

Article

Effectiveness of High-Solid Loading Treatments to Enhance Nutrient and Antioxidant Bioavailability in *Codium tomentosum*

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Abstract

Macroalgae have low nutrient bioavailability, often requiring pretreatments—physical, chemical, or biological—typically using low-solid loading hydrolysis, which produces separate liquid and solid phases. In contrast, high-solid loading hydrolysis offers a single-phase alternative, though it remains underexplored for macroalgae. This study evaluated the effectiveness of high-solid loading hydrolysis for breaking polysaccharides and increasing the availability of nutrients and antioxidant compounds in *Codium tomentosum*. Treatments using mixtures containing 25% dry biomass and 75% water or 0.5N and 1N NaOH, autoclaved for 30 or 60 min, were performed. Among the tested treatments, high-solid loading alkaline autoclaved treatment (1N NaOH, 60 min) was most effective in reducing neutral detergent fiber and enhancing the availability of bioactive compounds, particularly soluble proteins and phenols. Based on these results, a sequential enzymatic hydrolysis with Natugrain[®] at 0.2 and 0.4% was also applied to pre-treated *C. tomentosum* with water or 1N NaOH. Enzymatic hydrolysis after autoclaving had no major effect on fiber, soluble protein, or ash, but increased phenol levels. In conclusion, high-solid loading alkaline treatment (1N NaOH) followed by enzymatic hydrolysis with Natugrain[®] enzyme reduced fiber content and enhanced soluble protein and phenolic compounds, thereby improving the nutritional and functional potential of *C. tomentosum* for inclusion in animal feeds.

Keywords: alkaline treatment; bioavailability enhancement; enzymatic hydrolysis; high-solid loadings; macroalgae; polysaccharide fractionation



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1. Introduction

Macroalgae play a crucial role in marine ecosystems as a vital food source for herbivores and act as a natural filter in the ocean, contributing significantly to reducing the overall levels of carbon dioxide, phosphorus, and nitrogen while releasing oxygen into the environment [1,2]. Macroalgae contain variable protein levels (ranging from 5 to 47%) and lipids ($5.8 \pm 0.2\%$) that are rich in long-chain polyunsaturated fatty acids, particularly omega-3, which have recently positioned them as a promising feed ingredient for fish [3–5]. Furthermore, macroalgae contain several bioactive compounds, such as vitamins, minerals, pigments, and phenols, known for enhancing antioxidant capacity, immunity, and overall animal health [6,7]. Thus, macroalgae are also considered potential functional ingredients.

Carbohydrates are the predominant macronutrient in macroalgae, constituting about 30–75% of dry matter, and are mainly composed of recalcitrant polysaccharides, such as mannans, xyloglucans, and alginates [8]. The structural complexity of these polysaccharides limits macroalgae's nutritional value for monogastric animals, such as fish, which cannot digest them [3,9].

Targeted biorefinery processes are a potential strategy to address this challenge and ensure macroalgae as a valuable feedstock by increasing their nutrient and bioactive compound availability [9,10]. Selecting an appropriate treatment capable of rapidly and efficiently hydrolyzing macroalgae cell walls is essential to enhance digestibility and improve antioxidant and immunostimulant properties [9,11].

Biorefinery studies showed that polysaccharides can be efficiently hydrolyzed through processes such as alkaline, acid, or hydrothermal treatments [11–17] and that combining treatments enhances hydrolysis efficiency [18,19]. The efficacy of these treatments is greatly dependent on the temperature, hydrolysis time, pH, and total solid content, which are necessary to maintain a high pressure to ensure that water is maintained in the liquid phase [20]. Technological processes for macroalgal hydrolysis have been extensively studied for biorefinery applications, being generally operated at high temperatures (100 to 260 °C), for short residence times (typically 15–60 min), and operating at an acid or alkaline pH [21]. These treatments often use a low solid-to-liquid ratio, which can lead to phase separation and loss of valuable components in the liquid phase when only the solid fraction is recovered [18,22,23]. Conversely, implementing high-solid loading hydrolysis offers many advantages, such as producing a single phase, which facilitates downstream processing, and allows the utilization of partially hydrolyzed biomass. These processes enhance nutrient bioavailability, thereby increasing nutritional value, reduce production costs, and are considered more environmentally friendly compared to the low-solid loading hydrolysis [24–26]. Therefore, implementing treatments with high-solid loading represents a strategy for increasing biomass valorization. However, to date, this approach has not been applied to macroalgae.

Codium tomentosum (Chlorophyta, Bryopsidales) is a green macroalgae belonging to the Codiaceae family, characterized by its distinctive bushy appearance and velvety texture [27,28]. Its favorable nutritional profile, including moderate protein content and high levels of bioactive compounds, makes it a promising ingredient for animal feed [29], and it has attracted growing interest for this application. However, its cell wall complex matrix hinders the availability of nutrients and bioactive compounds, devaluing *C. tomentosum* use as a feedstuff [3,9,30]. Accordingly, this study aimed to enhance the bioavailability of *C. tomentosum* nutritional and bioactive compounds by applying combined treatment methods under high-solid loading hydrolysis conditions, thus contributing to the valorization of this macroalgae as a feed ingredient.

