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# Integral valorization of *Sargassum muticum*

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## ABSTRACT

Marine macroalgae represent an excellent material to be used as biogas producer, adsorbent, biostimulant and fertilizer for soils, or feedstock. The success in the exploitation of seaweeds depends on their characteristics, and the approach used to separate their specific active components. In the context of circular economy, invasive species are a good candidate for exploitation, and biorefinery a key valorization technique. Here we investigate a novel biorefinery scheme for a fuller valorization of the alien species *Sargassum muticum.* An initial pressing stage allowed the production of a Sap fraction, which showed potential as a plant biostimulant, increasing both root development and shoot/root ratio, especially when used at a dose of 0.1 g/L lyophilized Sap. The solids after pressing were processed by non isothermal autohydrolysis, using pressurized hot water under subcritical conditions (120-210 ºC), previously optimized to solubilize the fucoidan and phlorotannin fractions. The residual solids remaining after pressing and autohydrolysis stages were evaluated for the production of biogas. The obtained value (150 mL $CH_4$/g residual solids at 150 ºC) is significantly higher than that found for the raw seaweed. The optimal autohydrolysis temperature (150 ºC) is compatible with the production of the fucoidan fraction, although the phenolic content is favoured at stronger operation conditions. We also discuss the possibility of preparing adsorbents for pollutant removal and mineral amendments from the autohydrolysis waste solids.

**Keywords:** seaweed, biorefinery, biostimulants, biogas

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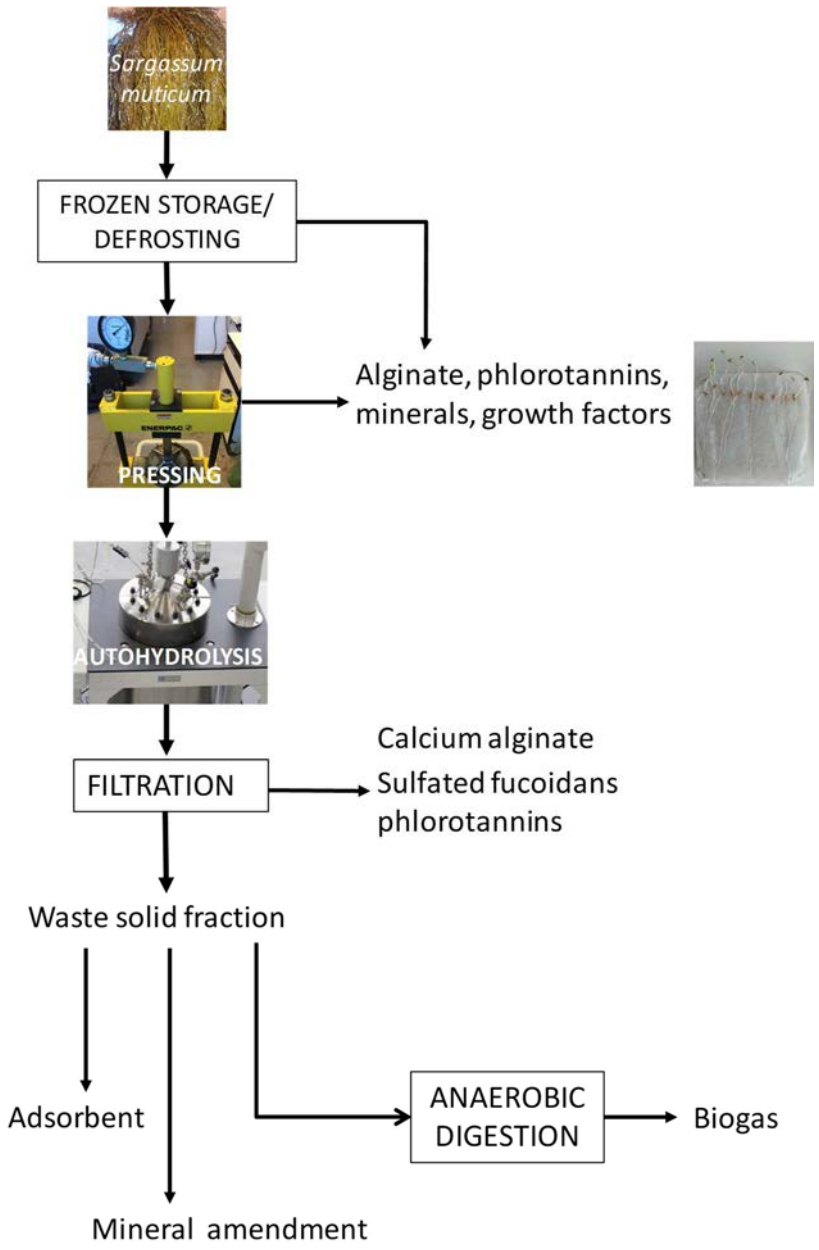
Sargassum muticum
FROZEN STORAGE/ DEFROSTING
PRESSING
Alginate, phlorotannins, minerals, growth factors
AUTOHYDROLYSIS
FILTRATION
Calcium alginate
Sulfated fucoidans
phlorotannins
Waste solid fraction
Adsorbent
ANAEROBIC DIGESTION
Biogas
Mineral amendment

# 1. Introduction

Seaweeds can be used as feed, for sheep [1] and goat [2], and for fish in aquaculture [3]. Yet their most common applications are to be spread on agricultural land as fertilizer, and composting [4].

The use of algae as a fertilizer is very common since ancient times due to numerous benefits for crops and soils. Seaweeds, especially brown algae, are an excellent fertilizer, rich in macro- and micronutrients, with a high potassium concentration, and similar nitrogen content to most types of animal manures [5, 6]. The organic matter and phycocolloids content of macroalgae increases the water retention and cation exchange capacity of soils, and modifies their structure, which improves the soil quality. In addition, seaweeds also show biostimulant effects as anticipated based on their content of complex polysaccharides, fatty acids, phenolics, vitamins and phytohormones [7-9]. Since the mid-20$^{th}$ century the use of seaweed as biostimulants has been receiving a special attention in the modern agriculture [7, 10, 11]. This biostimulant effect influences the physiology of plants with important benefits such as improved germination, nutrient absorption, or yield and crop quality, and general plant development [7, 12]. There are a range of examples in literature that demonstrate the beneficial effects of seaweeds on germination, increasing the percentage of germinated seeds, the length of the roots and the root/shoot ratio [13, 14]. In particular, there are several studies conducted with biostimulants specifically prepared using *Sargassum* species [15, 16]. In this context, the dose of biomass required is very small, and the fertilizer effect of the algae is therefore limited.

In biorefinery processes, the liquid phase obtained from mechanical pressing of the milled washed fresh seaweed, termed Sap, is often used as a biostimulant. Sap can be used to substitute part of the recommended dose of conventional fertilizers. As a result, it confers resistance to plant diseases, and enhances the growth, yield and quality of crops, especially when applied as a foliar spray [17-19]. Although Sap extraction from red seaweed is frequently reported, further research is needed, particularly involving invasive species [20].

