrate ratio, and clarify the mechanisms of macroalgae cell wall degradation as influenced by different enzyme combinations and ratios.

5. Conclusions

In summary, high-solids loading alkaline autoclaving hydrolysis, especially with 1 N NaOH, resulted in the most significant reductions in NDF, ADF, and crude protein content, while increasing soluble and Folin–Ciocalteu total phenolic content (GAE), indicating an increase in these compounds. Sequential enzymatic hydrolysis, with 0.4% Natugrain® TS-Feed Enzyme, BASF, did not further reduce fiber and protein content but increased Folin–Ciocalteu total phenolic content (GAE). These results suggest that high-solids loading alkaline autoclave treatment is a promising approach for cell wall disruption in *P. palmata;* however, further optimization of the process is required in subsequent studies.

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Abbreviations

The following abbreviations are used in this manuscript:

NaOH	Sodium hydroxide
NAT	Natugrain® TS-Feed Enzyme from BASF
H_2O	Water-autoclaved
Alk	Alkaline autoclaved
H_2OEnz	Water-enzymatic autoclaved
AlkEnz	Alkaline-enzymatic autoclaved
ADF	Acid detergent fiber
NDF	Neutral detergent fiber
GAE	Gallic acid equivalents

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