2. Materials and Methods

Codium tomentosum produced under Integrated Multitrophic Aquaculture (IMTA) conditions was supplied by Algaplus (Ílhavo, Aveiro, Portugal) in the form of unwashed dried powder (<0.250 mm). The macroalga was subjected to a two-step treatment process. In the first step, the samples were pre-treated by autoclaving under two different conditions: in water (H₂O) or in an alkaline solution of NaOH (Alk). In the second step, the most effective water- and alkaline-autoclaved treatments were selected for sequential enzymatic hydrolysis using specific enzymes to degrade complex polysaccharides. All treatments were performed in triplicate.

2.1. High-Solids Loading Water and Alkaline Autoclave Treatment

Dried *C. tomentosum* was mixed with water or 0.5N or 1N NaOH solutions at a solid-to-liquid ratio of 1:3 (25% solid and 75% liquid, *w/w*). After mixing, the pH of the slurry mixture was measured and confirmed to be approximately 11 for both alkaline solutions. The mixtures were autoclaved at 121 °C for 30 or 60 min, dried for 48 h at 60 °C, ground to 1 mm, and stored in sealed plastic flasks at room temperature until analysis. The resulting treatments were labeled as H₂O-30, H₂O-60Water, Alk(0.5N)30, Alk(1N)30, Alk(0.5N)60, and Alk(1N)60, respectively.

2.2. Enzymatic Hydrolysis Following High-Solids Water and Alkaline Autoclave Pretreatment

The water- and alkaline-autoclaved pre-treatments showing the highest yield of soluble protein and reduction in the fiber fraction, performed in Section 2.1, were selected for sequential enzymatic hydrolysis. A commercially available enzyme, Natugrain[®] TS-Feed Enzyme (BASF, NAT), containing highly purified endo-1,4-β xylanase (5600 TXU/g) and endo-1,4-β-glucanase (2500 TGU/g) was dissolved in 4 M phosphate buffer (pH 5.5) before use.

Pre-treated *C. tomentosum* (autoclaved with water or 1N NaOH, 1:3 solid-to-liquid ratio, 121 °C for 60 min, as described in Section 2.1) were subsequently submitted to a sequential enzymatic hydrolysis. After the pre-treatment, the mixture was cooled to 40 °C, and the pH was adjusted to 5.5 using 5 N HCl before the NAT enzyme addition. The NAT enzyme solution was then added at concentrations of 0.2% and 0.4% (*w/w* dry matter basis), referred to as AlkEnz0.2% and AlkEnz0.4%, respectively. These concentrations corresponded to 1120 and 2240 U of xylanase, and 2240 and 4480 U of glucanase per gram of dry *C. tomentosum*, respectively.

The mixtures were incubated at 40 °C for 6 h with manual stirring every 30 min to ensure homogeneous mixing. A negative control was included, in which water- or alkaline-autoclaved *C. tomentosum* was treated with a 4 M phosphate buffer without enzyme (NAT), designated H₂O and Alk treatment, respectively. At the end of the incubation, samples were dried for 48 h at 60 °C, ground to 1 mm, and stored in sealed plastic flasks at room temperature until analysis.

2.3. Proximate Composition

The chemical composition of untreated and treated *C. tomentosum* was determined as follows: moisture content by drying samples at 105 °C until constant weight; ash content by incineration in a muffle furnace at 505 °C for 2 h; total protein content (Nx6.25) after digestion with sulphuric acid (>95%) using a Kjeltex system (Tecator systems, Höganäs, Sweden, models 1026 and 1015, respectively); neutral detergent fiber (NDF) and acid detergent fiber (ADF) according to [31] using a Dosi-Fiber apparatus (J.P.Selecta, model 0361432).

For soluble protein and total phenolic compounds analysis, samples were mixed with distilled water (1:5 *w/v*) for 30 min at room temperature, centrifuged at 6500× *g* for 5 min, and the supernatant was stored at −20 °C until analysis. Soluble protein content was determined as described by [32].

Total phenolic compounds were determined according to the Folin–Ciocalteu's method [33], using caffeic acid as a standard, and the results are expressed in mg caffeic acid equivalents per gram of dry *C. tomentosum*.

2.4. Scanning Electron Microscopy

To evaluate *C. tomentosum*'s microscopic structure, samples were affixed to aluminum pin stubs using electrically conductive carbon adhesive tape (PELCO Tabs™), and the excess tape was removed using compressed air. The coated samples were then positioned on a Phenom Standard Sample Holder and examined in a desktop scanning electron microscope equipped with Phenom ProX and an EDS detector (Phenom-World BV, Eindhoven, The Netherlands). The microscopic analysis was carried out at 5 kV with a spot size of 3.3 µm for imaging. All images were captured and processed using ProSuite software version 3.0.