*Sargassum muticum* (Yendo) Fensholt, is an invasive macroalga on European and American West coasts [21]. Its eradication with conventional techniques, for example handly, trawl or cut, has been unsuccessful, and seasonal harvesting was therefore proposed as a control strategy [22].

*Sargassum muticum* has been successfully used for the adsorption of heavy metals [23], phenols [24], dyes [25], the treatment of industrial wastewaters [26], and the recovery of valuable elements, such as gold [27,28] .

Despite seaweeds are a suitable biomass for anaerobic digestion, different authors have confirmed the low methane potential of *S. muticum* (166-208 mL $CH_4$ per gram of volatile solids), and its limited efficiency due to the accumulation of non-biodegradable solids [29]. Milledge et al. [30, 31] have found that alginic acid and its sodium salt were recalcitrant to anaerobic digestion, and also confirmed that the presence of polyphenols is the cause of the low yields of biogas during the anaerobic digestion of *S. muticum*. An antimicrobial effect was described for low molecular weight phlorotannins [32], and also for the extracts obtained by washing the surface of seaweeds, which are probably produced by bacteria living on the algae surface [33]. However, the removal of components present on the surface of seaweeds did not improve the methane yield; indeed, surface washing procedures can result in delayed biomethane production [30]. Milledge et al. [31] have suggested that a preliminary treatment for the breakage of recalcitrant polymers may improve biogas production, although the associated energy cost can be high.

The separate valorization of *S. muticum* constituents following a green biorefinery approach has been proposed using a hot pressurized water processing (autohydrolysis) of the wet biomass [34, 35]. This technique allows higher solubilization yields than ultrasound-assisted [36, 37] and enzyme-assisted extractions [38]. Furthermore, autohydrolysis proved suitable as a pretreatment to enhance enzymatic susceptibility before bioethanol production from *S. muticum* [39] del Río et al., 2019). Therefore, a multistage processing of *S. muticum* following a biorefinery technique to separate different bioactives would thereby provide economic benefits [40]. In addition, the biogas production could be enhanced by this approach, which involves the removal of alginate and provides with a lower salt concentration of pretreated solids compared to the raw seaweed [29] . The influence of the temperature of the autohydrolysis on the solubilization of bioactives from *S. muticum* has been previously evaluated [34, 35], although the impact on the energetic and soil applications are still unknown.

Here we perform a detailed investigation to evaluate the potential of a biorefinery of *Sargassum muticum*, after initial stages aimed at extracting a Sap fraction with agricultural applications, and obtaining high pressure water soluble extracts with phenolic, alginate and fucoidans fractions. In the context of the circular economy, the remaining waste solids were evaluated for biogas production, and their use as mineral amendment in agriculture was also discussed.

## 2. Materials and methods

### 2.1. Materials

*Sargassum muticum* was collected in Praia da Mourisca, Pontevedra, Spain (42.22°N, 8.77°W) in Summer 2018, washed with tap water, ground and stored in plastic bags at -18 °C until use.

### 2.2. Pressing

The drained liquid phase separated during pressing at 2 MPa (Enerpac RC106, Wiscosin, USA) was processed separately from the liquid phase released during defrosting at room temperature. The first fraction was lyophilized and stored until use. The alginate presents in the Sap fraction was rheologically analysed. Aqueous dispersions of the alginate precipitated with $CaCl_2$ (1% w/w) were formulated at a commonly used biopolymer concentration (2.0 g/L) [41]. The alginate in the liquid phase was precipitated by adding 1% (w/w) $CaCl_2$ (Acros Organics), and separated by centrifugation at 4500 rpm for 40 minutes (Rotixa 50RS, Hettich Zentrigugen, Germany). Viscoelastic features were determined on a controlled stress rheometer (MCR302, Paar Physica, Austria) with a plate-plate geometry (25 mm diameter, 1 mm gap) at 25 °C. Frequency sweeps (from 0.1 to 10 Hz) were made at linear viscoelastic regime (1.2 Pa).

### 2.3. Autohydrolysis

The pressed solids (Smp) were contacted with water at a liquid:solid ratio of 30:1 (wt) and the suspension was heated up to the final selected temperature, in the range from 120 to 220 °C in a pressurized stirred reactor (Parr Instr., Il., USA). After reaching the final heating temperature, the reactor was cooled and the suspension was vacuum filtered.

### 2.4. Anaerobic digestion

Anaerobic assays were carried out in bottles of 126 and 50 mL of liquid volume following the head-space gas analysis method described by Soto et al. [42]. All assays were carried out in duplicate at 30 °C in a thermostatic chamber on a shaker at 150 rpm.

The medium for all assays was prepared with distilled water, a volume of anaerobic sludge calculated to obtain 2 g VS/L, macro and micronutrients at a ratio of 1 mL of the stock solutions defined by Ferreiro and Soto [43] per L of final media. 100 mg/L of

$Na_2S \cdot 9H_2O$ and 2 g/L of $Na_2CO_3$ were also added to the assay medium, in order to obtain an anoxic medium and sufficient buffer capacity, respectively. Diluted solutions of HCl and NaOH were used to regulate the pH to the range of 7.0–7.1. The inoculum used was a mixture of two anaerobic sludges obtained from a pilot plant treating domestic wastewater and a full-scale plant treating fish canning wastewater. 50 mL of this assay medium was transferred to each assay bottle. Then, the substrate corresponding to each test bottle was added, the head space was bubbled with nitrogen, and the bottle was closed with a septum lid.

A volatile fatty acid (VFA) mixture (acetic acid, 1.01 g/L; propionic, 0.30 g/L and n-butyric, 0.24 g/L) was used as substrate for the control assays, while blank assays contained the assay medium without substrate. An alga residual solids (RS) concentration of 3 g TS/L was used as substrate in the assays for biological methane potential (first feeding). Parallell assays were carried out for raw *Sargassum muticum*. A second feeding was carried out by renewing the VFA substrate of the control assays and adding 5 g TS/L of alga residual solids to the treatment assays. Prior to the second feeding, the pH of all assay media was corrected to the range of 7.0-7.1.

The composition of gas phase samples (0.5 mL) was determined on a gas chromatograph equipped with a thermal conductivity detector. Each feeding lasted about 40 days. The specific methane potential (SMP) of each substrate sample was obtained from the final cumulative methane production after subtracting the blank value and dividing by the amount of dry alga residual solids. An additional correction factor was obtained from the control assays, consisting of the ratio between the final cumulative methane production in the control assays, after subtracting the blank value, and the theoretical methane production of the VFA added as substrate. For the present study, the Control correction factor was 0.95 for both the first and second feeding.

The maximum methane production rate for each assay was obtained from the slope of cumulative methane production curves during the exponential phase of methane production after each feed [42]. The methane production rate was expressed as the percentage of each treatment assay referred to the control assay, and also as the percentage of the second feeding to the first feeding as well. Finally, the percentage of the methane produced during the exponential phase, referred to the final methane production, was obtained.