2.5. Data Analysis

Data were checked for normality and homogeneity of variances and normalized when necessary. Water, alkaline, water-enzymatic, and alkaline-enzymatic treatments were analyzed by two-way ANOVA, with solvents, time, and enzyme sections as fixed factors. Tukey's multiple range test was used to determine significant differences among means. If a significant interaction was detected, one-way ANOVA was performed to compare solvent effects within each time and to assess time effects within each solvent. Non-orthogonal contrasts were used to compare the untreated control with treatments. The probability level of 0.05 was used to reject the null hypothesis. All statistical analyses were performed using the IBM SPSS Statistics software version 26 (IBM, Armonk, NY, USA).

Two hierarchical clustering heatmaps were generated to integrate and discriminate the results and reveal the relationships among the variables. The first heatmap was based on data from the high-solids loading water and alkaline autoclave hydrolysis treatments, and the second was based on data from enzymatic hydrolysis following high-solids water and alkaline autoclave pretreatments. Data from three replicates per treatment were log-transformed prior to analysis. Both heatmaps were created using the free online tool ClustVis (<https://biit.cs.ut.ee/clustvis/>, accessed on 2 May 2025) [34].

3. Results

3.1. Effects of High-Solids Loading Water and Alkaline Autoclave Hydrolysis Treatment

The proximate composition of untreated and high-solids loading water- and alkaline-treated *C. tomentosum* is presented in Table 1.

Compared to the untreated algae, H₂O-30 and H₂O-60 treatments increased NDF and ash content (contrasts 1 and 2). The Alk(0.5N)30 treatment increased NDF and ash but decreased phenol content (contrast 3), while the Alk(0.5N)60 treatment led to a reduction in ADF and crude protein and an increase in ash content (contrast 4). Both Alk(1N)30 and Alk(1N)60 treatments decreased NDF, ADF, and crude protein, while increasing soluble protein, phenol levels, and ash content (contrasts 5 and 6).

Solvent and autoclaving time affected all parameters measured except soluble protein, which was influenced only by solvent and not by autoclaving time.

The Alk(1N) treatment led to a reduction in NDF and crude protein contents. ADF values also decreased in both Alk(1N) and Alk(0.5N) treatments, with Alk(1N) showing the highest levels of soluble protein and phenols. Increasing NaOH concentration resulted in higher ash content. While soluble protein and ash contents were unaffected by autoclaving time, NDF, ADF, crude protein, and phenol contents were altered. Specifically, NDF, ADF, and crude protein contents decreased, whereas phenol content increased after 60 min of autoclaving. A significant interaction between solvent concentration and autoclaving time was observed for ADF and ash contents: higher NaOH concentration combined with longer autoclaving led to lower ADF and higher ash values.

Table 1. Proximate composition of untreated and treated *Codium tomentosum* 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).

Treatment	Time	Solvent	NDF (%)	ADF (%)	Crude Protein (%)	Soluble Protein (mg/g)	Phenols (mg/g)	Ash (%)
Untreated	—	—	22.04 ± 0.4	6.35 ± 0.4	21.22 ± 0.8	4.10 ± 0.1	0.92 ± 0.0	36.51 ± 0.8
H ₂ O-30		H ₂ O	29.08 ± 0.7	7.01 ± 0.2 ^c	21.44 ± 0.3	3.85 ± 0.1	0.69 ± 0.2	38.94 ± 1.4 ^a
Alk(0.5N)30	30 min	0.5N NaOH	25.03 ± 0.6	5.57 ± 0.7 ^{b,B}	20.26 ± 0.5	4.18 ± 0.2	0.65 ± 0.2	44.28 ± 0.5 ^b
Alk(1N)30		1N NaOH	14.39 ± 0.7	4.22 ± 0.4 ^{a,B}	19.10 ± 1.6	7.36 ± 0.5	2.46 ± 0.2	51.23 ± 2.4 ^c
H ₂ O-60	60 min	H ₂ O	27.46 ± 0.7	6.71 ± 0.9 ^b	20.63 ± 0.4	4.03 ± 0.0	0.95 ± 0.1	38.86 ± 0.1 ^a
Alk(0.5N)60		0.5N NaOH	20.65 ± 0.6	3.41 ± 0.3 ^{a,A}	19.66 ± 0.3	4.59 ± 0.4	1.26 ± 0.1	42.58 ± 1.1 ^a
Alk(1N)60		1N NaOH	13.58 ± 0.2	2.50 ± 0.3 ^{a,A}	16.77 ± 0.3	8.68 ± 1.4	3.86 ± 1	57.24 ± 2.5 ^b
Non-orthogonal contrast ¹								
		NDF	ADF	Crude Protein	Soluble Protein	Phenols	Ash	
1. Untreated vs. H ₂ O 30		0.000	0.140	0.726	0.624	0.055	0.041	
2. Untreated vs. H ₂ O 60		0.000	0.403	0.353	0.893	0.842	0.046	
3. Untreated vs. Alk(0.5N)30		0.023	0.088	0.139	0.873	0.027	0.000	
4. Untreated vs. Alk(0.5N)60		0.253	0.000	0.022	0.340	0.066	0.000	
5. Untreated vs. Alk(1N)30		0.000	0.000	0.004	0.000	0.000	0.000	
6. Untreated vs. Alk(1N)60		0.000	0.000	0.000	0.000	0.000	0.000	
Two-way ANOVA								
		Variable Source ²				Solvent		
		Solvent	Time	Interaction	H ₂ O	Alk0.5	Alk1	
NDF		***	*	ns	c	b	a	
ADF		***	***	*	b	a	a	
Crude Protein		***	*	ns	b	b	a	
Soluble Protein		***	ns	ns	a	a	b	
Phenols		***	***	ns	a	a	b	
Ash		***	ns	*	a	b	c	

Values represent the mean ± standard deviation ($n = 3$). NDF: neutral detergent fiber; ADF: acid detergent fiber. ¹ Non-orthogonal contrast comparing untreated *C. tomentosum* with treated samples for 30 min (contrasts 1, 3, 5) and 60 min (contrasts 2, 4, 6). ² Two-way ANOVA: ns, non-significant ($p > 0.05$); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Means with different lowercase letters differ between solvents ($p < 0.001$); means with different uppercase letters differ between times within each solvent ($p < 0.01$).

The heatmap analysis of the proximate composition of the water and alkaline treatments identified two clusters (Figure 1). The first cluster grouped the untreated *C. tomentosum*, both H₂O treatments, and Alk(0.5N)30, based on lower soluble protein, phenolic, and ash contents and higher crude protein, NDF, and ADF values. The second cluster grouped the Alk(0.5N)60 and both Alk(1N) treatments based on higher soluble protein, phenol, and ash content and lower crude protein, NDF, and ADF values.

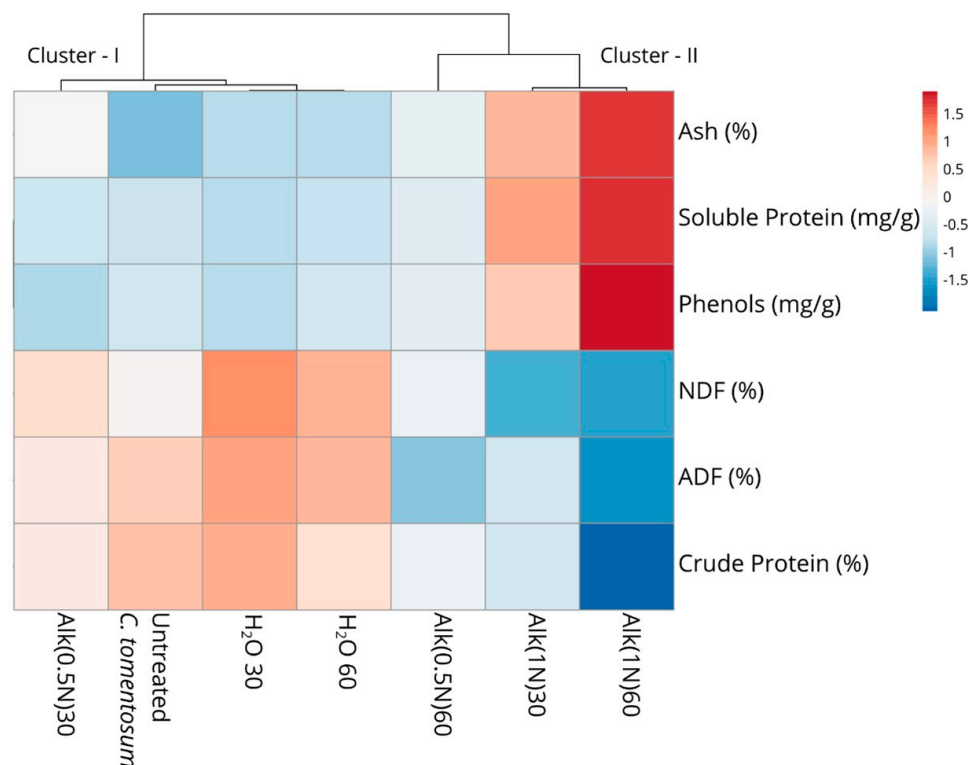


Figure 1. The heatmap illustrates the hierarchical clustering of traits (column-wise) and *Codium tomentosum* untreated, water autoclaved (H₂O), or alkaline autoclaved (Alk) for 30' and 60' treatments (row-wise). The rows and columns represent the traits and treatments, respectively. Colors are representative of a relative scale of −1.5 to 1.5, which is derived from the data normalization of the proximal composition of *C. tomentosum* untreated, water autoclaved, and alkaline autoclaved hydrolysis values. The dark blue indicates lower values, while the dark red indicates higher values.

3.2. Effects of Enzymatic Hydrolysis Following High-Solids Water and Alkaline Autoclave Pretreatment

The proximate composition of *C. tomentosum* submitted to high-solids water- and alkaline-based autoclaving followed by enzymatic hydrolysis is presented in Table 2.

Regardless of the sequential enzymatic hydrolysis, the type of solvent (water vs. 1N NaOH) affected NDF, crude protein, soluble protein, phenol, and ash contents, while ADF content remained unchanged. Alkaline-pretreated samples led to higher soluble protein, phenolic compounds, and ash contents, and lower NDF and crude protein values when compared to water-based treatments, independent of enzymatic supplementation.