### 2.5. Plant growth stimulant effect

To assess the potential of the Sap as a biostimulant, a germination test was carried out following the UNE EN 16086-2 standard [44]. For this purpose, four dilutions of the

lyophilized pressing liquid were used: 1 g/L; 0.1 g/L; 0.01 g/L; 0.001 g/L, and 0 g/L as control.

For each treatment, three Petri dishes with perlite were prepared. A sheet of filter paper was placed on them and was moistened uniformly with 50 mL of the respective solution. Ten cress seeds (*Lepidium sativum*) were placed on the filter paper and incubated by placing them between 70º and 80º in relation to the horizontal, in the dark at 25 ± 5 ºC for 72 h. After this time, the following parameters were determined:

RL: average root length per plant (mm)
SL: average shoot length per plant (mm)
R/S: rooth:shoot ratio
GD (%): germination degree of each treatment (% respect to total seeds)
RI (%): root Index, root development compared to control
MLV (%): Munoo-Liisa vitality Index, which compares the product of the germination degree (GD) by the average root length (RL) in the samples with the control.

$$MVL(\%) = \frac{GDs \times RLs}{GDc \times RL\,c}\ x\ 100$$

where:

GDs is the degree of germination of each replica of a treatment
GDc is the average germination degree of the control
RLs is the average length of each replica of a treatment
RLc is the average length of the three replicates of the control treatment

**2.6. Analytical methods**

The sample of *Sargassum muticum* was introduced in a laboratory oven at 105 °C for 24-48 hours, and moisture content was subsequently determined by gravimetric assay. Ash content was determined after calcination of the samples at 575 °C for 6 hours.

The content of oligosaccharides in the seaweed and in its solid residue obtained by autohydrolysis were determined after a post-hydrolysis step using sulfuric acid (4%) at 121 °C for 60 min. After post-hydrolysis the solid residue and liquid phase were filtered, the samples of liquid phase were analysed once filtered through 0.45 µm membranes. The saccharide composition was determined in a 1100 series Agilent equipment. Glucose, rhamnose, fucose, acetic acid and formic acid, also galactose (gal) + xylose (xyl) + mannose (man) were determined using an Aminex HPX-87H column (BioRad, Hercules, CA) operating at 60 °C with 0.003 M $H_2SO_4$ at 0.6 mL/min. The solid residue after post-hydrolysis was studied gravimetrically and quantified as acid insoluble residue (AIR).

Nitrogen content was measured in the seaweed samples using the Kjeldahl method. Minerals and heavy metals were determined as followed. At first, an acid digestion using nitric acid was necessary being the operation conditions: 1600W, 15 minutes and 200 °C for 10 minutes (Marsxpress-CEM Corporation, USA). Sodium and potassium were determined by atomic emission spectrophotometry (AES). Calcium, cupper, magnesium, manganesium, mercury, chromium, cadmium, lead, iron and zinc were determined by atomic absorption spectrophotometry (AAS). In both cases, the equipment used was 220 Fast Sequential Spectrophotometer (Varian, USA). All samples were analysed in triplicate.

Ultraviolet absorbance (ABS215) of anaerobic digested medium was measured in a 1 cm quartz cell at 215 nm, at pH 6 (0.2 M $KH_2P0_4$ buffer) by diluting samples to levels lesser than 0.8 absorbance units, as described previously [45]. A similar procedure was followed to determine the color, but operating at 440 nm (ABS440). In both cases the samples were previously filtered through a membrane of 0.45 μm. Others analysis were carried out following Standard Methods [46].

## 3. Results and discussion

### 3.1. Liquid phases

Frozen storage could be an approach for the integral and yearly-around utilization of *Sargassum muticum*. This storage and stabilization strategy avoids degradation of bioactives that occur with thermal drying [47]. A multistage water-based process consisting on the separation of Sap from the defrosted seaweed, and the subsequent autohydrolysis of the solid phase has been previously developed [41] and is the first stage of the proposed process (Figure 1).

#### 3.1.1. Sap

Seaweed Sap was extracted by pressing the defrosted seaweed with a yield of 0.1 L/kg of fresh *Sargassum muticum*; subsequently, the liquid obtained was lyophilized, achieving a yield of 199 g/L liquid Sap. Therefore, the final ratio was 20 g of lyophilized Sap per kg of *Sargassum muticum*. The characterization of the lyophilized Sap is shown in Table 1. The electrical conductivity, used as a measurement of the salt content, was 756; 85.6; 19.4; 3.5 and 2.1 μS/cm for Sap doses of 0.1; 0.01; 0.001 and 0 g/L, respectively.

It should be noted that this extraction and conservation process allows the correct preservation of biostimulant compounds that may be present, since these can be altered or denatured during storage, especially auxins that are heat sensitive [48]. The Sap provides an alginate fraction after precipitation with calcium chloride, which exhibited similar viscoelastic features (G', elastic modulus: 0.2 Pa and G'', viscous modulus: 2 Pa, Figure 2) to the alginate commercially available, in agreement with previously reported values [41]. However, in the present study, no separation of alginate from Sap was done, with the aim of using the whole liquid fraction for agricultural purposes.

As fertilizer, the Sap is particularly notable for its content in K. Indeed, there is a strong preconcentration of this element during the preparation process of the Sap, with percentages of K increasing from 2.3% in the raw material to 11.6% in the final fraction (Table 1). A fertilizer obtained from Sap would therefore be of interest in potato and fruit crops, whose demand for K is very high. However, due to the low yield of the process (2% in dry weight), it is more convenient to explore the use of Sap as a plant biostimulant, since the dose as biostimulant is much lower than the required as fertilizer.

The response of the stem development to the Sap treatment presents a significant and positive linear correlation between the dose used and its growth (Pearson 0.258; sig, 0.01), with significant differences relative to untreated controls at doses of 0.1 and 1 g/L. In contrast, the effect of the Sap dose in the root suggests a parabolic growth-dose correlation, with an apparent maximum around 0.1 g/L (Figure 3). This dose and also 0.01 g/L produced significantly higher root development relative to controls. For doses higher than 0.1 g/L a decrease on the root length was observed, although the growth fall below the control value. The highest root:shoot index occurred with a dose of 0.01 g/L, which was significantly higher than the obtained with 1 g/L and the control value. A high root:shoot index allows plants to be more effective in extracting nutrients from the deeper layers of the soil and influences the maturity of the crop as a whole [16].

Seaweed contains precursors of germination-inducing compounds [5]. However, no statistically significant responses to any germination treatment relative to unamended controls were found (Figure 4). This was mostly due to increased germination rates in all the treatments, including the control.