Enzymatic hydrolysis following high-solids autoclaving influenced only crude protein and phenol levels, with no significant effects observed on NDF, ADF, soluble protein, or ash levels. The AlkEnz0.2% treatment resulted in the highest crude protein content, while the AlkEnz0.4% treatment showed the highest phenol content.

Table 2. Proximate composition of *Codium tomentosum* following high solids loading autoclave pretreatments (121 °C, 60 min) with water or alkaline solution (NaOH, 1N), followed by sequential enzymatic hydrolysis using Natugrain® TS (dry matter basis).

Treatment	Solvent	Enzyme	NDF (%)	ADF (%)	Crude Protein (%)	Soluble Protein (mg/g)	Phenols (mg/g)	Ash (%)
H ₂ O	H ₂ O	—	33.5 ± 0.3	8.69 ± 3.5	21.6 ± 0.7	4.48 ± 0.1	0.83 ± 0.1	40.4 ± 0.0
H ₂ OEnz0.2%		0.2%	31.0 ± 1.7	6.56 ± 3.7	19.8 ± 0.4	3.99 ± 0.1	0.81 ± 0.1	38.1 ± 0.7
H ₂ OEnz0.4%		0.4%	25.6 ± 9.3	4.74 ± 2.1	20.7 ± 0.5	3.92 ± 0.1	2.20 ± 0.5	39.6 ± 1.8
Alk	1N NaOH	—	18.1 ± 0.0	3.40 ± 0.3	18.4 ± 0.3	7.54 ± 0.4	0.83 ± 0.2	44.8 ± 0.0
AlkEnz0.2%		0.2%	17.3 ± 1.5	3.96 ± 1.3	16.6 ± 0.6	7.28 ± 0.7	0.90 ± 0.1	47.4 ± 0.5
AlkEnz0.4%		0.4%	17.4 ± 0.9	3.15 ± 0.2	17.3 ± 0.4	8.27 ± 1.2	3.10 ± 0.7	47.0 ± 1.5
Two-way ANOVA	Variable Source ¹							
	Solvent	Enzyme	Interaction		0	Enzyme	0.4%	
NDF	**	ns	ns		-	-	-	
ADF	ns	ns	ns		-	-	-	
Crude Protein	***	**	ns		b	a	ab	
Soluble Protein	***	ns	ns		-	-	-	
Phenols	**	***	ns		a	a	b	
Ash	***	ns	ns		-	-	-	

Values represent the mean ± standard deviation ($n = 3$). NDF: neutral detergent fiber; ADF: acid detergent fiber. ¹ Two-way ANOVA: ns, non-significant ($p > 0.05$); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

The heatmap analysis based on the proximate composition of *C. tomentosum*, untreated or following high-solids water and alkaline autoclave pre-treatments, with or without subsequent enzymatic hydrolysis, highlighted two clusters (Figure 2). The first cluster grouped the alkaline treatments, with or without subsequent enzymatic hydrolysis (Alk, AlkEnz0.2%, and AlkEnz0.4%). This cluster was grouped based on lower crude protein, NDF, and ADF contents and higher phenol, soluble protein, and ash levels. The second cluster included the untreated *C. tomentosum* and the water autoclaved samples with or without subsequent enzymatic hydrolysis (H₂O, H₂OEnz0.2%, and H₂OEnz0.4%). This cluster is based on higher crude protein, NDF, and ADF contents, and lower soluble protein, phenol, and ash contents.

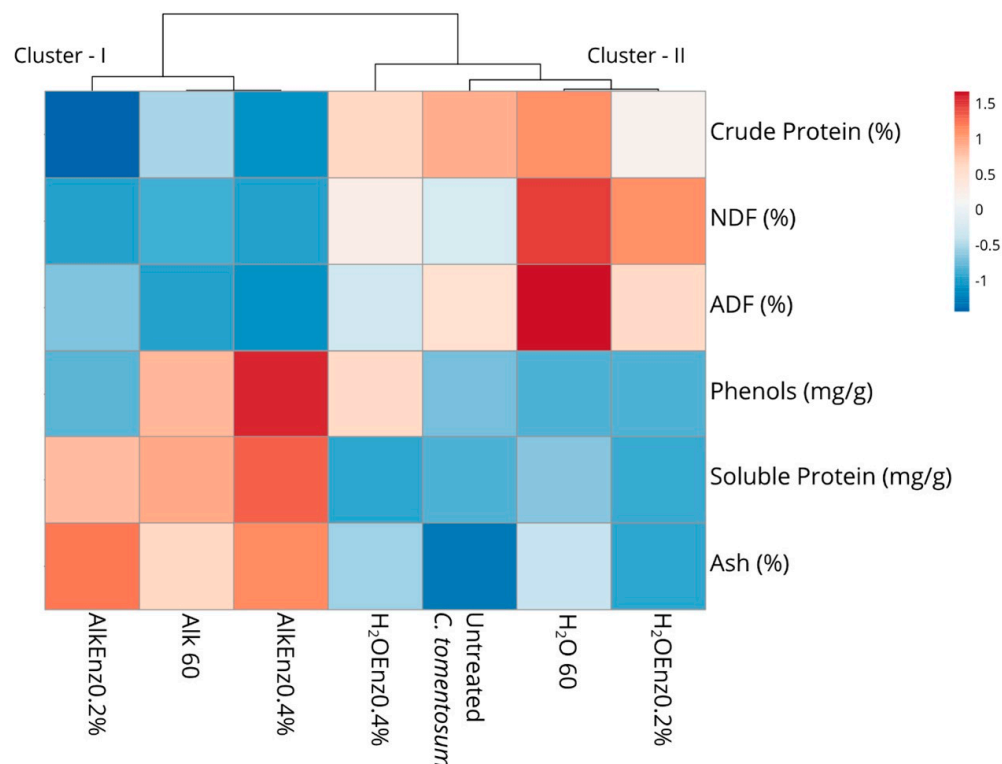


Figure 2. The heatmap illustrates the hierarchical clustering of traits (column-wise) and *Codium tomentosum* untreated, water autoclaved (H₂O), alkaline autoclaved (Alk), water enzymatic autoclaved (H₂OEnz), or alkaline enzymatic autoclaved (AlkEnz) treatments (row-wise). The rows and columns represent the traits and treatments, respectively. Colors are representative of a relative scale of −1.5 to 1.5, which is derived from the data normalization of the proximal composition of *C. tomentosum* untreated, water-enzymatic autoclaved, and alkaline-enzymatic autoclaved hydrolysis values. The dark blue indicates lower values, while the dark red indicates higher values.

3.3. Microscopic Structural Morphology

The surface structure of *C. tomentosum* before and following selected treatments is presented in Figure 3. The selection of this analysis was dependent on the results presented in Tables 1 and 2, of which only those that demonstrated the most promising results for the application in animal feed of this macroalgae were selected.

The untreated *C. tomentosum* exhibited a thoroughly coated surface with visible salts and displayed a highly uneven structure (Figure 3a). Following the Alk(1N), the *C. tomentosum* surface showed several cracks (Figure 3b,c), with a relatively milder presence of cracks when Alk(1N)60 was implemented (Figure 3c).

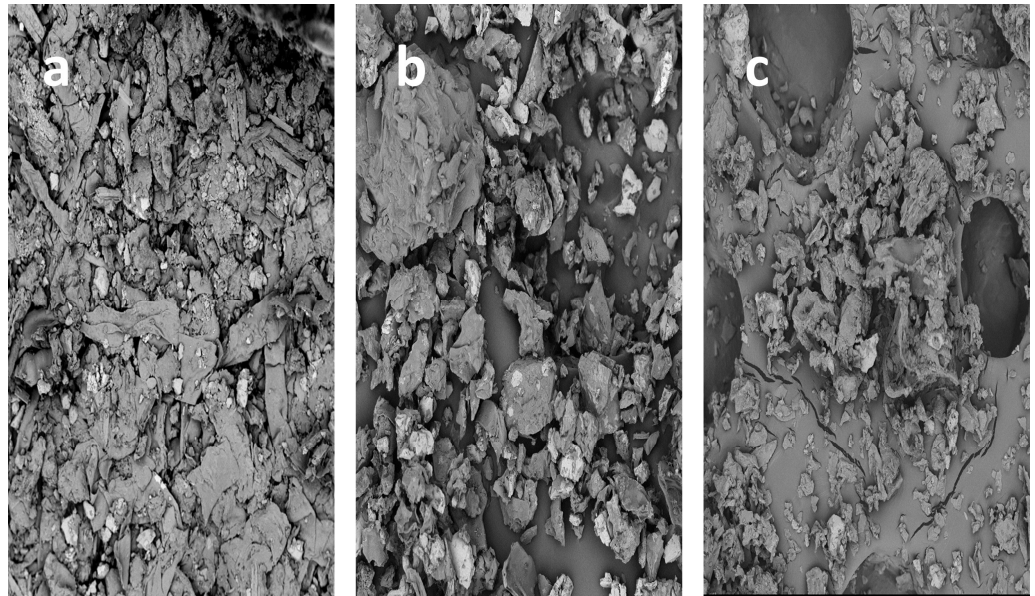


Figure 3. Scanning Electron Microscopy images (100 μm) of *Codium tomentosum*: (a) untreated; (b) high-solid loading alkaline autoclaved treatment with 1N NaOH for 30 min; (c) high-solid loading alkaline autoclaved treatment with 1N NaOH for 60 min.

4. Discussion

Macroalgae represent a renewable and sustainable animal-feed commodity with a favorable nutritional profile, a moderate protein level, and a high concentration of bioactive compounds that can boost animal health. However, their value as a feed ingredient is often limited by the high content of indigestible compounds, primarily associated with the resistant cell wall [7,35]. To fully unlock the nutritional benefits of macroalgae, it is therefore essential to employ processing methods that modify the resistant cell wall structure and improve the bioavailability of these valuable compounds [8]. So far, processing methods typically yield two fractions with distinct compositions and industrial applications: a liquid fraction, containing solvent-soluble components such as phenolic compounds and soluble proteins, and a solid fraction, rich in proteins and lipids and residual insoluble carbohydrates [3].