A similar trend as for the length of the roots was observed for the root index (RI%). Improved growth occurs as the dose increases up to 0.1 g/L, achieving a 30% increase relative to control, whereas with a higher dose (1 g/L), this improvement was only 8%, with no significant differences relative to control (Figure 4). It should be appreciated

that there was no growth inhibition with any of the tested doses. Indeed, when the root length and germination parameters are jointly evaluated using the Munoo-Liisa vitality index (MLV%), the 0.01 and 0.1 g/L doses provided the highest promotion of root growth.

The beneficial effect of seaweed on germination may be due to the biostimulant compounds that algae contain, including brassinosteroids, and phytohormones such as auxins and cytokinins [49]. Yet the mechanisms of action of these bioactive compounds are still not well resolved [12]. In fact, some authors such as Wally et al. [50] concluded that the hormone-like activity, after the application of algae, is due to an alteration in the biosynthesis of endogenous phytohormones rather than to the contribution of exogenous phytohormones present in the extracts.

The decline in the beneficial effect of biostimulants when increasing the dose has been reported in several previous studies [15, 16, 51, 52]. In our study, this could either be a result of i) increased salinity values between treatments at 0.1 and 1 g/L dose, or ii) an excess of the biostimulant substances of the algae. We observed a clear increase in conductivity with values from 0.09 to 0.75 dS/m. Most plants are especially sensitive to salinity during germination and the onset of development [53]. For example, Hernández-Herrera et al. [16] observed a decline in the development of tomato seeds treated with extracts of *Sargassum liebmannii* that was attributed to the increase in salinity. Alongside salinity effects, the decrease observed at the highest dose may be due to dose effects. Biostimulants promote growth at low concentrations, while it is inhibited under high dose values [7].

### 3.1.2. Liquid extract produced by autohydrolysis of pressed solids

The solids that remained after pressing were processed by autohydrolysis at different temperatures (120-210 ºC). This treatment was previously proposed for the simultaneous extraction and depolymerization of fucoidan and for the extraction of the phenolic fraction [34, 35]. The optimal autohydrolysis temperature varies significantly depending on the target compound. Highest fucoidan and sulfate content in the liquid phase was obtained at heating temperatures of 175 ºC, whereas the phlorotannin extraction yield and concentration in the extract present a maximum at 220 ºC [35]. Further fractionation and concentration of the fucoidan fraction using membrane technology provided products with more than 80% fucoidan content that present antioxidant and antitumoral properties [54]. Furthermore, phlorotannin enriched products can be obtained by adsorption-desorption onto polymeric resins [38] or with ethyl acetate [41].

The application of a previous pressing stage led to slightly lower extraction yields during autohydrolysis, although the phenolic concentration in the extracts was enhanced [41]. Therefore, the decision on the optimal autohydrolysis conditions should considered both the production of bioactives (fucoidan, phlorotannins) and the energetic or agricultural valorization of the residual solids, which has not been previously evaluated.

### 3.2. Solid streams

#### 3.2.1. Biogas

**Evolution of methane production**

The evolution of methane production in all assays is shown in Figure S1 (supplementary material). The latency phase varied between 5 and 45 h in the first feeding and was virtually nil in the second feeding. The exponential production phase lasted between 350 and 700 h in the first feeding, depending on the assay, and was slightly shortened in the second feeding (range 290-525 h). The final methane production was obtained after 982 h and 956 h in the first and second feeds, respectively.

The blank assay recorded higher methane production in the first feeding (13 mL $CH_4$) than in the second feeding (5 mL $CH_4$). The VFA control trial yielded a final amount of methane, once blank methane production was discounted, of 105.6% and 106.5% of the theoretical expected from the added VFA. A correction factor of control of 0.95 was derived from both values.

The final methane production from the duplicate assays was very similar in each feeding, with coefficients of variation in the range of 1.7-5.7% (mean 3.6 ± 1.8%) in the first feeding, and 0.4-6.7% (mean 3.0 ± 2.5%) in the second feeding. Thus, accurate measurements of SMP were obtained.

**Specific methane potential**

The SMP of the tested substrates is shown in Figure 5a. Statistical differences between first and second feedings did not exist at a level of 0.01, except for RS120 ($p = 0.045$), the residual solids obtained at heating temperatures of 120 ºC. On the other hand, considering the mean values of both feedings, SMP was statistically different for all substrates at a p-level of 0.05, except for *S. muticum* and RS120 ($p = 0.07$) and for RS120 and RS190 ($p = 0.59$). The differences even persist between most substrates at a p-level of 0.01 (Figure 5a).

The raw alga *S. muticum* reached 85 mg $CH_4$/g TS, which equals 102 mL $CH_4$/g VS. Soto et al. [29] reported SMP for algae harvested in the same region ranging from 83 to 138 mL $CH_4$/g TS (or 166 to 208 mL $CH_4$/gVS). Thus, SMP for the raw alga used in the present study was in the lower part of the reported data, even when considering a large literature review worldwide (100-225 mL $CH_4$/gVS) [29, 30, 55-58] . Milledge and Harvey [58] claimed that methane yield from *S. muticum* is low and proposed further research to establish the reasons.

SMP for all residual solid substrates was higher than for *S. muticum*, showing the large increases of 76.4% and 55.4% for RS150 and RS170, respectively. RS120 and RS190 showed reduced increases of 14% (not significant) and 18%, while the SMP for RS210 was 36% higher than that of *S. muticum*. Thus, the maximum SMP of 149 mL $CH_4$/g TS (170 mL $CH_4$/g VS) corresponded to RS150.

Soto et al. [29] reported an average chemical oxygen demand (COD) content of 0.53 g/g TS for three *S. muticum* samples collected in the region. Considering the same factor for the substrates in this study, methane yield would be within 42-75% of theoretical yield. The methane potential for *S. muticum* (42% of the theoretical) was in the range of 37-62% reported by Soto et al. [29] while the methane potential for some of the residual solids was clearly higher, as indicated by the values of 70% and 75% for RS170 and RS150, respectively.

The higher methane yield of residual solids can be attributed to the effect of thermal hydrolysis pre-treatment. Several biomass pre-treatment methods are available to modify the substrate structure and increase the bioavailability of polysaccharides for their hydrolysis to sugars. These included mechanical pre-treatments such as washing and size reduction, thermal, chemical and biological pre-treatments or a combination of various methods [59]. These authors reported that methane yields improve between 19% and 68% after the use of some of these pre-treatments for the breakdown of biomass structures. However, in some cases null or even negative effects of pre-treatment on SMP have also been registered [57, 59].

There are very few studies about the effect of pre-treatments on anaerobic digestions of *S. muticum*. Milledge et al. [30] reported that washing did not affect methane yield from *S. muticum*, but that methane production was delayed, probably due to loss of readily digested substrates or removal of hydrolytic bacteria from seaweed surfaces. In the same way, ensiling did not show significant effect on methane yield [58]. Various types of thermal pre-treatment (hydrothermal, steam explosión, microwave…) have been reported for different algae spp. but not for *S. muticum* [59, 60]. On the

other hand, higher methane yield for microalgae residual cake was also reported after oil extraction [61, 62].

In the present study, *S. muticum* undergone washing, grinding, and frosting (-18 °C) and defrosting, while residual solids were obtained after additional pressing for alginate extraction and thermal autohydrolysis for fucoidan and phenolic fraction extraction. Despite the reduction of these compounds, the residual solids produced significantly higher methane volumes. According to Milledge et al. [30] (2018), alginic acid and sodium alginate present low methane yields while phenolic compounds can cause hydrolysis inhibition.

**Methane production rate**

After the latency phase, methane production in all assays showed a constant rate for a time that is identified as the exponential phase (linear correlation, $R^2 > 0.9$, mean value 0.99). Subsequently, when the substrate is depleted, the accumulated production of methane tends progressively to its maximum value corresponding to the methane potential of the substrate under consideration. The exponential phase allows us to obtain the maximum methane production rate, as well as the percentage of the total methane potential obtained at the high exponential rate.

The rate of methane production in the exponential phase increased in all assays during the second feeding relative to the first, but remaining practically constant when expressed as percentage of the control (Figure 5b). However, substrates RS120 and RS170 showed significant differences ($p < 0.02$) between the first and second feedings, as shown in Figure 5b. This behavior indicates a general absence of toxicity, which was to be expected given the low concentrations tested. Some residual solids showed high methane production rates, with averages of 3.4 (RS210), 3.7 (RS170) and 4.2 (RS150) times the rate obtained for *S. muticum*, while for RS120 and RS190 were only 1.4 and 2.4 times higher. This means that RS150 and RS170 not only offer the highest SMP but also allow it to be obtained at a higher rate than the other substrates and particularly than the raw alga *S. muticum*.

The percentage of methane potential reached in the exponential phase was 76.2 ± 7.8% and 72.5 ± 5.0% of the final SMP, at the first and second feeding, respectively. The mean value for both feedings was 74.3 ± 5.8%, but clearly higher for RS150 and RS210 (mean 81.2 ± 3.5%) than for *S. muticum* and the other residual solids RS120, RS170 and RS190 (mean 70.9 ± 2.1%). There were no significant differences within these two groups ($p > 0.25$), but between them ($p = 0.000$). The control assay achieved a mean of 93.3%, significantly higher than that of any of the other substrates ($p < 0.002$).

**Substrate solubilisation and biodegradability**

Anaerobic digestion involved the solubilisation of some organic compounds that appeared not further biodegradable in anaerobic conditions and accumulate in the digestion medium. At the end of the second feeding, soluble COD accumulated in all assays in different amounts and correlated with absorbance at 215 ($R^2$=0.975) and 440 nm ($R^2$= 0.908). The highest soluble COD and absorbance was obtained for RS210, with 1175 mg COD/L, 32.5 $ABS_{215}$ units and 3.4 $ABS_{440}$ units. The digested medium RS210 presented a strong red colour, followed by RS190. The lower COD and absorbance corresponded to RS170 followed by RS150. As phenolic compounds show high ability to absorb UV light, the results suggest that a large amount of phenols can accumulate in the digested medium of RS190 and RS210.

The total solubilized COD was obtained as the sum of the remaining (refractary) soluble COD and the produced methane expressed as COD. Figure 6 shows the total solubilized COD and the two accounting fractions.

Several studies indicate that the process step that limits methane yield is not methanogenesis but hydrolysis [29, 30, 62]. In fact, several pre-treatment methods aim to hydrolyse algae polymers and polysaccharides for its subsequent transformation in methane [58]. For example, alginic acid and sodium alginate reached low methane yields of approximately 25 % of their theoretical potential [31]. These authors attributed the low methane yield to the recalcitrance of these substrates and to the inhibition of hydrolysis by phenolic compounds.

The results shown in Figure 6 indicate that thermal hydrolysis at 120 and 190 ºC had a reduced effect on substrate solubilisation, whereas pre-treatment at 150, 170 and 210 ºC strongly increased the solubilized COD by 30-56%. However, most of the solubilized COD at 150 ºC, and particularly at 170 ºC was converted to methane, while a large amount (32.4%) of solubilised COD at 210 ºC behaved as refractory to methanogenesis. Further research is required to characterize the refractory COD fraction solubilised or produced after anaerobic digestion of residual solids pre-hydrolysed at 210 ºC. If this COD fraction has no beneficial properties, the optimum hydrolysis pre-treatment would be that of 150 to 170 ºC.

### 3.2.2. Mineral amendment

Table 1 summarizes the mineral content of the solids remaining after autohydrolysis. The residual solid phase after autohydrolysis of *S. muticum* has a low calorific value (3.5 kcal/g) and high ash content. Utilization of these solids as a mineral amendment

was suggested based on the presence of N, P, K, Ca, Mg, Cu, Fe, and Zn, although the content in some components was lower than in the pristine alga [34].

Both the pristine material and the residual phase of the hydrolysis have N and K contents close to those required by the Spanish fertilizer law (RD999/2017) [63] to be classified as “Organic fertilizer NK”. However, none of the analyzed materials reaches the required value of 2% in N and 3% in $K_2O$. Yet to take advantage of their high nutrient and C content, the materials investigated here could be marketed as organo-mineral fertilizers, as long as they present at least 1% of the N in organic form.

The balance of nutrients in the pristine material (10:1:18) is conditioned by the high $K_2O$ content (Table 1). As the treatment temperature increases, the ratio N:P:K changes, with N being the main nutrient for temperatures higher than 170 °C. The content in CaO and MgO is also very important, although the concentrations of both compounds tend to decrease as the hydrolysis temperature increases. This ratio of nutrients (N:P:K), makes its optimal to use in fruit crops, where the extractions of N and especially K, are much higher than P. However, it will always be necessary to combine it with some other fertilizer that provides this nutrient.

Nevertheless, it should be acknowledged the limitations for the use of raw seaweeds and their processing products in applications related to human nutrition and health. This is due to the elevated levels of toxic heavy metals, especially arsenic, known to occur in some algae. Arsenic is predominantly found in seaweeds as an organic compound, with less associated poisonous effects than inorganic arsenic, although potentially both species have the ability to form toxic species [64].

Alternatively, the utilization of marine macroalgae, e.g. *Sargassum* species, for pollutant removal is a known process in water treatment termed biosorption [65]. The underlying mechanisms of mineral amendment using seaweed, or its solid residues from biorefinery, present a clear similarity with biosorption processes. In particular, the mechanism of ion exchange exerts a fundamental control, at least for the retention of specific metals. In this context, the elevated proton binding capacity of *Sargassum muticum* (2.40-2.61 mol/kg of alga) anticipates success of micro-macronutrient amendments using this alga or its residues [66]. For example, *S. muticum* collected from the same geographical area as the specimens used for the present study showed adsorption capacities ranging between 1.12-1.42 mmol of metal/g of alga [23]. This great capacity of *S. muticum* for metal removal is associated to the polysaccharides present in the algae structure, i.e. alginates and fucoidans. Indeed, dealginated seaweed only provided maximum adsorption capacities of 0.3-0.8 mmol/g [65]. Therefore, it is expected that algae residues without alginate or fucoidans (e.g. the

solid residue fraction obtained in this study) present declined adsorption capacities. This topic will be the subject of future work.