The present study implemented a high-solid loading hydrolysis approach to valorize the entire *C. tomentosum* biomass using minimal to no free water. This method results in a single, nutrient-concentrated fraction, retaining all biomass components and avoiding phase separation that commonly occurs in conventional treatments with low solid-to-liquid ratios. The resulting slurry can be directly incorporated into feed formulations, eliminating the need to recover separate liquid and solid hydrolysates. High-solid loading also has other advantages, including lower use of solvents, reduced energy consumption, and improved environmental sustainability [36]. However, the limited availability of free water under these conditions may compromise hydrolysis efficiency by increasing viscosity, reducing mass transfer, and hindering enzyme-substrate interactions. Despite these potential limitations, this strategy presents a promising and underexplored approach for macroalgae bioprocessing, especially in applications such as animal feed, where concentration and sustainability are key considerations [24,37,38].

Macroalgae cell walls are complex and rich in polysaccharides, with a substantial proportion of dietary fibers [39]. In green macroalgae such as *Codium spp.* (Chlorophyta, Bryopsidales), the cell wall is particularly complex, containing sulphated polysaccharides with structurally diverse backbones [40]. Unlike Ulvales or Ulotrichales, which are rich in uronic acids and rhamnose, members of the Bryopsidales are characterized by sulphated

xyloarabinogalactans with little to no detectable uronic acids or rhamnose [41]. This biochemical complexity may hinder enzymatic accessibility, making hydrothermal or chemical pretreatments essential for biomass valorization [40].

In the present study, the high-solids loading water and alkaline autoclave hydrolysis of *C. tomentosum* showed that, compared with untreated biomass, water-based high-loading autoclaved hydrolysis increased NDF content, while ADF content was unaffected. The higher NDF content observed after the water-based high-loading autoclaved hydrolysis may be due to alterations in the solubility of fiber components. For instance, water-soluble non-starch polysaccharides and pectins may become insoluble after autoclaving, increasing the overall NDF content [42,43].

Previously, using the green macroalgae *Ulva rigida*, it was shown that hydrolytic autoclave treatment (121 °C for 30 min, using a solid/liquid ratio of 1:5 *w/v*) decreased NDF and increased ADF content compared with untreated *U. rigida* [3]. The disparity between these two studies may be associated with differences in cell wall polysaccharide composition in the two macroalgae, since *U. rigida* has a higher content of specific polysaccharides, such as ulvan, while *C. tomentosum* contains more mannans and xyloglucans. Additionally, variations in the amount of water used during hydrolysis may also contribute to the differences observed. Water not only serves as a reaction medium but also plays a critical role in facilitating hydrolytic reactions through its physicochemical properties. Under certain thermal conditions, changes in water's density, dielectric constant, and ionic product can significantly affect the depolymerization of biomass, influencing the solubilization and breakdown of polysaccharide structures [44]. The different compounds and chemical bonds may explain the differences in the solubilization of neutral components [30,45]. Further, the molecular transformation of dietary fiber and polysaccharides at high temperatures (90–130 °C) can produce lower molecular weight compounds, as shown in brewers' spent grain [46]. In brewers' spent grain, the conversion of insoluble to soluble fractions can occur due to the weakening of the bond between lignin and phenolic groups, affecting the dietary fiber of the product and increasing the lignin solubility, leading to an increase in NDF and ADF content [47].

The predominant treatment methods for macroalgae commonly involve a synergetic application of thermal and chemical processes for their convenience, efficiency, and cost-effectiveness [48,49]. Several heating techniques have been employed in treating cellulose and hemicellulose, with moist heat under pressure in an autoclave considered one of the most effective methods. In fact, the application of pressure is essential to maintaining water in a liquid state at temperatures higher than 100 °C, ensuring a proper heat transfer across the algal biomass. Autoclave treatment reduces the crystallinity of cellulose, making it more susceptible to chemical treatment [50,51].

Both acid and alkaline treatments have been used as thermochemical treatments of macroalgae to improve hemicellulose breakdown and consequently increase the release of reduced sugars, antioxidants, and phenolic compounds [52–56]. Recently, it was observed that, compared to acid treatment, alkaline treatment, without autoclave, and using a solid/liquid ratio of 1:5 *w/v*, was more efficient for increasing soluble protein, phenolic compounds, and antioxidant activity of *Ulva rigida* [3]. Similarly, the present study observed that, compared to the high-loading autoclaved hydrolysis using water or NaOH, the alkaline hydrolysis decreased NDF and ADF contents while increasing soluble protein and phenolic concentration. Further, the Alk(1N) hydrolysis led to higher soluble protein and phenolic compounds than the Alk(0.5N) hydrolysis. Previous research has reported that macroalgae require extensive technological processing to recover high-value compounds, including phenolic constituents [57]. For green macroalgae, alkaline treatments have been shown to effectively disrupt the polysaccharide matrix of the cell wall, enhancing polyph-

nol release and antioxidant capacity, as observed in *Caulerpa cylindracea* [58]. Accordingly, increased alkaline concentration has been positively correlated with reductions in hemicellulose and cellulose levels [59]. NaOH is particularly effective in breaking the ester bonds between hemicellulose and cellulose, preventing the fragmentation of hemicellulose polymers [60]. A study with *Ulva fasciata* reported significant alterations in cellular morphology after NaOH pre-treatment (solvent-to-solid ratio of 25:1 *v/w*), including cell wall degradation, increased porosity, and a larger surface area [61]. These effects are attributed to removing hemicellulose, which disrupted the cellulose-hemicellulose network and weakened the hydrogen bonds, stabilizing the cellulose structure [62].