## CONCLUSIONS

The results of the present study confirm the potential integration of different environmentally friendly stages for the sequential fractionation of valuable components obtained from raw *Sargassum muticum* biomass. The extraction process is valid for either frozen or fresh biomass. A Sap fraction, separated by physical means, stimulated root development and shoot/root ratio. The remaining solids were processed by autohydrolysis to solubilize the fucoidan and phenolic fractions with yields and properties comparable to those obtained from raw biomass in previous studies. The residual solids from the autohydrolysis stage were more suitable substrates for anaerobic digestion than the raw seaweed. Alternatively, these solids could be proposed as an organic fertilizer due to their high nitrogen and potassium content or could serve as adsorbents for the selective separation of heavy metals. The biorefinery scheme proposed here represents a complete approach for the production of components with interest for food, cosmetic, agricultural and energetic applications.


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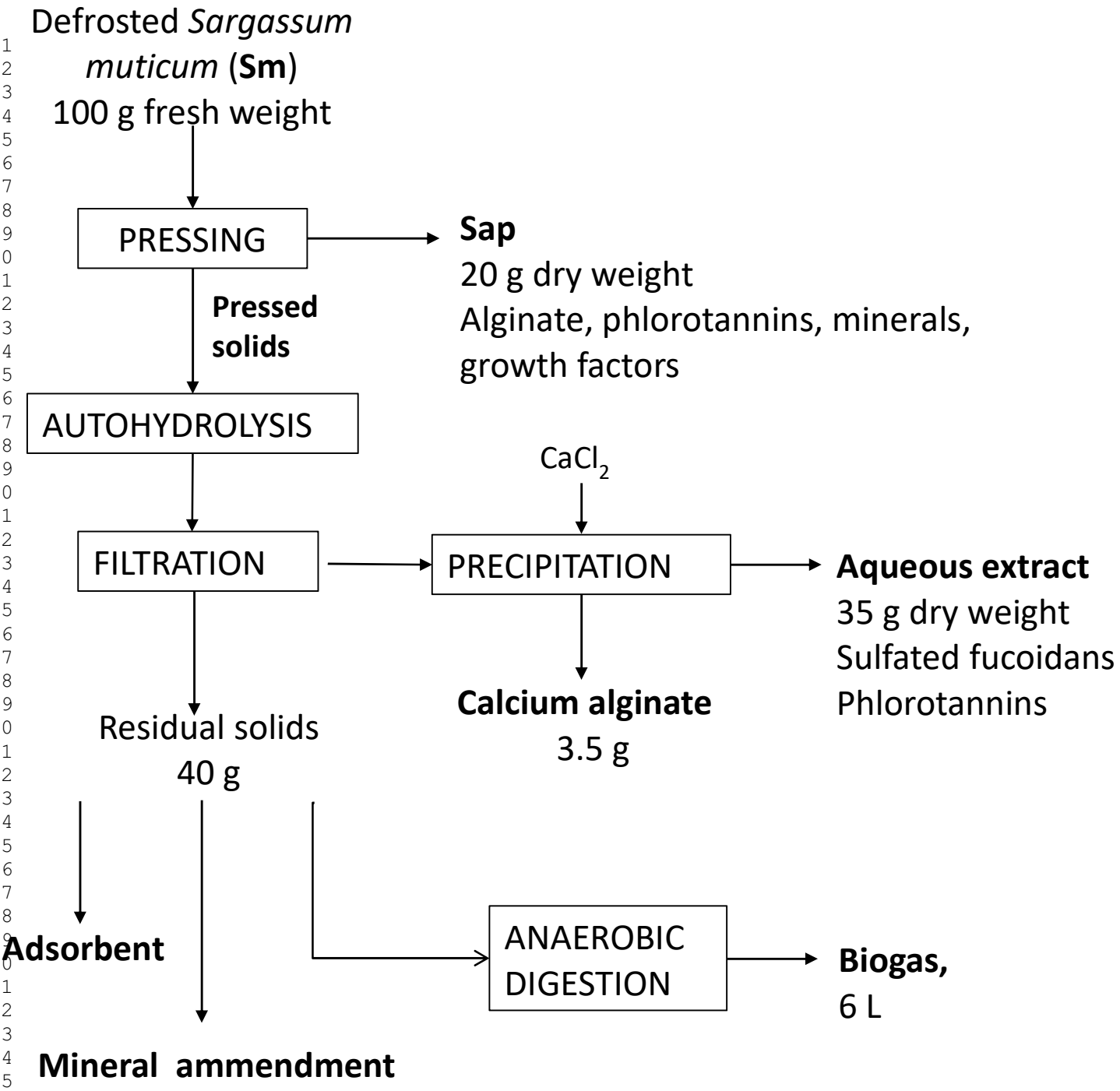


Figure 1. Flow diagram of the proposed process for the utilization of *S. muticum* components.

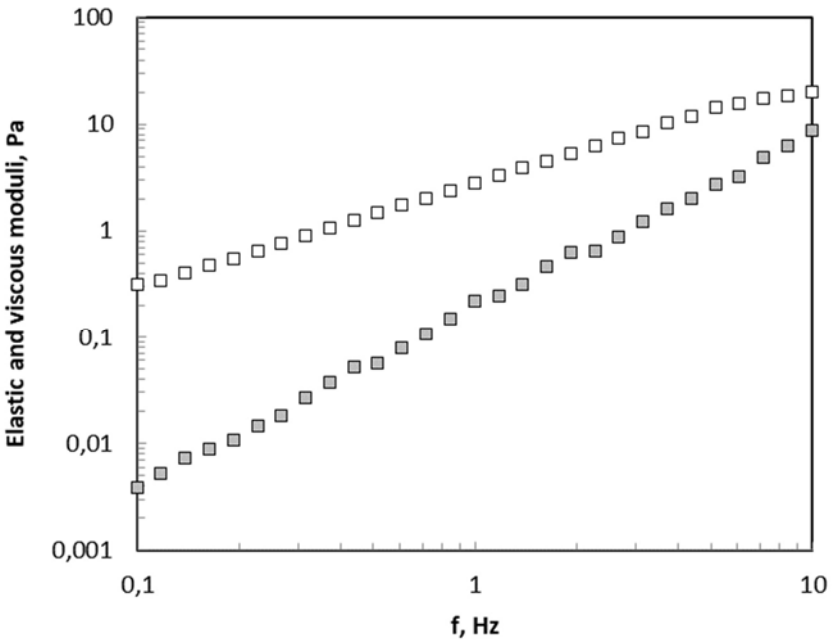


**Figure 2**. Viscoelastic properties of tested alginate precipitated from *S. muticum* pressing liquid at 25ºC. Symbols: G', elastic modulus and G'', viscous modulus.

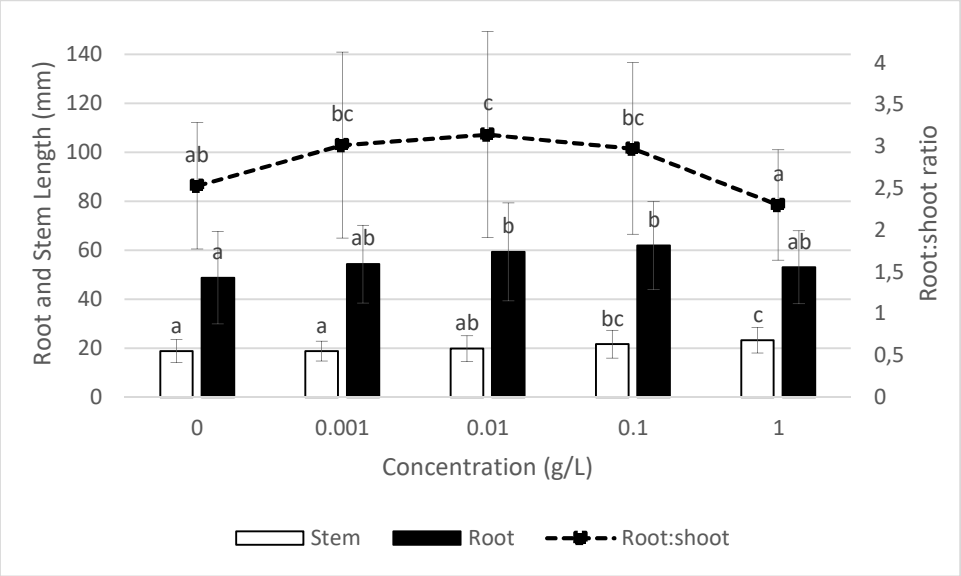


Figure 3. Evaluation of watercress seed growth at different doses of *S. muticum* Sap.

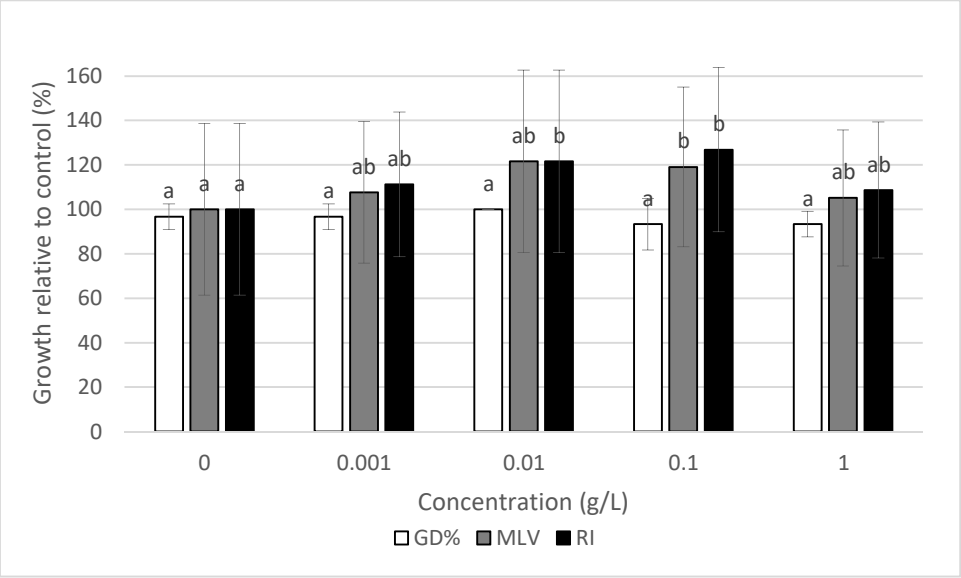


Figure 4. Growth promotion according to the biostimulant dose. GD: germination degree (%); RI: Root index (%); MVL: Munoo-Liisa vitality test (%).