Macroalgae are beneficial feed sources due to their rich abundance of essential nutrients such as vitamins, proteins, carbohydrates, trace minerals, and other bioactive compounds [63]. Some proteins in macroalgae are intricately bound to non-protein constituents, including polyphenols and polysaccharides, thereby negatively impacting protein bioavailability [64]. In the current study, high-loading autoclaved hydrolysis with 1N NaOH increased soluble protein while decreasing the crude protein content. This may be attributed to the hydrolysis of the complex and robust cell wall, facilitating protein extraction and the partial hydrolysis of proteins into more soluble forms [64]. In *Ulva rigida*, alkaline treatment with 1N NaOH and a high biomass-solvent ratio at 1:10 also led to the highest protein concentration [63].

Different macroalgae have been explored for their protein quantity and quality [65]. Studies showed that the extractability and availability of proteins can differ according to the applied method, macroalgae species, and macroalgae season [66,67]. In this study, high-loading autoclaved hydrolysis of *C. tomentosum* with 1N NaOH, independently of the autoclavation duration, presented the most promising results. Scanning electron microscopy is a powerful tool for evaluating modifications in the superficial structure before and after treatments. In the present study, *C. tomentosum* treated with Alk presented a modification of the cellular structure, notably evident by the several cracks observed. Previously, laser scanning confocal microscopy images of *C. tomentosum* submitted to thermal alkaline treatment (4 wt% NaOH, 80 °C, 2 h) showed the disintegration of fibers into distinct microfibrils with smaller and uniform diameters, indicating the complete removal of non-cellulosic substances, thus resulting in the presence of individual micro-sized cellulose fibers [68]. Similarly, alkaline treatments in *Ulva rigida* [3], *Porphyra umbilicalis*, *Ulva linza*, and *Laminaria digitata* [54] resulted in several cracks in the surface, indicating the destruction of the fiber structure.

Enzymatic hydrolysis can be applied to macroalgae as a unique treatment [69] or in combination with other treatments [3] to improve nutrient bioavailability. However, a lack of studies on the structures of sulfated polysaccharides from green macroalgae makes it challenging to select the most indicated enzyme capable of disrupting their cell wall [70]. For instance, *Codium* spp. contains different cell-wall carbohydrates depending on the species, including xylan, cellulose, and sulfated polysaccharides (e.g., sulfated galactan; sulfated mannan) [70].

The present study used NAT commercial enzymatic complex, rich in non-starch carbohydrate enzymes (NSPase), to treat selected hydrolyzed samples. The NAT enzymatic complex, containing xylanase and glucanase, likely facilitates cell wall breakdown. The results of the present study indicate that the enzymatic hydrolysis following high-solids water and alkaline autoclave pretreatment with 0.4% NAT efficiently increases phenol content in *C. tomentosum*. Although these results are promising, the enzymatic composition of NAT may not be optimal for hydrolyzing macroalgae cell wall carbohydrates. Additionally, the high-solid loading approach implemented in this study—characterized by a reduced liquid-to-biomass ratio—may have limited enzyme accessibility to the substrate, potentially

compromising hydrolysis efficiency [71]. Exploring more specific enzyme complexes for degrading *C. tomentosum* cell walls will be the next step toward more effective biological treatment. Optimization of this process will involve adjusting enzyme dosage, incubation period, and liquid-to-biomass ratio [72].

5. Conclusions

In conclusion, the present study indicates that high-solid loading alkaline treatment (1N NaOH) followed by enzymatic hydrolysis with Natugrain® enzyme is the most effective method for hydrolysis of *C. tomentosum*, resulting in reduced fiber content and increased concentrations of soluble protein and phenolic compounds. Furthermore, complementing this high-solid loading alkaline with enzymatic hydrolysis using a 0.4% (*w/w*) NAT enzyme complex further enhanced the release of phenols.

Future studies should aim to optimize key processing parameters, such as NaOH concentration, retention time, and the composition of the enzymatic complex, to further improve the degradation of *C. tomentosum* polysaccharides. Additionally, the effect of dietary incorporation of *C. tomentosum* pre-treated with the high-solid loading alkaline treatment (1N NaOH) should be evaluated to determine its effects on feed digestibility and fish zootechnical performance. Moreover, due to the enhanced release of phenolic compounds, the functional potential of this hydrolyzed biomass when incorporated into aquafeeds requires further investigation.

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Abbreviations

The following abbreviations are used in this manuscript:

NaOH	Sodium hydroxide
NAT	Natugrain® TS-Feed Enzyme from BASF
H ₂ O	Water-autoclaved
Alk	Alkaline autoclaved

H ₂ OEnz	Water-enzymatic autoclaved
AlkEnz	Alkaline-enzymatic autoclaved
ADF	Acid detergent fiber
NDF	Neutral detergent fiber

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