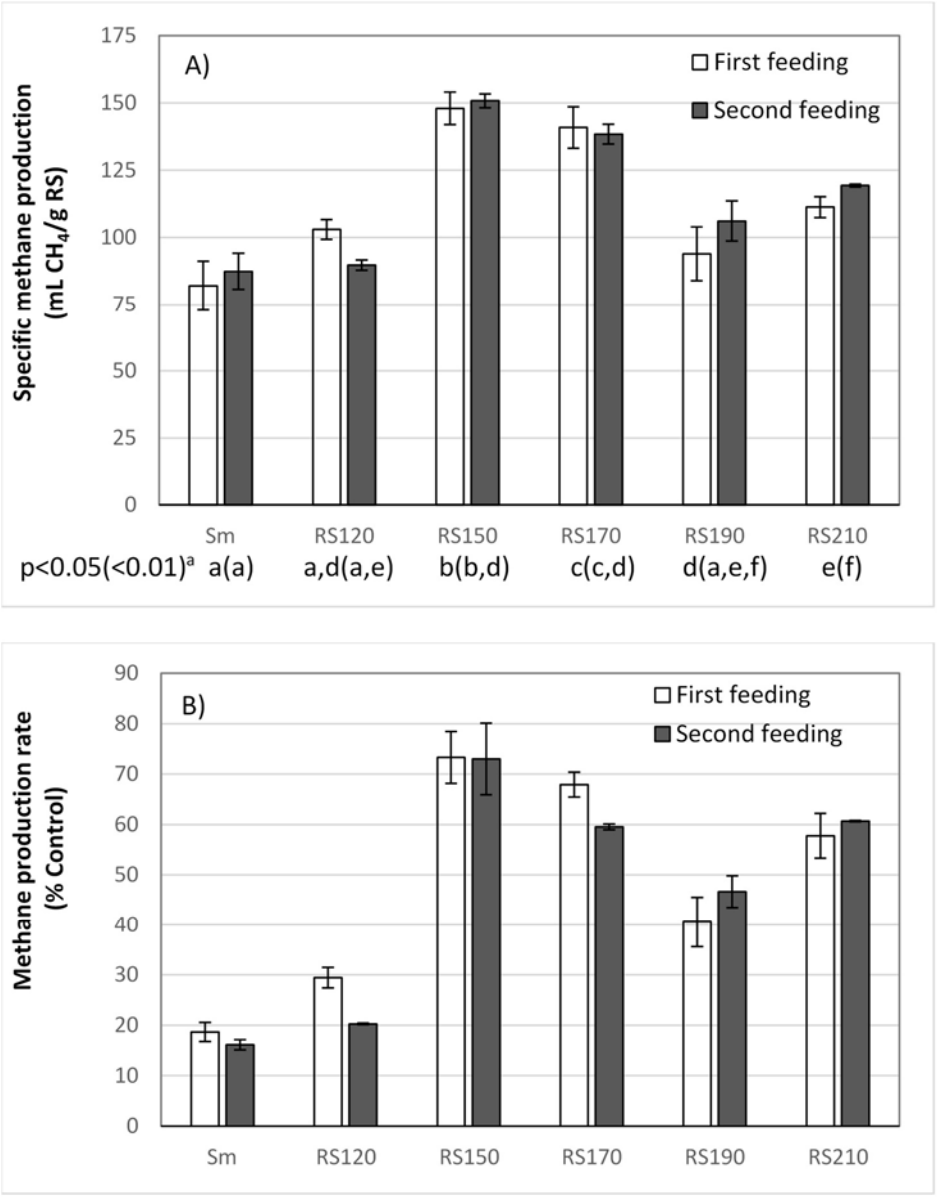


Figure 5. Specific methane production (A) and methane production rate (B) for the untreated sample *S. muticum* (Sm) and the waste solid fraction remaining after autohydrolysis at different final heating temperatures. Note: [a] Different letters indicate statiscal differences for the different substrates at p level of 0.05 (or 0.01, in brackets).

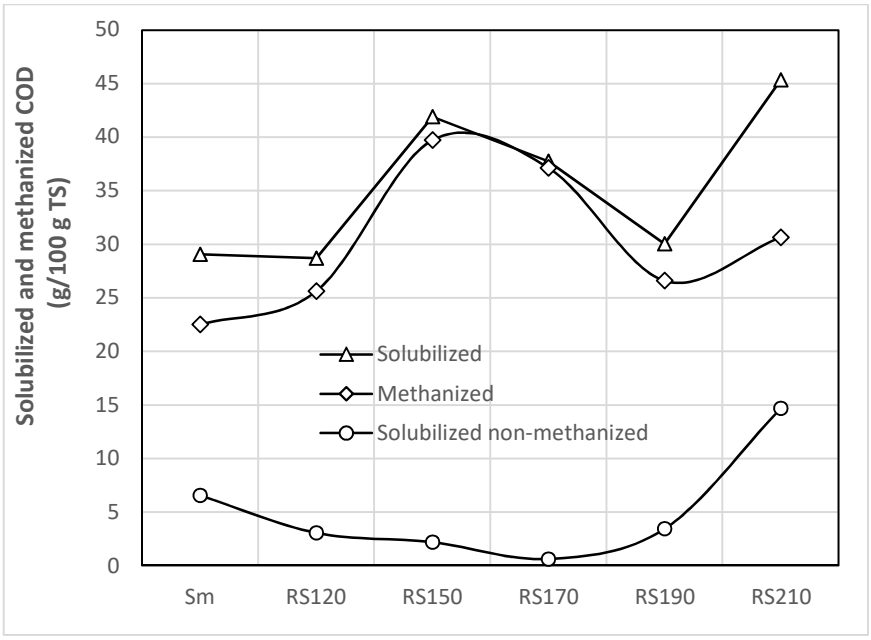


Figure 6. Solubilized COD in anaerobic digestion of *S. muticum* Sm and residuals solids and its distribution between methane and soluble refractory COD.

Table 1.- Elemental and mineral composition of *Sargassum muticum* (Sm), the Sap obtained by the pressing stage and effect of the autohydrolysis final temperature on the elemental and mineral composition of the remaining residual solids.

| Composition (%) | Sm | Smp | Sap | Autohydrolysis treatment (RS) | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | 120 °C | 150 °C | 170 °C | 190 °C | 210 °C |
| Ash | 17.15 | 24.80 | | 12.43 | 11.54 | 10.12 | 9.87 | 8.57 |
| N | 1.57 | 1.62 | 0.86 | 1.9 | 1.7 | 1.9 | 2.1 | 2.1 |
| Carbon | 25.9 | 35.55 | 18.90 | 36.56 | 37.83 | 41.10 | 46.16 | 47.58 |
| Glucose | 10.18 | 17.89 | | 15.49 | 20.68 | 22.45 | 25.73 | 25.40 |
| Xyl+Gal+Man | 6.75 | 9.66 | | 6.83 | 5.08 | 3.44 | 2.38 | 1.01 |
| Fucose | 6.00 | 7.98 | | 6.96 | 4.29 | 2.35 | 1.69 | 0.64 |
| Acetyl groups | 0.33 | 0.70 | | 0.46 | 0.39 | 0.35 | 0.33 | 0.42 |
| AIR* | 31.1 | 28.63 | | 37.45 | 41.19 | 55.12 | 59.26 | 58.93 |
| **Element** | | | | | | | | |
| K | 2.33 | 3.12 | 11.65 | 2.04 | 1.72 | 1.05 | 0.64 | 1.49 |
| Ca | 1.88 | 1.42 | 0.57 | 1.42 | 1.69 | 1.76 | 1.36 | 0.99 |
| Na | 0.73 | 1.26 | 5.63 | 0.81 | 0.63 | 0.29 | 0.18 | 0.58 |
| Mg | 0.75 | 0.54 | 1.37 | 0.54 | 0.35 | 0.30 | 0.24 | 0.25 |
| P | 0.07 | 0.12 | | 0.05 | 0.06 | 0.04 | 0.03 | 0.07 |
| I | 0.03 | 0.08 | | 0.02 | 0.03 | 0.03 | 0.03 | 0.02 |
| Fe | 0.01 | 0.03 | < 1 | 0.01 | 0.02 | 0.02 | 0.02 | 0.04 |
| Zn | <1 | 0.01 | < 1 | 0.01 | 0.02 | 0.01 | 0.01 | 0.03 |
| Cu (mg/kg) | <7 | 5.50 | < 1 | 3.2 | 4.2 | 6.5 | 9.1 | 6.0 |
| Pb (mg/kg) | <2 | 0.64 | < 2 | 0.60 | 0.36 | 0.34 | 0.19 | 0.17 |
| Hg (µg/kg) | <2 | <0.8 | < 1 | <34.9 | 32.3 | 37.5 | <34.6 | <34.1 |
| Cd (mg/kg) | <2 | <0.5 | < 1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 |
| N:P:K ratio | 10:1:18 | - | | 17:1:22 | 12:1:15 | 21:1:14 | 30:1:11 | 13:1:11 |
| **Compound (% dry weight)** | | | | | | | | |
| $P_2O_5$ | - | 0.2 | | 0.1 | 0.14 | 0.1 | 0.1 | 0.2 |
| $K_2O$ | - | 2.8 | | 2.4 | 2.1 | 1.2 | 0.8 | 1.8 |
| CaO | - | 2.6 | | 2.0 | 2.4 | 2.5 | 1.9 | 1.4 |
| MgO | - | 1.2 | | 0.9 | 0.6 | 0.5 | 0.4 | 0.4 |

*AIR: acid insoluble residue